

## A STUDY OF BIOFILM ECOLOGY, DEVELOPMENT AND DYNAMICS IN A RECIRCULATING AQUACULTURE SYSTEM AS A POSSIBLE VECTOR FOR FISH DISEASE

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### ABSTRACT

Biofilms are biological structures that coat the surfaces of submerged substrates and are endemic in recirculating aquaculture systems (RASs). They can be a vector for disease, both human and piscine and a range of species may inhabit a biofilm forming a trophic web that is sensitive to operator intervention. Aside from protozoan and bacterial components very few other species have been assessed for their influence on fish health and their pathogenic potential. The purpose of this study was to assess the influence of biofilm on fish health and as a vector for disease in RAS containing Murray cod (*Maccullochella peelii peelii*). Most species were identified at least down to family level and their pathogenicity and influence on biofilm mechanics was assessed. Apart from *Saprolegnia diclina-parasitica*, *Aphanomyces* spp. and *Trichodina* spp. fish pathogens were largely absent from the biofilm. The biofilm displayed a relative diversity of biota considering the low habitat complexity of such an ecosystem. This may have had a protective effect against pathogenic proliferation through the mechanism of competitive exclusion. It can therefore be concluded that the biofilm in this particular system is of little risk to stock and may even have a shielding effect through trophic processes that create equilibrium in community make-up.

**KEY WORDS:** Biofilm, recirculating aquaculture system (RAS), microorganisms, protozoans, metazoans, fish diseases, pathogenic proliferation, Murray cod

### INTRODUCTION

Recirculating aquaculture systems (RASs) are a modern food production technology ideally suited to many parts of the world including Australia. As it takes place indoors with a minimal amount of water exchange, it is relatively insulated from environmental uncertainties (Timmons and Ebeling, 2007). However, because of their highly intensive nature, disease outbreaks can happen with devastating rapidity and large fish kills must be avoided for any venture to be successful (Summerfelt *et al.*, 2009). Maintaining a healthy population of fish in a RAS requires intervention by the operator to control a number of water quality parameters, such as the removal of suspended and dissolved solids from the culture water (Timmons and Ebeling, 2007). However even the most hygienic systems will form biofilms wherever a solid substrate is submerged (Bourne *et al.*, 2006; Michaud, 2007). Biofilms are biological structures that coat the surfaces of submerged substrate and can constitute a response by microorganisms to changing environmental conditions and sub-lethal doses of inhibitory substances (Brown and Gilbert, 1993; Sasahara and Zottola, 1993; Yu and McFeters, 1994). Communities of bacterial, fungal, protozoan and metazoan species are known residents of biofilms associated with different systems (Kinsey *et al.*, 2003; Wei *et al.*, 2003; Parry *et al.*, 2007). As stated by Donlan and Costerton (2002), these communities interact within a protective extracellular matrix exuded by the bacterial component. As noted by King *et al.*, (2004), biofilms are endemic in aquaculture systems and indeed the entire process of biofiltration would be impossible without the succession of sessile nitrifying bacteria. However, they also found that biofilms may act as a vector for disease (human and piscine) within a system. Other researchers have come to the same conclusion (Karunasager *et al.*, 1996; Leonard *et al.*, 2000; Bourne *et al.*, 2006). By virtue of the natural protection provided by the film, Brown and Gilbert, (1993) found microbial cells in the matrix are more resistant to treatments and predators than their free-living planktonic counterparts.

Most studies on biofilms in aquaculture conclude that a more thorough understanding of the species present in aquaculture system would enable more effective management protocols to be enacted (Sugita *et al.*, 2005; Michaud *et al.*, 2006; Bourne *et al.*, 2006). As stated by Michaud *et al.* (2009), it may also help identify local bacterial strains with probiotic capabilities that are already naturalised within aquaculture systems. As little is known of the other components within RAS biofilm communities, knowledge on how other taxa interact with bacterial assemblages is

likely to improve management processes even further (Michaud, 2007). The aim of this study was to provide a critical analysis of RAS hygiene protocols in relation to biofilm formation. In particular micro-organisms and their abundance, distribution and species composition were investigated. By placing microscope slides in strategic points of the system, the development and recruitment of micro-organisms within the biofilm of the culture units at the Melbourne Polytechnic's RAS system was observed and enumerated over a period of six weeks. The effects of standard treatments such as harvest and addition of salt to the system, were also assessed in the context of biofilm function and pathogenic potential. The aim of this study was to investigate if biofilm development within a recirculating aquaculture system for Murray cod (*Maccullochella peelii peelii*) a vector for fish disease through the mechanisms of direct infection and indirect environmental stress. The specific objectives of this aim were to be achieved through: bacterial inventory and enumeration; protozoan inventory and enumeration; metazoan inventory and enumeration; and Saprolegnian inventory and enumeration

### MATERIALS AND METHODS

The study was conducted at Melbourne Polytechnic's Aquaculture Training and Research Centre (ATARC) in Epping for 6 weeks from July to August. As well as providing educational opportunities, the centre also produces advanced stocking juveniles for the aquaculture industry, for selling to irrigators in the country regions of Victoria Australia. The RAS in ATARC consists of two separate systems only one of which was employed for the study. The system consists of 20 x 2000L culture vessels which run through a screen filter to remove large solids and a Polygiser filter for biofiltration and removal of fine suspended solids. Three full time staff members are generally employed by the institute to provide on-going maintenance, husbandry and data collection services.

