The Effect of Dissolved Oxygen on the Development of Manganese Oxidizing – and Reducing Biofilms in the Lower Blyde Irrigation System

by

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Declaration

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Summary

The Blyderiver dam in Mpumalanga is an important source of water for the irrigation of a variety of produce on farms in Limpopo and Mpumalanga. Water from this dam is used for gravity fed irrigation to farms via the Lower Blyde Irrigation System (LBIS), a network of pipelines of approximately 150 km. Biofilm development in the system causes a reduction in hydraulic capacity, leading to a reduction in water delivery for irrigation.

It was hypothesized that high concentrations of manganese (Mn) in the water of the Blyderiver dam could potentially contribute to the development of these biofilms. Dissolved oxygen (DO) and Mn measurements taken during four sampling events from 2015 to 2017 indicated that the water of the Blyderiver dam has a distinct profile, with DO and Mn concentrations showing a strong inverse correlation. DO concentration typically remained constant between 8 and 9 mg l^{-1} in the upper 30 + meters of the water column, where after it decreased rapidly to below 2 mg l^{-1} at deeper depths. In contrast, total Mn concentration remained constant between 10 and 100 µg l^{-1} in the upper regions of the water column, followed by a rapid increase to higher than 8000 µg l^{-1} near the bottom. The current point of extraction for the LBIS is located near the bottom of the dam in this water with high Mn content.

Mn concentration decreased with distance along the LBIS pipeline. For instance, results of the May 2016 sampling showed a decrease in the bulk aqueous phase Mn concentration from 8631 µg l^{-1} at the extraction point to 134 µg l^{-1} at 23 km downstream, while a decrease in Mn concentration could also be seen from 30105.4 mg kg^{-1} biofilm biomass at 4.5 km downstream to 13727.7 mg kg^{-1} at 28.4 km downstream. This decrease suggests ongoing incorporation of Mn into the biofilm, and thus potentially further loss in hydraulic capacity.

Laboratory simulation experiments of the LBIS pipeline were conducted in which biofilms supplied with growth medium with different DO and Mn concentrations were cultivated. These biofilms were analysed by determining cell release into the effluent, Mn measurements, scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM/EDS), automated ribosomal intergenic spacer analysis (ARISA) as well as confocal laser scanning microscopy (CLSM). Results obtained from colony counts and ARISA indicated that statistically significant differences did exist between biofilms cultivated under different growth conditions in some cases, but that these differences could not be attributed to the effects of different DO and Mn concentrations and were instead due to physical conditions such as shear and flux. These findings were confirmed by CLSM analysis. No statistically

significant decrease in Mn concentration could be observed throughout the flow system, indicating that very little to no Mn was incorporated into the biofilm structure under any of the growth conditions tested in the laboratory simulations. SEM/EDS analysis of these biofilms further confirmed that the decreases in Mn concentration observed in both the bulk aqueous phase and biofilms with distance in the LBIS pipeline could not be replicated in these laboratory simulated flow systems. A number of factors were considered to explain this conclusion, including the time period of the experiment, nutrients used in the growth medium and biofilm sorption capacity. Future experimentation should include flow systems in which biofilms will be cultivated for longer time periods, and the use of microfluidic flow channels to provide shear values closer to that in the LBIS.

Opsomming

Die Blyderivierspoort dam in Mpumalanga is ‘n belangrike bron van water vir die besproeiing van ‘n verskeidenheid vrugte produkte op plase in Limpopo en Mpumalanga. Water van hierdie dam word gebruik vir gravitasie aangedrewe besproeiing op plase en word voorsien deur middel van die Laer Blyde Besproeiing Sisteem (LBBS), ‘n pyplyn netwerk van ongeveer 150 km. Die ontwikkeling van biofilms in hierdie pyplyn is egter verantwoordelik vir ‘n verlaging in hidroliese kapasiteit, wat verder lei tot verlaagde water voorsiening vir besproeiing.

Die hipotese was dat hoë konsentrasies van mangaan (Mn) in die water van die Blyderivierspoort dam moontlik kan bydra tot die ontwikkeling van hierdie biofilms. Opgeloste suurstof (OS) en Mn metings wat geneem is tydens vier monsternemings periodes tussen 2015 en 2017 het aangedui dat die water van die Blyderivierspoort dam ‘n baie spesifieke profiel het, waar OS en Mn konsentrasies ‘n sterk inverse korrelasie toon. OS konsentrasies het tipies konstant gebly tussen 8 en 9 mg l^{-1} in die boonste 30 + meter van die water kolom, gevolg deur ‘n skerp daling na minder as 2 mg l^{-1} by dieper dieptes. In teenstelling hiermee het totale Mn konsentrasies konstant gebly tussen 10 en 100 µg l^{-1} in die boonste dele van die water kolom, waarna dit skielik gestyg het na hoër as 8000 µg l^{-1} naby aan die bodem van die dam. Die ekstraksiepunt van die LBBS pyplyn is huidiglik naby aan die bodem van die dam in hierdie water met hoë Mn konsentrasie.

Mn het afgeneem in konsentrasie met die lengte van die LBBS af. Die resultate van die Mei 2016 monsterneming het byvoorbeeld ‘n afname getoon in die Mn konsentrasie van die vloeistoffase vanaf 8631 µg l^{-1} by die ekstraksie punt tot 134 µg l^{-1} teen 23 km verder stroomaf. Biofilms het ook ‘n afname getoon in Mn konsentrasie vanaf 30105.4 mg kg^{-1} biofilm biomassa by 4.5 km stroomaf tot 13727.7 mg kg^{-1} by 28.4 km stroomaf. Hierdie afname dui moontlik op die aanhoudende inkorporering van Mn in die biofilm struktuur en dus verdere verlies in hidroliese kapasiteit.

Laboratorium simulerings eksperimente van die LBBS pyplyn is opgestel waarin biofilms voorsien van groeimedium met verskillende OS en Mn konsentrasies opgegroei kon word. Hierdie biofilms is geanaliseer deur die sel vrystelling in die uitvloeisel te bepaal, Mn metings, skanderings elektronmikroskopie met energie verspreidende X-straal spektroskopie (SEM/EVS), ge-outomatiseerde ribosomale intergeniese spasie analise (GRISA), sowel as konfokale laser skanderings mikroskopie (KLSM). Die resultate verkry vanaf die tel van kolonies en GRISA het wel aangedui dat statisties relevante verskille in sommige gevalle bestaan het tussen biofilms wat onder verskillende groeitoestande opgegroei is, maar dat hierdie verskille nie toegeskryf kon word aan die

effek van verskillende OS en Mn konsentrasies nie, maar eerder ‘n gevolg is van fisiese oorsake soos skeuringskragte en vloeistoestande. Hierdie bevindings is bevestig deur KLSM analise. Mn metings het aangedui dat geen statisties relevante afname in Mn konsentrasie waargeneem kon word regdeur die sisteem nie, wat aandui dat baie min of geen Mn in die struktuur van die biofilm geïnkorporeer is onder enige van die groeitoestande nie. SEM/EVS analise op die biofilms het verder bevestig dat die afname in Mn konsentrasie wat waargeneem is in beide die vloeistoffase en die biofilms met afstand in die LBBS pyplyn nie gerepliseer kon word in hierdie laboratorium simulerings eksperimente nie.

’n Verskeidenheid faktore is in ag geneem om hierdie gevolgtrekking te verduidelik, insluitend die tydsduur van die eksperiment, die voedingstowwe gebruik in die groeimedium en die biofilm se kapasiteit vir opname. Toekomstige eksperimente behoort onder andere te kyk na vloeisisteme waarin biofilms vir ‘n langer tydsduur opgegegroei kan word en die gebruik van mikrovloeikanaal sisteme met skeuringskragte nader aan dit wat in die LBBS waargeneem word.

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List of Abbreviations

Al	Aluminium
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
ARISA	Automated ribosomal intergenic spacer analysis
CAF	Central analytical facility
CLSM	Confocal laser scanning microscopy
CFU	Colony forming units
DO	Dissolved oxygen
EPS	Extracellular polymeric substance
Fe	Iron
H_2O_2	Hydrogen peroxide
HSD	Honestly significant difference
ICP-MS	Inductively coupled plasma mass spectrometry
LBIS	Lower Blyde Irrigation System
MCO	Multicopper oxidaze
Mn	Manganese
Mn^{2+}	Manganous
MnO_2	Manganese dioxide
NMDS	Non-metric multidimensional scaling
O_3	Ozone
OD	Optical density
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
$P_2O_7^{4-}$	Pyrophosphate
PVC	Polyvinyl chloride
ROS	Reactive oxygen species
SEM/EDS	Scanning electron microscopy with energy dispersive X-ray spectroscopy
SGW	Synthetic ground water
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet

Chapter 1

General introduction and objectives

1.1. General introduction

The Blyderiver dam, located in Mpumalanga, South Africa, is a major source of water used for a variety of produce on farms in Limpopo and Mpumalanga. Water from this dam is supplied to farms for irrigation via the LBIS, a network of pipelines of approximately 150 km. The pipeline varies in diameter from 1500 mm to 250 mm. In 2010 it was discovered that biofilm formation inside the pipeline was the reason for the gradual loss in hydraulic capacity that had been observed over a number of years. This led to concerns as water is only propelled through the LBIS network under gravity and reduced water delivery for irrigation to farms may have a significant economic impact as a large percentage of South Africa's export produce is grown in this region. Although biocides could potentially offer a solution to this problem, their use does not guarantee success and it is usually accompanied by a number of factors that need to be considered. Biocides are widely used to combat surface contamination, however a diverse range of microorganisms often survive and continue to thrive after biocide dosing (Bagge-Ravn et al., 2003). Moreover, an increased microbial resistance to biocides has been detected n biofilm communities (Bressler et al., 2009; Vestby et al., 2009). Additionally, hydrogen peroxide (H_2O_2), a commonly used biocide considered for use in the LBIS, dissipates in water and with the high flow rates and extent of biofilms already developed inside the pipeline, H_2O_2 and other oxidizing biocides do not offer an economically feasible solution to control the fouling. This is compounded by the fact that H_2O_2 dissipation is catalyzed by the presence of Mn oxides (Knol et al., 2015).

In order to understand biofilm development inside the LBIS network, the large scale of the system, as well as the operations and complex chemical processes occurring in the Blyderiver dam need to be considered. Water from the Blyderiver dam contains high concentrations of Mn and iron (Fe), which is especially prevalent in the deeper regions of the water column where it remains in the reduced/soluble form as described by Gantzer et al. (2009) in their study of Mn concentrations in the Carvins Cove reservoir in South West Virginia, USA. Although three possible extraction points were built into the Blyderiver dam wall, water is currently only extracted from the lowest point. The two additional extraction points are spaced 10 m apart vertically, allowing for extraction from 20 m above the bottom of the dam (Swart, 2017).

In large water bodies, DO has been identified to play a key role in the processes associated with Mn oxidation and reduction, where high DO concentrations allow for Mn oxidation, while low DO concentration favours Mn reduction. Low DO concentration and resulting high soluble Mn concentration in the hypolimnion of the Carvins Cove reservoir were addressed by the installation of

a full scale hypolimnetic oxygenation system/diffuser to control Mn concentrations during summer periods when stratification was most significant. Results from the study indicated that total Mn concentration in the hypolimnion remained lower after diffuser operation started (Gantzer et al., 2009), confirming the important role that DO concentration plays in Mn oxidation. Although the temperature profile of the Blyderiver dam does not follow the typical thermal stratification pattern, persisting low DO concentrations in the deeper regions of the water column result in the reduction of precipitating oxidized Mn in the deeper zones. Due to the current extraction depth of the LBIS pipeline, water is extracted from the Mn rich zone. The high concentration of Mn entering the LBIS network could potentially contribute to the selection of microorganisms capable of oxidizing and reducing this element as these microorganisms are typically present in environments containing high concentrations of Mn (Tebo et al., 2005).

The occurrence of Mn in water distribution systems have been reported both locally and internationally (Mouchet, 1992; Stauffer, 1986), where it is often associated with unpleasant smells and causes discolouration and staining (Cerrato et al., 2010; Gantzer et al., 2009). Although conventional treatment methods typically include the use of chemicals as oxidizers of Mn, several limitations are associated with these methods. As such, water treatment facilities have started to implement alternative methods such as biological oxidation, where optimal environmental conditions are created for microorganisms capable of oxidizing Mn to carry out their natural processes. Due to the complex redox kinetics of Mn, it is not easily chemically oxidized in environments with a pH of 6 – 8, such as that observed in natural water, requiring Mn oxidizing bacteria such as *Pseudomonas manganoxidans*, *Crenothrix* and *Leptothrix*, among others, to catalyze its oxidation (Gantzer et al., 2009).

