**UNIVERSITY OF IOANNINA**

**SCHOOL OF HEALTH SCIENCES**

**DEPARTMENT OF BIOLOGICAL APPLICATIONS AND TECHNOLOGY**

MICROBIAL ECOLOGY LAB

# MICROBIAL FOOD WEBS IN EXPERIMENTAL AQUAPONICS AND AQUACULTURE SYSTEMS

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Δεν θα μπορούσα να παραλείψω και τα υπόλοιπα μέλη του εργαστηρίου Μικροβιακής Οικολογίας, τους υποψήφιους διδάκτορες Σοφία Ματσίνγκο και Οδυσσέα Πιπεράγκα για τις συμβουλές και τη στήριξη τους στα πρώτα μου εργαστηριακά βήματα. Επίσης ευχαριστώ τον διδάκτορα Θεόφιλο Βανικίωτη από το Εργαστήριο Βοτανικής για τις συμβουλές του και τις σοβαρές και μη συζητήσεις μας κατά τις επισκέψεις του. Ακόμη ευχαριστώ την Χαρίς Μελά, μέλος Ε.Τ.Ε.Π. του τΒΕΤ για την παρέα της στο εργαστήριο Υδροβιολογίας που έκανε τις πολλές ώρες στα μικροσκόπια και στα φοιτητικά εργαστήρια να περνούν πιο εύκολα.

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## Abstract

The present thesis explores the potential role of the neglected microeukaryotic component of suspended microbial communities in experimental recirculating aquaculture and aquaponics systems by utilizing both microscopy-based and metagenomics-based approaches, while also highlighting the metabolic potential of the prokaryotic community through identifying key genes involved in the cycling of major nutrients.

As global population increases there is a growing demand for food. Aquaculture production has recently surpassed the production of capture fisheries for the first time in history. Recirculating aquaculture systems (RAS) have shown promise as sustainable food production methods, especially when coupled with hydroponics set ups to form aquaponics systems. Aquaponics systems allow the simultaneous production of fish and edible plants with the benefits of minimal water usage, higher feed utilization efficiency and reduced environmental impact. Although such systems are not a new concept, their designs are undergoing constant optimization to offset their inherent drawbacks. In this context, particular interest has been shown in the bacterial communities of aquaponics biofilters (i.e., specialized compartments where prokaryotes are employed to carry out nitrification to maintain water quality), farmed fish intestines and to a lesser extent to the suspended bacterial communities in aquaponics tanks. However, the eukaryotic and archaeal fractions of microbial communities in such systems remain scarcely studied, despite playing a potentially critical part in shaping bacterial communities through predation or resource competition respectively. Furthermore, the focus on bacteria in RAS and aquaponics systems has been on nitrifying taxa, but there are other key metabolic functions such as nutrient cycling and organic matter degradation that remain unexplored.

Natural abundances and growth rates of the main heterotrophic prokaryotic groups, Ciliophora (ciliates) and heterotrophic nanoflagellates (HNF), were measured using microscopic counts and *in situ* growth experiments after size fractionation of system water from different compartments of RAS and aquaponics systems. Natural

abundances were found generally low, similar to those of rivers and oligotrophic to mesotrophic lakes. Growth rates in the aquaponics system were high, comparable to those in natural assemblages or isolated cultures. Fish tank environment allowed faster ciliate and HNF growth compared to drain tanks where water is collected after plant fertigation. Based on microscopic counts and observations, ciliate genera such as *Cyclidium*, *Halteria*, *Paramecium*, *Litonotus*, *Vorticella* and *Euplotes* were the most observed, while *Oxytricha*, *Stylonychia* and *Stentor* were the most abundant genera according to metagenomics sequence counts. The need for employing both methods to complement each other was also evident in other microbial groups such as amoebae which were clearly observed and identified under the microscope but not detected through metagenomics. Meanwhile, ciliate and HNF abundance was lower in the artificial seawater RAS and both populations declined during the incubation. Thus, it is implied that heterotrophic protists may be more influential over prokaryotic communities in aquaponics and less relevant in the constantly flowing, turbulent water of artificial seawater RAS.

Several observations using both metagenomics and microscopy derived data indicated that fish tanks and drain tanks constituted two major niches in the aquaponics systems. Specifically, drain tanks contained an increased abundance of autotrophic groups such as diatoms (e.g., *Fragilariopsis*) and algae (e.g., *Chlorella*) likely due to higher sunlight and nutrient availability, as well as amoebae (e.g., *Euglypha*) and rotifers (e.g., *Lecane*). Drain tanks also harbored higher richness of bacterial and archaeal genera whereas fish tank microeukaryotic communities were more diverse. Furthermore, although archaeal nitrifying genera were present in all aquaponics compartments, archaeal nitrification genes were mainly present in drain tanks, likely due to the higher ammonia concentration and the presence of sludge providing them better growth conditions. Overall, NMDS and clustering analyses indicated that microeukaryotic and archaeal communities were shaped by compartment type, whereas bacterial communities were more distinct between different aquaponics systems regardless of compartment type. In fact, different bacterial genera were dominant in tomato and cucumber growing systems, with

*Flavobacterium* and *Limnohabitans* being more abundant in the former and *Polynucleobacter* and *Mycobacterium* being more abundant in the latter system. Another system-wide difference was the presence of methanotrophic genera only in the samples of the parsley growing system.

In terms of relevant microeukaryotic genera in aquaponics, the presence of *Oxytricha*, *Stylonychia* and *Stentor* in high relative abundance indicates healthy microbial community composition. In addition, both pathogen-containing genera such as *Aphanomyces* and *Phytophthora* (Oomycota) and potentially beneficial taxa for fish and plant growth such as *Aspergillus* (Ascomycota) and *Chlorella* (Chlorophyta) were detected. The identification of key prokaryotic genes involved in sulfur oxidation suggested the presence of sulfur oxidizing bacteria throughout the aquaponics systems. Carbohydrate degradation gene abundance seemed to reflect the increasing content of plant based organic matter in fish feed. Another key observation was the presence of genes responsible for anaerobic ammonia oxidation (anammox) in the mature aquaponics communities, with the implication that anammox may take place in other aquaponics compartments apart from specialized anaerobic bioreactors. The relative abundance of bacterial nitrification genes peaked in the mature communities in all systems, whereas archaeal nitrification genes were abundant in the drain tanks of the tomato growing system.

In the artificial seawater RAS, ammonia oxidizing archaea dominated archaeal communities alongside methanogens, although no archaeal nitrifying genes were detected, highlighting the need for more complete genomic databases going forward with archaeal research. In addition, the RAS fish tank biofilm community was more enriched in nutrient cycling and organic matter degradation genes, as well as harboring a higher microeukaryotic diversity, implying it may serve as a hotspot for ecological processes in RAS. Several Ascomycota genera were among the dominant microeukaryotic groups in RAS fish tank water, biofilm and biofilter and may be a potential candidate for future RAS research.

# Περίληψη

Η παρούσα διατριβή διερευνά τον πιθανό ρόλο της παραμελημένης μικροευκαρυωτικής συνιστώσας των πλαγκτονικών μικροβιακών κοινοτήτων σε πειραματικά συστήματα υδατοκαλλιέργειας και ενυδρειοπονίας, χρησιμοποιώντας προσεγγίσεις που βασίζονται τόσο στη μικροσκοπία όσο και στη μεταγονιδιωματική ανάλυση, ενώ παράλληλα αναδεικνύει τις μεταβολικές δυνατότητες της προκαρυωτικής κοινότητας μέσω της ταυτοποίησης κύριων γονιδίων που εμπλέκονται στον κύκλο των βασικών θρεπτικών συστατικών.

Καθώς ο παγκόσμιος πληθυσμός αυξάνεται υπάρχει και αυξανόμενη ζήτηση για τρόφιμα ενώ πρόσφατα η παραγωγή μέσω υδατοκαλλιέργειας ξεπέρασε την παραγωγή μέσω αλιείας για πρώτη φορά στην ιστορία. Τα συστήματα υδατοκαλλιέργειας που στηρίζονται στην ανακύκλωση του νερού (RAS) έχουν αποδειχθεί πολλά υποσχόμενα ως μια βιώσιμη μέθοδος παραγωγής τροφίμων, ειδικά όταν συνδυάζονται με υδροπονικές εγκαταστάσεις συνιστώντας έτσι συστήματα ενυδρειοπονίας. Τα συστήματα ενυδρειοπονίας επιτρέπουν την ταυτόχρονη παραγωγή ψαριών και βρώσιμων φυτών με κύρια πλεονεκτήματα την ελάχιστη δυνατή χρήση νερού, την υψηλότερη αποδοτικότητα στην αξιοποίηση των ζωοτροφών και της μειωμένης περιβαλλοντικής επιβάρυνσης. Αν και τέτοια συστήματα δεν αποτελούν ακριβώς νέα ιδέα, ο σχεδιασμός τους βελτιστοποιείται διαρκώς για να αντισταθμιστούν τα εγγενή μειονεκτήματά τους. Σε αυτό το πλαίσιο, έχει εκδηλωθεί ιδιαίτερο ενδιαφέρον για τις βακτηριακές κοινότητες των βιοφίλτρων (δηλαδή των εξειδικευμένων διαμερισμάτων όπου προκαρυώτες μετατρέπουν την αμμωνία σε νιτρικά ιόντα, επιτρέποντας τη διατήρηση της ποιότητας του νερού), του πεπτικού συστήματος των εκτρεφόμενων ψαριών και σε μικρότερο βαθμό για τις πλαγκτονικές βακτηριακές κοινότητες. Ωστόσο, οι ευκαρυώτες και τα αρχαία που συμπληρώνουν τις μικροβιακές κοινότητες σε τέτοια συστήματα παραμένουν ελάχιστα μελετημένα, παρά το γεγονός ότι διαδραματίζουν δυνητικά κρίσιμο ρόλο στη διαμόρφωση των βακτηριακών κοινοτήτων μέσω της θήρευσης ή του ανταγωνισμού για διαθέσιμους πόρους αντίστοιχα. Επιπλέον, οι μελέτες των βακτηρίων στα συστήματα RAS και στην

ενυδρειοπονία έχουν επικεντρωθεί στις νιτροποιητικές ομάδες, αλλά υπάρχουν και άλλες βασικές μεταβολικές λειτουργίες όπως η ανακύκλωση θρεπτικών στοιχείων και η αποικοδόμηση της οργανικής ύλης που παραμένουν ελάχιστα μελετημένες.

Οι φυσικές αφθονίες και οι ρυθμοί αύξησης των κύριων ετερότροφων προκαρυωτικών ομάδων, δηλαδή των εκπροσώπων του φύλου Ciliophora (βλεφαριδωτά) και των ετερότροφων νανομαστιγωτών (HNF), μετρήθηκαν μέσω τεχνικών μικροσκοπίας και *in situ* πειραμάτων αύξησης μετά από κλασμάτωση δειγμάτων νερού από διαφορετικά διαμερίσματα πειραματικών συστημάτων RAS και ενυδρειοπονίας. Οι φυσικές αφθονίες βρέθηκαν γενικά χαμηλές, παρόμοιες με εκείνες που καταγράφονται σε ποταμούς και σε ολιγότροφες έως μεσότροφες λίμνες. Οι ρυθμοί ανάπτυξης στο σύστημα ενυδρειοπονίας ήταν υψηλοί, συγκρίσιμοι με εκείνους σε φυσικές κοινότητες ή καθαρές καλλιέργειες. Το περιβάλλον των δεξαμενών εκτροφής των ιχθύων επέτρεψε ταχύτερη ανάπτυξη των βλεφαριδωτών και των ετερότροφων νανομαστιγωτών σε σχέση με τις δεξαμενές αποστράγγισης όπου το νερό συλλέγεται μετά το πότισμα των φυτών. Με βάση τις παρατηρήσεις και μετρήσεις μέσω μικροσκοπίας, γένη βλεφαριδωτών όπως τα *Cyclidium*, *Halteria*, *Paramecium*, *Litonotus*, *Vorticella* και *Euplotes* ήταν τα πιο συχνά παρατηρούμενα, ενώ τα *Oxytricha*, *Stylonychia* και *Stentor* ήταν τα πιο άφθονα γένη σύμφωνα με την συχνότητα εμφάνισης των αντίστοιχων μεταγονιδιωματικών αλληλουχιών. Η ανάγκη χρήσης και των δύο μεθόδων λόγω της συμπληρωματικότητας τους ήταν επίσης εμφανής και σε άλλες μικροβιακές ομάδες όπως οι αμοιβάδες, οι οποίες παρατηρήθηκαν και ταυτοποιήθηκαν με ευκολία μέσω μικροσκοπίας αλλά δεν ανιχνεύθηκαν μέσω της μεταγονιδιωματικής ανάλυσης. Παράλληλα, η αφθονία των βλεφαριδωτών και των HNF ήταν χαμηλότερη στα RAS που λειτουργούσαν με τεχνητό θαλασσινό νερό, ενώ και οι δύο πληθυσμοί μειώθηκαν κατά την επώαση. Επομένως, φαίνεται ότι τα ετερότροφα πρώτιστα μπορεί να έχουν μεγαλύτερη επιρροή πάνω στις προκαρυωτικές κοινότητες των συστημάτων ενυδρειοπονίας και λιγότερο καθοριστικό ρόλο στο πιο τυρβώδες περιβάλλον των RAS που λειτουργούν με τεχνητό θαλασσινό νερό.

Αρκετές παρατηρήσεις που βασίστηκαν σε δεδομένα της μεταγονιδιωματικής ανάλυσης και της μικροσκοπίας έδειξαν ότι οι δεξαμενές εκτροφής των ψαριών και οι

δεξαμενές αποστράγγισης του νερού αποτελούσαν τα δύο κύρια περιβάλλοντα στα συστήματα ενυδρειοπονίας. Συγκεκριμένα, οι δεξαμενές αποστράγγισης χαρακτηρίζονταν από αυξημένη αφθονία αυτότροφων μικροευκαρυωτών όπως διάτομα (π.χ., *Fragilariopsis*) και φύκη (π.χ., *Chlorella*) πιθανώς λόγω της αυξημένης συγκέντρωσης θρεπτικών και έκθεσης στην ηλιακή ακτινοβολία, αλλά και αμοιβάδες (π.χ., *Euglypha*) και τροχοφόρα (π.χ., *Lecane*). Οι δεξαμενές αποστράγγισης φιλοξενούσαν επίσης μεγαλύτερο αριθμό γενών στις περιπτώσεις των βακτηρίων και των αρχαίων, ενώ οι μικροευκαρυωτικές κοινότητες των δεξαμενών εκτροφής των ψαριών είχαν μεγαλύτερη ποικιλότητα. Επιπλέον, αν και νιτροποιητικά γένη αρχαίων ήταν παρόντα σε όλα τα διαμερίσματα των συστημάτων ενυδρειοπονίας, τα νιτροποιητικά γονίδια των αρχαίων ανιχνεύτηκαν κυρίως στις δεξαμενές αποστράγγισης, πιθανώς λόγω της υψηλότερης συγκέντρωσης αμμωνίας και της ύπαρξης σωματιδιακής ύλης που πιθανώς προσεφέρουν ένα πιο ευνοϊκό περιβάλλον για την αύξηση των νιτροποιητικών αρχαίων. Συνολικά, οι αναλύσεις NMDS και συσταδοποίησης έδειξαν ότι οι κοινότητες των μικροευκαρυωτών και αρχαίων διαμορφώνονταν ανάλογα με τον τύπο του διαμερίσματος, ενώ οι βακτηριακές κοινότητες ήταν πιο διακριτές μεταξύ διαφορετικών συστημάτων ενυδρειοπονίας ανεξάρτητα από τον τύπο του διαμερίσματος. Πράγματι, διαφορετικά βακτηριακά γένη κυριαρχούσαν στα συστήματα καλλιέργειας ντομάτας και αγγουριού, με τα *Flavobacterium* και *Limnohabitans* να είναι πιο άφθονα στο πρώτο και τα *Polynucleobacter* και *Mycobacterium* να είναι πιο άφθονα στο δεύτερο σύστημα. Μια άλλη διαφορά μεταξύ ολόκληρων συστημάτων ήταν η παρουσία μεθανότροφων βακτηριακών γενών μόνο στα δείγματα του συστήματος καλλιέργειας μαϊντανού.

Όσον αφορά τα σημαντικά μικροευκαρυωτικά γένη στην ενυδρειοπονία, η παρουσία των *Oxytricha*, *Stylonychia* και *Stentor* σε υψηλή αφθονία υποδήλωνε την υγιή σύνθεση της μικροβιακής κοινότητας. Επιπλέον, ανιχνεύθηκαν τόσο γένη που περιέχουν παθογόνα στελέχη όπως τα *Aphanomyces* και *Phytophthora* (Oomycota) αλλά και δυνητικά ωφέλιμα για την ανάπτυξη ψαριών και φυτών όπως τα *Aspergillus* (Ascomycota) και *Chlorella* (Chlorophyta). Η ταυτοποίηση κύριων προκαρυωτικών γονιδίων που εμπλέκονται στην οξείδωση θειικών ενώσεων υποδηλώνει την παρουσία

θειοξειδωτικών βακτηρίων σε όλα τα διαμερίσματα των συστημάτων ενυδρειοπονίας. Η αφθονία των γονιδίων που σχετίζονται με την αποικοδόμηση υδατανθράκων φάνηκε να αντανακλά την αυξανόμενη περιεκτικότητα οργανικής ύλης φυτικής προέλευσης στις ιχθυοτροφές. Μια άλλη βασική παρατήρηση ήταν η παρουσία γονιδίων που είναι υπεύθυνα για την αναερόβια οξείδωση αμμωνίας (anammox) στις ώριμες μικροβιακές κοινότητες των συστημάτων ενυδρειοπονίας, με το συμπέρασμα ότι η διαδικασία anammox μπορεί να λαμβάνει χώρα και σε άλλα διαμερίσματα των συστημάτων ενυδρειοπονίας εκτός από τους εξειδικευμένους αναερόβιους βιοαντιδραστήρες. Η σχετική αφθονία των βακτηριακών νιτροποιητικών γονιδίων έφτασε στις μέγιστες τιμές της στις ώριμες κοινότητες σε όλα τα συστήματα, ενώ τα νιτροποιητικά γονίδια των αρχαίων ήταν πιο άφθονα στις δεξαμενές αποστράγγισης του συστήματος καλλιέργειας ντομάτας.

Στην περίπτωση των RAS που λειτουργούσαν με τεχνητό θαλασσινό νερό, τα αμμωνιοξειδωτικά αρχαία κυριάρχησαν στις κοινότητές τους παράλληλα με μεθανογόνα γένη, αν και δεν ανιχνεύθηκαν νιτροποιητικά γονίδια αρχαίων, γεγονός που υπογραμμίζει την ανάγκη για πιο ολοκληρωμένες γονιδιωματικές βάσεις δεδομένων για μελλοντικές έρευνες πάνω στα αρχαία. Επιπλέον, η βακτηριακή κοινότητα των βιοϋμενίων στις δεξαμενές εκτροφής των ψαριών στα RAS ήταν πιο εμπλουτισμένη σε γονίδια που συμμετέχουν στην ανακύκλωση θρεπτικών συστατικών και στην αποικοδόμηση οργανικής ύλης, ενώ η αντίστοιχη μικροευκαρυωτική κοινότητα παρουσίαζε και υψηλότερη ποικιλομορφία, υποδηλώνοντας ότι τα βιοϋμένια αυτά μπορεί να είναι σημείο κλειδί για πολλές οικολογικές διεργασίες στα RAS. Ακόμη, αρκετά γένη του φύλου Ascomycota ήταν μεταξύ των κυρίαρχων μικροευκαρυωτικών ταξινομικών ομάδων στο νερό, τα βιοϋμένια και το βιοφίλτρο των RAS και επομένως το φύλο αυτό πιθανώς χρήζει μελλοντικής έρευνας στο πλαίσιο των RAS.

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## List of abbreviations

| | |
|---|---|
| Anammox | Anaerobic ammonia oxidation |
| AOA | Ammonia oxidizing archaea |
| AOB | Ammonia oxidizing bacteria |
| AP | Aquaponics |
| BB | Biofilter biofilm of the artificial seawater RAS |
| CIL | Ciliates |
| Comammox | Complete ammonia oxidation |
| CU | Cucumber growing aquaponics system |
| DR | Drain tanks |
| DO | Dissolved oxygen |
| FEB | 1$^{st}$ protist growth experiment in the aquaponics system |
| FT | Fish tanks |
| FTB | Fish tank biofilm of the artificial seawater RAS |
| FTW | Fish tank water of the artificial seawater RAS |
| HNF | Heterotrophic nanoflagellate(s) |
| JUN | 2$^{nd}$ protist growth experiment in the aquaponics system |
| NMDS | Non-metric multidimensional scaling |
| NOB | Nitrite oxidizing bacteria |
| PA | Parsley growing aquaponics system |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| PERMANOVA | Permutational multivariate analysis of variance |
| PGPF | Plant growth promoting fungi |
| PGPR | Plant growth promoting rhizobacteria |
| RAS | Recirculating aquaculture system(s) |
| RAS1, RAS 2 | Replicate loops of the artificial seawater RAS |

| | |
|---|---|
| SIMPER | Similarity percentage |
| SUMP | Clear buffer tank of the tomato growing aquaponics system |
| TO | Tomato growing aquaponics system |
| TOM | Drain tanks of the tomato growing aquaponics system |
| UPGMA | Unweighted pair group method with arithmetic mean |

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# 1. General Introduction

## 1.1. Microbial community components in aquatic environments and their interactions

Microorganisms are single-cell organisms with a widespread distribution, inhabiting soil, sediment, freshwater and marine environments, while they also naturally live alongside multicellular organisms and other microorganisms as symbionts. In terms of taxonomy, microorganisms consist of members of all three domains (or superkingdoms) of life, i.e. Bacteria, Archaea and Eukarya. Bacteria and Archaea are characterized by their small cell size, typically <10 μm, and the lack of a structured nucleus which is why they are termed prokaryotes. On the other hand, eukaryotic microorganisms are relatively larger, with an average size range of 1 – 100 μm, and their genetic material is packed into a nucleus (or more than one in some cases). The main representatives of eukaryotic microorganisms are protists and fungi. In addition to their cell structure and evolutionary relationships, microorganisms are also categorized based on their function within the ecosystem they inhabit. Thus, microorganisms generally fall into one of two broadly defined groups, namely the autotrophs who carry out photosynthesis or chemosynthesis and are the primary producers of organic matter, and the heterotrophs who either feed directly on primary producers (also called grazers) or feed on grazers themselves. There is also another group of microorganisms called mixotrophs who combine both heterotrophic and autotrophic functions to different degrees. Microorganisms are an indispensable component of the ecosystem as they are involved in numerous processes that are necessary for ecosystem function such as oxygen production, organic matter remineralization, nutrient cycling and degradation of various pollutants (Kirchman, 2018).

Aquatic habitats consist of water bodies that contain particulate and dissolved organic and inorganic matter such as various salts and micronutrients. Typically, these habitats are divided based on their salinity (i.e. NaCl concentration measured as total dissolved solids – TDS) into freshwater environments such as lakes

and rivers (0.02 – 1 g $L^{-1}$), marine environments such as oceans (~ 35 g $L^{-1}$), and brackish environments such as estuaries (1 – 35 g $L^{-1}$) (Saccò et al., 2021). Each of these types of habitats is characterized by different physicochemical properties and nutrient concentrations. However, as a general rule, the water column in aquatic habitats has a lower concentration of organic matter compared to the sediment, and open ocean water columns are typically the poorest in terms of nutrients. These intrinsic differences have led to the prevalence of distinct microbial groups that have adapted to the respective environmental conditions. Furthermore, lacustrine and oceanic water columns tend to have similar prokaryotic abundances, mainly controlled by nutrient availability and viral lysis (Clasen et al., 2008).

These aquatic microorganisms are frequently studied to answer questions related to environmental quality, human health and ecological function. Commonly, researchers utilize data such as dissolved oxygen and nutrient concentrations to draw insights about microbial communities but microbial cell abundance and microbial community composition can also be studied to lead to more direct conclusions. The main method of estimating microbial cell abundance is the direct counting of cells using epifluorescence microscopy following appropriate DNA staining, or automatic cell counting using a flow cytometer. Both methods have replaced the older agar plate culture-based enumeration techniques, thus overcoming the issue arising from a high percentage of unculturable environmental microorganisms. Additionally, quantitative polymerase chain reaction (qPCR) can be used to detect and count specific microbial genes to measure microbial abundance. Meanwhile, to determine which phylogenetically related groups (or taxa) form a microbial community and their relative abundance, current methods rely on the extraction of the community's total DNA, the amplification through PCR of certain gene targets, the high throughput sequencing of these gene targets and then taxonomic classification based on existing sequence databases. This approach, known as amplicon sequencing, utilizes highly conserved genes such 16S rRNA for the classification of Bacteria and Archaea, and 18S rRNA for the classification of Eukaryotes. Other genes can be targeted as well to reveal specific metabolic functions, such as the amoA gene for identifying ammonia-oxidizing taxa. In

the case of the shotgun sequencing approach, the whole community's genome (metagenome) is sequenced, providing both taxonomic and functional information based on identified genes and gene pathways.

### 1.1.1. Prokaryotes

Bacteria are single-celled organisms without a structured nucleus or other organelles. In natural aquatic systems, bacteria are usually spherical shaped with a diameter of about 0.5 µm. Despite their small size, Bacteria can carry out important metabolic processes such as atmospheric nitrogen ($N_2$) fixation and can utilize a wide range of energy sources due to the specialization of different taxa. In terms of diversity, Bacteria contain more major phylogenies compared to Archaea and Eukarya (Hug et al., 2016). However, the fact that bacterial diversity is typically high in aquatic habitats (Kemp and Aller, 2004) is not reflected in an equal contribution of bacterial taxa to the total bacterial community composition. Instead, it is not uncommon for a small fraction of the taxa present to make up most of the community, while thousands of taxa make up the remaining community. This group of numerous, low-abundance bacterial taxa is referred to as the rare biosphere (Sogin et al., 2006). The coexistence of so many taxa that would seem counterintuitive in larger, multicellular organisms is likely the result of a combination of factors. One of these factors is the aforementioned metabolic diversity of bacterial taxa which allows them to utilize different energy sources and persist despite the heavy competition for available nutrients. Another major factor that shapes community composition is bacterial mortality caused by either predation (grazing) by heterotrophic protists or viral lysis. This phenomenon is collectively known as top-down control of the bacterial community. Thus, when the opportunity arises for a particular taxon to start dominating the community, the pressure caused by its natural predators will increase and limit its abundance until a new equilibrium is reached (kill the winner strategy, Maslov and Sneppen, 2017).

Pseudomonadota (or Proteobacteria) is one of the main bacterial phyla in aquatic ecosystems. Within this phylum, Betaproteobacteria is the dominant class in

freshwater communities, followed by Gammaproteobacteria and Alphaproteobacteria. Meanwhile, Alphaproteobacteria is the dominant class in marine communities where Betaproteobacteria abundance is limited. Besides Pseudomonadota, Bacteroidota (or Bacteroidetes) are commonly abundant in natural aquatic ecosystems, and Actinomycetota (or Actinobacteria) are mainly encountered in freshwater samples. Another commonly abundant phylum in environmental samples, Bacillota (or Firmicutes) are also well-known inhabitants of animal intestines (Liu et al., 2021). In lower taxonomic levels, marine water bacterial communities are dominated by the ubiquitous SAR11 clade (order *Candidatus* Pelagibaterales) whose members are so successful due to their small cell size and minimal DNA content that leads to limited nutrient requirements (Wang et al., 2023b). On the other hand, *Limnohabitans* and *Polynucleobacter* (Betaproteobacteria) are among the commonly abundant genera in freshwater communities (Nuy et al., 2020).

Archaea are also prokaryotic microorganisms and have similar size, shape and appearance to Bacteria, though they make up a different domain of life due to several major differences in cell structure and metabolic function. While it was previously thought that Archaea were only found in extreme environments (e.g., high temperature, high pressure, high acidity), it was later shown that they are present in nearly all natural environments, although typically in lower abundance compared to Bacteria. In fact, deep ocean water is one of the few habitats where the abundance of Archaea is increased, likely due to the oligotrophic conditions in such environments. Archaea are commonly represented by a limited number of taxa compared to Bacteria, usually within the phyla Nitrososphaerota (previously Thaumarchaeota) and the disputed Euryarchaeota or Methanobacteriota. Nitrososphaerota are able to grow autotrophically by oxidizing ammonia (Stahl and De La Torre, 2012). These ammonia oxidizing archaea (AOA) can become more abundant than ammonia oxidizing bacteria (AOB) in nutrient limited environments such as deep ocean water, where dissolved organic matter is scarce, likely outcompeting heterotrophic bacterial taxa (Offre et al., 2013). On the other hand, bacteria are more numerous than archaea in surface water

(Teira et al., 2006), where their metabolism allows them to grow more efficiently in the presence of available organic matter.

### 1.1.2. Heterotrophic nanoflagellates and ciliates

Protists is a paraphyletic group of unicellular eukaryotic microorganisms with a wide variety of ecological roles and enormous genetic diversity. Different protists exhibit different modes of feeding such as heterotrophy (i.e., feeding on other protists), bacterivory (i.e., feeding on prokaryotes), autotrophy through photosynthesis or mixotrophy. Due to their diversity and small size (1 – 200μm), protists have a widespread distribution in aquatic and terrestrial environments, where they play an important part in regulating prokaryotic abundance and contribute to the nutrient remineralization process.

The first major group of bacterivorous protists in aquatic ecosystems are heterotrophic nanoflagellates (HNF). These protists are small, with an average diameter of 1 – 5 μm, and they rely on one or more flagella for their motility. Their typical abundance in these systems is between $10^3$ – $10^4$ cells $ml^{-1}$. Due to their size, HNF are considered as the main bacterivores in aquatic environments, although there are also nanoflagellates capable of photosynthesis as well (Mostajir et al., 2015).

Ciliates are another important group of protists that feed on bacteria, as well as algae and HNF. Ciliate cells fall within a size range of 5 to over 200 μm (Mostajir et al., 2015), and are characterized by the presence of cilia, hair-like structures which they typically use to capture their prey and move around in the aquatic medium or slide along surfaces. Taxonomically, ciliates are classified into the Ciliophora phylum, within the Alveolata clade. The most common ciliates in freshwater are members of the Spirotrichea class, while in seawater Oligohymenophora are known to be among the dominant ciliate taxa. Ciliates are usually heterotrophic or mixotrophic, in the latter case combining grazing with limited photosynthesis to fulfil their energy requirements. For example, *Strombidium* is a ciliate genus that can retain the chloroplasts from its

algal prey and use them until they degenerate. These mixotrophic protists can make up a large fraction of their community and thus contribute to both grazing and primary production within aquatic ecosystems (Maselli et al., 2020; Mostajir et al., 2015). Due to their size, ciliates have traditionally been studied using microscopy to describe their morphology. However, morphological similarity between two ciliate taxa is not necessarily associated with phylogenetic similarity. Next generation sequencing techniques are also not completely reliable when investigating protist communities, mainly due to the high variability of the number of 18S rRNA gene copies in the genome of different taxa, combined with the lack of information regarding protistan 18S rRNA gene sequences (Martin et al., 2022).

### 1.1.3. Microbial food web interactions

In a typical aquatic ecosystem, cyanobacteria and algae are the primary producers of organic matter through photosynthesis. These primary producers can be grazed on by HNF which are in turn consumed by larger protists like ciliates. Ciliates may also directly feed on primary producers (Karayanni et al., 2005). As relatively larger microorganisms, ciliates are then consumed by metazoan plankton such as crustacean copepods, allowing organic matter and energy to flow into higher trophic levels occupied by macroorganisms such as fish (Caron and Goldman, 1988; Corliss, 2002; Mostajir et al., 2015). Furthermore, due to their active metabolism and fast growth rate, protists can significantly contribute to nutrient recycling within the food web even when representing a small fraction of its total biomass (Massana and Logares, 2013; Mostajir et al., 2015). Meanwhile, as cyanobacteria and algal cells naturally die, they sink deeper into the water column, while also releasing small amounts of dissolved organic matter (DOC). Heterotrophic prokaryotes are able to utilize this dead organic matter to facilitate their own growth, thus keeping a large amount of organic matter and energy (that would be otherwise removed) available within the ecosystem. These prokaryotes are grazed on by HFN and ciliates, and are also targeted by viruses which are abundant in the aquatic environment. Viral lysis of

prokaryotic cells, along with the organic debris left over during protistan grazing allow the recycling of organic matter and nutrients within this so called microbial loop, as new prokaryotic cells absorb and utilize the dissolved organic matter (Bonkowski, 2004). In short, the aquatic microbial community is the basis on which aquatic ecosystems rely on, and its components are critical for the production of organic matter and the recycling of nutrients required for sustaining both the microbial food web and higher trophic levels.

Prokaryotic abundance and community composition are shaped by bottom-up and top-down control. The term bottom-up refers to the availability of nutrients. For example, nitrogen concentration is usually the limiting factor in the marine environment while phosphorus is the limiting factor in freshwater lakes (Correll, 1999; Li et al., 2018). Meanwhile, top down control refers to the pressure grazers exert on their prey. In this case, HNF and ciliates are the main grazers of the prokaryotic community. Heterotrophic protists are typically either filter feeders such as members of the ciliate genera *Paramecium*, *Colpidium*, *Cyclidium*, *Euplotes*, and *Halteria*, or active predators that chase after their prey (Montagnes et al., 2008). The grazing process is usually shaped by the abundance ratio of prey and predator, their cell size ratio, and also by prey preference.

## 1.2. Recirculating aquaculture technologies

### 1.2.1. Recirculating aquaculture systems

Over the past decades, production of aquatic species from capture fisheries and aquaculture systems has been on the rise (Figure 1.1), due to an increasing demand for high quality food. In fact, recent data (FAO, 2024) has shown that aquaculture production has surpassed capture fisheries production for the first time in history (Figure 1.2). Thus, the aquaculture industry is becoming increasingly relevant, and its sustainability needs to be optimized.

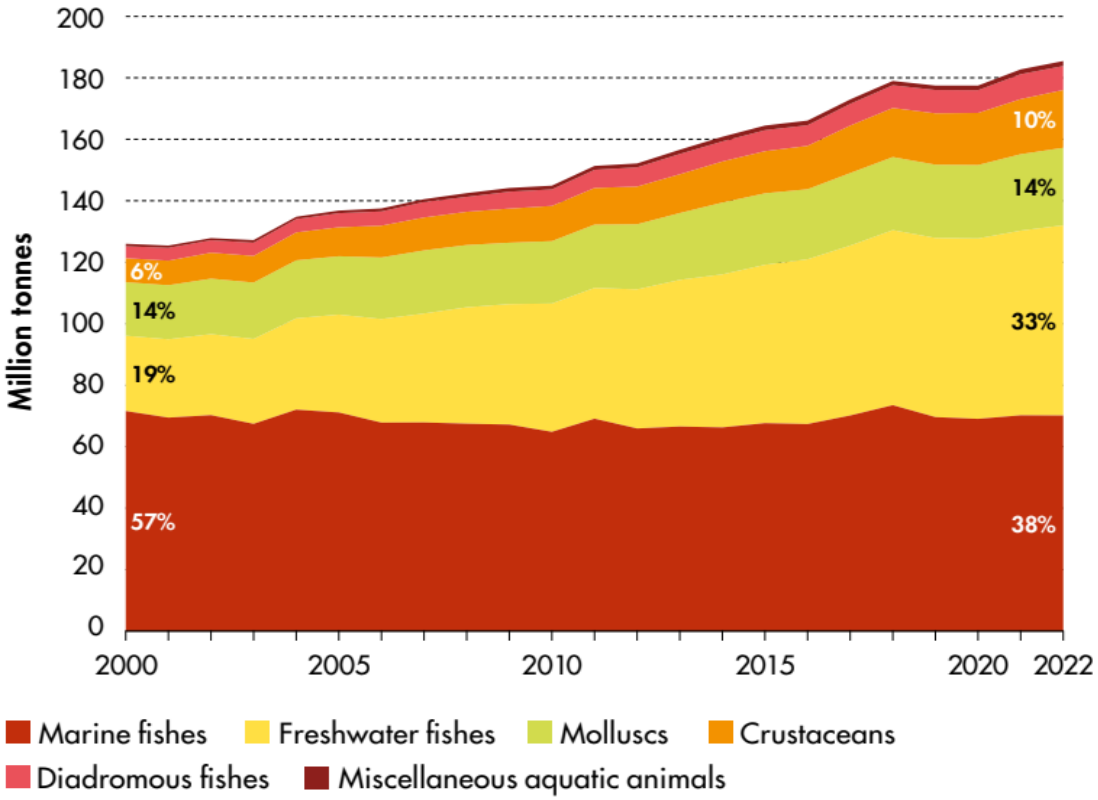



**Figure 1.1.** Worldwide production (in million tonnes) of aquatic species groups from capture fisheries and aquaculture systems. Adapted from FAO, 2024.

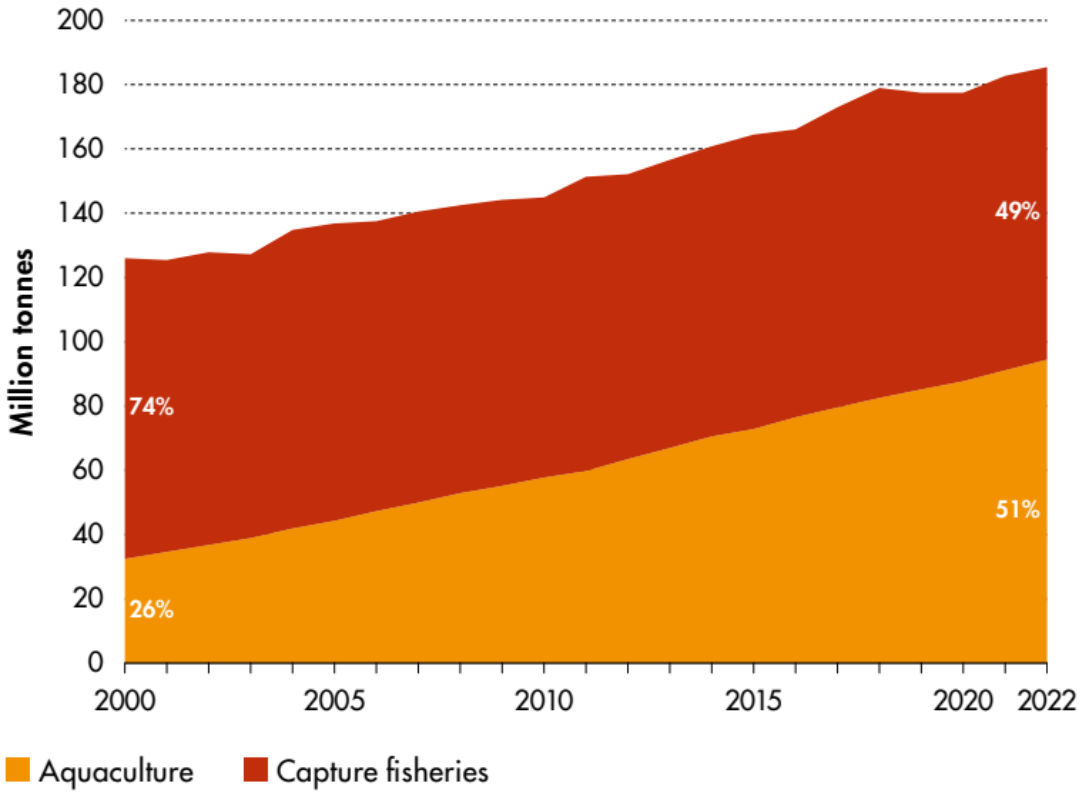



**Figure 1.2.** Worldwide production (in million tonnes) of aquatic organisms from capture fisheries (red) and aquaculture systems (orange). Adapted from FAO, 2024.

Recirculating aquaculture systems (RAS) are artificial systems for intensive farming of aquatic species, whose function relies on water reuse by utilizing physical, chemical and biological processes to maintain water quality. Typically, RAS designs aim to allow for the recirculation of at least 90% of system water per unit time (Bjørgen et al., 2025). However, as water circulates with minimal replenishment, issues arise from the accumulation of potentially toxic substances such as dissolved carbon dioxide ($CO_2$) released through respiration of farmed animals (Laine et al., 2024), ammonia ($NH_3$) produced by fish metabolic processes (Altinok and Grizzle, 2004), and mineral ions such as phosphorus, potassium, copper and zinc (Prabhu et al., 2017). That is why, apart from the rearing tanks (Figure 1.3), RAS designs usually include devices for the removal of solid particles (Couturier et al., 2009), nitrification biofilters for ammonia oxidation (Gutierrez-Wing and Malone, 2006), gas exchange devices for

water aeration (Moran, 2010; Summerfelt, 2003), and disinfection devices such as UV irradiators and ozonators (Sharrer and Summerfelt, 2007).

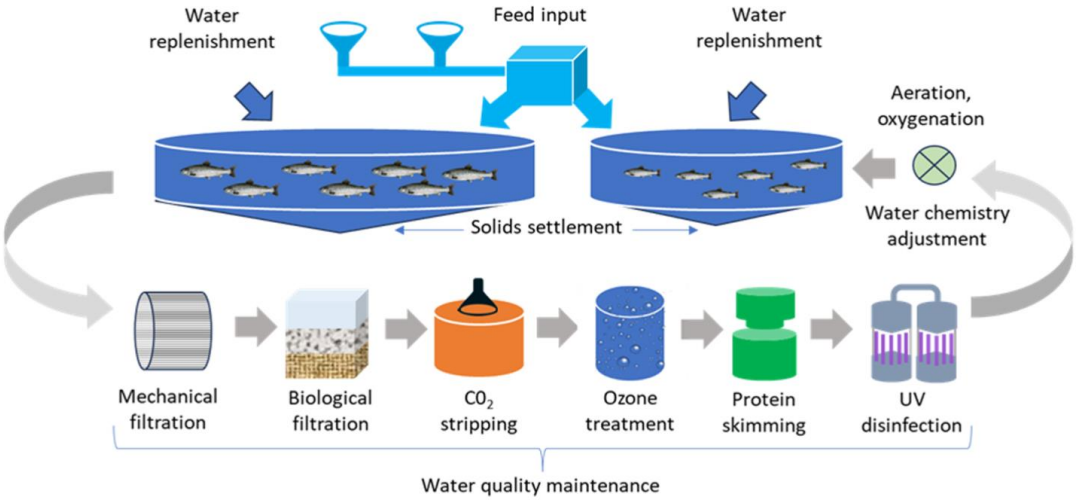



**Figure 1.3.** Schematic representation of a typical freshwater RAS. Adapted from Brown et al., 2025.

Dissolved oxygen (DO) is the most critical water quality parameter monitored in RAS, as a lower than adequate concentration is harmful to fish and decreases the efficiency of the nitrification biofilter, thus resulting in the accumulation of ammonia. Furthermore, dissolved and particulate organic matter that derives from the added fish feed, fish excretions and biofilms (Ebeling et al., 2009; Yildiz et al., 2017) is also monitored to prevent excessive growth of the heterotrophic bacterial community that would lower dissolved oxygen concentration, inhibit the nitrification process (Michaud et al., 2006), and increase water turbidity. Another important parameter monitored in RAS is ammonia concentration, in its non-ionized ($NH_3$) and ionized ($NH_4^+$) forms. To prevent its toxic accumulation, nitrifying biofilters are integrated into RAS, in the form of attached or suspended growth (biofloc) systems (Pfeiffer and Malone, 2006). The most common designs of attached biofilters in RAS include moving bed biofilters (Rusten et al., 2006), fluidized sand bioreactors (Davidson et al., 2008) and fixed bed biofilters (Emparanza, 2009; Zhu and Chen, 2002). These biofilters offer a substrate where the nitrifying prokaryotic community can grow and oxidize dissolved ammonia into less harmful nitrate ($NO_3^-$). This nitrifying community mainly consists of chemolithotrophic bacteria such as *Nitrosomonas*, *Nitrosococcus*, *Nitrospira*,

*Nitrobacter,* and *Nitrococcus* as well as archaea such as *Nitrosopumilus*, *Nitrososphaera*, '*Candidatus* Nitrosopelagicus' (Moschos et al., 2022; Rurangwa and Verdegem, 2015). As water is processed, two main types of discharge are produced. The first one contains sludge collected through sedimentation and particle filtration, and is rich in organic matter and phosphorus, while the other type of discharge is more dilute and contains the $NO_3^-$ which is produced in the nitrification biofilters (Laine et al., 2024). RAS discharge can be processed in wastewater treatment plants or be used in agriculture as fertilizer.

RAS have been operating worldwide for several decades, prominently used in hatcheries (Fudge et al., 2023), but presently they are also used for farming fish like Atlantic salmon (*Salmo salar*) up to marketable size in various counties such as Denmark, China and the USA (Brown et al., 2025). In fact, estimations show that as much as 70% of post-smolt Atlantic salmon in sea pens in Norway are grown in RAS (Meriac, 2019). Additional countries like Canada, France and South Korea have also shown interest in developing RAS for inland rearing of Atlantic salmon (Laine et al., 2024). Apart from Atlantic salmon, other marine and freshwater fish commonly reared in RAS are respectively seabass (*Dicentrarchus labrax*), sole (*Solea* spp.), turbot (*Scophthalmus maximus*), and rainbow trout (*Oncorhynchus mykiss*), eel (*Anguilla anguilla*) and Nile tilapia (*Oreochromis niloticus*) among others (Dalsgaard et al., 2013; Espinal and Matulić, 2019). As the global demand for animal protein production increases and fishing stocks are in danger of being depleted, aquaculture through RAS may prove to be a reliable alternative (Brown et al., 2025).

There are several arguments to be made in favor of developing RAS over relying on traditional aquaculture methods (e.g. raceway ponds or open sea pens). First off, water recirculation leads to minimal water input requirement, allowing the establishment of RAS even in areas with limited water availability, possibly closer to several markets. In this case, food transport costs as well as carbon emissions can be reduced while products can become available to consumers faster, remaining fresher (Brown et al., 2025). In addition, nutrient-rich aquaculture discharge is easier to manage in RAS, compared to other aquaculture systems which release organic matter

and nutrients from fish feeds and fish excretions directly into the water bodies they are in contact with, promoting eutrophication and oxygen depletion (Brown et al., 2025; Laine et al., 2024). Furthermore, fish are better protected from pathogens, parasites and pests in RAS due to the control of water intake, while control of the system's discharge prevents the infection of local fish populations with aquaculture-associated parasites, and also prevents the escape and proliferation of non-endemic fish species (Laine et al., 2024). The reduced requirement for antibiotics and pesticides (such as azamethiphos for sea lice) compared to traditional aquaculture, coupled with the degradation of antibiotic traces in RAS water through ozonation, also leads to decreased ecological disruption from RAS discharge (Brown et al., 2025).

Although RAS offer many advantages, several issues still remain to be solved before these systems can become unquestionably efficient. For example, water recirculation may indeed limit the need for replenishment but still as RAS scale increases to match the production needs, daily water consumption increases significantly. Thus, the location of inland RAS establishment needs to be carefully chosen to provide adequate clean water supply and wastewater treatment or utilization options (Brown et al., 2025). In addition, RAS may require three times as much electricity to power necessary equipment and maintain water temperature (especially for cold water species like Atlantic salmon) compared to traditional aquaculture systems, more than doubling their global warming potential (Laine et al., 2024; Philis et al., 2019). Another possibly harmful effect of water recirculation in RAS is the build-up of taste-tainting chemicals in the farmed fish. These chemicals include geosmin and 2-methyl-isoborneol, and are released into the water by certain groups of bacteria like Actinomycetota and Cyanobacteriota that grow within biofilms in RAS compartments (Schrader and Summerfelt, 2010). Tainting can be overcome with the timely removal of such biofilms, and by depurating the farmed fish before slaughter in a process that is costly and reduces the dietary value of the product to be sold (Brown et al., 2025). Furthermore, fish steroid hormones may also accumulate in RAS water, contributing to early maturation in salmon and negatively affecting fish health (Davidson et al., 2021). Finally, the relatively high establishment cost and extensive

planning required for RAS operation still stand in the way of this promising technology acquiring social license (Brown et al., 2025), while planning permission for commercial RAS is still unavailable in several countries due to outdated legislation (Urošević et al., 2021).

