FACULTY OF BIOSCIENCE ENGINEERING

NOVEL STRATEGIES FOR REMOVAL OF GEOSMIN AND 2MIB RELATED OFF-FLAVOUR IN RAS PRODUCTION

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List of Abbreviations

2-MIB	2-methylisoborneol
ANOVA	Analysis of variance
ARC	Aquaculture and Artemia Reference Centre
BLAST	Basic Local Alignment Search Tool
CASO	Tryptic Soy Agar
CFU	Colony forming unit
CSID	Chemspider identification
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DW	Drinking Water
EBCT	Empty Bed Contact Time
FITC	Fluorescein isothiocyanate
FSC	Front Scatter
HRT	Hydraulic Retention Time
ILVO	Institute for Agricultural and Fisheries Research (Instituut voor Landbouw-en Visserijonderzoek)
LB	Luria-Bertani
LEU	leucine pathway
M	Mean
MA	Marine Agar
MEP	2-methylerythritol-4-phosphate pathway

MEV	mevalonate pathway
MM	Mineral Medium
MTT	Thiazolyl Blue Tetrazolium Bromide
NA	Nutrient Agar
NA	Not Analysed
NR	Not Relevant
OD	Optical density
PC5.5	PerCP-Cy™5.5
PE	R-phyco- erythrin
PI	Propidium Iodide
RAS	Recirculated aquaculture systems
rpm	Revolutions per minute
SD	Standard deviation
SPME	Solid-Phase Micro Extraction
SPSS	Statistical package for the social sciences
SSC	Side Scatter
TO	Thiazole orange
UV	Ultra Violet

Abstract

Off-flavour of aquaculture RAS products is a major problem for the industry that affects market demand and prices. Off-flavour originates from geosmin and 2-methylisobornoel (2-MIB). In this study, micro-organisms, capable of degrading geosmin, were selected, identified, and evaluated on applicability in RAS systems. In a first step, micro-organisms capable of degrading geosmin were selected by inducing growth on the off-flavour molecule as sole carbon source based on the protocol of Then, thirteen selected strains of bacteria and fungi were evaluated on several criteria related to applicability in RAS. Growth potential was established on Luria-Bertani (LB) media. Toxicity of the strains was tested on Artemia. BIOLOG™ assays were performed on all strains. Strains were identified using 16s sequencing. Cell metabolic activity on geosmin was tested using Thiazolyl Blue Tetrazolium Bromide (MTT) assays. Finally, five most promising strains were evaluated for their geosmin degradation potential using low geosmin concentrations as sole carbon source. Geosmin degradation was evaluated using gas chromatography in combination with SPME. The bacterial growth on geosmin was evaluated using different techniques namely flowcytometry, formation of flocculants, OD measurements and CFUs.

After 96 hours in LB, all the strains reached stationary phase, indicating that all selected strains are particularly slow growing micro-organisms. Mortality of *Artemia* caused by the different strains in challenge tests varied widely both between strains and between repeated experiments. Among the tested strains only 5 strains proved not to be toxic ($p<0.05$). Identification of the 5 strains through 16s sequencing revealed 4 genera: *Bosea* sp., *Mycobacterium* sp., *Roseomonas* sp. and *Brevundimonas* sp.. BIOLOG™ assays indicated that the 5 strains varied significantly in affinity for certain C-sources, indicating that strains are metabolically different from each other. MTT assays clearly showed that *Mycobacterium* sp. and *Bosea* sp. F2 showed an increased metabolic activity when grown in a with geosmin enriched environment as compared to negative control. Growth of micro-organisms in media with only geosmin as carbon source resulted in overall slow growth with *Mycobacterium* sp. performed best compared to the others. *Roseomonas*sp. *and Brevundimonas*sp. showed no growth at all over the duration of the experiment. On the other hand, based on the result of geosmin degradation test, *Mycobacterium*sp. and *Brevundimonas*sp. were the only strains

capable to degrade geosmin, with implication on different mechanisms. Results from this study are promising, with at least 3 strains (*Mycobacterium* sp., *Bosea* sp. F2 and *Brevundimonas* sp.) proven to be able to remove geosmin in at least one test. To be able to apply these strains in biofilters for aquaculture purposes, further research on toxicity and mechanism of action is required.

In addition to this microbiological approach, physical removal of geosmin and 2MIB was tested with a specially designed zeolite filter. Tests were carried out in a semi-commercial and commercial fish farm, but the zeolite filter proved to be ineffective in removing geosmin and 2-MIB from RAS water.

Keyword: geosmin, 2-methylisoborneol, *Mycobacterium, Brevundimonas*, flowcytometry

Chapter 1. Introduction

1.1 Background information

Off-flavour of aquaculture products is a major problem for the industry that affects market demand and prices (Tucker, 2000). The undesirable taste and odor are linked to the presence of certain metabolites such as aliphatic hydrocarbons, sulfur-containing compounds, aldehydes, ketones and especially geosmin and 2-methylisoborneol (2-MIB) (Jüttner, 1988; Kenefick *et al*, 1986). Repulsive taste and odor are encountered in many species such as common carp (*Cyprinus carpio*) (Tucker, 2000), channel catfish (*Ictalurus punctatus*) (Lovell *et al*., 1986; Martin *et al*, 1988), rainbow trout (*Oncorhynchus mykiss*) (Schrader *et al*, 2010) and Nile tilapia (*Oreochromis niloticus*) (Yamprayoon & Noomhorm, 2000) and in almost all species cultured in recirculation aquaculture systems (RAS) (Azaria & van Rijn, 2018).

Geosmin and 2-MIB can be produced by several different species belonging to the genus of *Streptomycetes* (Gerber & Lechevalier, 1965; Medsker *et al*, 1969) and Cyanobacteria (Safferman *et al*.,1967; Tabachek & Yurkowski,1976). Furthermore, geosmin and 2-MIB are potentially produced by other organisms including *Myxobacteria* (Breheret *et al*,1999; Dickschat *et al*, 2005), fungi (Smith *et al*, 2008)and amoeba of the genus *Vanella* (Hayes *et al*, 1991).

Unlike most other production problems in aquaculture, off-flavour does not affect the growth or health of the animals (Tucker, 2000), however, its economic impact is high, making it one of the most severe challenges of the aquaculture industry worldwide (Jonns et al., 2017). Depuration-keeping fish in running fresh water without feeding- is the most adapted practice to eliminate off-flavour. Not only increasing production cost by US$ 8 million, this is also a risky procedure, as delays in harvest result in additional feed costs and forfeiture of income from foregone sales since producers are forced to delay the restocking of ponds. Furthermore, loss of weight and mortality of fish during the holding period is possible due to disease, water quality deterioration, and bird predation (Tucker, 2000).

Although being a serious problem faced by the aquaculture industry, especially in RAS systems, only a few studies have been carried out to find out the possible causes and remediate this problem in so far.

1.2 Research problem identification and justification.

As RAS systems provide the ideal condition for micro-organism that produce off-flavour to thrive, fish produced in RAS has been plague by the odor of geosmin and 2MIB ever since. Different removal methods have been used including activated carbon, both granulated and powdered (Azaria & van Rijn, 2018; Chen *et al.,* 1997; Lalezary *et al.*, 1988) oxidation by UV light. These methods, although working well in drinking water treatment, performed poorly in the high organic loaded and high turbidity environment of a RAS system (Cook *et al*., 2001; Zoschke *et al.*, 2011). As physical and chemical methods proved unreliable and inapplicable in RAS systems, biological methods have been turned to as an alternative. Guttman & van Rijn (2009), McDowall *et al* (2009) and Hsieh *et al* (2010) observed that sludge derived from the digestion basin of a marine system possesses the ability to absorb and degrade both geosmin and 2-MIB.

Because of their flexibility and ease of application in the biofilter of a RAS system, micro-organism has been investigated more and more recently. Microbes capable of degrading geosmin and 2MIB have recently been isolated (Azaria & van Rijn, 2018). This study focusses on a protocol to isolate micro-organism that can remove geosmin and evaluate certain characteristics of isolates in order to decide they can be applied in aquaculture.

1.3 Objective.

This study has as objective to develop the protocol to isolate micro-organisms that are able to degrade geosmin and to test the isolates for their growth, toxicity and metabolic activity. Furthermore, the ability of zeolite to remove geosmin and 2-MIB from RAS water was also tested.

Chapter 2. Literature review

2.1. Off-flavour in Aquaculture.

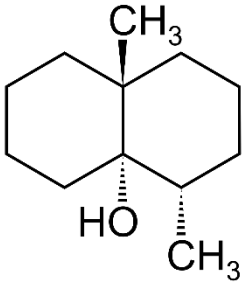
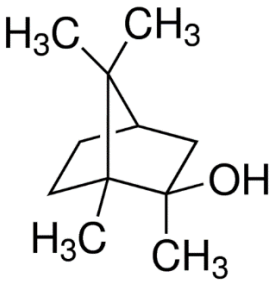
Off-flavour of aquaculture products is a major problem for the industry that affects market demand and prices (Tucker, 2000). Off-flavour can originate from the animal's diet or post-harvest management strategy but is mainly caused by odorous microbial metabolites which are absorbed from the water environment and deposited in edible tissues(Tucker, 2000; Schrader & Rimando, 2003). The undesirable taste and odor are linked to the presence of certain metabolites such as aliphatic hydrocarbons, sulfur-containing compounds, aldehydes, ketones and especially geosmin and 2-methylisoborneol (2-MIB) (Jüttner, 1988; Kenefick *et al*, 1986). The most common description of off-flavour is the musty, earthy smell which originates from geosmin and 2-MIB, which has been described by Mallevialle & Suffet (1987) and Suffet *et al,* (1999). In aquaculture, geosmin and 2-MIB related undesirable taste and odor have been observed as early as in the mid-16th century in common carp (*Cyprinus carpio*) (Tucker, 2000), other recorded affected species includes channel catfish (*Ictalurus punctatus*) (Lovell *et al*., 1986; Martin *et al*, 1988), rainbow trout (*Oncorhynchus mykiss*) (Schrader *et al*, 2010) and Nile tilapia (*Oreochromis niloticus*) (Yamprayoon & Noomhorm, 2000) cultured in open ponds and in almost all species cultured in RAS systems (Azaria & van Rijn, 2018). As an interesting anecdote, repulsive taste and odor are not only encountered in water and aquaculture products but also in others such as wine (Darriet *et al*, 2000; Lisanti *et al*, 2014), fruits (La Guerche *et al*, 2005) beans (Buttery *et al*, 1976).

2.2. Introduction to geosmin and 2-methylisoborneol (2-MIB).

Both geosmin and 2-MIB are tertiary alcohols, each of which exists as (+) and (−) enantiomers and odor outbreaks are caused by biological production of the naturally occurring (−) enantiomers (Jüttner & Watson, 2007) while Polak & Provasi (1992) has stated that (−) geosmin has 11 times lower detection threshold than its (+) enantiomers. Both geosmin and 2-MIB are very potent flavour-impairing chemicals with geosmin being described as smelling muddy, earthy while 2-MIB as musty, camphor, moldy and basement-like (Suffet *et al*., 1999). Human detection threshold for these compounds is as low as 10 to 30 $ng.L^{-1}$ in water (Srinivasan & Sorial, 2011) and 6 $\mu g.kg^{-1}$ in fish (Yurkowski & Tabachek, 1974) However, the human perception of geosmin and 2-MIB greatly varies

among individuals and species of fish (Persson, 1980) because both compounds are lipophilic and are being deposited in the fatty tissue of the cultured animals.

Table 1. Chemical and physical characteristics of geosmin and 2-MIB

Parameter	geosmin	2-MIB	reference
Molecular formula	C12H22O	C11H20O	CSID:27642 CSID:16024
Molecular weight (g Mol-1)	182.33	168.28	CSID:27642 CSID:16024
Boiling point (°C at 760 mmHg)	270	207 to 209	CSID:27642 CSID:16024
Aqueous solubility (mg L-1)	150	195	Pirbazari *et al*, 1992
Enthalpy of vaporization (kJ Mol-1)	59.0	52.69	Li, 2015
Log Kow Octanol/water partition coefficient	3.57	3.31	Howgate, 2004
Chemical structure			Li, 2015

CSID: Chemspider Identification

Source: (Dissanayake, 2018)

Geosmin and 2-MIB were first isolated from Streptomycetes. sp. (aerobic filamentous Actinomycetes) (Gerber & Lechevalier, 1965; Medsker *et al*, 1969). Cyanobacteria were also confirmed to produce geosmin and 2-MIB shortly after

by Safferman *et al*. (1967)and Tabachek & Yurkowski (1976). Furthermore, geosmin and 2-MIB are potentially produced by other organisms including Myxobacteria (Breheret *et al*,1999; Dickschat *et al*, 2005), fungi (Smith *et al*, 2008), amoeba of the genus *Vanella* (Hayes *et al*, 1991) and interestingly also in the plant (*Symphyogyna brongniartii*) (Spörle *et al*, 1991). Most odor producing Cyanobacteria species produced either geosmin or 2-MIB, however, there are records of both compounds been produced simultaneously in strains of *Oscillatoria* (Tsuchiya & Matsumoto, 1999; Wu & Jüttner, 1988) and *Phormidium* sp. (Izaguirre, 1992).

Although both Actinomycetes and Cyanobacteria can produce odorous compounds, it is adopted that Cyanobacteri*a* are the cause for most off-tasting and odorous problems in nutrients enriched environment such as aquaculture pond (Jüttner, 1995), with geosmin and 2-MIB as a secondary metabolites related to photosynthesis and pigment synthesis. As Cyanobacteria thrive in nutrient-rich environments, the seasonal variation of Cyanobacteria is linked closely with the fluctuation in water quality, making them easier to be identified as compared to Actinomycetes (Watson *et al*., 2007). Tucker (2000) stated that because of the light-limited nature of eutrophic ecosystem, free living organisms that are living high in the water column have a competitive advantage as compared to the benthic or substrate attached organism such as Actinomycetes.

On the other hand, Actinomycetes growth is poorly correlated with the production of geosmin and 2-MIB. Although first identified as a producer of odorous compounds, the capacity to produce odorous compounds varied greatly between different strains (Kenefick *et al*., 1992). Cross (1981) has suggested that Actinomycetes are relatively inactive in most aquatic environment. This, combined with the fact that they can be outcompeted by Cyanobacteria in open aquaculture system, makes it harder to detect and identify them.

According to Jüttner & Watson (2007), these two compounds are believed to be produced along three different pathways in Streptomycetes and Myxobacteria: 2-methylerythritol-4-phosphate (MEP) pathway, mevalonate pathway (MEV) and/or the leucine pathway (LEU).

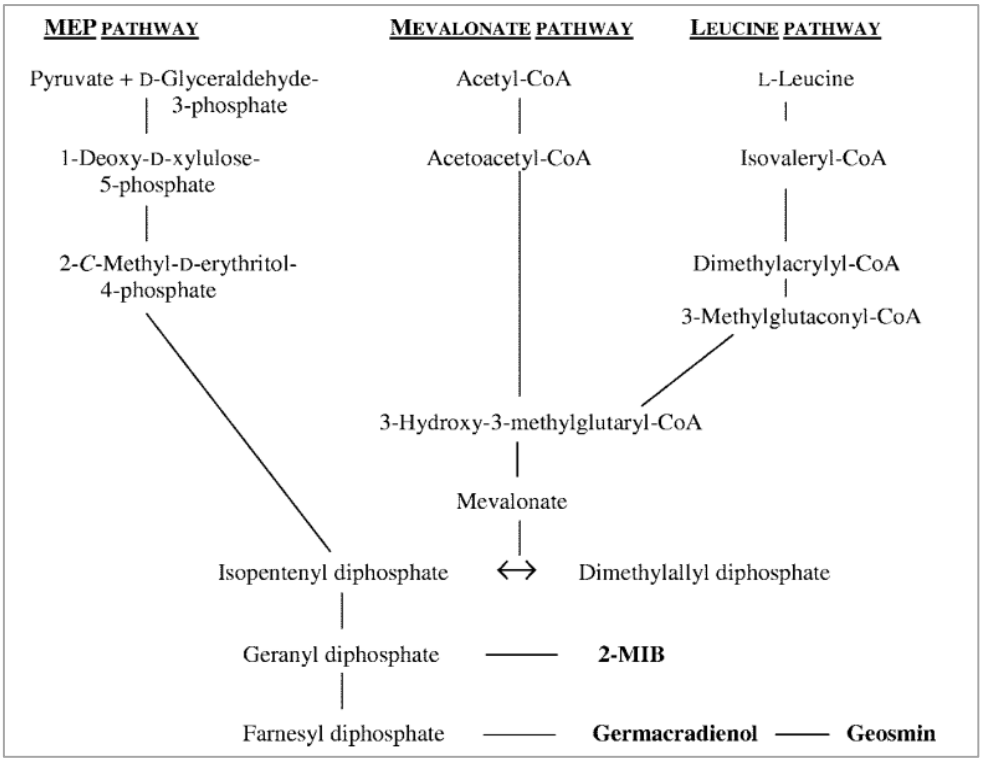


Figure 1. The biochemical pathways for the formation of geosmin and 2MIB in *Streptomycetes* and *Myxobacteria* (Jüttner & Watson, 2007)

According to Jüttner & Watson (2007), MEP is the major isoprenoid biosynthetic pathway in many bacteria. On the other hand, in some groups of Myxobacteria, MEV pathway is active in the production of isoprenoids including geosmin (figure 1). MEV pathway also contributes to geosmin production in the stationary growth phase of Streptomycetes. Furthermore, there are evidences that MEP is active during growth and MEV in the stationary growth in Actinomycetes (Seto *et al.*, 1998; Seto *et al.*, 1996)

2.3. Presence of geosmin and 2-MIB in aquaculture systems.

Until recently, it is established that for conventional aquaculture systems such as ponds, Actinomycetes and Cyanobacteria are the main contributors of odorous compounds with cyanobacteria dominating in nutrients-rich, outdoor ponds with direct sunlight (Jüttner, 1995). Observations by Lanciotti, Santini *et al*(2003) have shown that during winter, Actinomycetes is the main the producer of off-tasting compounds.

RAS is a technology for intensive aquaculture, based on the filtration and recirculation of water, thus limiting discharge to as low as 1%. RAS is also versatile and can be applied for both indoor and outdoor, and both for marine and fresh

water species. Like conventional pond systems, RAS cultured animals are also subjected to off-tasting problems.

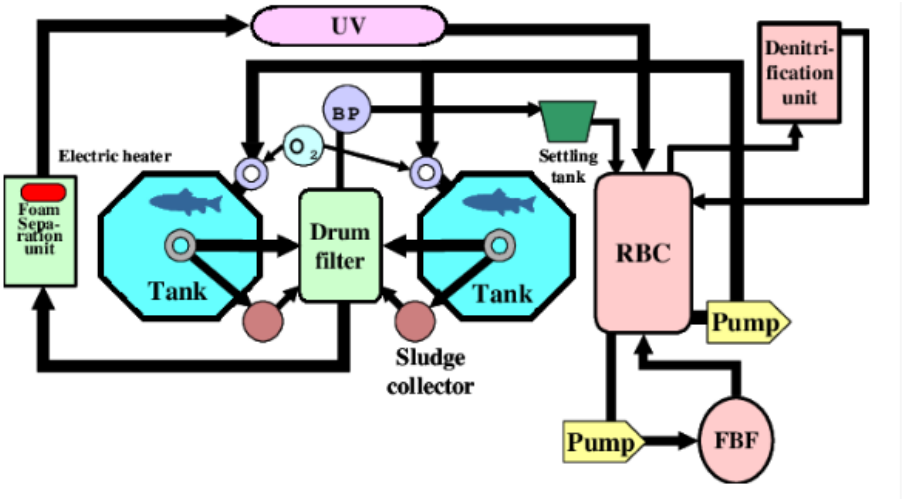


Figure 2. Schematic of a RAS system (adapted from Yoshino *et al.*, 1999) FBF- Floating Bead Filters; BC- Biological Filter; UV- Ultra Violet; RBC – rotating biological contactor

Since the RAS environment is high in nutrients and high in turbidity, combined with the availability of substrate and biofilm in the filtration system, Actinomycetes play a key role in the production of geosmin and 2-MIB. A recent study of Azaria & van Rijn (2018) has shown that Actinomycetes accounted for half of the microorganism identified in a RAS system and they are found in almost all components of the system.

On the other hand, Lukassen *et al.*, 2017 indicated that Myxococcales, Actinomycetales, and genus *Sorangium* were the main geosmin producing bacteria in European RAS. Specifically, four species of Actinomycetes *(Nocardia cummidelens, Nocardia fluminea, Streptomyces albidoflavus*, and *Streptomyces luridiscabiei*) were isolated from biosolids from a RAS used for rainbow trout production (Schrader & Summerfelt, 2010). Relatively high geosmin and 2-MIB concentrations and higher in vitro production was reported in the aerobic components (drum filter and a trickling filter) of the RAS (Guttman & van Rijn, 2008).

Table 2. Geosmin and 2-MIB producing bacteria found in RAS (adapted from (Azaria & van Rijn, 2018)

Bacteria	Fish	Location in RAS	Reference
Nocaradia cummidelends	Rainbow trout	Biofilter bed	Schrader & Summerfelt, 2010
Nocardia fluminea		Culture tank	
Streptomyces luridiscabiei		Heat exchanger	
Streptomyces albidoflavus		Drum filter	
Streptomyces roseoflavus	Hybrid tilapia	Trickling filter	Guttman & van Rijn, 2008
Streptomyces thermocarboxydus			
Streptomyces anulatus	Brook trout	Culture water	Auffret *et al*., 2011
Streptomyces flavogriseus			
Myxococcus xanthus			
Sorangium sp.	Rainbow trout	Trickling filter	Auffret *et al*., 2013
Nannocystis sp.			
Sorangium sp.	Not reported	Moving bed filter	Lukassen *et al*., 2017
Actinobacteria spp.			
Myxobacteria sp.			

2.4. Uptake of geosmin and 2mib in fish.

Off-flavour can be taken up in fish tissue via several routes. Fish can ingest microbial cells containing intracellular storages of geosmin or 2-MIB, as indicated by the detection of geosmin-producing bacteria in the stomach, skin and intestinal mucus layer (Gutierrez *et al.*, 2006; Lukassen *et al*., 2017; Tucker, 2000; Watson *et al.*, 2016). A major uptake route is passive diffusion through water (From & Hørlyck, 1984). According to Tucker (2000), most uptakes occurred across the gills as their structure and functions enhance diffusion of substances between water and blood. As with most odor-causing compounds, geosmin and 2-MIB are lipophilic and upon uptake, are transported throughout the animal's body via the blood stream before being concentrated and stored in lipid-rich tissues such as skin or visceral fat (Tucker, 2000).

As a result of their lipophilic nature, uptake of geosmin and 2-MIB is relatively rapid but elimination is much slower (Persson, 1984; Rurangwa & Verdegem, 2015). As both substances are very potent odor inducers, exposing fish for only a few minutes can cause sufficient accumulation to impair flavour. On the other hand, elimination process through depurations can take days or even weeks (Tucker, 2000). According to Johnsen & Lloyd (1992), accumulation of 2-MIB is effected by the fat content and water temperature being more important.