The system is generally self-sufficient in terms of stock recruitment with a yearly Murray cod breeding program reducing the need to bring in stock from other systems. This has positive biosecurity implications and contributes to the low incidence of serious disease events within the facility. Glass microscope slides were placed in two locations of the RAS system as a substrate for biofilm development. Three slides for each of the six weeks were attached to a plastic tray utilizing aquarium grade silicon and placed on the bottom of a tank containing Murray cod (*Maccullochella peelii peelii*). An identical tray was placed in the inlet of the screen filter. This gave a total of 36 slides and 16 in each location. Three slides were removed weekly from each of the positions and transported in a beaker of culture water.

**Table 1.** Method of culture and enumeration of bacterial species and for the enumeration of protozoan and microscopic metazoan populations

Action	Explanation
<b>Collection of biofilm</b>	One slide was scraped free of biofilm (the top surface only) using a scalpel. The biofilm was diluted in a known volume of deionised water and homogenised using a magnetic stirrer for 15 minutes. The homogenised diluted biofilm was used for the culture and isolation of bacteria, oomycetes and fungi on agar.
<b>Ten-fold dilution of sample</b>	Four small beakers were each filled with 9mls of deionised water. 1ml of the sample was added to the first beaker and mixed thoroughly. 1ml of this solution was added to the next beaker and so on. This gave four beakers with the dilution factors of x10; x100; x1000; x10,000
<b>Inoculation of agar for bacterial culture</b>	Bacteria were cultured on the general medium, tryptic soy agar (TSA) (Buller 2004; King <i>et al.</i> 2004; Whitman 2004) using the streaking method, as described by Whitman (2004) for colony forming unit (CFU) counts and isolation of pure colonies. Plates for each dilution were inoculated in triplicate.
<b>Inoculation of agar for the culture of Oomycota</b>	Oomycetes ( <i>Saprolegnia</i> spp) were selected for, using a glucose – yeast agar (GYA) with an organic form of sulphur added (10% HCL L-Cystiene) (Webster and Weber 2007; Stueland <i>et al.</i> , 2005). The formulation is described in Appendix. 1. No attempt was made at enumeration due to the low reproducibility of results when working with filamentous organisms due to their ability to form new colonies from fragmentation (Kinsey <i>et al.</i> , 2003; Shearer <i>et al.</i> , 2004).
<b>Inoculation of agar for the culture of aquatic hyphomycetes</b>	Potato-dextrose agar (PDA), a good all-purpose medium for isolation of true fungi (Shearer <i>et al.</i> , 2004), was also prepared and inoculated using the same sample as for the bacteria. Enumeration was not attempted due to the same problems cited previously.
<b>Incubation of plates</b>	Agar plates were incubated at 25°C for 48 hours (Whitman, 2004). They were checked at 24 hours for rampant fungal growth.
<b>Determination of colony-forming units (CFU)</b>	A darkfield colony counter was employed for enumeration of CFU using the plates with TSA. Initial and secondary dilutions were multiplied to ascertain the conversion factor
<b>Inventory of Saprolegniaceae</b>	All plates were checked for hyphal growth which was recorded. Oomycota were plated onto GY and PDA plates. Saprolegnious species were identified using the keys provided by Johnson <i>et al.</i> , (2002)
<b>Isolation of bacterial colonies</b>	Bacterial species were sorted according to their morphological features, Grams stain (and 3% KOH test), catalase and oxidase tests. They were identified using 'Bergey's Manual of Determinative Bacteriology' (Bergey & Holt 1994), Buller (2004) and API 20 strips <sup>2</sup> (Biomerieux 2003)

One slide from each position was prepared for direct observation (microscopy) of protozoan and metazoan species. Annelid species were counted under the cover-slip at 40x magnification and were identified using the keys provided by Pinder (2010). The biofilm was filmed and photographed using Motic 2.0<sup>1</sup> hardware and associated software. Agar technique as described by Whitman (2004) was used for the detection and enumeration of culturable organisms. The full process is explained in more detail on Table 1.

Protozoans and tiny metazoans were enumerated by using the original homogenised biofilm and inoculating a Sedgewick-Rafter cell for examination at 100x magnification. A conversion factor was applied by multiplying total divisions counted with the initial dilution. Ciliates were identified using the generic keys provided by Serrano *et al.* (2008). Other protozoans were identified using Patterson and Burford (2001). Texts used for the identification of protozoans and metazoans include Ingram *et al.*, (1997), Pinder (2010). The final slides from each position, had their non-exposed surfaces scrapped clean and wiped with a tissue. These were added to a beaker of deionised water with standard floating baits (boiled hemp seed and squares of boiled snakes skin) as described by a number of authors (Rossman *et al.*, 1998; Webster and Weber, 2007; Shearer *et al.*, 2004). Baits were examined every 24 hours for the presence of filamentous growth and any hyphae were to be isolated onto PDA. Statistical analysis consisted of paired T-tests performed on a Linux system using LibreOffice 3 Calc software. Shannon-Wiener diversity indices were performed using LibreOffice Calc spreadsheets and a scientific calculator for Inverse functions.