### 1.2.2. Aquaponics systems

Aquaponics is an emerging food production technology that aims to integrate tank-based animal aquaculture with soilless, hydroponic plant production. The basis of this integration is the sharing of water and dissolved nutrients between the aquaculture and hydroponic compartments, mediated by microbiological processes, to simultaneously produce marketable fish and plant products. While aquaponics technologies come in many variations (Baganz et al., 2022; Kushwaha et al., 2025), the term is commonly used to describe the combination of RAS with aquatic or hydroponic plant cultivation (Espinal and Matulić, 2019; Lennard and Goddek, 2019). Fully recirculating aquaponics systems (Figure 1.4 A), also known as coupled aquaponics systems, rely on fish feed as the main source of nutrients for the whole system. As fish consume and metabolize the feed, they produce particulate and dissolved waste (Yildiz et al., 2017), which in turn is used as the main nutrient source for the cultured plants. Of course, additional plant nutrients are supplemented directly into the system to cover the plant's needs and to control the aquatic medium's pH (Espinal and Matulić, 2019). On the other hand, in decoupled aquaponics systems (Figure 1.4 B), nutrient supplementation is more extensive and can be tailored to the specific requirements of the cultured plant as water does not circulate back to the RAS component after it has been through the hydroponic component (Espinal and Matulić, 2019).

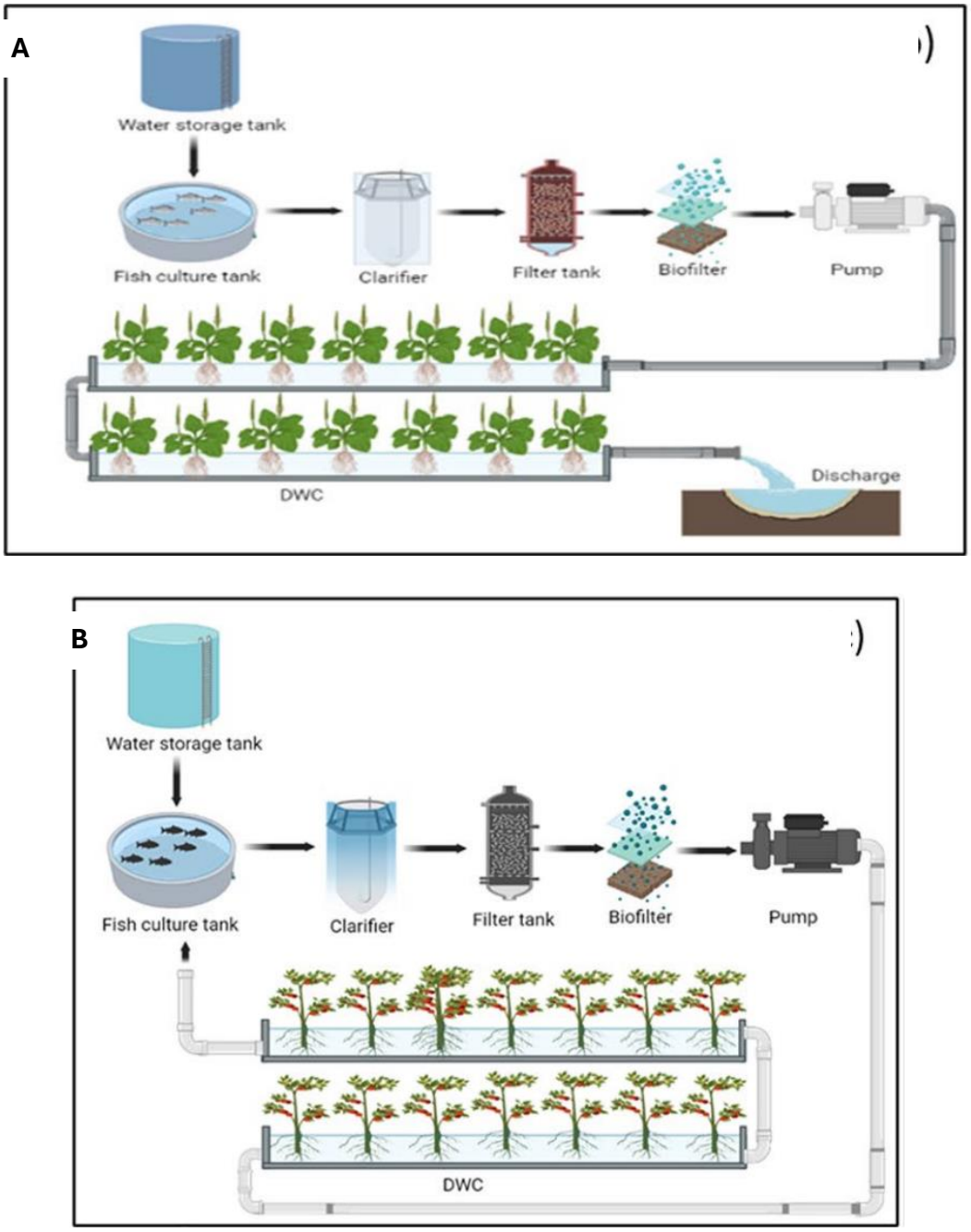



**Figure 1.4.** Schematic representation of the basic aquaponics system designs. **A**: compartments of a standard decoupled aquaponics system, **B**: compartments of a standard coupled aquaponics system. Adapted from Kushwaha et al. (2025).

Due to the separation of the RAS and hydroponic compartments, decoupled aquaponics systems are easier to set up and manage, although they are associated with reduced nutrient utilization and waste management efficiency (Kushwaha et al.,

2025). Furthermore, the designs of decoupled aquaponics systems allow the RAS compartment to operate at an appropriate pH level to accommodate fish health and achieve maximum nitrification rate of ammonia by the nitrifying prokaryotic community without having to compromise between the optimal pH level required for the plants' nutrient uptake efficiency (Aslanidou et al., 2023; Yildiz et al., 2017). While coupled aquaponics systems are more environmentally friendly, it has been shown that decoupled aquaponics systems can be more productive than coupled aquaponics while still having a lighter environmental impact compared to hydroponics, and upon further optimization of their design decoupled aquaponics show greater promise in becoming a staple for sustainable food production technology (Aslanidou et al., 2024; Aslanidou et al., 2023).

As in the case of RAS, microbial community function is essential for the successful operation of aquaponics systems (Baganz et al., 2022). Similar biological filter set-ups are employed to enhance ammonia and nitrite ($NO^{2-}$) oxidation, likely harboring similar nitrifying microorganisms, although the overall microbial community composition may differ between the RAS and hydroponic compartments (Kasozi et al., 2021a). In fact, the microbial community of an aquaponics system can be shaped by numerous factors such as the choice of fish species, fish stocking density, plant species, pH, water temperature and nutrient availability (Santoferrara et al., 2022). All these factors need to be taken into consideration to promote a balanced microbial community, thus achieving efficient nutrient cycling and maximum plant growth (Kushwaha et al., 2025).

A recent study (Raulier et al., 2023) noted 140 professional aquaponic systems in Europe, mainly in France, Belgium, Germany, the UK, but also in several other countries including the Netherlands and Sweden. Based on further investigation of a subset of 46 of these aquaponics entities, it was reported that 37% of the systems were decoupled, 30.4% of the systems were coupled, and the remaining 32.6% included both coupled and decoupled sub-systems. Interestingly, the authors noted an increase in the number of decoupled systems compared to an earlier study and suggested that this increase was due to better risk management and the prospect of

higher yields associated with decoupled systems. Regarding the farmed fish, trout, carp, and tilapia were the most common choices, while nearly half of the aquaponics systems were used for the farming of more than one fish species. On the other hand, herbs, tomatoes and lettuce were the most represented cultured plants in the studied systems. Annual production of these systems was variable, with 26% of the systems occupying a relatively small surface (each up to 1500 $m^2$) and producing between 1-49 kg of fish per year, and only one system producing 20,000 kg, while 15.2% of systems (each occupying a surface up to 500 $m^2$) produced 1-49 kg of vegetables, and 8.7% of systems exceeded 20,000 kg respectively.

Since aquaponics is a promising alternative to traditional aquaculture and plant cultivation practices, its potential benefits and challenges should be discussed. Firstly, both water and nutrient input requirements are minimized through water recirculation and fertigation, while less discharge is produced. In addition, aquaponics systems are artificial systems with limited input and thus farming and cultivation environmental parameters such as temperature, water quality, and flow rate are more easily controlled and maintained at optimal levels, ensuring the best conditions for growth. This way growth rates and yields are easy to estimate and achieve. Furthermore, aquaponics systems can be set up almost anywhere where there is access to clean water and electricity, as all farming and cultivation takes place indoors. After a substantial initial investment, aquaponics systems require little manual labor that mostly includes adding fish feed and keeping an eye on water quality parameters. Additionally, there is less chance of pest infestation and disease outbreaks, and thus less need for the use of pesticides and antibiotics (Okomoda et al., 2023). However, plants grown indoors can still be affected by several diseases and pests that usually cause problems in traditional field crops, and solutions like developing more resistant plant varieties or relying on the biological control of pathogens may prove critical. Also, within the RAS compartment of an aquaponics system, in the unlikely case of a disease outbreak pathogens can spread rapidly due to water recirculation, while pathogens may persist in the system after the diseased fish have been removed, and even infect the next batches of fish introduced to the system.

Another major hurdle yet to be overcome is the establishment capital for new aquaponics systems, as the operation equipment (pumps, aerators, filters) can be costly and power consuming, while there has been no observed relation between the amount of funds invested for aquaponics systems and their profitability. This initial spending can be reduced by using locally available materials for construction and possibly by creatively improvising the new systems' design. Of course, as fish and plants are vastly different organisms, their nutrient requirements also differ, rendering the use of certain nutrient supplements necessary. To be more in line with the environmentally friendly concept of aquaponics, system designs should be optimized to ensure that both fish and plants grow under the best possible conditions without the need for additional nutrient inputs, and to determine the most efficient fish to plant ratio for optimal yield (Kushwaha et al., 2025; Okomoda et al., 2023).

### 1.2.3. Nitrification in aquaponics and recirculating aquaculture systems

Nitrogen is a necessary nutrient for every life form since it is found in proteins, nucleic acids, adenosine phosphates like ATP, pyridine nucleotides such as NAD+, and pigments (Hagopian and Riley, 1998). Typically, fish species are able to utilize about 20-30% of the nitrogen they acquire through feeding. The rest of the nitrogen enters the fish's circulatory system after protein digestion and is excreted through the gills (branchial diffusion) in the form of ammonium ($NH_4^+$). Heterotrophic bacteria can also produce $NH_4^+$ as they break down protein-rich organic matter such as uneaten fish feed (Roosta and Hamidpour, 2011). Within a recirculating system, this constant production of $NH_4^+$ would lead to its accumulation up to a concentration that is toxic for fish, as in the case of salmonid species where 0.2 mg $L^{-1}$ $NH_4^+$ is enough to cause acute toxicity (Hagopian and Riley, 1998). Instead, biological nitrification is utilized to convert $NH_4^+$ to nitrate ($NO_3^-$), which is less harmful to fish. Nitrate-rich water can then be discharged from standalone RAS and be used as fertilizer solution in agriculture, or it can be directly used as a nutrient source for plants in aquaponics systems, as $NO_3^-$ is readily absorbed by plant roots. Thus, biological nitrification is essential for the proper

function of aquaponics systems, being the main biological process that maintains water quality for fish health, and allows fish-feed derived nitrogen to become available to plants (Kasozi et al., 2021a).

Biological nitrification is carried out by several well studied prokaryotic genera (Table 1.1) that are purposely added in RAS and aquaponics systems and are allowed to grow on surfaces in special compartments known as nitrification biofilters (Espinal and Matulić, 2019). Some common biofilter set-ups include moving bed biofilm reactors, trickling filters, fluidized sand biofilters and submerged biofilters (Moschos et al., 2022). These biofilters can be seeded with commercial nitrifier inocula that contain prokaryotic communities with a predetermined ratio of known nitrifiers, or receive transplanted nitrifier biofilms from existing, mature RAS or aquaponics systems. Bacterial nitrifiers can be grouped into categories based on the substrate they oxidize. Ammonia oxidizing bacteria (AOB) convert $NH_4^+$ and $NH_3$ to nitrite ($NO_2^-$) in a process called nitritation. The most common AOB in the environment and within recirculating aquaculture biofilters belong to the genera *Nitrosomonas*, *Nitrosovibrio* and *Nitrosospira* (family Nitrosomonadaceae, class Betaproteobacteria) as well as genus *Nitrosococcus* (family Chromatiaceae, class Gammaproteobacteria). In the next step of the nitrification process, $NO_2^-$ is converted to $NO_3^-$ by nitrite oxidizing bacteria (NOB) through nitratation. NOB include the genera *Nitrobacter* (class Alphaproteobacteria), *Nitrococcus* (class Gammaproteobacteria), '*Candidatus* Nitrotoga' (class Betaproteobacteria), *Nitrospina* (class Nitrospinia) and *Nitrospira* (class Nitrospiria) in RAS and aquaponics biofilters (Kasozi et al., 2021a; Moschos et al., 2022). In addition, *Nitrospira* species typically identified in RAS can also perform both steps of the nitrification process and are thus able to carry out complete ammonia oxidation (comammox), oxidizing $NH4^+$ and $NH_3$ to $NO_3^-$. In fact, the comammox reaction is regarded as more efficient in nutrient limited aquatic systems (Bartelme et al., 2017). Apart from Bacteria, there are also Archaea with the ability to oxidize $NH_4^+$. Specifically, ammonia oxidizing archaea (AOA) such as genera *Nitrososphaera*, *Nitrosopumilus* and '*Candidatus* Nitrosopelagicus' within the phylum Nitrososphaerota have been identified in RAS biofilters (Bartelme et al., 2019; Xu et al.,

2020). There is evidence hinting that archaeal nitrifiers may be able to outcompete their bacterial counterparts in environments with low $NH_4^+$ concentration, due to higher affinity to this substrate that offers them an advantage (Hatzenpichler, 2012). On the other hand, Bacteria are expected to be the more efficient and abundant nitrifiers in nutrient rich environments like aquaculture water. Thus, the contribution of the archaeal biofilter community to nitrification in RAS has been questioned (Hüpeden et al., 2020) and further investigation is required to clarify the significance of these obscure nitrifiers in the context of recirculating aquaculture.

**Table 1.1.** Nitrifying prokaryotic genera identified in RAS and their function. Adapted from Moschos et al. (2022).

| Genus | Function | Superkingdom |
|---|---|---|
| '*Candidatus* Nitrosopelagicus' | Ammonium oxidation | Archaea |
| *Nitrosococcus* | Ammonium oxidation | Bacteria |
| *Nitrosomonas* | Ammonium oxidation | Bacteria |
| *Nitrosopumilus* | Ammonium oxidation | Archaea |
| *Nitrososphaera* | Ammonium oxidation | Archaea |
| *Nitrosospira* | Ammonium oxidation | Bacteria |
| *Nitrosovibrio* | Ammonium oxidation | Bacteria |
| '*Candidatus* Nitrotoga' | Nitrite oxidation | Bacteria |
| *Nitrobacter* | Nitrite oxidation | Bacteria |
| *Nitrococcus* | Nitrite oxidation | Bacteria |
| *Nitrospina* | Nitrite oxidation | Bacteria |
| *Nitrospira* | Nitrite oxidation, comammox | Bacteria |

In terms of metabolism, prokaryotic nitrifiers are chemoautotrophs, able to use inorganic $NH_4^+$ or $NH_3$ as an electron source to incorporate inorganic carbon into biomass. These chemoautotrophs are also aerobic, requiring oxygen ($O_2$) as the final

electron acceptor (Hagopian and Riley, 1998). Furthermore, the nitrifying community has specific pH requirements with an optimum recommended value of 7.8, while growth and activity of these prokaryotes decrease significantly in acidic conditions. The nitrifying community is also mesophilic, with an optimum water temperature of 25°C (Hagopian and Riley, 1998). Even when achieving the optimal conditions for the proliferation of the nitrifier community (given the compromise due to the individual requirements of fish and plant species in aquaponics systems), the maximum specific growth rate of nitrifying bacteria is still relatively slow compared to aerobic heterotrophic bacteria that also dwell in the system and break down organic matter. This slower growth can be attributed to the lower efficiency of the energy producing pathways of nitrifiers. In addition, nitrifiers typically have fewer copies of the ribosomal RNA (rrn) operon in their genome compared to heterotrophic bacteria which can also explain their slower growth rates due to a slower rate of protein synthesis (Moschos et al., 2022; Roller et al., 2016). Within the nitrifying community itself, AOB can grow at a faster rate compared to NOB at optimum temperature (Kushwaha et al., 2025). More specifically, NOB and AOB have shown a doubling time of 60 h and 26 h respectively, and even the fastest doubling time calculated in optimal growth conditions is still an order of magnitude slower than the typical doubling time of aerobic heterotrophic prokaryotes (Hagopian and Riley, 1998). Due to this significant difference in growth rate, heterotrophic prokaryotes usually are more abundant compared to nitrifiers. That is why it is critical that organic matter and DO concentration is controlled in RAS and aquaponics systems, to prevent the complete dominance of heterotrophic taxa over the nitrifying community (Kasozi et al., 2021a).

Additional forms of nitrifiers can also be found in freshwater aquaculture biofilters. For instance, heterotrophic ammonia-oxidising bacteria (HAOB) can also carry out nitrification by oxidizing $NH_4^+$ to hydroxylamine which is in turn oxidized to $NO_2^-$. In addition, certain prokaryotes can perform anaerobic ammonium oxidation (anammox), oxidizing $NH_4^+$ by using $NO_2^-$ as an electron acceptor and releasing $N_2$ (Kasozi et al., 2021a). Of course, since recirculating aquaculture water is kept well oxygenated to accommodate fish health and allow AOB and NOB to carry out

nitrification, these anammox nitrifiers can only be found in anaerobic micro-niches within biofilters. Another way to remove nitrogen from RAS and aquaponics is through the biological process of denitrification. Denitrification is carried out by heterotrophic, facultative anaerobic bacteria, which utilize a carbon source to turn excess $NO_3^-$ to $N_2$ (Joyce et al., 2019). Dissolved organic carbon is used as an electron donor and $NO_3^-$ as an electron acceptor. Denitrification takes place in the presence of an adequate carbon source and under anaerobic conditions, hence in the context of recirculating aquaculture special denitrification bioreactors can be set up to offer such an environment. To provide an adequate carbon source, ethanol, methanol, glucose or molasses is commonly added to these denitrification bioreactors (Espinal and Matulić, 2019). The most common denitrifying genera include *Bacillus*, *Flavobacterium*, *Acinetobacter*, *Pseudomonas*, *Micrococcus*, *Proteus*, *Aerobacter* and *Achromobacter* (Kasozi et al., 2021a). These denitrifying taxa can inhabit anoxic microenvironments within aquaponics systems such as mineralized zones of gravel media beds. While denitrification rates may depend on the available carbon source type, it can be responsible for the removal of more than half of the total nitrogen available in an aquaponics system (Kasozi, et al., 2021a).

Given that the function of the nitrifying community is essential for RAS and aquaponics systems' operation, its presence on the nitrification biofilter must be guaranteed. Depending on biofilter design, biofilm carriers or biofilm sections from mature pre-existing biofilters can be transferred to newly set-up biofilters. Otherwise, nitrifying communities can be transferred into biofilters through the use of carefully designed, commercially available nitrifying inocula (Moschos et al., 2022). Several bacterial phyla like Proteobacteria, Nitrospirae, Bacteroidetes Planctomycetes and Chlorobi have been identified as dominant in commercial inocula, although their proportions may be altered in the biofilter communities that form over time (Brailo et al., 2019). Comparison of mature biofilm transfer and nitrifying inocula, has shown that the former method is more efficient in certain systems. While inocula may contain higher proportions of nitrifying Archaea (Nitrososphaerota), mature biofilm carriers harbor nitrifying communities which are dominated by the bacterial genera *Nitrospira*,

*Nitrosomonas*, *Nitrosococcus* and '*Candidatus* Nitrotoga' (Navada et al., 2021; Roalkvam et al., 2020).

### 1.2.4. Microbial diversity in aquaponics and recirculating aquaculture systems

Most microorganisms in aquaponics and RAS are heterotrophic (Kasozi et al., 2021a; Moschos et al., 2022; Rurangwa and Verdegem, 2015) and they can affect the performance of the nitrifying community, as well as fish and plant health and product value. Fermentative and hydrolyzing bacteria can break down organic macromolecules such as carbohydrates, lipids and peptides. By breaking down these complex compounds typically found in fish feces and uneaten feed, these prokaryotes remineralize dead organic matter and allow the recirculation of micronutrients which become available to other heterotrophic prokaryotes, autotrophic nitrifiers and plants. However, as mentioned in the previous section, the mostly aerobic heterotrophic community competes for DO with the also aerobic nitrifying community (Rurangwa and Verdegem, 2015). Thus, excessive growth of heterotrophic prokaryotes within RAS may inhibit proper nitrification processes in biofilters and the monitoring of parameters including DO concentration and total organic carbon to $NH_4^+$ ratio is crucial for RAS function (Kasozi et al., 2021a).

As expected, heterotrophic prokaryotes tend to congregate in places where organic matter such as solid waste accumulates within RAS and aquaponics systems. In fact, although water circulates through the various compartments, each compartment constitutes a specific habitat for the development of distinct microbial communities, both in terms of diversity and abundance (Kasozi et al., 2021a; Moschos et al., 2022; Rurangwa and Verdegem, 2015). RAS biofilters have been shown to carry the highest prokaryotic abundance (about $10^{10}$ cells $g^{-1}$ of filter material) compared to other compartments (Rurangwa and Verdegem, 2015), and these communities are dominated by the classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Flavobacteriia and by the phylum Nitrospirota (Moschos et al., 2022). Though less studied, aquaponics biofilters have also been found to be

dominated by Proteobacteria and harbor a higher relative abundance of Nitrospirae compared to the rest of the aquaponics compartments (Schmautz et al., 2017). Specifically, the oligotrophic class Alphaproteobacteria has been reported to amount to more than 35% of biofilter bacterial population in a recent aquaponics study (Schmautz et al., 2022), while at the family level Planctomycetaceae, Hyphomicrobiaceae, Cytophagaceae, Chitinophagaceae and Sphingomonadaceae were the most represented groups. The same study showed that the biofilter archaeal community was mostly represented by only a few families within the phylum Methanobacteriota, namely Methanobacteriaceae, Methanosarcinaceae, Methanoregulaceae, and another taxon from the order Thermoplasmatales.

Similar bacterial taxa have been characterized as dominant in water samples of numerous RAS studies. Apart from the metabolically diverse classes of organic matter remineralizers such as Alphaproteobacteria, Gammaproteobacteria and Flavobacteriia, the phylum Actinomycetota has also been found to be a significant component of prokaryotic communities in RAS water. Certain members of Actinomycetota produce beneficial bioactive molecules and excrete enzymes such as peptides and polyketides with antimicrobial and antiviral properties. Still, Actinobacteria have been associated with geosmin production, a substance whose build up in fish tissue is known to cause an undesirable earthy odor and taste, adversely affecting their market value and overall quality. Down to the genus level, studies have reported that water samples from freshwater RAS are usually dominated by chemoorganoheterotrophic taxa including *Comamonas*, *Novosphingobium*, *Zoogloea*, *Hydrogenophaga*, *Sphaerotilus*, *Pseudomonas* (Proteobacteria) and *Sphingobacterium* (Bacteroidota). Meanwhile, in the case of marine or brackish RAS, *Donghicola*, *Gemmobacter*, *Leucothrix*, *Sphaerotilus*, *Thalassomonas* (Proteobacteria), *Kordia*, *Polaribacter*, *Sediminocola* (Bacteroidota) and *Rubritalea* (Verrucomicrobiota) have been reported among the dominant bacterial genera (Moschos et al., 2022). *Phaeobacter* is another prominent bacterial genus in RAS water, characterized by antagonistic properties against common aquaculture bacterial pathogens such as members of genus *Vibrio*. Interestingly, the composition of the

suspended prokaryotic community generally differs from the composition of the community that develops attached to solid surfaces (e.g. tank walls, sediment particles) within RAS. Chemorganoheterotrophic genera such as *Aestuariibacter*, *Paracoccus*, *Phaeobacter*, *Roseovarius*, *Ruegeria* (Proteobacteria), *Muricauda*, *Psychroserpens* (Bacteroidota), *Blastocatella* (Acidobacteria) *Persicirhabdus* (Verrucomicrobia), *Planctomyces* (Planctomycetes) and *Mycobacterium* (Actinomycetota) were identified as dominant in RAS biofilms (Moschos et al., 2022). Meanwhile, fish tank biofilms from aquaponics systems harbor prokaryotic communities dominated by families such as Rhodobacteraceae, Alcaligenaceae Planctomycetaceae, Gammaprotebacteria genera such as *Dokdonella* and *Thermomonas*, and usually identified freshwater taxa like *Limnohabitans*, *Pirellula* and *Lysobacter* (Schmautz et al., 2017, 2022).

Apart from the microbial communities that grow on inorganic surfaces or are suspended in the system water, microorganisms also inhabit fish associated surfaces including gills, skin and the intestinal tract. It is known that fish microbiome plays a crucial role in nutrition, immune response, and the development and modulation of gut epithelium cells (Yu et al., 2021). As the least studied communities, gill-associated samples from freshwater RAS fish have been shown by (Minich et al., 2020) to be dominated by the genus *Limnohabitans*, while skin related communities are enriched in other genera such as *Acinetobacter*, *Rhodobacter* and Pseudomonas (Proteobacteria). Furthermore, analyses of intestinal tissue samples have revealed the dominance of anaerobic chemoorganotrophic genera including *Aeromonas*, *Ralstonia*, *Salmonella*, *Shewanella*, *Vibrio* (Proteobacteria) *Bacillus*, *Lactobacillus*, *Lactococcus*, *Megamonas*, *Acinetobacter*, *Leuconostoc* (Bacillota), *Bacteroides* (Bacteroidota) and *Methanosphaera* (Methanobacteriota) in the intestinal microbiome of examined fish (Minich et al., 2020; Moschos et al., 2022). Most of these genera have been widely studied due to their interactions with fish. For instance, different species of *Aeromonas*, *Bacillus*, *Pseudomonas*, *Shewanella* and *Vibrio* can act as either pathogens or putative probiotics (Moschos et al., 2022), while genera *Lactobacillus*, *Lactococcus* and *Leuconostoc* are members of the group of lactic acid bacteria,

known for their beneficial role against fish pathogens through the secretion of bacteriocins (Ringø and Gatesoupe, 1998). In addition, communities that grow on fish feces particles were shown to be dominated by members of the phylum Fusobacteriota, mainly represented by the genus *Cetobacterium*, as well as phyla Bacteroidota, and Bacillota (Schmautz et al., 2022).

In the case of aquaponics systems, microbial communities also develop on surfaces associated with the cultivated plants, such as the root system or the hydroponics substrate. These prokaryotes make up the rhizosphere microbiome, whose function increases the bioavailability of nutrients derived by uneaten feed and fish feces to plants (Goddek et al., 2016). Furthermore, several bacterial taxa within phyla such as Proteobacteria, Actinomycetota, and Bacillota can enhance plant growth, while species like *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens* have the genetic potential for synthesizing antibiotics and siderophores, as well as neutralizing various toxins (Kasozi et al., 2021a). The few published aquaponics rhizosphere studies that have been published examined the root microbiome in systems for the cultivation of lettuce (*Lactuca sativa*) and the rearing of Nile tilapia (*O. niloticus*). Specifically, root-associated prokaryotic communities were reportedly dominated by the families Herpetosiphonaceae (phylum Cloroflexota), Planctomycetaceae (phylum Planctomycetota) and Comamonadaceae (class Betaproteobacteria). At the genus level, the chemoheterotrophic *Herpetosiphon* was clearly the most abundant, while other rhizosphere genera such as *Pseudomonas*, *Acidovorax*, *Sphingobium* and *Flavobacterium* were also more abundant in root samples compared to the rest of the compartments, indicating a likely selection of certain genera by the plant root habitat (Schmautz et al., 2017, 2022).

Apart from Bacteria and Archaea, the microbial communities of aquaponics systems and RAS also consist of microeukaryotic organisms such as ciliates (phylum Ciliophora), fungi (kingdom Fungi), oomycetes (phylum Oomycota), algae (e.g. phyla Chlorophyta and Bacillariophyta) and the paraphyletic group of nanoflagellates. Although these eukaryotic microorganisms can form complex relationships with the prokaryotic communities (predation – top down control, competition for nutrients) as

well as fish (pathogens or beneficial symbionts) and plants (pathogens, enhancers of nutrient absorption or promoters of plant growth), very few studies have focused on their identification and possible function within these types of aquaculture systems. For instance, the investigation of microeukaryotic communities in RAS for the farming of sole (*S. senegalensis*) and turbot (*S. maximus*) by Boaventura et al. (2018) showed the dominance of the Stramenopiles clade, followed by Cilliophora (dominated by the genus *Zoothamnium*), Choanoflagellata (dominated by the species *Choanoeca perplexa*), Apicomplexa, Cercozoa, Lobosa and Fungi. Another study by Khalil et al. (2021) revealed the prominence of certain fungal genera in a RAS for the rearing of Nile tilapia. These genera included *Yarrowia*, *Acremonium*, *Harposporium*, *Aureobasidium* and *Phaeoacremonium* in the rearing tanks and *Candida*, *Trichosporon*, *Bettsia*, *Sporobolomyces*, *Penicillium*, *Entomocariticium*, *Cryptococcus*, *Preussia*, *Macrophomina*, *Sterigmatomyces*, *Microascuss* and *Enyodonium* in the RAS wastewater. The same authors noted the presence of beneficial fungal taxa such as *Acremonium* and *Penicillium* which are considered to act as promoters of plant growth or plant pathogen antagonists. However, as putative pathogenic genera including *Fusarium*, *Candida* and *Cryptococcus* were also detected in the same systems, further investigation is clearly required to more accurately describe the actual role of the fungal community within RAS and aquaponics.

## 1.3. Aim of the study

The main purpose of the present study is to start filling in the knowledge gap regarding the role of protists in recirculating aquaculture systems (RAS) and aquaponics systems, and to highlight prevalent prokaryotic taxa and their function in the context of these aquaculture systems. To achieve this, several specific goals were set:

- The investigation of changes in diversity, abundance and potential growth rates of heterotrophic protists in different compartments of experimental aquaponics systems and RAS (Chapter 3)
- The investigation of temporal changes in prokaryotic community composition in different compartments of aquaponics systems and RAS (Chapter 4)
- The description of the main taxa associated with nitrification and nutrient cycling in different compartments of aquaponics systems and RAS (Chapter 5)

# 2. Materials and Methods

## 2.1. Study systems

The present study was based on data from samples collected at an experimental aquaponics system and an experimental RAS (images are available in Appendices 1 and 2 respectively). Both systems were operated by the School of Agricultural Sciences, University of Thessaly, Greece.

The aquaponics system (Figure 2.1) was located in Velestino, Magnesia regional unit, (latitude 39°440' N , longitude 22°790' E, elevation 85 m) housed within the climate-controlled greenhouse of the Laboratory of Agricultural Constructions and Environmental Control of the University of Thessaly. Its RAS subsystem comprised three fish rearing tanks of 1.3 $m^3$ each, a 0.7 $m^3$ buffer tank, a mechanical drum filter (Combi Bio 15, ProfiDrum, Retford, UK) of 0.5 $m^3$, a 0.7 $m^3$ biological nitrification filter containing 15 mm ceramic rings and 1 mm K1 Kaldness media, as well as a clear buffer water tank of 2.3 $m^3$. RAS water continuously flowed downstream to the clear buffer tank by gravity and was pumped back to the fish tanks. Thus, the approximately 6.6 – 7.1 $m^3$ of water in the system was recirculating constantly with a steady flow rate of 6 $m^3$ $h^{-1}$. RAS water was replenished daily at about 3.3% to 16.9% using tap water as occasionally RAS water from the clear buffer tank was pumped to the central hydroponic mixing tank for the preparation of the nutrient solution used for plant fertigation. Fish tanks were stocked with red tilapia (*Oreochromis* spp.) at an average of 8.09 kg $m^{-3}$, and fish were hand-fed three times a day to satiation with Prodac Pondsticks Color feed. According to the manufacturer, the feed consisted of cereals, soya, fish and fish by-products, crustaceans, 0.20% spirulina algae, vegetables and by-products of vegetable origin. Water temperature was maintained at 26 °C.

Meanwhile, the hydroponics subsystem covered an area of 352 $m^2$ and consisted of the central mixing tank, six stock solution tanks, 6 fertigation solution storage tanks, 18 crop channels and 6 drainage tanks (Figure 2.1). The nutrient solution was prepared in the central mixing tank by receiving the required amount of nutrients

stored in the stock solution tanks and by being injected with acid to achieve the required pH. Once prepared, the nutrient solution was stored in the 500 L fertigation storage tanks to be used for fertigation according to the specific crop needs via drippers at a flow rate of 2.3 L $h^{-1}$. Following fertigation, the drainage solution flowed through gravity to the corresponding 120 L drainage tank. The 18 channels were divided equally into three blocks, with every single channel per block connected to a different fertigation solution storage tank and drainage tank. The hydroponics system operated from May 2020 to June 2022 completing several sequential production cycles, cultivating basil (*Ocimum basilicum* cv. Genovese), cucumber (*Cucumis sativus* cv. Aisopos), parsley (*Petroselinum crispum*) and tomato (*Solanum lycopersicum* cv. Kabrera) alongside the farming of red tilapia in the RAS subsystem. Over the course of these production cycles, plants were fertigated with three distinct treatments, namely the hydroponic, the decoupled and the coupled treatment. Drainage from the coupled treatment was collected, sterilized and pumped back to the RAS subsystem, while the hydroponic and decoupled treatment drainage was removed from the aquaponics system.

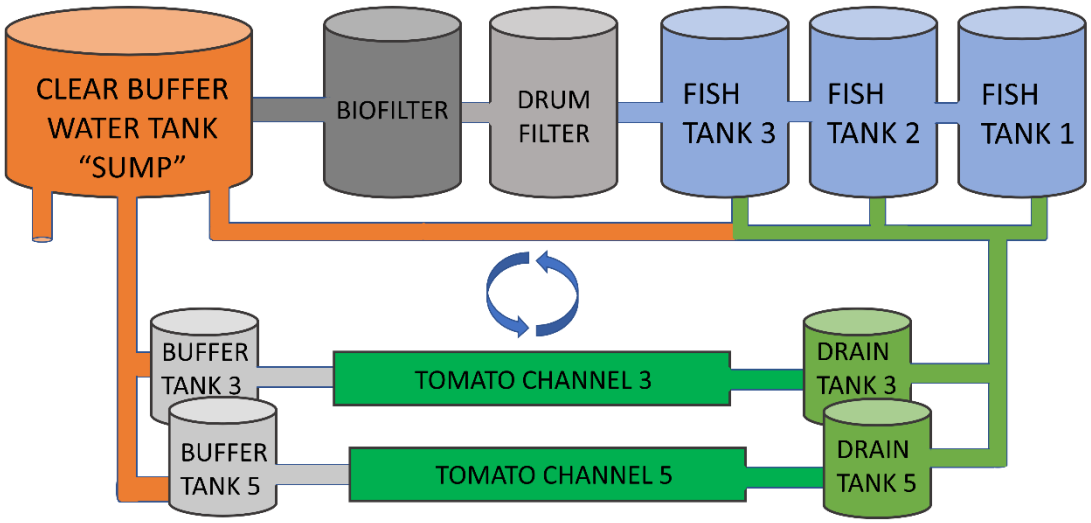



**Figure 2.1.** Aquaponics system scheme. Simplified representation of the compartments of the aquaponics system investigated in the present study. System water flowed in a counterclockwise direction, based on the scheme's depiction. Water circulation time was ~ 1 h. Adapted from Moschos et al. (2022).

The experimental RAS system was located indoors at the campus of the Department of Ichthyology and Aquatic Environment, University of Thessaly, in Volos (39° 23' N, 22° 56' E). The system's set up consisted of three independent parallel lines, each equipped with four fish rearing tanks upstream of a biofilter tank. Fish tanks were stocked with sea bass (*D. labrax*) individuals weighing about 3 – 4 g at the time of sampling. The system operated with artificial seawater (30 g $L^{-1}$ salinity), maintained at a temperature of 22 °C. The biofilter tank was packed with plastic biofilm carrier pieces, and the microbial community attached to them was considered mature since the system had been previously used for the rearing of sea bass. Conventional fish feed was added to the fish tanks by hand.

## 2.2. Samplings for molecular analysis

To investigate the diversity of the suspended microbial community in the aquaponics system described above, water samples were collected from fish tanks and drain tanks of the coupled aquaponics treatment. Samplings were performed at different time points in order to compare temporal shifts in community composition when different combinations of plants and fish were grown (Table 2.1). For each sample, equal volumes of water from replicate tanks were mixed to a total volume of 2 L, collected in clean pre-sterilized plastic containers that had been previously thoroughly rinsed with system water. Water samples were kept cool and in the dark for 3-4 h until they were processed in the laboratory. Adequate volumes (100-500 ml) from each sample were filtered through 0.2 μm Isopore Membrane Filters (diameter 47 mm) using a vacuum pump at low pressure < 5 mmHg, until the filters were clogged. Filters were then carefully placed into sterile 5 ml cryotubes and stored at -20 °C until DNA extraction was performed. Likewise, 3L of water were collected from fish tanks of two of the independent seawater RAS lines and then 1L from each sample was filtered through 0.2 μm filters. Additionally, swab samples from the fish tank biofilm and

biofilm carriers from the biofilter tanks were collected, placed in cryotubes and stored at -20 °C. All DNA extractions were performed on the same day (7/11/2023).

**Table 2.1.** List of sample codes and key information regarding each sample.

| Sample code | System type | Reared fish | Cultivated Plants | Compartment | Date | Day of operation | Metagenomics data | Material type | Microscopy data | Growth experiment | Replicates |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CU_FT_1 | Aquaponics | Tilapia | Cucumber | Fish tank | 25/7/2020 | - | Yes | Water | No | No | - |
| CU_DR_1 | Aquaponics | Tilapia | Cucumber | Drain tank | 25/7/2020 | - | Yes | Water | No | No | - |
| CU_FT_2 | Aquaponics | Tilapia | Cucumber | Fish tank | 14/11/2020 | - | Yes | Water | No | No | - |
| CU_DR_2 | Aquaponics | Tilapia | Cucumber | Drain tank | 14/11/2020 | - | Yes | Water | No | No | - |
| PA_FT | Aquaponics | Tilapia | Parsley | Fish tank | 25/1/2021 | - | Yes | Water | No | No | - |
| PA_DR | Aquaponics | Tilapia | Parsley | Drain tank | 25/1/2021 | - | Yes | Water | No | No | - |
| TO_FT_1 | Aquaponics | Tilapia | Tomato | Fish tank | 17/2/2022 | Day 17 | Yes | Water | No | No | - |
| TO_DR_1 | Aquaponics | Tilapia | Tomato | Drain tank | 17/2/2022 | Day 17 | Yes | Water | No | No | - |
| TO_FT_2 | Aquaponics | Tilapia | Tomato | Fish tank | 8/6/2022 | Day 127 | Yes | Water | No | No | - |
| TO_DR_2 | Aquaponics | Tilapia | Tomato | Drain tank | 8/6/2022 | Day 127 | Yes | Water | No | No | - |
| RAS_FTW | RAS | Sea bass | - | Fish tank | 21/2/2023 | - | Yes | Water | No | No | 2 |
| RAS_FTB | RAS | Sea bass | - | Fish tank | 21/2/2023 | - | Yes | Biofilm | No | No | 2 |
| RAS_BB | RAS | Sea bass | - | Biofilter | 21/2/2023 | - | Yes | Biofilm | No | No | 2 |
| FEB_FT_t0 | Aquaponics | Tilapia | Tomato | Fish tank | 17/2/2022 | Day 17 | No | - | Yes | Yes | 2 |
| FEB_FT_t1 | Aquaponics | Tilapia | Tomato | Fish tank | 18/2/2022 | Day 18 | No | - | Yes | Yes | 2 |
| FEB_FT_t2 | Aquaponics | Tilapia | Tomato | Fish tank | 19/2/2022 | Day 19 | No | - | Yes | Yes | 2 |
| JUN_FT_t0 | Aquaponics | Tilapia | Tomato | Fish tank | 8/6/2022 | Day 127 | No | - | Yes | Yes | 2 |
| JUN_FT_t1 | Aquaponics | Tilapia | Tomato | Fish tank | 9/6/2022 | Day 128 | No | - | Yes | Yes | 2 |
| JUN_FT_t2 | Aquaponics | Tilapia | Tomato | Fish tank | 10/6/2022 | Day 129 | No | - | Yes | Yes | 2 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| FEB_SUMP_t0 | Aquaponics | Tilapia | Tomato | Sump tank | 17/2/2022 | Day 17 | No | - | Yes | Yes | 2 |
| FEB_SUMP_t1 | Aquaponics | Tilapia | Tomato | Sump tank | 18/2/2022 | Day 18 | No | - | Yes | Yes | 2 |
| FEB_SUMP_t2 | Aquaponics | Tilapia | Tomato | Sump tank | 19/2/2022 | Day 19 | No | - | Yes | Yes | 2 |
| JUN_SUMP_t0 | Aquaponics | Tilapia | Tomato | Sump tank | 8/6/2022 | Day 127 | No | - | Yes | Yes | 2 |
| JUN_SUMP_t1 | Aquaponics | Tilapia | Tomato | Sump tank | 9/6/2022 | Day 128 | No | - | Yes | Yes | 2 |
| JUN_SUMP_t2 | Aquaponics | Tilapia | Tomato | Sump tank | 10/6/2022 | Day 129 | No | - | Yes | Yes | 2 |
| FEB_TOM_t0 | Aquaponics | Tilapia | Tomato | Drain tank | 17/2/2022 | Day 17 | No | - | Yes | Yes | 2 |
| FEB_TOM_t1 | Aquaponics | Tilapia | Tomato | Drain tank | 18/2/2022 | Day 18 | No | - | Yes | Yes | 2 |
| FEB_TOM_t2 | Aquaponics | Tilapia | Tomato | Drain tank | 19/2/2022 | Day 19 | No | - | Yes | Yes | 2 |
| JUN_TOM_t0 | Aquaponics | Tilapia | Tomato | Drain tank | 8/6/2022 | Day 127 | No | - | Yes | Yes | 2 |
| JUN_TOM_t1 | Aquaponics | Tilapia | Tomato | Drain tank | 9/6/2022 | Day 128 | No | - | Yes | Yes | 2 |
| JUN_TOM_t2 | Aquaponics | Tilapia | Tomato | Drain tank | 10/6/2022 | Day 129 | No | - | Yes | Yes | 2 |
| RAS_t0 | RAS | Sea bass | - | Fish tank | 21/2/2023 | - | No | - | Yes | Yes | 2 |
| RAS_t1 | RAS | Sea bass | - | Fish tank | 22/2/2023 | - | No | - | Yes | Yes | 2 |
| RAS_t2 | RAS | Sea bass | - | Fish tank | 23/2/2023 | - | No | - | Yes | Yes | 2 |

2.3. Protist growth experiment set up

Growth experiments were carried out *in situ* to calculate the growth potential of heterotrophic protists, specifically ciliates and HNF in both types of aquaculture systems. In the case of the aquaponics system, growth experiments took place twice over the production cycle of tomatoes alongside red tilapia rearing. The first time point was the 17$^{th}$ day (16/02/2022) of the system's operation, shortly after the transplantation of the tomato plants into the hydroponics compartment, and the second time point corresponded to the 127$^{th}$ day (08/06/2022) of operation, just before the end of the tomato production cycle. To estimate ciliate growth rates, system water from fish tanks (FT), clear buffer tank (SUMP) and coupled treatment drain tanks (TOM) after tomato plant fertigation was gravity filtered through a mesh filter with a pore size of 64 μm into duplicate clear polycarbonate bottles (Nalgene, USA) up to their full volume (2 L). These incubation bottles had been treated with 1 % HCl solution and then thoroughly rinsed with distilled water prior to their use. In the case of fish tanks and drain tanks, the bottles were filled with a mix of equal volumes of the respective duplicate tanks, while in the sump's case, replicate bottles were filled with water from the singular sump tank. Once filled, bottles were capped and left to incubate in their respective tanks to ensure in situ light and temperature conditions. The incubation lasted 48 h, during which time water samples (250 mL) were taken from the polycarbonate bottles and transferred into amber glass bottles at three time points: the start of the incubation (t0), at 24 h (t1) and at the end of the incubation at 48 h (t2). These samples were then fixed with the addition of adequate volume of acid Lugol's solution to a final concentration of 0.4 % (Karayanni et al., 2004), and stored in the dark at 4 °C for at least three days before microscopic inspection. The same procedure was carried out simultaneously for the HNF growth experiments, with a few distinctions. In this case, the incubation bottles were filled with system water that had been filtered through a 10 μm pore size mesh filter. Sample volume was approximately 50 mL, and samples were transferred into 50 mL plastic falcon tubes, then fixed with ice-cold glutaraldehyde (previously filtered through a 0.2 μm syringe filter to remove particles) to a final concentration of 1% (Karayanni et al., 2008). Furthermore, unfiltered system

water from the three examined compartments was also sampled (500 mL) and fixed with acid Lugol's solution to confirm that no larger sized ciliate taxa were excluded by the filtration through the 64 μm pores. Unfiltered water samples (50 mL) were also taken and fixed with a final concentration of 1% formaldehyde for the estimation of the natural abundance of prokaryotic cells in the different compartments through microscopy. The same overall process was repeated on 21/02/2023 at the seawater RAS, where two out of the three parallel fish tank subsystems were examined separately (RAS1 and RAS2) and incubation bottles were filled with a mix of equal volumes of different fish tanks of the same RAS subsystem.

### 2.4. Enumeration of prokaryotes and protists

After being transported to the laboratory, fixed ciliate samples were left undisturbed for at least three days at 4 °C so that preserved cells could settle at the bottom of the amber glass bottles before microscopic inspection (Karayanni et al., 2004). Before the inspection of each sample, water was carefully siphoned out from the surface of the samples to prevent perturbation and mixing, until about 100 mL remained at the bottom of the amber glass bottle. Subsequently, bottles were capped and gently inverted by hand several times to thoroughly homogenize the remaining sample without harming the preserved cells. Then a 50 mL subsample was transferred into a Utermöhl sedimentation chamber (HYDRO-BIOS, Germany) for overnight sedimentation (Edler and Elbrächter, 2010) to ensure that all ciliate cells would settle on the bottom of the chamber. The following day concentrated samples were inspected with brightfield and phase contrast microscopy using an inverted microscope (Olympus IX73). Due to high cell abundances, half of the sedimentation chamber's bottom surface was counted at 400X magnification and ciliate abundances were calculated as cells $L^{-1}$.

To measure HNF abundance, adequate volumes of 30–50 mL of glutaraldehyde fixed water samples were filtered with the use of a vacuum pump on 0.8 μm pore size black polycarbonate filters (Whatman Nucleopore Track-Etch

Membrane, 25mm) and stained with DAPI (4',6-diamidino-2-phenylindole, ThermoFisher, USA) for 5–10 min in a final concentration of 2 μg·mL$^{-1}$ (Livanou et al., 2019). Each filter was transferred on a previously labelled slide where a drop of immersion oil (Leica Microsystems) was then added on it, and it was carefully covered with a cover slip. Stained filters were briefly inspected to ensure the success of the staining process, then stored in the dark at − 20 °C until counting. Microscopic examination of the filters was performed using epifluorescence microscopy (Leica DM2000 LED, Germany) at 1000X magnification with an immersion lens, after a drop of immersion oil was added on each filter's cover slip. The excitation wavelength was set to 365 nm (UV) for DAPI. To obtain a representative measurement of HNF abundance from each sample, 10–15 optical fields equally distributed across the surface of each filter were randomly chosen and counted. Similarly, natural prokaryotic cell abundance was measured by filtering formaldehyde fixed water samples (1% final concentration) through 0.2 μm pore size filters (Whatman Nucleopore Track-Etch Membrane, 25mm), DAPI staining and epifluorescence microscopy (Porter and Feig, 1980). To minimize statistical error, a total of at least 300 prokaryotic cells were directly counted in 10 randomly chosen optical fields equally distributed across the surface of each filter. Both HNF and prokaryotic abundances were calculated as cells ml$^{-1}$ based on microscopic counts using the formula $A = N(S_F/S_C)\,/V$ where $A$ is the abundance expressed as cells ml$^{-1}$, $N$is the average number of cells counted across all optical fields in a sample, $S_F$ is the total filtration surface, $S_C$ is the total surface of the optical fields counted and $V$ is the volume of the sample.