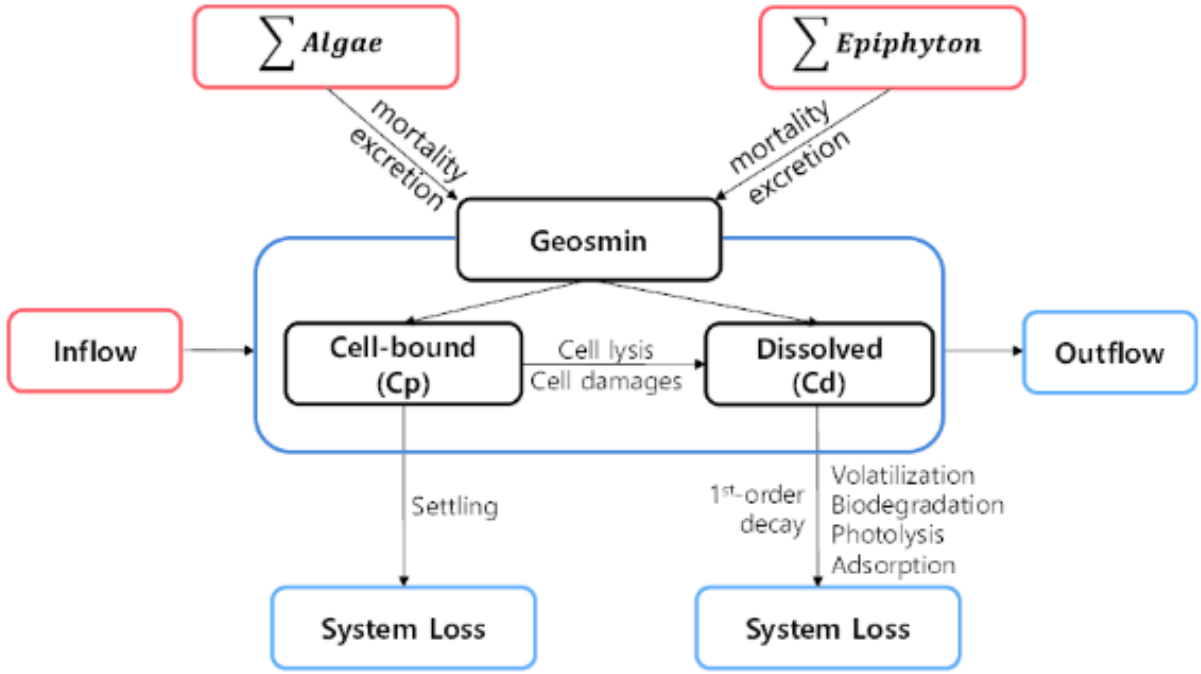


Figure 3. Schematic description of the geosmin occurrences and transport of geosmin (Chung *et al.*, 2016)

2.5. Disadvantages due to the taste and odor compounds in aquaculture.

Unlike most other production problems of aquaculture, off-flavour does not affect the growth or health of the animals (Tucker, 2000), however, it's economic impact is high, making it one of the most severe difficulty in aquaculture industry worldwide (Jonns *et al*., 2017). Most losses are incurred when depurating the fish, which can go up to 30% of the potential revenues (Tucker, 2000). Products with off-flavour can hardly find acceptance from the consumers. Furthermore, a first time buyer may assume that off-flavour is inherent in aquaculture products in general (Tucker, 2000), making it extremely difficult to compete with wild caught fisheries products. This all makes market expansion more difficult.

Off-flavour has impacted economically both US and European aquaculture. Production cost of catfish has been reported to increase by US$47 million in 1999 (Mississippi State University/MAFES). Problems caused by off-flavour are estimated to increase production cost by 0.25 US$ per kilogram of fish (Hanson *et al.*, 2003) Losses from off-flavour in the United States catfish aquaculture industry range from US$0.04 to US$0.26 per kg of catfish (farm gate price per kg of catfish was US$2) (Engle *et al.*, 1995). Catfish farmers are estimated to have lost up to 12% annual revenue due to off-flavour in catfish (Kinnucan *et al.*, 1988).

In Europe, it is estimated that the cost arising from depuration has reach the value of 8 million Euros; this has a serious impact on the profit margin of the farmers utilizing RAS. The three main causes for economic damage to the European aquaculture industry related to off-flavour are consumer rejection of off-flavoured fish, reduction of market volumes and prices and costs of depurating off-flavour from fish crops (CORDIS, European Commission, 2018).

2.6. Removal of geosmin and 2-MIB

2.6.1. Depuration.

As human detection level for geosmin and 2-MIB is very low (Tucker, 2000), the removal process must be done very well. There are several ways to remove geosmin and 2-MIB in aquaculture: depuration, physical or chemical removal and biological removal.

Depuration is the most common method used, based on the fact that the diffusion of odorous compounds is driven by the difference in gradient between the environment and the fish body, thus removing the fish from tainted water and placing it in clean water can facilitate the movement of substances from the fish flesh, through the gills and epithelial surfaces into the water (Azaria & van Rijn, 2018). On the other hand, the stripping of geosmin and 2-MIB proceeds at a much slower pace as opposed to their accumulation (Persson, 1984; Rurangwa & Verdegem, 2015), making depuration a long and potentially risky business, as delays in harvest that result in additional feed costs and forfeiture of income from foregone sales because producers are forced to delay restocking ponds. Furthermore, loss of fish can occur during the holding period from disease, water quality deterioration, and bird depredation (Tucker, 2000).

Depuration is a very disadvantageous process as it contradicts the concept of RAS by consuming huge quantities of clean water in a flow through system. Also, to minimize bacterial growth, fish are usually fasted, and this would lead to weight loss and deteriorated fillet quality (Azaria & van Rijn, 2018; Burr *et al.*, 2012; Palmeri *et al*., 2008).To reduce depuration time, several methods are applied such as using pre-disinfected basins with no aeration (Davidson *et al*., 2014) and induced exercise to increase water movement through the gill thus increase elimination rate (Schram, Schrama, Kusters *et al.,* 2016). The depuration systems and required time for various fish species are shown in table 3.

Table 3. Depuration systems and required time for various fish species (adapted from Azaria & van Rijn, 2018)

Species	Depuration time (hours)	Purging system	Pre-depuration geosmin ($\mu g.kg^{-1}$)	Post depuration geosmin ($\mu g.kg^{-1}$)	Reference
Rainbow trout (*Oncorhynchus mykiss*)	36	Flow through	1.68	0.9	Robertson *et al.,* 2005
	60	Flow through	2.98	0.9	Robertson *et al.,* 2005
	120	Flow through	6.25	0.9	Robertson *et al.,* 2005
Atlantic salmon (*Salmo salar*)	240	Recirculating tank	0.26-0.51	0.07-0.26	Davidson *et al*., 2014
	360	Flow through	0.2-0.3	0.012	Burr *et al*., 2012
European eel (*Anguilla anguilla*)	192	Flow through	13-19	9.5-13	Schram *et al*., 2016
Tilapia (*Oreochromis niloticus*)	384	Static tank	31.76	1	Yamprayoon & Noomhorm, 2000

2.6.2. Physical/Chemical removal.

As they are hydrophobic, physical filtration of geosmin and 2-MIB by adsorption materials such as activated carbon is achievable in both granular and powdered form (Azaria & van Rijn, 2018; Chen *et al.,* 1997; Lalezary *et al.*, 1988). The effectiveness of this method has been validated in the drinking water industry with low organic matter content (Cook *et al.,* 2001; Drikas *et al.*, 2009; Herzing *et al.*, 1977). On the other hand, the effectiveness of activated carbon is greatly diminished when applied to water with high organic content (Cook *et al*., 2001; Zoschke *et al.*, 2011) e.g. RAS water, which usually operate at relatively high organic residue level (>10mg.L^{-1}) (Azaria & van Rijn, 2018). The explanation for the reduction in efficiency is the competitive adsorption of organic particles in RAS water quickly clogged up the pores of activated carbon (Newcombe *et al.*, 2002). Furthermore, other factors have been proven to affect performance of activated carbon such as pore volume (Yu *et al*., 2007). The presence of humic substances in water also reduces the adsorption capacity of both geosmin and 2-MIB while pH level has no significant impact on the process (Herzing *et al*., 1977). Activated carbon particle size also matter as adsorptive removal of geosmin improved with decrease in activated carbon particle size down to 1 mm but further particle size reduction produced little improvement (Matsui *et al.*, 2009). Azaria & van Rijn (2018) stated that because the capability of manipulating water quality in RAS is better than in most other aquaculture system, the removal of geosmin and 2-MIB via activated carbon adsorption is feasible if one can manage the water quality properly.

Apart from activated carbon, the removal of geosmin and 2-MIB can be achieved using different materials such as hydrophobic substances such as polystyrene or paraffin (Kelly *et al.*, 2006). Chen *et al* (2011) has demonstrated the ability of tablet ceramic adsorbent (TCA) in the removal of geosmin, stating several advantages such as long lifecycle, effective regenerative performance and almost no second contaminations. Zeolite is a microporous, aluminosilicate mineral commonly used as commercial adsorbent and catalyst (Grace, 2010). The removal of geosmin and 2-MIB in drinking water by an ultra-stable form of zeolite has been validated by Ellis & Korth (1993). According to the authors, adsorption by zeolite is not affected by water hardness and the presence of low concentration of humic acid. However, removal of geosmin by zeolite has only been reported in laboratory

experiments a so far, no commercial application has been reported (Lindholm-Lehto & Vielma, 2019).

Oxidation by UV, ozone and peroxide is another technology to remove geosmin and 2-MIB, with experimental reports of removal rate in drinking water as high as 90% for geosmin and 60% for 2-MIB (40–43 $ng.L^{-1}$ initial concentration) at the UV dose of 1200 $mJ.cm^{-2}$ with 6 $mg.L^{-1}$ H_2O_2 (Collivignarelli & Sorlini, 2004; Jo *et al.*, 2011). Similar to adsorption, the efficiency of this method is greatly diminished in water with high organic matter, both in drinking water and aquaculture water (Ho *et al.*, 2007; Schrader *et al*., 2010). Furthermore, the fact that this method incurs high energy and capital costs (Srinivasan & Sorial, 2011), in combination with the production of toxic by-product (Tango & Gagnon, 2003), greatly reduces its applicability. A recent study of Nam-Koong *et al* (2016) introduced an alternative method of using ultrasonic induced cavitation to remove off-flavour compound, independent from the organic load of the water and different water type (tap water, RAS fresh water, RAS sea water). Chemical approach was also considered in the form of using biocide to control the growth of *Actinomycetes* and *Cyanobacteria*, but given the operating nature of RAS system, this is implausible (Schrader & Summerfelt, 2010)

2.6.3. Biological removal.

As physical and chemical methods proved unreliable and poorly applicable in RAS, a novel method is required to control off-flavour in aquaculture. Biological degradation of geosmin and 2-MIB was first reported by Silvey & Roach (1964). Further research has identified strains of *Bacillus cereus* and *Bacillus subtilis* to be responsible for the process (Narayan & Nunez, 1974; Silvey *et al.*, 1970). Most progress in biological removals of odorous compounds is made in the water treatment industry (Saito *et al.*, 1999), with biological geosmin and 2-MIB removal examined in different reactors. Different components of a water treatment plant and RAS can be utilized as a geosmin and 2-MIB removal unit, for example the sand filter was used successfully to treat tainted water by McDowall *et al.*, (2009)and Hsieh *et al.,* (2010), with the former enriching the filter with geosmin-degrading Proteobacteria. Combination of adsorption and biodegradation of geosmin was achieved by 2 different filter types of activated carbon and expanded clay (Persson *et al.*, 2007). These components can be easily integrated into a RAS system. Guttman & van Rijn (2009) found that sludge derived from the digestion

basin of a marine RAS system possesses the ability to absorb and degrade both geosmin and 2-MIB. A summary of the biological removal of geosmin and 2-MIB in different filter materials is shown in Table 4. Bioflocs produced from solid waste of RAS was tested *in vitro* for the geosmin and 2-MIB biodegradable capability (Ma *et al.,* 2016). However, the removal of off-flavour compounds was underlain by mainly adsorption (>90% of total removal).

Table 4. Biological removal of geosmin and MIB in filters and by different materials (Adapted from Azaria & van Rijn (2018))

Filter type	Water type	EBCT (min)/HRT (hours)	Initial level ($ng.L^{-1}$)		Removal (%)		References
			Geosmin	MIB	Geosmin	MIB	
Filter							
Sand filter	DW	15 min	50-200	50-200	>85	>85	Ho *et al* (2007)
Sand filter	DW	15 min	100	NA	75	NA	McDowall *et al* (2009)
Sand filter	DW	173 min	40-130	55-126	87-96	48-69	Hsieh *et al* (2010)
RAS sludge	RAS water	4.34 hours	NA	800	NA	70	Azaria *et al* (2017)
Activated Carbon	DW	30 min	20	20	96	96	Persson *et al* (2007)
Expanded clay	DW	30 min	20	20	88	82	Persson *et al* (2007)
Biomaterial							
RAS sludge	MM	NR	400	450	83	95	Guttman & van Rijn (2009)
Biofilm	DW	NR	2500	NA	90	NA	Xue *et al* (2012)
Biofloc	MM	NR	1990	968	93	98	Ma *et al* (2016)

EBCT: Empty Bed Contact Time

HRT: Hydraulic Retention Time

DW: Drinking Water

NA: Not Analysed

MM: Mineral Medium

NR: Not Relevant

2.6.4. Geosmin and 2-MIB degrading bacteria

As research on the biodegradation of geosmin and 2-MIB has recently started, very little information regarding the mechanism and microbes involved can be obtained. Early reports by Silvey *et al*., (1970) and Narayan & Nunez (1974) on the degradation of geosmin and 2-MIB by strains of *Bacillus cereus* and *Bacillus subtilis* prove unreliable as further experiments by MacDonald *et al.* (1987) and Danglot *et al* (1983) cannot replicate the results when using the same strains. A 2-MIB degrading consortium of seven *Pseudomonas* species was isolated by Izaguirre *et al* (1988) from sediment samples of MIB-tainted lakes. The consortium was discovered to be actively degrading MIB when added as the sole carbon source. On the other hand, it was found that the degradation process can be greatly enhanced with the addition of another, readily available carbon source. Similarly, Saito *et al* (1999) found that geosmin is extremely difficult to biodegrade when it was used as the sole carbon source. In his experiments, an acceleration of the reaction was achieved by adding ethanol. Furthermore, McDowall *et al* (2009) were able to utilize a geosmin degrading consortium comprised of Proteobacteria. Three bacterial geosmin-degrading species of the Proteobacteria phylum, taxonomically related to *Sphingopyxis alaskensis*, *Novosphingobium stygium*, and *Pseudomonas veronii* were reported by Hoefel *et al* (2006). These findings imply that biodegradation of geosmin and 2-MIB is not accomplished by a single strain of microbes but a consortium of bacteria and in this consortium, the degradation process relied on metabolic cooperation between the consortium members (Azaria & van Rijn, 2018). On the other hand, single bacteria degradation of 2-MIB was achieved with *Pseudomonas* sp*.* and *Enterobacter* sp*.* isolated from the backwash water of a water treatment plant (Tanaka *et al*., 1996), proving that single strain degradation is not impossible. Experiments performed by Luo *et al.* (2016) showed that the amount of 2-MIB removal in the inoculated reactors was significantly greater than that of geosmin, suggesting that the removal of 2-MIB is more efficient than that of geosmin. These findings differ from earlier results of Ho *et al. (*2012) which demonstrate that geosmin appears to be degraded more easily than 2-MIB by the bacteria within the sand filters and bioreactors. A summary of different geosmin and 2-MIB degrading bacteria can be seen in Table 5.

Biodegradation of geosmin and 2-MIB does not mean elimination of the repulsive odor and taste, as the end products of several bacteria when degrading 2-MIB is 2-methylcamphene and 2-methylenebornane or camphor (Eaton, 2012; Tanaka *et al*., 1996). These compounds also have the same muddy smell and taste as 2-MIB, making the biodegradation process meaningless from the point of removing off-flavour. Furthermore, several 2-MIB hydroxylation products were identified as 3-hydroxy-2-MIB, 6-hydroxy-2-MIB or 5-keto-2-MIB and 5-hydroxy-2-MIB. These products, while being less volatile than MIB, still provoke a muddy smell (Eaton, 2012).

Table 5. Summary of geosmin and 2-MIB degrading bacteria (adapted from Azaria & van Rijn (2018))

Species	Compound	Range (ng/L)	Source	Reference
Pseudomonas aeruginosa, Pseudomonas paucimobilis Pseudomonas pseudoalcaligenes, Pseudomonas mendocina, Pseudomonas vesicularis, Pseudomonas diminuita, Moraxella osloensis	2-MIB	290-2×10^6	Lake water	Izaguirre *et al* (1988)
Pseudomonas sp., Enterobacter sp.	2-MIB	NA	Water treatment biofilter	Tanaka *et al* (1996)
Bacillus fusiformis, *Bacillus sphaericus*	2-MIB	25-20×10^6	Lake water	Lauderdale *et al* (2004)
Micrococus sp., Flavobacterium spp., Brevibacterium spp., Pseudomonas sp.	2-MIB	515-4.2×10^6	Water treatment biofilter	Yuan *et al* (2012)

Pseudomonas putida G1, Rhodococcus ruber T1, Rhodoccocus wratilaviensis	2-MIB	0.125×10^6-0.5×10^6	Various	Eaton & Sandusky (2009)
Pseudomonas sp., Sphigomonas sp.	2-MIB	NA	Soil	Eaton (2012)
Rhodococcus sp., Variovorax sp., Comamonas sp.	Geosmin and 2-MIB	5000-0.25×10^6	RAS	Guttman & van Rijn (2012)
Sphingopyxis alaskensis, Novosphingobium stygiae, Pseudomonas veronii	Geosmin	40-20×10^6	Sand filter	Hoefel *et al* (2006)
Sphingopyxis sp.	Geosmin	100-1000	Sand filter	Hoefel *et al* (2009)
Chryseobacterium gambrini, Sinorhizobium morelense, Stenotrophomonas maltophilia	Geosmin	560-2×10^6	Activated carbon filter	Zhou *et al* (2011)
Pseudomonas sp., Rhodococcus wratislaviensis	Geosmin	9.4×10^6	Activated sludge	Eaton & Sandusky (2010)
Shinella zoogloeoides, Bacillus idriensis, Chitinophagaceae bacterium	2-MIB	20×10^3	Sand filter	Du *et al* (2016)

NA: Not Analysed

Chapter 3. Materials and methods

3.1. Section 1: Pilot experiment.

3.1.1 Aim.

The aim of this experiment is to test the feasibility of utilizing bacteria from biofilter sludge to degrade geosmin in laboratory conditions.

3.1.2 Experimental set up.

The pilot experiment was carried out in the Laboratory of Aquaculture and Artemia Reference Centre (ARC), Faculty of Bioscience Engineering, Ghent University, Belgium (Table 6). Sea water sludge samples and fresh water sludge samples were collected from ARC's *Macrobrachium rosenbergii* and *Litopenaeus vanamei* RAS culture systems respectively. Samples were filtered through a 30µm sieve, and inoculated into 6 * 500mL sterilized glass reactors (sealed airtight) containing 100 mL of mineral medium (Table 7 & 8, Guttman & van Rijn, 2012) leading to a final concentration of 10^5 cell.mL^{-1} without replicate. Bacterial densities in sieved samples were measured using a flow cytometer (Beckman Coulter CytoFLEX™, Fig. 7). A geosmin stock was prepared containing 5mg of pure geosmin (Wako chemicals GmbH) in 625mL of autoclaved distilled water. A total of 0.33µg of geosmin (0.04 mL of stock solution) was added to the glass reactor. This amount was calculated to provide enough carbon for micro-organism to grow from 10^5 cells.mL^{-1} to 10^8 cells.mL^{-1} (based on data of Troussellier*et al.,* 1997 (ANNEX I).

Table 6.Setup of the pilot experiment aiming at selecting micro-organisms that can grow on geosmin as sole carbon source. Geosmin was added in a concentration of 33ng.L^{-1}

Label	Content
F++	Mineral medium, Fresh water sludge, geosmin
F+	Mineral medium, Fresh water sludge
F	Mineral medium
S++	Mineral medium, Sea water sludge, geosmin, 0.3g NaCl
S+	Mineral medium, Fresh water sludge, 0.3g NaCl
S	Mineral medium, 0.3g NaCl

Table 7. Content of mineral medium (adapted from Guttman & van Rijn, 2012)

Mineral	Amount (L^{-1})
NH_4Cl	0.05 g
$MgSO_4 \cdot 7H_2O$	0.05 g
$CaCl_2 \cdot 2H_2O$	0.02 g
K_2HPO_4	0.1g
$FeCl_3 \cdot 6H_2O$	0.001 g
trace element solution	0.5 ml

Table 8. Trace element solution (Stanier *et al*,. 1971)

Mineral	Amount (L^{-1})
H_3BO_3	2.86g
$MnCl_2.4H_2O$	1.81g
$ZnSO_4.7H_2O$	0.222g
$Na_2MOO_4.2H_2O$	0.39g
$CuSO_4.5H_2O$	0.079g
$Co(NO_3)_2.6H2O$	0.0494g

The reactors were incubated in the dark, preventing algal growth. Temperature was maintained at 28°C and reactors were shaken continuously (Fig. 4). Every 6 days, 100µL of geosmin stock (0.8µg) was spiked to the reactors to replenish losses via evaporation and micro-organism's consumption. After 10 days, the cultures were diluted back to the original concentration of 10^5 cell L^{-1} by inoculating part of the old cultures into fresh media in autoclaved reactors. The experiment was carried out for the duration of 15 days. Sampling was carried out every 2 days to monitor bacterial density with flow cytometer. Water samples were collected using sterilized syringes and needles, then filter sterilized through 0.2 µm pore filter. Capped glass vials were filled completely with the filtrate to prevent air contact (Fig. 5) then stored in -4°C for a maximum of 13 days awaiting analysis.

3.1.3 Geosmin analysis.

At the end of growth experiment on day 15^{th}, the selected bacteria were spiked again with geosmin 0.33µg. Water samples were taken according to "3.1.1" at 0H, 24H and 96H and sent for Institute for Agricultural and Fisheries Research (Instituut voor Landbouw-en Visserijonderzoek) – ILVO- for geosmin analysis.

For analysis of geosmin and 2-MIB, 10 ml of water was transferred to a SPME-vial and 4.17 g of NaCl was added together with 20 µl stock solution of internal standards geosmin-D5 and 2-methyl-d3-isoborneol. Samples were incubated at 80°C for 15 min, followed by extraction at 80°C for 25 min by a DVB/CAR/PDMS SPME-fiber at an agitator speed of 500 rpm. Separation and detection took place on an Agilent GC-MS, temperature programmed from 70°C to 230°C. Injection temperature was 200°C. Separation was done on a select PAH column (Agilent, 30m, 0.25mm, 0.25 µm) with helium as carrier gas. Detection was done by electron impact ionization in single ion mode with transfer line temperature at 340°C, ion source temperature at 300°C and quadruple temperature at 150°C. All analyses were done ISO/IEC 17025 accredited.

Figure 4. Set-up pilot experiment

Figure 5. Glass capped vial for geosmin sampling

3.1.4 Flow cytometer measurement.

Protocol for microbial density measurements was adapted from Van Nevel *et al,.* 2013. A 96 wells plate was used for measurement of cell density with a dead/alive assay. A total of 100µL of sample was mixed with 5µL of 17µM thiazole orange (TO) and 5 µL of 1.9mM propidium iodide dye (PI). Dyes were well mixed with the samples by pipet-mixing and the plates were incubated in the dark for 5 minutes before measurement. Measurement channels were chosen based on the compatibility of the detection bandwidth and the emission peak of the dye. In this

case Fluorescein isothiocyanate (FITC) channel was used to measure TO and PerCP-Cy™5.5 (PC 5.5) channel was used to measure PI emission.

The flow cytometer detector sensitivity (gain) was set up as shown in Table 9, with threshold put on FITC channel at 10^3. Particles were detected using the 'area' option rather than the 'height' option of the signal.

Table 9. Flow cytometer gain setup for microbial density measurement.