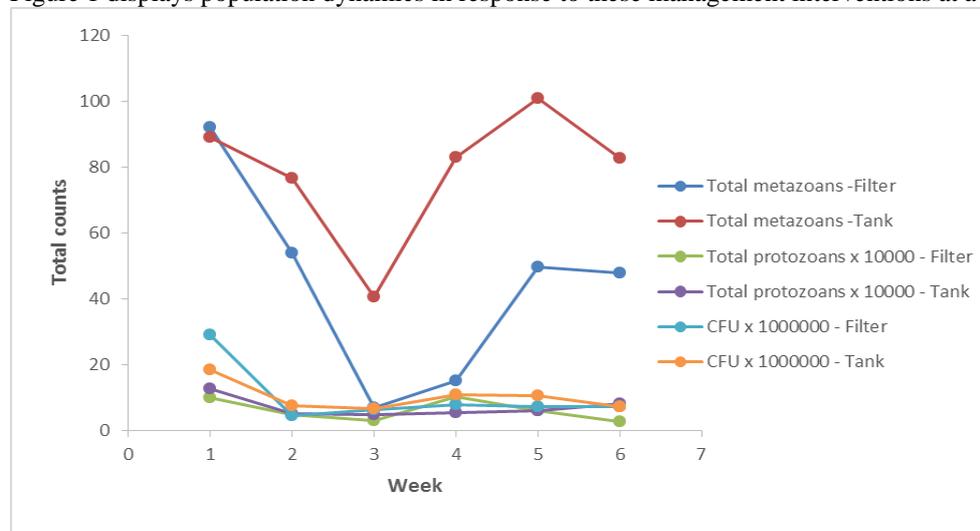
## RESULTS

### Water Quality Parameters

There were two major events that had a bearing on water quality parameters in the system. On the day the first slide was due to be removed 50% of the total biomass of the system was harvested. As a result, a quarter of total water volume was topped up and feed additions also dropped by 50%. A paired T-test (95% confidence) showed that there was a significant difference between bacterial populations before and after harvesting (Tank;  $P > 0.032$ , Filter;  $P > 0.0008$ ).

Between weeks 2 and 3, an outbreak of the free-living ciliate *Chilodonella hexasticha* occurred and salt levels were boosted to overcome this threat to stock. This organism is thought to have been introduced with some silver perch (*Bidyanis bidyanis*) being housed in another system. It should be noted that this species was not detected in the biofilm.

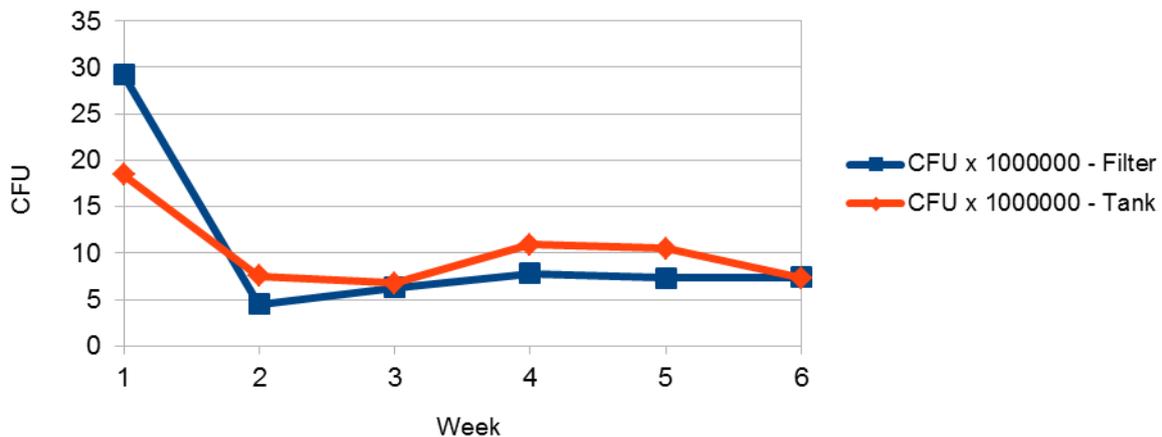
Figure 1 displays population dynamics in response to these management interventions at a Kingdom level.



**Figure 1.** Total counts for each taxonomic subgroup at a Kingdom level. Major drops in populations were associated with a partial harvest in the second week (50% of system biomass) and the addition of salt (0.23 – 5.44ppt) in the 3rd week

### Bacterial CFU counts

The results of counts of bacterial CFU from tryptic soy agar plates are charted in Figure 2.



**Figure 2.** A comparison of total CFU x 10<sup>5</sup> per cm<sup>2</sup> for the biofilms associated with filter and the tank over a six-week period. Bacterial proliferation was greatly impeded following harvest during week 2

### Pathogenicity and Ecological function of Bacterial species identified

Obligate pathogens of fish species were absent from the inventory. Of the species that were uncovered somewhere found to be opportunistic infectious agents in humans and many of these were associated with hospital biofilms. *P. multocida* was the only serious pathogen discovered. It is known to cause serious respiratory disease in mammals and is known to proliferate in naked amoebas which are lysed to allow the bacterium to escape. Table 2 displays pathogenic potential and ecological and metabolic function in species identified.

**Table 2.** Pathogenic and ecological function of identified bacterial species within the biofilm and the RAS at large

Species	Pathogenic potential	General comments	Influence in the system
<i>Brevundimonas vesicularis</i>	Can cause infection, mainly septicemia in immunocompromised patients (Karadag <i>et al.</i> , 2012)	Environmental organism generally found in streams (Buller 2004). Known to produce biofouling in paper mills (Verhoef <i>et al.</i> , 2002). It has bioremediation potential as a bio-absorbent for lead contamination in wastewater streams (Resmi <i>et al.</i> , 2010)	A factor in biofilm formation. Lead is not known to be a problem in the system, but maybe useful in systems where this is an issue
<i>Chryseobacterium indologenes</i>	Cause of infection and at least one death in immunocompromised patients and burns victims (Casio <i>et al.</i> , 2005)	<i>C. indologenes</i> is a facultative anaerobe that can utilize nitrate as a terminal electron acceptor (Hsueh <i>et al.</i> , 1996). It is metabolically chemo-organotrophic and is a common environmental organism (Hsueh <i>et al.</i> 1996).	Able to remove nitrogen from the system through denitrification, although only in anaerobic zones. Chemo-organotrophic potential means that it could act as a carbon dioxide sink potentially improving buffering capacity against pH swings
<i>Pasteurella multocida</i>	Cause of pasteurellosis in humans mainly following	Facultative anaerobe mainly associated with the upper	Its occurrence in the system coincided with the appearance of