Protist cell abundances were then used to estimate protist growth rates. Natural logarithm transformed abundances were plotted against the incubation time and growth phases were selected accordingly after examination of each plot (Appendices 3 and 4). Growth rates were then calculated using the formula $\mu = \ln(N_t/N_0)\,/t$ where $N_0$ and $N_t$ are ciliate or HNF abundance at the beginning and end of the incubation, respectively; μ (d$^{-1}$) is the intrinsic rate of increase; and t is the duration of the growth phase (Frost, 1972; Karayanni et al., 2008; Weisse and Stadler, 2006).

## 2.5. Morphology-based ciliate classification

During microscopic examination at 400X (Appendix 5), ciliates were categorized into morphotypes based on morphological characteristics and size, and images were captured using a microscope mounted camera and the cellsSens Entry software (Olympus). Morphotypes were then associated with ciliate genera according to well established identification keys (Foissner and Berger, 1996). Ciliate genera were then grouped to subclasses based on the latest NCBI taxonomy. Based on these observations, calculation of ecological diversity indices (Shannon, Simpson) for the ciliate community and the overall morphology related data statistical analysis was performed using the PAST ver. 4.09 software (Hammer et al., 2001). Furthermore, abundances of other heterotrophic eukaryotes such as amoebae and rotifers were also determined and their morphotypes were assigned to taxa based on Tsyganov et al. (2017), and the online key "An Image-Based Key To The Zooplankton Of North America", Version 5.0 (https://cfb.unh.edu/cfbkey/html/rotifers.htm), respectively. For HNF, while morphology-based classification was not possible as DAPI only stains HNF nuclei, blue light excitation was used to rule out the presence of chlorophyll and thus exclude autotrophic nanoflagellates from the counts (Appendix 6).

## 2.6. DNA extraction, sequencing and metagenomics analysis

Prior to DNA extraction, sample material (i.e. filters, swabs, biofilm carriers) was left to thaw at room temperature. Filters and biofilm carriers were then sliced into smaller pieces using a sterile scalpel. DNA extraction was performed using the Qiagen DNeasy PowerSoil Pro Kit (Hilden, Germany), following the manufacturer's instructions. The duplicate samples from RAS fish tank water, RAS fish tank biofilm and RAS biofilm carriers were pooled after the extraction. Sample quality (absorbance ratio at 260/280 nm) and DNA concentration were estimated using a UV spectrophotometer (Q3000 Quawell DNA/Protein Analyser, Quawell Technology Inc, CA, USA). The average

absorbance ratio of all samples was 1.68 (SD = 1.66) and DNA concentration ranged between 5.2 and 46.5 ng $\mu L^{-1}$. Appropriate volumes of each sample (50-70µl) were then shipped to Eurofins Genomics (Konstanz, Germany) for metagenomic sequencing (INVIEW Metagenome, 2 × 150 bp paired-end Illumina sequencing). Metagenomic raw data was then received in fastq format and subjected to bioinformatics analysis.

Retrieved sequences were quality assessed, trimmed (for a min Phred Q of 20 at a sliding window size of 4 bases, retaining reads of 70 bp min length) and read-pairs were attempted to be assembled to their inserts of origin with the fastp v0.21.0 software (Chen et al., 2018). Assembled reads together with the forward reads of the unassembled read pairs, and the forward or reverse reads of read-pairs with one read surviving the quality control, were annotated by comparison with a range of databases. Comparison was performed with diamond v2.0.15.153 (Buchfink et al., 2015) with cutoff e value and identities of 1e-10 and 70% respectively, over a minimum length of 30 amino acids of translated sequence. The SEED database was used for generic functional gene annotation (Overbeek et al., 2014) using the BacMet v2.0 pipeline (Pal et al., 2014) for calling the diamond comparisons and annotation parsing. Kaiju v1.7.3 (Menzel et al., 2016) was used for the taxonomic annotation of prokaryotes, eukaryotes and viruses. Parallel BLAT v36x2 (Wang and Kong, 2019) was used for searching against the SILVA v138 database (Prüsse, 2011) for counting 16S rRNA genes, for hit normalization purposes.

Furthermore, the Nonpareil algorithm (Rodriguez-R and Konstantinidis, 2014b, 2014a) was implemented (software version v3.304) in junction with the Nonpareil data analysis package v3.3.1 (Rodriguez-R et al., 2018) of R v4.2.2 (R Core Team, 2023) on the quality controlled sequences for assessing the achieved coverage given the sequencing effort.

To facilitate the comparison of the relative abundance of bacterial genera between samples, read counts were normalized based on the total 16S rDNA sequence counts in each respective sample (Zemb et al., 2020).

## 2.7. Data analysis

### 2.7.1. Protist data analysis

Data analysis was performed using MS Excel 365, RStudio (version 2024.04.1 – 2025.05.1, R Core Team, 2023) and PAST (version 4.09). The Shapiro-Wilk test was used to determine whether the data were normally distributed. The non-parametric Kruskal-Wallis test was used to check for statistically clear differences between observations from the three compartments studied (FT, SUMP, TOM), and then Dunn's post hoc test was used for pairwise comparisons. Pearson and Spearman correlation coefficients were calculated to investigate linear and non- linear relationships between the abundances of microeukaryotic groups. Standard alpha diversity indices based on morphological data were calculated using PAST. Based on the relative abundances of microeucaryotic taxa, the Bray-Curtis similarity index was calculated and used for UPGMA (unweighted pair group method with arithmetic mean) based clustering. SIMPER (similarity percentages) analysis was carried out in PAST to determine statistically clear differences in the abundance of microeukaryotic groups across aquaponics systems. RStudio "vegan" (Oksanen et al., 2025) and "tidyverse" (Wickham et al., 2019) packages were used to generate rarefaction curves based on metagenomic data, estimate Good's coverage index for the samples, calculate alpha diversity indices for the microeukaryotic community and perform statistical tests such as t-test and Wilcoxon test. In addition, RStudio was used to perform PERMANOVA (permutational multivariate analysis of variance) using the Adonis2 function, and NMDS (non-metric multidimensional scaling) to explore microeukaryotic beta diversity based on metagenomic data. Figures were prepared using MS Excel 365, RStudio, PAST and Venny 2.1 ({HYPERLINK "https://bioinfogp.cnb.csic.es/tools/venny/index.html"}).

### 2.7.2. Prokaryotic diversity data analysis

Data analysis was performed using MS Excel 365, RStudio (versions 2024.04.1 – 2025.05.1, R Core Team, 2023) and PAST (version 4.09). Specifically, Shapiro-Wilk normality tests, Kruskal-Wallis tests, Dunn's post hoc tests, Pearson and Spearman correlation coefficients' calculation, Bray-Curtis based clustering, and SIMPER analysis were carried out in PAST. RStudio was used to generate rarefaction curves based on metagenomic data, estimate Good's coverage index for the samples, calculate alpha diversity indices for the prokaryotic community using the vegan package, perform statistical tests such as t-tests and Wilcoxon tests. In addition, RStudio was used to perform PERMANOVA and NMDS to explore prokaryotic beta diversity based on metagenomic data, as well as visualize the correlations between microbial groups in the form of networks using the "igraph" package (Csárdi and Nepusz, 2006). Figures were prepared using MS Excel 365, RStudio, PAST and the website Venny 2.1 ({HYPERLINK "https://bioinfogp.cnb.csic.es/tools/venny/index.html"}).

### 2.7.3. Prokaryotic function data analysis

Data analysis was performed using MS Excel 365, RStudio (versions 2024.04.1 – 2025.05.1, R Core Team, 2023) and PAST (version 4.09). Specifically, Shapiro-Wilk normality tests, Kruskal-Wallis tests, Dunn's post hoc tests, Pearson and Spearman correlation coefficients' calculation, Bray-Curtis based clustering analysis were carried out in PAST. Figures were prepared using MS Excel 365, RStudio and PAST. Based on published literature, key prokaryotic functional genes (Chapter 5, Table 5.1) were selected for the investigation of metabolic processes related to nutrient cycling.

# 3. Heterotrophic protist abundance, growth rates and diversity

## 3.1. Introduction

Heterotrophic protists are regularly encountered in multiple trophic levels within the aquatic food web and serve as a critical link between the prokaryotic community and larger zooplankton in pelagic habitats. As such, their part is considered essential for nutrient, biomass and energy flow through the food web (Caron and Goldman, 1988; Corliss, 2002; Mostajir et al., 2015). Despite their relatively low biomass compared to the other food web components, protists can significantly contribute to nutrient cycling within the ecosystem due to their inherent capacity for high metabolic activity and rapid growth rate (Massana and Logares, 2013; Mostajir et al., 2015). HNF are widely regarded as the most important consumers of prokaryotic picoplankton, especially in oligotrophic environments (Livanou et al., 2019), while their significance for planktonic food web dynamics has been highlighted for decades (Azam et al., 1983; Pomeroy, 1974). Apart from grazing on prokaryotes, HNF facilitate the transfer of organic carbon from bacteria and small algae to larger protists such as ciliates and dinoflagellates, as well as mesozooplankton (Vargas et al., 2012).

Ciliates also contribute to energy and nutrient flow to higher trophic levels (Pitta et al., 2016) and are characterized by diverse morphology, size and feeding behavior. Thus, ciliates can feed through bacterivory, herbivory, or by feeding on smaller protists and zooplankton (Mostajir et al., 2015). In addition to being part of the microbial food web, certain ciliate taxa can also affect fish through pathogenic or parasitic interactions, and inhibit or promote other microbial or viral infections by neutralizing or protecting fish pathogens (Pinheiro and Bols, 2013). Ciliate interactions with other microorganisms and fish are of great significance in the context of aquaculture, both for economic and scientific reasons. As the trend of increasing fish production leads to increasing stocking densities of farmed fish in aquaculture systems, disease outbreaks become more common due to an imbalance between microbial community diversity, farmed fish and environmental factors (Kotob et al.,

2016). As RAS and aquaponics systems designs are undergoing continuous improvement and optimization to offset their high investment cost, temporal and spatial dynamics of the microorganisms within these systems must be elucidated (Schreier et al., 2010). Heterotrophic protists in particular still remain uncharacterized despite their significant role. As such, the first specific goal of the present study was to investigate shifts in diversity, abundance and potential growth rates of heterotrophic protists in different compartments of experimental RAS and aquaponics systems, as the first step in the process of describing the functional role of heterotrophic protist communities in these rapidly developing food production technologies.

## 3.2. Results

### 3.2.1. Aquaponics relative abundance

The natural abundance of heterotrophic protists was measured through microscopy (indicative images are presented in Appendices 5 and 6) twice during the tomato production cycle in the aquaponics systems, specifically on the 17$^{th}$ day of operation (FEB) and on the 127$^{th}$ day (JUN), in three compartments, namely the fish tanks (FT), the clear water buffer tank (SUMP) and the drain tanks after tomato fertigation (TOM). Total ciliate abundance in FEB was estimated at 1063 ± 23 cells $L^{-1}$ in FT, 732 ± 21 cells $L^{-1}$ in the SUMP, and 4582 ± 907 cells $L^{-1}$ in TOM (Figure 3.1.A). At the time of the second sampling, abundances were 1867 ± 400, 5451 ± 118, and 851 ± 34 cells $L^{-1}$ respectively in the three compartments sampled. Meanwhile, HNF natural abundance (Figure 3.1.B) appeared more consistent in FT and SUMP samples on both dates, starting at 147 ± 18 and 124 ± 6 cells $mL^{-1}$ respectively in FEB, and shifting to 139 ± 6 and 121 ± 12 cells $mL^{-1}$ in JUN. In TOM samples, HNF abundance more than doubled, increasing from 58 ± 8 cells $mL^{-1}$ to 133 ± 12 cells $mL^{-1}$. Additionally, prokaryotic abundance measurement via epifluorescence microscopy revealed 1.199 ± 0.005 × $10^6$ bacteria $mL^{-1}$ in aquaponics FT and SUMP water samples, and 6.24 × $10^5$ cells $mL^{-1}$ in RAS fish tanks.

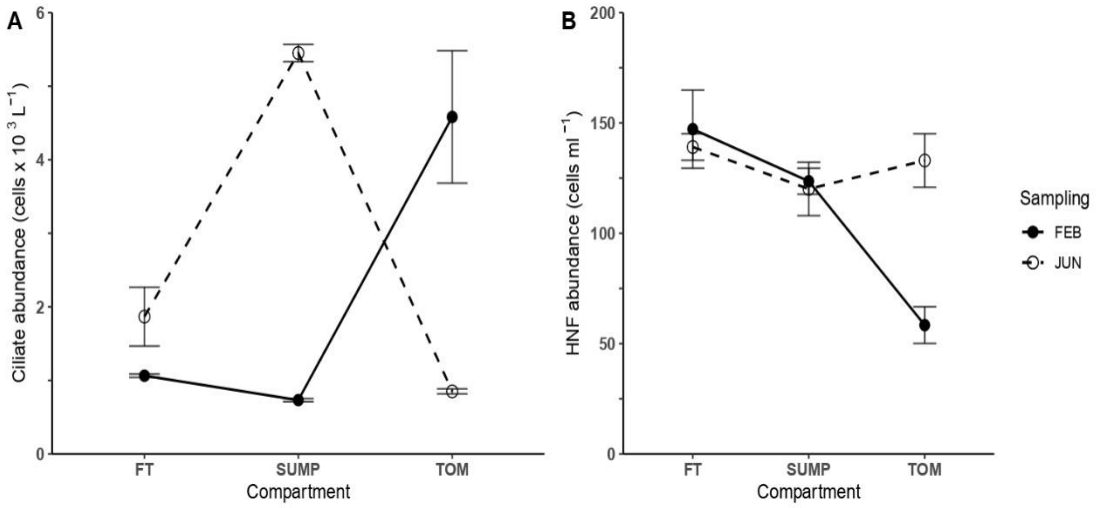



**Figure 3.1**. Natural abundance of heterotrophic protists in the aquaponics system. **A**: Ciliates (cells × $10^3$ $L^{-1}$) and **B**: HNF (cells $ml^{-1}$), in the three aquaponics compartments and two sampling months. FT: fish tanks, SUMP: clear water buffer tank, TOM: drain tanks after tomato fertigation. Error bars represent uncertainty defined as [(max-min) / 2]. Adapted from Moschos et al. (2024).

### 3.2.2. RAS relative abundance

Water samples were also examined to determine the natural protist abundance in the fish tanks of the seawater RAS. Sampling was conducted at a single time point, at two independent RAS loops, RAS1 and RAS2. The average total ciliate natural abundance was 203.27 (SD = 47.94) and 78.46 (SD = 28.52) cells $L^{-1}$ respectively, with the maximum value being 243 cells $L^{-1}$ (SD = 66.26) in RAS1. HNF were scarcely encountered in the samples, preventing an accurate estimation of their abundance in the RAS.

### 3.2.3. Growth experiments

Ciliate and HNF abundances during the incubation were natural logarithm transformed and plotted against incubation time to obtain the respective growth curves (Figure 3.2, Appendices 3 and 4). Within the aquaponics system, total ciliate growth rates were clearly higher in FT samples compared to the other compartments (Kruskal–Wallis test for equal medians, $p < 0.05$, followed by Dunn's post hoc test) for both FEB and JUN samplings, at 1.90 ± 0.01 and 1.26 ± 0.13 $d^{-1}$ respectively (Table 3.1). In the SUMP, total ciliate growth rate was lower in JUN, having decreased from 0.78 ± 0.27 to 0.57 ± 0.04 $d^{-1}$, while in TOM samples total ciliate growth increased from 0.54 ± 0.03 to 0.79 ± 0.05 $d^{-1}$. A similar pattern was observed for total HNF growth rates (Figure 3.2) as they were clearly lower in TOM samples compared to the other compartments (Kruskal–Wallis test, $p < 0.05$, Dunn's post hoc test). Specifically, growth rates were 0.5 ± 0.3 $d^{-1}$ in FEB and 1.37 ± 0.05 $d^{-1}$ in JUN showing an increase over time, while they decreased both in FT and SUMP samples, from 6.03 ± 0.34 to 4.09 ± 0.11 $d^{-1}$ and from 5.67 ± 0.22 to 4.64 ± 0.11 $d^{-1}$ respectively. No statistically clear correlation was found between HNF and ciliate abundances despite the negative Pearson coefficient ($r = -0.54$, $p = 0.07$). Meanwhile, total ciliate abundance decreased in both seawater RAS loops, with the lowest abundance measured in RAS2 after the 48 h incubation (13 cells $L^{-1}$, SD = 3.28). Furthermore, no detectable increase in HNF abundance was observed during the incubation period.

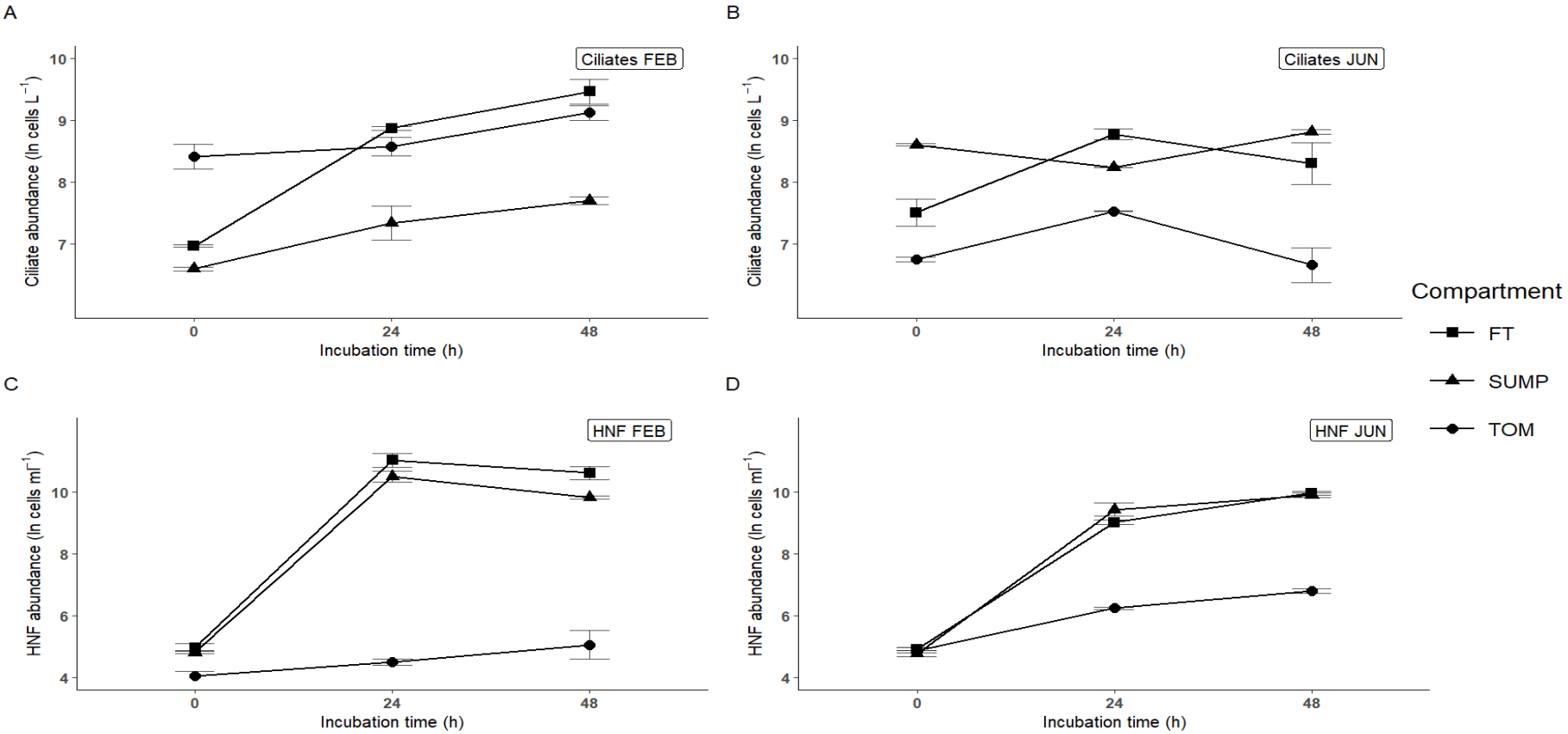

A
Ciliates FEB
Ciliate abundance (ln cells L$^{-1}$)
10
9
8
7
0
24
48
Incubation time (h)
B
Ciliates JUN
Ciliate abundance (ln cells L$^{-1}$)
10
9
8
7
0
24
48
Incubation time (h)
Compartment
FT
SUMP
TOM
C
HNF FEB
HNF abundance (ln cells ml$^{-1}$)
10
8
6
4
0
24
48
Incubation time (h)
D
HNF JUN
HNF abundance (ln cells ml$^{-1}$)
10
8
6
4
0
24
48
Incubation time (h)

**Figure 3.2.** Growth curves of the total ciliate (**A – B**) and HNF (**C – D**) communities in the three aquaponics compartments (FT: fish tanks, SUMP: clear water buffer tank, TOM: drain tanks) and two growth experiment dates (FEB, JUN). Ciliate and HNF abundances were natural logarithm transformed [(ln cells $L^{-1}$) and (ln cells $ml^{-1}$) respectively]. Error bars represent uncertainty defined as [(max-min) / 2]. Adapted from Moschos et al. (2024).

**Table 3.1.** Average total growth rates ($d^{-1}$) of heterotrophic nanoflagellates (HNF) and ciliates (CIL) in FEB (day 17) and JUN (day 127) growth experiments in the studied aquaponics compartments, with uncertainty [(max–min)/2] in parenthesis. FT: fish tanks, SUMP: clear water buffer tank (sump), TOM: drain tanks. Adapted from Moschos et al. (2024).

| Compartment | FEB | | JUN | |
|---|---|---|---|---|
| | HNF | CIL | HNF | CIL |
| FT | 6.03 (0.34) | 1.90 (0.01) | 4.09 (0.11) | 1.26 (0.13) |
| SUMP | 5.67 (0.22) | 0.78 (0.27) | 4.64 (0.11) | 0.57 (0.04) |
| TOM | 0.5 (0.3) | 0.54 (0.03) | 1.37 (0.05) | 0.79 (0.05) |

With regard to morphological characteristics, ciliate individuals that were clearly observed and counted were then categorized into morphotypes. These morphotypes were then associated with described genera. Thus, in the aquaponics system's compartments genera such as *Cyclidium*, *Halteria*, *Litonotus*, *Euplotes*, *Paramecium*, *Vorticella*, and *Chilodonella* were the most observed ciliates (Figure 3.3). In fact, the *Cyclidium* associated morphotype was the most common one, with an abundance range of 572 ± 5 cells $L^{-1}$ in TOM natural community samples to 12228 ± 2684 cells $L^{-1}$ in FT samples of  at the end of the 48 h incubation in FEB. This morphotype also proved capable of rapid growth, especially in the FT incubation samples where it achieved rates of 2.12 ± 0.01 $d^{-1}$ in FEB and 1.29 ± 0.09 $d^{-1}$ in JUN, clearly higher (Kruskal–Wallis test, $p < 0.05$) than growth rates measured in the respective SUMP and TOM samples. The *Euplotes* morphotype was often encountered in various samples with a maximum abundance of 139 cells $L^{-1}$ in JUN SUMP samples, although no clear sign of growth over time was recorded. *Litonotus* was a morphotype that usually became detectable at 24 or 48 h of incubation, peaking in abundance in FEB SUMP samples with 134.5 ± 42.5 cells $L^{-1}$. *Halteria* and *Paramecium* were mainly detected in FEB TOM samples. *Halteria* grew at a rate of 0.55 ± 0.08 $d^{-1}$ to a maximum of 5438 ± 100 cells $L^{-1}$, whereas *Paramecium* abundance was variable during

incubation, ranging from 108 to 467 cells $L^{-1}$. *Chilodonella* cells were chiefly observed in FEB samples with an abundance between 11 to 40 cells $L^{-1}$.

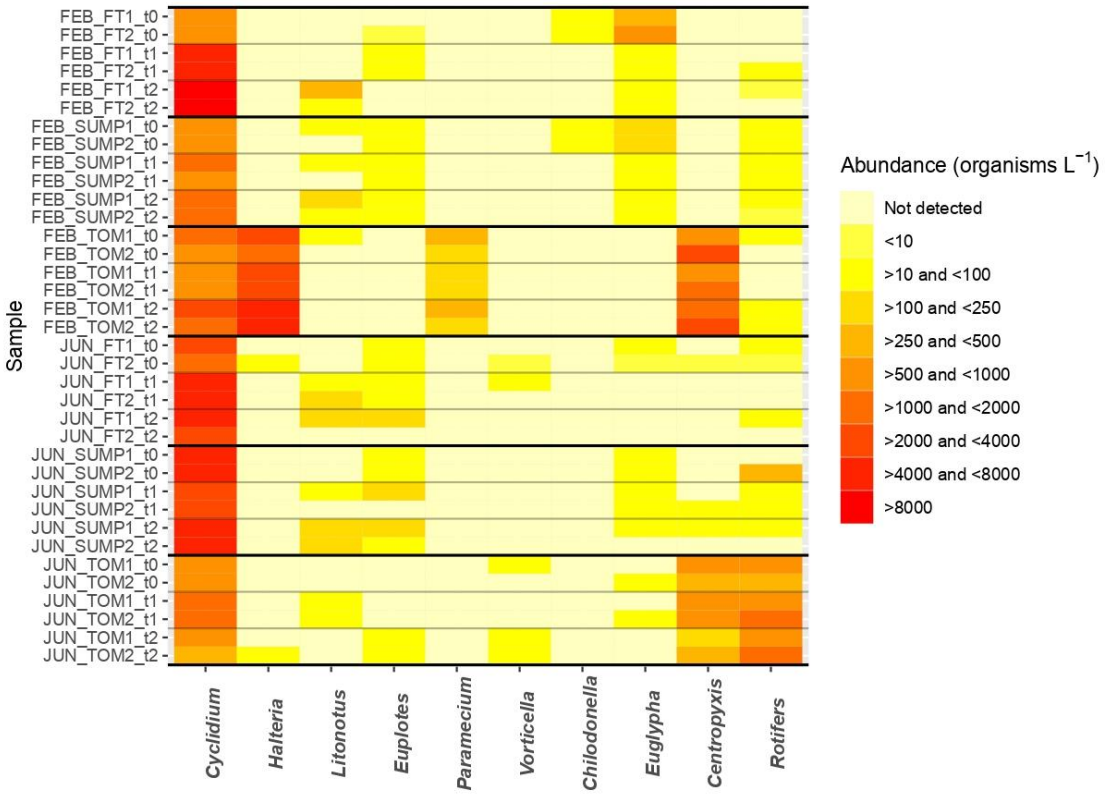



**Figure 3.3.** Abundance (organisms $L^{-1}$) heatmap of major observed heterotrophic microeukaryotic groups in all samples from the growth experiments. FT: fish tanks, SUMP: clear water buffer tank, TOM: drain tanks, FEB: first sampling, JUN: second sampling, t0: start of incubation, t1: 24 h, t2: 48 h. Adapted from Moschos et al. (2024).

The most common ciliate morphotypes in the seawater RAS fish tanks were attributed to genera *Cyclidium*, *Aspidisca*, *Euplotes*, *Litonotus*, *Colpoda*, and *Chilodonella*. Just like in the aquaponics system, the *Cyclidium* morphotype was the most abundant in both RAS subsystems, at 155.8 ± 16.7 and 26.9 ± 19.2 cells $L^{-1}$ in RAS1 and RAS2 respectively. *Aspidisca* individuals were only detected at the start of the incubation, with an abundance of 29.6 ± 13.9 cells $L^{-1}$ in RAS1 and 19.2 ± 11.5 cells $L^{-1}$ in RAS2. *Colpoda* and *Euplotes* were also present in both subsystems at low

concentrations. Furthermore, *Litonotus* was detected only in RAS1 while *Chilodonella* was present only in RAS2.

Other eukaryotic microorganisms were also detected in high abundance in the aquaponics system. Firstly, testate amoebae of the genus *Euglypha* were commonly identified in water samples (Figure 3.3), though with higher abundance in FEB FT samples (519 ± 39 cells $L^{-1}$, excluding empty amoeba tests). Another genus of testate amoebae, *Centropyxis aculeata*, was mostly present in TOM samples (Fig. 5), with average abundances of 1453 (SD = 645.17) and 481 cells $L^{-1}$ (SD = 163) in FEB and JUN, respectively. In both cases however, no increasing trend was observed in their abundance, and instead *Euglypha* abundance started decreasing in FEB FT (567 ± 87 to 39 ± 9 cells $L^{-1}$) and SUMP (211 ± 11 to 79 ± 24 cells $L^{-1}$) samples over the first 24 h of incubation. Phytoplankton was also represented in the aquaponics system. Specifically, different diatoms (phylum Bacillariophyta) were observed in TOM samples during both growth experiment dates, and *Scenedesmus* (class Chlorophyceaea) colonies were ubiquitous in JUN samples with a noticeable prominence in the drain tanks. In addition, rotifers were also detected in the aquaponics compartments, with a more than tenfold increase in abundance in JUN samples (23 ind $L^{-1}$ (SD = 29.80) to 293.83 ind $L^{-1}$ (SD = 399.04)). At the time of the second sampling, a negative Pearson correlation was observed between rotifer and ciliate abundance ($r = -0.639$, $p < 0.01$). In terms of taxonomy, rotifers were represented by two genera, *Lepadella* and *Lecane* (phylum Rotifera, order Ploima).

Conversely, commonly observed microorganisms in the seawater RAS fish tanks included a dinoflagellate morphotype associated with the genus *Amphidinium* (class Dinophyceaea) as well as various menotactic amoebae. As in the case of RAS ciliates, *Amphidinium* abundance also decreased over the incubation period, from 361.2 ± 47.5 to 230.4 ± 162.5 cells $L^{-1}$ in RAS1 and from 273 ± 67.9 to 153.8 ± 39.5 cells $L^{-1}$ in RAS2. Rotifers were once again observed, represented by the genus *Colurella* (order Ploima), with a decreasing abundance over time, between 133.3 ± 55 and 73.3 ± 5.3 cells $L^{-1}$ in RAS1, and 67.9 ± 3.6 and 29 ± 9 cells $L^{-1}$ in RAS2. As both rotifer and

ciliate populations decreased during the incubation, their abundances were positively correlated (Pearson r = 0.59, $p < 0.05$).

### 3.2.4. Morphology based microeukaryotic diversity

To assess morphology based ciliate diversity in the different compartments of the aquaponics systems, commonly used ecological indices were calculated. Specifically, the Shannon (H) diversity index ranged between 1 in natural community drain tank samples in FEB and 0 in JUN samples taken at the end of the 48 h incubation. Meanwhile Simpson (1-D) index was 0.7 in FEB drain tank natural community samples to 0 in JUN samples after the incubation. Comparison of system-wide average indices of the natural ciliate communities revealed higher values for Shannon (H) and Simpson (1-D) indices in FEB samples, while the Dominance D index was higher in JUN samples (two-sample t-test, $p < 0.01$). A similar observation was made when all growth experiment samples of each sampling date were considered (Mann–Whitney test). Furthermore, drain tank samples had a higher Evenness (e^H/S) index compared to FT and SUMP samples (Kruskal–Wallis test, $p < 0.01$, followed by Dunn's post hoc test). In the seawater RAS, Shannon index ranged between 1.57 in RAS1 natural community samples to 0.58 in RAS2 samples after the 48 h incubation. Similarly, the Simpson index decreased from 0.74 in RAS1 natural community samples to 0.3 in RAS2 after the incubation. In addition, RAS1 was found harboring a higher natural abundance of ciliates compared to RAS2 (Wilcoxon test, $p < 0.05$).

Furthermore, cluster analysis of the ciliate communities was performed for natural ciliate communities and ciliate communities during the growth incubation. Regarding the natural ciliate communities (Figure 3.4.A), TOM samples of each sampling date were grouped apart from FT and SUMP samples. Specifically, the TOM community in FEB was the most different from the natural communities in the rest of the compartments on both dates. When the clustering included all growth experiment samples, it was shown that the respective TOM samples of each date had distinct

communities from each other and from the other compartments even after the 48 h incubation (Figure 3.4.B).

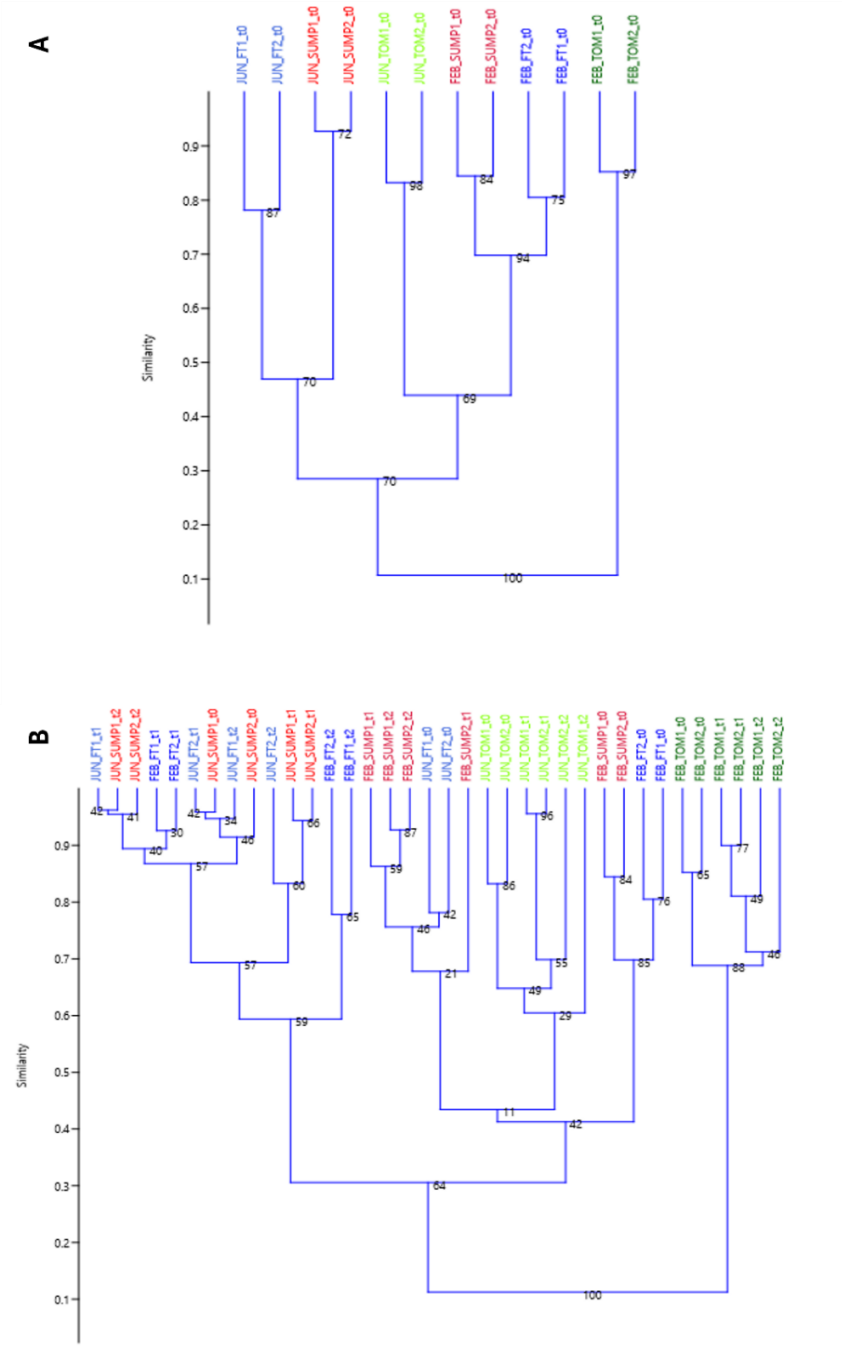



**Figure 3.4.** Clustering of samples based on microeukaryotic community composition using Bray–Curtis similarity index and the UPGMA method. Bootstrap values (N=1000) are displayed at each node. **A**: Natural community samples at the start of incubation, **B**: All samples over the 48 h incubation. FT: fish tanks, SUMP: clear water buffer tank, TOM: drain tanks, FEB: first sampling, JUN: second sampling, t0: start of incubation, t1: 24 h, t2: 48 h. Adapted from Moschos et al. (2024).

### 3.2.5. Metagenomics based microeukaryotic diversity

Metagenomic analysis of the microbial communities of the aquaponics and RAS samples revealed that Eukaryota reads made up about 0.1% – 2% of the total. The rarefaction curves for Eukaryota were saturated for most samples (Appendix 7), and Good's coverage was > 0.99 for all samples, indicating an adequate sequencing depth and a reliable representation of eukaryotic diversity. At the genus level, richness (Table 3.2) ranged between 395 and 799 in aquaponics samples and between 541 and 642 in the seawater RAS. The Shannon (H) diversity index ranged between 2.79 and 5.45 while the inverse Simpson (1/D) index ranged between 3 to 142 in the aquaponics samples. Total Eukaryota reads were between 9316 and 101567 across the aquaponics samples. Statistical analysis indicated a clearly higher number of eukaryotic reads (Figure 3.5.A, Wilcoxon test, $p = 0.032$, effect size $r = 0.694$) obtained from drain tank samples (average = 55930.8, SD = 30720.8) compared to fish tank samples (average = 19268.4, SD = 11164.6). Meanwhile, based on the inverse Simpson index, eukaryotic diversity was higher (Figure 3.5.B, t-test, $p = 0.033$) in the fish tanks (average = 87.3, SD = 42.8) compared to the drain tanks (average = 28, SD = 21.5).

**Table 3.2.** Alpha diversity metrics of the eukaryotic community at genus level in aquaponics and RAS samples based on metagenomic data. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling, BB: biofilter biofilm, FTB: fish tank biofilm, FTW: fish tank water.

| Sample | Richness | Shannon (H) | Inverse Simpson (1/D) | Reads |
|---|---|---|---|---|
| CU_DR_1 | 568 | 4.47 | 25.3 | 22365 |
| CU_DR_2 | 778 | 5.06 | 62.6 | 53886 |
| CU_FT_1 | 395 | 4.97 | 80.5 | 9316 |
| CU_FT_2 | 678 | 5.13 | 51.4 | 32004 |
| PA_DR | 741 | 2.79 | 3.73 | 101567 |
| PA_FT | 609 | 5.45 | 142 | 14710 |
| TO_DR_1 | 682 | 4.31 | 20.5 | 34929 |
| TO_DR_2 | 799 | 4.60 | 28.1 | 66907 |
| TO_FT_1 | 589 | 4.72 | 43.2 | 30524 |
| TO_FT_2 | 479 | 5.23 | 119.6 | 9788 |
| RAS_BB | 541 | 5.44 | 150.2 | 17630 |
| RAS_FTB | 591 | 5.52 | 157.5 | 17341 |
| RAS_FTW | 642 | 3.75 | 8.40 | 72440 |

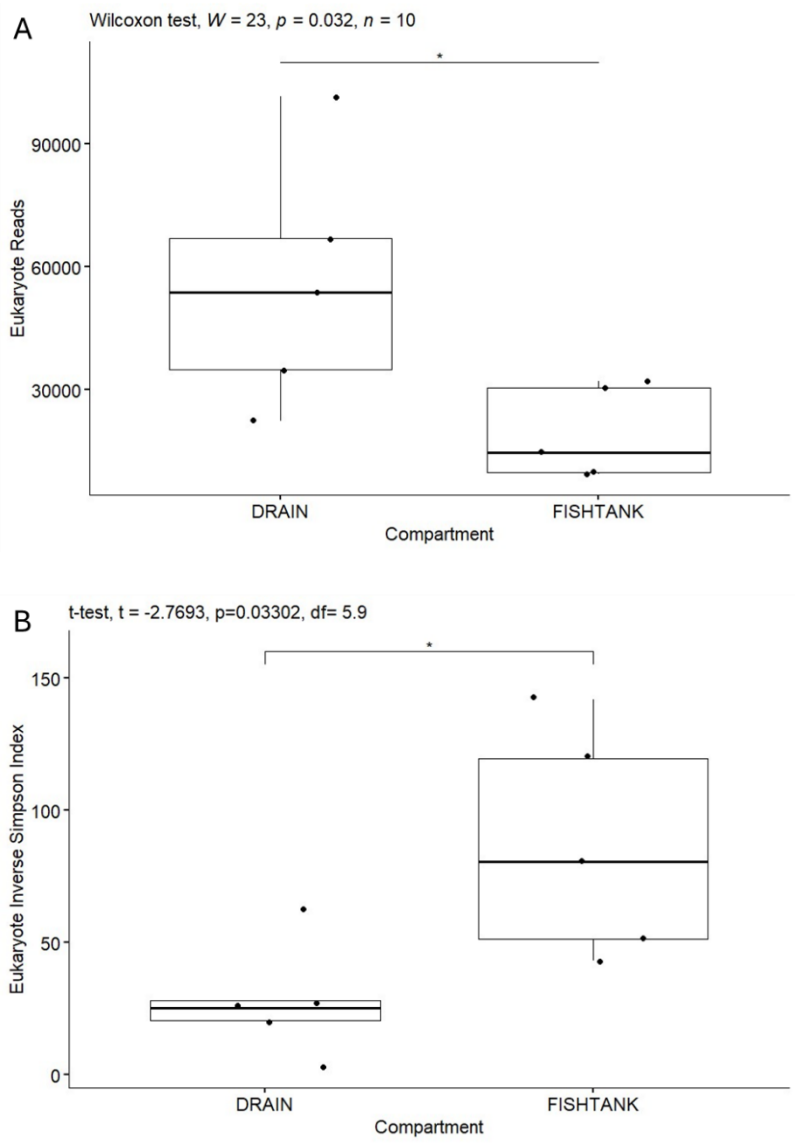



**Figure 3.5.** Box-plot distribution of aquaponics samples based on number of reads (**A**) and inverse Simpson index values (**B**) of the eukaryotic community, with test results for statistically clear differences between the fish tank and drain compartments.

Looking into the beta diversity of the microeucaryotic communities, PERMANOVA yielded a p value < 0.001, indicating a difference between sample group composition, with $R^2 = 0.307$. Subsequently, NMDS analysis was performed, showing a grouping of communities from aquaponics drain tank samples and fish tank samples

respectively (Figure 3.6). Furthermore, clustering analysis based on Bray-Curtis similarity using the UPGMA method (Figure 3.7) indicated that drain tank communities were generally similar with the exception of the parsley cultivation system (PA) which harbored the most unique community. In addition, attached microeukaryotic communities in RAS biofilters (BB) and fish tanks (FTB) were very similar (80%), and very different from the suspended community in RAS fish tank water (FTW).

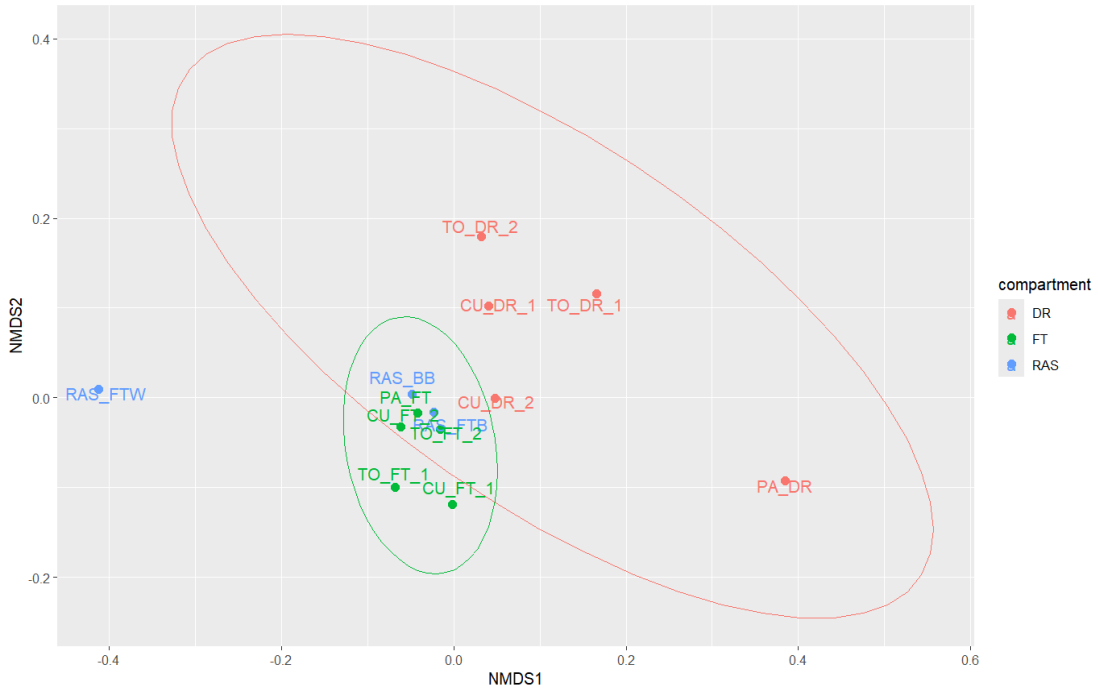



**Figure 3.6.** NMDS plot depicting the grouping of microeukaryotic communities based on the compartment sampled (DR: drain tanks, FT: fish tanks, RAS: RAS fish tanks, TO: tomato aquaponics, CU: cucumber aquaponics, PA: parsley aquaponics, 1: first sampling time, 2: second sampling time, FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm).

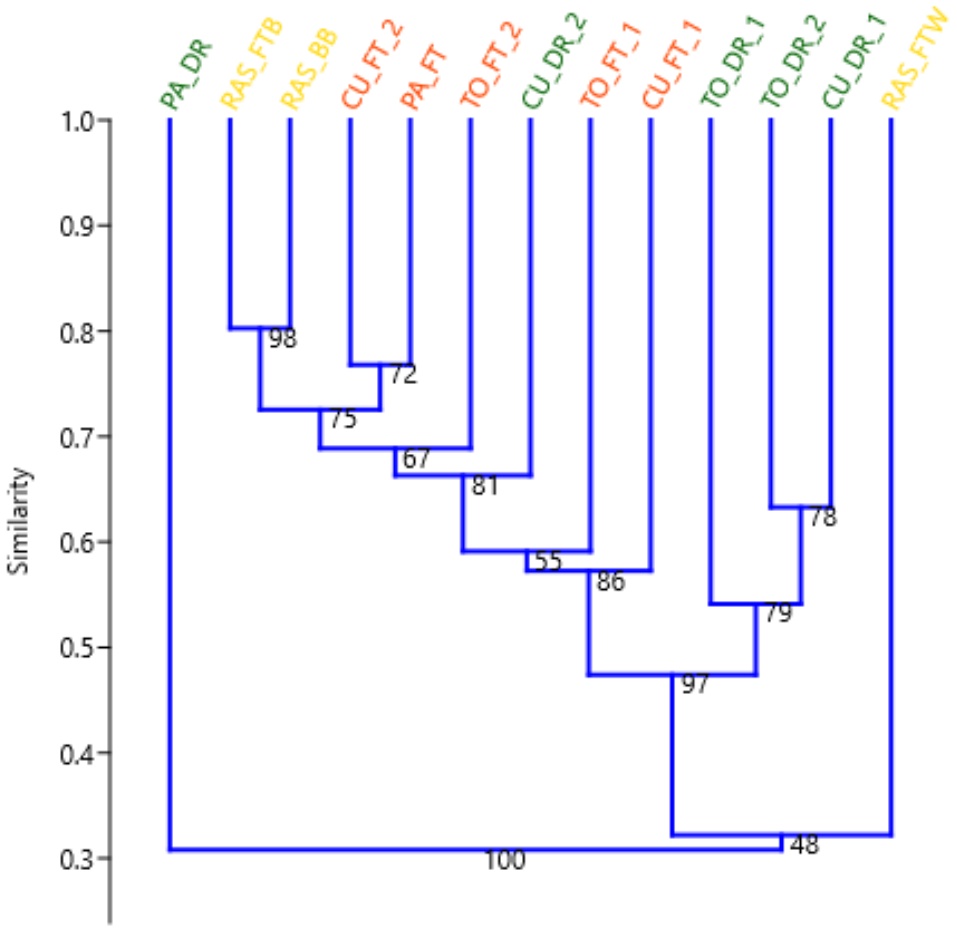



**Figure 3.7.** Clustering of samples based on microeukaryotic community composition using the Bray–Curtis similarity index and the UPGMA method. Bootstrap values (N=1000) are displayed at each node. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling, BB: biofilter biofilm, FTB: fish tank biofilm, FTW: fish tank water.