Gain	Setting
Front Scatter (FSC)	165
Side Scatter (SSC)	400
Fluorescein isothiocyanate (FITC)	240
R-phyco- erythrin (PE)	180

Sample measurements were performed at 60µL.Min^{-1}(high) flowrate for a time period of 60 seconds, with a fixed volume of 60µL. All samples were loaded in triplicate, followed by a distilled water well to remove the carry-over of microorganisms between samples (Fig. 6). Bacterial density was determined via gating on PC 5.5 versus FITC dot plot (Fig. 8).

Table 10. Excitation and emission peaks of TO and PI dyes (nm) and Detection bandwidth of FITC, PE and PC5.5 channels (nm)

Dye/Channel	Excitation peak	Emission peak	Detection bandwidth
TO	513	532	
PI	534	617	
FITC			525±40
PE			585±42
PC5.5			690±50

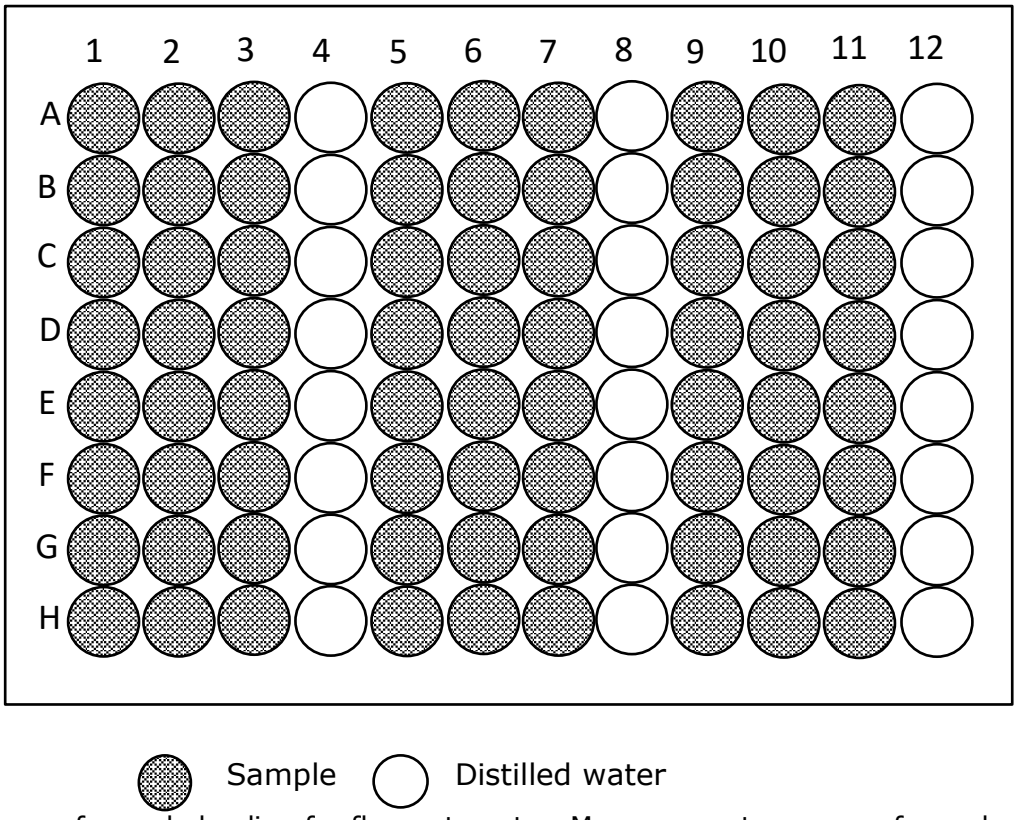


Figure 6. Scheme of sample loading for flow cytometry. Measurements were performed per row.

Figure 7. Beckman Coulter CytoFLEX™ flow cytometer

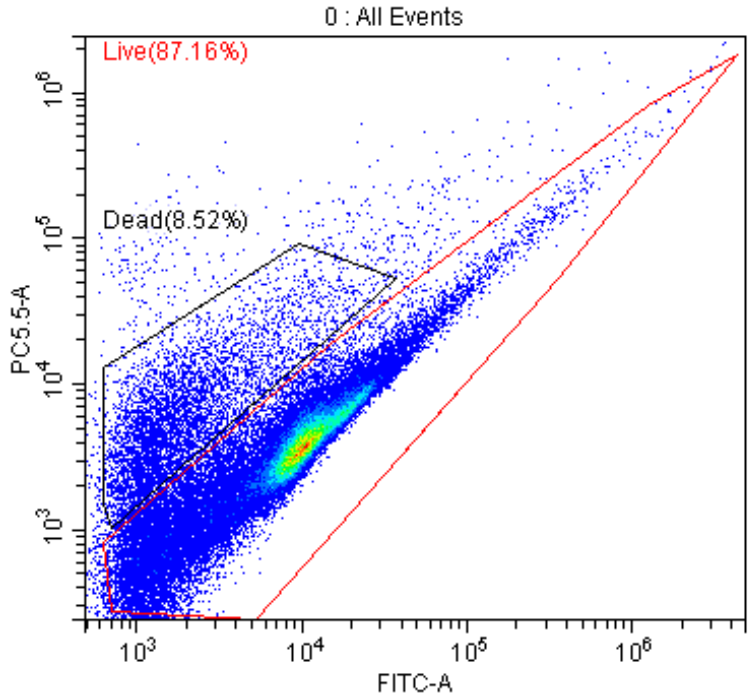


Figure 8. Example of gating for bacteria density estimation using PC5.5 and FITC

3.1.5 Testing bacterial growth on different agar media

After the selection period of 15 days, the growth of the selected micro-organism was tested on different general-purpose agar media in order to choose the medium best suited for isolation during follow-up experiments. The agar media were chosen based on growth potential (CFU's) and bacterial variety: Nutrient Agar (NA); Luria-Bertani (LB); Marine Agar (MA) and Tryptic Soy Agar (CASO).

A total of 50µL of sample from each reactor was taken every 2 days and plated on 4 different agar media to find out the most suitable for these micro-organisms. Three dilutions of 10^{-2}, 10^{-3} and 10^{-4} were made in triplicate. Plates were incubated in 28°C. Microbial growth was monitored visually at 24, 48 and 72 hours.

3.2. Section 2: Selection for geosmin-degrading fresh water micro-organism.

The aim of this experiment is to select micro-organism that can utilize geosmin as the sole carbon source for growth. As off-flavour in freshwater is more prominent in fresh water RAS, also for practicality reason, only fresh water sludge was used.

3.2.1 Selection.

A subsequent experiment was carried out on freshwater micro-organism whereby 2 treatments were used, F++ and F (control) in triplicate. A modification was made

in the mineral media, substituting NH_4Cl with $NaNO_3$ to eliminate the possibility of selecting nitrifying bacteria (Table 12). Sludge from the *Macrobrachium rosenbergii* RAS system was filtered through 30µm sieve, then inoculated to 6 * 500mL reactors in order to reach 10^5 cells. mL^{-1}. Every 6 days, 100µL of geosmin stock (0.8µg) was spiked to the reactors to replenish losses via evaporation The selection was carried out for the duration of 15 days. Live bacteria density was monitored daily using flow cytometry following "3.1.4". The formation of flocculants was determined every 4 days by optical density at 550nm wavelength using the fluorescent spectrophotometer Tecan Infinite® 200 PRO (Fig. 10). 25 measurement were made at different locations for each well, following 5 seconds of orbital shaking. Water samples from each reactor were also plated on LB agar every 4 days using a spiral plating machine L.E.D Techno Spiral System® (Fig. 9).Three dilutions were made: 10^{-2}, 10^{-3} and 10^{-4}. All measurement was carried out in triplicate.

Figure 9. L.E.D Techno Spiral System® Spiral plating machine

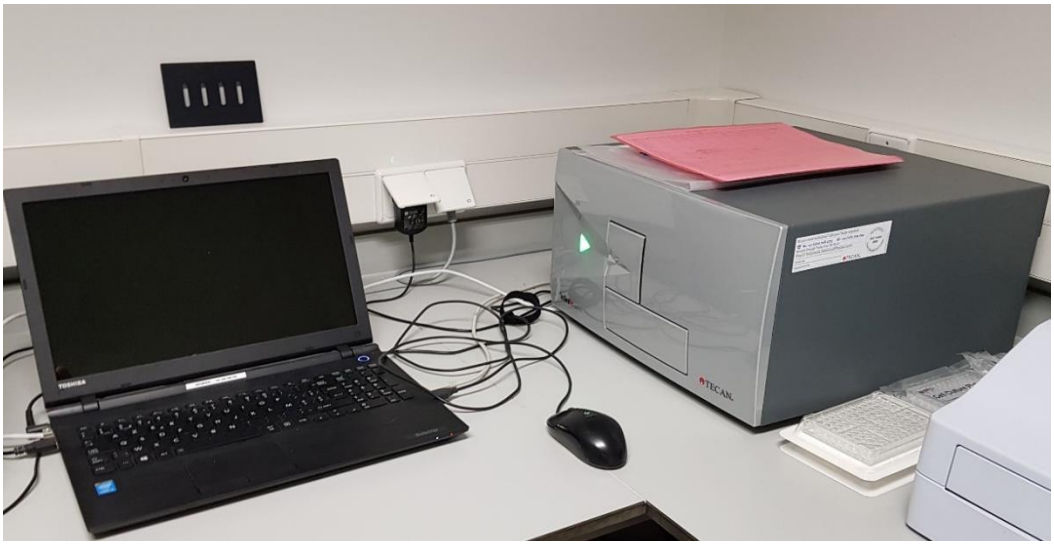


Figure 10. Tecan Infinite® 200 PRO machine

Table 11. Content of modified mineral medium (adapted from Guttman & van Rijn, 2012) (L^{-1})

Mineral	Content
$NaNO_3$	0.08g
$MgSO_4 \cdot 7H_2O$	0.05 g
$CaCl_2 \cdot 2H_2O$	0.02 g
K_2HPO_4	0.1g
$FeCl_3 \cdot 6H_2O$	0.001 g
trace element solution	0.5 mL

3.3. Section 3: Applicability of isolated strains.

In this section, bacteria community from both the pilot (Section 1) and selection experiments (Section 2) were isolated, growth in LB broth, put through a toxicity screen with *Artemia* nauplii. The non-toxic strains were selected and further tested for metabolic activities with BIOLOG™ and MTT-formazan assay.

3.3.1. Isolation of geosmin-degrading micro-organism strains.

Geosmin-degrading micro-organisms from the mixture resulting from the selection experiments, were isolated and a collection of pure cultures was built that will be used in follow-up experiments.

13 single colonies were picked from the freshwater plates of the pilot experiment (6 strains) and sellection experiment (7 strains), based on external morphologies and used to inoculate sterile Erlenmeyer's containing 20mL of LB broth. 50µL of each strain was also plated on LB agar to further confirm the isolation result. For microscopic observation, 50µL of culture broth was heat-fixed on a glass slide, followed by Gram staining and observed under the microscope at 40x and 100x magnification.

Growth curves of the isolated micro-organism was constructed by incubating single strains in the 96 wells microplate in LB broth in 8 replicates. OD at 550nm wavelength was measured at 25 different position per well at 12 hours intervals for a total of 96 hours using the Tecan Infinite® 200 PRO.

3.3.2. Preliminary toxicity screen of isolated strains using *Artemia.*

The aim of this experiment is to test the toxicity of the isolated strains on aquatic animals, utilizing *Artemia franciscana* nauplii as a robust model animal. The non-toxic strains for *Artemia* were selected for further analysis.

3.3.2.1. Preparation.

Artemia nauplii was prepared following Kumar et al. (2018).A total 30mg *Artemia* cyst (INVE Ocean Nutrition™) were rehydrated in 10mL distilled water for 1 hour, follow by decapsulation by adding 330µL NaOH and 8mL NaClO for a maximum of 2 minutes. The decapsulation solution was neutralized by adding 10mL $Na_2S_2O_3$ and washed with 400mL of sterile seawater. The decapsulated cysts were put in 30mL of sterile sea water and incubate for 24 hours for hatching.

Stock cultures of 14 isolated strains was prepared by inoculating 20mL LB broth with 20µL of pure bacteria solution. The cultures were incubated for 96 hours at 28°C under continuous shaking, to make sure all strains have reached stationary phase.

3.3.2.2. Assay.

5 artemia nauplii were selected and put into each well of a 96 well plate, containing 100 µL of 10^9 cells.mL^{-1}bacterial stock, 150 µL of sterile seawater and 1.7 µL (10^7 CFU.mL^{-1}) of autoclaved Artemia feed strain LVS3 to provide feed for the nauplii for the duration of the experiment. There were 2 control treatments: *Artemia*

nauplii were kept in sterile LB broth (control 1) and sterile seawater (control 2). All treatment was repeated 12 times. The plates were incubated at 28°C and mortality was recorded at 24 and 48 hours post inoculation.

3.3.3. Identification of isolated strains.

Micro-organism DNA samples of 5 strains which shown highest *Artemia* survival rate from the toxicity screen was sent to LGC Genomics GmbH for 16S rRNA extraction and sequencing. Sequenced results were compared with online database using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov) to identify the micro-organism.

3.3.4. BIOLOG™ assay.

To generate further information regarding the ability of each isolated strain's to utilized different carbon source, a BIOLOG™ assay was carried out. BIOLOG is an assay to measure the metabolism of the microorganism on 31 different carbon sources. The mechanism of this test is based on the MTT-formazan reaction. If the carbon source in a well is metabolized, the Mitochondrial Reductase enzyme from the micro-organism will also reduce the yellow Thiazolyl Blue Tetrazolium Bromide (MTT) dye to the purple formazan.

3.3.4.1 Micro-organism preparation.

The 5 micro-organisms strains selected by the *Artemia* assay were cultured in LB broth for the duration of at least 5 days at 28°C under continuous shaking. The microbe cells were separated from the media by centrifugation at 4000rpm for 15 minutes at room temperature and discarding the supernatant. The pellets were resuspended in sterile distilled water and further centrifuged to wash the cells. The supernatant was discarded, and the pellet re-suspended in 1mL sterile distilled water.

3.3.4.2. Assay.

Following the instructions of the manufacturer, 100µL of bacteria suspended in distilled water was added to each well of the BIOLOG EcoPlate^{tm}. The plates were incubated in 28°C and optical density at 590 nm was measured at 4H, 24H, 48H and 96H after inoculation respectively. The changing colors of each plate were also recorded.

3.3.5. MTT formazan assay.

The goal of this experiment is to determine the ability of selected micro-organism to metabolize geosmin as the only carbon source. Cell metabolic activities are detected by measuring the ability of their Mitochondrial Reductase enzyme to reduce the yellow Thiazolyl Blue Tetrazolium Bromide (MTT) dye to the purple, insoluble formazan.

The micro-organisms were prepared according to "3.2.5.1". For the assay, the 96 wells plates were used. As negative control, 100µL of 5 pure strains of bacteria were loaded into 5 columns, 8 replicates each. To study the bacterial affinity for geosmin as sole carbon source, a similar setup was used; with the only difference that 10µL of 0.66µgµL^{-1} geosmin stock solution were added to each well. Positive controls were prepared by inoculating the same micro-organism in easily to metabolize, general purpose media, in this case LB broth was used. 2 rows of blank media and blank media with geosmin were also included. To exclude the metabolic activity based on the minerals in mineral media, the test was repeated in distilled water. 10µL of MTT dye was added to each well, both control and treatment. Plates were incubated for 4 hours at 37°C in dark condition.

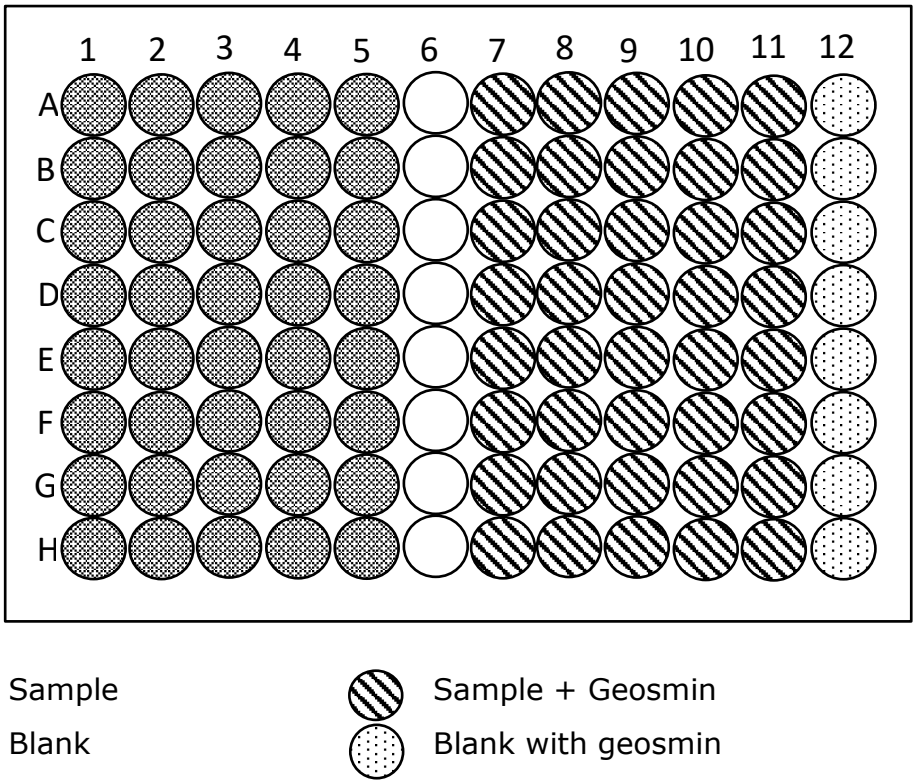


Figure 11. Scheme of sample loading in MTT plate. Samples were loaded in rows, from left to right: F1 *Bosea* sp., F2 *Bosea* sp., F4 *Mycobacterium* sp., F9 *Roseomonas* sp., F10 *Brevundimonas* sp.

After 4 hours, formazan crystals precipitate on the side of the well. Therefore, the suspension was removed from each well, taking care not to disturb the formazan crystal that were formed. 100µL DMSO was added to each well and mixed well to dissolve the formazan crystals. The plates were incubated for 5 minutes, followed by optical density measurement at 570nm wavelength. A purple color forms if metabolic activity had taken place.

3.3.6. Growth and geosmin degradation activity of isolated strains.

The aim of this experiment is to determine the growth of the selected single strains micro-organism in media with geosmin as the only carbon source. Furthermore, the ability to degrade geosmin was also evaluated.

3.3.6.1. Micro-organism preparation.

Pure cultures of selected micro-organism were cultured in LB broth for the duration of 5 days at 28°C and continuous shaking. The microbe cells were separated from the media by centrifugation at 4000rpm for 15 minutes at room temperature and discarding the supernatant. The pellets were resuspended in sterile mineral media (table 12) and further centrifuged to wash the cells. The supernatant was

discarded, and pellet resuspended in 1mL sterile mineral media. A serial dilution was made, and live bacteria enumerated on the flow cytometer following procedure of "3.1.4" to determine the cell density.

3.3.6.2. Assay.

7 treatments of 5 strains, 1 mixture and 1 blank control were prepared in triplicates using 21 sterilized capped glass vials, containing 10mL mineral media, 0. 033µg.L^{-1} geosmin and 10^5 cells.mL^{-1} single strain micro-organism. The reactors were then incubated at 28°C on a rotator. Sampling was made initially, at 24H, 48H, 96H and 144H respectively using sterile syringes and needles. Cell density in the samples was then analyzed using flow cytometer, Tecan reader and plating on LB agar plates.

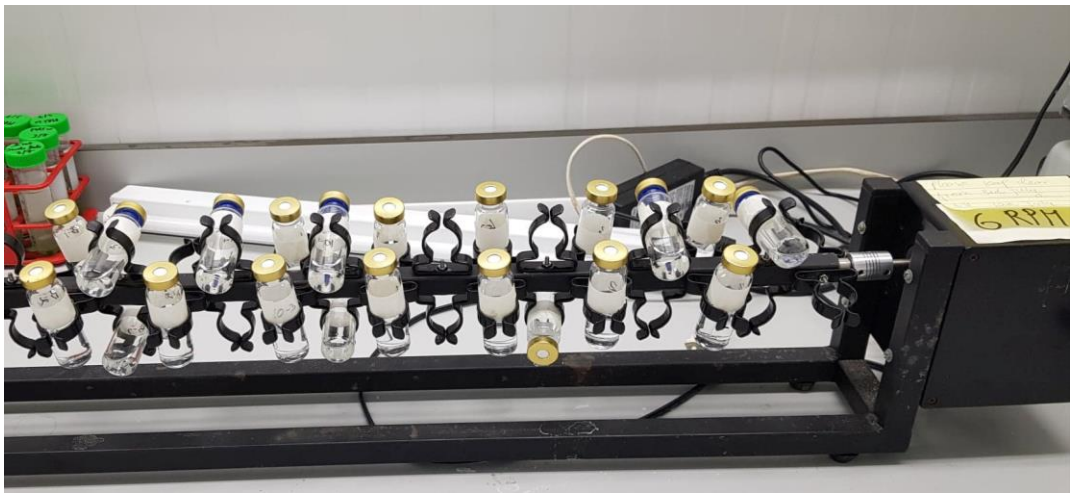


Figure 12. Reactors on rotator.

To measure the geosmin degradation capacity of each strain, samples were collected according to the protocol described in "3.1.2" at the start of the experiment and after 96H. All samplings were made in triplicate.

3.4. Section 4: Evaluation of geosmin and 2-MIB removal capacity of zeolite filter using different flow rates.

The aim of this experiment is to test the capability of a zeolite filter to remove geosmin and 2-MIB from a RAS system.

The filter was design following Figure 13, with the inlet pipe divided into two 0.6L chambers, each having an independent valve for flow rate control. One chamber was filled with 200g granulated zeolite material with maximum size of 6mm, the other was left empty.

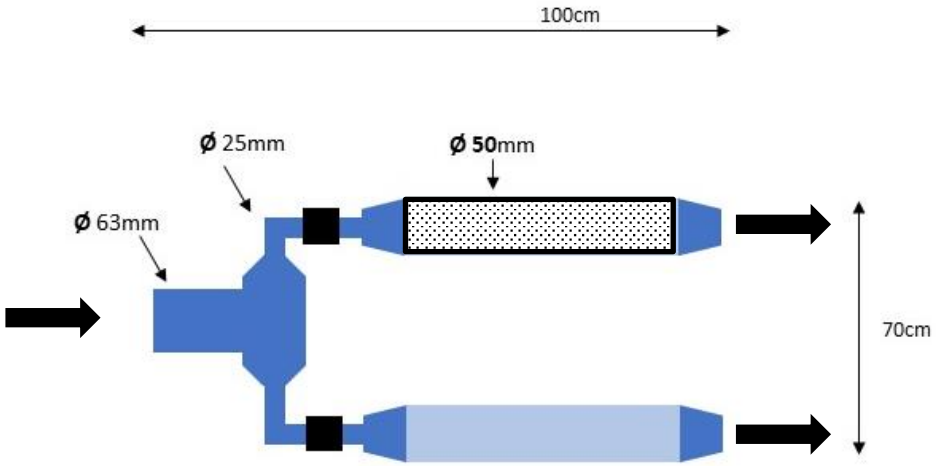


Figure 13. Zeolite filter design, input (left) and 2 outflows (right)

Figure 14. Zeolite filter flow rate measurements

The experiment was carried out in the sturgeon farm of AquaBio in Dottenij (www.aquabio.be) Water was taken directly from the culture system and pumped through the filter. The valve was manipulated to produce a desired flowrate, which was measured using a graduated beaker and a timer. Flow rate was estimated by measuring the time it takes to fill up 1L, follow by these calculations:

$$Flow\ rate\ (L/sec) = \frac{1L}{Time\ to\ fill\ up\ 1L\ (sec)}$$

$$Retention\ time\ (sec) = \frac{Time\ to\ fill\ up\ 1L\ (sec) \times zeolite\ reactor\ volume\ (L)}{1L}$$

The flow rate and associated retention time are shown in Table 12.