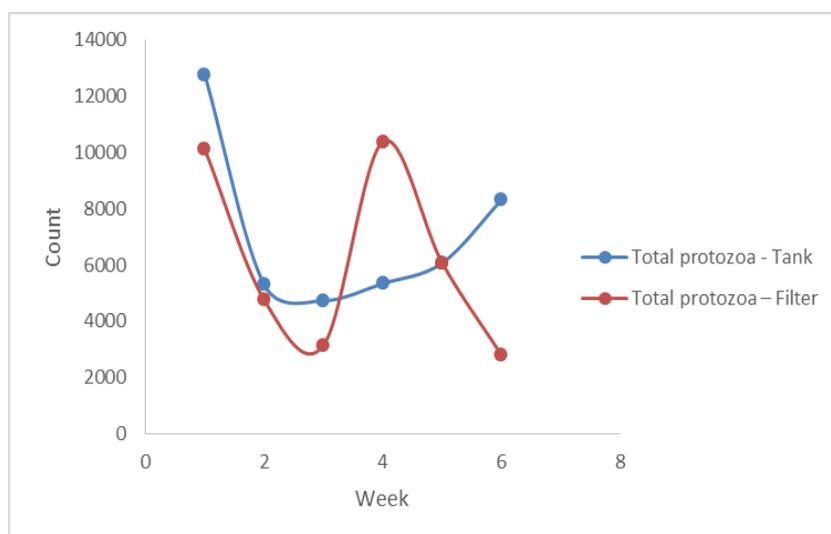
	animal bites but also the cause of avian cholera. Commonly found in other mammals where it occasionally causes disease (Myers <i>et al.</i> , 2012)	respiratory tract of mammals (Myers, Ward & Myers 2012). <i>P. multicolor</i> known to proliferate inside amoebas as a vector of avian cholera (Hundt and Ruffolo, 2005).	naked amoebas. Not likely to persist as amoeba are lysed as they leave their host and the amoeba occur in very small numbers. May limit the proliferation of amoebas within the system. A potentially dangerous human pathogen.
<b><i>Pseudomonas oryzae</i></b>	Implicated in clinical infection on immunocompromised patients. A cause of bacteraemia in patients with catheters and open wounds, sometimes leading to septicemia. Can cause peritonitis and meningitis (Marin <i>et al.</i> , 2000)	First isolated in rice paddies, this species is able to form biofilms in underground aquifers. Its resistance to chlorine once in the biofilm means it is commonly found in drinking water (Dussart <i>et al.</i> , 2003). One domestic vector of human infection is bath sponges (Marin <i>et al.</i> , 2000)	Definitely a factor in biofilm formation but no reports of piscine disease therefore not likely to have much influence
<b><i>Pseudomonas putida</i></b>	A single case of ulcerative fin disease in rainbow trout (Altinok <i>et al.</i> , 2006). Generally classed as non-pathogenic in humans, although rare cases have occurred (Perz <i>et al.</i> , 2005)	A saprophytic species with massive potential for bioremediation. It can degrade most hydrocarbons pesticides such as atrazine (Fernández <i>et al.</i> , 2012) and can convert styrene oil into Polyhydroxyalkanoates (PHA) which are environmentally-friendly, biodegradable plastics (Ward <i>et al.</i> , 2005). It is an important and beneficial species within rhizosphere interactions (Romano and Koulter, 2005)	Able to deal with a variety of low-level pollutants which may buffer the system against chronic build-up. This species could pose a problem if the media in the Polygeiser filters were ever changed to polystyrene balls. May be of great service in the aquaponics industry as protection against insidious fungal agents in the rhizosphere
<b><i>Serratia plymuthica</i></b>	Has been isolated from moribund trout but not considered to be pathogenic to fish (Austin and Stobie, 1992) Although has been isolated from sick patients there is no real evidence that this is species is pathogenic to humans (De Vleeschauwer and Hofte, 2007)	Environmental organism. A species that provides protection from a range of pathogenic fungi by colonising the rhizosphere of plants. Some of these hosts include brassicas, strawberries corn, wheat, rice, grapes, melons, beans, cucumbers, potatoes and tomatoes (De Vleeschauwer and Hofte, 2007)	Very little influence suspected. Would possibly be a useful species for aquaponic research
<b><i>Shewanella putrefaciens</i> group</b>	Very rarely implicated as a human pathogen (Pagani <i>et al.</i> 2003). Known to cause dermal necrosis, fin damage and exophthalmia in rabbit fish (Buller, 2004)	Facultative anaerobe that can use iron, magnesium (Beliaev <i>et al.</i> 2001) and even uranium as terminal electron acceptors (Min <i>et al.</i> 2005). Produces trimethylamines which cause rotten fish smell and are acknowledged as a major spoilage organism, even during cold storage (Lu and Levin, 2010)	As fish are mainly produced for advanced stocking and not direct consumption, the presence of spoilage organisms is not likely to have any great effect on the system