Delving into the microeukaryotic community composition, a handful of superphylum level clades made up most of the observed diversity (Figure 3.8). Specifically, Opisthokonta was the most abundant clade in all but one sample, with an average relative abundance of 12 – 60%, followed by Alveolata (4 – 70%), and

Stramenopiles (8 – 43%). Alveolata was the dominant superphylum clade only in the drain tank sample of the parsley aquaponics system. Meanwhile, at the phylum level (Figure 3.9) Ascomycota was the dominant group (relative abundance 5 – 53%), followed by Ciliophora (2 – 67%), Chlorophyta (2 – 44%), Basidiomycota (3 – 12%), Bacillariophyta (0.9 – 23%) and Oomycota (1 – 12%). Chlorophyta were generally more prevalent in drain tanks than fish tanks, while Ciliophora was by far the dominant phylum (67%) in the drain tank of the parsley aquaponics system. Pearson correlation coefficients were calculated to investigate linear correlation between eukaryotic phyla, indicating positive relationships between Ascomycota and Basidiomycota ($r = 0.95$), Basidiomycota and Euglenozoa ($r = 0.65$) and Oomycota and Eustigmatophyceae ($r = 0.8$). Meanwhile, Spearman correlation coefficient was also calculated to measure the strength and direction of monotonic association between the relative abundance of phyla, indicating positive correlation between Ascomycota and Basidiomycota ($r_s = 0.95$), Ascomycota and Euglenozoa ($r_s = 0.7$), Basidiomycota and Euglenozoa ($r_s = 0.73$), Oomycota and Euglenozoa ($r_s = 0.8$). Negative Spearman correlation was detected between Bacillariophyta and Ascomycota ($r_s = -0.7$) and Bacillariophyta and Basidiomycota ($r_s = -0.76$). Regarding the RAS samples, the fish tank suspended community was dominated by Ascomycota (53%) and Eustigmatophyceae (26%), while the biofilm communities of the fish tank walls and biofilter were very similar to each other and more diverse than the suspended community.

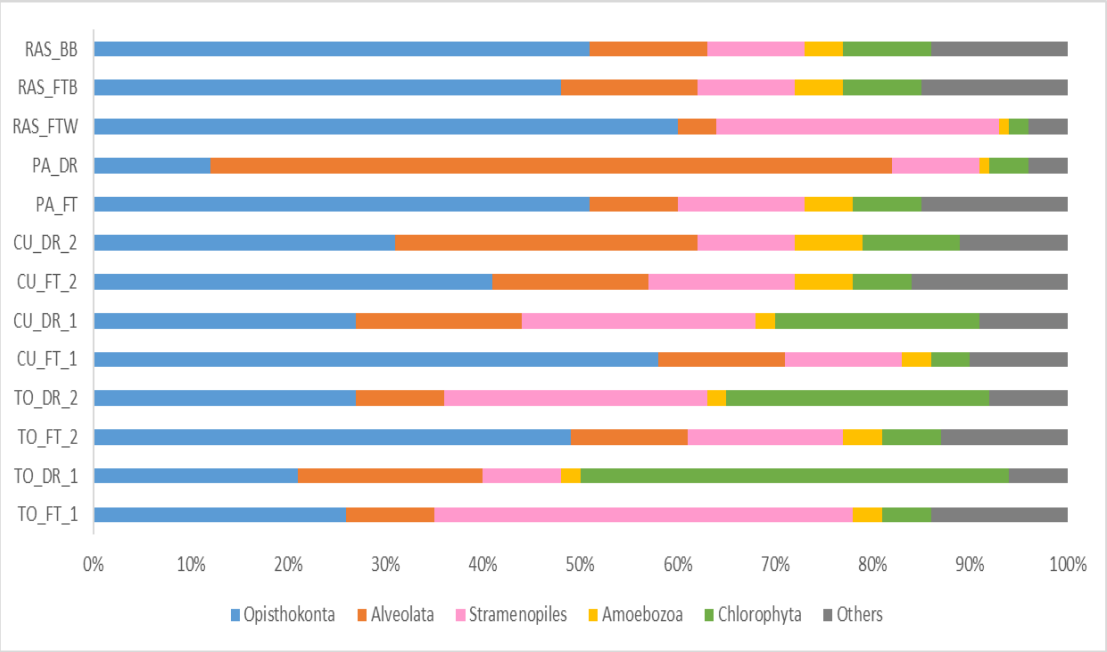



**Figure 3.8.** Relative abundance (%) of microeukaryotic clades at superphylum taxonomic level. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling, BB: biofilter biofilm, FTB: fish tank biofilm, FTW: fish tank water.

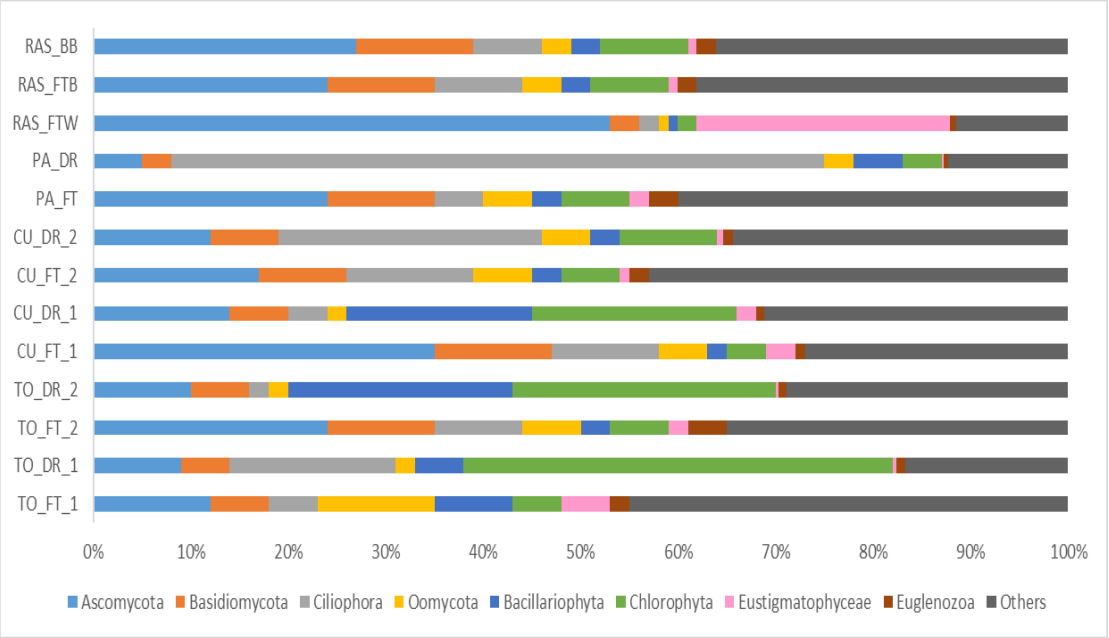



**Figure 3.9.** Relative abundance (%) of microeukaryotic clades at phylum taxonomic level. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling, BB: biofilter biofilm, FTB: fish tank biofilm, FTW: fish tank water.

Due to the very high number of taxa at the genus level (395-799) and their low relative abundances, genera were characterized as dominant if they were present in samples at a relative abundance ≥ 0.01% (Kurm et al., 2019). To make comparisons between communities in the aquaponics systems clearer, samples of the parsley aquaponics system were excluded from further analyses due to the lack of samples from a second sampling time in their case. Further analyses focused on samples from the fish tanks and drain tanks of the tomato and cucumber systems, taken near the start and conclusion of each production cycle. Using Venn diagrams (Figure 3.10) it was shown that all tomato and cucumber drain tank communities shared 16 abundant microeukaryotic genera, while all fish tank communities also shared 16 dominant genera. Among these groups of 16 genera, seven were shared, meaning that they were abundant in all aquaponics samples investigated. These were *Phaeodactylum*,

*Fragilariopsis*, *Thalassiosira* (Bacillariophyta), *Dictyostellium*, *Acanthamoeba* (Amoebozoa), *Aspergillus* (Fungi) and *Guillardia* (Cryptophyceaea). Furthermore, ANOSIM based on Bray-Curtis similarity between fish tank and drain tank communities revealed statistically clear differences ($p < 0.05$), which were then further examined using SIMPER. The main contributors to observed community differences were *Chlorella* (6.9%) and the dinoflagellate *Durinskia* (4.2%), as well as a few ciliates such as *Stentor* (4%), *Stylonychia* (2.7%) and *Oxytricha* (2.5%). Of the above, only *Stentor* was on average more abundant in the fish tanks compared to the drain tanks. Venn diagrams were also used to look into the shared genera in all tomato and cucumber aquaponics samples respectively. In this case, 10 dominant genera were shared in all tomato aquaponics samples, while 12 dominant genera were shared in all cucumber aquaponics samples. The subsequent ANOSIM showed no statistically clear difference ($p > 0.05$) between community composition in the two aquaponics systems, i.e. tomato and cucumber.

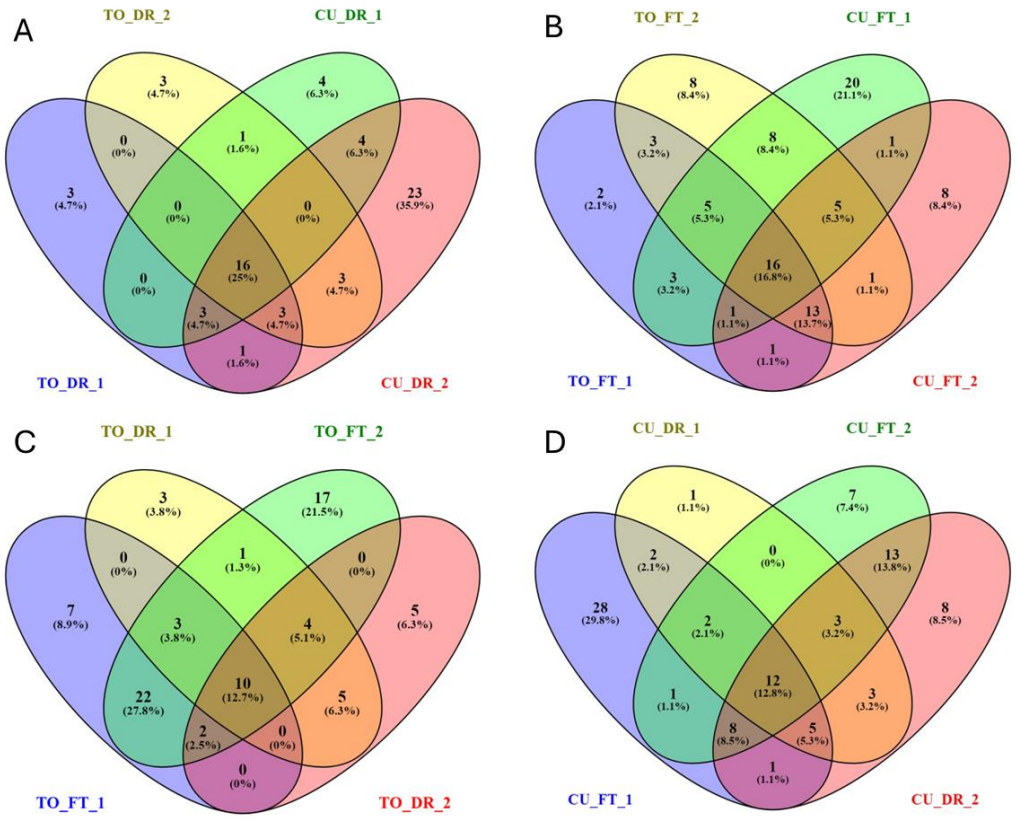
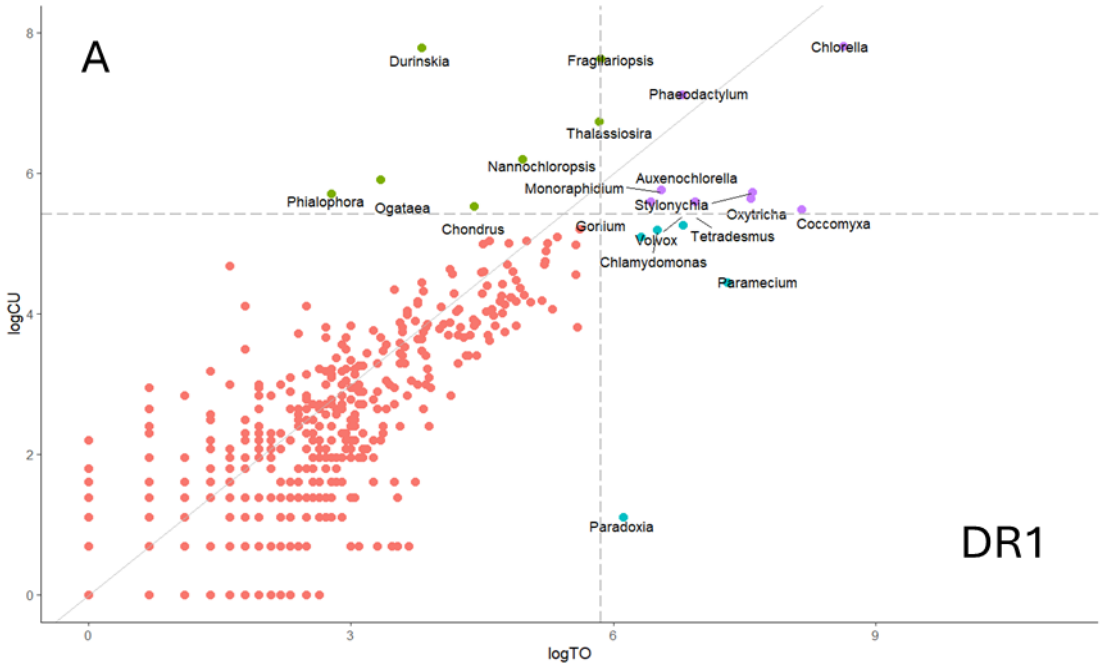
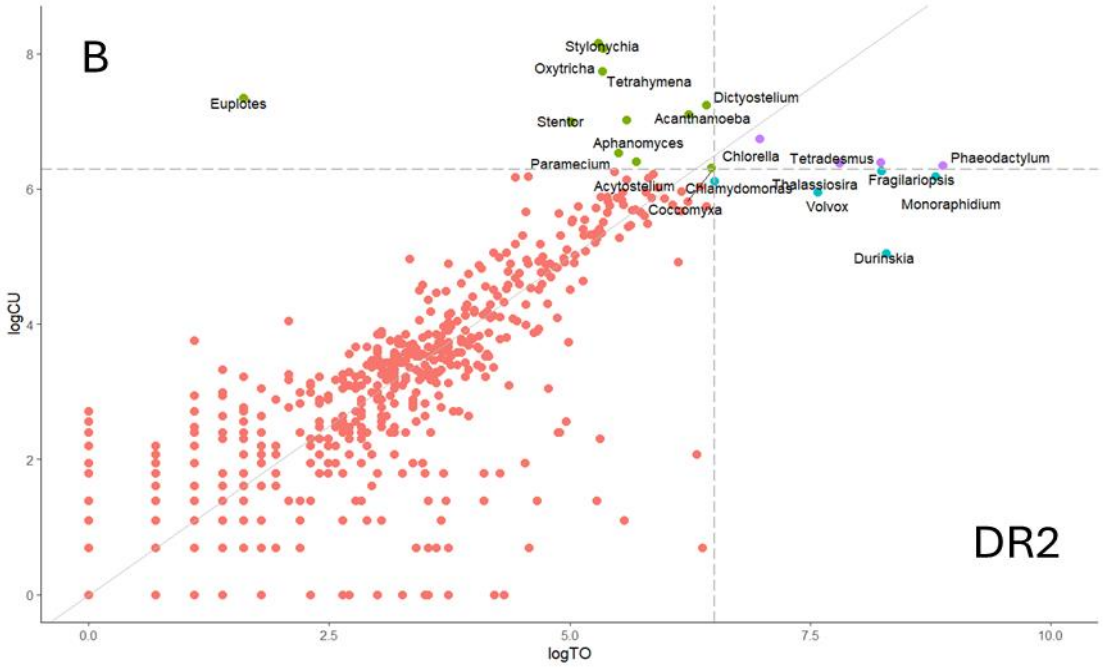



**Figure 3.10.** Number of shared dominant (≥ 0.01% relative abundance) microeukaryotic genera in aquaponics samples. **A**: drain tanks, **B**: fish tanks, **C**: tomato aquaponics, **D**: cucumber aquaponics. CU: cucumber aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

To compare the dominant microeukaryotic genera in the two aquaponics systems, looking into the respective compartment (i.e., fish tank and drain tank) and time point (i.e., start and conclusion of the cultivation period), abundances were natural logarithm transformed and relative abundances were plotted (Figure 3.11). Thus, *Chlorella*, *Tetradesmus* and *Phaeodactylum* were the dominant autotrophic microeukaryotes in all drain tank samples, with *Thalassiosira* becoming abundant over the course of the cultivation period. In addition, ciliates like *Stylonychia* and *Oxytricha* (family Oxitrichidae) were abundant in the drain tanks at the start of the cultivation period. The same ciliate genera were also abundant in the fish tanks of both systems

at the time of the first sampling, alongside *Nannochloropsis*, *Dictyostelium* and *Aphanomyces*. However, at the time of the second sampling, there were more abundant genera shared by both systems, including *Stentor*, *Naegleria*, *Dictyostelium*, *Nannocloropsis*, *Acanthamoeba*, *Thalassiosira*, *Phytophthora* (Oomycota), *Aphanomyces*, *Aspergillus*, *Ectocarpus* and *Aureococcus* (Stramenopiles). To further look for patterns in the abundance of dominant microeukaryotes, abundant genera were also presented in the form of a heatmap (Figure 3.12). Inspection of the heatmap showed that while *Chlorella* was among the most abundant genera in both systems' drain tanks at the time of the first samplings, its abundance decreased to an unremarkable level by the time of the second samplings. In addition, other photosynthetic genera like *Tetradesmus*, *Phaeodactylum* and *Monoraphidium* were found in higher abundance in the drain tanks of the tomato aquaponics system, compared to the cucumber aquaponics system. Furthermore, while *Stylonichia* and *Oxytricha* were commonly abundant ciliate genera in both systems, *Stentor* was clearly more abundant in the fish tanks of the cucumber aquaponics system.


A
Durinskia
Fragilariopsis
Chlorella
Phaeodactylum
Thalassiosira
Nannochloropsis
Auxenochlorella
Monoraphidium
Phialophora
Ogataea
Stylonychia
Oxytricha
Coccomyxa
Chondrus
Gorlium
Volvox
Tetradesmus
Chlamydomonas
Paramecium
Paradoxia
DR1
logCU
logTO
0
2
4
6
8
0
3
6
9


B
Stylonychia
Oxytricha
Tetrahymena
Euplotes
Dictyostelium
Stentor
Acanthamoeba
Aphanomyces
Chlorella
Tetradesmus
Phaeodactylum
Paramecium
Thalassiosira
Fragilariopsis
Acytostelium
Chlamydomonas
Volvox
Monoraphidium
Coccomyxa
Durinskia
DR2
logCU
logTO
0
2
4
6
8
0.0
2.5
5.0
7.5
10.0

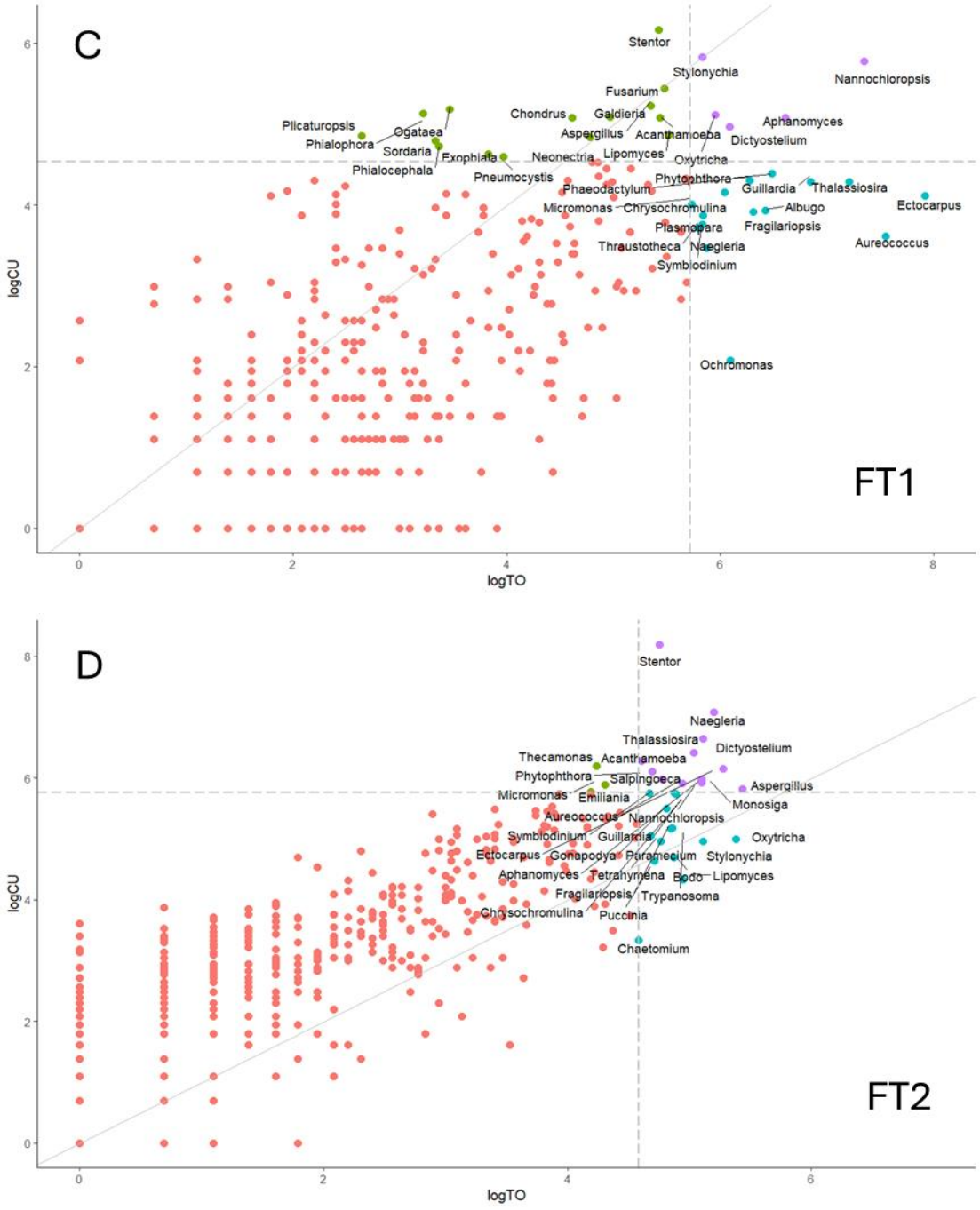



**Figure 3.11.** Natural logarithm transformed abundance of microeukaryotic genera in the cucumber (CU) and tomato (TO) aquaponics systems. **A**: Drain tank, first sampling, **B**: Drain tank, second sampling, **C**: Fish tank, first sampling, **D**: Fish tank, second sampling. Genera with relative abundance ≥ 0.01% (dashed grey lines) in both TO and CU samples (purple dots), only in CU samples (green dots) and only in TO samples (blue dots). Genera along the diagonal line had equal abundance in both aquaponics systems. Red dots indicate non-abundant taxa.

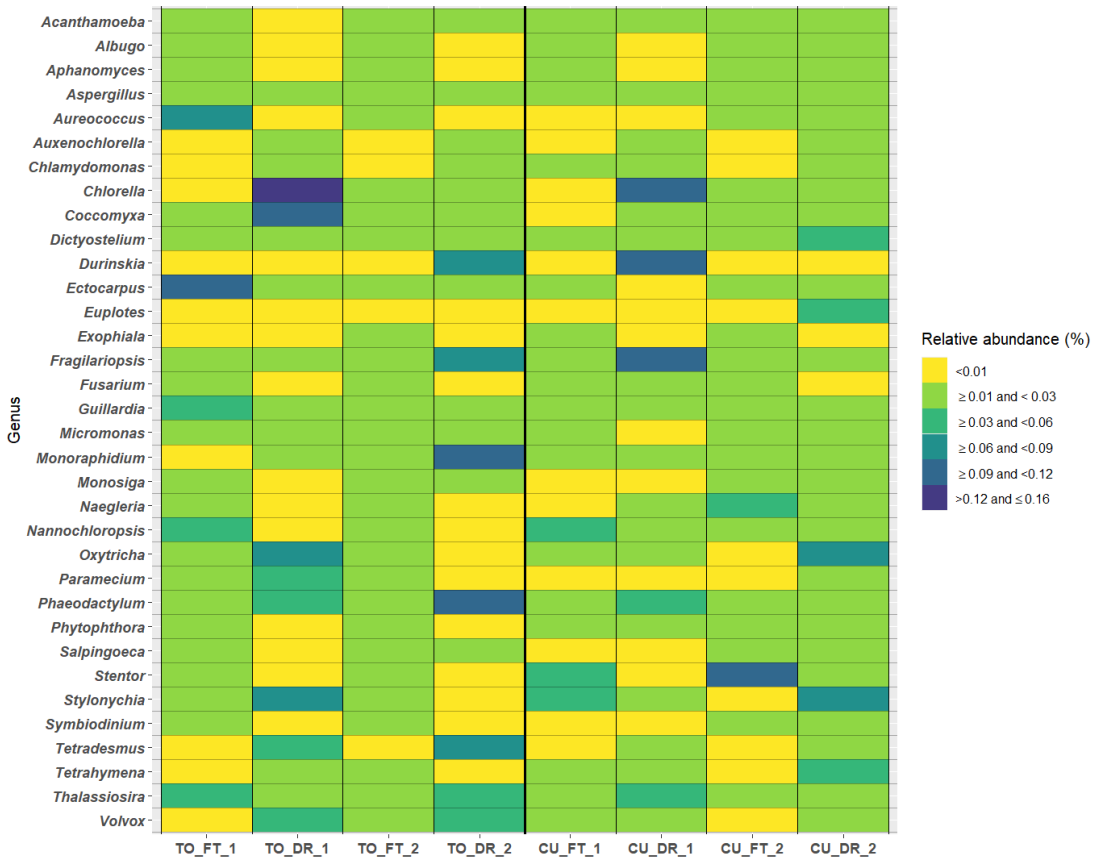



**Figure 3.12**. Relative abundance (%) heatmap of microeukaryotic genera with relative abundance ≥ 0.01% in at least one sample of the studied aquaponics systems. CU: cucumber aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

In the case of the RAS microeukaryotic communities, only five genera (Figure 3.13) were abundant in all three types of samples (i.e., fish tank water, fish tank biofilm and biofilter biofilm). Four of these genera were Fungi, namely *Aspergillus*, *Colletotrichum*, *Fusarium* and *Penicillium*, with *Nannocloropsis* (Stramenopiles) being the fifth. Fish tank and biofilter biofilm communities shared 41 abundant genera, about 54% of the total abundant genera in all three sample types. The fish tank biofilm exclusively harbored 9 abundant genera, namely *Galdieria* (Rhodophyta), Ichthyophthirius, Pseudocohnilembus (Ciliophora), *Bathycoccus*, *Chlamydomonas*, *Gonium* (Chlorophyta), *Plasmodium* (Apicomplexa), *Albugo* (Oomycota) and *Naegleria*

(Heterolobosea). Lastly, the biofilter attached community was exclusively enriched in the genera *Porphyra*, *Cyanidioschyzon* (Rhodophyta), *Fonsecaea*, *Hesseltinella*, *Mortierella*, *Schizosaccharomyces* (Fungi) and *Trypanosoma* (Euglenozoa).

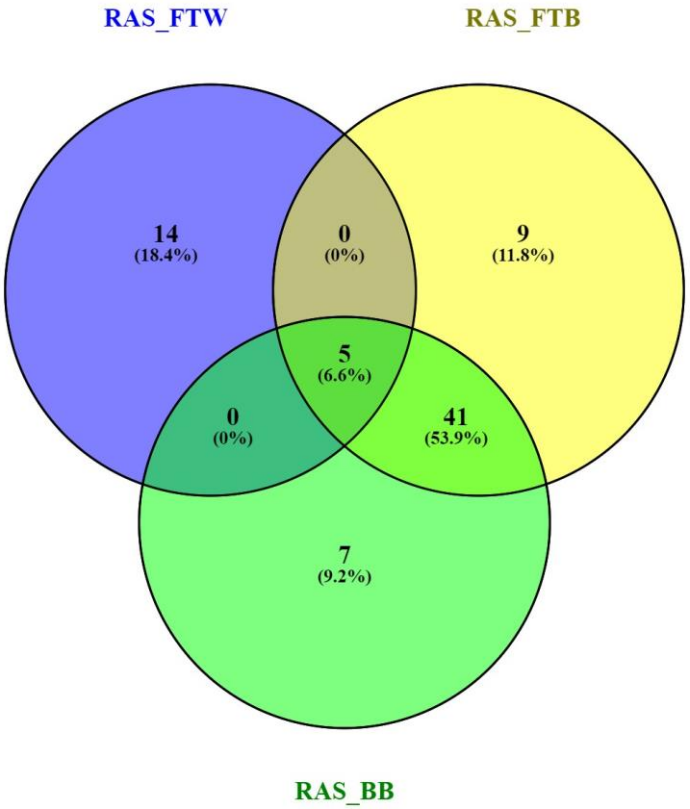



**Figure 3.13.** Number of unique and shared dominant (≥ 0.01% relative abundance) microeukaryotic taxa in all three RAS samples. FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm.

## 3.3. Discussion

In the present study, in situ growth experiments were carried out in experimental aquaponics and RAS compartments to measure ciliate and HNF possible growth rates, while metagenomic analysis was performed on DNA samples from various aquaponics and RAS systems compartments to characterize their microeukaryotic diversity.

Total ciliate abundance ranged between 732 to 5451 cells $L^{-1}$ and exhibited high variability across samples from different compartments. Similarly low abundances have been previously reported by studies on rivers (e.g., Rychert, 2009; Scherwass and Arndt, 2005) and mesotrophic or oligotrophic lakes (e.g., Kalinowska, 2004; Macek et al., 2006; Šimek et al., 2000). Ciliate growth rates were clearly higher in the fish tanks of the tomato aquaponics system in both growth experiments, reaching 1.9 ± 0.01 and 1.26 ± 0.13 $d^{-1}$, respectively, near the start and near the conclusion of the tomato production cycle. The growth rates estimated in the present study are within the range of observations previously published (i.e., 0.10 – 2.40 $d^{-1}$) regarding freshwater ciliate communities or isolated species (Carrias et al., 2001; Rychert, 2016; Weisse et al., 2021; Weitere et al., 2005). Furthermore, the higher growth rates in the aquaponics fish tanks can be attributed to the rapid growth of certain ciliate taxa such as the *Cyclidium* morphotype which indeed grew clearly faster in the fish tanks, with a growth rate that ranged between 0.55 ± 0.04 to 2.12 ± 0.01 $d^{-1}$ across all compartments. Indeed, *Cyclidium* species have been shown to be capable of rapid growth (1.92 to 5.00 $d^{-1}$, Rychert, 2016), and it's likely that during bottle incubation *Cyclidium* cells can benefit by attaching on bottle surfaces (Esteban and Fenchel, 2020) and increasing their filter-feeding efficiency (Macek et al., 1996). Apart from the *Cyclidium* morphotype which dominated the fish tank incubation bottles, *Halteria* was another morphotype which achieved rapid growth (i.e., 0.55 ± 0.08 $d^{-1}$) in drain tank incubation bottles. *Halteria* were shown capable of comparable growth rates in eutrophic freshwater systems (0.42 ± 0.25 $d^{-1}$, Macek et al., 1996) where they act as filter-feeders preying on bacteria and small HNF (Šimek et al., 2000).

Meanwhile, HNF abundance ranged between 58 ± 8 and 147 ± 18 cells $mL^{-1}$ in the different aquaponics compartments. These relatively low values can be also found in natural environments such as mesotrophic and oligotrophic lakes (e.g., Berdjeb et al., 2011; Cai et al., 2020) as well as rivers (e.g., Bouvy et al., 2010; Kiss et al., 2009; Scherwass et al., 2010). HNF growth rates in the drain tanks were clearly lower compared to the other compartments, ranging from 0.5 ± 0.3 to 1.37 ± 0.05 $d^{-1}$. These values were on par with growth rates previously measured in natural HNF assemblages

in freshwater habitats (e.g., Šimek et al., 2018). However, much higher growth rates were measured in FT and SUMP incubation bottles, between 4.09 ± 0.11 and 6.03 ± 0.34 $d^{-1}$. Such rapid growth is usually associated with lab cultures and species-specific growth rates (e.g., Eccleston Parry and Leadbeater, 1994). It is likely that this clear difference in growth rates is a result of the reduced efficiency of the size fractionation method (Rychert, 2013) in removing all putative predators of HNF in the drain tanks. Specifically, the increased presence of *Halteria* is a possible indicator that the known small-sized ciliate bacterivore was outcompeting the HNF population in the drain tanks.

Overall, based on ciliate and HNF natural abundance and putative growth rates, there are indications that different compartments offer different niches for the persistence and proliferation of different heterotrophic protists. Fish tanks appear to mainly promote the growth of ciliates, while drain tank conditions seem to limit the growth potential of HNF. Nevertheless, although there is potential for rapid ciliate and HNF growth due to the lack of larger predators of protists and an adequate concentration of prokaryotic prey to sustain growth (Esteban and Fenchel, 2020; Weisse and Montagnes, 2021), ciliate and HNF natural abundance remains relatively low as in the case of riverine ecosystems, possibly indicating that continuous water flow may also be disrupting feeding and growth of heterotrophic protists in aquaponics compartments. As for the RAS, total ciliate and HNF abundance showed no increase over time during the incubation and instead ciliate population declined. Several reasons can potentially explain this observation. Firstly, the RAS operated with artificial seawater, meaning that the natural planktonic microeukaryotic community of the source groundwater likely had restricted chances to adapt to the increased salinity and soon perished after being introduced to the system. In particular, salinity has been established as a major driver of ciliate abundance and diversity (Maicá et al., 2012). Secondly, heterotrophic protist population decline could be the result of bottom-up limitation, as prokaryotic abundance in the RAS was $6.24 \times 10^5$ bacteria $mL^{-1}$, below the reported minimum concentration ($10^6$ bacteria $mL^{-1}$) required to sustain ciliate and flagellate bacterivorous communities in natural aquatic environments (Esteban and

Fenchel, 2020; Weisse and Montagnes, 2021). Thirdly, heterotrophic protists in the RAS could be subject to increased top-down control through predation by rotifers which were detected in the samples. Fourthly, several ciliates including *Halteria* can be negatively impacted by incubation because of the fragility of their cells, leading to negative growth rates in similarly set up experiments (Carrias et al., 2001; Sime-Ngando et al., 1990). Still, based on total and specific ciliate natural abundances, the two RAS lines examined in the present study appeared to be very dynamic systems that may develop distinct ciliate communities over time, despite being considered replicates in principle.

Based on all the microscopy derived data of the present study, it was concluded that fish rearing tanks and drain tanks after plant fertigation were the two main distinct niches in the aquaponics systems, and thus their microbial communities were worth investigating through metagenomics analysis. As usual, fish tanks and biofilter tanks were the main niches in the RAS. Statistical analysis of metagenomic data revealed a clearly higher number of microeukaryotic reads in the drain tank samples, indicating a higher abundance of eukaryotes in the drain tanks compared to the fish tanks. This observation can be explained by the notable presence of abundant photosynthetic taxa such as *Chlorella*, *Tetradesmus* and *Phaeodactylum* in the drain tanks, all of which were also detected through microscopy as well in the tomato aquaponics drain tanks. In addition, ciliates like *Oxytricha* and *Stylonychia* were on average more abundant in drain tanks, also contributing to the total higher abundance of eukaryotic taxa. *Oxytricha* is among the most diverse ciliate genera, and it presently includes approximately 50 species (Han et al., 2025). Its presence has been detected in various environments such as soil, freshwater and hot springs (Zhu et al., 2021), and it seems to be an important component of the heterotrophic community in aquatic microbial mats (Mieczan and Adamczuk, 2016). In addition, the high abundance of *Stylonychia* in the drain tanks could be another indication of the proper function of the aquaponics system, as this genus cannot proliferate in environments with high biochemical oxygen demand (BOD), organic content and ammonia concentration (Agrawal, 2017). Despite the lower microeukaryotic abundance in the fish tanks,

eukaryotic diversity was higher based on the inverse Simpson index. As the fish tank community is characterized by higher diversity and lower abundance, it could be described as more stable and balanced, better able to withstand a likely perturbation. The good ecological health of the fish tanks can also be highlighted by the fact that *Stentor* was the main abundant ciliate which was found in higher abundance in fish tank samples compared to drain tanks. *Stentor* has been associated with stable wastewater communities and balanced biomass environments (Kuśnierz et al., 2022).

Apart from ciliates and phytoplankton, a couple of Oomycota genera were abundant in the late-stage aquaponics samples. Specifically, the genus *Phytophthora* which consists of nearly 200 mostly pathogenic species is well known for disrupting crops worldwide. While fortunately certain *Phytophthora* pathogens are specialized, other species such as *Phytophthora cactorum* can infest a wide range of plant targets (Chen et al., 2023). *Aphanomyces* was another genus detected in high proportion in the aquaponics systems. This genus contains about 40 species which act as either plant and animal pathogens or saprotrophs. For example, *Aphanomyces cladogamus* can cause disease in tomato plants among several other crops (Wikström et al., 2025). Therefore, certain *Aphanomyces* species can potentially cause disease outbreaks in aquaponics systems, and the available options for controlling pathogenic *Aphanomyces species* in agriculture are still limited (Wikström et al., 2025). However, putative plant growth promoting fungi (PGPF) like *Aspergillus* (Ascomycota) were also present in high abundance in the aquaponics samples of the present study. PGPF generally enhance plant growth by increasing the bioavailability of nutrients and help protect plants such as tomatoes against pathogens including *Fusarium* spp. (Abdel-Motaal et al., 2020; Attia et al., 2022). Thus, given their increased abundance and possible functional role as pathogens or PGPF in aquaponics, Fungi and Oomycota should be further investigated in the future.

Investigation of the microeukaryotic community in RAS samples showed major differences to the results of the only previously published study in the same context (Boaventura et al., 2018), where authors reported the dominance of Stramenopiles and Metazoa in sole and turbot RAS. Instead, the communities

examined in the present were shown to be dominated by Opisthokonta (mainly Ascomycota and Basidiomycota) followed by Alveolata and Stramenopiles. Fish tank biofilm and biofilter biofilm harbored similar taxa and differed in composition compared to the suspended fish tank community. This distinction was evident both at the phylum level and in the composition of abundant genera. Interestingly, four out of five shared abundant microeukaryotic genera in all RAS niches belonged to Ascomycota. Furthermore, fish tank biofilm contained a couple of putatively pathogenic genera in abundance, namely *Ichthyophthirius* and *Pseudocohnilembus*. *Ichthyophthirius multifiliis* is a widespread parasitic ciliate which causes the white spot disease in a multitude of freshwater fish species (Yang et al., 2023). Meanwhile *Pseudocohnilembus* is part of the scuticociliate group, an assortment of common non-specialized culture fish parasites and capable of causing scuticociliatosis, a prevalent parasitic disease with severe impact in aquaculture production. *Pseudocohnilembus persalinus* has been previously isolated from infected sea bass individuals but the disease mainly affects other fish (Wang et al., 2025). As in the case of the aquaponics systems, the dominant ciliate genera in the RAS biofilms were *Stylonychia*, *Oxytricha* and *Stentor*, once again different genera from the abundant *Zoothamnium* reported by Boaventura et al. (2018), while no ciliates were present in > 0.01% relative abundance in the fish tank water community. It would seem that biofilms forming on RAS surfaces allow the persistence and growth of several ciliate, fungal and Oomycota genera despite the fact that most of the microeukaryotic community evidently cannot proliferate in the artificial seawater environment, as indicated by the difference in relative abundances, the low natural abundance of ciliates and HNF measured through microscopy and the negative growths rates during the incubations.

# 4. Prokaryotic diversity in different aquaponics and RAS compartments

## 4.1. Introduction

The function of aquaponics systems relies on the performance of their nitrifying prokaryotic community, which in turn is affected by environmental factors as well as interactions with the rest of the microbial community including heterotrophic Bacteria, Archaea and microeukaryotes. While it is relatively simple to manage physicochemical parameters to promote optimal system performance, predicting and controlling biological interactions within the microbial community is a far more complicated task. The first step in this process is the characterization of the microbial community present in aquaponics systems. Regarding the prokaryotic taxa, a handful of studies have so far investigated the diversity, composition and function of Bacteria in different niches such as farmed fish intestine (Gao et al., 2022a) and feces (Schmautz et al., 2022), fish tank water (Kasozi, et al., 2021a) and cultured plant roots (Oliveira et al., 2020). Apart from the well-studied nitrifiers, certain bacterial taxa such as *Pseudomonas* spp. have been associated with plant growth promotion (Eck et al., 2019b), biocontrol through the excretion of antimicrobial compounds or nutrient competition (Schmautz et al., 2017), or increasing fish tolerance against other pathogenic bacteria (Moghaddam et al., 2012). Furthermore, potential fish pathogenic taxa from the genera *Aeromonas*, *Acinetobacter*, *Arcobacter*, *Clostridium* and *Flavobacterium* have been detected in such aquaculture systems (Rieder et al., 2023; Xiong et al., 2015). In addition, specific bacterial taxa have exhibited correlation with the presence of antibiotic resistance genes (ARG) whose levels can be high in aquaponics systems even without the use of antibiotics (Kampouris et al., 2022).

Despite this pioneer work in understanding microbial community structure and function in the various aquaponics niches, there is still a lack of information regarding both the archaeal and microeukaryotic community structure and function. Archaea have long been overlooked due to their low abundance in less extreme

environments but their potential to outcompete Bacteria in nutrient limited conditions makes them prime candidates for further investigation, making use of ever-expanding genome databases. Similarly, while the prokaryotic community in aquaponics and RAS has been extensively studied, there has been no characterization of its interactions with the microeukaryotic community of these systems (Kushwaha et al., 2025). Finally, functionally important low abundant prokaryotic taxa within aquaponics (Changey et al., 2025; Kasozi et al., 2021a) still need to be described in future studies.

## 4.2. Results

Metagenomic analysis of the aquaponics systems' prokaryotic communities showed that bacterial reads made up 98% – 99% of the total amount while archaeal reads were represented by a much lower percentage, between 0.05 – 0.6%. Rarefaction curves were well saturated for all samples in the case of Bacteria and in nearly all samples for Archaea (Appendices 8 and 9 respectively), Good's coverage was > 0.99 for all samples, and the estimated coverage was adequate (Appendix 10) showing that the sequencing depth was enough to obtain a reliable representation of prokaryotic diversity. Richness of bacterial genera (Table 4.1) ranged between 1989 and 2051 in all aquaponics samples and total bacterial reads were $2.7 \times 10^6$ to $6.5 \times 10^6$ across samples. In fact, bacterial richness was clearly higher (t-test, $p < 0.05$, Figure 4.1.A) in the drain tank samples (average = 2049.2, SD = 10.3) compared to the fish tanks (average = 2015, SD = 22.5). In terms of alpha diversity, the Shannon (H) index ranged between 3.32 and 5.47 while the inverse Simpson (1/D) index ranged between 5.54 and 61.5. Meanwhile, archaeal richness (Table 4.2) was between 93 and 132 genera and archaeal reads ranged between 1734 and 35145. Both richness and number of reads were clearly higher (Wilcoxon test, $p < 0.05$, effect size $r \approx 0.83$, Figure 4.1.B-C) in the drain tanks, with 130.4 genera on average (SD = 1.5) and 15234.4 reads (SD = 14057) compared to the fish tank samples which had 114.6 genera (SD = 13.3) and 2862.2 reads (SD = 949) respectively.

**Table 4.1.** Alpha diversity metrics of the bacterial community at genus level in the aquaponics samples based on metagenomic data. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

| Sample | Richness | Shannon (H) | Inverse Simpson (1/D) | Reads |
|---|---|---|---|---|
| CU_DR_1 | 2035 | 4.56 | 17.0 | 5006584 |
| CU_DR_2 | 2048 | 3.99 | 5.86 | 5769068 |
| CU_FT_1 | 1989 | 3.78 | 7.48 | 6439003 |
| CU_FT_2 | 2039 | 4.46 | 9.87 | 4497869 |
| PA_DR | 2051 | 4.94 | 22.3 | 4005227 |
| PA_FT | 2034 | 5.47 | 61.5 | 2745755 |
| TO_DR_1 | 2048 | 5.05 | 30.7 | 2863344 |
| TO_DR_2 | 2064 | 5.30 | 46.3 | 4763128 |
| TO_FT_1 | 1995 | 3.32 | 5.54 | 6523429 |
| TO_FT_2 | 2018 | 4.60 | 23.1 | 4636531 |

**Table 4.2.** Alpha diversity metrics of the archaeal community at genus level in the aquaponics samples based on metagenomic data. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

| Sample | Richness | Shannon (H) | Inverse Simpson (1/D) | Reads |
|---|---|---|---|---|
| CU_DR_1 | 128 | 3.42 | 11.3 | 4859 |
| CU_DR_2 | 131 | 3.47 | 8.55 | 7151 |
| CU_FT_1 | 93 | 3.58 | 23.1 | 2050 |
| CU_FT_2 | 127 | 4.15 | 34.8 | 3852 |
| PA_DR | 130 | 4.16 | 34.6 | 4021 |
| PA_FT | 124 | 5.16 | 33.2 | 2992 |
| TO_DR_1 | 131 | 1.65 | 2.68 | 24996 |
| TO_DR_2 | 132 | 1.79 | 2.81 | 35145 |
| TO_FT_1 | 114 | 3.04 | 8.12 | 3683 |
| TO_FT_2 | 115 | 3.94 | 28.2 | 1734 |

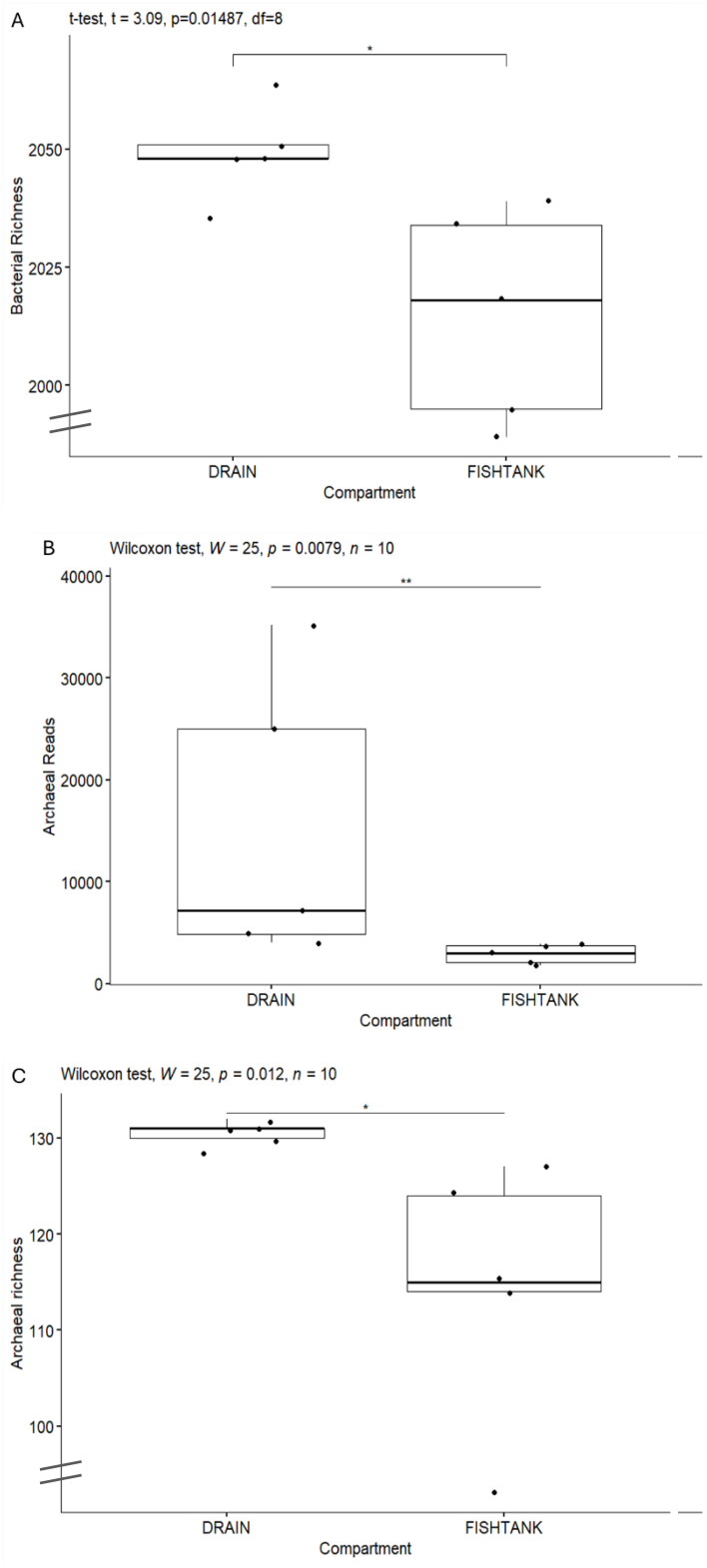



**Figure 4.1.** Box-plot distribution of aquaponics samples based on bacterial richness (A), archaeal read count (B) and archaeal richness (C), with test results for statistically

clear differences between the fish tank and drain compartments. In subfigures B and C there is a break in the y axis to highlight the small variations.