Table 12. Different flow rate and retention time used in zeolite experiment

ID	Flow rate (Ls^{-1})	Retention time (s)
1	0.16	36
2	0.0038	155
3	0.0022	273
4	0.0014	456
5	0.0003	1983

For each flow rate, water coming out of the zeolite chamber was sampled by totally filling a glass vial to avoid any air bubble inside. The vial was subsequently capped. A "blank" sample was taken from the water flowing through the empty chamber. All sampling was done in triplicate. The samples were then stored at 4°C and sent for analysis following the protocol described in chapter "3.1.3"

3.5. Section 4: Statistical analysis.

Differences between treatment were analyzed using one-way ANOVA and two-way ANOVA with significant differences assumed at $p < 0.05$ level and post-hoc analysis was performed using Tukey HSD test. Q-Q plot and Levene's-test were used to determine homogeneity of variance. All statistical analysis was conducted using SPSS version 21.

Chapter 4. Results

In this chapter, results of the experiments are displayed in 3 major sections in line with the structure of Chapter 2: Materials and methods. Section 1,2 and 3 explain the results of the pilot experiment, the main selection experiment and the follow-up experiments while Section 4 displays the result of geosmin and 2-MIB removal using zeolite filter.

4.1. Section 1: Pilot experiment.

During the pilot experiment the method for selecting micro-organisms that can degrade geosmin, was verified. Bacterial growth was monitored during the course of the 14 days enrichment with geosmin.

4.1.1. Growth monitoring.

The microbial density during the experiment was monitored by flow cytometry and is shown in Figure 15. Only treatment with sludge inoculum and geosmin are shown.

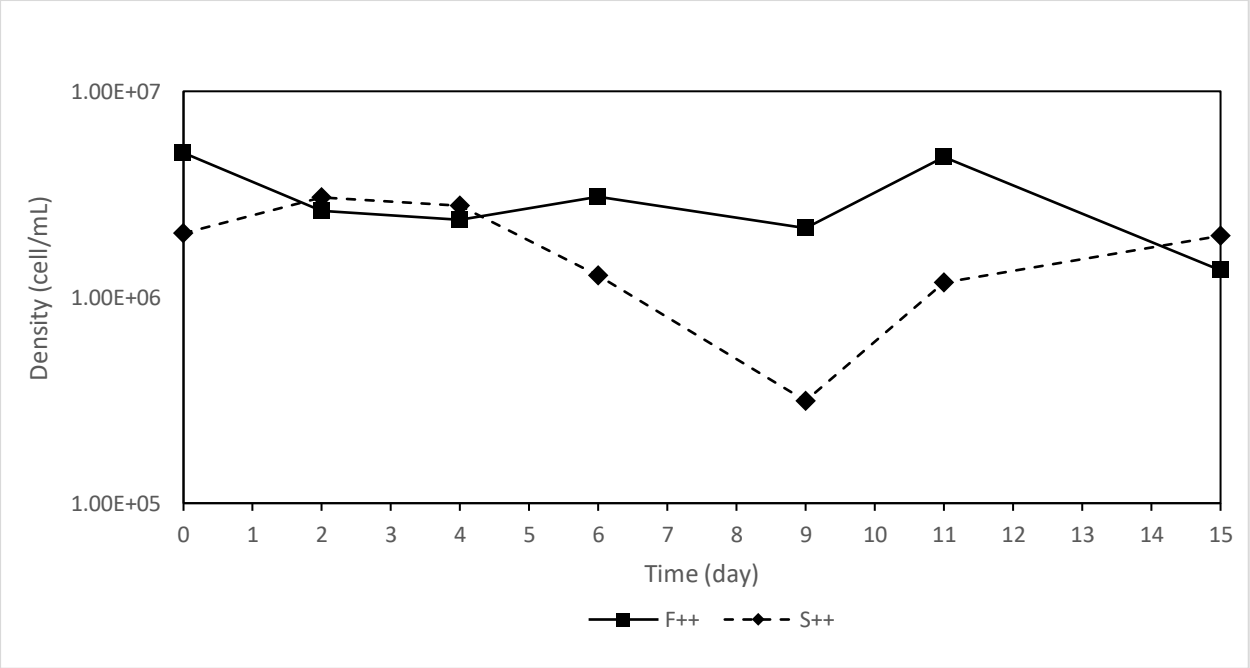


Figure 15. Microbial density in fresh mineral water (F++) and salt mineral water (S++) with geosmine as sole carbon source (33 ng.L^{-1}) monitored by flowcytometry during 2 weeks.

F++: mineral media, fresh water sludge, geosmin; S++ mineral media, seawater sludge, geosmin.

From Figure 15. It is shown that the initial density of fresh water (F++) and sea water (S++) is higher than the intended 10^5 CFU.mL^{-1}. Microbial density is relatively stable throughout the entire experiment duration with the dilution on day 10 seemed ineffective on fresh water microbial density. During the culture period, small particles of flocculant were observed to form inside the both treatment reactors (Figure 16), although unable to be measured, this can be an interesting indicator of microbial activity.

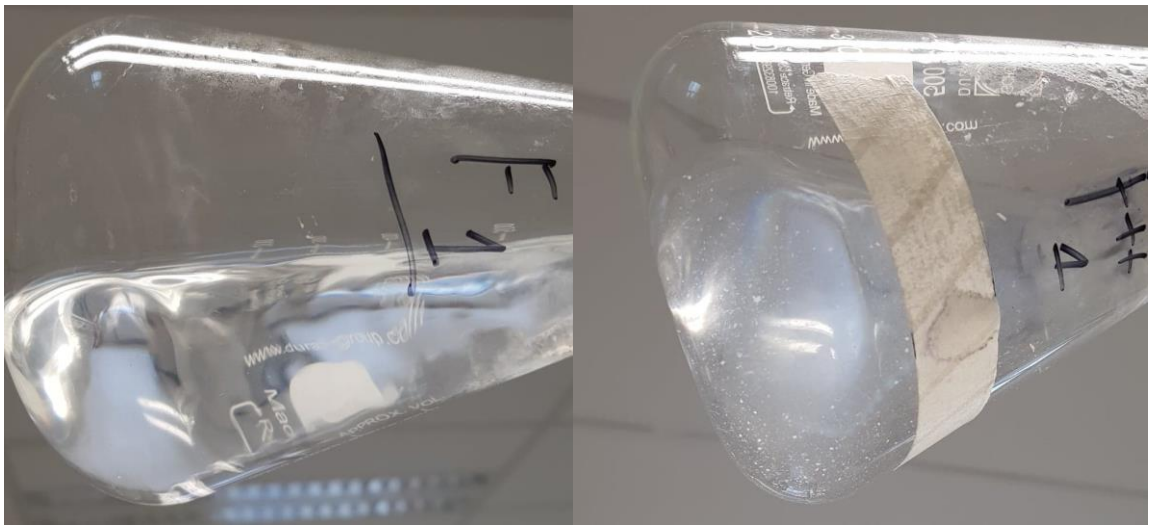


Figure 16. flocculant forming in treatment reactor (right) and no flocculant forming in blank (left)

4.1.2. Geosmin degradation test.

In this experiment, geosmin was spiked into all reactors after the selection period of 15 days, water samples were taken immediately at 0H, 24H and 96H respectively. Sample from each reactor was used to fill up a capped glass vial, avoiding air contact. The vials were then analyzed for geosmin using protocol in chapter 3.1.3.

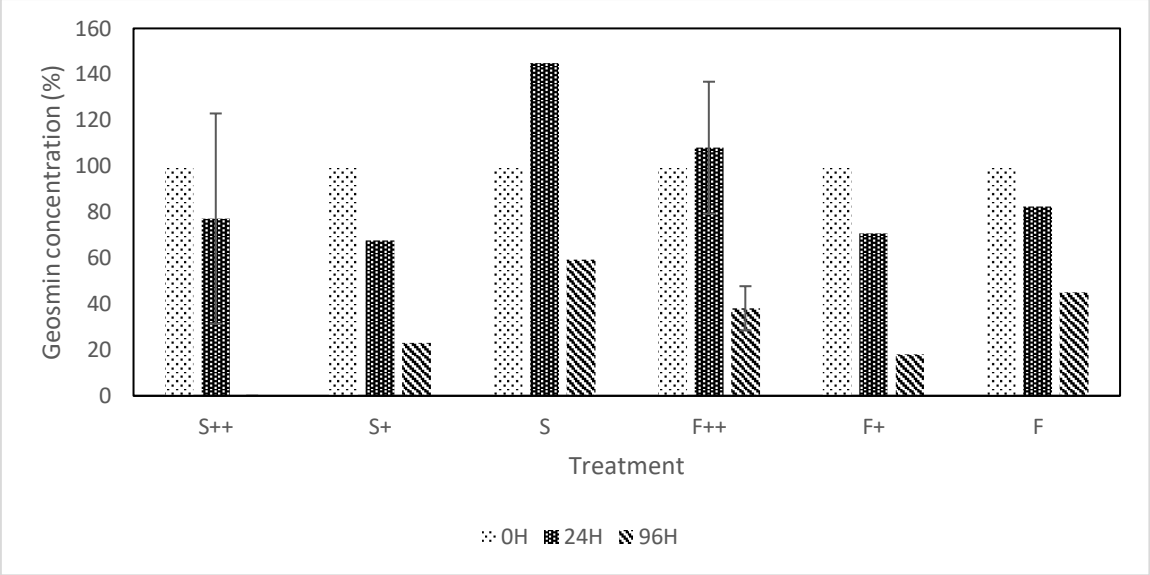


Figure 17. Potential of mixed bacterial population to breakdown geosmin (0.8µg at 0H) after 15 days of growth on selective media.

Presence of standard deviation indicate the average value of 3 technical replicates. Treatment S++ has geosmin concentration below detection level at 96H, therefore is not shown in this graph. F++: mineral medium, fresh water sludge, geosmin; F+: mineral medium, fresh water sludge; F: mineral medium; S++: mineral medium, sea water sludge, geosmin; S+: mineral medium, sea water sludge; S: sea water mineral medium.

Over the course of the experiment, it is observed that geosmin concentration has gradually reduced over time in all treatment, except for the seawater blank. The most prominent reduction was seen in treatment "S++", which has significantly decreased ($p<0.05$) to below detection limit. "F++" treatment also shown significantly reduction ($p<0.05$), but at a smaller magnitude as compared to the seawater samples, reaching 36% compared to the original concentration. Interestingly, geosmin was detected going as high as 144% in sea water blank. As all treatment shown reduction in geosmin between 24 and 96H, the effect of microbial activity on geosmin is not proven.

4.1.3. Testing bacteria growth on different agar media.

After 15 days after first inoculation, micro-organisms were plated on 4 different media: NA, LB, MA and CASO to indicate what media can best be used for isolation of organisms.

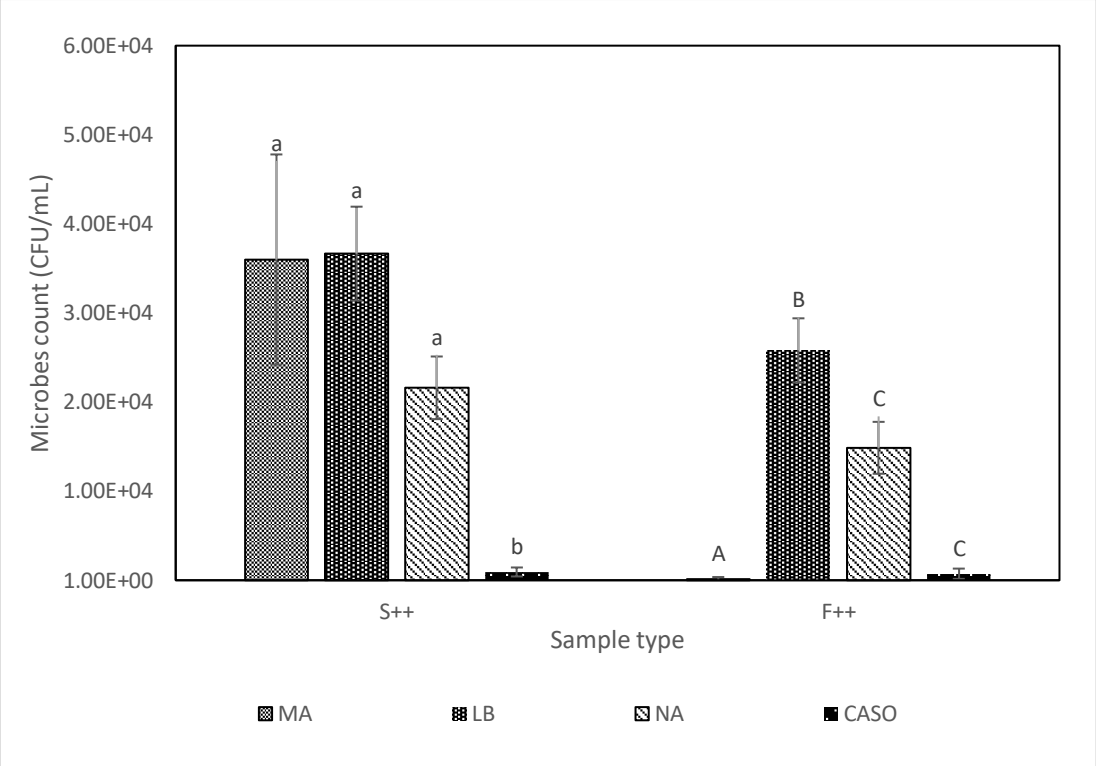


Figure 18. Bacterial count on 4 different agar media after 72H of culture, starting after 15 days of selection.

MA: marine agar, LB: Luria-Bertani, NA: nutrient agar, CASO: tryptic soy agar. Small letter denotes differences in sea water (S++) treatments while capital letter denotes differences in fresh water (F++) treatments. F++: mineral medium, fresh water sludge, geosmin; F+: mineral medium, fresh water sludge; F: mineral medium; S++: mineral medium, sea water sludge, geosmin; S+: mineral medium, sea water sludge; S: sea water mineral medium.

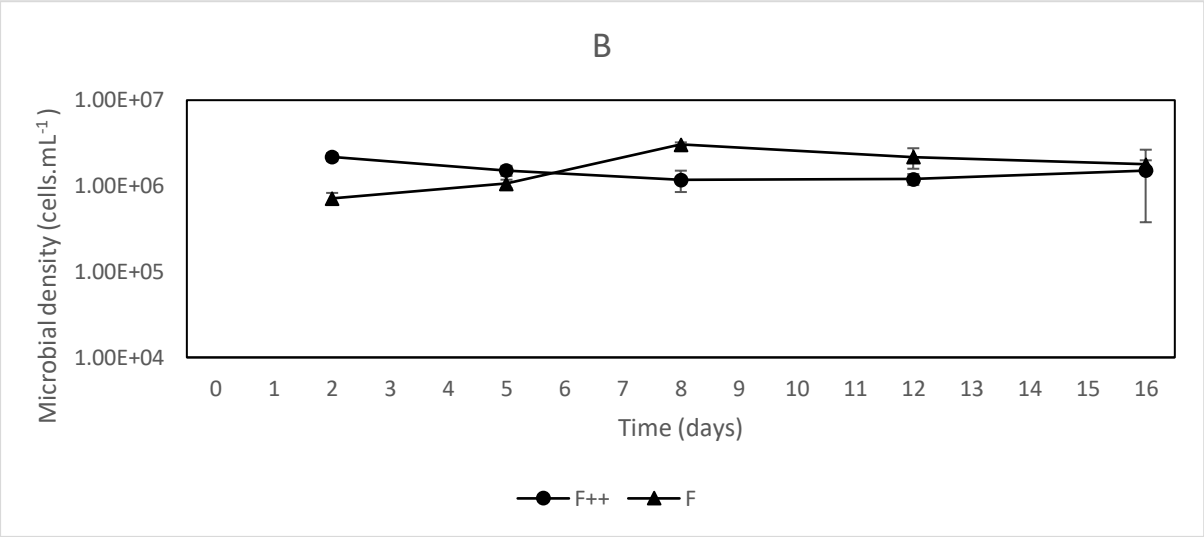
Results from the media test are shown in Figure 18. It is clearly shown that Luria Bertani is a superior media for growing bacteria from water selected on geosmin, exhibiting significantly higher bacteria count in freshwater samples ($p<0.05$), while showing comparable performance with NA and MA in sea water samples. On the other hand, nutrient agar shown good colony forming potential for species selected from seawater biofilter. CASO, while a very popular general-purpose media, shows virtually no microbial growth.

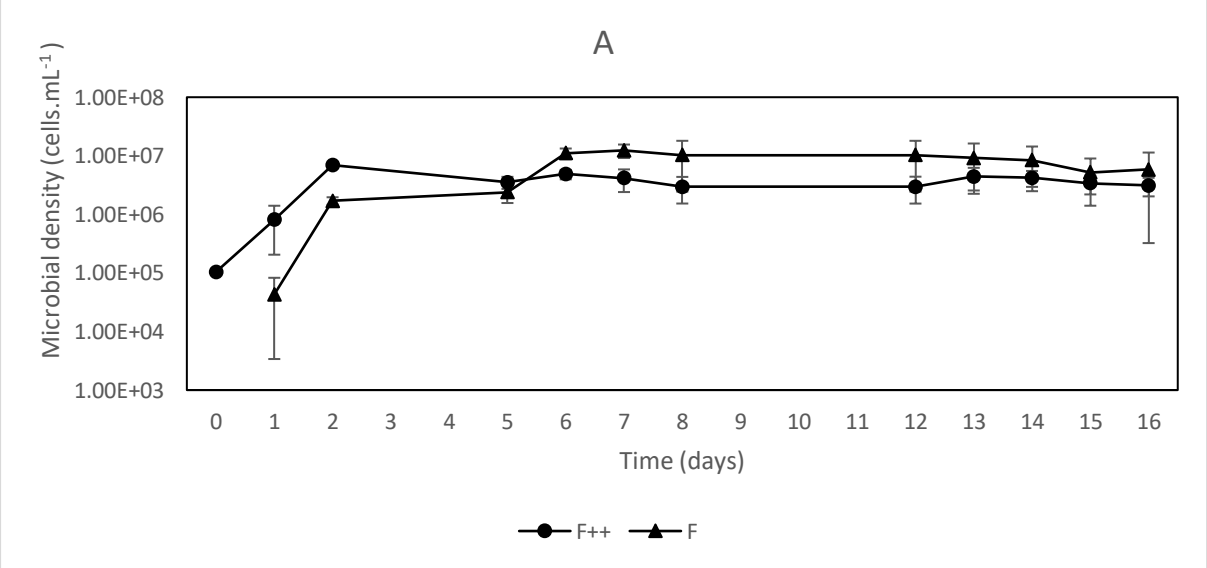
4.2. Section 2: Selection for geosmin-degrading fresh water micro-organism.

In this experiment, geosmin-degrading freshwater micro-organisms were selected using the method verified by the pilot experiment. The fresh water micro-organisms were chosen on the ground of practicality and the fact that off-flavour problem is more severe in fresh water than sea water RAS. The selected micro-

organisms were then isolated, identified and put on several screening test in order to determine their toxicity for *Artemia franciscana* nauplii, the ability to utilize different carbon sources, the ability to metabolize geosmin and finally to degrade geosmin test.

4.2.1. Selection experiment.





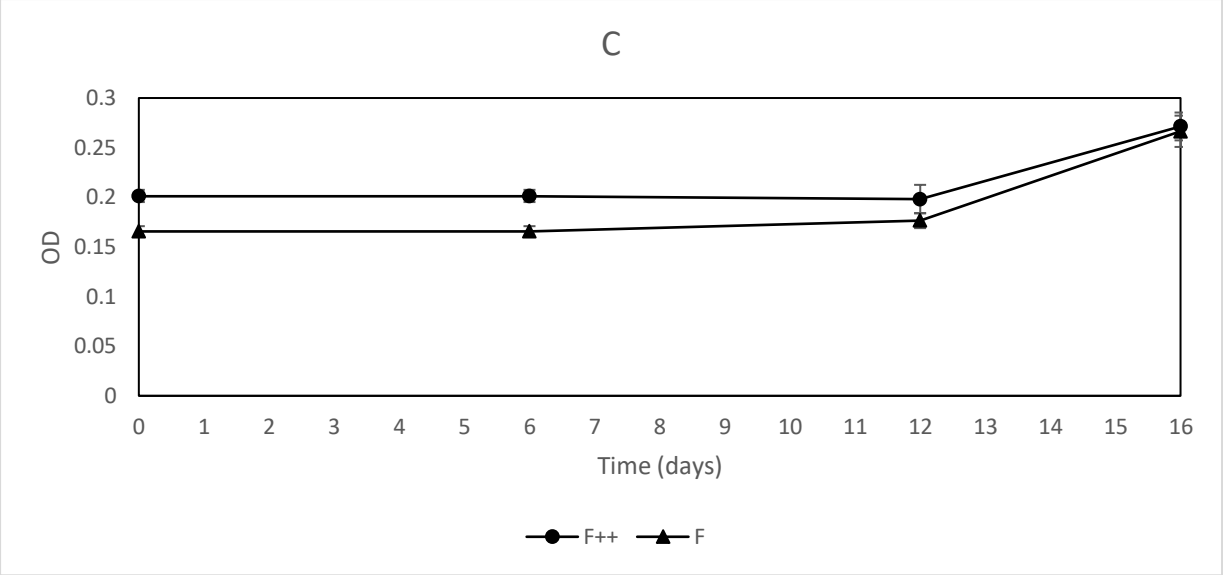


Figure 19. Microbial density of freshwater treatment monitored by flow cytometry (A), plate counting (B) and OD (C), with addition of geosmin (0.8μg) at 6th and 12th day.

Data point which microbial density was too low to be detected were not shown. F++: mineral medium, fresh water sludge, geosmin; F+: mineral medium, fresh water sludge; F: mineral medium; S++: mineral medium, sea water sludge, geosmin; S+: mineral medium, sea water sludge; S: sea water mineral medium

Microbial density of the fresh water treatment reactor (F++) and the control reactor (F) are shown in Figure 19. Overall, it is observed that both methods of monitoring show similar trends, with plate counting displaying 1 log lower than flowcytometry. Optical density (figure 19 C), on the other hand, did not gave much information, showing a slight increase at the 12^{th} day. A contamination in all 3 replicates was detected in the control treatment immediatly after experiment started and increased up to more 10^7 before stabilizing on the 6th day.

Similar with the pilot experiment, flocculant formation was observed in the treatment (F++) reactors but not in the control. Plating also reaveals that there are different types of colonies in F++ samples while only 1 single type of colony formed in F samples.