Tebo et al., (2005) showed that the presence of Mn oxidizing microorganisms can accelerate Mn oxidation to occur faster than it would when solely driven by chemical reactions. Additionally, the ability to oxidize Mn may be beneficial for Mn oxidizing microorganisms, protecting them against reactive oxygen species (ROS). The exact mechanisms through which these organisms oxidize Mn is still unknown, but a number of likely processes involving enzymes and biopolymers involved in the oxidation process have been suggested (Mouchet, 1992). Although these processes generally involve the oxidation of manganous (Mn^{2+}) to manganese dioxide (MnO_2), this oxidation path has been proven to require a Mn^{3+} intermediate and is not a direct process as previously hypothesized (Webb et al., 2005). It is thus clear that Mn oxidation is a complex process dependent on multiple parameters that need to be optimal for this process to occur. Fe, in contrast, is much easier to oxidize

and although this element was not the focus of the current study, it is included in the discussion as this element often occurs concurrently with Mn in water sources (Gantzer et al., 2009).

1.2. Hypothesis

It was hypothesized that the water profile of the Blyderiver dam would contain low concentrations of Mn in the upper layers, followed by an increase with depth. Additionally, this increase will be mirrored by DO concentration, decreasing with depth, resulting in decreased oxidation of soluble Mn^{2+} to insoluble MnO_2. As a result, high concentrations of soluble Mn^{2+} are introduced into the LBIS pipeline, which may lead to the growth of biofilms that accumulate Mn.

1.3. Overall goal of the project

The overall goal of the project was to investigate and determine why biofilms formed on the inside of the LBIS pipelines and what role they may potentially play in Mn oxidation and reduction. It was therefore necessary to obtain DO and Mn profiles of the Blyderiver dam to determine whether high concentrations of soluble Mn entering the pipeline could contribute to the development of these biofilms and to elucidate the contribution of DO concentration in this process.

1.4. Aims

1. To investigate the relationship between DO and Mn concentration and how this changes with an increase in depth in the Blyderiver dam.
2. To determine whether Mn is incorporated into the structure of biofilms growing in the LBIS pipeline network.
3. To determine if DO and Mn concentrations have a significant effect on biofilm growth and development using laboratory scale flow systems.

1.5. The specific objectives for this project

1. To conduct a number of sampling events over two years to record DO and Mn measurements at selected locations in the Blyderiver dam to constitute a full depth profile.
2. To collect water and biofilm samples from the inside of the LBIS pipeline for analysis to identify whether biofilms act as a catalyst or nucleation site for Mn oxidation and deposition and if so, whether Mn precipitation leads to enhanced biofilm formation.

3. To optimize an airtight linear laboratory flow system in which DO and Mn concentration can be adjusted.
4. To compare the effects of different DO and Mn concentrations on the growth and development of potential Mn oxidizing and reducing, mixed community biofilms.

Chapter 2

Literature review

2.1. The chemistry of Mn

The occurrence of Mn as a persisting contaminant in water distribution systems is not uncommon and globally, this problem has been reported several times (Bryant et al., 2011; Cerrato et al., 2010; Gantzer at al., 2009; Mouchet, 1992; Stauffer, 1986). In these systems, Mn usually occurs in the soluble/reduced (Mn^{2+}) form, especially under anoxic conditions. Several methods of treatment exist; however, these methods are often not cost effective nor capable of completely solving the problem. Treatments using chemical oxidation involves the use of oxidants such as ozone (O_3), chlorine, chlorine dioxide and potassium permanganate, often accompanied by pH manipulation (Budd et al., 2007). However, this method does not address the anoxic conditions naturally formed within large water bodies during stratification. As such, oxidized Mn particles may settle out and resolubilize upon reaching anoxic regions deeper down in the water body. To address this issue, anoxia has been managed using methods such as oxygenation where pure oxygen is used as oxygen supply, as well as aeration, where compressed air is used as oxygen supply (Beutel and Horne, 1999; Gantzer et al., 2009; Singleton & Little, 2006).

Another contributing factor is its complex redox kinetics, making it difficult to chemically oxidize Mn in environments with a pH of 6 – 8, typically observed in natural waters, which implies that microbial communities capable of oxidizing Mn are often required to facilitate oxidation (Baden et al., 1995; Kristiansen et al., 2002; Roitz et al., 2002). Microbial processes associated with Mn oxidizing bacteria are responsible for oxidizing Mn^{2+} to Mn^{4+} (Fig. 2.1) up to five orders of magnitude faster than oxidation via abiotic oxidation observed in surface water (Tebo et al., 2005; Webb et al., 2005). In environments with low DO concentration, Mn reducers can use oxidized Mn as an electron acceptor to obtain energy for anaerobic growth (Nealson and Myers, 1992). In contrast, Fe is easier to control as it can be easily oxidized chemically in the presence of oxygen (Gantzer et al., 2009; Kristiansen et al., 2002). It should be noted that the primary focus of this study was the process of Mn oxidation and how it is affected by bacteria capable of oxidizing it. However, Fe oxidation is also discussed as Mn and Fe often occur together in high concentrations in water sources.

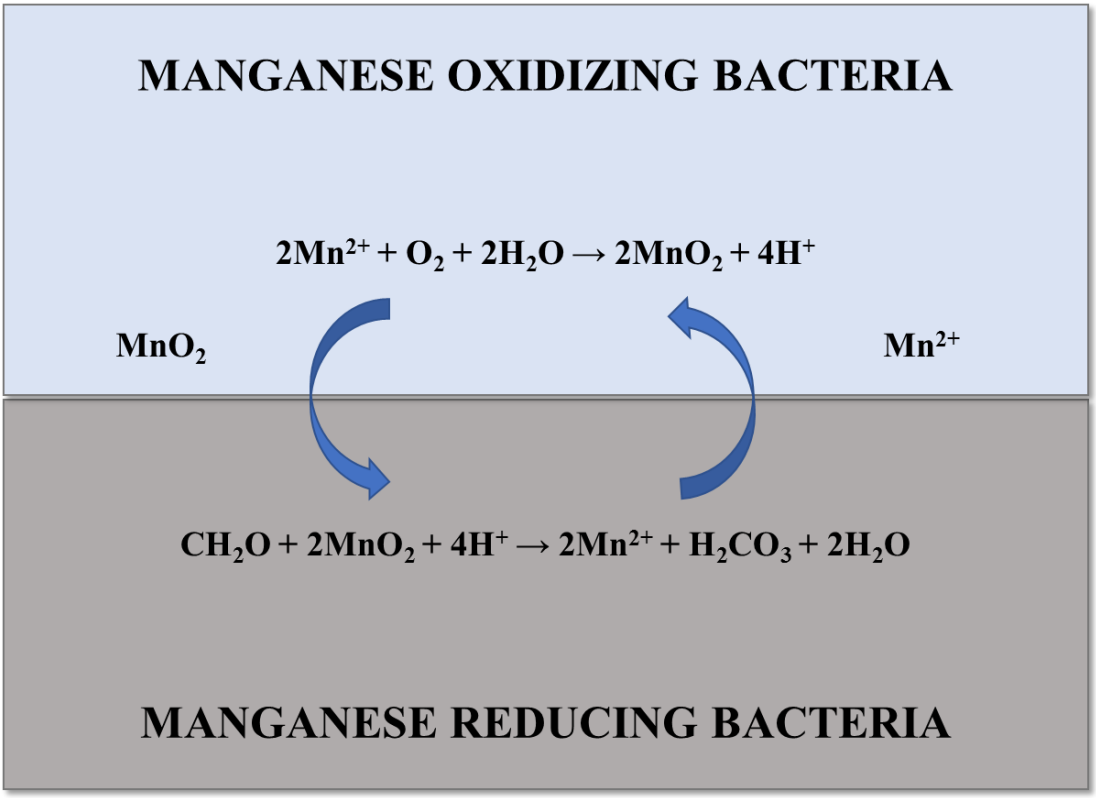

Fig. 2.1 - The Mn cycle as carried out by Mn oxidizing bacteria and Mn reducing bacteria (Adapted from De Schamphelaire et al., 2007).

2.2. Why do bacteria oxidize Mn?

Mn oxidizing bacteria are widespread in nature and can therefore be detected in almost all environmental samples. Their presence is especially notable when the environment contains high concentrations of Mn^{2+} (Tebo et al., 2005). Environments prone to harbour these organisms include water pipes and oxic-anoxic boundaries. The exact mechanisms associated with Mn^{2+} oxidation are still unknown and has been the subject of ongoing research. However, the presence of Mn oxidizing bacteria does seem to accelerate the oxidation of Mn^{2+} to MnO_2 compared to chemical oxidation. It is generally accepted that Mn^{2+} will be oxidized to MnO_2 in favourable environments, where after it precipitates and forms part of the sediment and the Mn cycling processes occurring there. However, Mn may also be recycled during the precipitation process (Tebo et al., 2005). The formation of Mn oxides further seems to play a role in other biogeochemical cycles, where it can react with reduced substances and become reduced in the process.

Although the reason for Mn oxidation has been questioned in the past, there are several benefits for bacteria to carry out this process. Although it has been suggested that Mn oxidation may simply be an evolutionary trait with minimal purpose, the large number of bacterial species that possess the

ability to oxidize Mn signifies that this process is beneficial for bacteria. Mn oxidation seems to have both intracellular as well as protective functions for the bacteria. An important advantage of Mn oxidation is the protection against ROS. Intracellular Mn may act as a scavenger of ROS such as superoxide, thereby serving as an antioxidant. The reaction of Mn^{2+} with superoxide results in oxidation to either Mn^{3+} or Mn^{4+} as it occurs in MnO_2 and this reaction is thought to be especially beneficial to bacteria such as *Deinococcus radiodurans*, where it provides a mechanism for resisting high concentrations of ionizing radiation. Mn is an important trace nutrient that is required for several cellular processes and therefore it is possible that Mn oxidation occurs as a consequence of its role in cellular functioning. Bacteria may also oxidize Mn to derive energy for chemolithoautotrophic growth, but even though this reaction is thermodynamically favourable and thus possible, there is no evidence to prove that this is a reason for Mn oxidation (Tebo et al., 2005).

The possibility exists that Mn oxidizing bacteria carry out this process as Mn oxides are needed as an end product for protection in an unfavourable environment. By oxidizing Mn^{2+}, Mn oxidizers can coat themselves in the resultant Mn oxides, protecting them from ultraviolet (UV) radiation, heavy metal toxicity or even viral attacks (Brouwers et al., 2000). Furthermore, Parikh & Chorover (2005), showed that monoculture biofilms capable of oxidizing Mn accumulated biomass at a faster rate in the presence of Mn oxides than in its absence. Mn oxides are also known to degrade humic substances to compounds of low molecular weight through oxidation, thereby enabling microbes to use these substances for microbial growth (Sunda et al., 1994). In conditions where carbon and other energy sources are scarce, it was proposed that Mn oxides may serve as a storage unit of electron acceptors until carbon becomes more readily available (Tebo et al., 2005). Although the specific reason for Mn oxidation is not yet known and fully understood, the widespread presence of Mn oxidizers in a wide variety of environments could suggest that the diversity of Mn oxidizers will continue to increase.