Looking into the beta diversity of the aquaponics bacterial communities, PERMANOVA was performed in R using the Adonis2 function, indicating a statistically clear difference between sample group composition ($p = 0.002$, $R^2 = 0.48$). Following NMDS analysis, it was shown that bacterial communities (Figure 4.2) were grouped by aquaponics system (i.e., different cultured plant) whereas archaeal communities were grouped by compartment ($p = 0.021$, $R^2 = 0.407$) in the same samples (Figure 4.3). Furthermore, clustering analysis of the bacterial community based on Bray-Curtis similarity using the UPGMA method (Figure 4.4) showed that community composition in all aquaponics samples shared a base similarity of about 40%, and while the starting community of the cucumber growing system (CU) was more similar to the tomato growing system's (TO) community (55% similarity), by the end of the production cycles the cucumber system's community was more similar to the parsley system's (PA) community (60% similarity). This could indicate either a temporal shift of the bacterial community of the cucumber system towards the community of the parsley system or a further shift of the tomato system's community away from the communities of both other systems. Meanwhile, clustering analysis of the archaeal communities (Figure 4.5) showed that drain tank samples from the tomato and cucumber systems had a base similarity of 52.5%, and these respective communities remained similar over time. Furthermore, fish tank archaeal communities in the three aquaponics systems were 65.7% similar near the end of their respective production cycles, and among these the cucumber and parsley systems' fish tank communities were 82.5% similar.

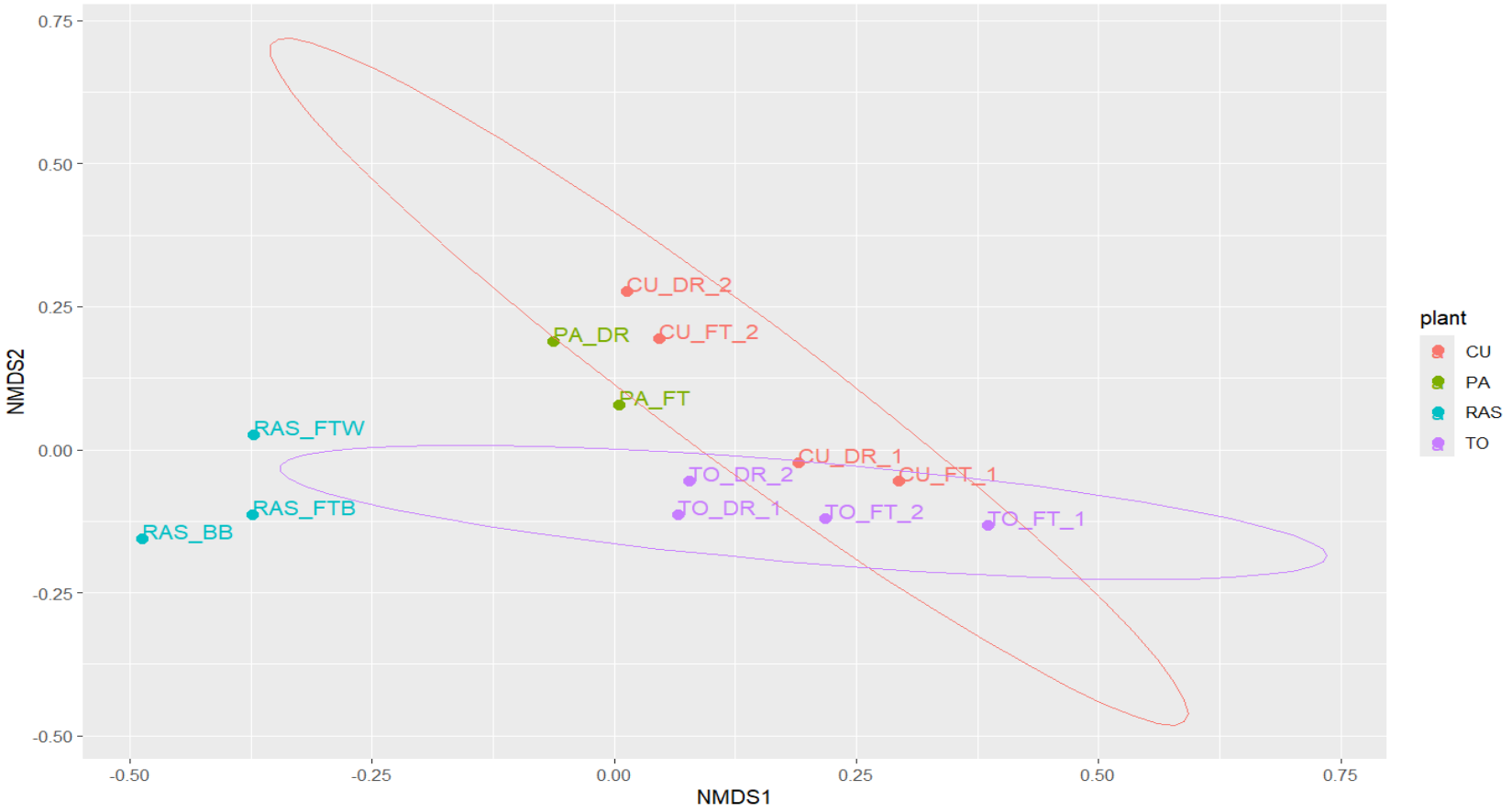

0.75
0.50
0.25
0.00
-0.25
-0.50
NMDS2
-0.50
-0.25
0.00
0.25
0.50
0.75
NMDS1
CU_DR_2
PA_DR
CU_FT_2
PA_FT
RAS_FTW
CU_DR_1
JO_DR_2
CU_FT_1
RAS_FTB
JO_DR_1
JO_FT_2
JO_FT_1
RAS_BB
plant
CU
PA
RAS
TO

**Figure 4.2.** NMDS plot depicting the grouping of bacterial communities based on the aquaponics system sampled (TO: tomato aquaponics, CU: cucumber aquaponics, PA: parsley aquaponics, DR: drain tanks, FT: fish tanks, 1: first sampling time, 2: second sampling time, FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm).

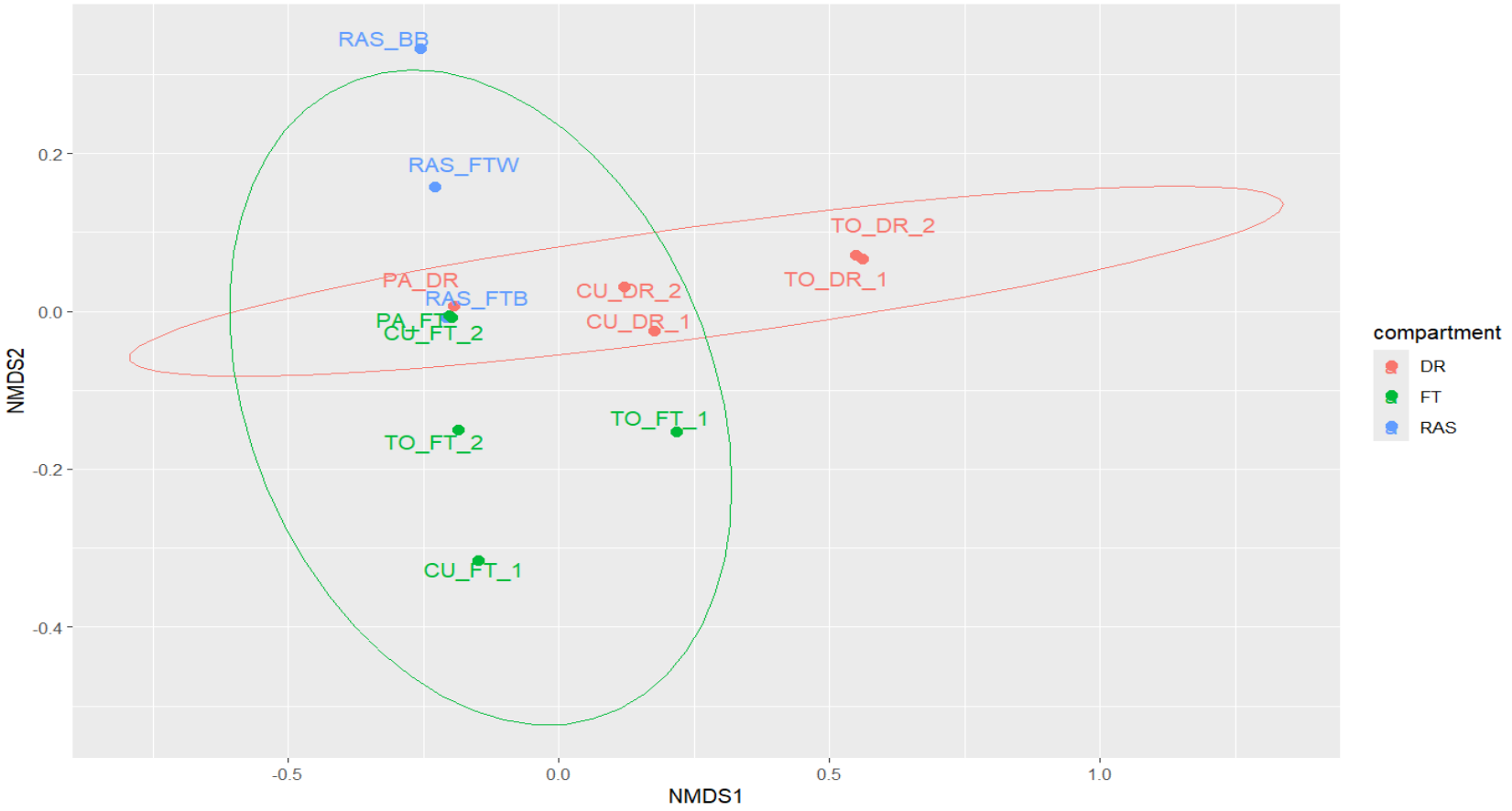

RAS_BB
RAS_FTW
PA_DR
RAS_FTB
PA_FT
CU_FT_2
TO_FT_2
CU_FT_1
CU_DR_2
CU_DR_1
TO_FT_1
TO_DR_2
TO_DR_1
0.2
0.0
-0.2
-0.4
NMDS2
-0.5
0.0
0.5
1.0
NMDS1
compartment
DR
FT
RAS

**Figure 4.3.** NMDS plot depicting the grouping of archaeal communities based on the aquaponics compartment sampled (DR: drain tanks, FT: fish tanks, TO: tomato aquaponics, CU: cucumber aquaponics, PA: parsley aquaponics, 1: first sampling time, 2: second sampling time, FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm).

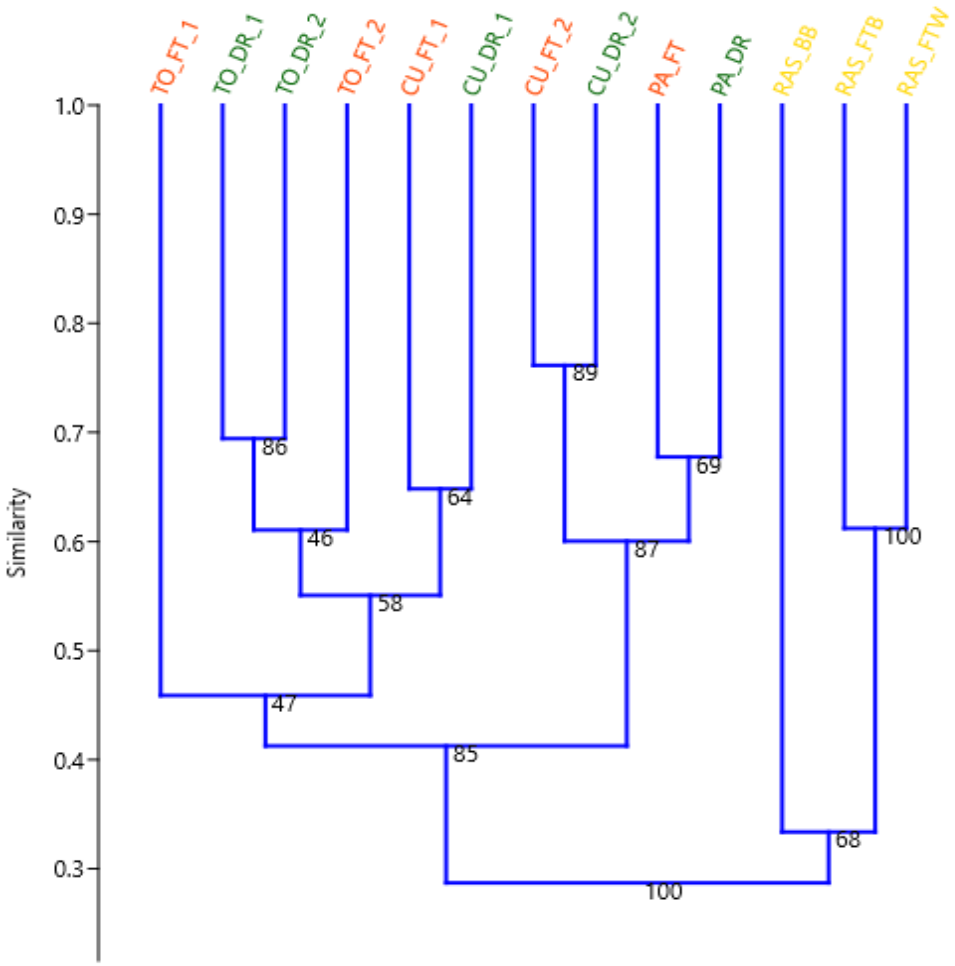



**Figure 4.4.** Clustering of samples based on bacterial community composition using the Bray–Curtis similarity index and the UPGMA method. Bootstrap values (N=1000) are displayed at each node. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling, FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm.

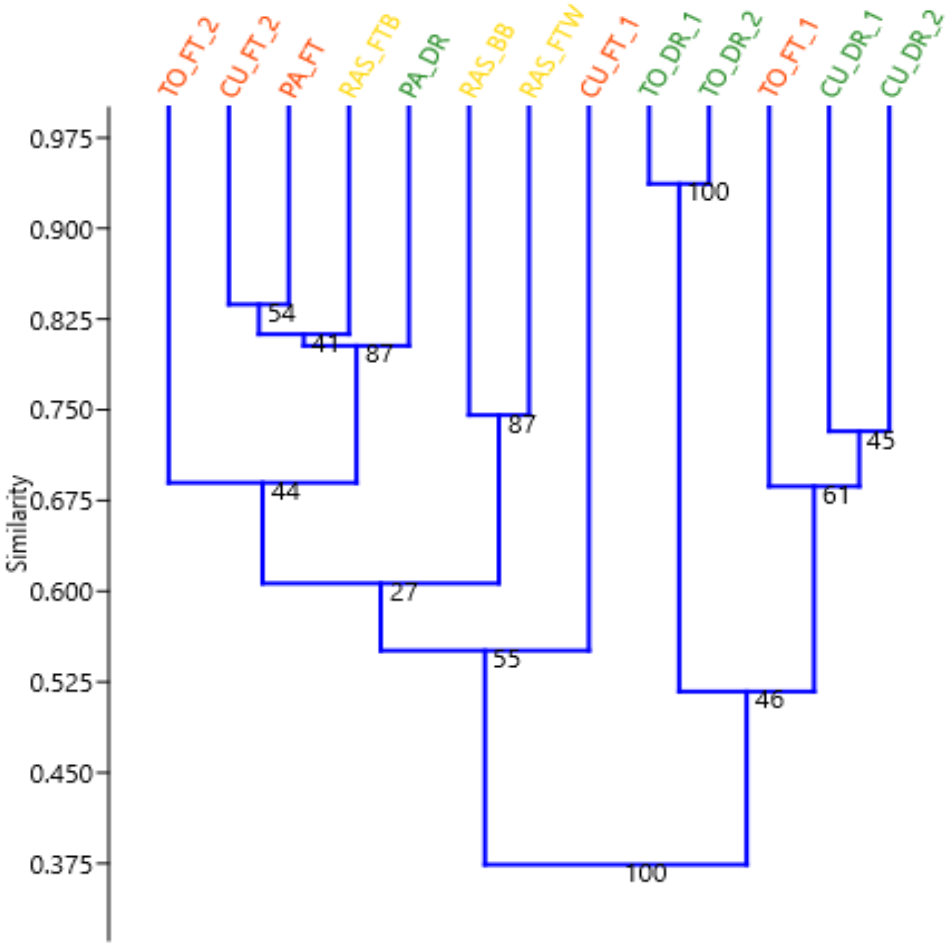



**Figure 4.5.** Clustering of samples based on archaeal community composition using the Bray–Curtis similarity index and the UPGMA method. Bootstrap values (N=1000) are displayed at each node. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling, FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm.

At the phylum level (Figure 4.6), archaeal communities largely consisted of Methanobacteriota (14 – 76%), Nitrososphaerota (5 – 79%), *Candidatus* Pacearchaeota (0.5 – 15%), Thermoproteota (0.8 – 4%) and *Candidatus* Micrarchaeota (0.5 – 5%). Simultaneously, bacterial communities were mainly represented by the phyla (Figure 4.7) Pseudomonadota (23 – 70%), Bacteroidota (6 – 57%),

Actinomycetota (5 – 45%), Verrucomicrobiota (0.2 – 6%), Planctomycetota (0.1 – 4%) and Cyanobacteriota (0.09 – 1%). Comparison of the relative abundance of these phyla across samples showed a clearly higher proportion of Bacteroidota in the tomato aquaponics samples ($p = 0.03$, Mann-Whitney test) compared to the cucumber aquaponics samples. In addition, Cyanobacteriota abundance was clearly higher ($p < 0.01$, t-test) in drain tanks compared to fish tanks despite their overall low representation. At the same time, the prominent bacterial classes (Figure 4.8) in the aquaponics samples were Betaproteobacteria (11 – 39%), Actinomycetes (5 – 45%), Alphaproteobacteria (5 – 28%), Flavobacteriia (2 – 55%), Gammaproteobacteria (2 – 14%), Chitinophagia (0.2 – 8%), Cytophagia (0.3 – 6%), Verrucomicrobiia (0.2 – 6%), Sphingobacteriia (0.1 – 6%) and Deltaproteobacteria (0.2 – 5%).

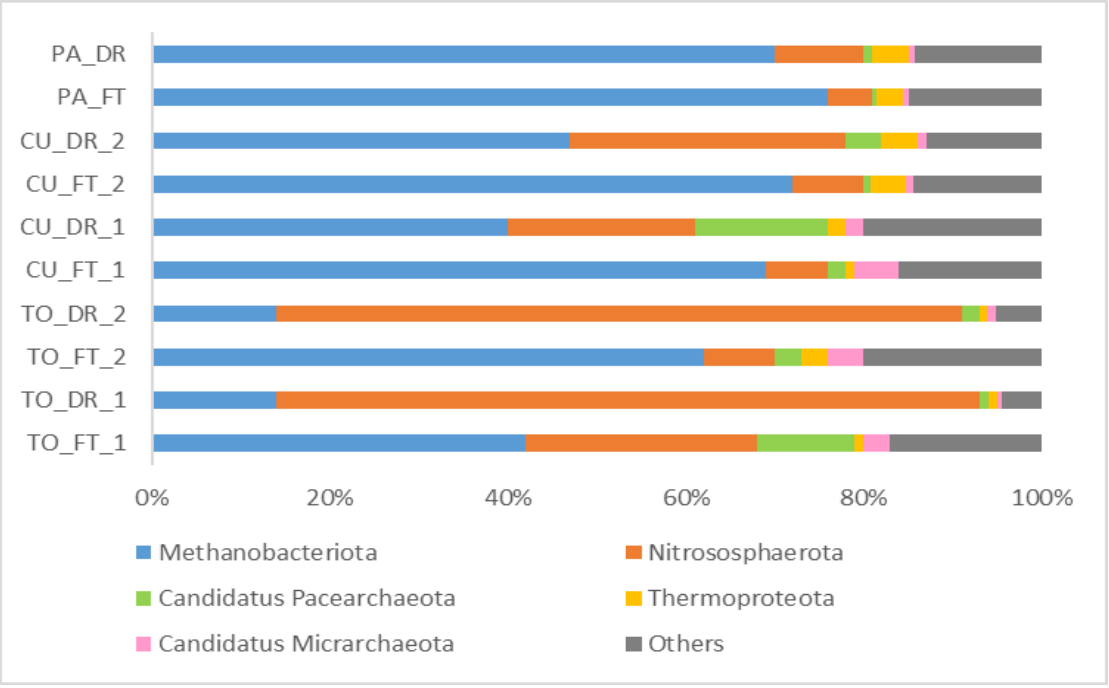



**Figure 4.6.** Relative abundance (%) of archaeal phyla in aquaponics samples. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

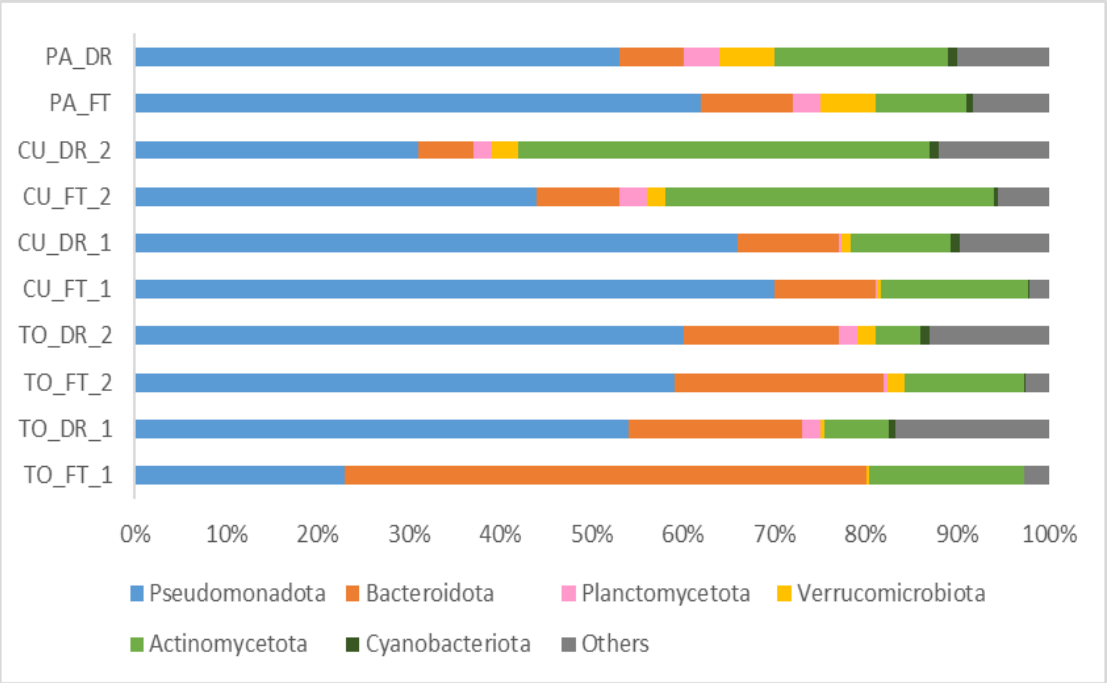



**Figure 4.7.** Relative abundance (%) of bacterial phyla in aquaponics samples. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

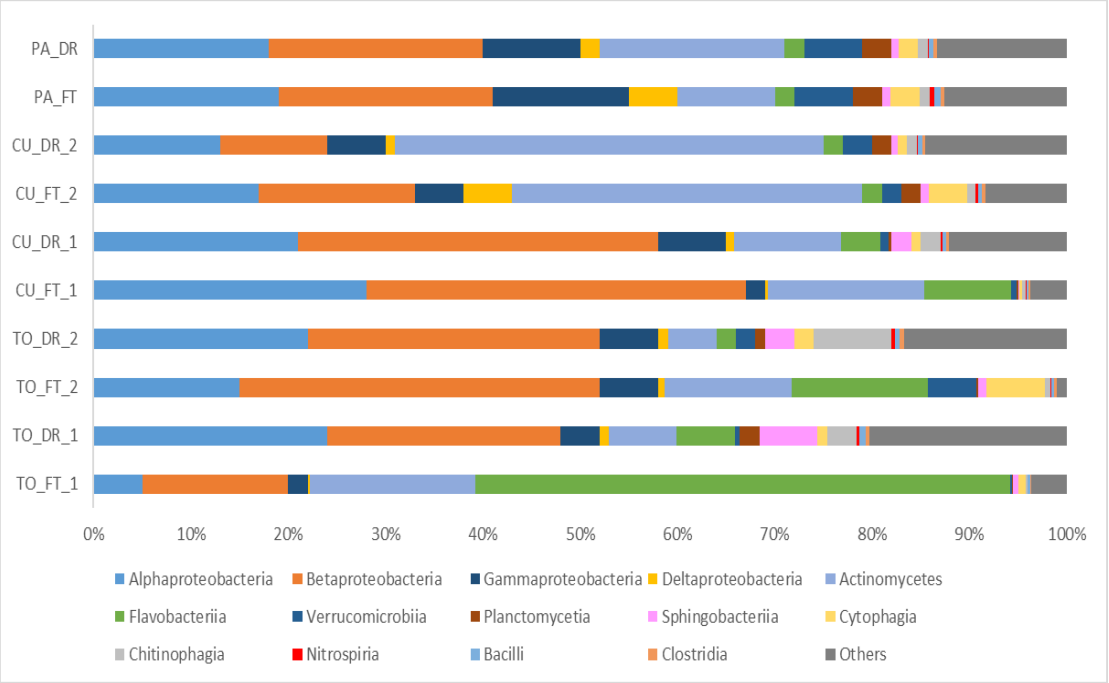



**Figure 4.8.** Relative abundance (%) of bacterial classes in aquaponics samples. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

The relative abundance of main bacterial classes was used to investigate correlations between these groups across the aquaponics samples. As represented in the form of correlation networks, there were statistically clear ($p < 0.05$) correlations between several classes when calculating both the Pearson (Figure 4.9) correlation coefficient for investigating linear relationships between relative abundances assuming normally distributed data and the Spearman (Figure 4.10) correlation coefficient which was used to interpret non-linear relationships and is less affected by outliers in the data. Specifically, when considering the Pearson correlation, the only negative correlations were found between Flavobacteriia and Bacillota (represented by Clostridia and Bacilli in approximately equal proportions), Flavobacteriia and Alphaproteobacteria, as well as between Actinomycetes and Betaproteobacteria. Similarly, Flavobacteriia and Actinomycetes were the two classes showing only negative correlations, the former with Bacillota, Deltaproteobacteria and Planctomycetia, and the latter with Alphaproteobacteria and Sphingobacteria.

Interestingly, Nitrospiria, which is a class that contains nitrifying taxa critical for the function of aquaponics systems, positively correlated with Bacillota and Deltaproteobacteria based on both Pearson and Spearman coefficients.

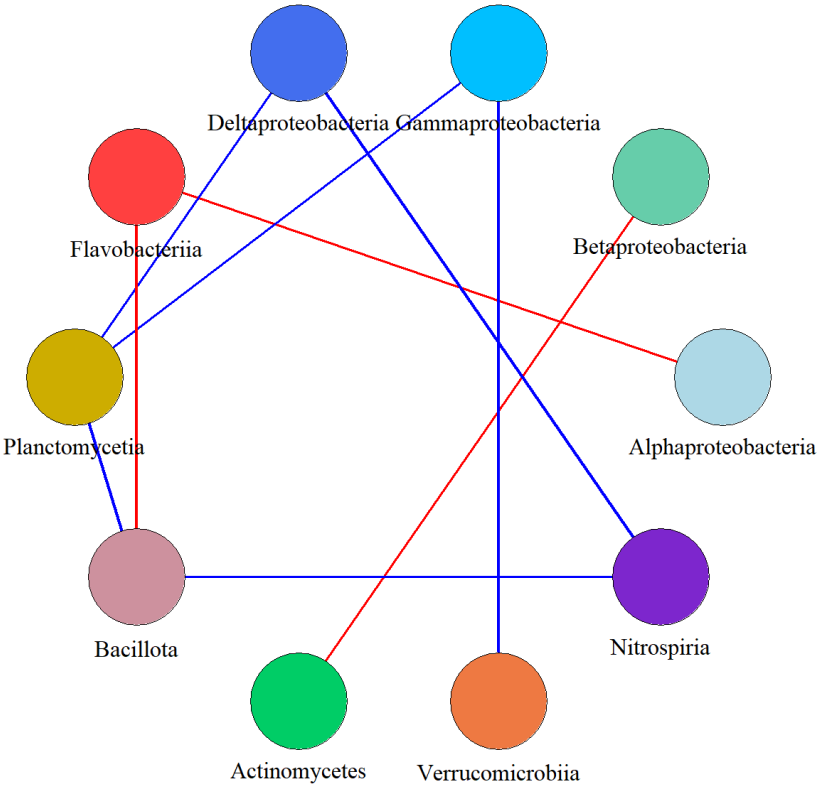



**Figure 4.9.** Pearson correlation network based on the relative abundance (%) of bacterial classes in aquaponics samples. Only statistically clear ($p < 0.05$), strong ($r > 0.6$ or $< -0.6$) correlations are shown as either blue (positive) or red (negative) colored lines.

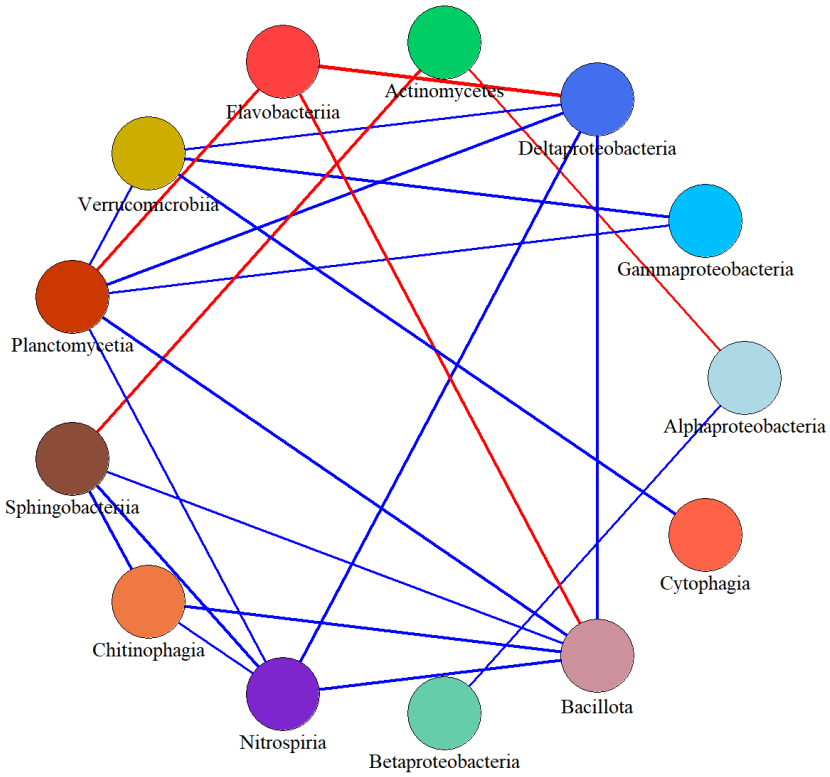



**Figure 4.10.** Spearman correlation network based on the relative abundance (%) of bacterial classes in aquaponics samples. Only statistically clear ($p < 0.05$), strong ($r_s > 0.6$ or $< -0.6$) correlations are shown with either blue (positive) or red (negative) colored lines.

Based on the very high number of prokaryotic genera who were present in low relative abundance, genera were considered dominant if they were represented with relative abundance ≥ 0.01%. To facilitate meaningful comparisons between communities at this taxonomic level, the samples of the parsley aquaponics system were excluded and subsequent analyses focused on the samples from the fish tanks and drain tanks of the tomato and cucumber growing systems, as each of these systems was sampled twice, firstly near the start and secondly near the conclusion of their production cycle.

Starting with the dominant archaeal genera (Figure 4.11), all drain tank communities from the tomato and cucumber aquaponics shared 5 dominant genera, namely '*Candidatus* Nitrosotenuis', '*Candidatus* Nitrosoarchaeum', *Nitrosopumilus* (Nitrososphaerota), *Methanosarcina* and *Methanobacterium* (Methanobacteriota). The fish tank samples harbored more shared abundant genera (15 compared to five which were shared by the drain tank samples) including '*Candidatus* Nitrosotenuis', *Methanosarcina*, *Nitrosopumilus* and *Methanobacterium* as in the case of the drain samples, as well as genera '*Candidatus* Methanoperedens', *Haloarcula*, *Methanobrevibacter*, *Methanocella*, *Methanococcoides*, *Methanococcus*, *Methanoculleus*, *Methanohalophilus*, *Methanolobus*, *Methanosaeta* and *Thermococcus* (Methanobacteriota). Bray-Curtis similarity was used to perform ANOSIM between fish tank and drain tank communities showing statistically clear differences ($p < 0.05$). Further investigation using SIMPER revealed that community composition differences were largely explained by the nitrifying genera '*Candidatus* Nitrosotenuis' (26.3%) and '*Candidatus* Nitrosoarchaeum' (13.8%) which were both present in higher relative abundance in the drain tanks. '*Candidatus* Nitrosotenuis' and '*Candidatus* Nitrosoarchaeum' had relative abundance ranges of 0.01 – 0.31% and < 0.01 – 0.03% in the fish tanks and 0.22 – 0.53% and 0.02 – 0.33% in the drain tanks respectively. Similarly, '*Candidatus* Nitrosotenuis', *Nitrosopumilus*, *Methanosarcina* and *Methanobacterium* were the four abundant genera shared by all samples from the tomato aquaponics system, while 22 genera were only abundant at the time of the second fish tank sampling. Conversely, 21 abundant genera were shared by all samples of the cucumber aquaponics system, indicating a more uniform community across this system. SIMPER once again indicated that community composition differences were mainly due to genera '*Candidatus* Nitrosotenuis' (25.2%) and '*Candidatus* Nitrosoarchaeum' (13.9%) which were both present in higher relative abundance in the tomato aquaponics samples.

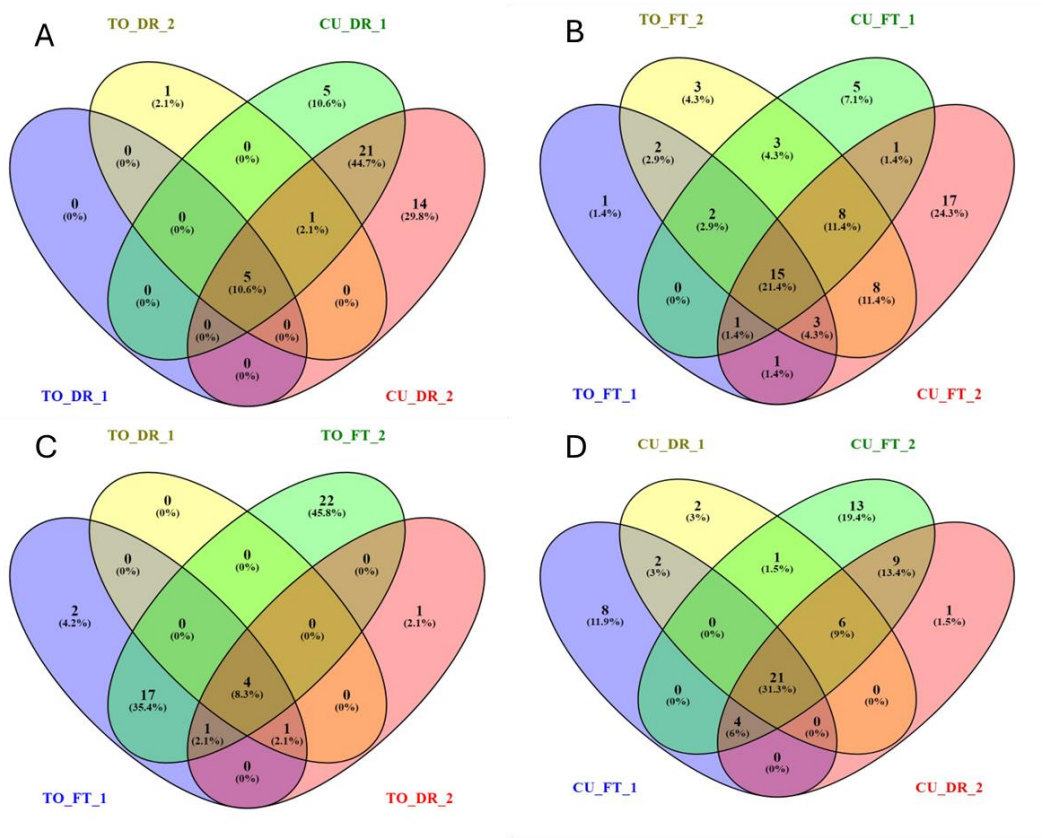



**Figure 4.11.** Number of shared dominant (≥ 0.01% relative abundance) archaeal genera in aquaponics samples. **A**: drain tanks, **B**: fish tanks, **C**: tomato aquaponics, **D**: cucumber aquaponics. CU: cucumber aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

To compare the dominant archaeal genera in the two aquaponics systems, abundances were natural logarithm transformed and relative abundances were plotted for the respective compartments and sampling time points. Thus, when looking at the dominant genera during the first samplings of the fish tank communities (Figure 4.12.A), 10 genera were found abundant in both systems. Among these shared dominant genera, *Methanosarcina*, *Methanobacterium*, *Methanobrevibacter*, *Nitrosopumilus* and *Thermococcus* were approximately equally abundant in the two systems, whereas '*Candidatus* Nitrosotenuis', '*Candidatus* Methanoperedens', '*Candidatus* Nitrosoarchaeum', *Natrinema* and *Methoanococcus* had higher relative abundance in the tomato system. However, at the time of the second samplings only a

couple of genera including *Nitrosopumilus* and *Methanohalophilus* were about equally abundant in both systems while the rest of the shared dominant genera were more abundant in the cucumber system (Figure 4.12.B). These genera included *Methanosarcina*, *Methanoculleus*, *Methanococcoides*, *Methanobacterium*, *Thermococcus*, *Methanolobus*, *Haloferax*, *Halococcus*, *Nitrososphaera*, *Methanobrevibacter*, *Haloarcula*, *Halorubrum*, *Methanocella*, *Methanosaeta*, '*Candidatus* Methanoperedens', and '*Candidatus* Syntrophoarchaeum'.

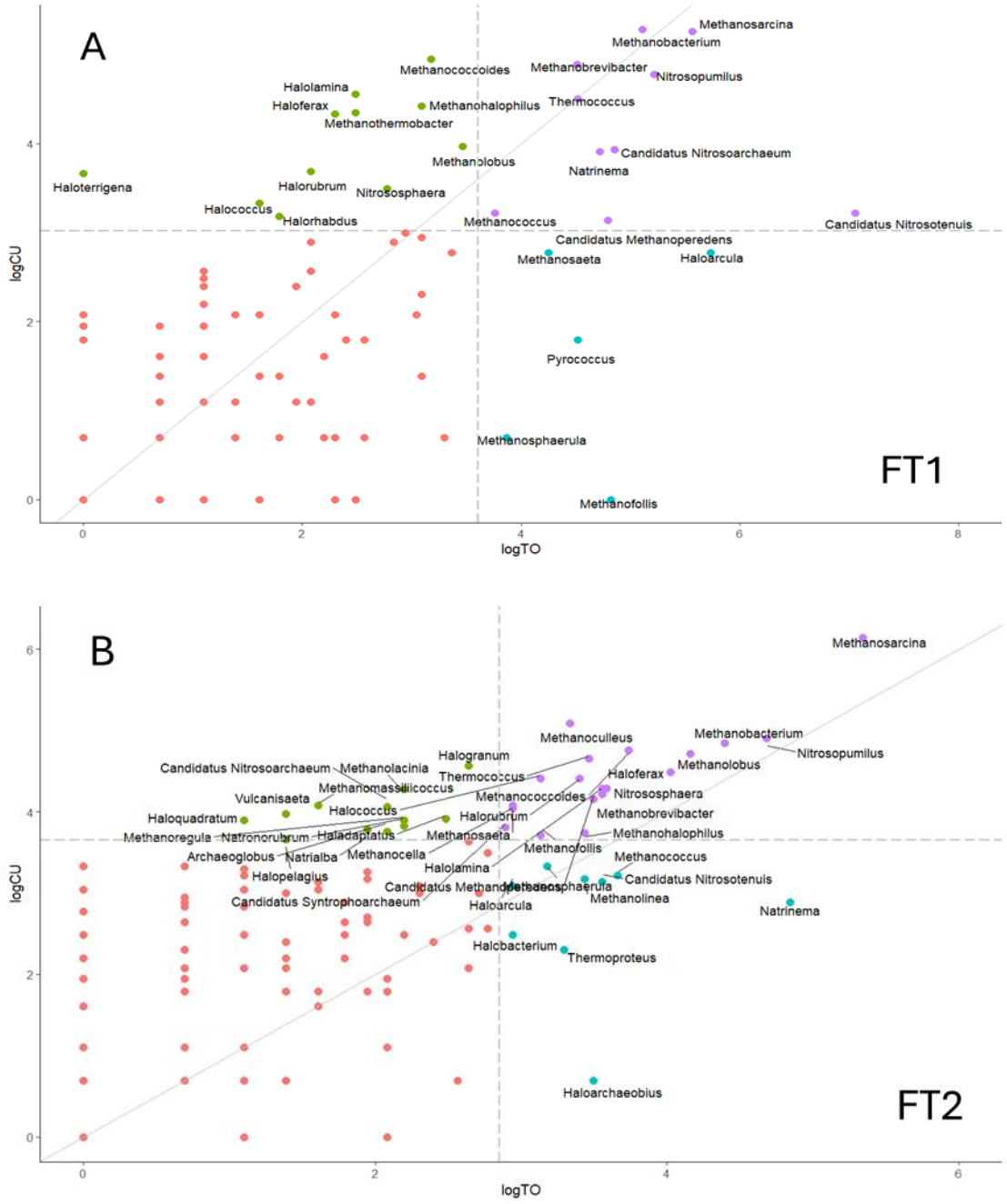



**Figure 4.12**. Natural logarithm transformed abundance of archaeal genera in the cucumber (CU) and tomato (TO) aquaponics systems. **A**: fish tank, first sampling, **B**: fish tank, second sampling, Genera with relative abundance ≥ 0.01% (dashed grey lines) in both TO and CU samples (purple dots), only in CU samples (green dots) and only in TO samples (blue dots). Genera along the diagonal line had equal abundance in both aquaponics systems. Red dots indicate non-abundant taxa.

Looking into the distribution of dominant bacterial genera (Figure 4.13.A-B), six (6) genera were dominant in all drain tank communities, specifically *Flavobacterium* (Flavobacteriia), *Aurantimicrobium*, *Mycobacterium* (Actinomycetes), *Polynucleobacter*, *Hydrogenophaga* (Betaproteobacteria), and *Pseudomonas* (Gammaproteobacteria). In the case of the fish tank communities, once again there were six (6) shared dominant genera, namely *Flavobacterium*, *Aurantimicrobium*, *Mycobacterium* and *Polynucleobacter* as in the drain tanks and additionally *Novosphingobium* and *Sphingomonas* (Alphaproteobacteria). Among these genera, *Polynucleobacter*, *Aurantimicrobium* and *Flavobacterium* were generally more abundant in the fish tank samples compared to the drain tanks (Figure 4.14). When looking at the dominant bacterial genera of each aquaponics system separately (Figure 4.13.C-D), it seems that in the tomato growing system there were 12 shared genera including *Flavobacterium* (Flavobacteriia), *Polynucleobacter*, *Limnohabitans*, *Hydrogenophaga*, *Acidovorax*, *Curvibacter*, (Betaproteobacteria), *Aurantimicrobium*, *Mycobacterium* (Actinomycetes), *Novosphingobium*, *Sphingomonas*, *Erythrobacter* (Alphaproteobacteria), *Pseudomonas* (Gammaproteobacteria), whereas in the cucumber growing system's compartments there were only 4 shared dominant genera, specifically *Polynucleobacter*, *Flavobacterium*, *Aurantimicrobium* and *Mycobacterium*. Between these two aquaponic systems, the relative abundances of *Flavobacterium* and *Limnohabitans* were higher in the tomato growing system while the relative abundances of *Polynucleobacter* and *Mycobacterium* were higher in the cucumber growing system (Figure 4.14).