<i>Sphingobacterium spiritivorum</i>	Has been isolated from humans but not thought to cause disease (Buller, 2004)	Known to assimilate hydrocarbons in bioremediation studies (Chaineau <i>et al.</i> 1999)	Very little influence except in the off-chance of background hydrocarbon pollution
<i>Sphingomonas paucimobilis</i>	Can cause a wide variety of infections in humans. Although rarely considered a problem, in a hospital environment its importance is only starting to be appreciated (Ryan and Adley, 2010)	Treated as an environmental organism (Buller, 2004). Metabolically flexible and able to degrade a variety of pollutants including hexachlorocyclohexane (Nishiyama <i>et al.</i> , 1992). Commonly found in shower curtain biofilms (Kelley <i>et al.</i> 2004)	A major influence on biofilm development. Unlikely to cause any problems for workers or students
<i>Stenotrophomonas maltophilia</i>	In healthy individuals may cause pneumonia, urinary tract infection and septicemia. In immunocompromised patients other forms of infection may manifest (McGowan, 2006). It is naturally resistant to many antibiotics due to the function of unique enzymes (Denton and Kerr, 1998)	Ubiquitous in soil and water. Also known to form biofilms (Huang <i>et al.</i> 2006)	Very little influence except in the formation of biofilm. Although able to cause disease in healthy individuals, incidence seems to be extremely low

Using a paired T-test, significant difference between tank and filter bacterial enumerations gave the result of  $p = 0.4837507817 > p = 0.05$ , therefore there was no significant difference between the two bacterial communities in terms of CFU.

### Protozoans Inventory and Enumeration

Protozoan numbers fluctuated during week two due to biomass reduction and during week three with the addition of salt. However, the drop during week three may have been caused by trophic cascade. Figure 3 charts total protozoan numbers associated with tank and filter biofilms over the course of the study.



**Figure 3** - Total protozoans populations for Tank vs. Filter. The filter population seems particularly unstable in comparison to the tank population which follows a more predictable growth curve in relation to treatments before counts in weeks two and three

Table 3 displays pathogenicity and ecological function of protozoan species identified in the system.

**Table 3.** Characteristics of protozoan inhabitants of the ATARC biofilm. They have been divided into functional groups with flagellates consolidated for easy reading

### Stalked ciliates

Species	Pathogenic potential	General comments	Influence in the system
<i>Epistylus spp.</i>	Often found attached to the skin and fins, but can also be isolated from gills (Pouder <i>et al.</i> , 2005). Because anchor points damage the skin, <i>Epistylus spp.</i> may increase prevalence of saprolegniosis and bacterial disease (Rowland <i>et al.</i> , 2007)	Colonial species of peritrich ciliate. Its presence in large numbers indicates overload of organics and large numbers of bacteria (Patterson & Burford, 2001). Commonly found with <i>Aeromonas spp.</i> (Pouder <i>et al.</i> 2005) which is a known factor in fin rot (Buller, 2004)	Slight pathogenic potential, possibly leading to more serious secondary complications. Likely to exert a positive effect as it is effective at countering bacterial turbidity (Madoni, 2003). When present in large numbers known to deplete dissolved oxygen (Patterson and Burford, 2001)
<i>Vorticella spp.</i>	Same as <i>Epistylus spp.</i>	Non-colonial peritrich with contractile stalk (Serrano <i>et al.</i> , 2008).	Similar influence as <i>Epistylus sp.</i> although different species indicate different nutrient loads (Madoni, 2003; Serrano <i>et al.</i> , 2008)

### Crawling ciliates

Species	Pathogenic potential	General comments	Influence in the system
<i>Aspidisca spp.</i>	None reported	Small crawling hypotrich ciliate. Commonly found over a range of nutrient loads but is generally associated with environmental stability (Serrano <i>et al.</i> , 2008)	Likely to reduce bacterial load in the system and improve oxygen penetration of the biofilm surface through ciliary currents
<i>Euplotes spp.</i>	None reported	Hypotrich ciliate that is larger than <i>Aspidisca spp.</i> Associated with older stabilized sludge in treatment plants (Serrano <i>et al.</i> , 2008).	Same as <i>Aspidisca spp.</i>

### Swimming ciliates

<i>Trichodinia spp.</i>	Common gill and skin parasite and the cause of trichodinosis. Mortality often occurs via a secondary infection (Rowland <i>et al.</i> , 2007)	Ciliated protozoan with characteristic 'flying saucer' whirling motion. Large ventral denticles for attachment to substrate (Klinger and Floyd, 1987)	Likely to have entered the system with a shipment of silver perch ( <i>Bidyanis bidyanis</i> ) brood stock. Less virulent on <i>B. bidyanis</i> than other species of fish (Rowland <i>et al.</i> , 2007), but pathogenicity of this particular strain likely to be low as no problems were observed
<i>Litonotus spp.</i>	None reported	Pleurostomid ciliate which is free swimming but also associated with floc surfaces	Not likely to have a huge impact on stock in the system, but a positive influence on bacterial load could be expected

### Total Flagellates

<i>Paranema spp.</i>	None reported	Free swimming flagellate. In wastewater processes generally negatively correlated to increases in nitrate levels (Papadimitriou <i>et al.</i> , 2010)	Likely to have a very small impact on stock and system parameters
<i>Bodo spp.</i>	None reported although a related parasitic flagellate <i>Ichthyobodo necator</i> can be the primary cause of mortality in a range of fish species (Rowland <i>et al.</i> , 2007)	Very small free swimming flagellate that is associated with young systems as it is quickly out competed by bacterivorous ciliates	Not very effective at bacterial limitation and generally associated with a high oxygen demand (Madoni, 2003)

### Amoebas

<i>Arcella spp.</i>	None reported	A common species of testate amoeba. In wastewater plants it is indicative of proper function and effluent of an excellent quality (Madoni, 2003)	Little influence as they have slow growth and metabolisms in comparison to other protozoans (Madoni, 2003)
Naked Amoeba Unknown <i>spp.</i>	<i>Neoparamoeba perurans</i> associated with amoebic gill disease in Atlantic salmon ( <i>Salmo salar</i> ) (Adams <i>et al.</i> , 2012)	Amoebas are able to enter the biofilm and can become keystone species	Even if they were more common, their influence on stock and the function of the system would likely be minimal. May be a carrier of <i>Pastuerella multocida</i> as both appeared during the same week (Hundt and Ruffolo, 2005)

Using a paired T-test, significant difference between tank and filter protozoan enumerations gave the result of  $p = 0.2837200534 > p = 0.05$ , therefore there was no significant difference between the two protozoan communities in terms of overall number.