2.3. The mechanisms associated with bacterial oxidation of Mn

While the reason for Mn oxidation is still disputed and remains the focus of many studies, the question also remains as to how Mn is oxidized. The exact biochemical mechanism of this process is still unknown, but several details of this process have emerged through various studies. Several methods by which Mn can be oxidized have been proposed and it has been speculated that the growth of Mn oxidizing bacteria may lead to increased pH levels in its environment (Czekalla et al., 1985), allowing for the indirect oxidation of soluble Mn^{2+} through the process of chemical oxidation as environmental conditions become more favourable. However, in most cases this is not the process by which Mn is oxidized as biocatalytic processes are mostly responsible for this process (Czekalla et al., 1985;

Ghiorse, 1984; Gounot, 1986; Mouchet, 1992; Rittman, 1984; Schweisfurth, 1972). Furthermore, oxidation processes are not always the same and may occur by different means and at different locations depending on the bacteria. Three different methods are thought to explain this process: (1) Soluble Mn^{2+} is oxidized intracellularly through the action of an enzyme (Dubinina, 1979). (2) Soluble Mn^{2+} may be adsorbed to the surface of the cell membrane where after it is oxidized by enzymatic action as described above. During this process, the positively charged Mn^{2+} forms a bond with the negatively charged extracellular polymers produced by the oxidizing bacteria (Ghiorse, 1984). (3) Mn oxidizing bacteria may secrete biopolymers capable of oxidizing Mn in the vicinity of the bacterial cell. In all three processes the resulting product is MnO_2, which typically occurs as a black precipitate on the bacterial cells or sheaths of the filamentous bacteria. Precipitate forming as a result of biological oxidation tend to yield increased physical-chemical properties compared to that formed through the process of chemical oxidation. This is attributed to the produced MnO_2 being captured inside the extracellular polymeric substances (EPSs) during biological oxidation (Czekalla, 1985; Ghiorse, 1984; Mouchet, 1992; Tuschewitzki, 1983).

The oxidation of Mn^{2+} to MnO_2 is not a direct process but requires an intermediate in the form of Mn^{3+}. Studies with *Bacillus* sp. strain SG1 spores indicate that this process most likely occurs at the cell surface in vegetative cells. Studies on bacteria capable of oxidizing Mn have found genes encoding the multicopper oxidase enzyme. The specific process involved in Mn oxidation and the role that multicopper oxidazes (MCOs) play in this process have been questioned. As the oxidation of Mn^{2+} to MnO_2 requires the loss of two electrons and MCO mediated oxidation occurs through a one electron process, the role of MCOs in the biochemical process has never conclusively been demonstrated. Current knowledge of MCOs dictate that these enzymes oxidize substrates through a one electron process, which in the case of Mn would require more than one oxidation step, oxidizing Mn^{2+} to Mn^{3+} (Webb et al., 2005).

Three pathways have been proposed through which Mn^{2+} can be oxidized upon binding to the MCO: (1) Mn^{2+} is directly oxidized to MnO_2 through a one step process during which two electrons are removed. No Mn^{3+} intermediate is produced during this process. This pathway is considered unlikely as the two electrons that needs to be removed during this process are not energetically equivalent. (2) The second pathway is also unfavourable due to the energetics required and involves the oxidation of Mn^{2+} to Mn^{3+}, followed by dissociation of the produced Mn^{3+} from the enzyme binding site. Upon dissociation, the produced Mn^{3+} undergoes rapid disproportionation, the simultaneous oxidation and reduction of one substance, to produce both Mn^{2+} and Mn^{4+}. This pathway is both unlikely and energetically complex as a net input of energy would be required to produce free Mn^{3+}, or

alternatively an enzyme with a low potential Mn^{3+} binding site would be required. However, a low potential Mn^{3+} binding site may lead to increased Mn^{3+} stability, decreasing its ability to readily disproportionate. (3) Oxidation of Mn^{2+} occurs via two one electron transfer reactions which is enzymatically mediated. Mn^{2+} is first oxidized to Mn^{3+}, losing one electron in the process. The second electron transfer allows for the oxidation of Mn^{3+} to Mn^{4+} as it occurs in MnO_2. Mn^{3+} must therefore exist as an intermediate state in the two electron transfers, thus the third pathway seems most likely to occur, either on its own or in combination with the second pathway. To investigate this process further, chemical methods were used to trap Mn^{3+} intermediates produced during the oxidation reaction in spectroscopic experiments. Firstly, it was stated that Mn^{3+} has a short lifetime in aqueous solutions due to its ability to disproportionate rapidly to Mn^{2+} and Mn^{4+}. To detect the presence of Mn^{3+}, it was thus necessary to stabilize the Mn^{3+} intermediate should it be present. Pyrophosphate ($P_2O_7^{4-}$) was selected as a ligand binding complex, due to its ability to form a strong and stable complex with Mn^{3+}, as well as the strong coloured complex it forms in the UV visible spectrum. The results indicated that Mn^{3+} - pyrophosphate complexes did form during the oxidation reactions and that an enzymatic step was the cause of the Mn^{3+} - pyrophosphate complex decrease, resulting in the oxidation of Mn^{3+} to Mn^{4+}. Abiotic controls did not show a decrease of this complex (Webb et al., 2005).

Spectroscopic as well as kinetic results demonstrated the presence of Mn^{3+} intermediates during the oxidation of Mn^{2+} by the *Bacillus* sp. strain SG-1 spores. This in turn implies that oxidation of Mn^{2+} by bacteria occurs in two steps of one electron transfer reactions each, both mediated by enzymes. The exact mechanisms by which the Mn^{3+}-enzyme complex interaction functions and both oxidation steps occur still remains to be confirmed (Webb et al., 2005). Although this study only focused on Mn^{2+} oxidation by *Bacillus* sp. strain SG-1 spores, it is possible that the process described here is employed by other Mn oxidizing bacteria, as genes homologous to the *mnxG* gene encoding similar putative multicopper Mn oxidases have been identified in other bacteria capable of oxidizing Mn^{2+} (Webb et al., 2005).

Microorganisms capable of oxidizing Mn are not only limited to the bacterial domain, as members of the archaeal as well as the fungal domains have also been identified as capable of oxidizing Mn. In the bacterial domain, phylogenetic lineages such as the Firmicutes, Proteobacteria and Actinobacter were identified as being able to oxidize Mn (Tebo et al., 2005). Studies done by Larsen et al (1999) have found that *Pedomicrobium* sp. ACM 3067 can oxidize Mn during the early-to mid-log phase, which is in contrast to most other strains that oxidize Mn during the stationary phase.

2.4. Mn removal in water treatment facilities

Water treatment facilities in France were amongst the first to change over from conventional treatment methods to biological removal of Mn and Fe from water. Conventional treatment methods used worldwide fall into seven categories. These typically include aeration combined with a filtration system, chemical oxidation and the use of sequestering agents such as sodium silicate. In some cases, these treatment methods may be combined to produce more efficient removal of unwanted elements such as Mn and Fe. Up until the 1980's, aeration and filtration was most commonly used in combination with chemical oxidants in treatment facilities to remove Mn and Fe. However, these conventional methods have limitations, such as high operating costs, poor optimization and filtration rates, and often produce modest results (Andersen et al., 1973; Mouchet, 1992).

Biological oxidation was implemented after it was discovered that bacterial growth in conventional treatment plants were responsible for Fe removal. Some conventional treatment plants produced water of satisfactory standard despite poor oxidation of Fe in raw water. Upon closer investigation it was discovered that the treatment facility had experienced massive growth of Fe bacteria such as *Gallionella ferruginea* and *Leptothrix discophora* (Mouchet & Magnin, 1979). Further studies and the establishment of pilot plants, along with observations and identification of these organisms and their operating parameters in various treatment plants throughout Europe, helped establish the parameters used in biological oxidation today. It is understood that these bacteria are widespread but that their requirements for growth are species dependent. For successful biological oxidation, an optimal environment is needed to select for growth of oxidizing bacteria. These parameters typically include pH, DO concentration and temperature (Star et al, 1981; Starkey 1945). Salinity and organic content are also factors that need to be considered. Bacteria associated with Mn oxidation include *Leptothrix, Crenothrix, Metallogenium* and *Hyphomicrobium* with most of these organisms capable of oxidizing both Mn and Fe, where as other species such as *Pseudomonas manganoxidans* are only capable of oxidizing Mn (Mouchet, 1992).

2.4.1. *Local examples of Mn removal during water treatment*

The Preekstoel water treatment facility in Hermanus was originally designed to treat surface water from the De Bos dam for the supply of potable water to the greater Hermanus area. High water demand and severe droughts in the area during September 2010 to April 2012 forced the Overstrand Municipality to seek alternative water sources. Two groundwater well fields were identified as suitable for the continuous water supply to the Municipality. However, water from these wells

contained high concentrations of Mn and Fe, reaching values of 4 mg l^{-1} and 30 mg l^{-1} respectively, which were significantly higher than the treatment plant's design limits of 0.5 mg l^{-1} and 2 mg l^{-1} for Mn and Fe respectively. To compensate for this, caustic soda was added as a measure for pH control along with potassium permanganate to oxidize Mn and Fe prior to reaching the treatment plant, where after it could then be blended with surface water in its oxidized state (Du Toit et al., 2015). This solution, however, was not ideal as high cost, supply limitations and the large scale of the operation made the process practically unfeasible. An alternative treatment using biological oxidation was suggested.

Biological oxidation refers to the establishment of an environment which is optimal for colonization by bacteria or other microorganisms capable of oxidizing certain elements, such as Mn and Fe in this situation. Biofilms containing Mn oxidizing microorganisms are used as an advantage by applying the oxidation processes to remove Mn from drinking water. Important advantages of biological oxidation over chemical oxidation include the significantly reduced need for operator input and the lower operational costs involved. According to du Toit et al. (2015), optimal Mn oxidation requires an environment with pH levels ranging between 7.5 – 8.3 and a DO concentration between 5 and 6 mg l^{-1}, while optimal Fe oxidation requires pH levels ranging between 5.5 – 7.5 and a DO concentration between 0 and 1 mg l^{-1}. However, although these DO ranges can be used as a guideline, the exact optimal DO concentration under which Mn and Fe oxidation occurs differs in literature.

A study by Cai et al. (2015) indicated that DO concentrations deliberately maintained at 0.1 mg l^{-1} prevented Fe oxidation. Treatment facilities employing biological oxidation of Mn and Fe remove these elements separately, allowing oxidation of each element under the respective optimal conditions needed. The Preekstoel water treatment facility is designed according to these parameters to ensure optimal Mn and Fe oxidation. Cascade weirs with bypass controls are used to aerate the water to achieve optimal DO concentrations, while in-line pH control meters are used to regulate pH levels. Rapid gravity sand filters are used as colonising surfaces for microorganisms capable of performing Mn and Fe oxidation. This process occurs in two steps, first removing Fe and then Mn under the respective optimal conditions required for both elements separately. Once the Mn oxidizing bacteria come in contact with soluble Mn, it oxidizes the element, where after it precipitates and adsorbs to the sand filters, separating it from the water. After the water has been filtered of both Mn and Fe, it is dosed with chlorine gas for disinfection, where after it is blended with disinfected surface water.

Biological oxidation was initialised by colonising the respective sand filters with Mn and Fe – oxidizing microorganisms and maintaining the favourable DO and pH conditions required by the

respective communities to become established. The optimum colonisation time required before water treatment could start was a few weeks for Fe and up to three months for Mn. Interestingly, regular backwashing of the sand filters during the early colonisation steps led to significant loss of biomass, thus increasing the time needed for the establishment of robust biofilms. The shorter time required for Fe oxidizing bacteria to colonise the sand filters allowed Fe oxidizing bacteria to become established before Mn oxidizing bacteria during this early colonisation stages. Between the establishment period of Fe oxidizers and Mn oxidizers, it was determined that Fe could be effectively removed from the water, but that Mn remained in the soluble state, despite optimal DO and pH conditions. This serves as indication that Mn oxidation under purely physico-chemical conditions is a slow process and does not happen readily without a critical mass of Mn oxidizing bacteria (Du Toit et al., 2015).

Another important observation made during the early optimisation stages of this treatment facility was that the Fe filters would be blocked regularly due to precipitate unable to pass through. Oxygen entering the pipeline used to transport the untreated water from the boreholes was speculated to drive chemical oxidation of Fe en route to the treatment facility. The same problem was not observed for Mn, possibly due to its more complex redox kinetics, preventing it from oxidizing readily without the assistance of biological oxidation. Once the treatment facility was fully operational, further investigation found that the Mn and Fe concentrations differed significantly between the wells selected for groundwater supply and that this could lead to variations in the Mn and Fe concentrations in the water that had to be treated by biological oxidation. Initially this led to insufficient treatment during periods of rapid increase in Mn and Fe, as biofilters would remove these elements through oxidation, but not sufficiently to meet regulatory limits. However, as the Mn and Fe bacterial communities became better established over time, rapid increases in Mn and Fe did not seem to affect the efficiency of the biofilters, producing water within regulatory standards. A further concern was the re-establishment that would be needed if water flow to the biofilters had to be stopped. However, when the facility became operational after periods when the system was not in operation due to pipe bursts or shortage of NaOH for pH control, Mn – and Fe oxidizing communities recovered within hours after operations began, indicating that very little biomass was lost during stagnant periods, demonstrating the resilience of these biofilms once established (Du Toit et al., 2015).