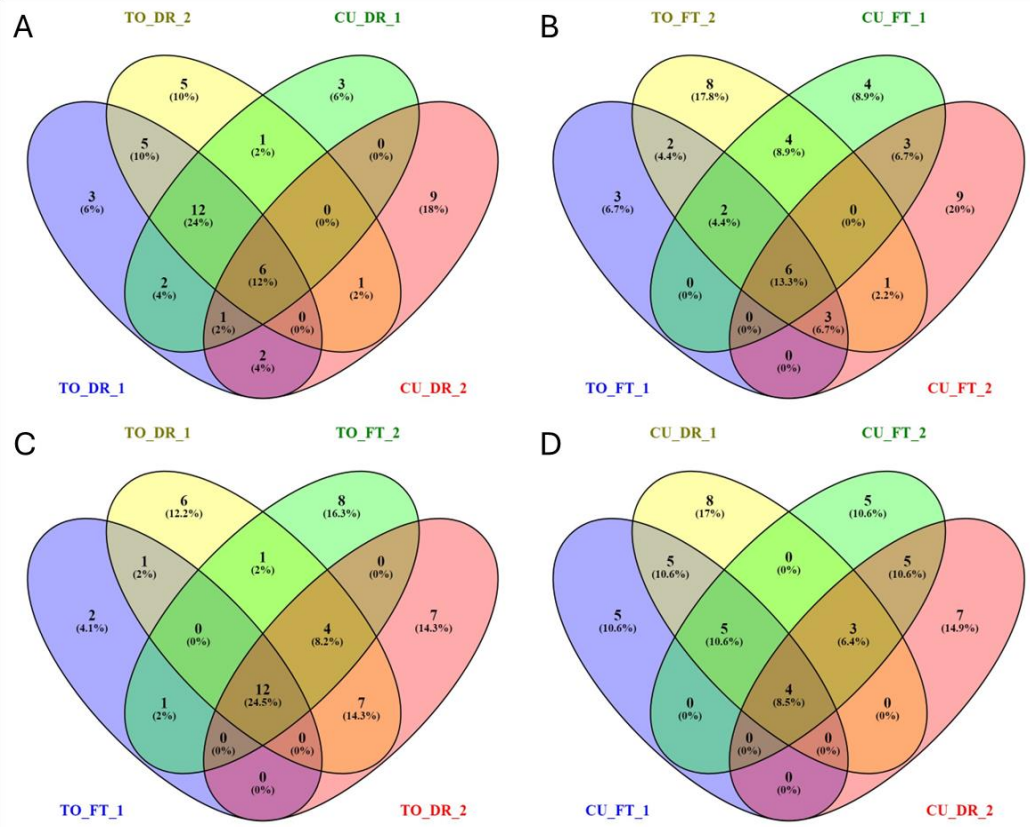



**Figure 4.13.** Number of shared dominant (≥ 0.01% relative abundance) bacterial genera in aquaponics samples. **A**: drain tanks, **B**: fish tanks, **C**: tomato aquaponics, **D**: cucumber aquaponics. CU: cucumber aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

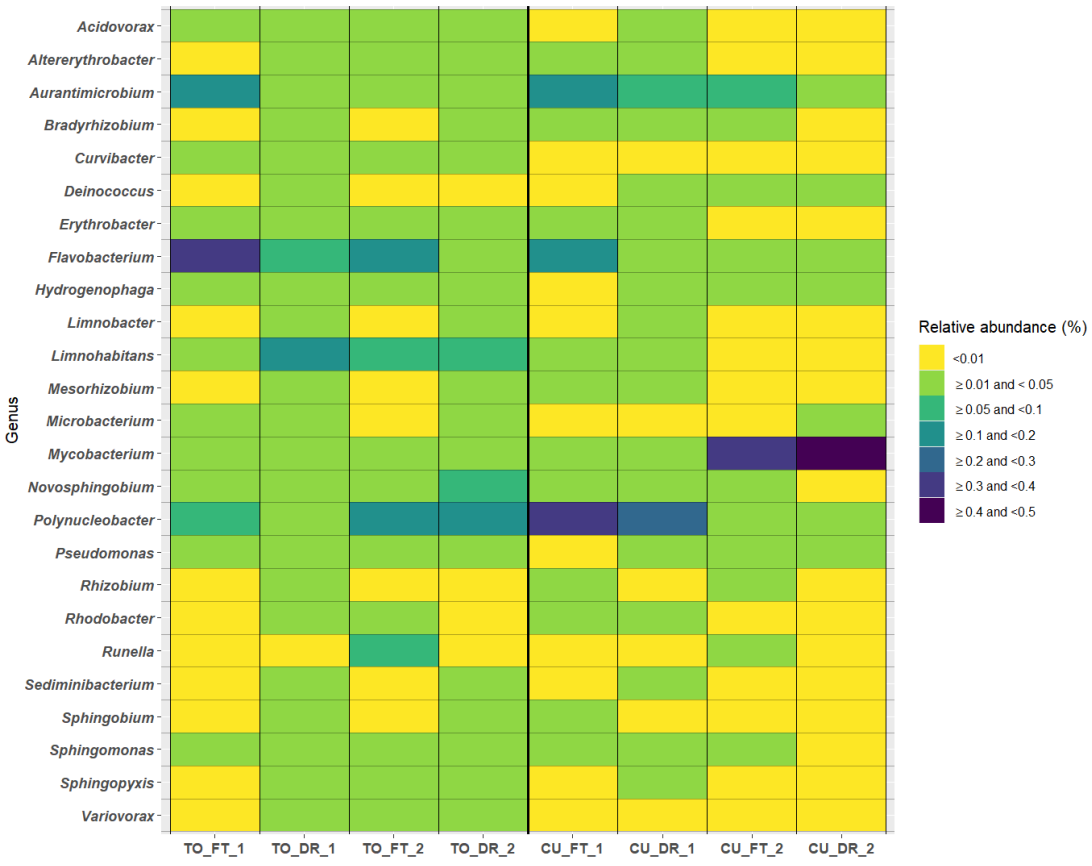
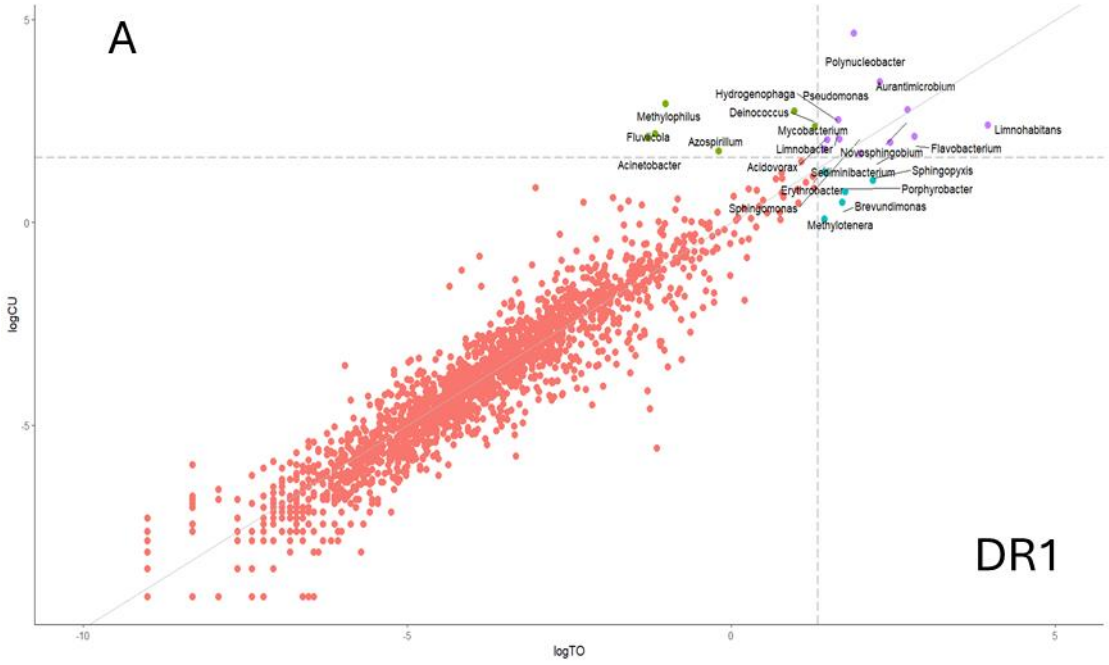
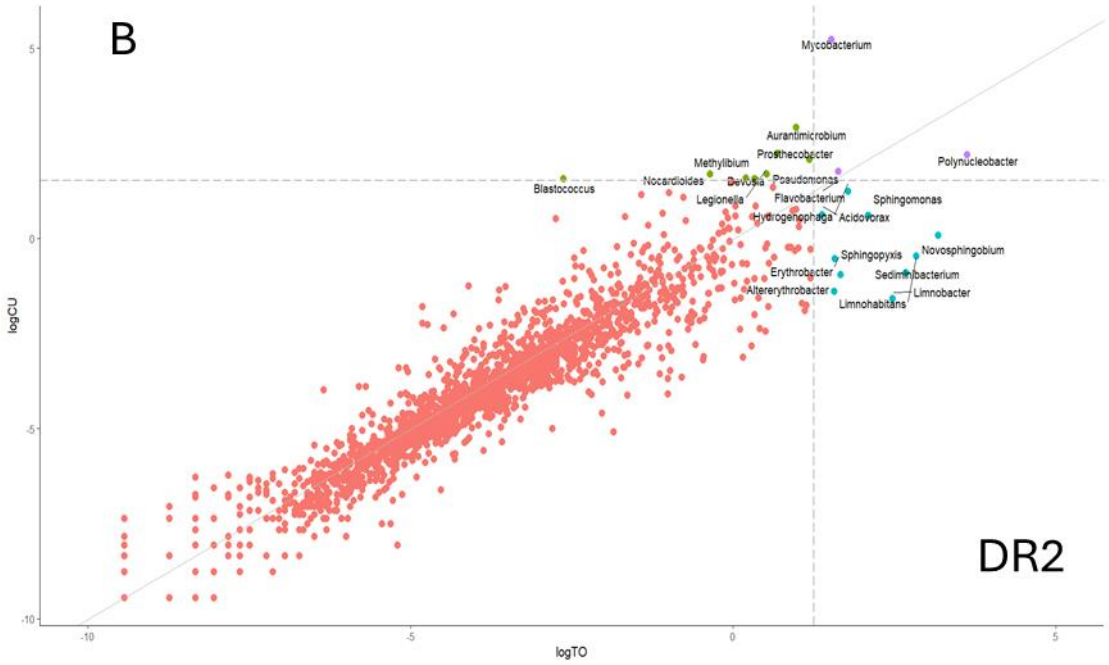



**Figure 4.14.** Relative abundance (%) heatmap of shared dominant (0.01%) bacterial genera in the aquaponics samples. CU: cucumber aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

Further analysis of the dominant bacteria shows that the number of shared dominant genera between the drain compartments of the two aquaponics systems was lower at the time of the second samplings, indicating a divergence of prominent taxa over time (Figures 4.15.A-B). In addition, while the number of shared dominant taxa was noticeably lower in the fish tanks at the time of the first samplings, the number of dominant genera at the time of the second sampling increased in the tomato aquaponics system's fish tanks (Figures 4.15.C-D). The genus *Runella* (Cytophagia) also became dominant in the fish tanks of both aquaponics systems even though its abundance was <0.01% in all other samples.


A
Polynucleobacter
Aurantimicrobium
Hydrogenophaga
Pseudomonas
Deinococcus
Methylophilus
Mycobacterium
Limnohabitans
Fluviicola
Azospirillum
Limnobacter
Novosphingobium
Flavobacterium
Acinetobacter
Acidovorax
Sediminibacterium
Sphingopyxis
Erythrobacter
Porphyrobacter
Sphingomonas
Brevundimonas
Methylotenera
DR1
logCU
logTO
5
0
-5
-10


B
Mycobacterium
Aurantimicrobium
Prosthecobacter
Methylibium
Polynucleobacter
Nocardioides
Blastococcus
Devosia
Pseudomonas
Legionella
Flavobacterium
Sphingomonas
Hydrogenophaga
Acidovorax
Novosphingobium
Sphingopyxis
Erythrobacter
Sediminibacterium
Altererythrobacter
Limnobacter
Limnohabitans
DR2
logCU
logTO
5
0
-5
-10

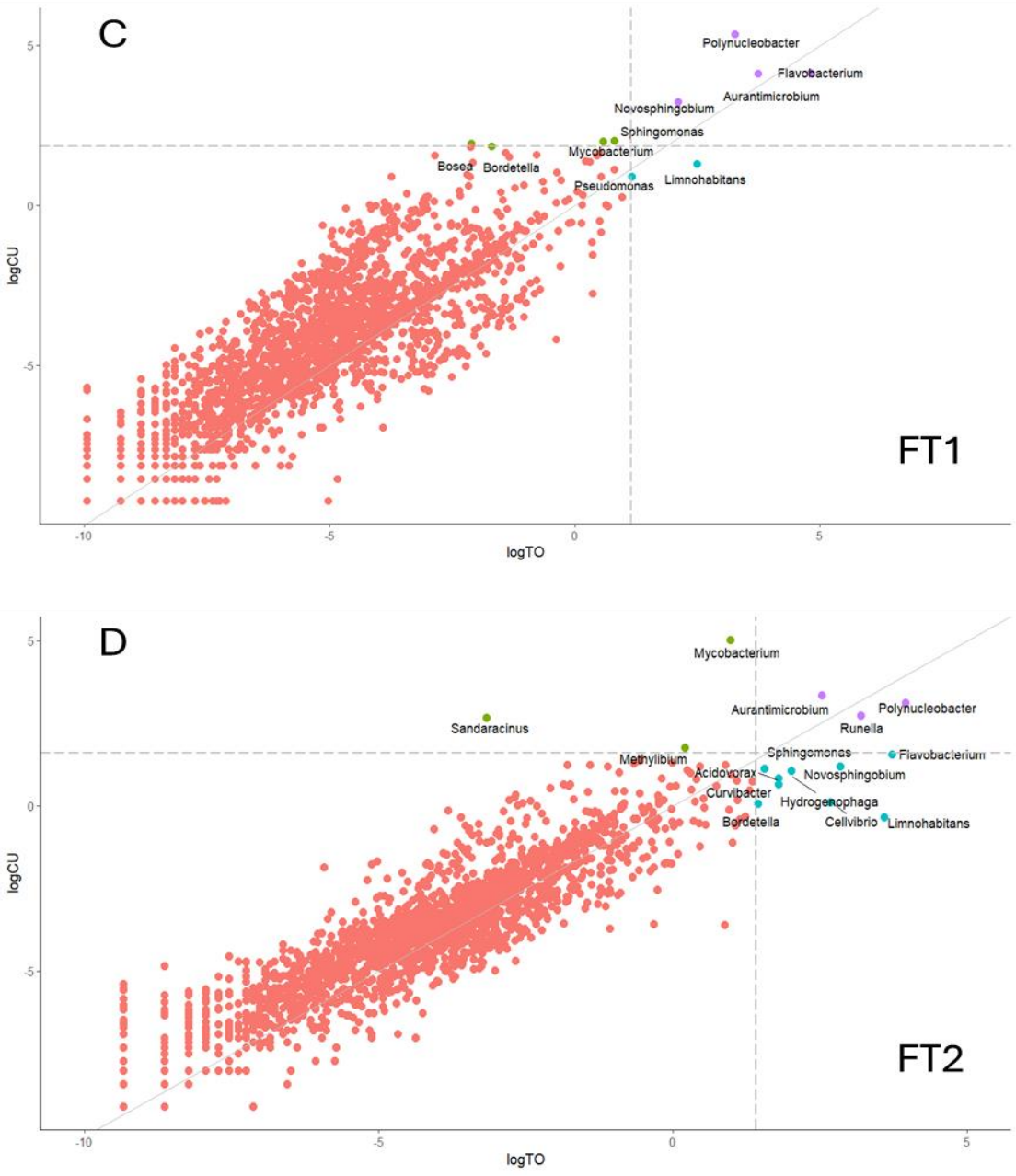



**Figure 4.15.** Natural logarithm transformed abundance of bacterial genera in the cucumber (CU) and tomato (TO) aquaponics systems. **A**: Drain tank, first sampling, **B**: Drain tank, second sampling, **C**: Fish tank, first sampling, **D**: Fish tank, second sampling. Genera with relative abundance ≥ 0.01% (dashed grey lines) in both TO and CU samples (purple dots), only in CU samples (green dots) and only in TO samples (blue dots). Genera along the diagonal line had equal abundance in both aquaponics systems. Red dots indicate non-abundant taxa.

Bacterial communities in the RAS samples mostly consisted of the phyla (Figure 4.16) Pseudomonadota (47 – 60% relative abundance) and Bacteroidota (18 – 30%) followed by Actinomycetota (3 – 11%), Planctomycetota (3 – 7%), Cyanobacteriota (0.7 – 1%) and Verrucomicrobiota (0.5 – 1%). Within these phyla, the most abundant classes (Figure 4.17) were Alphaproteobacteria (30 – 38%), Flavobacteriia (10 – 25%), Gammaproteobacteria (5 – 14%), Betaproteobacteria (2 – 11%), Planctomycetia (2 – 6%) and Cytophagia (1 – 4%). Furthermore, the relative abundance of the functionally relevant class Nitrospiria ranged between 0.7 and 3% and was higher in the fish tank biofilm. Down to the genus level, each niche was shown to be dominated by different taxa. Specifically, the fish tank water community was characterized by the high relative abundance of *Mycobacterium* (Actinomycetes) and *Hyphomonas* (Alphaproteobacteria) while the biofilm community was dominated by *Nitrosomonas* (Betaproteobacteria). Meanwhile *Nitratireductor* (Alphaproteobacteria) and *Muricauda* (Flavobacteriia) were the most abundant genera in the biofilter biofilm. Comparison of the composition of dominant (≥ 0.01% relative abundance) bacterial genera (Figure 4.18) revealed that 19.6% of the total dominant genera were abundant in all three RAS niches, while the fish tank had the highest amount (25% of the total) of dominant genera.

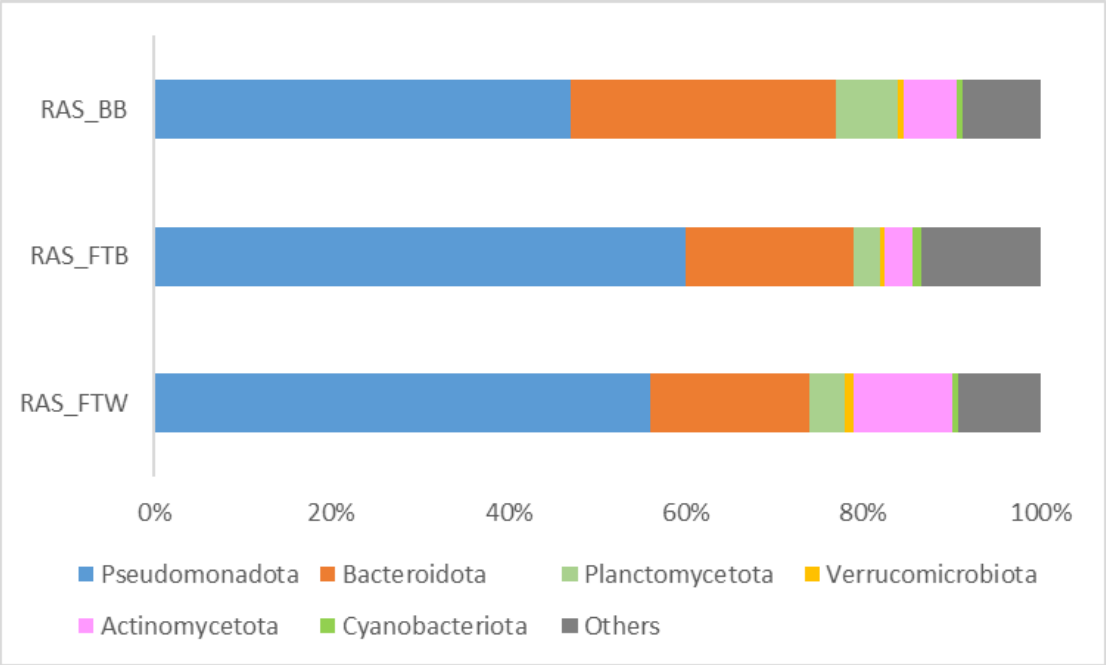



**Figure 4.16.** Relative abundance (%) of bacterial phyla in RAS samples. BB: biofilter biofilm, FTB: fish tank biofilm, FTW: fish tank water.

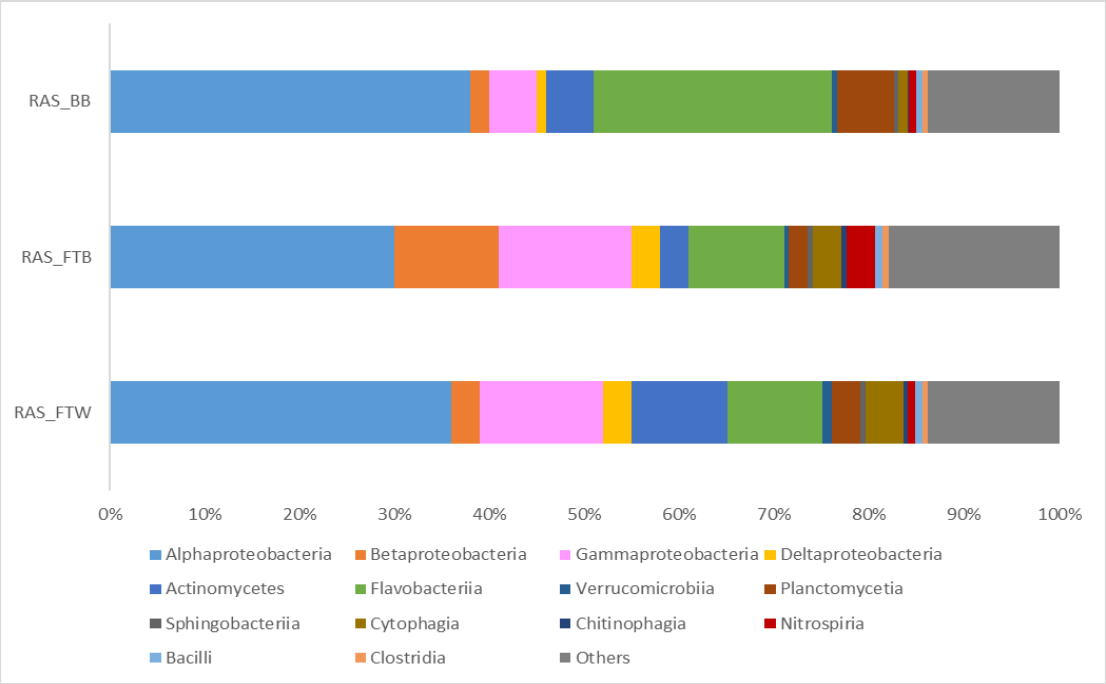



**Figure 4.17.** Relative abundance (%) of bacterial classes in RAS samples. BB: biofilter biofilm, FTB: fish tank biofilm, FTW: fish tank water.

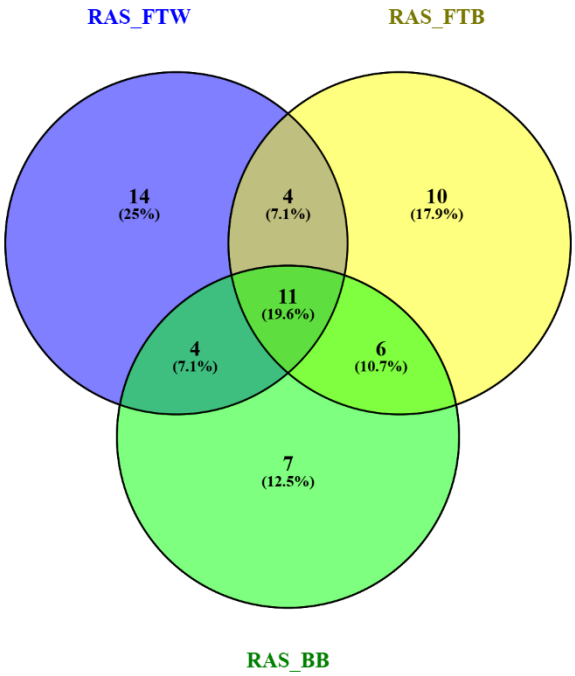



**Figure 4.18.** Number of unique and shared dominant (≥ 0.01% relative abundance) bacterial genera in all three RAS samples. FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm.

Archaeal RAS communities were mostly represented by the phyla (Figure 4.19) Methanobacteriota (62 – 82% relative abundance), Candidatus Pacearchaeota (2 – 14%), Nitrososphaerota (5 – 7%), Thermoproteota (2 – 3%) and Candidatus Micrarachaeota (0.3 – 0.9%). Fish tank water column and biofilter biofilm communities were both dominated by genera *Halococcus* (0.19 and 0.42% relative abundance respectively), *Methanosarcina* (0.07 and 0.04% respectively) and *Nitrosopumilus* (0.04 and 0.03% respectively). The fish tank biofilm community was more distinct, dominated by *Methanosarcina* (0.1%), *Halolamina* (0.04%), *Methanolobus* (0.04%) and *Methanosaeta* (0.04%). The fish tank biofilm was also characterized by the most exclusively abundant (Figure 4.20) archaeal genera (14% of the total) but overall samples shared the majority of the total abundant genera (61.4%).

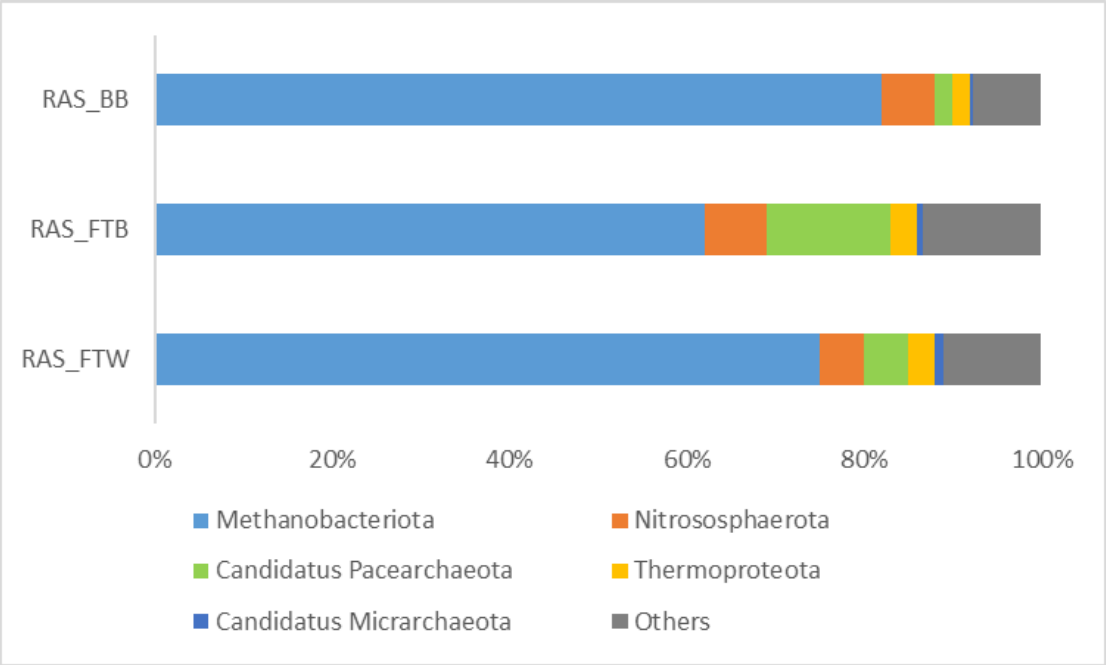



**Figure 4.19.** Relative abundance (%) of archaeal phyla in RAS samples. BB: biofilter biofilm, FTB: fish tank biofilm, FTW: fish tank water.

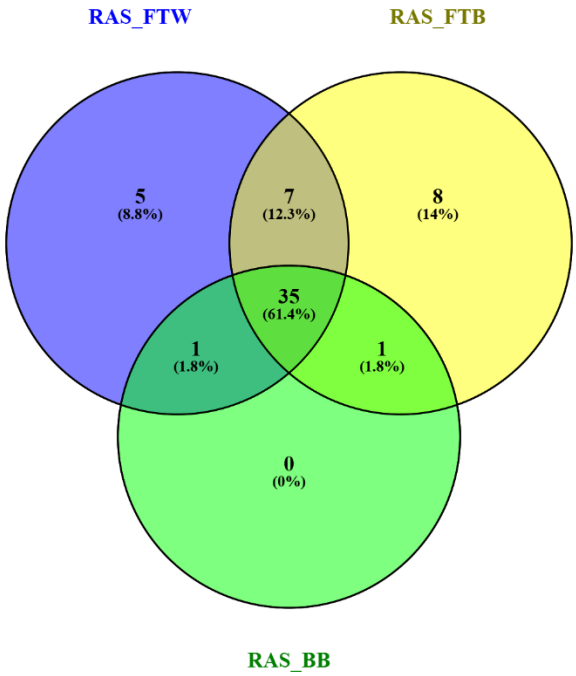



**Figure 4.20.** Number of unique and shared dominant (≥ 0.01% relative abundance) archaeal genera in all three RAS samples. FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm.

## 4.3. Discussion

In the present study, the taxonomic diversity of suspended —without distinguishing free-living and particle-attached communities— bacterial and archaeal communities was studied across water samples from fish tanks and drain tanks (after plant fertigation) in three aquaponics setups using metagenomic analysis.

The majority of the archaeal community in all samples consisted of three main phyla, Methanobacteriota, Nitrososphaerota and Candidatus Pacearachaeota. Methanobacteriota and Nitrososphaerota have been identified as major phyla in a previous study of tilapia pond water and sediment (Fan et al., 2018) as well as fish gut (in the case of Methanobacteriota) and in late-stage water samples from systems for the co-culture of lotus plants and crucian carp (Zeng et al., 2025). In the present study, there were several dominant Methanobacteriota methanogenic genera such as *Methanosarcina*, *Methanobacterium*, *Methanobrevibacter* in both the fish tank and drain tank compartments. *Methanosarcina* and *Methanobacterium* have also been detected as dominant in anaerobic sludge digester samples of an aquaponics system (Schmautz et al., 2022), grass carp aquaculture ponds (Liu et al., 2024), a sludge digester for a turbot-farming brackish aquaponics systems (Zhang et al., 2016) and the intestine of crucian carp co-cultured with lotus (Zeng et al., 2025). The other major phylum, Nitrososphaerota, was represented by two commonly dominant genera across samples, specifically '*Candidatus* Nitrosotenuis' and '*Candidatus* Nitrosoarchaeum'. Nitrososphaerota are known as key drivers of nitrification and critical participants in the carbon cycling (Nakagawa et al., 2025) and their functional role is discussed in greater detail in Chapter 5. However, it should be noted that their relative abundance was higher in the drain tanks, and generally higher in the tomato aquaponics samples compared to the cucumber aquaponics system. As for the third dominant phylum, '*Candidatus* Pacearchaeota', it has also been detected in a variety of natural and man-made environments such as anaerobic digesters, hot springs, deep sea water and hydrothermal vents (Takamiya et al., 2024). It has also been suggested that certain groups of archaea including '*Candidatus* Pacearchaeota' can form

consortia with methanogens under anoxic conditions and thus contribute to anaerobic carbon cycling (Liu et al., 2018).

Furthermore, archaeal alpha diversity based on the Shannon index ranged between 1.65 – 3.4 across the aquaponics samples and no statistically clear difference was observed between the two compartments or aquaponics systems. These Shannon index values are comparable with results from studies examining archaeal communities in fish ponds of other aquaculture setups such as crucian carp and lotus co-culture (2.19 – 3.4, Zeng et al., 2025) and tilapia fish ponds (3.5 – 5.5, Fan et al., 2018). Based on NMDS and clustering results, archaeal community composition appeared to be shaped primarily by the environment of different compartment types (fish tank – drain tank) rather than the environment associated with a different cultivated plant species (tomato – cucumber), in contrast to the composition of the bacterial community where the opposite was true. Drain tanks also harbored a clearly higher number of archaeal genera, as well as archaeal reads, indicating a higher natural abundance of archaea. AOA have been detected in aquaponics systems before (Bartelme et al., 2019) and specifically in the hydroponic compartment with low relative abundance (Schmautz et al., 2022), but this is the first time AOA and archaea in general have been studied in the drain tanks of the hydroponics subsystem in coupled aquaponics. The higher relative abundance of these nitrifying archaeal genera in drain tanks could be a further indication of their valuable contribution to the function of coupled aquaponics systems, given the water retention times of the drain tanks. These AOA are most likely autotrophic or mixotrophic (Wright and Lehtovirta-morley, 2023) and may be able to achieve higher relative abundances in the drain tanks where they can attach to solid particles that precipitate to the tanks' bottom, while they are likely outcompeted by heterotrophic prokaryotes in the fish tanks' water column. Furthermore, the archaeal community of the aquaponics drain tanks was particularly enriched in methanogens, despite the fact that methanogens are strictly anaerobic (Liu and Whitman, 2008). Anaerobic microorganisms have been detected in aerobic aquaponics compartments before (Schmautz et al., 2022). This recurring observation can be attributed to the nature of the recirculating systems that promotes

homogenization of the system water and its microbial communities and the fact that DNA based methods do not provide information on whether these organisms are actually metabolically active (Schmautz et al., 2022). In the case of the aquaponics systems investigated in the present study, the presence of sludge and particulate matter at the bottom of drain tanks could have allowed methanogenic taxa to proliferate by providing space for anaerobic niches to form (Pronk et al., 2015). Among these methanogens, autotrophic genera including *Methanosarcina* and *Methanobacterium* were more abundant but the acetoclastic genus *Methanosaeta* (renamed *Methanothrix*) was also among the dominant archaeal genera (Liu and Whitman, 2008; Wang et al., 2022). The presence of a functional methanogenic community can potentially be utilized in the establishment of coupled aquaponics that also include biogas production through anaerobic digestion of sludge, or in the case of decoupled aquaponics where the effluent can be mixed with other substrates such as cattle manure to increase biogas production efficiency (Lobo Paes et al., 2025).

Richness of the suspended bacterial community was clearly higher in the drain tanks compared to the fish tanks, indicating a distinction between the two compartments. In terms of taxonomy, Cyanobacteriota relative abundance was consistently higher in the drain tanks, likely because the drain tanks were exposed to sunlight whereas the fish tanks were housed indoors. Another reason that can possibly explain the higher abundance of Cyanobacteriota and the overall distinction between the fish tank and drain tank communities is the generally higher bioavailable nitrogen and phosphorus concentrations in the drain tanks (Table 4.3).

**Table 4.3.** Average concentrations (mg $L^{-1}$ ) of the main nutrients ($NO_3^-$, $PO_4^{2-}$, $NH_4^+$ , $K^+$ , $Ca^{2+}$, $Mg^{2+}$, $SO_4^{2-}$ and $Fe^{2+}$) in the fish tanks, and average concentrations (mg $L^{-1}$) ±SD of the main nutrients ($NO_3^-$, $PO_4^{2-}$, $NH_4^+$ , $K^+$ , $Ca^{2+}$ and $Na^+$) in the drain tanks of the aquaponics system during the cultivation period of each plant. Nutrient measurements were carried out on a weekly basis. Adapted from (Aslanidou et al., 2023).

| | Tomato aquaponics (TO) | | Cucumber aquaponics (CU) | | Parsley aquaponics (PA) | |
|---|---|---|---|---|---|---|
| | Fish tanks | Drain tanks | Fish tanks | Drain tanks | Fish tanks | Drain tanks |
| $NO_3^-$ | 113.7 | 70.5 ± 2.8 | 111.3 | 170.1 ± 95.2 | 92.4 | 81.9 ± 6.4 |
| $NH_4^+$ | 0.1 | 0.3 ± 0.0 | 0.1 | 0.8 ± 0.4 | 0.1 | 0.6 ± 0.2 |
| $PO_4^{2-}$ | 8.3 | 6.3 ± 0.8 | 11.7 | 23.4 ± 27.7 | 9.3 | 12.4 ± 1.6 |
| $K^+$ | 17.7 | 12.0 ± 2.2 | 19.3 | 35.7 ± 11.1 | 11.2 | 33.4 ± 3.3 |
| $Ca_2^+$ | 37.9 | 47.9 ± 2.7 | 36.7 | 42.1 ± 4.5 | 35.4 | 49.5 ± 4.3 |
| $Na^+$ | 43.8 | 58.7 ± 3.6 | 38.8 | 52.6 ± 2.0 | 41.6 | 54.3 ± 4.0 |
| $Mg_2^+$ | 42.5 | - | 41.4 | - | 37.5 | - |
| $SO_4^{2-}$ | 48.2 | - | 33.8 | - | 14.7 | - |
| $Fe^{2+}$ | 0.1 | - | 0.1 | - | 0.1 | - |

However, there was much more evidence suggesting that bacterial communities were mainly shaped by the environment of different aquaponics systems rather than by compartment type, contrary to the case of archaea. Specifically, both NMDS and Bray-Curtis cluster analysis showed that bacterial communities from the same aquaponics system were more similar compared to communities from the same compartment type across various aquaponics systems. Furthermore, bacterial communities in the tomato growing aquaponics were more enriched in Bacteroidota compared to communities in the cucumber growing system. Communities in these two systems also differed in terms of dominant genera, as *Flavobacterium* and *Limnohabitans* were more abundant in the tomato system while *Polynucleobacter* and *Mycobacterium* were present in higher relative abundance in the cucumber system. Other recent studies have reported the distinction of bacterial community composition in different aquaponics systems and compartments (Changey et al., 2025; Ruiz et al., 2023) and it is generally suggested that different compartments create different niches. Still, the system-wide grouping of samples in the present study can likely be explained by the increased similarity of suspended bacterial communities in all compartments of individual aquaponics systems compared to the attached communities in their respective compartments (Ruiz et al., 2023).

Overall, bacterial communities were mainly represented by a few dominant phyla such as Pseudomonadota, Bacteroidota, Actinomycetota, Verrucomicrobiota, Planctomycetota and Cyanobacteriota, and predominantly by classes including Betaproteobacteria, Actinomycetes, Alphaproteobacteria, Flavobacteriia, Gammaproteobacteria, Chitinophagia, Cytophagia, Verrucomicrobiia, Sphingobacteriia and Deltaproteobacteria. Pseudomonadota, Bacteroidota and Actinomycetota have been previously shown to dominate bacterial communities in aquaponics compartments (Kasozi et al., 2021a; Ruiz et al., 2023; Tiwari et al., 2025). In addition, Bacteroidetes and Proteobacteria have been characterized as microbial indicators of proper RAS and aquaponics function (Blancheton et al., 2013; Giatsis et al., 2015; Joyce et al., 2019) due to their metabolic capacity to degrade complex macromolecules (Kirchman, 2002; Wongkiew et al., 2018). Actinomycetes members

can also degrade organic matter derived from animal and plant sources using extracellular hydrolytic enzymes, and can survive even in nutrient limited environments (Zhang et al., 2019). At the class level, Flavobacteriia, Sphingobacteriia and Cytophagia were the main representatives of Bacteroidota as it has been previously observed in a previous study of aquaponics for the farming of Nile tilapia and lettuce (Menezes et al., 2021). The same authors also noted the prevalence of Alphaproteobacteria and Betaproteobacteria, also in agreement with the results of the present study. Furthermore, the relative abundance of the vital class Nitrospiria positively correlated with the abundance of Deltaproteobacteria and Bacillota based on both the Pearson and Spearman correlation coefficients. This observation is in line with the fact that *Bacillus* enriched aquaponics systems have been previously shown to harbor a higher relative abundance of *Nitrospira* compared to control systems (Kasozi et al., 2021b), overall indicating a positive interaction between these important taxa in the aquaponics environment. Further investigation of this possible relationship could provide another way to enhance the performance of the nitrifying community in aquaponics by relying on *Bacillus* utilization. On the other hand, Flavobacteriia which were mainly represented by the commonly dominant genus *Flavobacterium* in the samples of the present study only had negative correlation with several other bacterial classes. This could likely be the result of *Flavobacterium* dominance in community composition, due to its ability to degrade macromolecules such as cellulose and chitin, which make up the high molecular mass fraction of dissolved organic matter (Kirchman, 2002), allowing *Flavobacterium* to outcompete other planktonic bacteria and be among the dominant genera across aquaponics compartments (Bartelme et al., 2019). While containing taxa which function as decomposers of organic material and are found in various aquatic environments, several *Flavobacterium* species such as F. *psychrophilum* and F. *columnare* can cause diseases in freshwater fish like rainbow trout and coho salmon (Seo et al., 2024). Another possible function of *Flavobacterium* species in aquaponics is plant growth promotion, based on observations of its prominent presence in the rhizosphere (Carrión et al., 2019; Kwak et al., 2018). Although not well studied enough yet to be established as a PGPR like *Bacillus* and *Pseudomonas*, evidence shows *Flavobacterium* can prevent bacterial

and fungal diseases in plants such as tomato and even promote growth (Jung et al., 2021; Kwak et al., 2018). Also, the increased abundance of *Flavobacterium* in the tomato rhizosphere could explain the higher abundance observed in the tomato growing aquaponics system compared to the cucumber growing system. Two of the commonly dominant bacterial genera in the samples of present study, specifically *Polynucleobacter* and *Aurantimicrobium,* are often detected in natural freshwater environments and their increased presence in aquaponics systems compared to other artificial aquaculture systems like RAS may be an indication of a natural selection of bacterial taxa in aquaponics due to the presence of plants and algae associated nutrients just like in natural freshwater systems (Bartelme et al., 2019). *Polynucleobacter* presence in particular has been suggested as an index of water quality level regarding carbon and nitrogen cycling, showing promise within the context of aquaponics optimization (Kasozi et al., 2020). Apart from its presence in aquaponics compartments such as water, sump tanks and hydroponic media beds (Bartelme et al., 2019; Eck et al., 2021; Kasozi et al., 2020), *Polynucleobacter* has also been detected in high relative abundance in RAS systems as well (Eck et al., 2021). *Limnohabitans* which was among the dominant bacterial genera in the present study and was found particularly enriched in the tomato growing aquaponics system has been previously detected in aquaponics fish tank biolfilms (Schmautz et al., 2022) and has been reported as abundant in gill associated communities of Atlantic salmon in RAS hatcheries (Minich et al., 2020). As for *Mycobacterium*, while it contains many nontuberculous species which have been detected in the natural aquatic environments in water samples, biofilms and soil, several species are known to be capable of infecting various mammals and aquatic animals (Delghandi et al., 2020). There have also been cases of infections in RAS-reared fish such as gilthead seabream by *Mycobacterium marinum* (Davidovich et al., 2020). Finally, it is likely that plant choice may have led to the higher relative abundance of *Mycobacterium* in the cucumber growing system in the present study, since it has been reported that repeated cucumber cultivation can result in *Mycobacterium* enrichment of soil in intensive greenhouse agriculture (Liu et al., 2020).

The composition of bacterial communities in RAS samples investigated in the present study is similar to the results usually reported in literature (Moschos et al., 2022; Rurangwa and Verdegem, 2015). Chemorganotrophic genera *Mycobacterium* and *Muricauda* have been detected before in marine or brackish RAS water and biofilm samples (Moschos et al., 2022), as also has the sulfur oxidizing genus *Hyphomonas* (Ribičić et al., 2024). Interestingly, *Nitrosomonas* relative abundance was higher in the fish tank biofilm rather than the biofilter, indicating the fish tank biofilm community may also have a significant contribution to nitrification. The higher number of exclusively abundant genera in the fish tank water community can likely be attributed to the presence of fish and the increased organic matter load due to fish feeding. Regarding the archaeal component of the RAS prokaryotic community, the results of the present study showed that Methanobacteriota was the most abundant phylum in the biofilter and fish tank compartments, corroborating the observations of Schmautz et al. (2022). The dominance of Methanobacteriacea, represented by *Methanosarcina* and *Methanolobus* in the sample of the present study was another common observation with the reports of Schmautz et al. (2022). *Halococcus*, while not typically associated with aquaculture environments was among the most abundant archaea in the RAS investigated in the present study, and has recently been shown to be capable of producing chitinolytic and proteolytic enzymes (Pawar et al., 2025). In addition, *Halolamina* was another abundant halophilic genus of archaea that so far had not been associated with aquaculture systems, highlighting the ongoing characterization of archaeal diversity in man-made ecosystems. Both *Halococcus* and *Halolamina* are capable of phosphorus solubilization through the production of several organic acids (Li et al., 2022; Yadav et al., 2017) and can thus enhance plant growth in aquaponics systems.

Having discussed the taxonomic diversity of prokaryotic communities in the RAS and aquaponics systems investigated in the present study while placing the results in the context of well-established literature, the next chapter will focus on the still obscure subject of prokaryotic metabolic capacity in these systems.

# 5. Microbial community function in aquaponics and RAS

## 5.1. Introduction

The metabolic function of the microbial community is critical for the performance of RAS and aquaponics systems (Kasozi, et al., 2021a; Moschos et al., 2022; Rurangwa and Verdegem, 2015). While nitrogen metabolism and particularly nitrification is the most crucial process, as discussed extensively in chapter 1 of the present thesis, organic carbon metabolism such as the degradation of organic matter, as well as sulfur and phosphorous cycling are also important in the context of recirculating aquaculture. Fish feed is the main source of carbon, in the form of protein, starch or non-starch polysaccharides, and phosphorus. Nutrients can become inaccessible to fish and plants as a part of the fish feed is left uneaten and precipitates along with other debris (e.g., feces) and forms sludge which in most systems must be removed. This sludge can also contain high amounts of insoluble phytates bound with phosphorus or other micronutrients. The heterotrophic prokaryotic community can break down this organic matter by producing specific enzymes such as phytases and thus remineralize nutrients, making them bioavailable to other microorganisms and plants and limiting nutrient loss through sludge removal (Eck, et al., 2019a).

Several genes (Table 5.1) have been used to survey the metabolic function of prokaryotes regarding these main nutrients. Specifically, the presence of ammonia monooxygenase genes (*amoA, amoB, amoC*) and hydroxylamine oxidoreductase gene (*hao*) is required for the oxidation of ammonia to hydroxylamine and the oxidation of hydroxylamine to nitrite respectively by AOB. While both of these nitrification steps are carried out by AOA as well, hao is absent in AOA genomes. NOB function depends on the expression of the nitrite oxidoreductase gene (*nxrB*), which catalyzes the final step of nitrification, nitrite oxidation to nitrate (Preena et al., 2021). The enzymes expressed by *amoA*, *hao* and *nxrB* can also co-exist in *Nitrospira* species (van Kessel et al., 2015), allowing these bacteria to perform complete ammonia oxidation (comammox). Both NOB and comammox *Nitrospira* species have been detected in recirculating

aquaculture systems (Bartelme et al., 2017; Hüpeden et al., 2020). Another possibly beneficial process in the context of recirculating aquaculture is anaerobic ammonia oxidation (anammox), during which ammonium is oxidized to dinitrogen gas ($N_2$). Several genes are involved in the different stages of this process, but *hzo* codes the hydrazine oxidoreductase enzyme that oxidizes $N_2H_2$ to $N_2$ in the final stage. *hzo* is an important anammox marker gene as it defines the rate of the whole anammox process (Liu et al., 2022).

Apart from genes involved in nitrification, genes including *gdh* (glutamate dehydrogenase), *glnA* (glutamine synthetase), *ansB* (L-asparaginase II), *asnB* (asparagine synthetase), *glsA* (glutaminase A) and *ureC* (urease) play a major part in organic nitrogen metabolism (Jiang et al., 2023; Wan et al., 2023). Of course, organic matter consists primarily of carbon-rich macromolecules and several genes such as *xylA* (xylose isomerase), *manB* (phosphomannomutase) and *abfA* (α-L-arabinofuranosidase) are responsible for the degradation of different carbohydrates (Li et al., 2024). Other genes typically involved in the metabolism of methane such as *mmoX* (soluble methane monooxygenase) and *pmoA* (particulate methane monooxygenase) are typically involved to survey samples for potential methanotrophic prokaryotes (Li et al., 2024). Furthermore, organic matter can be mineralized by alkaline phosphatases encoded by genes such as *phoA* and *phoX* (Siles et al., 2022). Sulfur oxidation is another microorganism-mediated process that is a part of sulfur cycling, and is performed by sulfur oxidizing bacteria using the *sox* pathway (Jones and Santini, 2023). Specifically, the *soxB* gene which encodes a sulfate thiol esterase can be utilized as an index for the presence of sulfur oxidizing bacteria (Akerman et al., 2013; Jones and Santini, 2023).

As the prokaryotic community is responsible for such crucial metabolic processes taking place in RAS and aquaponics, it is deemed critical that the metabolic capacity of these functional groups is thoroughly examined across multiple systems, compartments and time points to highlight likely patterns that can be used to better control and exploit these valuable microbial groups.