### Metazoan Inventory and Enumeration

Total metazoan numbers between tank and filter communities were roughly correlated to each other and showed similar fluctuations and population growth as can be seen in Figure 4.

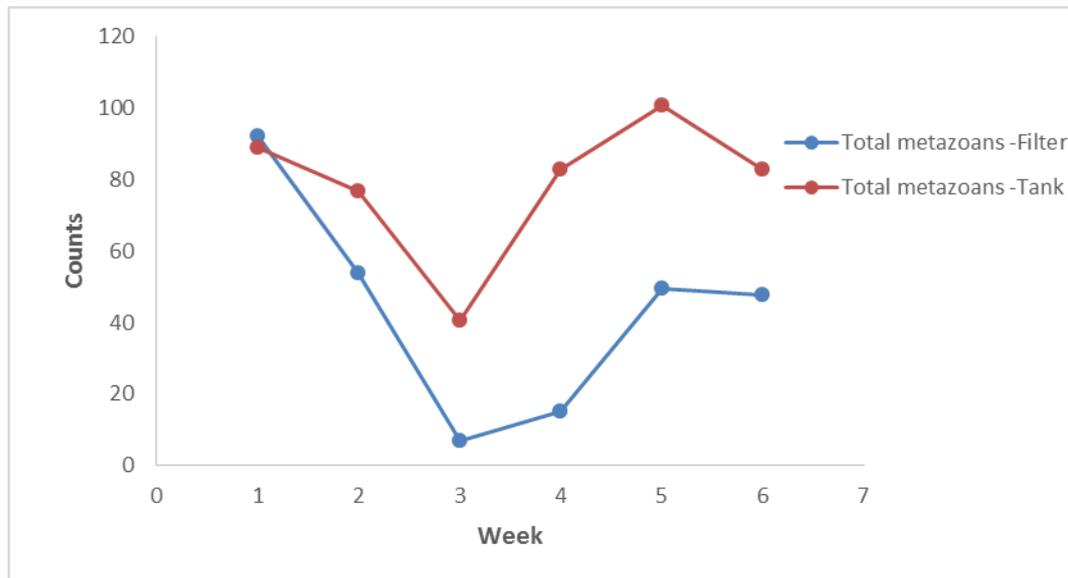


Figure 4 – Total metazoan numbers for tank and filter communities over the duration of the project. Biomass reduction had a big effect on total numbers, a likely effect of trophic cascade. Total numbers also dropped during the third week, a likely consequence of rising salinity, however because the events were so close together, trophic cascade cannot be ruled out as a factor. Many of the metazoan inhabitants were visible to the naked eye but microscopy was required for identification.

### Pathogenicity and Ecological function of Metazoan species identified

None of the metazoans identified have any parasitic potential according to the available literature. Table 5 is a summary of the ecological characteristics in terms of functional groupings.

**Table 5.** Ecological characteristics of metazoan functional groups observed in the ATARC biofilm

Metazoan functional group	Ecological characteristics
<b>Nematodes</b>	Non-parasitic nematodes are seen as a positive factors in a biofilm community for their attraction to anaerobic bacterial colonies (Wei <i>et al.</i> , 2003), as anaerobic processes are more likely to result in toxic metabolites (Avnimelech and Ritvo, 2003)
<b>Rotifers</b>	Although rotifers have long been considered pests in aquaculture systems, they are an important live feed for hatchery production of many important aquaculture species (Drillet, 2010). In wastewater processes, filter feeding rotifers can have a significant effect by reducing bacterial populations and helping to aggregate fine suspended particles (Lapinski and Tunnacliffe, 2003)
<b>Acari</b>	The available literature contains little regarding the influence of acari in aquaculture or wastewater processes. The acari in the system formed the top of the microscopic food chain as they were observed feeding on nematodes and <i>Aelosoma spp.</i>
<b>Copepods</b>	Copepods appeared in vary low numbers on the film and were probably associated with the surface only. They feed on detritus, protozoans and bacteria but probably have a limited effect on the biofilm due to their planktonic lifestyle (Drillet, 2010). Although considered one of the best possible live feeds for hatchery production (Drillet, 2010) this species like many, seemed

	to have low reproductive capacity in culture
<b>Annelids</b>	<i>Dero spp.</i> are relatively large filter feeders that can reduce bacterial and protozoan populations and help mineralise the nutrients in the biofilm which improves uptake capacity for heterotrophic bacteria (Wei <i>et al.</i> , 2003). Because of the sizeable biomass in many samples taken, annelids probably have a significant effect on biofilm rejuvenation

Using a paired T-test, significant difference between tank and filter metazoan enumerations gave the result of  $p = 0.2837200534 > p = 0.05$ , therefore there was no significant difference between the two metazoan communities in terms of overall number.