4.3. Section 3: Applicability of isolated strains.

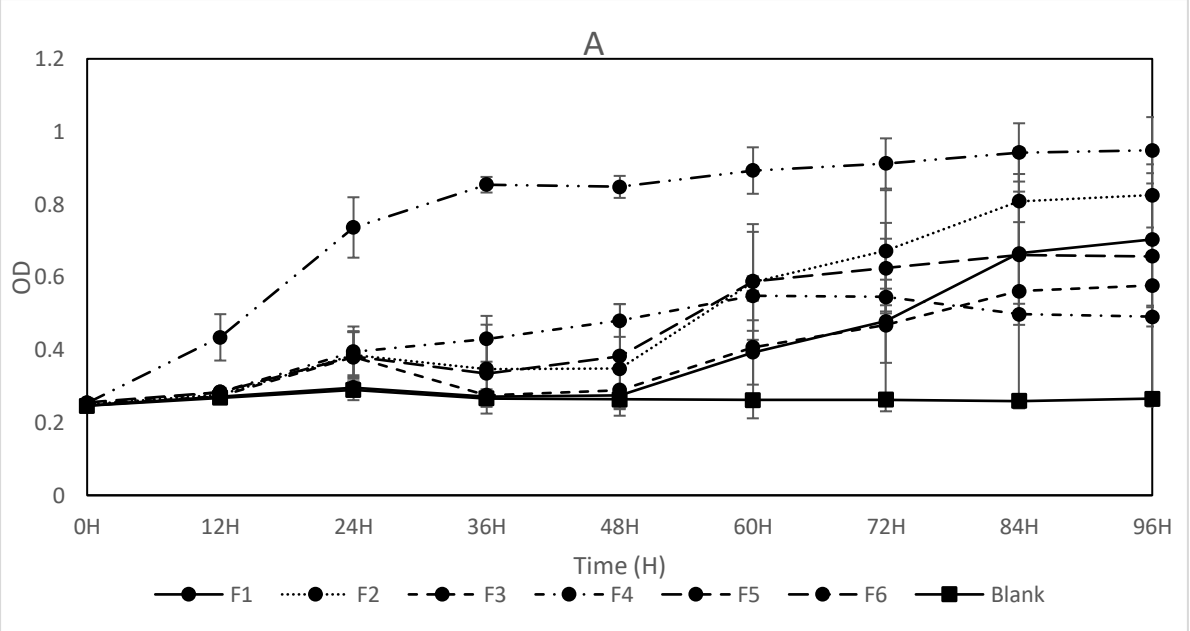
4.3.1. Growth of the selected micro-organism.

From the reactors, 14 strains were isolated by plating the mixture on LB plates, single colonies were picked out based on external morphology. The isolated strains were labeled F1 to F13. During the experiment, contamination occurred with strain

F7, so the strain was again isolated and re-named using the same number but with different letter (F7a and F7b).

Growth curves of all the isolated strains were constructed to estimate the duration it takes for each strain to reach stationary phase, so we were able to harvest the highest possible number of cells to be used in toxicity tests with *Artemia franciscana* nauplii.

The growth curves of fresh water strains are shown in Figure 20. From the curves, we can see that after 96 hours, all the strains have reached stationary phase. Some strains possess particularly fast growth compared to others such as F6, F7b and F11, reaching a plateau after 36 hours. On the other hand, strain F8 grew very slowly, only picking up after 96 hours. It is also observed that some strains have very similar growth pattern such as F7b and F11, indicating that these could be the same strains displaying different morphological differences.



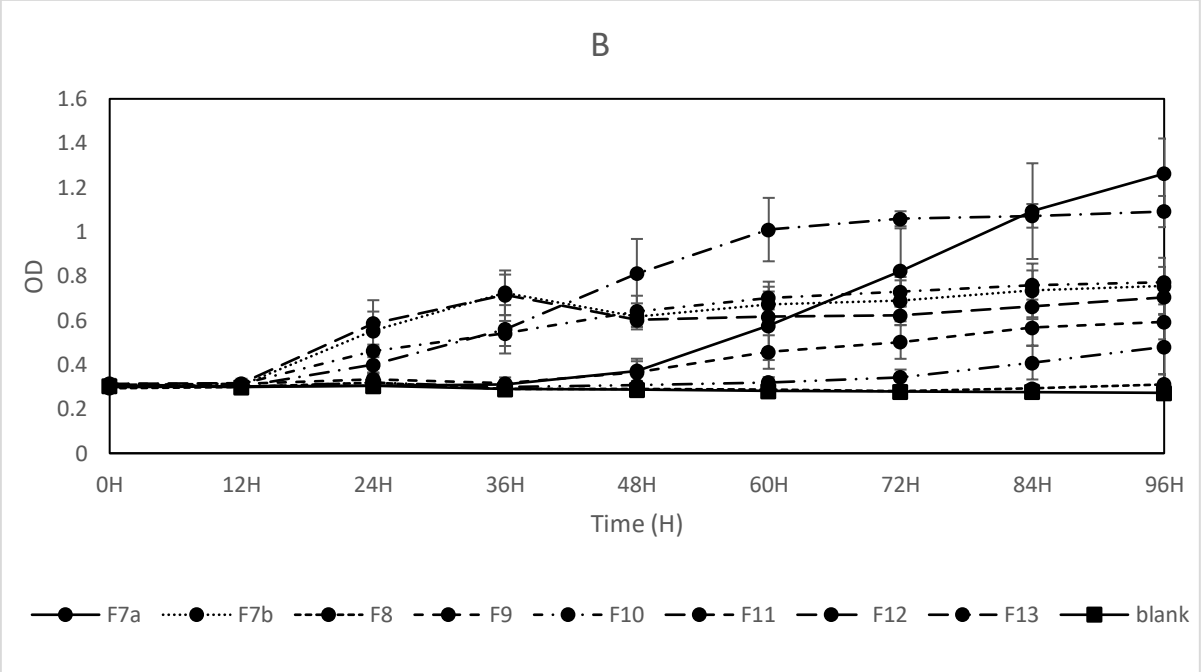


Figure 20. Growth curves in LB of isolated strains. Strain F1 to F6 (A) and Strains F7a to F13 (B)

4.3.2. Preliminary toxicity screen of selected strains using *Artemia.*

Survival rate of *Artemia* nauplii challenged with different isolated strains is shown in Figure 21.

In general, mortality rates varied widely both between different strains and between repeated experiments. Among the strains tested, strains F1, F2, F4, F9

and F10 have consistently shown significant higher survival rate ($p<0.05$) throughout 3 experiment replicates. Therefore, they were selected for further testing.

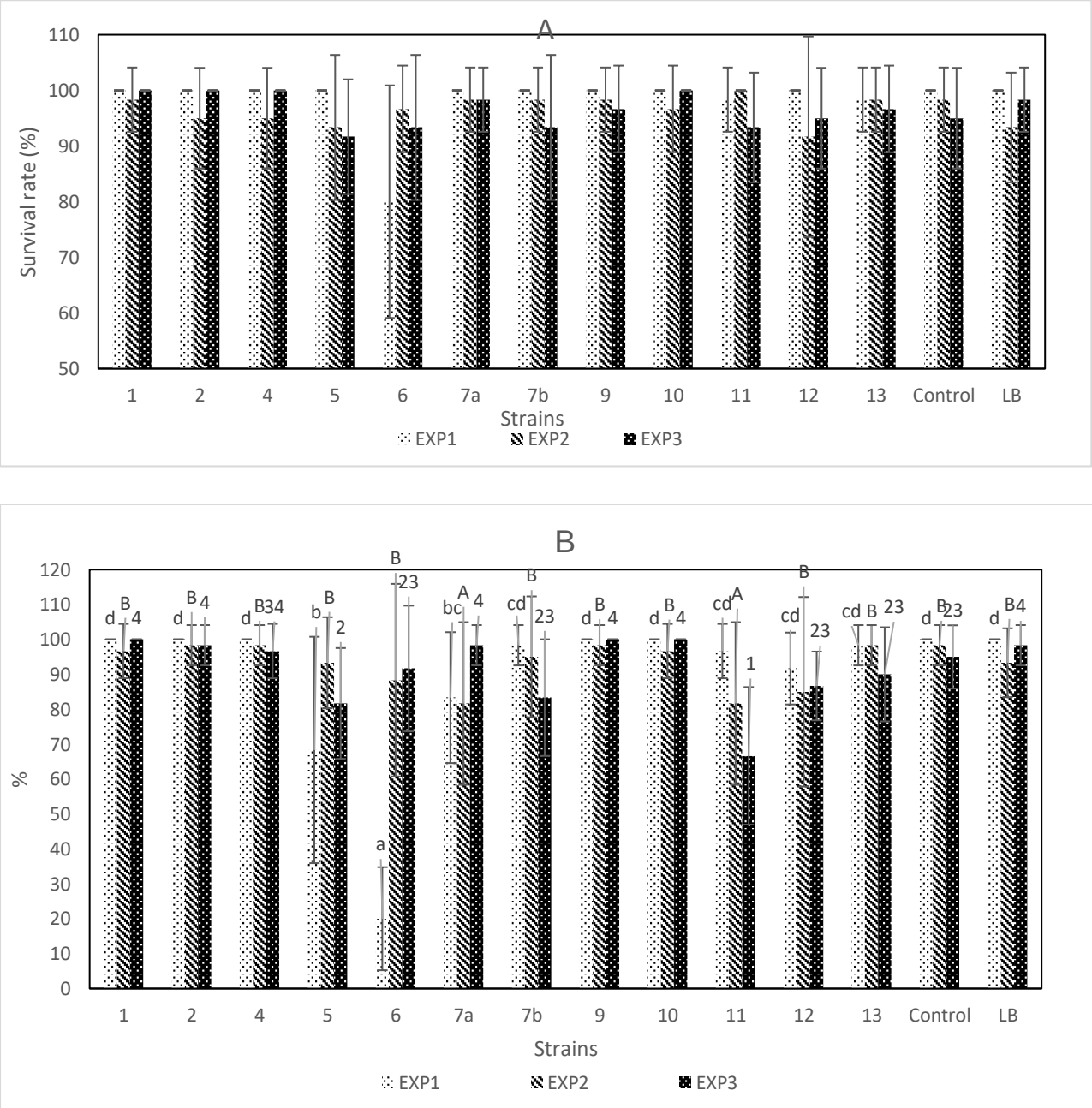


Figure 21. Survival rate of *Artemia franciscana* nauplii challenged with isolated microbiastrains after 24 hours (A) and 48 hours (B) (n=3).

Strains F3 and F8 is unable to be regrown from cryostorage so thereforediscarded. Small letters denotes difference in experiment 1, capital letters denotes differences in experiment 2, numbers denotes differences in experiment 3.

4.3.3. Identification of isolated strains.

Result from 16S identification from different selected strains are shown in Table 13. The 16S rRNA sequence of each strains can be found in ANNEX IV

Table 13. Identification result of the selected 5 bacterial strains.

No	Strain	Genus
1	F1	*Bosea* sp.
2	F2	*Bosea* sp.
3	F4	*Mycobacterium* sp.
4	F9	*Roseomonas* sp.
5	F10	*Brevundimonas* sp.

Figure 22. 5 isolated strains cultured in LB broth. From left to right: *Bosea* sp. F1; *Bosea* sp. F2; *Mycobacterium* sp.; *Roseomonas* sp. and *Brevundimonas* sp.

4.3.4. BIOLOG™ assay.

Result from BIOLOG is shown in table 14.

Table 14. BIOLOG™ result of 5 isolated and identified strains

Strain	Genus	Carbon source utilized
F1	*Bosea* sp.	▪ Pyruvic Acid Methyl Ester ▪ L-Asparagine ▪ L-Serine ▪ γ-Amino Butyric Acid ▪ Glycyl-L Glutamic Acid
F2	*Bosea* sp.	▪ L-Arginine ▪ Pyruvic Acid Methyl Ester

		▪ D Galacturonic Acid ▪ L-Asparagine ▪ L-Serine ▪ L-Threonine ▪ Glycyl-L Glutamic Acid ▪ α-Keto Butyric Acid ▪ D-Malic Acid
F4	*Mycobacterium* sp.	▪ L-Arginine ▪ Pyruvic Acid Methyl Ester ▪ Tween 40 ▪ i-Erythritol ▪ Tween 80 ▪ D-Mannitol ▪ α-Keto Butyric Acid ▪ Putrescine
F9	*Roseomonas* sp.	▪ D-Galactonic Acid γ-Lactone ▪ Pyruvic Acid Methyl Ester ▪ D-Xylose ▪ D Galacturonic Acid ▪ D-Mannitol ▪ 4-Hydroxy Benzoic Acid ▪ α-Amino Butyric Acid ▪ Glycyl-L Glutamic Acid ▪ α-Keto Butyric Acid
F10	*Brevundimonas* sp.	▪ γ-Amino Butyric Acid ▪ Glycyl-L Glutamic Acid ▪ α-Keto Butyric Acid

Identification and subsequence BIOLOG assay have yield some interesting results. Overall, we identified 5 different strains of bacteria, with each strain utilizing a different carbon source. There are similarities between strains in term of carbon source utilization. 4 in 5 strains can metabolize α-Keto Butyric Acid with strain *Bosea* sp. F1 being the exception. Glycyl-L Glutamic Acid is also a common carbon source for all except for *Mycobacterium* sp. Interestingly, strains F1 and F2 are

identified to be from the same genus of *Bosea* sp.. However, BIOLOG™ assay has shown that they are able to metabolize different Carbon source, with strain F2 being more flexible in carbon utilization.

4.3.5. MTT Formazan Assay.

The result from MTT-Formazan assay is shown in Figure 23 and 24

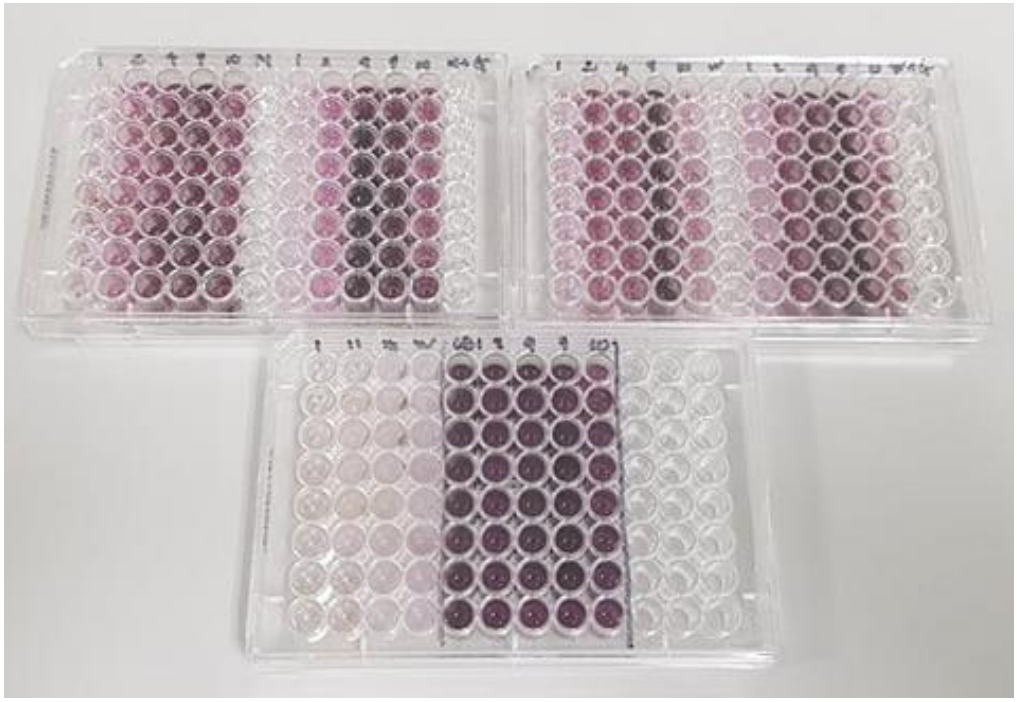
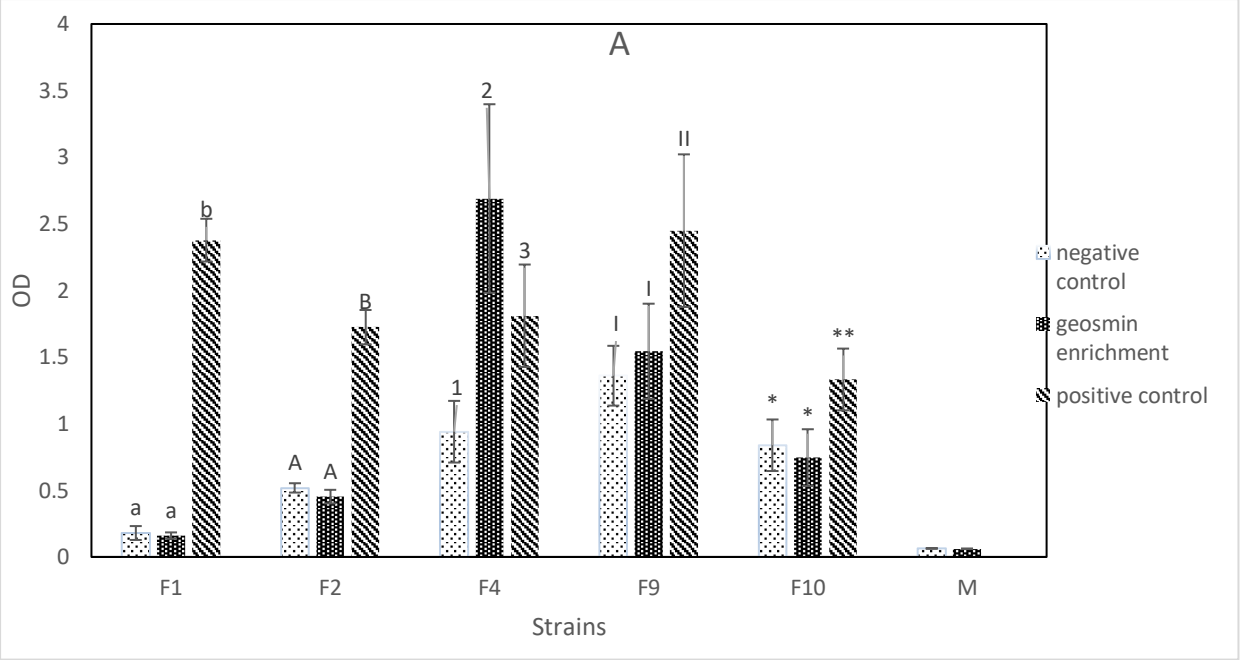


Figure 23. MTT formazan results of 2 different treatments: cells suspended in mineral media (upper left), cells suspended in distilled water (upper right) and positive controls (lower).

Strains were loaded from left to right: *Bosea*sp. F1; *Bosea* sp. F2; *Mycobacterium* sp. F4, *Roseomonas* sp. F9 and *Brevundimonas* sp. F10



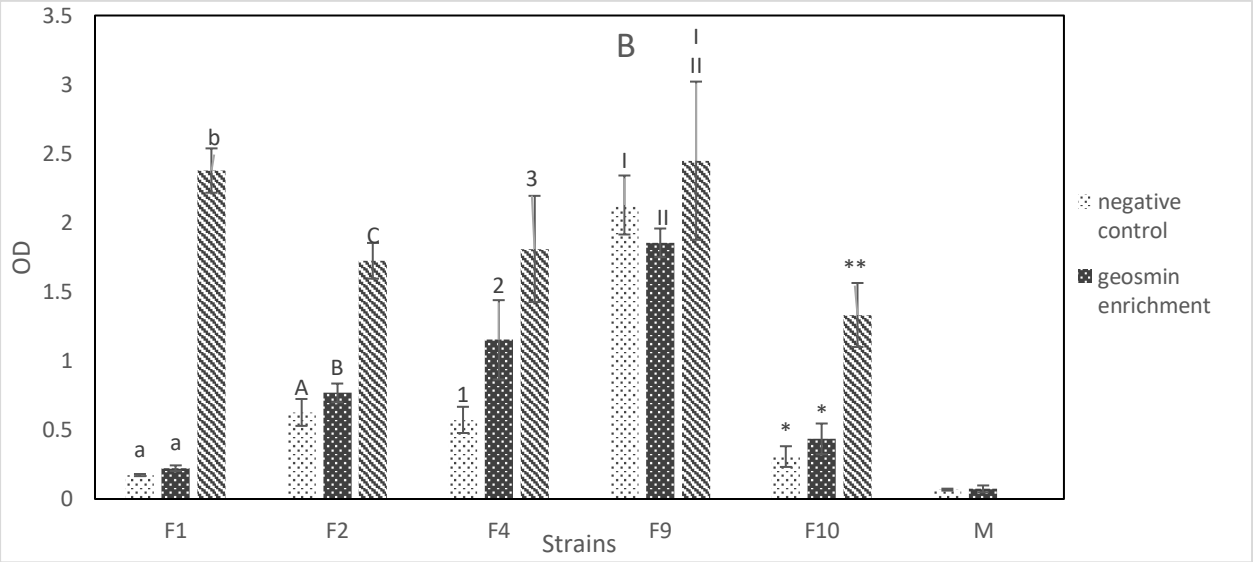


Figure 24. Optical density at 570nm of treatment with cells suspended in 2 different media: mineral medium (A) and distilled water (B).