**Table 5.1.** Functional prokaryotic genes investigated in the present study.

| Gene | Enzyme | Metabolic function | Representative genera | References |
|---|---|---|---|---|
| *amoA*, *amoB*, *amoC* | Ammonia monooxygenase | Ammonia oxidation | AOB (e.g., Nitrosomonas, Nitrosovibrio, Nitrosospira, Nitrosococcus) | Liu et al., 2022; Preena et al., 2021; van Kessel et al., 2015 |
| *hao* | Hydroxylamine oxidoreductase | Ammonia oxidation | | |
| *nxrB* | Nitrite oxidoreductase | Nitrite oxidation, comammox | NOB (e.g., Nitrobacter, Nitrococcus, Nitrospira, Nitrospina) and commamox Nitrospira | |
| *hzo* | Hydrazine oxidoreductase | anammox | *Candidatus brocadia*, *Candidatus kuenenia* | |
| *gdh* | glutamate dehydrogenase | Nitrogen Metabolism | | |
| *glnA* | glutamine synthetase | Nitrogen Metabolism | | Wan et al., 2023 |
| *ansB* | L-asparaginase II | Nitrogen Metabolism | | |
| *asnB* | asparagine synthetase | Nitrogen Metabolism | | |
| *glsA* | glutaminase A | Nitrogen Metabolism | | |

| | | | | |
|---|---|---|---|---|
| *ureC* | urease | Nitrogen Metabolism | | Jiang et al., 2023 |
| *xylA* | xylose isomerase | Carbohydrate metabolism | | |
| *manB* | phosphomannomutase | Carbohydrate metabolism | | Li et al., 2024 |
| *abfA* | α-L-arabinofuranosidase | Carbohydrate metabolism | | |
| *mmoX* | soluble methane monooxygenase | Methane metabolism | | Boada et al., 2020; Cupples and Thelusmond, 2022; Li et al., 2024; Smalley et al., 2015; van Spanning et al., 2022; Wang et al., 2023a |
| *pmoA* | particulate methane monooxygenase | Methane metabolism | *Methylococcus*, *Methylomonas*, *Mycobacterium*, *Methyloversatilis*, *Methylibium* | |
| *phoA* | alkaline phosphatase | Phosphorus metabolism | | Siles et al., 2022 |

| | | | | |
|---|---|---|---|---|
| *phoX* | alkaline phosphatase | Phosphorus metabolism | | |
| *soxB* | sulfate thiol esterase | Sulfur metabolism | sulfur oxidizing bacteria, mainly facultative chemolithotrophic Alphaproteobacteria e.g. *Paracoccus* | Akerman et al., 2013; Ghosh and Dam, 2009; Jones and Santini, 2023 |

## 5.2. Results

### 5.2.1. Nitrifying community composition

The AOB community in all aquaponics samples was dominated by *Nitrosomonas* and *Nitrosospira*, followed by *Nitrosovibrio* and *Nitrosococcus* (Figure 5.1). At the same time, NOB were mostly represented by *Nitrospira* and to a lesser degree by *Nitrobacter* (Figure 5.2). However, AOA community composition appeared to be more variable across samples (Figure 5.3), with genera such as '*Candidatus* Nitrosotenuis', '*Candidatus* Nitrosoarchaeum', *Nitrosopumilus* and *Nitrososphaera* being among the most abundant. *Nitrosospira* and *Nitrospira* abundance positively correlated with the abundance of most other AOB and NOB genera (Spearman $r_s \geq 0.73$, $p < 0.05$) while the archaeal genus *Nitrosopumilus* showed negative correlation with *Nitrospina* and '*Candidatus* Nitrosoglobus' and '*Candidatus* Nitrosoarchaeum' negatively correlated with two other AOA, specifically *Nitrososphaera* and '*Candidatus* Nitrosocosmicus'.

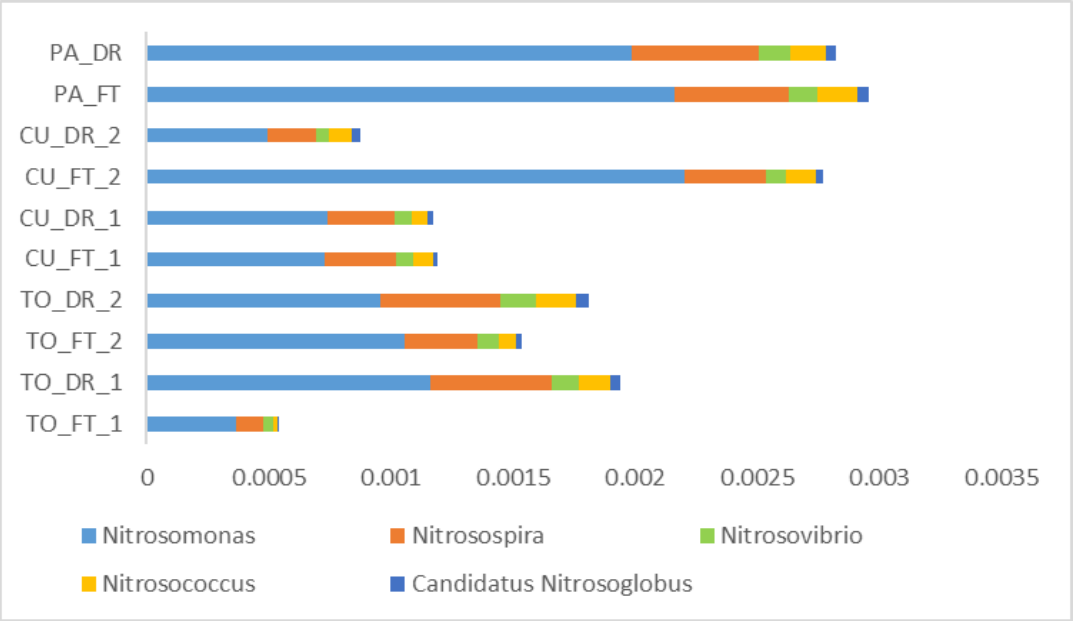



**Figure 5.1.** Relative abundance (%) of ammonia oxidizing bacteria (AOB) in the aquaponics water samples. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

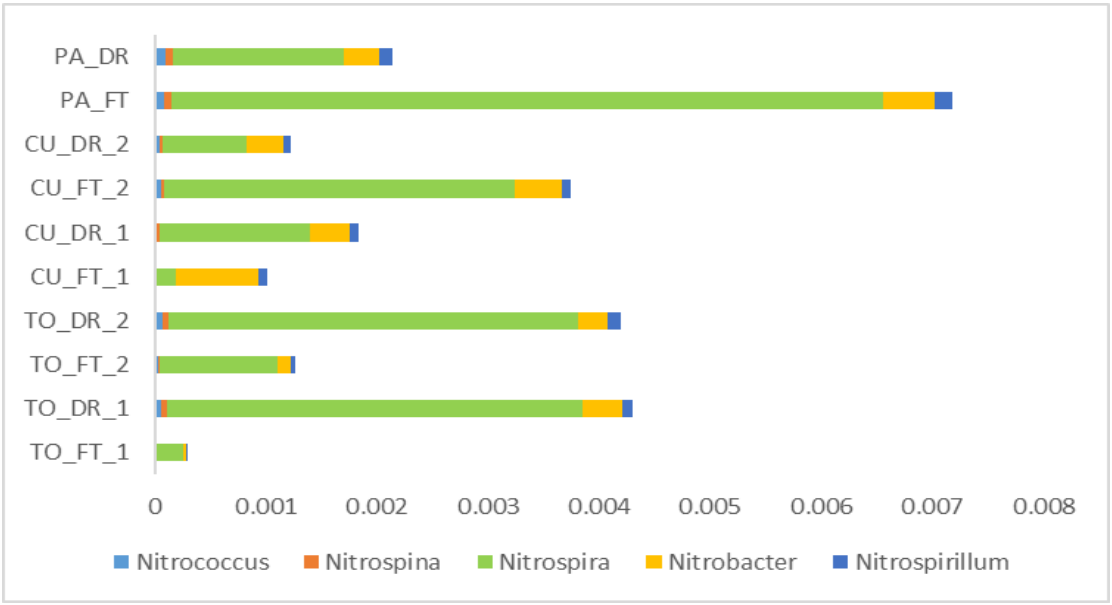



**Figure 5.2.** Relative abundance (%) of nitrite oxidizing bacteria (NOB) in the aquaponics water samples. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

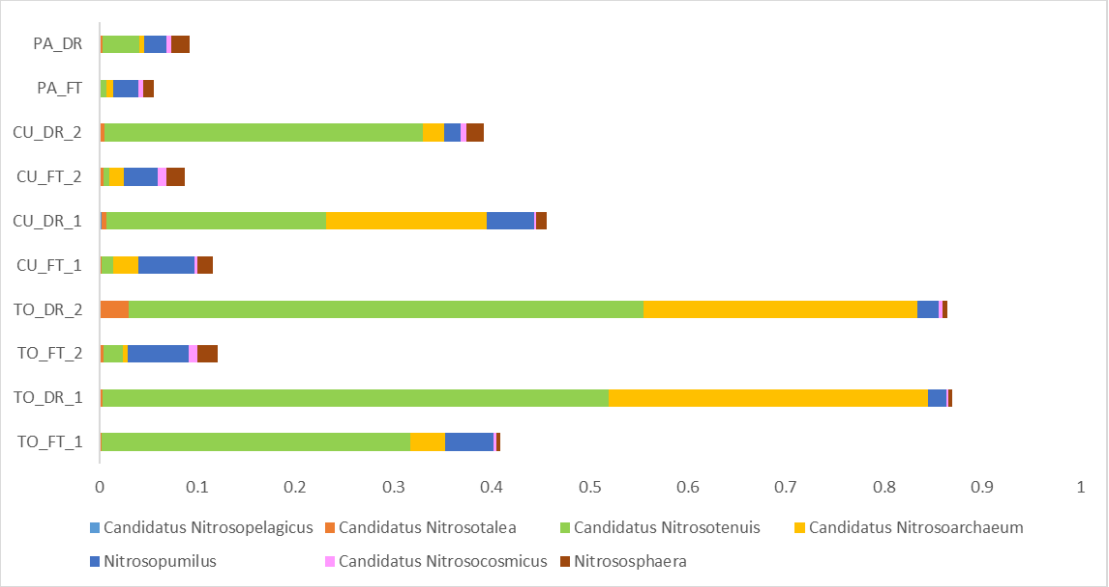



**Figure 5.3.** Relative abundance (%) of ammonia oxidizing archaea (AOA) in the aquaponics water samples. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

*Nitrosomonas* was the main AOB representative in the RAS samples as well, followed by *Nitrosospira* and *Nitrosovibrio* (Figure 5.4), and *Nitrospira* was the most abundant NOB (Figure 5.5). Relative abundance of AOB and NOB was about an order of magnitude higher in the fish tank biofilm community compared to both the fish tank water and biofilter biofilm communities, although AOA abundances were more similar in all three types of communities (Figure 5.6). Still AOA relative abundance was lower in the biofilter carrier communities compared to the fish tank associated communities. Contrary to the aquaponics samples, the dominant AOA genera in the RAS were *Nitrosopumilus*, *Nitrososphaera* while the rest of the AOA were represented with lower abundance.

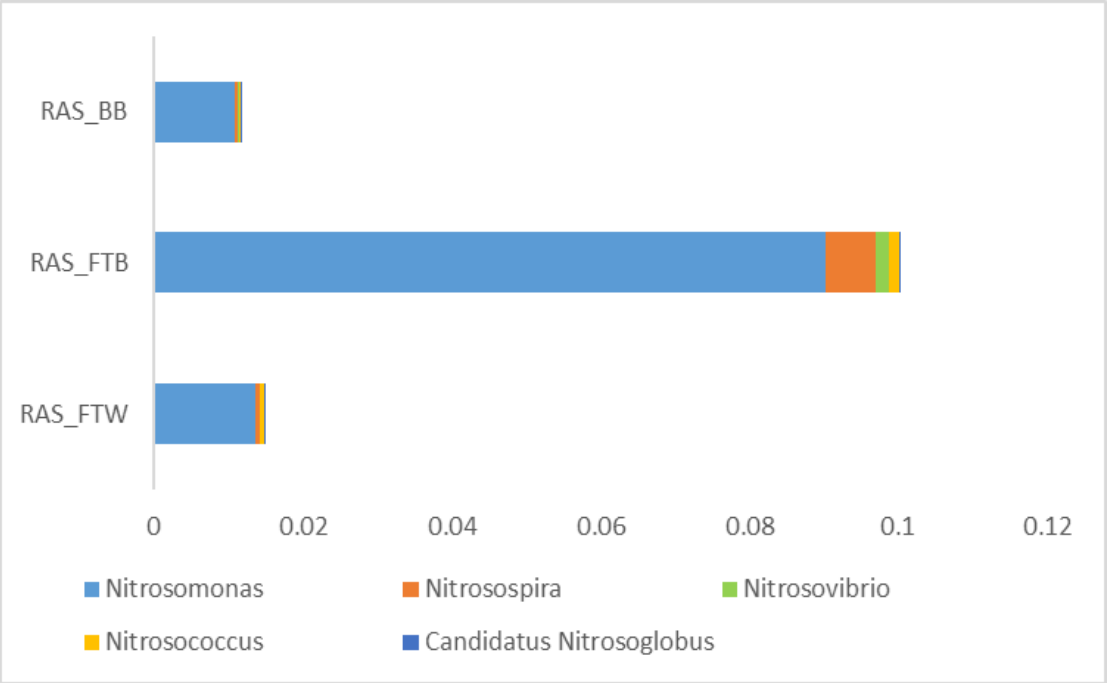



**Figure 5.4.** Relative abundance (%) of ammonia oxidizing bacteria (AOB) in the RAS samples. FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm.

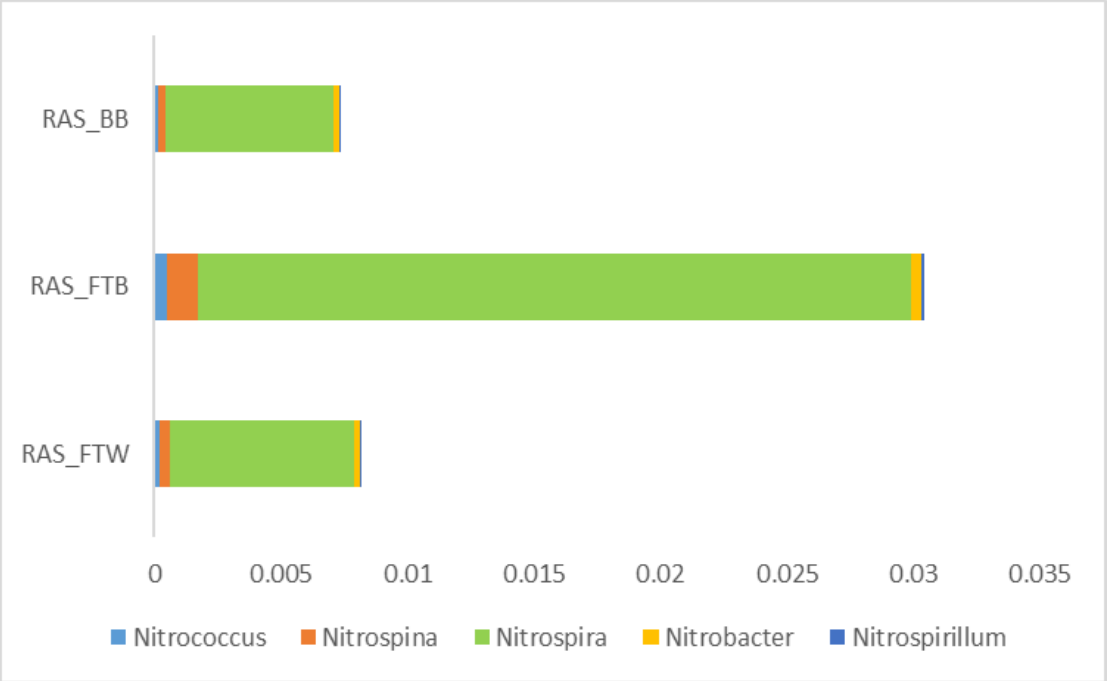



**Figure 5.5.** Relative abundance (%) of nitrite oxidizing bacteria (NOB) in the RAS samples. FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm.

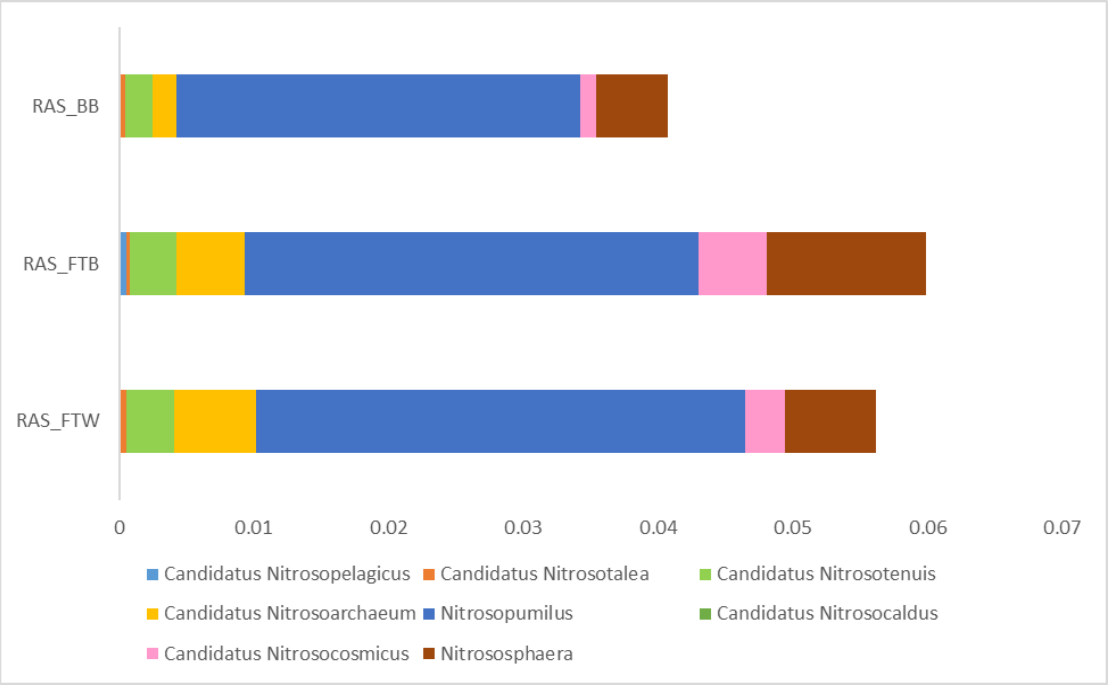



**Figure 5.6.** Relative abundance (%) of ammonia oxidizing archaea (AOA) in the RAS samples. FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm.

The occurrence and abundance of specific functional genes was also studied to assess the putative metabolic capacity of the nitrifying prokaryotic community across samples. All nitrification genes were present in low to non-detectable abundance in the fish tanks samples at the time of the first samplings in the tomato and cucumber growing systems, while the highest abundances were found in the fish tank samples taken near the end of the production cycle of the cucumber and parsley growing systems (Figure 5.7). Interestingly, *hzo* which is indicative of anammox bacteria was only detected in samples from the late stage aquaponics likely due to their slow growth rate (Wu et al., 2020), and in fact *hzo* was present in all three systems' fish tanks and the parsley systems drain tank in relatively high abundance. The abundance of genes associated with AOB (*amoA*, *amoB*, *amoC*, *hao*) and NOB (*nxrB*) peaked in the late stage cucumber and parsley systems' fish tank communities, although *nxrB* was also prominent in most drain tank samples. In the case of AOA, the archaeal *amoA*, *amoB* and *amoC* genes were mainly detected in the drain tanks of the

tomato and cucumber growing systems, and were clearly more abundant in the tomato system (Figure 5.8).

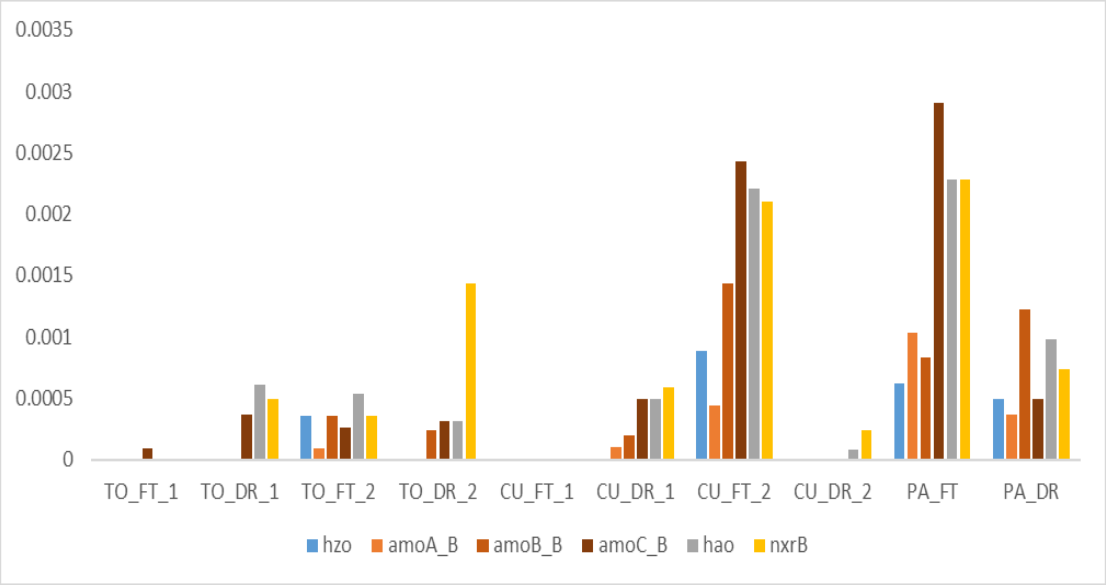



**Figure 5.7.** 16S-normalized relative abundance (%) of nitrification functional genes in the aquaponics samples. *hzo* : anammox; *amoA*, *amoB*, *amoC*, *hao* : AOB; *nxrB* : NOB. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

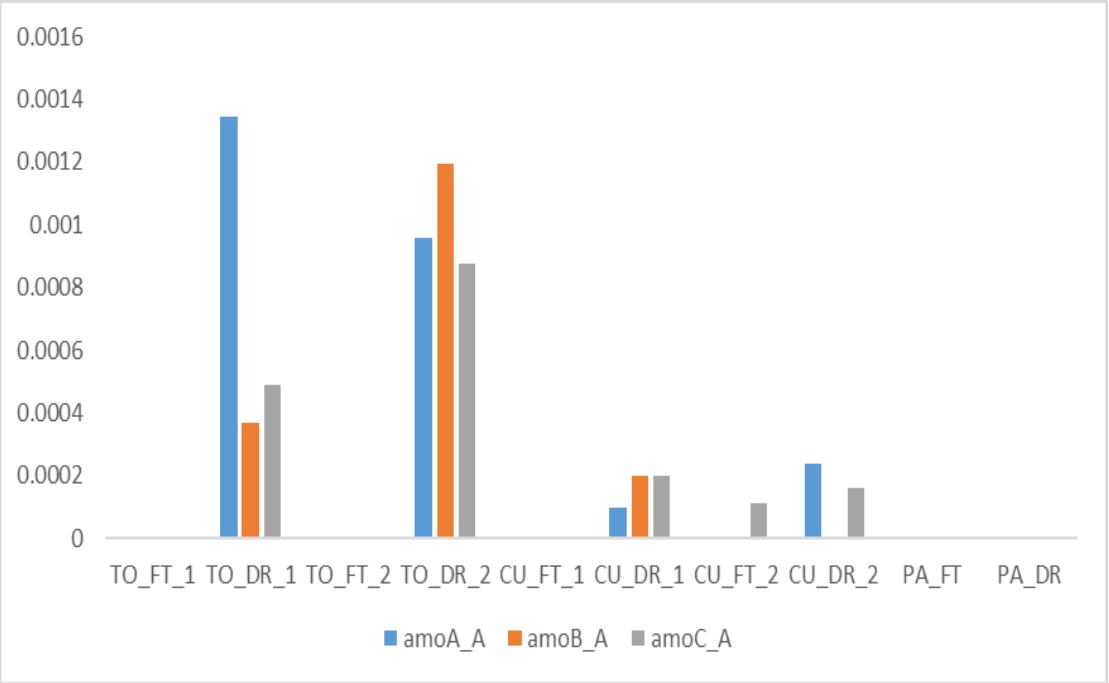



**Figure 5.8.** 16S-normalized relative abundance (%) of archaeal nitrification functional genes in the aquaponics samples. *amoA*, *amoB*, *amoC* : AOA. CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

Examination of the RAS samples revealed that no AOA functional genes were detected. The abundance of all nitrification genes was found to be highest in the fish tank biofilm community (Figure 5.9), unexpectedly higher than their abundance in the biofilter community sample. Among the nitrification genes investigated, *nxrB* was the most abundant in all three RAS niches sampled in the present study.

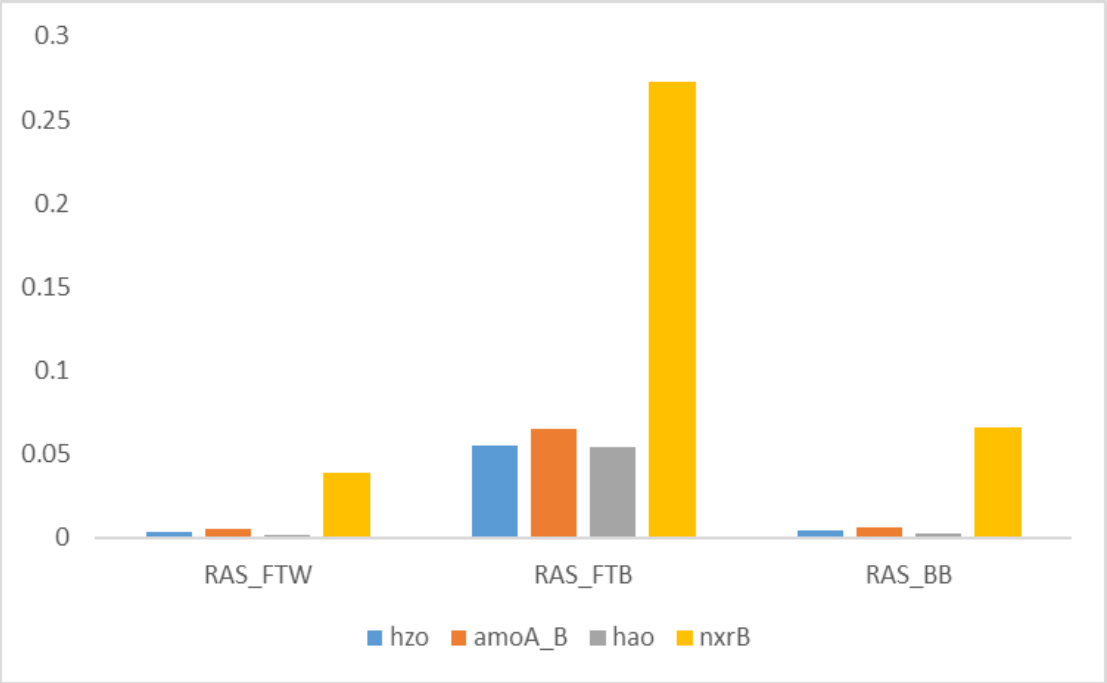



**Figure 5.9.** 16S-normalized relative abundance (%) of bacterial nitrification functional genes in the RAS samples. *hzo* : anammox; *amoA*, *hao* : AOB; *nxrB* : NOB. FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm.

5.2.2. Nutrient cycling gene composition

Apart from genes with a role in the nitrification process, the abundance of several other functional genes was also looked into to highlight metabolic processes related with nutrient cycling within recirculating aquaculture systems (Figure 5.10). Among the studied genes, *manB* was the most abundant of those associated with organic carbon degradation followed by *xylA* and both of these genes were more abundant in the cucumber growing system (Mann-Whitney test $p = 0.01$ and t-test $p < 0.01$ respectively). The *gdh* gene which takes part in both carbon and nitrogen metabolism was one of the most abundant, steadily well-represented across all samples. In addition, *glnA* which is another important gene for nitrogen metabolism was present with the highest abundance among the ones studied. Other commonly abundant genes that take part in nitrogen metabolism were *asnB* and *ureC*. Between the two genes involved in phosphorus metabolism, *phoA* was mainly detected in all

samples of the tomato system and drains tanks of the cucumber and parsley systems, whereas *ppx* was overall more abundant in the fish and drain tanks of the cucumber system near the start of its production cycle. Furthermore, the *soxB* gene was more abundant in fish tank samples compared to the respective drain tanks (Wilcoxon test, p= 0.04). Lastly, *mmoX* was only detected in the samples taken from the parsley growing system. Based on Spearman's index, negative correlation was observed between ansB and the sox genes, as well as between *abfA* and *mmoX* (Figure 5.11). However, most correlations were positive, especially in the case of *manB* which correlated with the *sox*, *glnA*, *ppx* and *abfA* genes. Also, *mmoX* and *ansB* genes ($r_s$ = 0.936) as well as *phoA* and *gdh* ($r_s$ = 0.939) respectively showed very strong positive correlation.

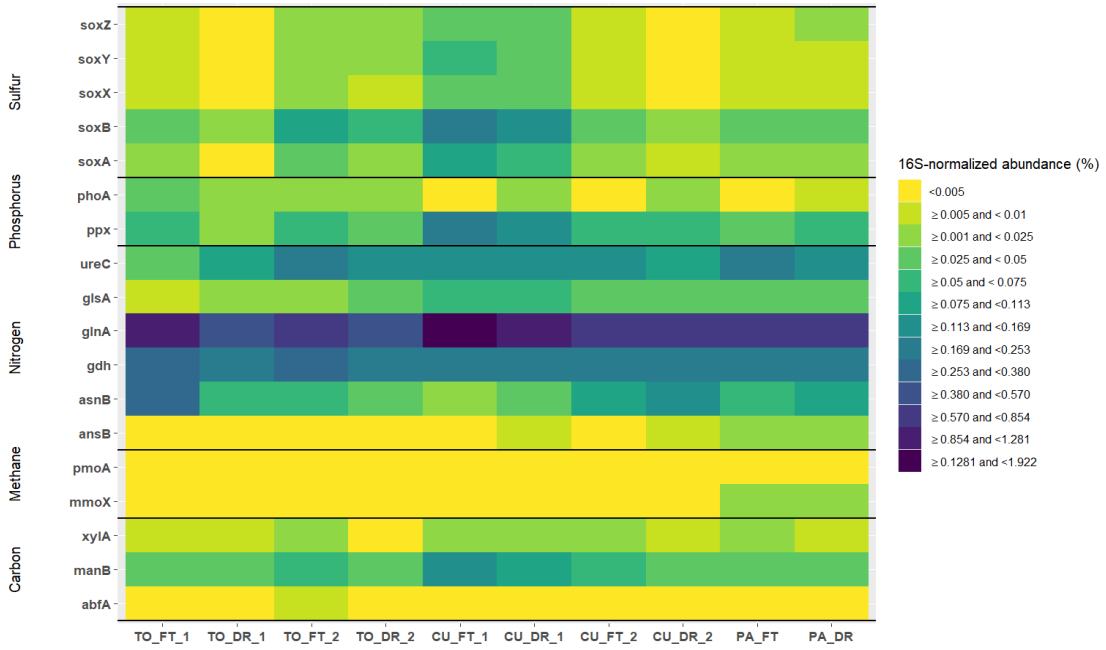



**Figure 5.10.** 16S-normalized relative abundance (%) heatmap of specific functional genes involved in prokaryotic community mediated nutrient cycling in the aquaponics samples. Carbon (C), Methane ($CH_4$), Nitrogen (N), Phosphorus (P), Sulfur (S). CU: cucumber aquaponics, PA: parsley aquaponics, TO: tomato aquaponics, FT: fish tanks, DR: drain tanks, 1: first sampling, 2: second sampling.

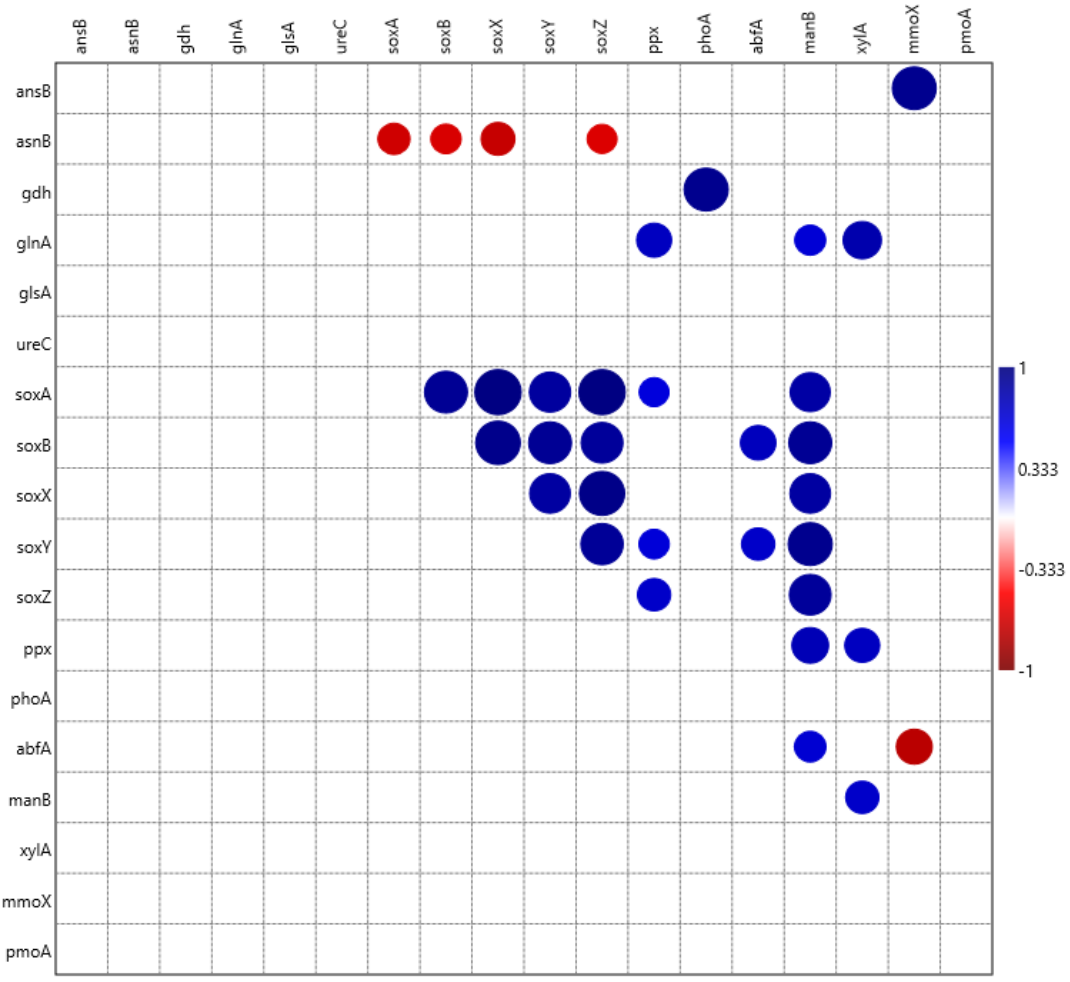



**Figure 5.11.** Correlation matrix based on Spearman's $r_s$ index using 16S-normalized relative abundance (%) of specific functional genes in the aquaponics samples. Only statistically clear ($p < 0.05$) correlations are shown. Blue color indicates positive correlation and red color indicates negative correlation.

Based on the occurrence and abundance of all the functional genes mentioned above, NMDS and PERMANOVA analysis was performed for the aquaponics and RAS samples based on Bray-Curtis similarity index (Figure 5.12). The results indicated that mature communities had a more similar metabolic potential compared to other communities from the same system or compartment type. Thus, it appears

that over operation time, aquaponics communities converged in terms of functional gene content, although they did not converge in terms of taxonomic diversity.

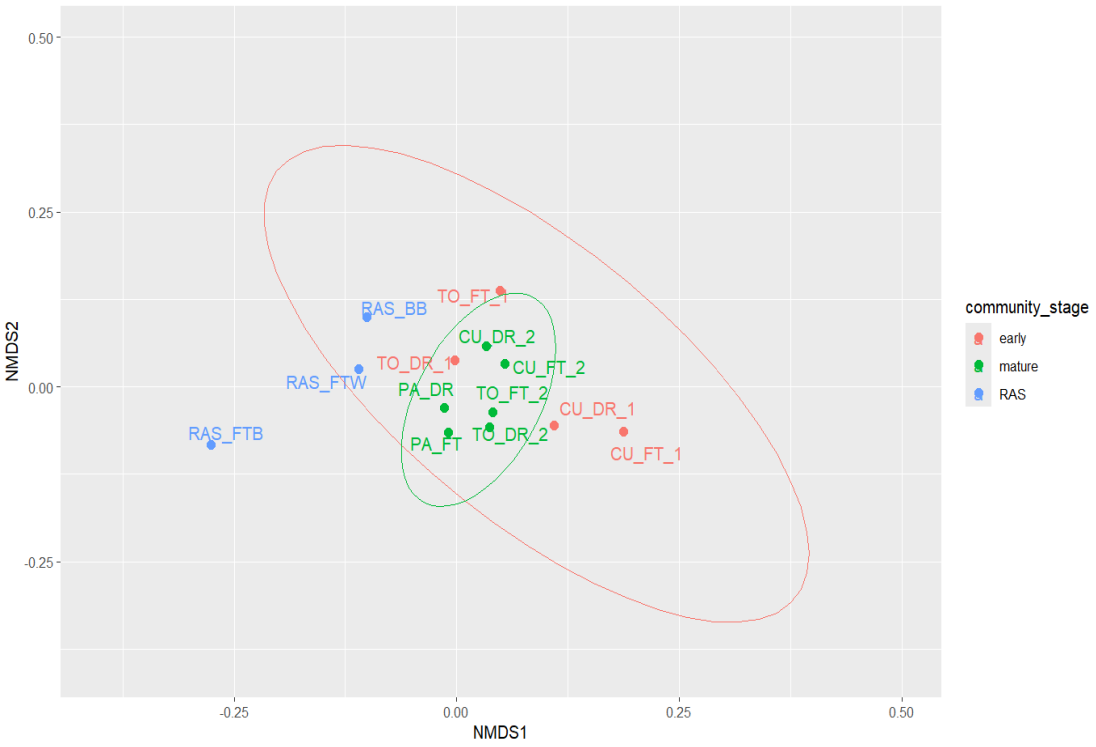



**Figure 5.12.** NMDS plot depicting the grouping of microbial communities by maturity stage based on the abundance of the functional genes investigated in the present study. (DR: drain tanks, FT: fish tanks, RAS: RAS fish tanks, TO: tomato aquaponics, CU: cucumber aquaponics, PA: parsley aquaponics, 1: first sampling time, 2: second sampling time, FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm).

Regarding the RAS samples, no genes involved in methane metabolism (*mmoX*, *pmoA*) were detected (Figure 5.13). In addition, *soxB*, *phoA* and *ppx* were similarly represented in all three RAS communities. The biofilter community harbored a higher abundance of *xylA* but a lower proportion of *manB* compared to the fish tank communities. Most of the genes involved in nitrogen metabolism were highly represented in all RAS communities, but still the biofilter sample had a higher abundance of *glnA*, *gdh* and *asnB* compared to the fish tank samples.

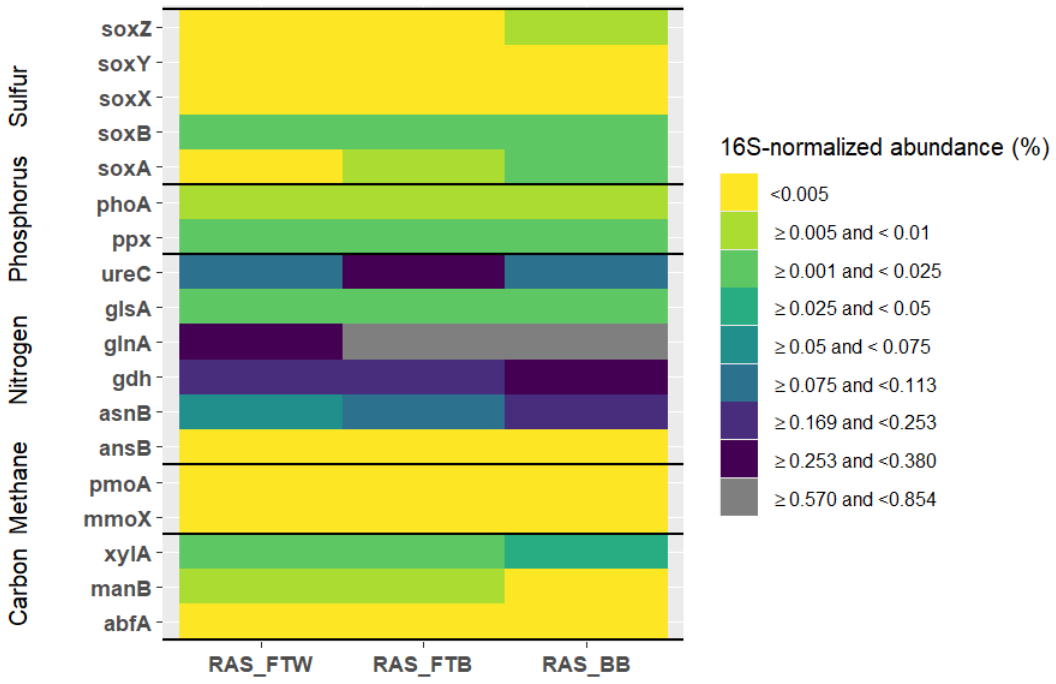



**Figure 5.13.** 16S-normalized abundance (%) heatmap of specific functional genes involved in prokaryotic community mediated nutrient cycling in the RAS samples. Carbon (C), Methane ($CH_4$), Nitrogen (N), Phosphorus (P), Sulfur (S). FTW: fish tank water, FTB: fish tank biofilm, BB: biofilter biofilm.

## 5.3. Discussion

Metagenomic analysis of the aquaponics samples revealed that the AOB communities mainly consisted of four genera, *Nitrosomonas* which was clearly dominant in every case, followed by *Nitrosospira*, *Nitrosovibrio* and *Nitrosococcus*. *Nitrospira* and *Nitrobacter* made up the majority of the NOB community. These observations corroborate the limited earlier reports (Bartelme et al., 2019; Eck et al., 2019b; Kasozi et al., 2021a; Rogge et al., 2024; Schmautz et al., 2022) that have shown *Nitrospira* and *Nitrosomonadaceae* as the main nitrifiers in aquaponics systems. Furthermore, the presence of nitrifiers like *Nitrospira* and *Nitrosomonas* in the aquaponics fish tanks and drain tanks supports the suggestion that nitrification also takes place in other compartments apart from the biofilter itself (Schmautz et al., 2022). At the same time, the most abundant AOA taxa across samples included '*Candidatus* Nitrosotenuis', *'Candidatus* Nitrosoarchaeum', *Nitrosopumilus* and *Nitrososphaera*. *Nitrososphaera* and *Nitrosopumilus* have been detected in high relative abundance in aquaponics systems before (Bartelme et al., 2019) and Nitrososphaeria have been found in aquaponics biofilter communities (Schmautz et al., 2022) although in low abundance. While '*Candidatus* Nitrosotenuis' has been identified in biofilter samples from freshwater aquaria (McKnight and Neufeld, 2021; Sauder et al., 2018) and is also widespread in freshwater lakes and rivers along with *'Candidatus* Nitrosoarchaeum*'* (Ren and Wang, 2022), this is the first time these candidate genera have been identified in aquaponics samples, and in a relatively high abundance (0.005 – 0.525% and 0.004 – 0.325% respectively) compared to the other archaeal nitrifiers as well. The low ammonia concentration in a successful aquaponics system may be driving the selection of NOB and comammox *Nitrospira* species as well as AOA which generally have a lower ammonia concentration requirement compared to most AOB (Bartelme et al., 2019; Preena et al., 2021). The overall differences in the relative abundance of nitrifiers observed in different aquaponics systems and compartments further support the suggestion that there cannot be one common optimum nitrifier consortium for all aquaponics systems (Bartelme et al., 2019).

As also reported in previous studies (Moschos et al., 2022; Rurangwa and Verdegem, 2015) *Nitrosomonas* was the dominant AOB in the RAS samples, followed by *Nitrosospira* and *Nitrosovibrio*, while *Nitrospira* was once again the most abundant NOB. However, *Nitrospina* was the second most abundant NOB in the RAS whereas *Nitrobacter* was more prominent in the aquaponics samples instead. This difference can likely be explained by the fact that *Nitrobacter* mainly consists of freshwater species although there are also taxa encountered in the marine environment, while *Nitrospina* is among the dominant NOB in the marine environment (Elling et al., 2022). Interestingly, the fish tank biofilm (FTB) was shown to harbor a higher relative abundance of both AOB and NOB compared to both the fish tank water (FTW) and the biofilter biofilm (BB) (Figures 5.4, 5.5), although by design the biofilter sample was expected to be the most enriched in nitrifiers. A similar observation was made by Schmautz et al. (2022) who reported a higher abundance of Nitrosomonadaceae in fish tank samples compared to biofilter samples. The different material used for DNA extraction (biofilm carriers and cotton swabs for the biofilter and fish tank biofilm respectively) could also be a contributing factor to the observed difference in relative abundance in the present study, as extraction efficiency may have been different for each material. Still, AOA were also less abundant in the biofilter sample compared to the fish tank samples, although the difference in this case wasn't as high. In contrast to the aquaponics AOA communities, the prevalent AOA in the RAS were *Nitrosopumilus* and *Nitrososphaera*, both associated more with marine habitats (Bei et al., 2024; Kraft and Canfield, 2022).

To further investigate the nitrifying community, the presence of functional genes that play a major role in different nitrification processes was also studied. All nitrification genes were either not present or under the detection level of the DNA analysis in the fish tank samples taken near the start of the production cycle of the aquaponics systems. Furthermore *amoA*, *hao* and *nxrB* abundance was also relatively low in the drain tanks at the time of the first samplings. However, the abundance of all nitrification genes increased by the time of the late-stage samplings and peaked in the fish tank samples of the cucumber and parsley growing systems. Thus, it is evident that

the abundance of nitrification genes increased over time in the aquaponics systems (Figure 5.7). The increase of *amoA* genes during the operation period of aquaponics systems has been also reported by (Derikvand et al., 2021). Additionally, the higher abundance of the *nxrB* gene in relation to the *amoA* gene in the samples of the present study is in line with the observation made by Gao et al. (2022b). In the same study, the authors noted that NOB dominated the nitrifying community, which was also true in the present study as *Nitrospira* was by far the dominant nitrifier with regard to relative abundance. The increase of both *nxrB* and *amoA* relative abundance in the late stages of the aquaponics systems could also indicate the increase of comammox *Nitrospira* species which possess both genes. As for the AOA component of the nitrifying community, archaeal amoA was only detected in the drain tank samples of the tomato and cucumber growing systems, and its relative abundance was higher in the former system. AOA were probably more abundant in the drain tanks due to the higher concentration of ammonia (Table 4.3) that enabled their growth in these compartments. In addition, the increased abundance of AOA in the presence of tomato plants was also noted by Carreras-Sempere et al. (2024) in soilless cultivation systems. Thus, plant choice and the optimization of water and nutrient parameters that go with it may promote or inhibit the growth of AOA within aquaponics. Apart from nitrification, another metabolic process associated with the nitrogen cycle is anammox. Anammox turns ammonium and nitrite into dinitrogen gas ($N_2$), effectively removing nitrogen from the aquaponics system (Liu et al., 2022). The *hzo* gene which plays a key part in the anammox pathway was detected in late stage fish tanks of the three aquaponics systems and the drain tank of the parsley system. At the same time, Planctomycetota which is among the main anammox phyla in RAS was also more abundant in the late-stage samples of the examined systems. The increased presence of anammox bacteria like Planctomycetota during the later stages of aquaponics production cycles can be explained by their relatively slow growth rate, and the time required for anoxic or low-oxygen microenvironments to form within developing biofilms in the aquaponics compartments, where Planctomycetota can coexist with nitrifying taxa (Preena et al., 2021).