### Detection of Saprolegnians

*Saprolegnia declina-parasitica* grew on TSA and PDA plates at dilution factors of 1X and 10X, where they were able to out compete bacterial colonies. Only asexual forms were observed possibly due to nutritional deficiencies in the substrate or because only one strain had the necessary metabolism to colonise, thus limiting the possibility for sexual reproduction (Johnston *et al.*, 2002). All specimens observed through direct microscopy of the slides were sexual forms.

*Aphanomyces spp.* was also identified through direct microscopy and was either out competed on agar or was unable to utilise it as a substrate. Table 6 below displays the occurrence of saprolegnians over the full six weeks.

**Table 6.** Detection and isolation of oomycetes on agar and through direct observation of the biofilm, weeks one through to six

Species, location and method of detection	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
<i>Saprolegnia declina-parasitica</i> detected on agar	Detected on 1 plate each of PDA and TSA both from tank. Sporangia present	Detected on 1 PDA plate from tank. Sporangia present	Nil	Nil	Detected on 1 PDA plate from tank. Sporangia present	Detected on 1 PDA plate from filter. Sporangia present
<i>Saprolegnia declina-parasitica</i> detected through direct observation	Oogonia observed on tank slide	Oogonia observed on tank and filter slide	Nil	Nil	Oogonia observed on tank slide	Nil
<i>Aphanomyces spp.</i> detected on agar	Nil	Nil	Nil	Nil	Nil	Nil
<i>Aphanomyces spp.</i> detected through direct observation	Oogonia observed on filter slide	Oogonia observed on filter and tank slides	Nil	Oogonia observed on tank slide	Oogonia observed on filter slide	Nil

### Baiting for oomycetes

This part of the experiment was unsuccessful due to the quick colonisation by protozoans and metazoans onto the baits. Within 24 hours the surface of both the hemp seeds and the snake skin had been colonized in full by *Epistylus spp.* and large numbers of *Aeolosoma spp.* were also present.

### Shannon-Wiener Diversity Indices (H)

Species diversity and equitability over the duration of the study was measured using the standard index of Shannon-Wiener (H) using the formula  $H = -\sum P_i(\ln P_i)$  with  $P_i$  representing the proportion of each species present. Results across each Kingdom are presented on Table 7.

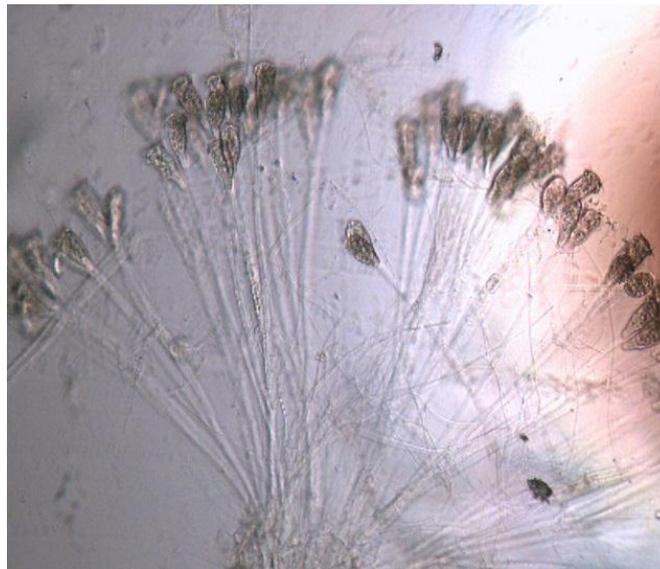
**Table 7.** Shannon-Wiener Entropy (H) for species present in the biofilm at Kingdom level

Week	1	2	3	4	5	6
<b>Bacterial (H) for Tank samples</b>	1.6074291	1.9275641	1.85225067	0.88090191	1.19084879	1.56453005
<b>Bacterial (H) for Filter samples</b>	1.63455723	1.74530099	1.08286349	0.42195715	0.6778918	1.67701084
<b>Protozoan (H) for Tank samples</b>	1.43389855	1.35174099	1.48161415	1.57236784	1.28749433	1.28056677
<b>Protozoan (H) for Filter samples</b>	1.46824776	1.58164987	1.54750484	1.84618534	1.27826267	1.77400925
<b>Metazoan (H) for Tank samples</b>	2.8337676	1.50327194	0.15442681	1.19052845	1.54622284	1.46348077
<b>Metazoan (H) for Filter samples</b>	1.93162129	1.67943586	0.30681585	1.00847775	1.7979431	1.82364159

Using the methodology outlined by Jayaraman (2000), H-values for each Kingdom grouping were analyzed for significant difference ( $p > 0.05$ ) between tank and filter communities using paired T-tests. Results are shown on Table 7.

### DISCUSSION

After a single week of submersion, the first slides to be extracted were covered in protozoan and metazoan life. *Epistylus spp.* (Fig. 5) were well represented in the sample and the presence of rotifers, copepods (Fig. 6) and annelids suggested that the biofilm was colonized from areas of mature film where trophic complexity had time to form (Madoni 2003).



**Figure 5.** Colonies of *Epistylus spp.* were the dominant protozoan species in most of the samples taken. Magnification x 100



**Figure 6.** Although insignificant in terms of total counts, the presence of the copepod *Australocyclops spp.* indicated that the biofilm during the first week had been colonised from mature assemblages from elsewhere in the system. Magnification x 40

As removal of the first slides coincided with a partial harvest of the tank, major disruption to the trophic web of the biofilm was observed. Bacterial numbers were affected in both tank (2.5-fold reduction) and in the filter populations (6.5-fold reduction). The disproportional drop in filter CFUs displays the unpredictability of disturbed ecosystems, even relatively linear ones such as aquaculture biofilms (Michaud, 2007). The benthic nature of cod in the system may have helped shield the biofilm from nutrient depletion and replenish it in tank populations, as the fish were frequently observed in direct contact with the slides. A paired T-test (95% confidence) showed that there was a significant difference between bacterial populations before and after (Tank;  $P > 0.032$ , Filter;  $P > 0.0008$ ). Therefore stocking density and total biomass of the system seem to be limiting factors on biofilm development.