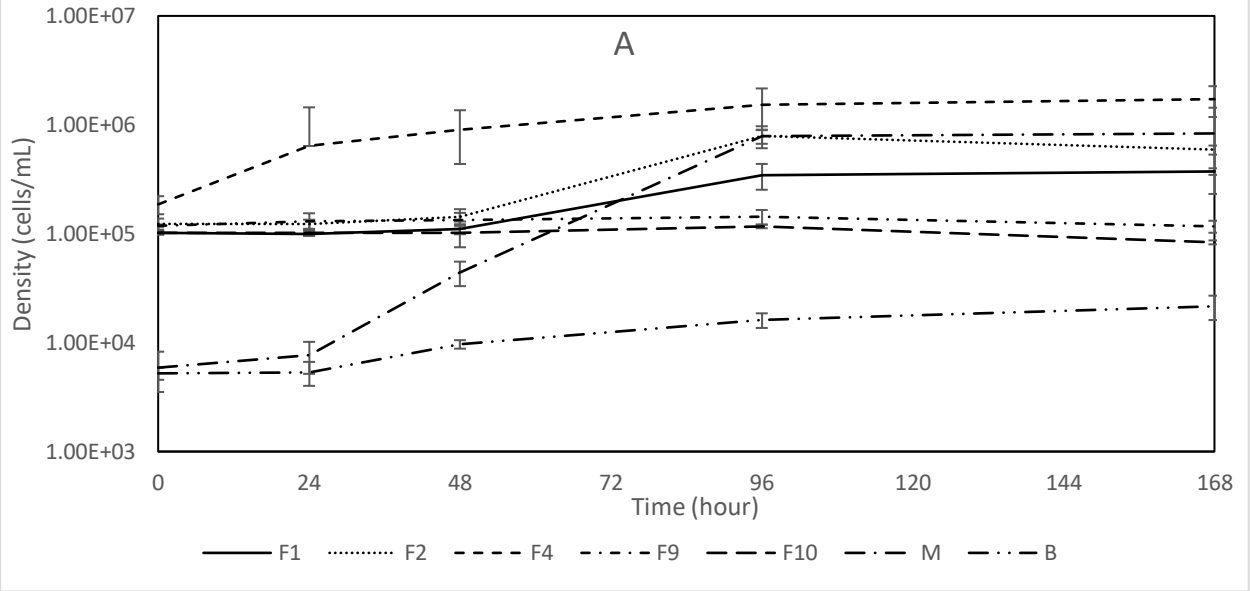
Negative control: bacteria cells suspended in mineral media or distiled water, Positive control: bacterial cells suspended in LB media. F1: Bosea sp., F2 Bosea sp., F4: Mycobacterium sp., F9: Roseomonas sp., F10: Brevundimonas sp., M: Media. Differences in growth on the different media for each strain are denoted by different small letters

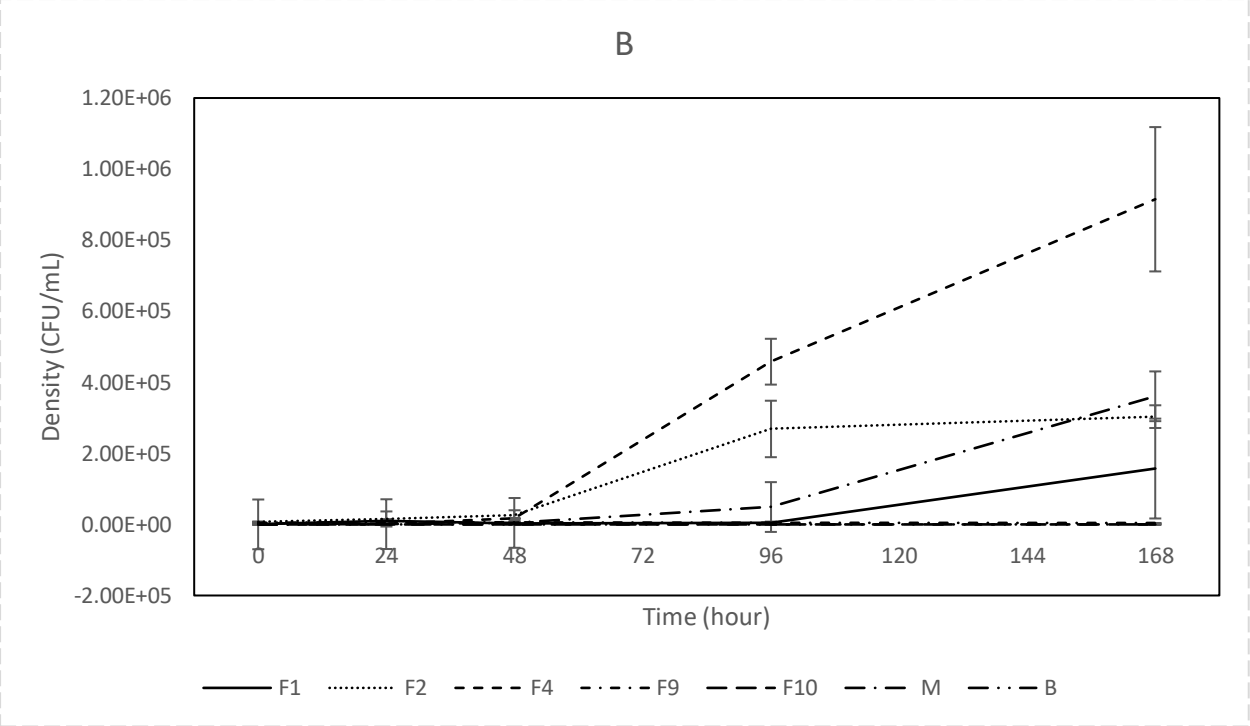
It is clearly shown that all strains show a significantly higher metabolic activity when grown in LB media ($p<0.05$) compared to the negative control and compared to geosmin enriched media with the exception for F4. Strain F1 (*Bosea* sp.) and F10 (*Brevundimonas* sp.) show no significant metabolic change in the precence of geosmin. Strain F4 (*Mycobacterium* sp.) shown significantly higher metabolic

activity with geosmin in both mineral media and distilled water and strain F2 (*Bosea* sp.) shown significant higher geosmin metabolism in distiled water but not in mineral media. Noted that strain F9 (*Roseomonas* sp.), while shown significantly lower metabolic activity with geosmin the distiled water ($p<0.05$), displayed no significant differences in mineral media.

4.3.6. Growth and geosmin degradation activity of isolated strains.

In this experiment, the 5 strains, 1 mixture of 5 strains and 1 blank control were prepared in triplicates using 21 sterilized capped glass vials, containing 10mL mineral media, 0.033µg.L^{-1} geosmin and 10^5 cells. mL^{-1} single strain micro-organism. The mixture treatment was prepared by mixing together 10^1 from each strain. The reactors were then incubated at 28°C on a rotator. Sampling were taken initially, at 24H, 48H, 96H and 144H respectively using sterile syringes and needles. Cell density in the samples was then analyzed using flow cytometer, Tecan reader and plating on LB agar plates.





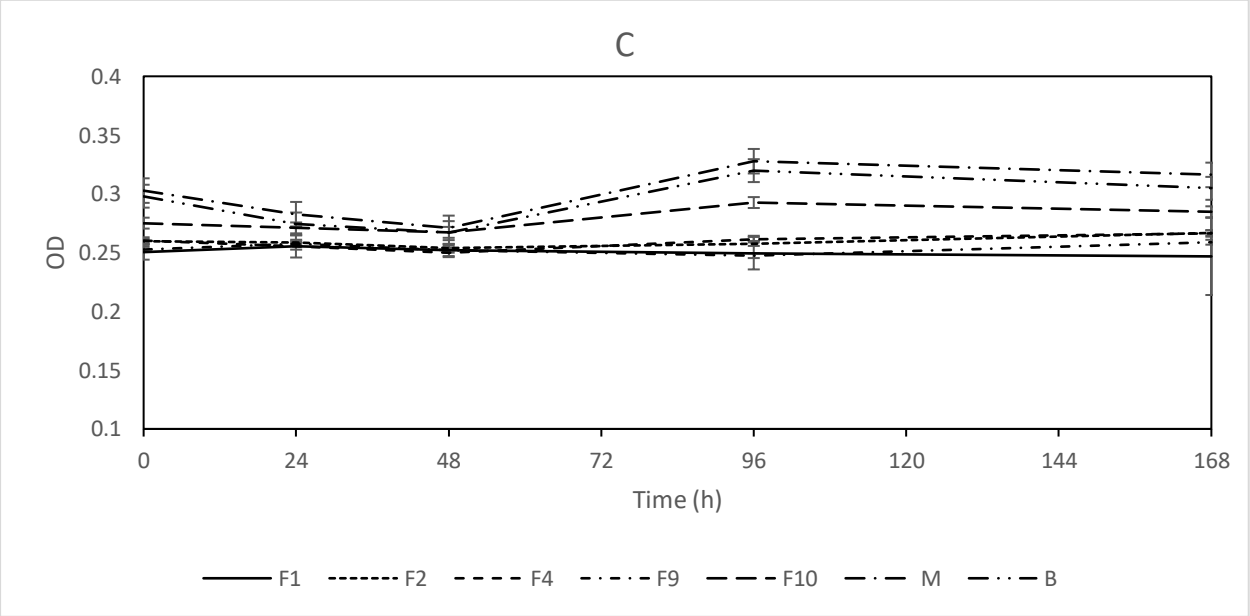


Figure 25. Microbial density of different single strain monitored by flow cytometry (A), plate counting (B) and OD (C).

F1: Bosea sp., F2 Bosea sp., F4: Mycobacterium sp., F9: Roseomonas sp., F10: Brevundimonas sp., M: Mixture of 5 strains, B: blank

5 bacteria strains that shown low toxicity toward *Artemia* were chosen for culturing in media spiked with geosmin. Growth of the 5 strains were monitored by flow cytometry, plate counting and OD and is shown in figure 25. All strain displays slow growth compared to the growth curves (figure 20) in LB; reaching stationary

phase only after 96 hours. Strain *Mycobacterium* sp. (F4) having slighly higher initial density shows best growth performance, reaching approximately $1.5\pm0.6*10^6$ cells.mL^{-1}. Strain *Bosea* sp. (F2) and treatment containing the mixed community show poorer performance, reaching stationary phase only at $7.9\pm1.8*10^5$ and $7.8\pm1.1*10^5$ cells.mL^{-1} respectively. Noted that the mixture being inoculated 1 log less than other treatment. Strains *Roseomonas* sp. (F9) and *Brevundimonas* sp. (F10) shown no growth while strain *Bosea* sp. (F1), being from the same genus with strain *Bosea* sp. (F2) shown significantly lower growth, reaching only $3.5\pm0.9*10^5$ at stationary phase. Contaminations was detected in the blank by flow cytometry, however this was not picked up by plate counting.

Growth curves monitored by plate counting show similar trends compared to growth measured by flow cytometry. Comparable to the previous results, strain F4 shows the best growth, reaching $4.5\pm0.6*10^5$after 96 hours followed by *Bosea* sp. (F2) and the mixture of 5 strains, reaching $2.6\pm0.8*10^5$ and $4.8\pm1.3*10^4$ cells.mL^{-1} respectively. Noted that the colonies formed in the mixture plates is dominated by F4 type. Strains *Roseomonas* sp. (F9) and *Brevundimonas* sp. (F10), in accordance with flow cytometry results, shown no growth. It is noted that plate counting method cannot detect growth in the first 48 hours, even when initial inoculation is 10^5 cells.mL^{-1}. Viable count result are nearly always 10 times lower that result given by flow cytometry.

A small ammount of flocculant was observed in F4 treatment but was not present in any other treaments.

As opposed to flow cytometry and plate counting method, optical density has yielded no result regarding the growth of the bacteria, showing no significant differences.

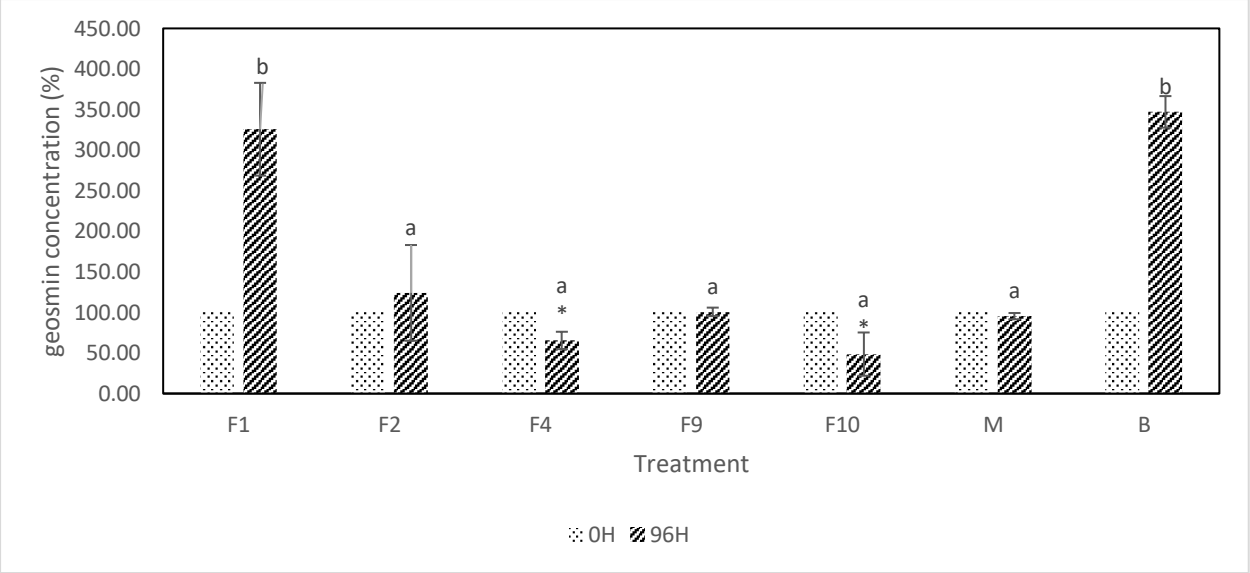


Figure 26. Changes in geosmin concentration over 96 hours (n=3).

F1: Bosea sp., F2 Bosea sp., F4: Mycobacterium sp., F9: Roseomonas sp., F10: Brevundimonas sp., M: Mixture of 5 strains, B: blank. Letters denotes significant difference between strains, "*" symbol denotes significant differences in geosmine concentration for 1 strain.

Results from geosmin degradation test show that among the 5 selected strains, only F4 (*Mycobacterium* sp.) and F10 (*Brevundimonas* sp.) show a significant reduction ($p<0.05$) in geosmin to 65.45±10.70 and 48.60±26.67, respectively. Strains F9 (*Roseomonas* sp.) and the Mixed population shown no reduction while, curiously strains F1 (*Bosea* sp.) and the Blank show significant increase in geosmin concentration as much as 350 %

4.4. Section 4: Evaluation of geosmin and 2-mib removal capacity of zeolite filter using different flow rate.

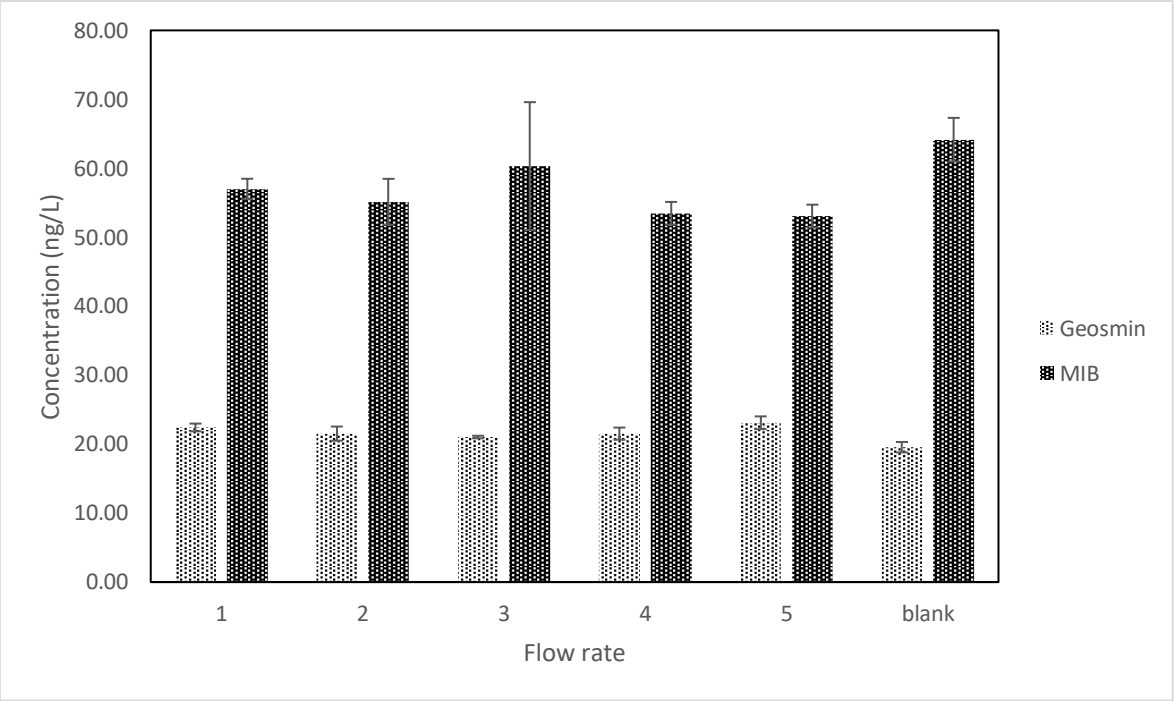


Figure 27. Geosmin and 2-MIB levels after zeolite filter at different flow rate.

Surprisingly, the zeolite filter was unable to remove either geosmin or 2-MIB, showing no significant different between the 5 different flow rate and the blank.

Chapter 5. Discussion

In this chapter, the results of the experiments are discussed in 3 major sections. This is done in accordance with the ordering of the Material and Methods and Result chapter. An additional section, translating findings of this study to application in aquaculture, is added to this chapter.

Section 1 discusses the practicalities of selecting geosmin degrading bacteria via the pilot experiment.

Section 2 discusses the selection of geosmin-degrading bacteria from fresh water RAS sludge.

Section 3 discusses the isolation and different tests on the isolated strains to evaluate: toxicity on *Artemia* nauplii, metabolic activities and growth and geosmin degradation test.

Section 4 discusses the performance of a zeolite filter to eliminate geosmin and 2-mib from cultivation water.

Section 5 elaborates on the possible application of the findings of this study.

5.1. Section 1: Pilot experiment.

Selecting microbes to degrade a substance by using it as the only carbon source has been used successfully in many instances such as pesticides (Aislabie & Lloyd-Jones, 1995; Chaudhry & Ali, 1988; Singh & Walker, 2006) and the hardly degradable lignin (López *et al.,* 2004). Recently, scientists have been fairly successful in isolating microbes that degrade geosmin, both in aerobic and anaerobic condition (Azaria & van Rijn, 2018; Guttman & van Rijn, 2012; Hoefel *et al.,* 2009).

Results from microbial density monitoring show that flow cytometry is effective in measuring density fluctuation. As shown in Figure 15, microbial density stabilized post-inoculation around the initial value of 10^5 cells.mL^{-1} in freshwater. Sea water treatment, however shows a continuous decrease of density until the dilution on the 10th day These observations prove that the selection process was happening. Strains that are unable to adapt to geosmin were eliminated while strains that can utilize geosmin to growth or maintain density remains. This observation is shared with Guttman & van Rijn (2012), on whose protocol this study is based. Furthermore, the presence of flocculant (Figure 16) in the treatment of both sea water and fresh water further indicate that micro-organism growth was taking place. Using the remaining microbes after the selection period, we were able to reduce geosmin level in some cases to beyond detection level (Figure 17). Furthermore, the treatments with sludge inoculum shows faster reduction of geosmin. Geosmin reduction was observed however in all the treatment, except the seawater control. This can be explained by the fact that geosmin is a very volatile substance and loss via evaporation is expected.

The results from this experiment show that the selection procedure was successful displaying stable microbial density after geosmin spiking, furthermore, there was a formation of flocculant in the reactor with micro-organism. It further implies the presence of geosmin-degrading micro-organism in the ARC's RAS system. The results also mean that this protocol adapted from Guttman & van Rijn (2012) is feasible in our condition. However, geosmin degradation test was inconclusive as all the treatment shown decrease in geosmin levels

5.1.1. Flow cytometry measurement.

Flow cytometry has seen applications in various fields of research, including bacteria enumeration (Gunasekera *et al.,* 2000; Van Nevel *et al.,* 2013). During the pilot experiment, results have shown that flow cytometry is a robust method to estimate microbial density in water sample. However, some limitations have been identified. Staining by Thiazole Orange (TO) and Propidium Iodide (PI) varied between different strains (ANNEX II), requiring case by case gating. Furthermore, accurate estimation can only be achieved if the bacteria density is within the range of 10^5 to 10^7 cells.mL^{-1} (ANNEX III). This may explain the inability to detect density reduction of fresh water microbes after spiking in the pilot experiment, since concentrations fell below 10^5 cells.mL^{-1}.

Observations during the experiment show the presence of flocculant in the reactors spiked with geosmin (figure 16). As flow cytometry require a homogenous sample to work accurately (Falcioni et al., 2006), flocculants can cause error in reading. In order to better visualize and quantify this phenomenon, measurement of optical density of samples was needed. Measurement via optical density at 550nm has been proposed as an effective method for flocculant quantification (Kurane et al., 1986; Toeda & Kurane, 1991; Yokoi et al., 1996). Furthermore, as flow cytometry is prone to errors when measuring unhomogenized samples, plate counting was also included as a third method.

5.1.2. Testing bacteria growth on different agar media.

Results from the media test show that LB is clearly more suitable for growing bacteria from the selection experiment as it provided significantly higher colony counts and higher variation in micro-organism. LB has been the media of choice in many selections for microbes degrading various substances such as trichloroethylene (Shields & Reagin, 1992), petroleum (Ueno *et al.,* 2006) and geosmin (Guttman & van Rijn, 2012). Marine agar, although showing similar performance as LB for marine microbes, lags significantly in the case of freshwater microbes. This can be explained by the fact that the osmotic stress is simply too much for some fresh water bacteria to grow, regardless of the nutrient provided. Interestingly, CASO, which is a very popular general-purpose media showed little growth for both sea water and freshwater inoculum. The poor performance of CASO media can be due to several reason such as the very slow growth rate of

the microbes and the unsuitability of the nutrients provided. Overall, LB media was chosen by the virtue of its performance as well as to simplify logistics

5.2. Section 2: Selection for geosmin-degrading freshwater microbes

5.2.1. Selection experiment.

Based on Figure 19A, we can see that bacterial density increased immediately post innoculation, reaching 2 log increment after 48H. This proves that the selection process is working. The stabilization after 2 days coincides with the formation of flocculants which further indicates microbial growth. Figure 19B also shows similar trends to Figure 24A, however, only asmall increment of bacteria density was observed immediately post-spiking of geosmin. This trend was picked up by flow cytometry but undetectable via plate counting. Furthermore, plate counting, while producing a similar trend as with flow cytometry, always gives results at least 1 log lower. This phenomenon is also observed by Hoefel et al. (2003) whose results show that flow cytometry reading is 2 to 4-log higher than viable count. As flow cytometry relies on homogenized samples and enumerates cells individually, while plate counting may count clumping cells as a single colony, this difference in reading is understandable. Furthermore, colony forming is an energy demanding process and will not be carried out by the bacteria when in less than optimum shape. Overall, all 3 measurements enforced each other and indicated that there was microbial growth in the selecting reactors. However, flowcytometry is shown to give the most detail.

A contamination was detected by both flow cytometry and plate counting, reaching densities equivalent to the treatment. However, there were some fundamental differences: There were no flocculants forming in the blank reactors, even though the microbial density is comparable to the treatment. Furthermore, plating also revealed differences in microbial community as the sample from the treatment reactor yielded several different types of colonies while the blank samples only yielded 1 single type of colony.

This result is shared by Guttman & van Rijn (2012) and is possibly the result of lack of nutrients from the mineral media as well as oxygen limitation. Replacing the mineral media with LB was an option as LB spiked with geosmin provided at least 4 times faster bacteria growth (Guttman & van Rijn, 2012). However, doing this comes with a risk of selecting non-related bacteria, as stated by the same

author. Oxygen limitation can be eliminated by aeration but as geosmin is a very volatile substance (Buttery *et al.,* 1976; Gerber & Lechevalier, 1965; Sunesson et al., 1995), aeration may cause non-bio mediated decrease in geosmin as was observed by Guttman & van Rijn (2012). In our experiment, loss via evaporation was prevented by using air tight reactors, with large headspace to provide enough oxygen without aeration.

5.3. Section 3: Applicability of isolated strains.

5.3.1. Isolation and growth of geosmin degrading bacteria in LB media.

The mixture of selected bacteria was plated on LB and the different strains were selected based on external colony morphology. Especially color, growth speed (size of colony) and colony surface pattern was used as parameters for characterization. The selected strains were grown in LB as this medium was proved to the be most optimal (see 4.1.3). A growth curve for each strain was constructed with the aim of determining the time taken for each strain to reach stationary phase, in preparation for the toxicity screen. Overall, the tested strains show a typical exponential growth curve with stationary phase achieved at different time points and different optical densities. Growth time of different strains also varied, with most strains requiring at least 60 hours to reach stationary phase. As this is a part of the screening process, standard curves were not needed. The logic behind this is that the density obtained growing in LB would be the highest possible density for each strain, highly unlikely to be achieved in normal RAS circumstances – as observed in the selection experiment.

5.3.2. Toxicity screen on *Artemia*.

This experiment is carried out to eliminate the toxic strains and select the non-toxic ones that could be useful for application in an aquaculture context. The logic behind this is that maximum growth was obtained on LB medium and *Artemia nauplii* were exposed to these high densities of bacteria. If the strain fails to kill *Artemia*, which is LB broth and sterile seawater, we can be assured that it is non-toxic for other aquatic organisms.

Artemia has been used as the subject organism for toxicity study for a long time (Kiviranta et al., 1991; Lewan et al., 1992; Marques et al., 2006) mainly due to the convenience of its easy hatching from dry cysts and year round availability (Sorgeloos et al., 1978). The use of pure bacteria culture in challenge test is due

to practicality reason, as this is a preliminary screening, analyzing and extracting toxins from those strains means unnecessary complications. Furthermore, using bacterial culture make it easier to repeat the screening process. In the toxicity screen, strains F1, F2, F4, F9 and F10 were selected as they showed significant higher or comparable with the control treatment. Strain 13 while giving the same positive result, could not be regrown after cryo-storage and therefore was discarded.