Among the functional genes examined in the present study, ammonia assimilation genes like *glnA*, *gdh* and *asnB* were the most abundant. Both the *glnA* and *gdh* genes have been reported among the dominant genes associated with the decomposition of organic nitrogen in freshwater environments (Wan et al., 2023). Specifically, *glnA* which encodes a glutamine synthetase enzyme plays a crucial role in the nitrogen metabolism of prokaryotic taxa including nitrifiers (Xiao et al., 2021). Another functional gene represented in high abundance across samples was *ureC*, which encodes the extracellular urease enzyme in prokaryotes and fungi, allowing them to hydrolyze urea to ammonium (Jiang et al., 2023). This urease encoding gene has been characterized as abundant in freshwater habitats before (Wan et al., 2023) and as it breaks down urea it leads to the formation of bicarbonate that can act as buffer, helping to counteract possible acidification of RAS and aquaponics water that would inhibit nitrification (Derikvand et al., 2021; Tyson et al., 2004). Furthermore, it has been suggested that marine AOA can utilize urea-derived ammonia for nitrification using their own *ureC* genes and also by relying on the *ureC* genes of other urea decomposing prokaryotes (Shiozaki et al., 2021).

Functional genes related to the cycling of other main nutrients were also investigated. Starting with sulfur, the *soxB* gene was detected in relatively high abundance across all samples, though it was more abundant in the fish tanks of the aquaponics systems. This particular gene is part of the Sox enzyme system, one of the few major pathways for sulfur oxidation in prokaryotes, and is used as a marker for the presence of sulfur oxidizing bacteria (Akerman et al., 2013; Krishnani et al., 2010). Oxidation of inorganic sulfur (e.g., sulphide, sulphite, thiosulphate) to sulphate is critical for providing bioavailable sulfur to microorganisms and plants, both of which are major components of aquaponics systems. The high abundance of the *soxB* gene in fish tanks indicates that sulfur oxidizing bacteria, mainly facultative chemolithotrophic Alphaproteobacteria (Tourna et al., 2014), are more abundant in that compartment likely due to the increased load of sulfur added through fish feed and excretions.

Phosphorus, another essential nutrient, can be introduced to aquaponics systems in the form of polyphosphates added either through fish feed or plant fertilizer. However, phosphorus is absorbed by plants predominantly in the form of orthophosphate (Kulakovskaya et al., 2012). Bacteria, which also require orthophosphate can produce enzymes such as alkaline phosphatases which hydrolyze orthophosphate monoesters and are expressed by the Pho regulon genes such as *phoA*, as well as exopolyphosphatases encoded by the *ppx* gene which release orthophosphate from a polyphosphate chain (Srivastava et al., 2022). The overall high relative abundance of the *ppx* gene, especially during the first sampling of the cucumber system, could be the result of increased polyphosphate concentration (Ramzan et al., 2025), and the presence of *ppx* across samples could indicate that phosphorus cycling can effectively take place in the aquaponics system.

Furthermore, *xylA* and *manB* which are genes associated with the degradation of organic carbon were also abundant across the aquaponics samples. As aquaculture feeds are increasingly relying on plant derived proteins to keep up with production demands and minimize costs, starch and non-starch polysaccharides are becoming more common in farmed fish diets (van Riel et al., 2023). Xylan is a prime example of non-starch polysaccharide in cereals, and as generally indigestible is of particular importance due to the negative impact it has on farmed animals and fish by decreasing nutrient availability and limiting growth (Yang et al., 2019) However, diets containing 1.25% xylan have also exhibited prebiotic effects in juvenile turbot by enhancing the intestinal mucosal barrier function and by regulating the gut microbiome (Yang et al., 2019). The presence of bacteria bearing the *xylA* gene which encodes the xylose isomerase enzyme could be an indicator of xylan degradation to xylose in the system, and further xylose utilization by the *xylA* bearing bacteria. The function of such enzymes in the fish gut is beneficial for fish digestion, and the role of xylan degrading gut bacteria such as *Lactobacillus* and *Bifidobacterium* species (Gufe et al., 2024; Poolsawat et al., 2021) may become increasing relevant in the future. Mannans are another type of plant polysaccharide and a known component of hemicellulose. The β-mannanase gene, *manB*, was also among the most abundant functional genes

examined in the present study. This mannan degrading enzyme has been already used in dietary supplementation to promote beneficial microorganisms like Pseudomonadota, Actinomycetota and Bacillota in fish gut and to aid digestion by reducing the viscosity of digesta (Adeshina et al., 2024). Given that the fish feed used in the aquaponics systems examined in the present study contained cereal, soy, and algal matter, the presence of *xylA* and *manB* genes with high relative abundance is justified. Finally, among the two genes commonly used for the detection of methanotrophs, *pmoA* and *mmoX* (Wang et al., 2023a), only the latter was identified and only in the samples of the parsley growing system. The presence of mmoX has been linked with genera such as *Methylococcus*, *Methylomonas* (Wang et al., 2023a) and also *Mycobacterium* (Cupples and Thelusmond, 2022; van Spanning et al., 2022). In the present study, samples from the parsley system were particularly enriched in representatives of the methylotrophic Betaproteobacteria genera *Methyloversatilis* and *Methylibium* (Boada et al., 2020; Smalley et al., 2015), as well as the commonly dominant *Mycobacterium*. There was also a higher relative abundance of Verrucomicrobiota in the parsley system, and this phylum is known to contain the most methanotrophic genera after Pseudomonadota (Wang et al., 2023a). Even though aquaponics systems can have low net carbon emissions due to optimal fish feed utilization and $CO_2$ uptake by plants, lots of emissions associated with system operation and maintenance have yet to be quantified (Kalvakaalva et al., 2022). Thus, the prospect of methylotrophic genera proliferating in aquaponics systems and further limiting methane emissions seems promising. Furthermore, methanotrophs can also degrade other types of short chain carbon compounds thanks to the low substrate specificity of the monooxygenase enzymes they produce (Wang et al., 2023a).

As for the RAS samples, the highest abundance of nitrification associated genes was detected in the fish tank biofilm, rather than the biofilter biofilm. Despite the presence of several known nitrifying archaea like *Nitrosopumilus* and *Nitrososphaera*, no archaeal *amoA* genes were identified. However, the clear dominance of *Nitrospira* in the nitrifying community was reflected in the high relative abundance of the *nxrB* gene. Phosphorus and sulfur cycling related genes were found

to be uniformly represented across all RAS samples and no methanotrophic taxa were found based on the lack of *pmoA* and *mmoX* genes. The biofilter community seemed to be the most enriched in nitrogen metabolism genes. Lastly, *manB* genes were more abundant in the fish tank communities, likely due to direct access of fish tank prokaryotes to the added fish feed and excretions.

# 6. General discussion and conclusions

## 6.1. General discussion

Recirculating aquaculture technologies and particularly aquaponics systems are considered a promising alternative to standard food production methods such as pond aquaculture and land-based cultivation, as they combine the production of different types of high quality food with minimal water usage and nutrient input requirements. Given that RAS and aquaponics systems' function depends on the metabolic activity of prokaryotic communities within the systems, and that these communities can be shaped by bottom-up and top-down control as well as inter-species interactions, it is clear that their whole microbial community calls for investigation. Thus, the characterization of microbial taxonomic and functional diversity can elucidate microbial interactions and contribute to the conceptualization of more efficient aquaponics designs or highlight ways to achieve biocontrol and improve production.

To address this, the present study involved the investigation of the microeukaryotic and prokaryotic communities across different aquaponics systems, focused on fish rearing and fertigation drainage tanks. Microeukaryotes, and particularly heterotrophic ciliates and HNF were quantified using microscopy, and their achievable growth rates were measured through *in situ* bottle incubations following size fractionation. Additionally, both the microeukaryotic and prokaryotic suspended community were studied using metagenomics to describe their taxonomic diversity, aiming to elucidate patterns across systems and compartments. The functional diversity of the prokaryotic diversity was also investigated, to demonstrate the capacity of the suspended community to perform nitrification and contribute to nutrient and organic matter cycling. Artificial seawater RAS samples were similarly examined to compare the suspended microbial community of fish tanks with the attached communities growing on fish tank and biofilter biofilms, looking to highlight microeukaryotic diversity and prokaryotic functional diversity.

Natural abundance of ciliates and HNF in the aquaponics systems was relatively low for freshwater systems though within the typical range encountered in rivers (e.g., Bouvy et al., 2010; Kiss et al., 2009; Scherwass et al., 2010) or oligotrophic to mesotrophic lakes (e.g., Berdjeb et al., 2011; Cai et al., 2020). This low abundance is contradicted by the high growth rate that these heterotrophs can potentially achieve, as shown in the growth experiments. The fact that the natural bacterial abundance was adequate to sustain the growth of heterotrophic protists, as evidenced by the results of the growth experiments and relevant literature (Esteban and Fenchel, 2020; Weisse and Montagnes, 2021), coupled with the lack of larger predators of ciliates confirmed through microscopy likely suggest that some other factor prevents the growth of heterotrophic microeukaryotes. Of course, water undergoes sterilization before it is returned to the fish tanks after plant fertigation, but several of the detected ciliate taxa such as *Cyclidium*, *Euplotes* and *Vorticella* can attach or move along surfaces to feed (Esteban and Fenchel, 2020; Macek et al., 1996), avoiding sterilization and thus becoming likely fast growing taxa on aquaponics tank biofilms. The prevalence of ciliates that feed on bacteria and the capacity of these ciliates and HNF to grow in aquaponics compartments suggests that these grazers do apply top-down pressure on prokaryotic communities in aquaponics systems. However, the even lower abundance of ciliates and HNF in RAS fish tanks along with the negative growth rates calculated, indicate that heterotrophic protists couldn't successfully grow in the constantly flowing artificial seawater RAS and thus didn't exert as much influence on its prokaryotic communities. It is likely that most ciliates which are introduced to the system through the source water cannot properly adapt to the increased salinity of the artificial seawater medium. In addition, ciliates have been shown to be negatively affected by increased water flow rate and turbulence through prevention of food capture and dispersal from food patches (Risse-buhl et al., 2009), leading to lower ingestion rates and slower growth (Dolan et al., 2003). Thus, fast ciliate growth may be largely inhibited in RAS fish tanks while aquaponics compartments such as drain tanks which aren't characterized by constant water flow can provide a more hospitable environment for ciliate growth.

Based on microscopic inspection of ciliates, the most notable genera in terms of abundance or growth rate were *Cyclidium*, *Halteria*, *Paramecium*, *Litonotus*, *Vorticella* and *Euplotes*. However, metagenomics analysis revealed that the most abundant ciliates across samples were *Oxytricha*, *Stylonychia* and *Stentor*. This discrepancy is somewhat expected as a result of limitations of both methods, especially when it comes to Ciliophora. Previous studies (Santi et al., 2021; Stoeck et al., 2014) have already addressed this disagreement of microcopy and DNA based methods, and have attributed it to incomplete genetic databases and the inherent difficulty of precise morphology-based classification of ciliate taxa. A similar disagreement of results between the two methods was also evident in the case of Amoebozoa, as genera with characteristic morphology such as *Centropyxis* and *Euglypha* were identified through microscopy in high abundance but were not detected or detected in very low abundance through metagenomic analysis. Despite not being an object of the present study, phytoplankton was more accurately detected with both methods, as in the case of *Chlorella* and *Scenedesmus* or *Tetradesmus*. Thus, the results of the present study further support the practice of using both microscopy-based and metabarcoding approaches to more accurately describe taxonomic diversity of planktonic microeukaryotes (Santi et al., 2021).

Several results of the present study point to the fact that aquaponics fish rearing tanks and fertigation drainage tanks constitute different niches for microbial settlement and growth. To begin with, both ciliate and HNF growth rates were clearly higher in the fish tanks compared to the drain tanks. Drain tanks also harbored a higher abundance of phytoplankton such as *Chlorella*, Scenedesmaceae, diatoms and Cyanobacteriota likely due to being located in the greenhouse and thus more easily exposed to direct sunlight through their open lids, compared to the fish tanks which were housed indoors. Although sunlight exposure was indeed limited in the drain tanks, it has been shown that shading can lead to increased phytoplankton abundance in freshwater ponds (Yamamichi et al., 2026). Another reason why phytoplankton was more abundant in the drain tanks was likely the higher average concentration of bioavailable nitrogen and phosphorus (Table 4.3). Additionally pH was consistently

lower in the drain tanks, closer to 7.5 compared to about 8 in the fish tanks, and most phytoplankton species grow optimally between 6.5 and 8.5 (Hinga, 2002; Lakane et al., 2025). Other heterotrophic microeukaryotes such as amoebae and rotifers were also mainly detected in the drain tanks. Furthermore, both archaeal and bacterial richness were clearly higher in the drain tanks while microeukaryotic diversity was higher in the fish tanks. Based on molecular data, community composition in samples from the same compartment was more similar in the case of microeukaryotes and archaea, whereas based on bacterial community composition, samples were more similar when they originated from the same aquaponics system. This could be explained through the higher relative abundance of bacteria and their ability to disperse and colonize different niches more easily (Echenique-Subiabre et al., 2025). In addition, the difference between the communities in the two compartments is also shown by the fact that different ciliates were more abundant in each, with *Oxytricha* and *Stylonychia* dominating the ciliate population in drain tanks and *Stentor* being the prominent ciliate in fish tanks. Apart from taxonomy, drain tanks samples were also the only ones harboring archaeal genes associated with nitrification, indicating a possible niche other than the biofilter allowing archaeal nitrification. Consequently, as system water may spend a significant amount of time in the drain tanks before undergoing sterilization and reintroduction to the fish tanks, the microbial community of the drain tanks can potentially influence its quality by performing metabolic functions and affect the RAS component even if the microorganism themselves may not carry over. For instance, bacteria can break down organic matter and contribute to nutrient cycling, or produce toxins, antimicrobial compounds and plant growth promoting enzymes (Carrión et al., 2019; Eck et al., 2019a; Kwak et al., 2018; Schmautz et al., 2017). At the same time, heterotrophic or mixotrophic microeukaryotes can graze on these prokaryotes and affect their community composition. Considering all the above, the microbial component of the drain tanks in coupled aquaponics systems should be considered an important factor in the system's function, alongside the fish tank community and of course the biofilter community itself.

Differences in community composition were also detected among different aquaponics systems examined in the present study, mainly regarding the bacterial component. While the samples of the tomato growing system showed a dominance of *Flavobacterium* and *Limnohabitans* in the bacterial community, *Polynucleobacter* and *Mycobacterium* were the common most abundant genera in the cucumber system. Such a difference could be the result of different plant choice, as *Flavobacterium* has been hinted as a putative growth promoting genus in tomato plants before (Kwak et al., 2018). The bacterial communities of different systems were not only taxonomically distinct but also functionally, based on the occurrence and abundance of key genes associated with nitrification, nutrient cycling and organic matter degradation. For example, samples from the fish tanks and drain tanks of the cucumber system were more similar to each other at the time of each sampling, than to samples from fish tanks or drain tanks from the other two systems. Furthermore, the parsley growing system was the only one that harbored a detectable amount of gene copies indicative of methanotrophic genera, regardless of compartment. This difference was also reflected in the higher abundance of methanotrophic genera such as *Methyloversatilis* and *Methylibium* as well as Verrucomicrobiota. The successful function of aquaponics systems can thus be achieved despite the establishment and proliferation of different abundant or functional planktonic bacterial taxa, and plant choice along with the compromise of optimal water parameters for fish and nitrifiers can lead to different community composition between aquaponics systems.

Multiple microbial groups detected in the present study can be characterized as relevant in the context of aquaponics and can help shape the direction of future research. First of all, known nitrifiers such as *Nitrosomonas* and *Nitrospira* were widespread in both the fish tanks and the drain tanks of all aquaponics systems. Similarly, nitrification associated genes were also detected in both compartments with an increase in abundance in the later stage of operation. Also in the later stage, the increase in the abundance of anammox genes implies that this process also takes place in these compartments, likely in anoxic layers of more mature biofilms. Thus, it appears that nitrification and nitrogen removal can also take place to the same degree

in other parts of the aquaponics systems, besides the nitrification biofilter and anammox bioreactors. In the case of archaea, nitrification genes were detected only in drain tanks which was likely the result of higher ammonia concentration compared to the fish tanks. Interestingly, the most abundant AOA were shown to be '*Candidatus* Nitrosotenuis' and '*Candidatus* Nitrosoarchaeum', both detected for the first time in aquaponics systems. Genes associated with nitrogen, sulfur and phosphorus cycling were also widespread across the aquaponics samples, indicating that the planktonic community contributes to the remineralization of nutrients and the breakdown of complex organic matter, thus assisting the growth of nitrifiers and improving the bioavailability of fish feed and its nutrients to fish and plants alike. Specifically, the presence of sulfur oxidizing bacteria can improve sulfur uptake via plant roots, while non-starch polysaccharide degrading bacteria can aid fish digestion. Additionally, methanotrophic genera can also proliferate in aquaponics systems under favorable conditions, and their implementation may become beneficial as a way of reducing carbon emissions associated with the operation of aquaponics systems. Apart from functionally relevant microorganisms, others can possibly be of use as indicators of good water quality and microbial community health. For example, ciliates such as *Stylonychia* and *Stentor* are sensitive to perturbation and their presence in high abundance as was the case in the systems examined in the present study implies an overall healthy ecosystem. Genera containing pathogenic species were also detected among the microeukaryotic and prokaryotic community. *Flavobacterium* was among the most abundant bacterial genera in the aquaponics samples which is also known to include pathogenic species, as well as putative plant growth promoting species. Meanwhile, *Aphanomyces* and *Phytophthora* (Oomycota) which contain pathogenic species to fishes or plants were detected in the examined aquaponics system, although no disease outbreak was recorded. Possible beneficial microeukaryotes were also detected, such as *Aspergillus* (Ascomycota) which enhances plant growth and *Chlorella* (Chlorophyta) which produces bioactive compounds with antimicrobial and antioxidant properties (Tiwari et al., 2023).

The investigation of the artificial seawater RAS microeukaryotic community showed major differences compared to the only previous similar study. These differences were evident both in the case of Ciliophora, where in the samples of the present study *Stylonychia*, *Oxytricha* and *Stentor* were the most abundant representatives determined through metagenomics analysis and *Cyclidium*, *Apsidisca* and *Colpoda* were prevalent under microscopic analysis, whereas *Zoothamnium* was dominant in the samples examined by Boaventura et al. (2018). Another major distinction was the high relative abundance of Ascomycota and Basidiomycota in the samples of the present study. These differences can probably be attributed to the different RAS setups and farmed fish species, as well as the ongoing update of genomic databases over time. The high relative abundance and ubiquitous presence of Ascomycota genera in the RAS samples suggest that these fungal communities may be worth looking into in the context of RAS optimization, further strengthening the argument that microeukaryotic communities should be taken into account in future RAS studies (Boaventura et al., 2018). Meanwhile, the low abundance of ciliates and HNF in the water samples based on microscopy and molecular data as well as their population decline during the growth experiments coupled with the increased abundance of ciliates in biofilm samples based on molecular data implies that ciliates are mainly active grazers within the biofilms. This is relevant as the biofilms, and especially the fish tank biofilms were found to harbor a relatively high abundance of nitrifying prokaryotes and bacterial nitrification associated genes. In addition, biofilm communities also contained a high relative abundance of genes involved in nutrient cycling and organic matter degradation. Thus, although ciliates may be scarce in the RAS water column, benthic ciliates can still interact and graze on the prokaryotic community within biofilms, which also happens to be the most metabolically active and as such important for RAS function. Another interesting finding regarding ciliates in RAS biofilms was the presence of the putatively pathogenic genera *Ichthyophthirius* and *Pseudocohnilembus*. All these observations suggest that future ciliate and fungal research in RAS should focus on biofilm communities, and that fish tank biofilm microbial communities may be more important than previously considered. Finally, the confirmed presence of known archaeal nitrifying taxa and the simultaneous

absence of common archaeal nitrification genes highlights the need for more investigation of these prokaryotes so that more complete genomic databases can allow better identification and detection in the future.

## 6.2. Conclusion

In conclusion, the present study highlights the need for characterization of the whole microbial community in RAS and aquaponics systems, by describing the diversity and growth potential of microeukaryotes as well as the likely implications of several taxa to the other major biotic components, namely prokaryotes, fish and plants in the case of aquaponics. Furthermore, the present thesis shows patterns of how different compartments and systems affect the community composition of each major microbial group (i.e., Bacteria, Archaea and microeukaryotes) to allow for better insights during the conceptualization of new RAS and aquaponics systems. Finally, nitrification and less studied metabolic processes carried out by prokaryotes like anammox, methane degradation and sulfur oxidation are shown as possible across aquaponics compartments, while taxa with putative beneficial effects on fish and plants are also highlighted.

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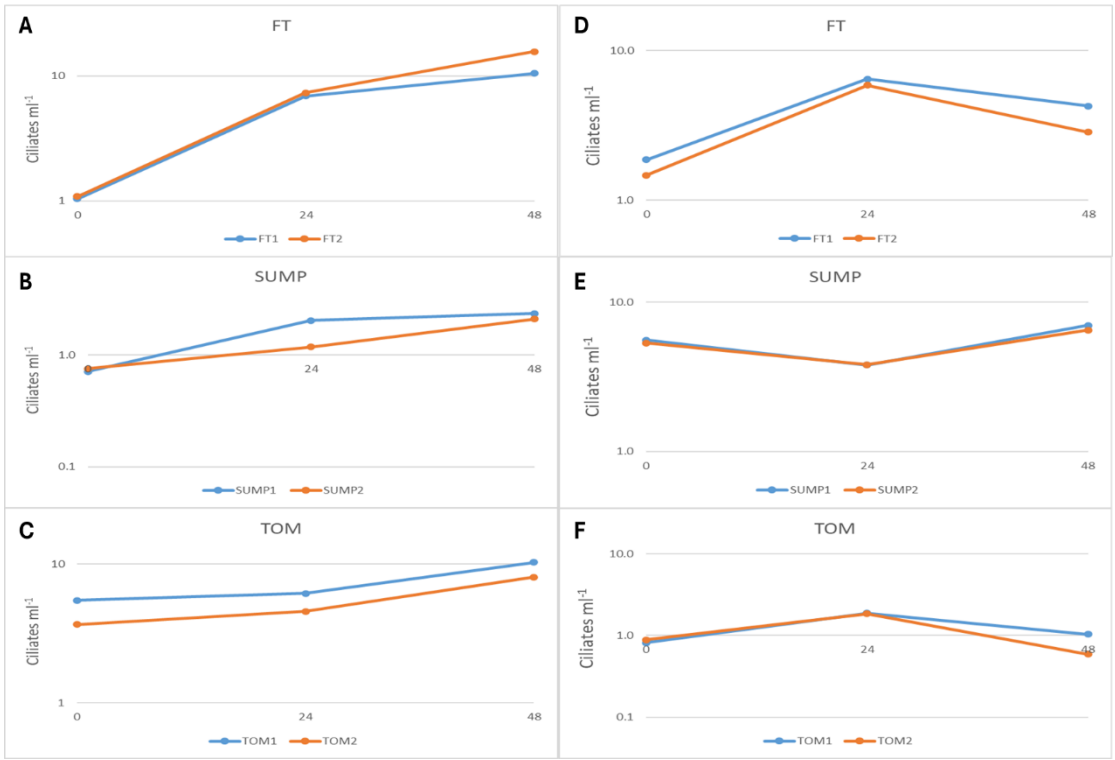
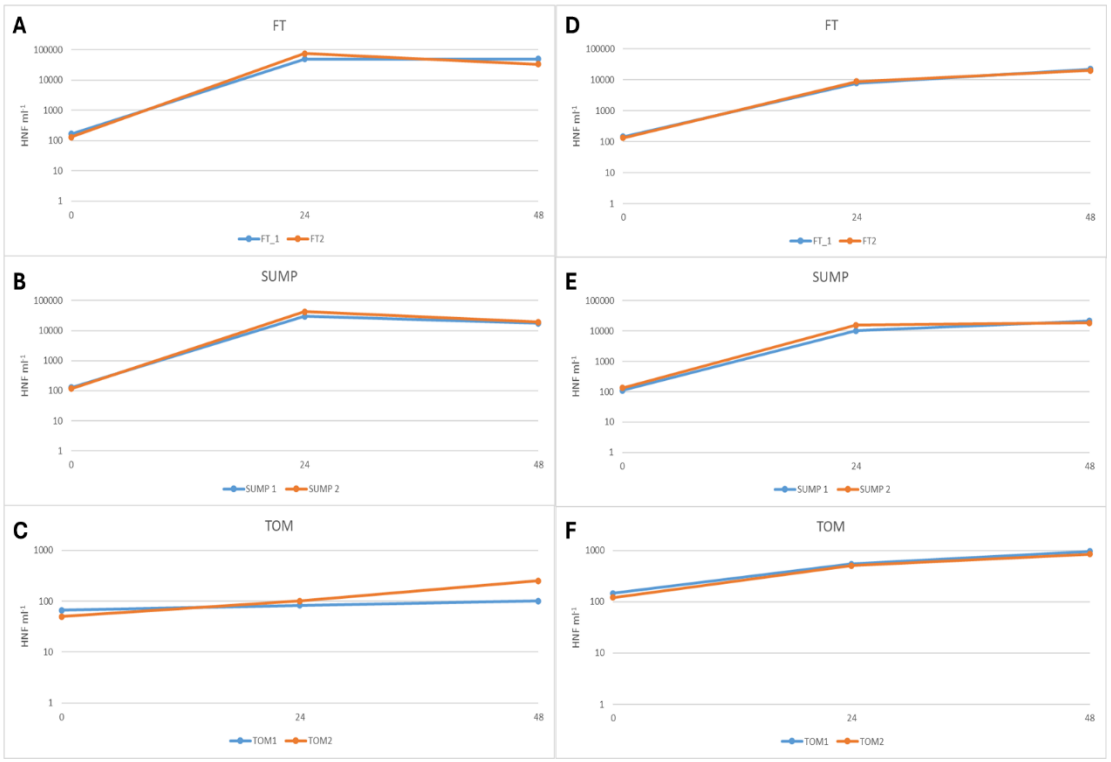
## Supplementary material


**Appendix 1.** Main compartments of the experimental aquaponics system operated by the School of Agricultural Sciences of the University of Thessaly in Velestino. **A**: Fish rearing tanks (in blue), **B**: sump tank, **C**: hydroponics compartment with tomato plants, **D**: drain tanks following plant fertigation.

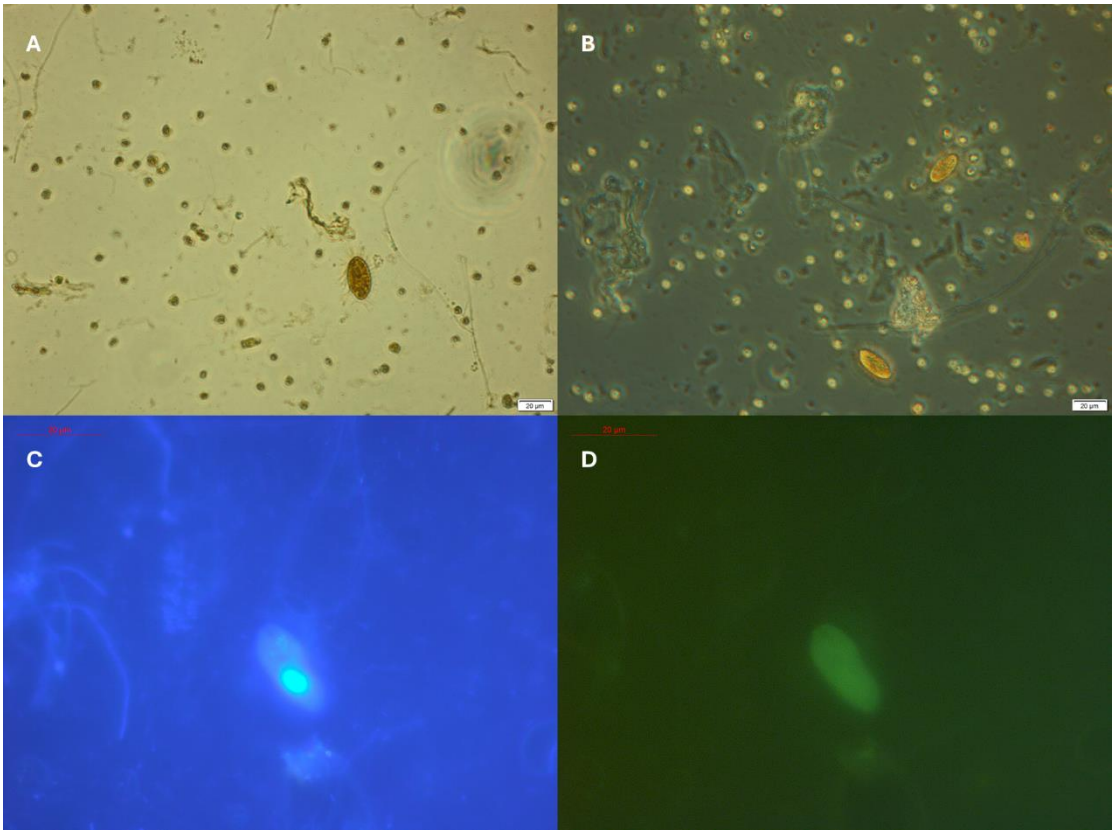

**Appendix 2.** Main compartments of the experimental recirculating aquaculture system operated by the Department of Ichthyology and Aquatic Environment of the University of Thessaly in Volos. **A**: Fish rearing tanks and biofilter. **B**: Fish tank with incubation bottle for the protist growth experiment.



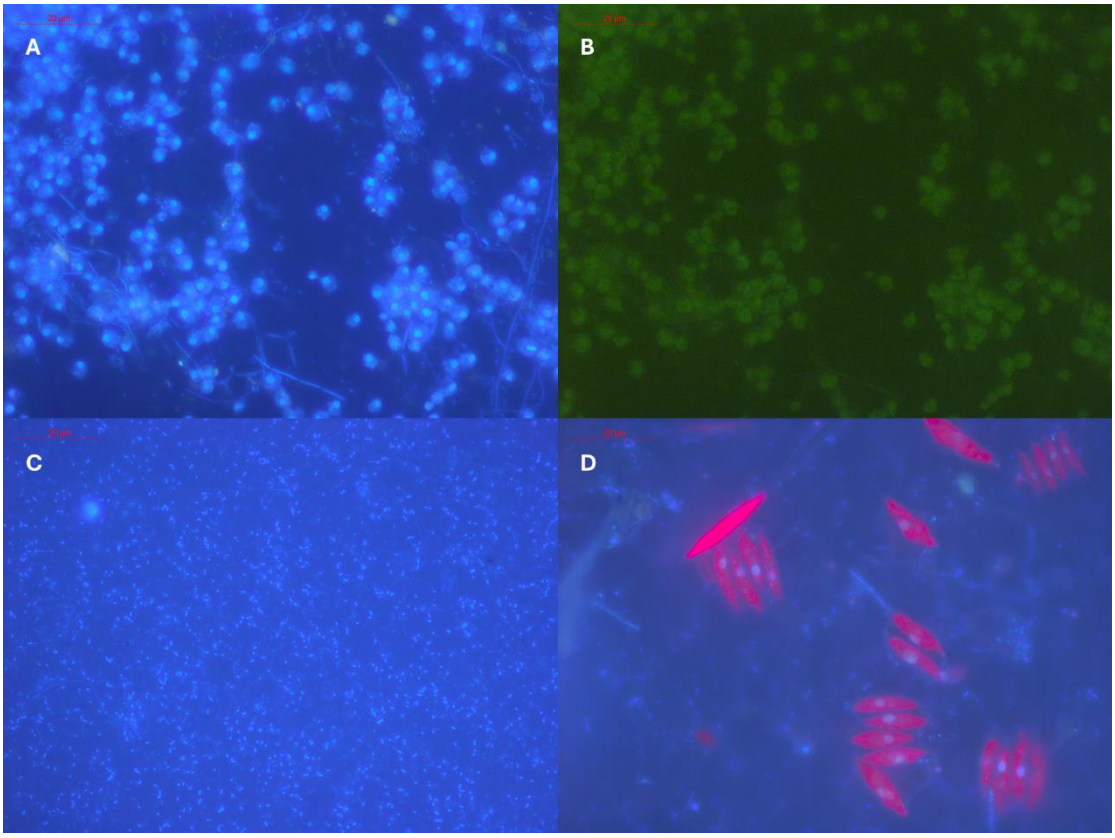

**Appendix 3.** Ciliate growth curves from the aquaponics growth experiments. **A – C**: FEB, **D – F**: JUN. Y axis in logarithmic scale. FT: fish tank, SUMP: sump tank, TOM: drain tanks.



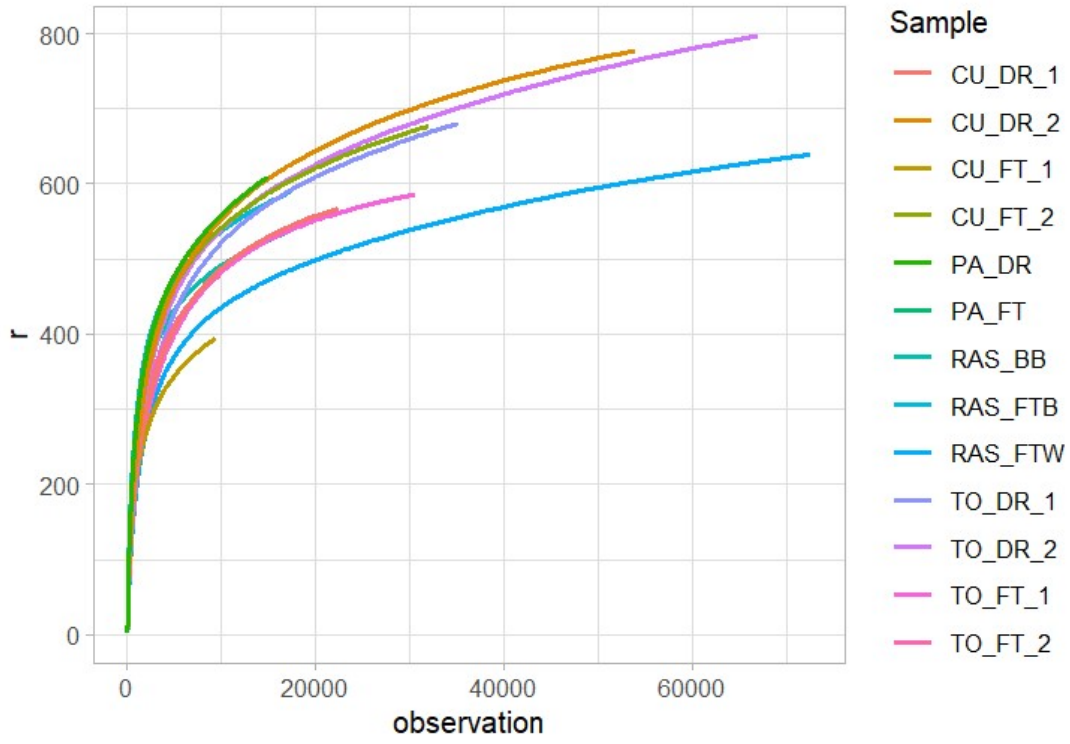

**Appendix 4.** HNF growth curves from the aquaponics growth experiments. **A – C**: FEB, **D – F**: JUN. Y axis in logarithmic scale. FT: fish tank, SUMP: sump tank, TOM: drain tanks.



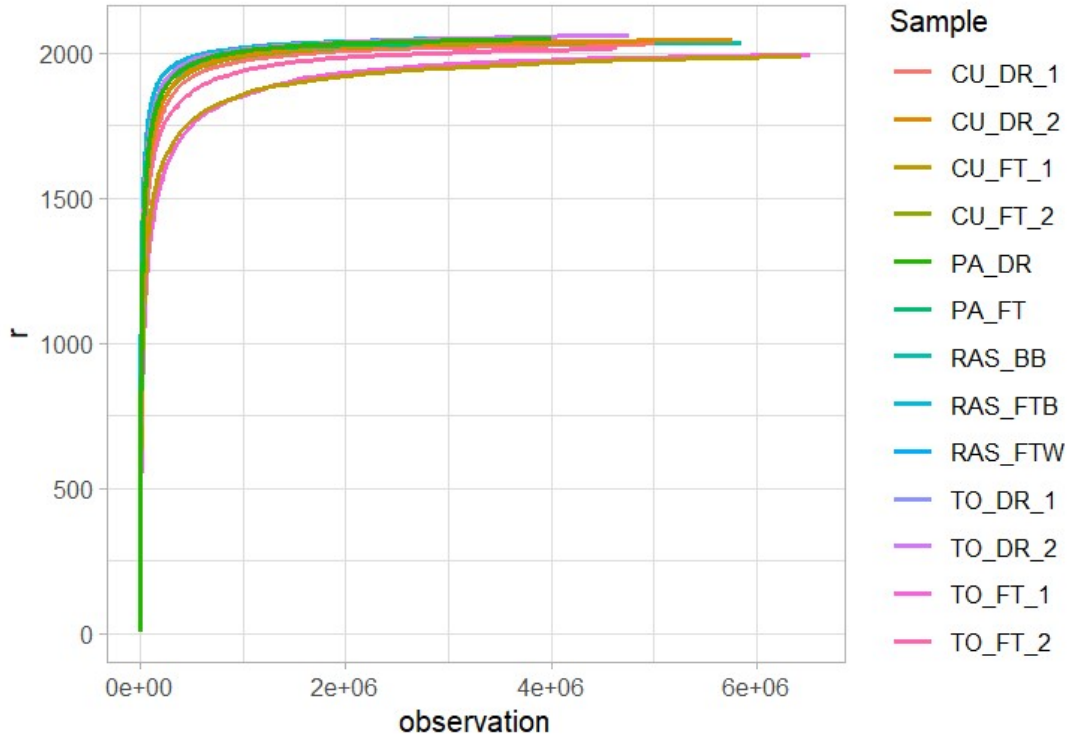

**Appendix 5.** Microscopy images of the *Cyclidium* morphotype. **A – B**: inverted microscopy images at 400X magnification after fixation with Lugol's solution, **C**: epifluorescence microscopy image at 1000X after DAPI staining and UV excitation, **D**: epifluorescence microscopy image at 1000X after DAPI staining and blue light excitation.



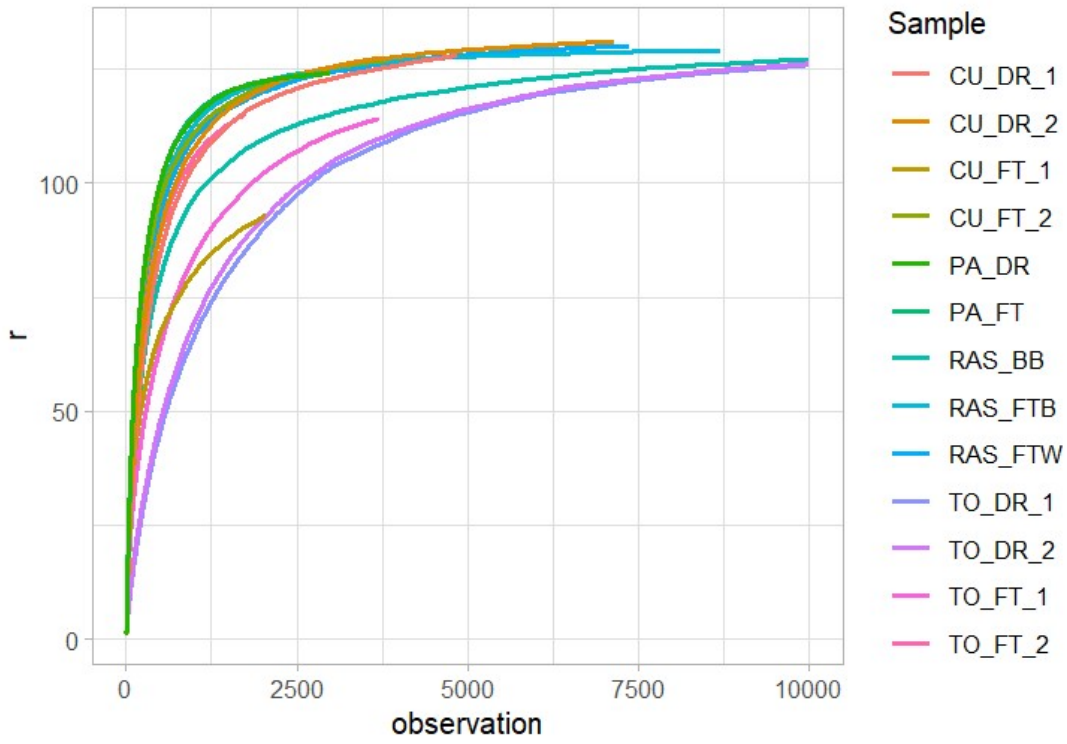

**Appendix 6.** Epifluorescence microscopy images at 1000X magnification. **A**: HNF stained with DAPI under UV excitation, **B**: HNF under blue light excitation, **C**: prokaryotic cells stained with DAPI under UV excitation, **D**: various microorganisms under simultaneous UV and blue light excitation.




**Appendix 7.** Rarefaction curves of the eukaryotic community.




**Appendix 8.** Rarefaction curves of the bacterial community.




**Appendix 9.** Rarefaction curves of the archaeal community.

**Appendix 10.** Coverage estimation of metagenomics samples.

| Sample | % of classified reads out of total | Estimated Coverage | Nonpareil Diversity (Nd) |
|---|---|---|---|
| RAS_FTW | 35.2 | 0.664 | 19.94 |
| RAS_FTB | 34.5 | 0.832 | 19.36 |
| RAS_BB | 41 | 0.868 | 18.74 |
| TO_FT_1 | 64.1 | 0.917 | 16.68 |
| TO_DR_1 | 47.5 | 0.587 | 20.16 |
| TO_FT_2 | 57 | 0.884 | 17.74 |
| TO_DR_2 | 40.9 | 0.625 | 20.41 |
| CU_FT_1 | 59.8 | 0.957 | 16.86 |
| CU_DR_1 | 50.2 | 0.797 | 18.66 |
| CU_FT_2 | 40.1 | 0.737 | 19.18 |
| CU_DR_2 | 46 | 0.696 | 19.78 |
| PA_FT | 40.9 | 0.667 | 19.91 |
| PA_DR | 36.8 | 0.68 | 20.24 |

**Appendix 11.** List of publications pertaining to the dissertation.

1) Moschos, S., Kormas, K. A., & Karayanni, H. (**2022**). Prokaryotic diversity in marine and freshwater recirculating aquaculture systems. *Reviews in Aquaculture*, *14*(4), 1861-1886. https://doi.org/10.1111/raq.12677

2) Moschos, S., Kormas, K. A., & Karayanni, H. (**2024**). Ciliate diversity and growth rates in experimental recirculating aquaculture and aquaponics systems using microscopy. *European Journal of Protistology*, *95*, 126113. https://doi.org/10.1016/j.ejop.2024.126113

**Appendix 12.** Poster presentation for the FEMS2023 international conference, Hamburg, 9-13 July 2023.

# Characterization of ciliate diversity and growth rates in a coupled aquaponic system using microscopy

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## Background

Aquaponics is a farming approach that enables the simultaneous rearing of fish and cultivation of edible plants by combining recirculating aquaculture systems (RAS) with hydroponics[1]. Due to their benefits regarding nutrient and waste cycling, limited water requirements, and reduced environmental impact, these systems are currently being studied and optimized. Aquaponic function is based around the prokaryotic microbial community that dwells on special biofilters, carrying out nitrification and organic matter degradation[1].

## Objective

The aim of this study is to describe for the first time the diversity, abundance and potential growth rate of planktonic ciliates that may prey on the prokaryotic community in an aquaponic system (Fig. 1).

## Study system

- Experimental, freshwater, coupled aquaponics
- School of Agricultural Sciences, University of Thessaly, Greece
- Nile tilapia (*Oreochromis niloticus*)
- Tomato (*Solanum lycopersicum*)
- Water circulation time: ~1h, Temperature: 26°C

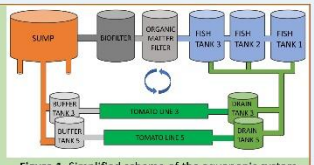


**Figure 1.** Simplified scheme of the aquaponic system investigated in the present study.

## Methods

- **3 compartments**
  - Fish tanks (**FT**)
  - Sump (**SUMP**)
  - Drain tanks (**TOM**)
- **2 time points**
  - Day 17 (**FEB**)
  - Day 127 (**JUN**)
- Ciliate growth experiments[2] (Fig. 2)
- Morphology based taxonomic identification[3]
- Ecological indices and statistical analysis using the PAST (v4.09) software[4]

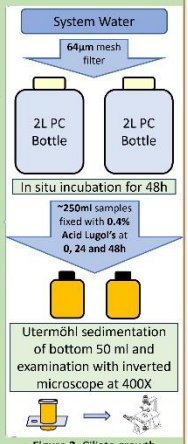


**Figure 2.** Ciliate growth experiment setup and observation method.

## Results

### Abundance

- Ciliate natural abundance (Fig. 3) was highest in TOM samples for FEB (4582 ± 907 cells $L^{-1}$), and SUMP samples for JUN (5451 ± 18 cells $L^{-1}$)
- *Cyclidium* was the most encountered morphotype (Fig. 4) with abundance of up to 12228 ± 2684 cells $L^{-1}$ in t2 samples

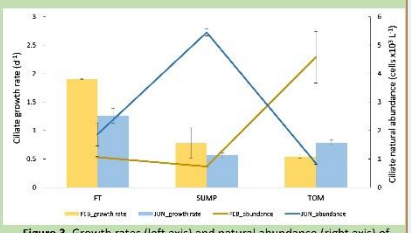


**Figure 3.** Growth rates (left axis) and natural abundance (right axis) of planktonic ciliates. FT: fish tanks, SUMP: sump, TOM: drain tanks.

### Growth rates

- Ciliate growth rate (Fig. 3) was higher in FT (Kruskal-Wallis $p < 0.05$) both in FEB (1.900 ± 0.006 $d^{-1}$) and JUN (1.260 ± 0.127 $d^{-1}$)
- *Cyclidium* exhibited the highest growth rate among ciliates (max 2.12 ± 0.01 $d^{-1}$)

### Observed diversity

- Ciliate morphotypes associated with 7 genera (Fig. 5(a-g))
- Other taxa: testate amoebae, rotifers, *Scenedesmus* (Fig. 5(h-j))
- Higher ($p < 0.05$) Shannon diversity in FEB (0.75 (SD=0.34) compared to JUN 0.2(SD=0.23)
- Equitability J also decreased from 0.52 (SD=0.21) to 0.2 (SD=0.16)
- More distinct protist communities in TOM based on cluster analysis (Bray-Curtis)
- Ciliate and rotifer abundance correlated in JUN (Pearson $r = -0.639$, $p < 0.01$)

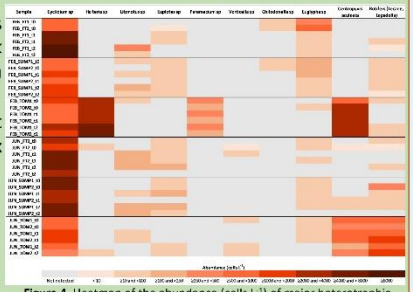

**Figure 4.** Heatmap of the abundance (cells $L^{-1}$) of major heterotrophic micro-eukaryotic groups. t0: start of incubation, t1: 24 h, t2: 48 h

**Figure 5.** Images of micro-eukaryotic community representative morphotypes inspected with inverted microscopy (400X). a) *Cyclidium*, b) *Halteria*, c) *Vorticella*, d) *Litonotus*, e) *Chilodonella*, f) *Euplotes*, g) *Paramecium*, h) *Euglypha*, i) *Centropyxis*, j) *Lepodella* and *Scenedesmus*

## Conclusions

- *Cyclidium* was ubiquitous, the most abundant and fastest growing ciliate morphotype
- Different compartments allowed different ciliate taxa to grow (Fig. 4)
- Larger ciliates were rare and didn't seem to increase in abundance during the 48h incubation (Fig. 4)
- Over time, heterotrophic micro-eukaryotic diversity decreased
- Rotifers and testate amoebae may also be important predators in aquaponic systems

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