**Figure 7.** Oogonia of *S. dielina - parasitica* photographed from a sample scraped from the tank. Magnification x 400

The addition of salt seemed to have a large effect on populations during week 3, however due to the possibility of trophic cascade caused by bacterial debasement nothing can be ascertained without further analysis. In the case of the rotifers and annelids it can be safely ascertained that salinities between 2.7 and 5.44 were detrimental to diversity, equitability and over all biomass. Acari on the other hand seemed to thrive, although they were always deep in the biofilm suggesting that this offered some degree of protection.

Only protozoans displayed any significant difference between tank and filter assemblages, and only in terms of species diversity and equitability. This indicates that, as in wastewater treatment plants (Madoni, 2003), protozoa could serve as useful indicators in assessing water quality trends in RAS systems.

The discovery of saprolegnians (Fig. 7 and 8) as participants in biofilm processes is significant. Being filamentous in nature, oxygen gradients in the biofilm are likely to be improved by their presence (Kinsey *et al.*, 2003). As they are considered ubiquitous in Murray cod populations due to their habitat preferences, they would likely be impossible to eliminate from the system (Ingram *et al.*, 2005) and their presence may even create a more complex habitat increasing protozoan diversity. This was certainly the case when samples of *Saprolegnia declina-parasitica* were kept in still water cultures for identification.



**Figure 8** – Sporangium of *S. declina – parasitica* from an agar culture. The grainy texture of this asexual organ is attributable to the presence of zoospores awaiting release. Magnification x 400.

The diversity of metabolic function within the bacterial community was unexpected and likely to be beneficial in terms of nutrient control and negation of any unforeseen pollutants in the water supply. This is particularly true in the case of aromatic hydrocarbons and pesticides, but also for heavy metal pollutants that maybe associated with ageing water distribution systems (Lessard and Le Bihan, 2003). Although heavy metal reduction is associated with uptake rather than degradation, maintaining constant numbers of organisms capable of this function would likely reduce the toxicity of these substances, particularly as the biofilm food chain has no trophic links to the target aquaculture species.

The large proportion of species which are already proven in bioremediation, both experimentally and in practice, warrants further investigation as to the specific enzymatic capabilities of these organisms. The discovery of new strains could greatly add to the tools available to bioremediators in the degradation of persistent pollutants, especially since many bioremediation organisms are currently off-limits to most practitioners due to patenting restrictions (Stamets, 2005). In this context the biofilm at ATARC can also be viewed as a potential bioresource.

Although nitrate is generally considered of low importance to fish wellbeing, as no process currently exists in standard RAS to deal with excessive levels, apart from water exchange (Timmons and Ebeling, 2007), the presence of preferential nitrate metabolisers is also significant. Excessive nitrate is a limiting factor on the environmental

credentials of aquaculture and a significant impediment to the development of zero-exchange systems (Rakocy and Bailey, 2003).

More research on the nitrate reducing efficiency of these bacteria is therefore warranted. If shown to be viable in a laboratory setting, this should be followed with experimentation on how to further incorporate these organisms into the standard biological processes of RAS, as other methods such as aquaponics, take the focus away from fish-farming due to the disproportionate ratio of plants needed to properly deal with nitrogen based waste products (Rakocy and Bailey, 2003).

The isolation of species that inhabit aquatic environments as well as providing protection in the rhizosphere of commercially important terrestrial plants implies that the study may also have positive implications for the aquaponics industry. It is worth investigating if these species have any part to play in overcoming bacterial and fungal problems associated with root rot in constantly submerged environments (Rakocy *et al.*, 1997). The lack of primary pathogens in the biofilm gives some weight to the conclusion of King *et al.* (2004) that even though biofilms can harbour disease causing organisms, each RAS can be thought of as unique in its microbial assemblage. Contrary to their position that pathogen reduction requires direct intervention by the operator, maintaining diverse populations of microbial species could alleviate many of the environmental problems with disinfection that they themselves identified in their conclusion.

In a linear ecosystem with little habitat diversity, species diversity is likely to have a significant effect on non-proliferation of pathogenic biofilm organisms (Michaud, 2007). This is probably due to increased competition between all present taxa for resources and substrate as a result of a limited habitat diversity and therefore niches within the biofilm ecosystem. Also, the biofilm ecosystem can probably be considered to be a nutrient store that if disrupted to a large extent could have negative consequences on water quality parameters as suggested by spikes in ammonia and to a lesser extent nitrite during weeks following harvest and salt addition.

## CONCLUSIONS

Though it may seem counter-intuitive, species diversity and biomass within the biofilm of a RAS seems to buffer the system against negative water quality issues and is likely to have a shielding effect against pathogenic proliferation through the mechanism of competitive exclusion on the substrate and competition for nutrient. The presence of metabolically diverse and useful organisms makes the biofilm at ATARC a possible resource for the bioremediation of polluted environments and more research in how to utilise these species is warranted. Furthermore, the aquaponic potential of environmental organisms that persist in both aquatic biomes and also play a protective role in the rhizosphere of plant communities warrants further investigation. In conclusion, the biofilm in the ATARC RAS is of very low risk in terms of pathogenicity to stock, and while disinfection is important and indeed unavoidable in many situations, a balance should be sought to limit major disruption of the biofilm ecosystem.

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