Research done by Thompson, et al (Unpublished technical report) at Umgeni Water – Process services obtained results comparable to that of the Preekstoel water treatment facility. Initial establishment periods were similar to that of the Preekstoel facility, with Fe oxidizing bacteria requiring two to three weeks and Mn oxidizing bacteria approximately two months before showing efficient removal of Fe

and Mn, respectively. Their research found that Fe removal did not seem to be negatively affected after a two-week shutdown of the plant, indicating that there was no significant loss of Fe oxidizing bacteria. However, at one of the treatment plants, it was found that efficient Mn removal could not be maintained by the biofilter after a shutdown period of two-months. It was speculated that potential bacterial die-off during the two-month shut down was the cause, however, their study did not investigate this matter further. It was noted that the establishment period after this shutdown was much faster than during the initial establishment period. The researchers concluded that the presence of biofilms on the filter sand may have accelerated growth during re-establishment. Lastly their study also focused on the detection of bacteria present in both the Mn and Fe filters, identifying organisms such as *Pseudomonas manganoxidans* in Mn oxidizing filters and *Leptothrix* bacteria in Fe oxidizing filters.

Although water treatment facilities are not the focus of this study, their management and the details surrounding it is of considerable interest, as these treatment facilities aim to optimally simulate the natural conditions during which Mn oxidation may occur. The natural oxidation processes that are encouraged inside these treatment facilities are similar to that occurring inside the LBIS pipeline network where optimal environmental conditions such as high Mn concentrations, large surface area for biofilm attachment and favourable DO concentrations allow Mn oxidizing biofilms to oxidize soluble Mn^{2+}. However, whereas this is a desirable process in water treatment facilities to remove Mn from water, this same process is detrimental for the operation of the LBIS due to loss in water delivery and loss in pressure. Even though it has been found that the dynamic interactions observed in nature together with a constantly changing environment is near impossible to simulate in a laboratory setup (Kielemoes et al., 2002), these observations in operational water treatment facilities provide valuable information regarding the optimal conditions needed for Mn oxidation processes to occur, serving as a guideline for laboratory based experiments.

Biofilm formation in these systems are complex and not yet fully understood. Very little research have been done on the development of Mn oxidizing – and reducing biofilms under flow conditions in pipelines. Kielemoes et al (2002), investigated the occurrence of biocorrosion and the biofilms associated with this process. In their study, pilot scale flow-through systems fed with brackish water were used, testing different flow conditions over the period of the study. Cultivation of bacteria from biofilms that formed on these surfaces on selective growth media indicated the presence of Mn oxidizing bacteria, while chemical and microscopic analyses was used to confirm the presence of high levels of Mn and Fe, as well as filamentous microorganisms capable of precipitating Mn. These organisms were speculated to also be *Leptothrix* species.

Further analysis revealed that filamentous microorganisms dominated in the biofilms. These microorganisms showed interesting growth characteristics during periods when flow conditions were stagnant, with precipitated metals occurring on the outside of the filaments, as well as entanglement of the biofilm and adhesion of organisms such as diatoms. Features that were observed under various flow conditions also included the formation of micronodules rich in Mn. These micronodules are known to cause corrosion (Kielemoes et al., 2002).

2.5. Seasonal lake stratification

DO plays a key role in the chemical processes associated with Mn oxidation. Mn oxidizing bacteria can oxidize Mn in the presence of high DO, while low DO is favourable for Mn reduction. As such, the occurrence of lower concentrations of DO at greater depths may lead to the occurrence of higher concentrations of soluble Mn and Fe as proved in studies by Gantzer et al. (2009). The decreased concentration of DO observed at greater depth in a water body can be attributed to a process called stratification.

The cyclic pattern of DO in a water body throughout the seasons can be attributed to the change in temperature density profile from one season to the next. Seasonal lake stratification refers to the changes that occur in the temperature profile with depth in a water body. The temperature profile of a water body changes from one season to the next, creating a cyclic pattern that is repeated each year. At the beginning of spring, the temperature of large water bodies is typically the same from the surface to the bottom. Wind allows for mixing and circulation, pushing oxygen rich surface water downward and allowing water at the bottom of the water body to rise to the surface. This allows large amounts of oxygen to reach the bottom of the lake and is called spring overturn (As the lake turns: The seasonal cycle of lake stratification, 2015; Quillen).

However, as summer approaches, air temperatures start to rise and heat from the sun begins to warm the water. As the sun heats the water from the surface downwards, the amount of solar radiation absorbed decreases with depth and a layer of less dense, warmer water called the epilimnion begins to form above the cooler water at the bottom of the water body called the hypolimnion (Kirillin & Shatwell, 2016). These two layers are separated by the metalimnion, forming three distinct layers during complete stratification. During the summer months, stratification prevents complete water mixing, as warm water from the epilimnion is unable to drive through the cooler water of the hypolimnion, allowing wind to only circulate warm water in the epilimnion. Insufficient mixing of the top and bottom water layers prevents DO from reaching the bottom of the water body (Gebhart

& Summerfelt, 1978). Microorganisms in the hypolimnion use the available oxygen in their metabolic processes during biodegradation of algal biomass when it sinks to the hypolimnion, causing the hypolimnion to become anoxic during this period (Beutel and Horne, 1999). The start of autumn is accompanied by a decrease in temperature, leading to less distinct water layers and ultimately causes the water body to lose its stratification (As the lake turns: The seasonal cycle of lake stratification, 2015; Quillen).

2.6. Mn management in open water bodies: Carvins Cove, West Virginia, USA as case study

Water treatment facilities around the world need to adhere to specific standards dependent on the country or region, as well as the end use of the water. These facilities, and the treatment methods they employ therefore often differ. Previous studies performed by Gantzer et al. (2009) investigated the use of a full scale hypolimnetic oxygenation system for the control of Mn concentrations in a drinking water supply reservoir. The Carvins Cove reservoir with a depth of 23 m is situated in South West Virginia, USA and is used to supply water to a treatment facility, where it is treated to be suitable for drinking. The authors reported increases in soluble Mn and Fe concentrations during summer months as a result of summer stratification, leading to increased concentrations of Mn entering the treatment facility, where it could not be sufficiently removed due to its high concentration. This subsequently lead to poor water quality as a result of high concentrations of Mn causing discolouration, staining and unpleasant smells in water (Cerrato et al., 2010; Gantzer et al., 2009). Monthly water samples were collected from 1999 to 2005 to record the concentrations of Mn and Fe in the reservoir and to determine whether it consistently remained high. To address summer anoxia experienced in the hypolimnion, a diffuser (oxygenation system) was installed by the Western Virginia Water Authority in 2005 to control the levels of soluble Mn and Fe by oxygenating the hypolimnion during the summer period using pure oxygen. Air flow rates were adjusted to maintain DO concentrations at or above 7 mg l^{-1} in the bulk water of the hypolimnion. The diffuser was continuously operated from August 2005 to June 2006, where after it was switched off for a month-long test period. The system was switched on again in July 2006 and has been in continuous operation ever since.

Data collected from 1999 to 2005 indicated that Mn levels did not consistently remain high throughout this six-year period. During summer months with prominent stratification and low DO concentration in the hypolimnion, soluble Mn and Fe concentrations typically increased. However, following fall turnover, sufficient water mixing led to an increase in DO concentration of the hypolimnion. During this period, soluble Fe concentrations typically decreased as expected. In

contrast, due to its complex redox kinetics, Mn oxidation was less predictable and controlled by these natural DO shifts (Gantzer et al., 2009). One of the main reasons for the installation of the diffuser is because of its ability to aid in the control of the oxic/anoxic boundary that forms close to the sediment. During winter periods when the water body is naturally mixed, the water column is typically isothermal. This causes the distribution of DO to be more uniform throughout the water body, leading to turbulence at the sediment–water interface, driving the oxic/anoxic boundary deeper into the sediment (Katsev et al., 2007). It was thus hypothesised that the decrease observed in Mn concentration during the winter months could be explained by the increase in DO concentrations in the hypolimnion, leading to oxidation and precipitation of Mn, a shift of the oxic/anoxic boundary deeper into the sediment, as well as a corresponding shift in the microbial communities responsible for oxidizing Mn. The inverse could then be assumed for summer stratification, where the lowered DO concentration in the hypolimnion effectively isolates the hypolimnion from the epilimnion, leading to a decrease in DO and favouring the growth of Mn reducing communities. During summer stratification when mixing occurs predominantly within the epilimnion, little DO rich water is transferred to the hypolimnion, causing the oxic/anoxic boundary to move up out of the sediment into the overlying water, where reduced, soluble Mn^{2+} can be released into the hypolimnion (Gantzer et al., 2009).

During the summer periods, the diffuser allowed for mixing in the hypolimnion. To determine if mixing induced by the diffuser led to turbulence, measurements of the diffusive boundary layer were taken in the reservoir at various locations, both near and close to the diffuser. The diffusive boundary layer is a viscous layer of water, found immediately above the sediment-water interface, that decrease in thickness with increased turbulence (Hondzo et al., 2005; Lorke et al., 2003). Measurements taken showed variations in the thickness of the diffusive boundary layer at the various sampling points, indicating the existence of turbulence within the hypolimnion due to mixing induced by diffuser operation. However, Gantzer et al. (2009) found that diffuser induced mixing was lower and less uniform than what would typically be observed during natural mixing in winter. Between June and July 2006, the diffuser was shut down for a period of one month in order to compare the effect of diffuser induced mixing in the hypolimnion with the conditions that would develop in natural conditions during the summer months. After the month-long shutdown, average DO concentrations in the hypolimnion decreased from above 7 mg l^{-1} to below 5 mg l^{-1}. Upon closer examination, it was discovered that the DO concentrations in the upper and lower hypolimnion were different, with DO concentrations in the lower hypolimnion decreasing to below 1 mg l^{-1}. Following this decrease, total Mn concentration in the upper hypolimnion decreased, while total Mn concentrations in the lower hypolimnion increased. It was determined that the increase in total Mn observed in the lower

hypolimnion was as a result of an increase in soluble Mn concentrations, corresponding to the decrease in DO concentrations in the lower hypolimnion.

After the diffuser had been switched on again, DO concentrations in the hypolimnion increased to 5 mg l^{-1} and above and was maintained for 10 days, while total Mn levels were evenly distributed throughout the upper and lower hypolimnion. However, soluble Mn concentrations in the hypolimnion continued to increase, despite the high DO concentrations. It was speculated that the benthic region (the lowest region in the water body including the sediment surface and some sub-surface layers) may be the source of soluble Mn (Kusky, 2010; Russel et al., 2011). It was concluded that the most likely explanation could be the horizontal transport of Mn from parts in the upper hypolimnion that are in contact with hypoxic sediments further away at shallower depth. Soluble Mn concentrations continued to remain high for one month after the diffuser had been switched on and only started to show a decrease once the average DO throughout the hypolimnion increased to 7 mg l^{-1} (Gantzer et al., 2009).

This research furthermore made some important conclusions, stating that even though the delay in Mn decrease in the hypolimnion could be due to the slow oxidation kinetics of Mn in natural water at a pH of 8.0, Mn oxidation by chemical reactions alone is unlikely. Furthermore, it was noted that the observed decrease in soluble Mn occurred during periods when organic matter entered the hypolimnion after summer algal growth. It was thus concluded that the well oxygenated hypolimnion combined with organic matter may have played a role in the oxidation of soluble Mn, causing it to precipitate (Gantzer et al., 2009). After continuous operation of the diffuser during 2007, both soluble and oxidized Mn concentrations were lower in the hypolimnion than in 2006 when the diffuser was switched off for one month. It was finally concluded that oxygenation by means of a diffuser was successful in maintaining a higher DO concentration throughout the hypolimnion and that this was successful in decreasing the concentration of soluble Mn concentrations in the hypolimnion. Although DO concentration plays a crucial role in Mn oxidation, it is not solely dependent on chemical oxidation but is also influenced by role players such as Mn-oxidizing microorganisms (Gantzer et al., 2009).