The results show that survival rate of *Artemia nauplii* exposed to different isolated strains varied both between strains and between replicate experiments. There was no significant mortality at 24 hours post exposure, while mortality increased dramatically for some strains after 48 hours. This result indicates that these strains do not possess high toxicity as oppose to other strains such as the cyanobacteria *Oscillatoria agardhii* and the bacteria *Vibrio harveyii*, which cause mortality even after 24 hours (Kiviranta & Abdel-Hameed, 1994; Kiviranta et al., 1991; Lee et al., 1999; Soto-Rodriguez et al., 2003). However, it must be noted that *Artemia* is a hardy animal and its sensitivity decreases as it grow (Sorgeloos et al., 1978). Furthermore, most study on toxicity done with artemia as a test subject use pure toxin as the treatment. As pure toxin potency and dosage is much higher than live bacteria, along with the fact that bacteria only produce toxin in some conditions, further in-depth test should be carried out to give definite confirmation of these strains' toxicity. Apart from artemia, different animals have been used as model organism for toxicity screening; such as rotifer (Juchelka & Snell, 1994; Preston & Snell, 2001; Snell & Janssen, 1995), *Daphnia* (Buikema et al., 1980; Nizan et al., 1986) and zebrafish (Parng, 2005; Sipes et al., 2011).

5.3.3. Identification.

Results from 16S sequencing show that the isolated bacteria strains belong to 4 different genera, with strain 1 and 2 belonging to the same genus *Bosea* sp., while strains 4,9,10 are identified to be *Mycobacterium* sp., *Roseomonas* sp. and *Brevundimonas* sp. respectively. These strains have not been listed in previous studies (Azaria & van Rijn, 2018; Guttman & van Rijn, 2012; Lindholm-Lehto & Vielma, 2019).

Bosea is a genus of recently discovered and scarcely studied gram-negative bacteria from the family *Bradyrhizobiaceae* (Marcondes de Souza et al., 2014).

Bosea strains are isolated from various water and waste water sources (Das et al., 1996; La Scola et al., 2003; Thomas et al., 2007) as well as from soil, they are also isolated in symbiotic relationship with legumes (Das et al., 1996; De Meyer & Willems, 2012; Safronova et al., 2015). Although they are strictly aerobic, *Bosea minatitlanensis* is proven to be able to survive in anaerobic digestor (Ouattara et al., 2003), this trait may help this strains to out-compete others in the condition of this experiment, in which oxygen maybe limited due to the tightly capped reactors. Interestingly, some species of *Bosea* sp. are proven to be able to reduce sulfur substances (Das et al., 1996).

Mycobacterium is a genus of Actinobacteria and consists of more than 190 species (King et al., 2017). This genus contains some serious pathogens for mammals and humans such as tuberculosis and leprosy (Ryan & Ray, 2004). The discovery of this species from the biofilter sludge of the ARC is a surprise. However, this is not unprecedented as there are reports of *Mycobacterium* presence in tropical aquaria (Barrow & Hewitt, 1971; Edelstein, 1994; Von Reyn et al., 1993). Interestingly, the *Mycobacterium* sp. strains isolated is proven to be non-toxic to Artemia (Figure 21). On the other hand, the presence of non-tuberculous *Mycobacterium* is becoming more and more understood with the advances in molecular biotechnology (Turenne, 2019). To better understandthis strain, a whole genome sequencing is reccomended.

Roseomonas is a genus of Gram -negative bacteria, this genus is named after the pink color of their colonies, a fact that was also observed in this study. *Roseomonas* sp. is proven to be an opportunistic pathogen for human (Rihs et al., 1993). An interesting fact regarding this genus is that it can adapt to extreme conditions such as gamma radiation (Kim et al., 2018) and acid (Guazzaroni et al., 2013). One species of *Roseomonas* sp. is proven to be able to survive on Mars (Cheptsov et al., 2018).

Brevundimonas is a genus of Gram-negative, aerobic bacteria, mainly isolated from the environment. Being an opportunistic pathogen, there has been cases of antibiotic resistance reported (Chi, Fung, Wong, & Liu, 2004; Gilad et al., 2000; Han & Andrade, 2005). Along with *Roseomonas* sp., this genus is also proven to be an extremophile, able to withstand exposure simulated Martian environment of high solar radiation, low temperature and desiccation (Dartnell et al., 2010). This

genus have shown promise in water treatment such as removal of nitrate and heavy metal (Kavitha et al., 2009; Resmi et al., 2010)

5.3.4. BIOLOG™ assay.

Result from BIOLOG assay show that different strains have different degrading capability for different carbon source. The ability to utilize different carbon sources can indicate the flexibility of the microbes. In this case, strain *Bosea* sp., *Mycobacterium* sp. and *Roseomonas* sp. seems to be the most flexible and promising (Table 15). Noticeably, strains F1 and F2, while both belongs to genus *Bosea*, have a different behavior in carbon utilization, indicating that they are two different species. This observation us further backed up by the difference in morphological feature (Figure 22). *Myco*bacterium stands out as it is the only strain that can utilize Tween 40, Tween 80 and Putrescine, this was stated in literature (Michaels & Kim, 1966; Smith et al., 1993). The ability to utilize inexpensive substrates such as manitol can be an advantage in enriching the microbes to the desired density. On the other hand, this is only a preliminary result done on ECO Plate™ with a limited amount of carbon sources (31 sources). Further in-depth study using specifically BIOLOG™ plate for identification of bacteria and applying mixed community in BIOLOG™ test may yield more interesting results.

5.3.5. MTT-formazan assay.

This experiment is designed to detect differences in metabolic activity between the basal metabolic rate in a lean environment and the metabolic rate in an environment where geosmin is added. The logic behind this is that geosmin utilization capability may be detected by the strains' heightened metabolic activity in geosmin spiked media as MTT can detect slight metabolic increases which other methods like density monitoring and geosmin concentration measurement cannot detect.

From the result of Figure 24, we can see that the strains of interest are *Mycobacterium sp*. and *Bosea* sp. F2, which show significantly higher metabolic activity in the presence of geosmin. Strain *Mycobacterium* sp. shows higher geosmin metabolism in both distiled water and mineral media. Furthermore, geosmin metabolism of this strain in mineral media is significantly higher than in distiled water. Strain *Bosea* sp. F2 shows significantly higher metabolic activity in

the presence of geosmin ($p<0.05$) in distilled water. However, metabolic activity of this strain is comparable to the negative control where geosmin is suspended in mineral media. The behavior of strain F2 (*Bosea* sp.) and F4 (*Mycobacterium* sp.) can be explained as the result of co-metabolism. It was proven that geosmin is better utilized when another carbon source, such as ethanol, was added (Saito et al., 1999). An idea about the underlying mechanism of geosmin degrading of these strains can be that *Bosea* sp. F2 can utilize geosmin as sole the carbon source while *Mycobacterium* sp. use geosmin as a single carbon source as well as in a co-metabolic pathway with other organic substances, mostly from dead bacterial cells, or even the mineral itself. The presence of mineral media can accelerate the metabolism activity as reported by (Zhong et al (2007). *Mycobacterium,* shown to be the most flexible as it can utilized geosmin both singly and co-metabolically, with co-metabolism greatly enhancing the process. Same observations have been reported with *Mycobacterium* able to degrade phenolic-ring substances both singly and co-metabolically (Guerin & Jones, 1988; Leys, Bastiaens, Verstraete, & Springael, 2005; Ren et al., 2016; Solano-Serena et al., 2000; Zeng, Lin, Zhang, & Li, 2010).

5.3.6. Growth and geosmin degradation activity of isolated strains

Results from the culture of pure strains in media with geosmin continues to show that flow-cytometry is a robust and sensitive method of detecting and monitoring microbial density. When comparing plate counting to flowcytometry during all growth experiments, it became clear that flow-cytometry cell counts show 1 log higher density than CFUs, in line with previous observations. Optical density still cannot detect flocculant formation, this may be due to the low-density nature of the experiment, preventing significant flocculant formation. Unsurprisingly, *Mycobacterium* sp. shows highest and fastest growth, reaching 1 log increment within 96 hours. This may partially be due to the slightly higher initial concentration of this strain compared to others. Furthermore, the observation of flocculants forming inside *Mycobacterium* sp.reactors and the fact that this strain is dominant in the mixture treatment, indicate that this strain can metabolize geosmin.

In line with previous BIOLOG™, MTT test and growth curve (Figure 20, 24; table 15), *Mycobacterium* is shown to be a very flexible microbe, able to metabolize many carbon sources, including geosmin. *Mycobacterium* sp. can remove 35%

geosmin within 96 hours (Figure 26), showing strong geosmin utilization ability. This is backed up by the MTT result (Figure 23), which shows *Mycobacterium* sp. As a very promising strain, as it can work efficiently in close to real-life conditions in the presence of geosmin and other substances. Furthermore, *Mycobacterium* has been proven to be able to degrade substances with phenolic ring, similar to geosmin (Boldrin et al., 1993; Ren et al., 2016; Zeng et al., 2010).

Interestingly, strain *Brevundimonas* sp., while showing no capability in MTT test and virtually no density increase during the experimental period, shows highest reduction of geosmin of more than 50 %. This can be due to the ability to adsorb- a process in which substances are taken up but not metabolized by the micrio-organism- a trait that has been proven be previous studies (Resmi et al., 2010). On the other hand, it must be noted that the geosmin measurement in this study has repeatedly shown increased geosmin level of more than 100% and up to 300% during degradation test, this put a doubt on the reliability of the analytic method used.

The geosmin-removal capacity of our strain *Mycobacterium* , while high in this study's context, is actually lower than for other strains that have been reported. Guttman & van Rijn (2009) reported reduction of 89 % after 48 hours and complete removal of maximum 250 $\mu g.L^{-1}$within 10 days in unidentified sludge culture.

To sum up, 3 strains have been proven to degrade geosmin via 2 tests of metabolic activity and geosmin degradation. However, only *Mycobacterium* sp. passed both of the test, while *Bosea* sp. F2 failed the degradation test and *Brevundimonas* sp. showed no interesting result in the metabolic activity test.

5.4. Section 4: Evaluation of geosmin and 2-mib removal capacity of zeolite filter using different flow rates.

This experiment is carried out as a follow-up of the previous experiment by INAGRO, whereby the level of geosmin and 2-MIB were reducedsignificantly even in shorter retention time (ANNEX IV). It is a surprising result that the use of zeolite cannot reduce geosmin and 2-MIB level in this experiment at Aquabio. Zeolite has been proven to be effective to remove both geosmin and 2-MIB in water (Ellis & Korth, 1993; Ghasemi et al., 2018; Wee et al., 2015). The failure to remove off-tasting substances in this experiment can be due to the failure to regenerate

zeolite material, as the zeolite pellet in this experiment was the same used in the previous test in INAGRO. To regenerate, zeolite pellets were heated to 540°C for 1H (Wang et al., 2006) but maybe this process was not successful. Studies have shown that zeolite's capacity reduced dramatically to 42% (Azaria et al., 2017) and 60% (Wang et al., 2006) post regeneration. To better understand this, further studies should be carried out to determine the most optimum method for zeolite regeneration.

5.5. Section 5: Implication for application in aquaculture

This study has yielded interesting results including the identification of 3 bacterial strains that are able to degrade geosmin. However, for these to be able to apply in aquaculture, there are a few considerations.

All the strains selected have low growth rate, even in nutrient rich media like LB (Figure 20). This gives them a disadvantage when inoculated into the biofilter. It is highly unlikely that these geosmin-degrading strains can compete with the faster growing nitrification bacteria, thus preventing them to reach a high enough density to make a difference. This, combined with the fact that some of these strains are opportunistic pathogens (Gilad et al., 2000; Han & Andrade, 2005), makes their use problematic. Although the *Mycobacterium* sp. strains isolated in this study is proven to be non-toxic for *Artemia* (Figure 21), extra precaution must be taken as random mutation can take place and cause the strain to become pathogenic. Furthermore, although non-toxic towards *Artemia*, *Mycobacterium* sp. can still cause serious health problems in humans (Barrow & Hewitt, 1971; Edelstein, 1994). As stated, further testing on different model animals is needed to give definite result on this strain. A better strategy is to sequence the entire genome of this strain.

The strains *Mycobacterium* sp., *Bosea* sp. F2 and *Brevundimonas* sp. have been proven to be able to reduce geosmin. On the other hand Figure 26 shows that the rate of removal is still lower than those reported by Guttman & van Rijn (2009). This can be mitigated by sheer density of the bacteria in the biofilter, which can only be achieved by using a separate bio-reactor to enrich these strains. It must be noted that the mixed treatment of this study showed poor performance both in growth and in geosmin degradation, a problem that may be caused by inter-species competition, therefore, a further study should be carried out to

determine the best combination of species that can achieve highest growth plus highest geosmin reduction.

Another proposition would be the use of a combined zeolite/biofilter, as proposed by Lindholm-Lehto & Vielma (2019)who were utilizing zeolite's high surface area as a substrate for bacterial growth. Although the result from this study show that zeolite has no effect on geosmin and 2-mib removal, this result is probably incidental, caused by the poor regeneration process of zeolite material. A combination of zeolite adsorption with microbial degradation can achieve good performance and help to reduce the frequency of renewal of zeolite materials.

To sum up, the application of the result from this study in aquaculture is promising. Although there are several gaps to fill in, the general scenario should be a separate bio-reactor in combination with aa zeolite filter and bacterial strains *Mycobacteria* sp., *Bosea* sp. and *Brevundimonas* sp. in pure or mixed community. Disinfection by UV light should be complemented or replaced by ozonation as *Brevundimonas* sp. is proven to be UV resistance. Before that, a thorough test on toxicity should be carried out to give confirmation of the toxicity of these strains.

Chapter 6 General conclusion

In the present study, the micro-organisms, capable of degrading geosmin, were selected, identified, and evaluated on applicability in RAS systems. Furthermore, a small test on the removal capacity of zeolite was also carried out.

Overall, this study's procedure to select microbes capable of degrading geosmin is feasible. A total of 14 pure strains were isolated from the biofilter of the ARC which can utilize geosmin as the only carbon source. Using toxicity screening with *Artemia*, the selection was narrowed down to 5 strains belonging to 4 genera: *Bosea, Mycobacterium, Roseomonas* and *Brevundimonas.* All selected strains show slow growth both in LB and mineral media spiked with geosmin, reaching stationary phase at 1 log density increase after 96 hours.

BIOLOG assay shows that these strains can utilize different carbon sources, with the most flexible bacteria being *Mycobacterium.* Strains F1 and F2, belonging to the same genus *Bosea*, display different carbon utilization which confirms they are different species. The ability of *Mycobacterium* to utilize cheap carbon sources like mannitol can be useful for enrichment.

MTT results imply different geosmin utilization pathway. *Mycobacterium* can utilize geosmin both singly and co-metabolically, with co-metabolism greatly enhancing the process. On the other hand, *Bosea* sp. F2 is most likely only able to degrade geosmin singly.

Regarding geosmin degrading capability, *Mycobacterium* and *Brevundimonas* have the best performance, able to reduce 35 and 50 % geosmin respectively within 96 hours. However, a different mechanism is implied as only *Mycobacterium* has increase in density while *Brevundimonas* shows no growth and no metabolic activity in the presence of geosmin.

In this study, zeolite pellets have been shown to be unable to significantly reduce geosmin and 2-MIB from RAS water, despite previously positive tests. This can be the result of a poor regeneration procedure.

Regarding analysis methods, flow cytometry has been proven to be both a sensitive and robust technique to detect microbial density in water. Flowcytometry consistently gave result at least 1 log higher than viable plate counting, with a suitable density ranging between 10^3 to 10^6. A protocol for using the cytoFLEX

flow cytometer has also been produced. SPME method to analyze geosmin concentration in aqueous sample has some inconsistency in reading, giving very high result of more than 300 %.

To conclude, results from this study is promising, with at least 3 strains proven to be able to degrade geosmin. However, to be able to apply this in aquaculture require further research on toxicity and mechanism of action.

Chapter 7: Recommendations for further research

Flowcytometry is a robust method, however, further tests and studies should be carried out to further develop this technique, especially in staining. In this study, flocculant proved to be very problematic for flowcytometry to measure. A method to break down and homogenize flocs should be developed.

Although proven to be non-toxic towards artemia, all strains proven as geosmin-degrading from this study should be test further on other model animals such as rotifer, daphnia or on fish. A full genome sequencing should be carried out on *Mycobacterium* strain to ensure it is safe for human.

Zeolite has been proven to be unable to remove geosmin and 2-mib. However, this is due to poor regeneration performance. Future research should be focus on how to efficiently regenerate zeolite. A combine adsorption-biodegradation of zeolite materials acting as both adsorption and bacteria substrate should also be notice and developed.

To be able to apply in aquaculture, a new bioreactor should be designed to accommodate these strains as their slow growth means they are unable to compete with bacteria living in biofilter.

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9. Appendixes

ANNEX I. Geosmin needed for cell growth calculations

Carbon content in one bacteria cells (Troussellier et al., 1997)

$$Carbon\ in\ 1\ cell = 26fg = 26 \times 10^{-15}g = 2.17 \times 10^{-15}mole$$

Number of cells needed

$$10^7 - 10^5 = 9900000\ cells$$

Weight of carbon needed

$$mC = 9900000 \times 26 \times 10^{-15} = 2.574 \times 10^{-7}g = 2.145 \times 10^{-8}mole$$

Amount of geosmin needed

$$nGeosmin = \frac{2.145 \times 10^{-8}}{12} = 1.7875 \times 10^{-9}\ mole$$

$$mGeosmin = 1.7875 \times 10^{-9} \times 182 = 3.3 \times 10^{-7}g = 0.33\mu g$$

ANNEX II. Flow cytometry protocol for cytoFLEX machine

- **Dye preparation**
  - Thiazole orange (TO) dye 17 µM (BD Biosciences Catalog No. 349483 or 349480), or equivalent at 8.1 µg/mL (FW 476.6) in DMSO
  - Propidium iodide (PI) dye, 1.9 mM (BD Biosciences Catalog No. 349483 or 349480), or equivalent, at 1.3 mg/mL (FW 668.4) in Phosphate buffered saline (PBS)
- **Sample preparation**
  - Sample should be well homogenized and diluted to within 10^3 to 10^6 cells.mL^{-1}
  - Avoid using nutrient media, cells should be suspended in distilled water instead.
- **Sample loading**
  - add 5µL of thiazole orange and 5µL of propidium iodide to each well. Dye must be protected from direct light and must be stored back to 4°C immediately

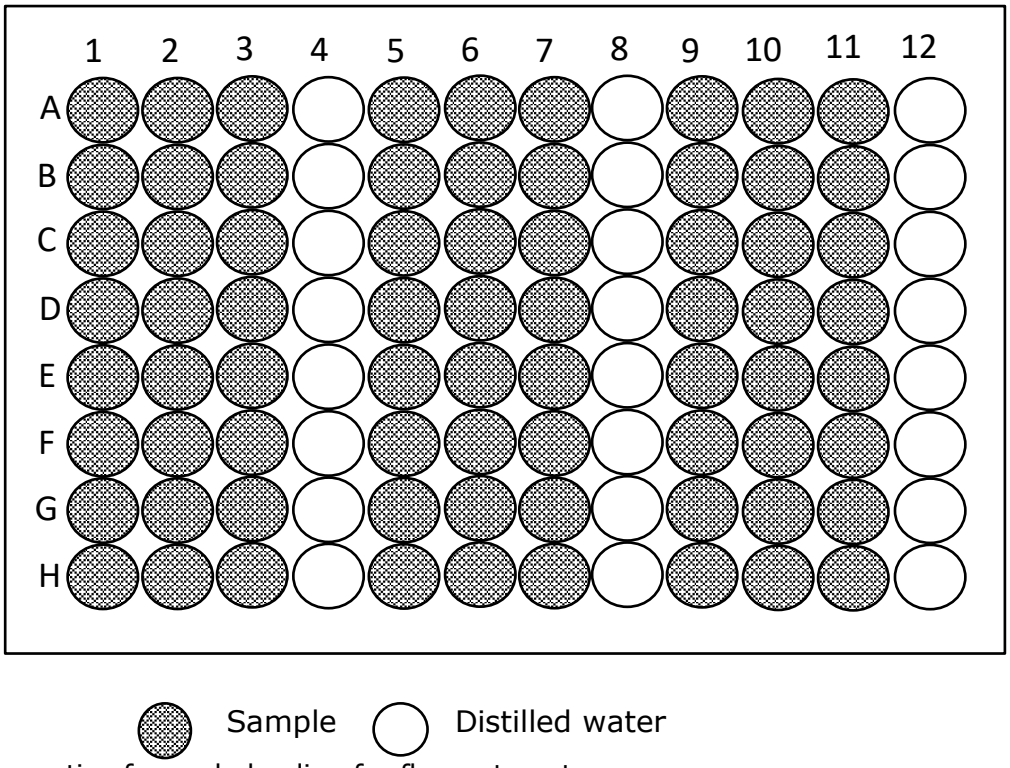

Figure 28. Schematic of sample loading for flow cytometry.