2.7. The Blyderiver dam and biofilm growth inside the Lower Blyde Irrigation System, Mpumalanga, South Africa

The Blyderiver dam, located in Mpumalanga, South Africa, is an important source of water for the production of a variety of produce in the surrounding area. The dam with a capacity of approximately 54 million cubic meters is mainly supplied with water from two incoming rivers, the Orighstad river and the Blyderiver, with contribution from the Treur river. Water from this dam is used for gravity fed irrigation to farms in the Mpumalanga and Limpopo provinces for irrigation purposes via the LBIS, a pipeline network of approximately 150 km. The pipeline varies in diameter from 1500 mm to 250 mm. Constructed between 1998 - 2003, the LBIS was originally developed to use the limited water resources optimally, thus reducing the amount of losses and providing a convenient, pressurised water supply to farmers in the surrounding area and managing the water allocations to the various farms. A decrease in water delivery, below design specifications has been observed. It was suspected that bacterial growth inside the pipeline in the form of biofilms could be a cause of the problem, and this view was supported following inspections of the inner walls of the pipeline (Fig. 2.2 and Unpublished internal reports; prepared by various contributors for MBB Consulting Engineers).

Biofilms can be described as surface associated, highly organized microbial communities that produce EPSs in which the microbial cells are encased and can be considered as one of the most successful forms of life on earth, capable of tolerating unfavourable conditions such as high concentrations of biocides (Flemming & Ridgway, 2008; Willey, Sherwood & Woolverton, 2011). Due to water flow only being propelled by gravity through the pipeline, a loss of water delivery results in less water availability for irrigation purposes, posing major problems for farmers as a sizeable percentage of South Africa's mangoes, tomatoes, papayas and avocadoes are produced in this region. Disturbances in the constant water supply needed for these farms could thus have severe consequences for the local economy (Unpublished internal reports; prepared by various contributors for MBB Consulting Engineers).

The inner wall of pipelines that convey surface waters offers an ideal location for biofilm development, providing a relatively large surface area to grow on as well as a constant nutrient supply through passing water. High levels of Mn in the water could potentially contribute to the growth of Mn oxidizing - and reducing microorganisms (Tebo et al., 2005). Although high Mn and Fe concentrations and their negative effects in water treatment facilities have been reported as described above, very little literature exist on the full-scale effects caused by biofilms capable of oxidizing and reducing Mn inside a pipeline such as that faced inside the LBIS network. Water treatment facilities

typically only focus on the removal of Mn and Fe and decreasing its concentration to below the drinking water limits, whereas limited attention is given to the concentrations of Mn and Fe eventually reaching the farms at the end of this pipeline, as this is solely used for irrigation purposes. By investigating previous case studies and using knowledge regarding the optimal environmental factors needed for Mn oxidation and reduction, comparisons can be drawn between the biofilms growing inside the pipeline compared to that observed in water treatment facilities. This leads to a better understanding regarding biofilm growth and development and how to reduce or treat these biofilms, instead of optimizing environmental conditions for enhanced growth.

Fig. 2.2 - Thick biofilms are present in sections of the LBIS network, leading to reduced water flow through the pipeline.

Although chemical disinfection methods such as biocides have been considered as treatment methods, these treatments are often accompanied by several obstacles that need to be taken into account. Biocides are known to remove most surface contamination, but in many cases some microorganisms survive and can continue to thrive (Bagge-Ravn et al., 2003). The resistance of these microorganisms are often associated with the presence of biofilms (Bressler et al., 2009; Vestby et al., 2009), where they are embedded in the biofilm matrix and are known to display specific properties, such as increased resistance to biocides (Nett et al., 2008; Smith & Hunter, 2008). Furthermore, although several standard protocols exist for the treatment of planktonic cells, the same is not true for the evaluation of biofilm susceptibly, making it difficult to adapt treatments to remove biofilms (Meylheuc et al., 2006; Ntsama-Essomba et al., 1997).

Commonly used biocides such as H_2O_2 and O_3 have been considered as treatment methods for the removal of biofilms from the inside of the LBIS pipelines. However, in water, H_2O_2 has a tendency to decompose to water and oxygen as these products are more stable than H_2O_2 (Petrucci et al., 2007). This reaction occurs as follows:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Mn oxide can act as catalyst, with its surface offering a favourable environment for this decomposition reaction. Therefore, H_2O_2 should not be used as a biocide in environments with high Mn concentrations as it reduces its biocide capability (Knol et al., 2015). O_3 may not offer an effective strategy for biofilm removal either. Firstly, O_3 is known to be a powerful oxidizing agent that can act rapidly on a wide spectrum of microorganisms (Robbins et al., 2005; Tachikawa et al., 2009; White, 1999). Due to its oxidizing capability, O_3 may thus induce oxidation of soluble Mn and Fe in the water and biofilms, thereby modifying or blocking the flow channels inside the biofilm structure and decreasing the amount of oxygen capable of entering the biofilm and stopping the O_3 mode of action. Secondly, O_3 may modify the outer layers of the EPS, which would also form a barrier and preventing oxygen from entering the biofilm. Thirdly, O_3 is unstable in water, with a half-life of only one hour at pH 8.0. This period is too short to ensure effective biofilm removal over a long time period (Cloete et al., 1998). It was thus concluded that O_3 may be used as a biocide to kill suspended cells and restrict further biofilm development, but that it would not be suitable for biofilm removal over a long time period. It should also be noted that even though biocides may kill or inhibit growth, remaining biofilm layers not removed by the biocides may still disturb water flow inside the pipelines, and serve as scaffold for renewed biofilm growth as well as a nutrient reserve. It is therefore often suggested that combinations of chemical and mechanical treatment methods should be employed to effectively kill microorganisms in biofilms and remove all biomass. However, complete removal of biofilms from water distribution networks is close to impossible, therefore attention should rather be focused at controlling it (Unpublished internal reports; prepared by various contributors for MBB Consulting Engineers; Flemming et al., 2011).

One of the major problems with studying biofilm growth inside water distribution lines is accessibility. Due to the high demand for water and continuous operation, shutting down the pipeline typically requires careful planning and is only allowed for a maximum period of 48 hours. Other factors such as depressurisation and opening of the pipeline also needs to be considered as this process take several hours to complete. To compensate for this problem, a separate experimental pipe rig was constructed and connected to the LBIS network and supplied with the same water, but could be

independently shut down and opened for biofilm collection without disrupting flow to the LBIS main pipeline. Biofilms were allowed to develop naturally in these experimental pipe rigs to investigate their growth dynamics over time. A polyurethane pigging/scraping device was constructed and used to scrape biofilms from the inside of the experimental pipe rigs for analysis. Biofilm development inside pipe rigs were monitored for one year to observe seasonal variation, and accumulated biofilm mass was observed to be highest during June, decreasing and reaching its minimum values during the summer period (Unpublished internal reports; prepared by various contributors for MBB Consulting Engineers).

The Blyderiver dam's hydraulic retention time was also monitored over four years by dividing the dam's capacity of 54 million cubic meters into the net flow rate (the difference between all the inflows and outflows from the dam) into the dam. It was found that the maximum retention time occurs during the winter months, with the lowest retention periods observed between January and April, typically the period within which this area receives its maximum rainfall. This region typically receives little rainfall during the winter months, resulting in less inflow and a higher average dam retention (Climate: Mpumalanga; Unpublished internal reports; prepared by various contributors for MBB Consulting Engineers).

Comparing the accumulated biofilm mass values to that of the dam's retention curve, it was observed that biofilm accumulation followed a similar pattern to that of the dam's retention curve, reaching its maximum accumulation mass during the winter months. The internal surface of the LBIS pipeline network also offered an ideal surface for biofilm development, with high flow conditions providing a constant nutrient supply. Similar results have been reported by previous studies (Lehtola et al., 2006). The turbulent flow experienced inside the pipeline allows for better diffusion of nutrients from the water to the biofilms. Furthermore, this study monitored the production of EPS and found that a decrease in nutrient availability resulted in an increase in EPS production. Following a rainy season, an increase in dissolved molecules and suspended solids in the water led to a higher attachment rate of these solids onto the biofilm, lowering its organic fraction. Bacterial growth in the biofilm is driven by the increase in dissolved molecules such as Mn, Fe and nutrients. The authors speculated that the resultant increase in biofilm growth following the rainy season led to increased sloughing, resulting in higher concentrations of EPS in the water that could collect and transport sediment entering the pipeline. Results indicated that sediment deposition followed similar patterns to that of biofilm accumulation and dam retention and played an important role in the biofilm's composition. Apart from this, it was concluded that flow velocity also impacts the rate at which biofilms develop and plays a role in nutrient transport as well as cause shear stress, both considered

to be critical factors affecting biofilm development (Lehtola et al., 2006; Ollos et al., 2003; Unpublished internal reports; prepared by various contributors for MBB Consulting Engineers).

To investigate the development and growth of biofilms inside the LBIS pipeline, biofilms were left to develop for an initial 28 days before being collected from both test pipe rigs by means of pigging over a period of approximately 200 days thereafter and dried. Results indicated that biofilm dry mass peaked between approximately 70 to 80 days after the start of pigging, where after it decreased rapidly and achieved a consistent mass. Biofilms collected during this time was also subjected to inductively coupled plasma mass spectrometry (ICP-MS), to measure Mn and Fe content in the biofilms. Both Mn and Fe concentrations inside the biofilms followed similar patterns to biofilm accumulation, increasing rapidly within the first 50 days and both reaching its maximum concentration after approximately 50 days. However, biofilm accumulation only achieved its maximum mass after 80 days. No possible explanation for this observation was given in the previous study. The observed results were consistent for biofilm accumulation, as well as Mn and Fe concentration in both test pipe rigs (Unpublished internal reports; prepared by various contributors for MBB Consulting Engineers).

2.7.1. *Biofilm accumulation on different pipe surfaces*

In another unpublished internal report to MBB consulting engineers, the formation of biofilms on different materials similar to that used in various sections of piping of the LBIS network was investigated. Using a Modified Pederson Device, biofilm formation on slides of asbestos cement, cast iron with an epoxy coating, galvanised steel and polyvinyl chloride (PVC) plastic was monitored over a period of 672 hours, with slides removed at different stages to investigate biofilm growth over time. Biofilms were subjected to epifluorescent microscopy, scanning electron microscopy as well as colony counts on agar plates.

Although biofilms formed on all the materials tested, the development of these biofilms differed between the different piping materials used, with some materials being more favourable for the adhesion of biofilms than others. Microscopic analysis indicated that biofilms formed rapidly on asbestos cement and PVC within the first 48 hours. The rate of biofilm increase on asbestos cement remained constant for the further duration of the experiment, whereas a decrease in biofilm formation could be observed on PVC after 48 hours. Biofilm development on the galvanized steel and coated cast iron were both higher overall than the other materials tested, with biofilm development remaining high throughout the entire test period. Coated cast iron showed high biofilm development during the

first few days and fluctuated thereafter (Unpublished internal reports; prepared by various contributors for MBB Consulting Engineers).

Colony counts indicated that biofilm development on all four materials tested followed the same growth pattern, following a gradual increase from day 0 up to day 7, where after growth rapidly accelerated, reaching its maximum for all four materials on day 11. After this period the colony counts decreased and stabilized for the remaining time period up to 24 days. The study found that results obtained corresponded to that seen inside the LBIS pipeline network, with biofilm growth notably present on the inside surfaces of both asbestos cement and epoxy coated cast iron pipes (Unpublished internal reports; prepared by various contributors for MBB Consulting Engineers). These results corresponded to that found in previous studies, with bacterial biomass being the highest on iron and lowest on PVC (Niquette et al., 2000).

2.8. Potential negative effects of Mn in irrigation water

Although water supplied by the LBIS pipeline network is used for irrigation purposes, attention should be given to the high concentration of certain elements present in the water. It has been shown that aluminium (Al) and elements such as Mn have growth limiting capabilities, where high concentrations of these elements in soil has the potential to be toxic to plant roots, leading to poor or even abnormal root development. When plants are subjected to high levels of these elements, their roots often become thick and stubby, with very little development of the fine root system. This leads to reduced plant growth due to less efficient uptake of water and nutrients. Plant growth is especially impacted in periods of drought due to decreased penetration of the root system in the soil sublayers, making less water available for plant consumption (Miles et al. 2013).

The studies performed at water treatment facilities and the Blyderiver dam along with the results obtained is of valuable interest to this study. These findings provide important information regarding the environments in which Mn oxidation occur, that can serve as guide to design laboratory simulation experiments to investigate the growth dynamics of these complex biofilms and how they react to changes in DO and Mn concentration. Although literature encompassing all the factors influencing Mn oxidation and biofilm development in the Blyderiver dam and LBIS pipeline network is still very limited, the combination of the literature captured here provides relevant information on many of the individual factors at play. This study aimed to expand the current knowledge of these processes.

Chapter 3

Potential role of dissolved oxygen and manganese concentration on the development of biofilms causing reduction in hydraulic capacity of a gravity-fed irrigation system*

***This chapter is currently in preparation for publication**

Potential role of dissolved oxygen and manganese concentration on the development of biofilms causing reduction in hydraulic capacity of a gravity-fed irrigation system

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Key Words: Manganese, Dissolved Oxygen, Biofilms, Oxidation

Highlights

- Biofilms with high Mn content reduced hydraulic capacity of the irrigation system
- Total Mn concentrations had a strong inverse relationship with measured DO profiles
- Reservoir's water column showed DO concentrations of ≤ 2 mg l^{-1} in deeper zones
- Water for the irrigation system is extracted from this low DO – high Mn zone

Abstract

Water from the Blyderiver dam in the Mpumalanga province, South Africa, is used for gravity-fed irrigation. Biofilm development in the pipelines causes a reduction in hydraulic capacity, estimated to be as high as 20%, resulting in water delivery below design capacity for the production of a variety of produce such as mangoes, tomatoes, papayas and citrus cultivated on nearly 7,000 hectares. The potential role of manganese (Mn) concentration on biofilm development is of interest, since the water is currently extracted at depth near the bottom of the reservoir where high Mn levels were measured during four sampling events spread over two years. Dissolved oxygen (DO) and Mn concentrations in the water yielded a strong, inverse correlation, with rapid decrease in DO at increased depth, mirrored by an increase in both total and soluble Mn. The depth of this inflection point was found to correlate with the reservoir's water level. DO concentrations typically remained constant between 8 and 9 mg l^{-1} in the upper 30 + meters of the water column, followed by a rapid decline to lower than 2 mg l^{-1} at deeper depths. Similarly, Mn concentrations remained constant with increasing depth, ranging between 10 and 100 µg l^{-1}, followed by a rapid increase once the depth is reached where DO levels started to decline, higher than 8000 µg l^{-1} near the bottom. The extraction point for the pipeline is located at this depth of approximately 45 m when the reservoir is at 100% capacity. Mn concentrations decreased with distance along the pipeline; inductively coupled plasma mass spectrometry (ICP-MS) analyses indicated a decrease from 8631 µg l^{-1} at the extraction point to 134 µg l^{-1} at 23 km downstream in the bulk aqueous phase, while biofilm biomass decreased from

30105.4 mg kg^{-1} at 4.5 km to 23501.9 mg kg^{-1} at 12.5 km, and 13727.7 mg kg^{-1} at 28.4 km downstream. This decrease in Mn concentration with distance indicates that there is a net ongoing deposit of Mn in the pipeline, suggesting incorporation of Mn in the biofilm's extracellular polymeric matrix and thus sustained biofilm formation that has not yet reached a steady state.

3.1. Introduction

The Blyderiver dam, located in Mpumalanga, South Africa, is a major source of water used for irrigation of a variety of agricultural produce. The dam with a capacity of approximately 54 million cubic meters is primarily supplied with water from two incoming rivers, the Orighstad river and the Blyderiver, with some minor contribution from the Treur river. Water from this dam is supplied under gravity to farms in the Mpumalanga and Limpopo provinces via the Lower Blyde Irrigation System (LBIS), a pipeline network of approximately 150 km. The pipeline varies in diameter from 1500 mm to 250 mm. Constructed between 1998 – 2003, the LBIS was originally developed to optimize the limited water resources, including eliminating leakages and evaporation from the original canal system, reducing energy consumption for pumping by providing water under pressure, and improved management of water allocations. A gradual loss in hydraulic capacity over a number of years were linked to biofilm formation in the pipe network (Unpublished internal reports; prepared by various contributors for MBB Consulting Engineers). This has serious implications for the local economy and agricultural output, as a notable share of the country's mangoes, citrus, papaya and avocadoes are produced on the ~7000 hectares of land being irrigated.

Although biocides may offer a potential solution to this problem, its use is accompanied by several factors that needs to be considered. The use of biocides such as H_2O_2 and O_3 have been proposed. However, the local geology is known for the presence of Mn. A previous study has identified oxisols with Mn content of up to 17% in the Graskop region of the dam's catchment, and attributed substantial Mn release to natural wet-dry cycles of topsoil (Dowding, 2004). Leaching of this Mn makes the use of biocides such as H_2O_2 and O_3 impractical as Mn acts as a catalyst for the decomposition of H_2O_2 to water and oxygen in the presence of water (Petrucci, 2007), while O_3 has powerful oxidizing capabilities which would contribute to Mn oxidation inside the pipeline, potentially worsening the problem (Robbins et al., 2005; Tachikawa et al., 2009; White, 1999). Indeed, biofilms could not be effectively removed using these biocides at dosages that would be cost effective during independent on-site pilot studies. For example, a field experiment that involved a 50 mg l^{-1} H_2O_2 treatment (flow rate had to be lowered from ~2000 l s^{-1} to below 500 l s^{-1} to achieve this) did not result in noticeable

biofilm detachment, and exhibited a marginal friction loss in a 6,000 m-long test section (Unpublished internal reports; prepared by various contributors for MBB Consulting Engineers).

Since it is known that high levels of Mn in water support the growth of Mn oxidizing and reducing microorganisms (Tebo et al., 2005), this became the focus as a potential cause of increased biofilm formation in the pipeline. The occurrence of Mn as a nuisance contaminant in water distribution systems is not uncommon and globally this problem has been reported several times (Bryant et al., 2011; Cerrato et al., 2010; Gantzer at al., 2009; Stauffer, 1986). Mn can occur in different forms in nature but due to its complex redox kinetics, it is difficult to chemically oxidize in environments with pH levels of 6 – 8 typically observed in natural waters. In conventional water treatment plants, Mn is removed through oxidation by the addition of oxidants such as chlorine dioxide or potassium permanganate (Bryant et al., 2011; Gantzer et al., 2009). However, Mn can also be oxidized by Mn oxidizing microorganisms, typically converting manganous (Mn^{2+}) to a Mn^{3+} intermediate before final conversion to Mn^{4+} as it occurs in Mn dioxide (MnO_2) through the process of biological oxidation (Webb et al., 2005). This process typically occurs in environments with high DO concentrations, while biological reduction from MnO_2 to Mn^{2+} typically occurs in environments with low DO concentrations as indicated in Fig. 3.1.

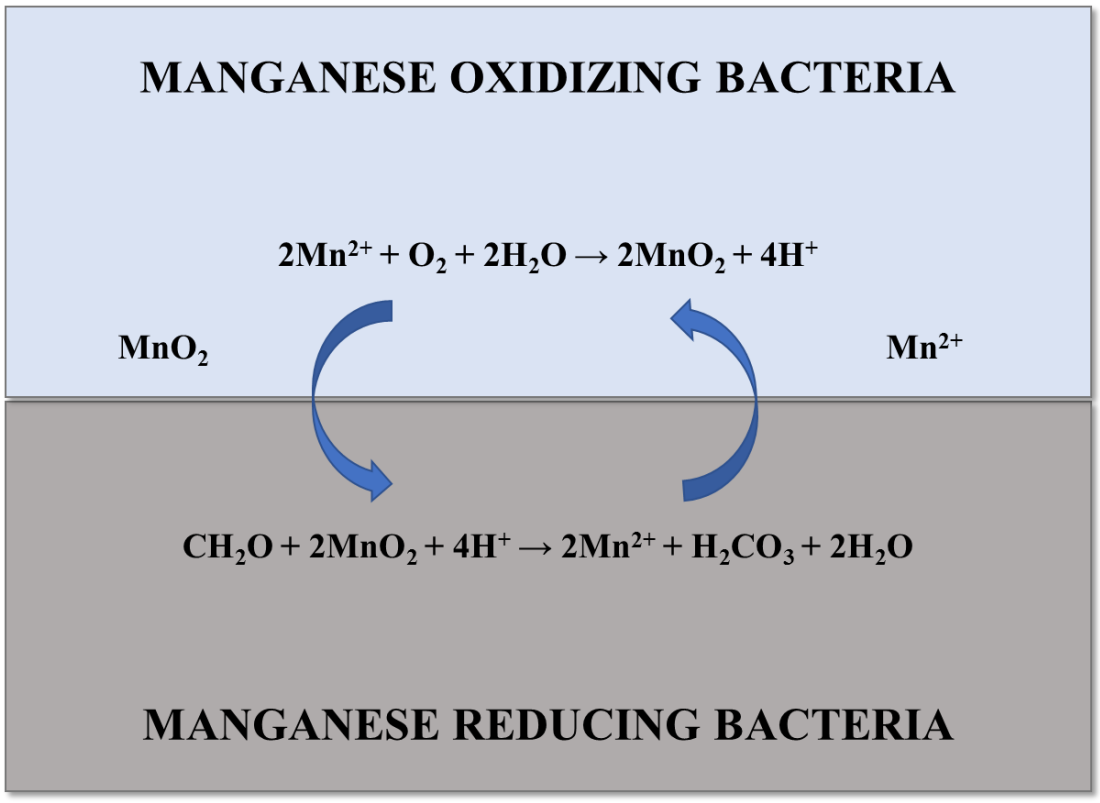

Fig. 3.1 - The Mn cycle as carried out by Mn oxidizing and reducing bacteria (Adapted from De Schamphelaire et al., 2007).

Gantzer et al., (2009) indicated that high total Mn concentrations persist in the hypoxic hypolimnion of a reservoir in Carvins Cove, West Virginia, USA, especially during summer stratification when DO concentrations often decrease to hypoxic levels in the deeper zones. Considering the high Mn content in the catchment soils, and preliminary results showing a high Mn content in the biofilms, we hypothesized that an improved knowledge of the Blyderiver dam's geochemistry may provide cues to be considered when devising strategies to mitigate the biofilm build-up in the irrigation pipeline. The objectives were therefore to i) investigate the relationship between DO and Mn concentration with depth in the water column, with specific focus on the depth of extraction, ii) determine Mn concentrations in both aqueous phase and biofilms with distance in the pipeline, and iii) perform laboratory experiments under defined conditions in an effort to better understand biofilm development.

3.2. Materials and methods

3.2.1. Sampling sites

The Blyderiver dam is in the Mpumalanga province in South Africa with coordinates 24º32'59"S 30º48'22"E and a depth of approximately 45 – 50 m when 100% full. Water samples were collected at different sites as indicated in Fig. 3.2. Additionally, samples were collected from the LBIS pipeline at various points downstream from the dam wall extraction point along the three pipeline routes stretching into the Limpopo province up to a distance of 31.7 km (Appendix A).