- Load 100µL of sample in triplicate, followed by 200µL of distilled water
- Incubate the plate for a minimum of 5 minutes

- **CytoFLEX Flow cytometer setup**
  - Turn on the power switch on the back cover of the instrument
  - Log on to the computer and start CytExpert software in desktop
- **QC**
  - Add 1 drop of Fluorespheres and 200µl distilled water into 1 well of the calibrate plate or 3 drops in 1mL of distilled water and add 200µL of the mixture to the QC well
  - QC menu → Start QC → Eject → Put plate onto the plate loader → Load → Lot No: Expire 04-06-2019 → Initialize → Start
- **Create experiment from a template**
  - Sellect File → New experiment from Template→ Experiment → Browse → Choose file to store the experiemnt → Template → Browse → file 'Live-dead_TO+PI' in desktop → Start
  - Create new experiment
  - Sellect File → New experiment from Template→ Experiment → Browse → Choose file to store the experiemnt

- **Plate setting:**
  - Select 'Add new plate' → Select option of Plate type corresponding to the used plate → Choose wells containing samples → 'Set as sample wells'
  - Threshold setting:
  - Threshold: Channel FITC → Manual: 1000 → Height
- **Gain setting**
  - Use default machine setting or as follows

Table 15. Gain setting for flow cytoFLEX flowcytometer

Gain	Setting
FSC	106
SSC	73
FITC	111
PE	119
PC 5.5	525

  - Set up plots to monitor the measurement
  - All plots are set on logarithmic scale

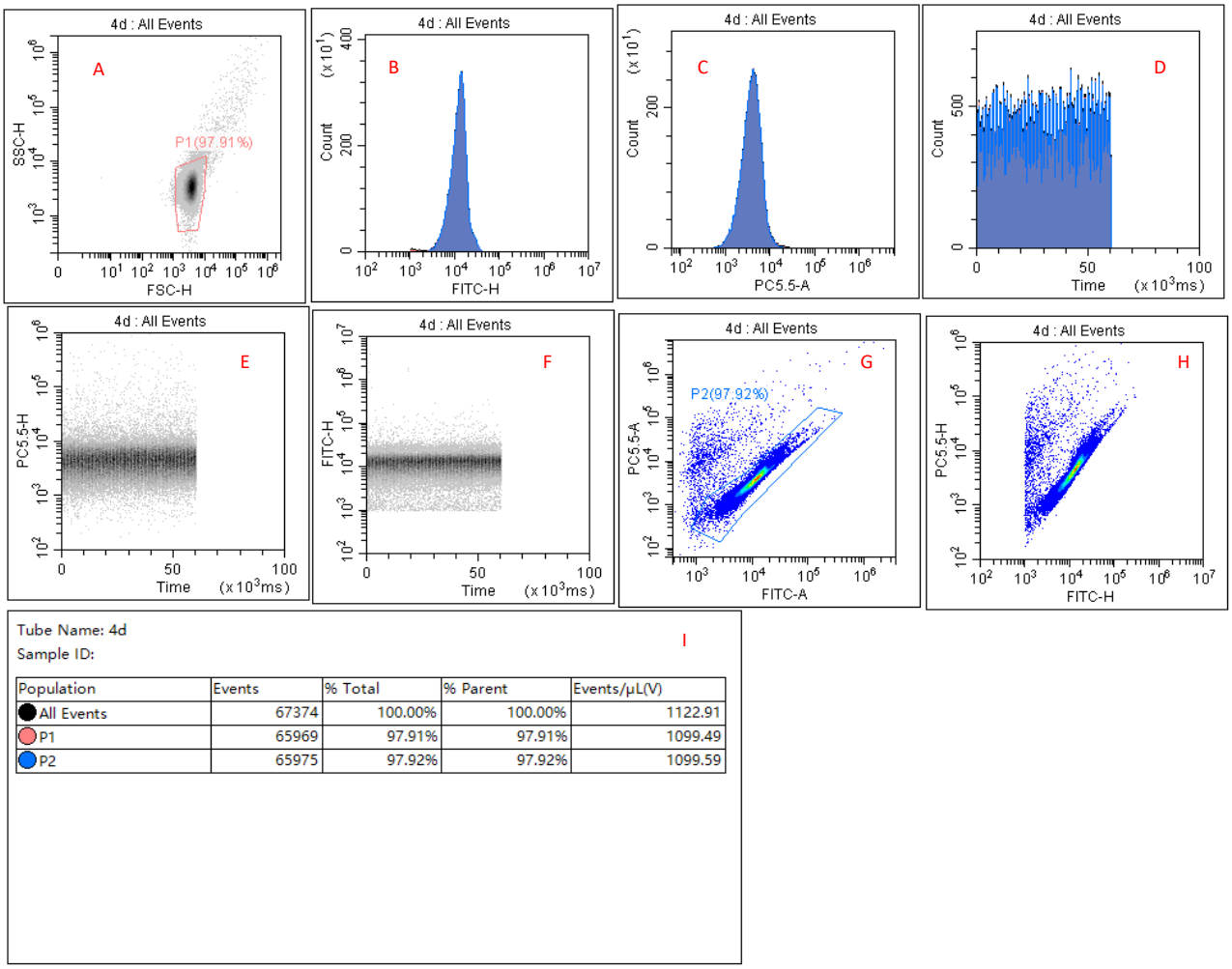

Figure 29. Screenshot of a typical flowcytometry measurement

Label	Plot	Reading
A	SSC-H versus FSC-H	Total population
B	Count of FITC	TO dye intensity consistency
C	Count of PC5.5	PI dye intensity consistency
D	Count versus time	Sample flow consistency
E	Time versus FITC	Sample TO dye flow consistency
F	Time versus PC5.5	Sample PI dye flow consistency
G	PC5.5-A versus FITC-A	Live and dead cells in different populations
H	PC5.5-H versus FITC-H	Live and dead cells in different populations
H	Statistics table	Shown number of different populations

- **Load plate to machine and measure**

- Select ‘Eject’→ Put plate onto plate loader → Load → Initialize → Auto record
- Select ‘Stand by’ when the measurement is finished

- **Cleaning and shut down**
  - Add 200µl distilled water (x5 wells)
  - Add 200µl cleaning solution (x5 wells)
  - Select ‘Cytometer’→ Daily clean…→ Set the default wells as ‘Empty wells’ → Set distilled water wells as ‘Deionized water wells’ → Set cleaning solution wells as ‘Cleaning reagent wells’
  - Select ‘Run’
  - Wait until cleaning is finished
  - Turn off the CytoFLEX

ANNEX III. Flow cytometry optimization

Carry over effect

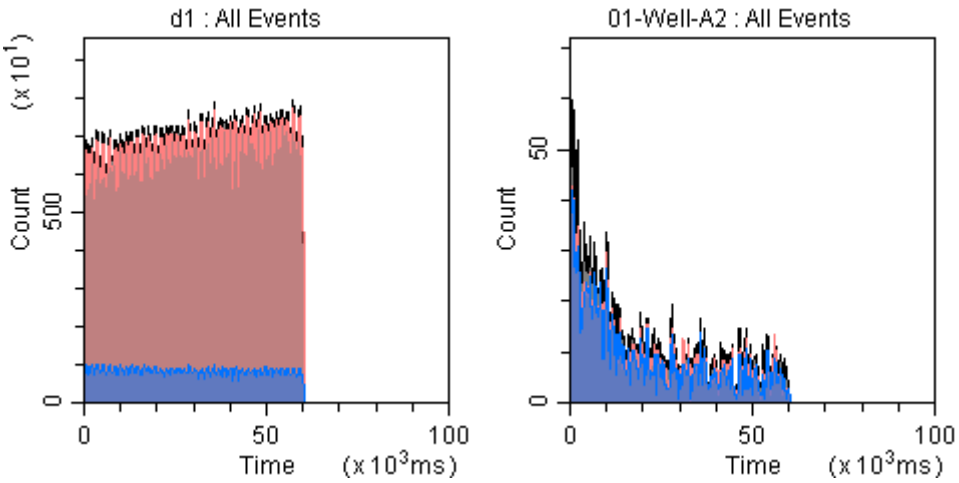

Figure 30. Normal sample flow (left), distilled water cleaning well (right)

We can see that the flow of normal sample is stable and horizontal. The flow of distilled water cleaning sample is shown gradually decreasing, indicating the carry over effect from previous sample.

Dye versus no dye

Table 15 Reading from dye and no dye sample with different dilutions (from a density of 10 cells.mL^{-1})

Dilution	Dye (cells.mL^{-1})	No dye (cells.mL^{-1})
10^{-3}	8520	7
10^{-4}	1034	7
10^{-5}	105	6
10^{-6}	11	8

We can see that reading from undyed sample is significantly lower than that of dyed samples

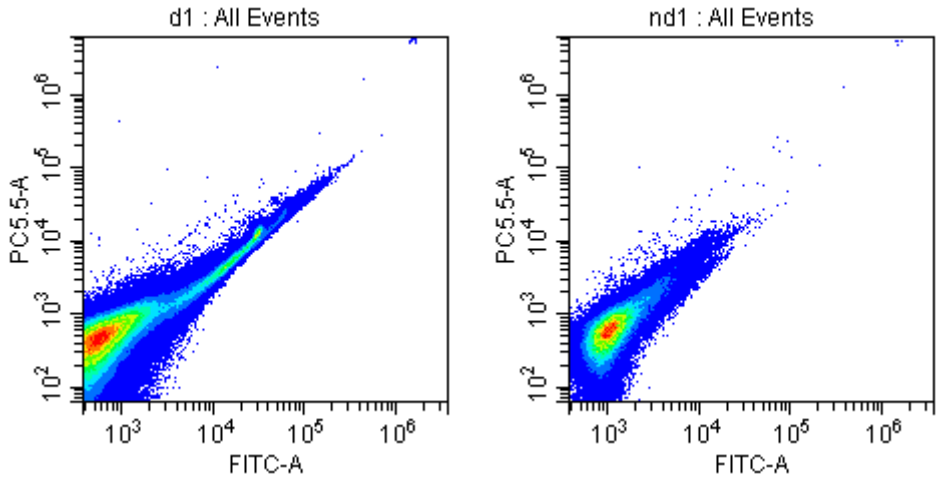

Figure 31. Strain F1, dye (left) versus no dye (right)

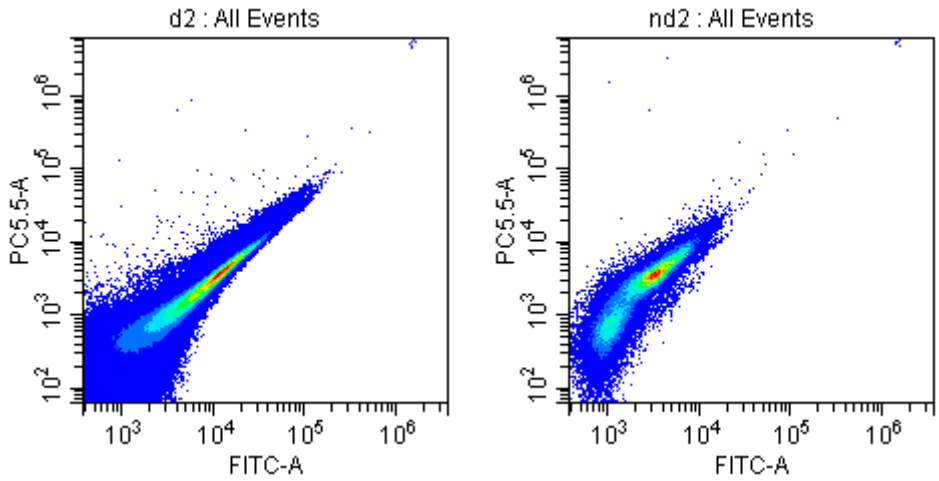

Figure 32. Strain F2, dye (left) versus no dye (right)

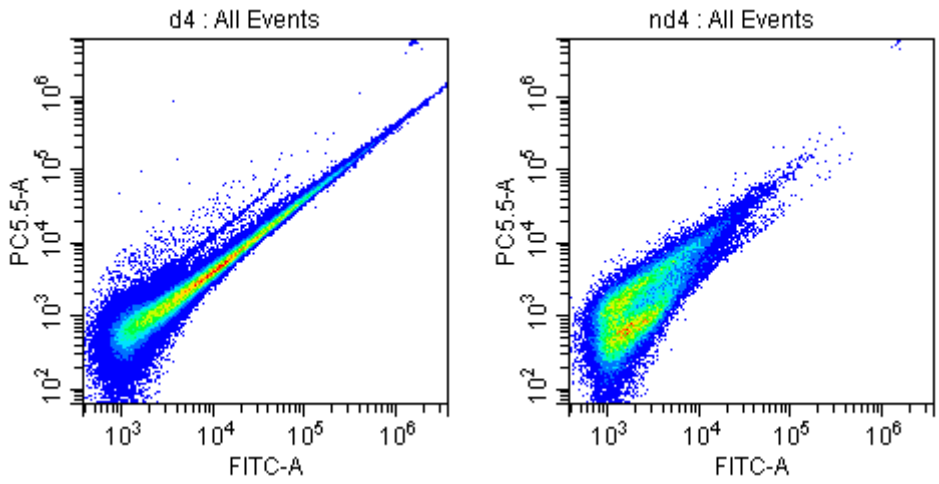

Figure 33. Strain F4, dye (left) versus no dye (right)

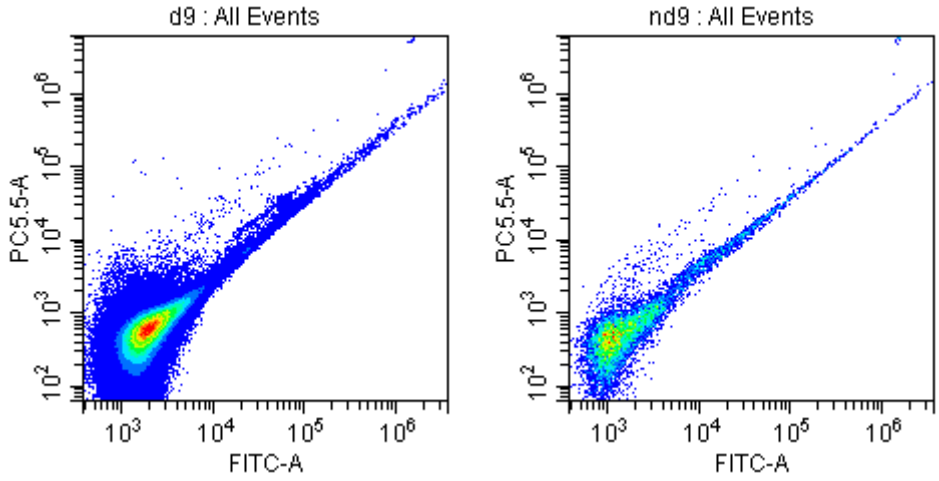

Figure 34. Strain F9, dye (left) versus no dye (right)

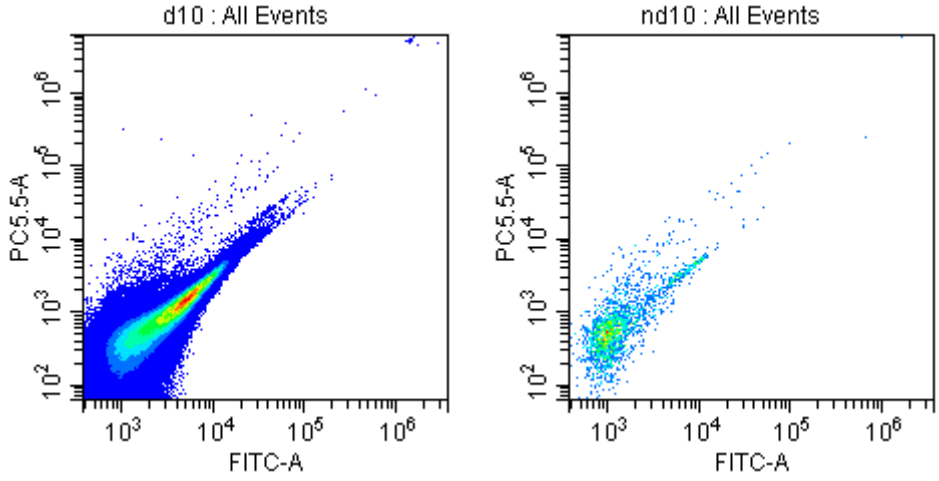

Figure 35. Strain F10, dye (left) versus no dye (right)

Dilutions

Table 16. Bacteria density at different dilutions from a density of 10^9 cells.mL^{-1}

Dilution	cells.mL^{-1}
1	716
10^{-1}	733
10^{-2}	8043
10^{-3}	8520
10^{-4}	1034
10^{-5}	105
10^{-6}	11
10^{-7}	2
10^{-8}	2
10^{-9}	1
10^{-10}	4

From the table, it is clear that readings are consistent with dilution level only between the range of 10^{-3} to 10^{-6}

ANNEX IV. 16S sequences of selected strains

F1

TGCAAGTCGAACGGGCACTTCGGTGCTAGTGGCAGACGGGTGAGTAACGCGTGGGAA
CGTGCCTTTCGGTTCGGAATAATCCAGGGAAACTTGGACTAATACCGGATACGCCCTTC
GGGGGAAAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAGCTAGTTGGTGAGGT
AATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGG
GACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATG
GGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGC
TCTTTTGTCCGGGAAGATAATGACTGTACCGGAAGAATAAGCCCCGGCTAACTTCGTGC
CAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAG
GGCGCGTAGGCGGACTCTTAAGTCGGGGGTGAAAGCCCAGGGCTCAACCCTGGAATT
GCCTTCGATACTGGGAGTCTTGAGTTCGGAAGAGGTTGGTGGAACTGCGAGTGTAGAG
GTGAAATTCGTAGATATTCGCAAGAACACCGGTGGCGAAGGCGGCCAACTGGTCCGAA
ACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGAATGCCAGCCGTTGGGGAGCTTGCTCTTCAGTGGCGCAGCTA
ACGCTTTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACC
TTACCAGCTTTTGACATGTCCGGTTTGATCGGCAGAGATGCCTTTCTTCAGTTCGGCTG

GCCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTCGCCCCTAGTTGCCATCATTCAGTTGGGAACTCTAG
GGGGACTGCCGGTGATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGC
CCTTACAGGCTGGGCTACACACGTGCTACAATGGCGGTGACAATGGGCAGCGAAAGG
GCGACCTCGAGCTAATCCCAAAAAGCCGTCTCAGTTCAGATTGTACTCTGCAACTCGAG
TACATGAAGGTGGAATCGCTAGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCC
CGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGGTTTACCCGAAGGCGTCG
CGCTAACCGCAAGGA

F2

GGAATAATTCAGGGAAACTTGGACTAATACCGGATACGCCCTTCGGGGGAAAGATTTAT
CGCCGATAGATCGGCCCGCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGG
CGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCC
CAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCC
AGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTTGTCCGGGAAG
ATAATGACTGTACCGGAAGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAA
TACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGTAAAGGGCGCGTAGGCGGAC
TCTTAAGTCGGGGGTGAAAGCCCAGGGCTCAACCCTGGAATTGCCTTCGATACTGGGA
GTCTTGAGTTCGGAAGAGGTTGGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATA
TTCGCAAGAACACCAGTGGCGAAGGCGGCCAACTGGTCCGATACTGACGCTGAGGCG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AATGCCAGCCGTTGGGGAGCTTGCTCTTCAGTGGCGCAGCTAACGCTTTAAGCATTCC
GCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCTTTTGAC
ATGTCCGGTTTGATCGGCAGAGATGCCTTTCTTCAGTTCGGCTGGCCGGAACACAGGT
GCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTCGCCCCTAGTTGCCATCATTCAGTTGGGAACTCTAGGGGGACTGCCGGTG
ATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACAGGCTGGG
CTACACACGTGCTACAATGGCGGTGACAATGGGCAGCGAAAGGGCGACCTCGAGCTAA
TCCCAAAAAGCCGTCTCAGTTCAGATTGTACTCTGCAACTCGAGTACATGAAGGTGGAA
TCGCTAGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACC
CCCCCCGTCACACCATGGGAGTTGGGTTTACCCGAAG

F4

TGCAAGTCGCACGGGCAGCAATGTCAGTGGCGGACGGGTGAGTAACGCGTAGGAAC
GTGTCCTGAGGTGGGGGACAACCCCGGGAAACTGGGGCTAATACCGCATATGGGCTG
AGGCCCAAAGCCGAGAGGCGCCTTTGGAGCGGCCTGCGTCCGATTAGGTAGTTGGTG
GGGTAAAGGCCTACCAAGCCTGCGATCGGTAGCTGGTCTGAGAGGACGACCAGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA
CAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGGGTGAAGAAGGTCTTCGGATCG
TAAAGCCCTTTCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAA
CTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATTACTGG
GCGTAAAGGGCGCGTAGGCGGCGGCCCAAGTCAGGCGTGAAATTCCTGGGCTCAACC
TGGGGACTGCGCTTGATACTGGGTTGCTTGAGGATGGAAGAGGCTCGTGGAATTCCC
AGTGTAGAGGTGAAATTCGTAGATATTGGGAAGAACACCGGTGGCGAAGGCGGCGAG
CTGGTCCATTACTGACGCTGAGGCGCGACAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGTGCGCTGGATGTTGGGGCCCATAGGGTCTCA
GTGTCGTAGCCAACGCGGTAAGCGCACCGCCTGGGGAGTACGGCCGCAAGGTTGAAA
CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAG
CAACGCGCAGAACCTTACCAGCCCTTGACATGGTCACGACCGGTCCAGAGATGGACTT
TCCTAGCAATAGGCGTGATGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCTCTAGTTGCCAGCATGTTC
GGGTGGGCACTCTAGAGGAACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACG
TCAAGTCCTCATGGCCCTTATGGGCTGGGCTACACACGTGCTACAATGGCGGTGACAG
AGGGAAGCCAGGTCGCGAGGCCGAGCCGATCCCGAAAAGCCGTCTCAGTTCGGATTG
CACTCTGCAACTCGGGTGCATGAAGGTGGAATCGCTAGTAATCGCGGATCAGCACGC
CGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGG
TTCTACCTTAAGTCGTTGCGCTAACCAGCGATGGG

F9

TGCAAGTCGCACGGGCAGCAATGTCAGTGGCGGACGGGTGAGTAACGCGTAGGAACG
TGTCCTGAGGTGGGGGACAACCCCGGGAAACTGGGGCTAATACCGCATATGGGCTGA
GGCCCAAAGCCGAGAGGCGCCTTTGGAGCGGCCTGCGTCCGATTAGGTAGTTGGTGG
GGTAAAGGCCTACCAAGCCTGCGATCGGTAGCTGGTCTGAGAGGACGACCAGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
ATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGGGTGAAGAAGGTCTTCGGATCGTAA
AGCCCTTTCGACGGGGACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTTC
GTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATTACTGGGCGT
AAAGGGCGCGTAGGCGGCGGCCCAGTCAGGCGTGAAATTCCTGGGCTCAACCTGGGG

ACTGCGCTTGATACTGGGTTGCTTGAGGATGGAAGAGGCTCGTGGAATTCCCAGTGTA
GAGGTGAAATTCGTAGATATTGGGAAGAACACCGGTGGCGAAGGCGGCGAGCTGGTC
CATTACTGACGCTGAGGCGCGACAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGTGCGCTGGATGTTGGGGCCCATAGGGTCTCAGTGTCGTA
GCCAACGCGGTAAGCGCACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCA
GAACCTTACCAGCCCTTGACATGGTCACGACCGGTCCAGAGATGGACTTTCCTAGCAAT
AGGCGTGATGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTCGCCTCTAGTTGCCAGCATGTTCGGGTGGGCAC
TCTAGAGGAACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCA
TGGCCCTTATGGGCTGGGCTACACACGTGCTACAATGGCGGTGACAGAGGGAAGCCA
GGTCGCGAGGCCGAGCCGATCCCGAAAAGCCGTCTCAGTTCGGATTGCACTCTGCAAC
TCGGGTGCATGAAGGTGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTCTACCTTAAGTC
GTTGCGCTAACCAGCGATGGG

F10

TCTTCGGACTTAGTGGCGGACGGGTGAGTAACACGTGGGAACGAGCCTTTAGGTTCGG
AATAACTCAGGGAAACTTGTGCTAATACCGAATGTGCCCTTCGGGGGAAAGATTTATCG
CCTTTAGAGCGGCCCGCGTCTGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCG
ACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCA
AACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTGACGCA
GCCATGCCGCGTGAATGATGAAGGTCTTAGGATTGTAAAATTCTTTCACCGGGGACGAT
AATGACGGTACCCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA
CGAAGGGGGCTAGCGTTGCTCGGAATTACTGGGCGTAAAGGGAGCGTAGGCGGACAT
TTAAGTCAGGGGTGAAATCCCGGGGCTCAACCTCGGAATTGCCTTTGATACTGGGTGT
CTTGAGTGTGAGAGAGGTATGTGGAACTCCGAGTGTAGAGGTGAAATTCGTAGATATT
CGGAAGAACACCAGTGGCGAAGGCGACATACTGGCTCATTACTGACGCTGAGGCTCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGATT
GCTAGTTGTCGGGATGCATGCATTTCGGTGACGCAGCTAACGCATTAAGCAATCCGCCT
GGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCACCTTTTGACATGCC
CGGACCGCCACAGAGATGTGGCTTTCNCTTCGGAGACTGGGACACAGGTGCTGCATG
GCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
CGCCATTAGTTGCCATCATTCAGTTGGGAACTCTAATGGGACTGCCGGTGCTAAGCCG

GAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACAGGGTGGGCTACACACGT
GCTACAATGGCGACTACAGAGGGTTAATCCTTAAAAGTCGTCTCAGTTCGGATTGTCCT
CTGCAACTCGAGGGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGG
TGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTCTACC
CGAAGGCGCTGCGCTGACCGCTAAGGAG

ANNEX V. Preliminaty test on zeolite

Table 16. result from zeolite filter test in INAGRO

Sample	Geosmine (ng/L)	2-MIB (ng/L)
Zeolite filter (160sec/l)	7.16	5.57
Zeolite filter (60sec/l)	10.35	5.73
Growout after biofilter	3.23	8.26
Broodstock after biofilter	4.90	<4.37
Control	14.36	9.51