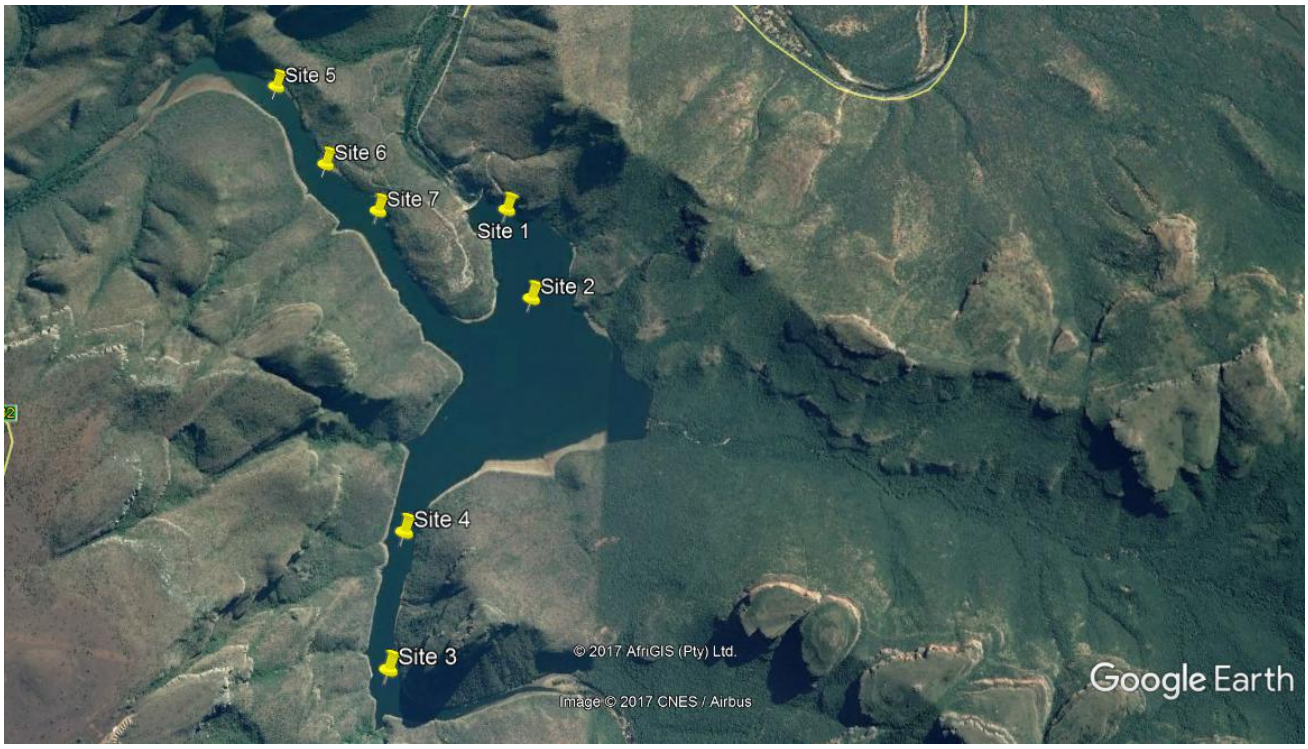

Fig. 3.2 - Sampling sites on the Blyderiver dam as indicated by yellow pins. Water samples were collected at 1 to 2 m depth intervals. Site 1 is nearest to the wall, and sites 3 and 5 are the sampling points closest to the influences of the Blyde and Orighstad rivers, respectively.

3.2.2. Sampling procedure

Four sampling events were undertaken over two years from October 2015 to February 2017 during different seasons. Water samples were collected using a custom-made water sampler, comparable to a Niskin sampler, at two-meter increments over the full depth profile of all sampling sites indicated in Fig. 3.2, except sites 3 and 6, with the dam's levels varying between 40% and 100% full over the two years. The samples were separated into filtered and unfiltered aliquots and acidified with 65% nitric acid (VWR Chemicals). Water samples were filtered through 0.22 µm syringe filters to determine soluble Mn concentrations. Both filtered and unfiltered samples were submitted for ICP-MS analysis at the Central Analytical Facility (CAF) at Stellenbosch University to measure soluble and total Mn concentrations, respectively. Although the presence of iron (Fe) was not the focus of this study, its concentration was measured during all sampling events and included in all data sets as it is suspected that Fe may contribute to the formation of Mn oxidizing – and reducing biofilms.

Continuous vertical profiles of DO and temperature concentrations through the full depth of the water column were done on-site using a YSI Professional Series DO meter. Sediment samples were collected at each sampling site and analyzed for total Mn via acid digestion and ICP-MS at CAF. In

addition to sampling of the reservoir's water columns, water and biofilm samples were collected at different locations along the LBIS pipeline network, along with DO measurements, and submitted for ICP-MS analysis. Biofilm samples were also dried at 26°C before visualization with scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM/EDS) for elemental analysis.

3.2.2.1. First sampling event – October 2015

Samples were only collected from sampling site 1 in two-meter increments down to the water-sediment interface approximately 240 m from the dam wall. Additionally, biofilm samples were collected from LBIS 1, 4.5 km downstream from the extraction point (Appendix A), where after they were dried at 26°C for 72 h before visualization with SEM/EDS.

3.2.2.2. Second sampling event – May 2016

Water samples were collected from sites 1, 2, 4, 5 and 7 (Fig. 3.2) up to the maximum depth of each site. Sites 1 and 2 were the deepest, with maximum depths of approximately 44 m and 35 m respectively. Sediment samples were also collected from all seven sampling sites and sent for ICP-MS analysis. Additional water samples were collected in the LBIS pipeline network at the LBIS 1 and LBIS 3 (approximately 23 km downstream from the dam wall) sampling sites. Biofilm samples were also collected from the LBIS 1 sampling site, where after it was dried and subjected to SEM/EDS analysis. SEM/EDS analysis on biofilms collected only during the first and second sampling events were performed using the Phenom ProX scanning electron microscope.

3.2.2.3. Third sampling event – October 2016

Water samples were only collected from sampling site 1 (Fig. 3.2) because, due to very little rainfall in 2016 and subsequently a low water level, sampling at the other sites was not possible.

3.2.2.4. Fourth sampling event – February 2017

The fourth sampling event involved water samples collected at sampling sites 1, 2, 4 and 7 (Fig. 3.2), as well as water samples along with DO measurements from the LBIS pipeline network at the following sampling sites downstream from the dam wall: LBIS 1 (4.5 km), LBIS 2 (6 km), LBIS 3 (23 km), LBIS 4 (26.3 km), LBIS 5 (25 km) , LBIS 6 (28.4 km), LBIS 7 (17.6 km), LBIS 8 (27.3 km) and LBIS 9 (31.7 km). The pipeline branches into various routes, with the main line

branching into routes 1 and 2 at LBIS 2 and route 2 subsequently branching into routes 2 and 3 approximately 4.5 km further.

Additional to the water samples, biofilm samples were also collected from the LBIS 1, LBIS 6 and LBIS 10 (12.5 km) sites (Appendix A). Biofilms were collected from these locations as they were the only sites where the pipeline could be opened for biofilm collection. Although these sampling sites are located on two different routes, the LBIS 6 sampling site occasionally receives water from route 1, depending on waterflow inside the pipeline. Biofilm samples were dried at 26°C for 72 h prior to acid digestion and ICP-MS analysis, as well as analysis by SEM/EDS using the Zeiss Merlin scanning electron microscope equipped with an energy dispersive X-ray spectrometer at CAF. All samples were coated with gold prior to SEM/EDS analysis. Total Mn mass per dry biomass was calculated.

3.3. Results and Discussion

3.3.1. Water profiles: DO and Mn concentration

3.3.1.1. First sampling event – October 2015

The water level of the dam was at approximately 80% and it was expected that DO levels near the bottom of the dam would be similar to that of the epilimnion, due to sufficient mixing between the two layers typically observed during turnover between seasons (Gantzer et al., 2009; Willey et al., 2011). However, as indicated in Fig. 3.3A, there was a marked DO stratification, with DO levels above 8 mg l^{-1} up to 10 m below surface, followed by a rapid decrease to 2.62 mg l^{-1} at 48 m depth. This unexpected result may be attributed to the region's temperate climate, with an average temperature of 14°C during the coldest month of June, causing seasonal turnover to be less significant than observed in cooler regions. Dams and large water bodies in this region may thus not follow typical stratification and seasonal turnover patterns, leading to persisting low DO conditions in deeper zones.

ICP-MS data for water samples collected during October 2015 show an inverse relationship between total Mn concentrations and DO concentration (Fig. 3.3A). At a depth of 2 m below surface, where the DO concentration was 8.47 mg l^{-1}, the total Mn concentration was only 1.47 µg l^{-1}. The inverse relationship continues as Mn concentrations increased with a decrease in DO concentration, finally reaching its maximum concentration of 730 µg l^{-1} at a depth of 44 m with DO concentration as low

as 2.06 mg l^{-1}. Temperature measurements indicated that water temperature declined gradually from 23.8°C at 2 m below surface to 17.8°C at 48 m depth. This gradual decline in water temperature indicates that this dam does not have a typical stratified temperature profile. It was concluded from this first sampling event that the extraction of water for the LBIS network at the depth with Mn-rich water may select for microorganisms that derive energy from the Mn cycle and that these microorganisms may potentially dominate in biofilms in the pipeline and serve as a catalyst for the build-up of a matrix composed of Mn and the extracellular polymeric matrix produced by these microorganisms. It was therefore decided to conduct follow-up sampling during different seasons, and with varying dam levels.

3.3.1.2. Second sampling event – May 2016

DO levels remained high (between 7 – 8 mg l^{-1}) for the upper ~25 m of the water column at site 1, followed by a decrease from 7.36 mg l^{-1} at 24 m depth to below 2 mg l^{-1} at 44 m depth (Fig. 3.3B). Inversely, Mn and Fe levels increased rapidly after 24 m depth, reaching concentrations of 8631 µg l^{-1} and 4921 µg l^{-1}, respectively at 44 m depth and confirming the inverse relationship observed in data from October 2015. Although this falls within the target water quality range of 5 mg l^{-1} for Fe in irrigation water, it drastically exceeds the target water quality range of 0.02 mg l^{-1} for Mn in irrigation water. Both concentrations also exceed 1.5 mg l^{-1}, considered the concentration where severe clogging of drip irrigation systems occur (DWAF, 1996). Analysis of data obtained from both the October 2015 and May 2016 campaigns are similar to findings by Bryant et al. (2011) that higher concentrations of DO in reservoirs' upper zones could result in the oxidation of Mn, converting it from soluble Mn^{2+} to the insoluble MnO_2 form, and allowing it to settle out to the sediment. Upon reaching deeper levels where DO concentrations are low, Mn can be converted back from MnO_2 to Mn^{2+}. To test the extent of this process in the Blyderiver dam, all water samples were separated into filtered and unfiltered aliquots to measure what percentage of the total Mn in the water sample was oxidized.

ICP-MS results showed that Mn exists primarily in the oxidized form in the upper bulk volume of the dam, with approximately 95% of the total Mn being oxidized in the upper zone up to 20 m below the surface. The percentage of total Mn oxidized decreased to approximately 56% and 6% at depths of 36 m and 42 m respectively, showing the link between Mn oxidation and DO concentration. It is also known that Mn oxidation is strongly affected by microbial activity, which is also influenced by DO concentration (Mouchet, 1992; Webb et al., 2005). Similar patterns were observed for Fe, with approximately 92% of total Fe content oxidized at 4 m depth, increasing to 94% at 36 m, where after

it decreased to 49% at 42 m. The low DO concentrations in the deeper regions of the water column effectively isolates the sediment from the oxygen-rich overlying water, which may result in the oxic/anoxic boundary expanding upwards from the sediment, releasing Mn^{2+} into the overlaying anoxic region as discussed by Katsev et al. (2007). Despite the low DO concentrations observed at deeper zones during May 2016, no significant decrease in temperature could be observed, with temperatures measuring approximately 20°C up to a depth of 36 m, where after it only declined to 18.9°C at a maximum depth of 44 m. Thus, there was no indication of thermal stratification.

Water samples collected from the maximum depths of sites 2, 4, 5 and 7 (Table 3.1) contained significantly lower concentrations of both Mn and Fe compared to what was measured at site 1 (8631 µg l^{-1} and 4921 µg l^{-1}, respectively). Both Mn and Fe concentrations were lower at sites further away from the dam wall (sites 4 and 5; it was not possible to collect samples at site 3 as the current prevented the sampler from reaching the bottom), with an increase in both Mn and Fe concentrations at sites closer to the dam wall, reaching concentrations of 1948.69 µg l^{-1} and 1181.74 µg l^{-1} respectively at site 2. The opposite is true for results obtained from sediment samples, where Mn and Fe concentrations were highest at sites furthest away from the dam wall (Sites 3 and 5; Fig. 3.4), which can possibly be explained by the high DO concentrations, measured at sites 4 and 5, driving the oxic/anoxic boundary toward the sediment, allowing only the release of MnO_2 into the overlying water, where higher DO results in precipitation. However, at the deeper zones of sites 1 and 2, DO concentrations decrease, expanding the oxic/anoxic boundary upwards from the sediment and releasing soluble Mn^{2+} into the overlying water.

Table 3.1 - Total Mn and Fe concentrations in water samples collected at sites in the Blyderiver dam located further away from the dam wall in May 2016.

Sampling site	Depth	Mn concentration (µg l^{-1})	Fe concentration (µg l^{-1})	DO (mg l^{-1})	Temperature (°C)
Site 2	35.5 m	1948.69	1181.74	3.81	20.4
Site 4	20 m	28.74	82.03	7.52	21.0
Site 5	5 m	33.82	98.95	8.14	20.6
Site 7	24 m	139.89	604.53	7.84	21.9

3.3.1.3. Third sampling event – October 2016

Sampling collection in October 2016 occurred during a period of drought, leaving the Blyderiver dam's water level at approximately 40%, and a maximum depth of approximately 35 m. ICP-MS results and DO measurements in Fig. 3.3C. indicated profiles similar to those observed during earlier sampling events, with DO concentrations of 8.93 mg l^{-1} at 1 m depth below surface and remaining above 8 mg l^{-1} up to a depth of 29 m, where after it decreased rapidly to 2.31 mg l^{-1} at 33 m depth. Although comparison between data from October 2015 (Fig. 3.3A) and October 2016 (Fig. 3.3C) showed this profile in both cases, DO measurements from October 2016 indicated that the DO concentration only decreased rapidly over the deepest 4 to 5 meters. The low rainfall and constant demand for water for irrigation resulted in faster extraction of water compared to inflow into the dam, allowing the dam's water level to decrease. A large portion of the hypoxic deeper region was extracted and DO rich water from shallower depths was subsequently drawn down, expanding the higher DO fraction throughout most of the water column.

Although the DO concentration only decreased in the last few meters near the bottom of the dam, similar patterns could be observed compared to previous sampling dates. As for water profiles from October 2015 and May 2016, total Mn and Fe concentrations increased following a decrease in DO concentrations, remaining relatively constant up to 29 m depth, where after they rapidly increased to 471.17 µg l^{-1} and 551.44 µg l^{-1} respectively. Interestingly, the Mn concentration, recorded in water with a DO concentration of 2.31 mg l^{-1}, was much lower compared to Mn concentration of 6989.3 µg l^{-1} but higher compared to an Fe concentration of 255.31 µg l^{-1}, at similar DO concentration in May 2016. This might be due to the decrease of incoming Mn rich water from the Blyde and Orighstad rivers, as well as a decrease in the transfer of Mn from sediment upstream in these rivers due to low rainfall during 2016.

Similar to data from May 2016, the percentage of oxidized Mn decreased with depth, with 94% of total Mn oxidized at 1 m below surface, decreasing to 27% at 33 m depth. However, the percentage of oxidized Fe showed a much smaller decrease, with 99% oxidized at 1 m depth and only decreasing to 91% at 33 m depth. The high percentage of oxidized Fe might have been the result of the overall high DO concentration present throughout a relatively larger portion of the water column compared to the previous two sampling events, and its ability to be oxidized more readily than Mn. Temperature measurements indicated that the temperature did not fluctuate significantly throughout the water column, peaking at 23.9°C at the surface and only declining to 19.2°C at 33 m depth.

3.3.1.4. Fourth sampling event – February 2017

The catchment of the Blyderiver dam experienced heavy rainfall in early 2017, with the dam water level at 100% during sampling in February 2017. It was expected that the water column would follow a similar profile as the previous sampling events, with an extensive upper layer having high DO concentrations, followed by a rapid decline in DO towards the water-sediment interface. However, the heavy rainfall and the dam's level rising from 33% in December 2016 to 100% in February 2017 resulted in sufficient mixing, with DO concentrations remaining mostly between 8 – 9 mg l^{-1} throughout the water column, only decreasing to a concentration of 5.41 mg l^{-1} at 43 m depth (Fig. 3.3D). The inverse was true for total Mn and Fe concentrations remaining mostly constant to approximately 40 m depth, followed by a rapid increase to 1147 µg l^{-1} and 6781 µg l^{-1} respectively at 43 m depth.

Aluminium (Al) measurements were added to this sampling event as farmers reported losses in crop production and it was suspected that this could be due to high Al concentrations in irrigation water. ICP-MS data from Fig. 3.3D indicated that total Al concentrations during February 2017 reached maximum values as high as 2225 µg l^{-1} at a depth of 43 m. Although Mn and Fe concentrations at 43 m depth both exceeded their target water quality range of 0.02 mg l^{-1} and 5 mg l^{-1} respectively, Al concentration lies within its water quality target range of 5 mg l^{-1} for irrigation water (DWAF, 1996). However, it has been reported that Al concentrations as low as 0.1 – 0.5 mg l^{-1} in soil solution can have toxic effects on several crops. Although these values cannot be directly extrapolated to irrigation water as chemical reactions in soil can modify Al species and concentration, these concentrations and reported losses are reason for concern (DWAF, 1996). High concentrations of Al can typically lead to abnormal root development in plants, causing roots to become thick and stubby and allowing for little development of the fine root system (Miles & Farina, 2013). Inefficient root development limits water and nutrient uptake, ultimately limiting yield. Due to the resultant poor root penetration in subsoil, Al toxicity magnifies the effects of drought (Miles & Farina, 2013).

The percentage of total Mn, Fe and Al that was oxidized showed minor decreases with increase in depth. Unlike data from previous sampling events, the percentage of total Mn oxidized only showed a decrease from 95% at 2 m depth below surface to 84% at 43 metres depth, compared to decreases of more than 60% observed during sampling in May and October 2016. The percentage of total Fe and Al concentrations oxidized both showed increases with increase in depth, with Fe increasing from 78% at 2 m depth to 97% at 43 m depth and Al increasing from 49% to 99% at the same depths. It is suspected that the high DO concentration throughout the water column influenced these oxidation

percentages, with high oxidation correlating to high DO concentrations. Similar to data from October 2016, the high DO concentration throughout most of the water column, combined with the rapid inflow of DO-rich water caused by high rainfall may have led to significant mixing at the sediment water interface, driving the oxic/anoxic boundary towards the sediment and decreasing the release of soluble elements such as Mn, Fe and Al as described by Katsev et al. (2007). Temperature measurements indicated a gradual decrease from 29.3°C at the surface to 23.7°C at 43 m depth indicating that the Blyderiver dam was not thermally stratified during this period.

Interestingly, throughout the four sampling events, no rapid decrease in temperature could be observed with increasing depth during any of the sampling events, indicating that the water column in this dam does not behave like water columns typically described in literature. Instead of forming the three typical thermal layers, the epilimnion, metalimnion and hypolimnion, this water column shows a fairly stable temperature profile throughout the water column. In contrast, DO profiles still correspond mostly to a water column that is thermally stratified, remaining high at the shallower depths and rapidly decreasing at deeper depths. This could possibly be explained by insufficient mixing of the water body due to the great depth of this dam, leading to hypoxic conditions at greater depths. In contrast, the mostly uniform DO profile observed during February 2017 could possibly be explained by extensive mixing of the water column due to heavy rain during the beginning of 2017.

Another factor to consider is the role of algal exudates and dead algal cells as microorganisms present in the deeper parts of the water column can use the available oxygen in their metabolic processes during biodegradation of algal biomass when it settles out to the bottom of the water column (Beutel & Horne, 1999). As the lower DO concentrations observed at deeper depths may be more favourable for the growth of anaerobic Mn reducing bacteria, oxidized Mn precipitating out may be reduced by both physico-chemical conditions as well as biological reduction. As this dam does not follow typical thermal stratification patterns observed in literature but still display the expected decrease in DO concentration at deeper depths, it can also be speculated that the activity of anaerobic bacteria/microorganisms are much greater in the Blyderiver dam than it would be in reservoirs typically studied in the Northern Hemisphere.

Table 3.2 – Total Mn, Fe and Al concentrations in water samples collected at sites in the Blyderiver dam located further away from the dam wall in February 2017.

Sampling site	Depth	Mn concentration (µg l^{-1})	Fe concentration (µg l^{-1})	Al concentration (µg l^{-1})	DO (mg l^{-1})	Temperature (ºC)
Site 2	34 m	19.4	111.87	35.95	8.37	27.9
Site 4	32 m	92.18	503.04	123.29	8.01	27.5
Site 7	27 m	1159.06	6248.88	1272.85	7.78	27.5

Total Mn, Fe and Al concentrations in water samples collected from sites 2, 4 and 7 (Table 3.2) did not follow the same pattern observed during sampling in May 2016 (Table 3.1), with total Mn, Fe and Al concentrations being higher at sites further away from the dam wall.

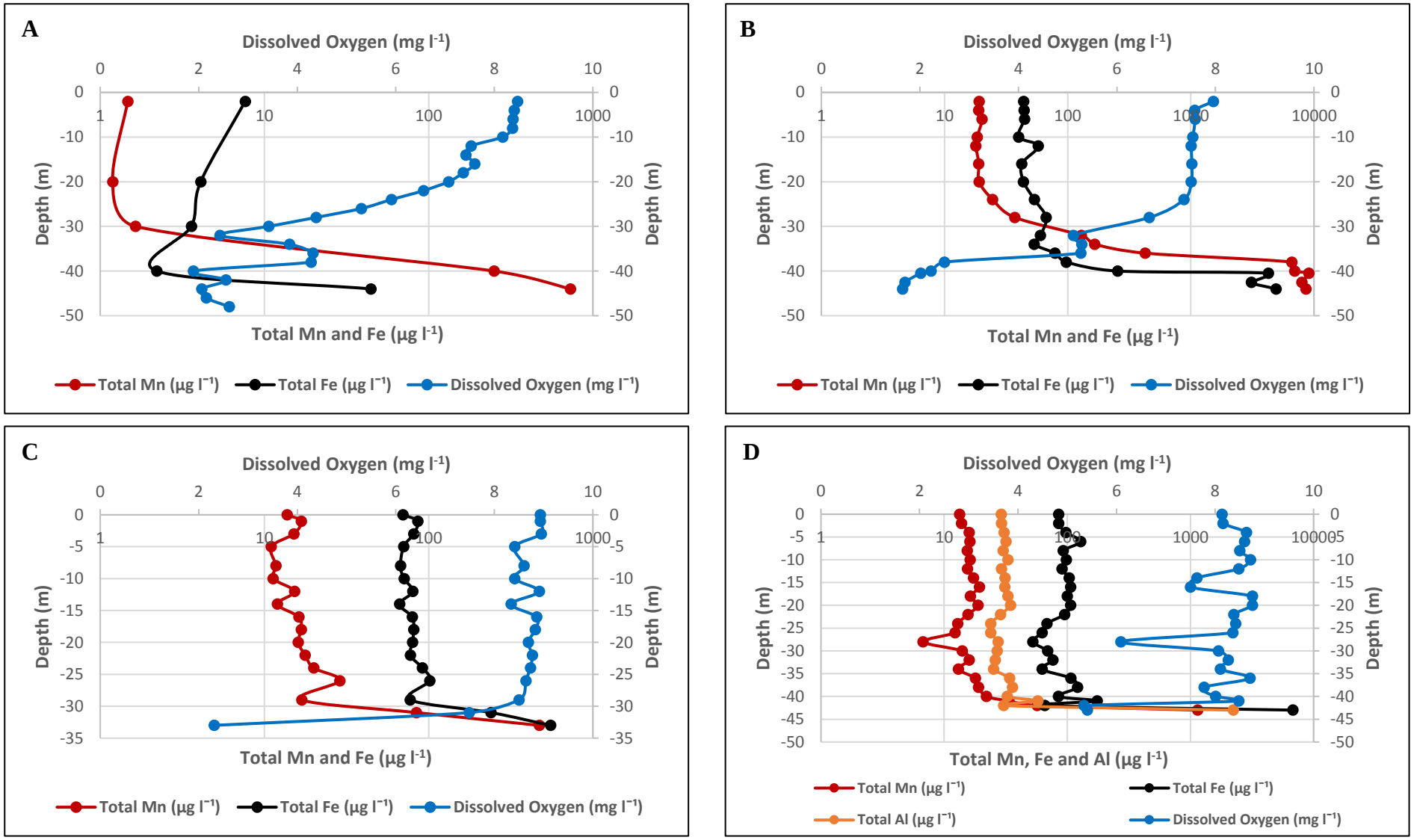

Fig. 3.3 - Depth profile of DO, total Mn and Fe at site 1 in the Blyderiver dam on A) October 2015; B) May 2016; C) October 2016; and D) February 2017. Al analyses are also included in D).

3.3.2. Total Mn and Fe in sediment

Sediment samples were collected from the sites indicated in Fig. 3.2 during sampling in May 2016. As shown in Fig. 3.4., Mn and Fe concentrations varied considerably amongst the sampling sites, being highest in the sediment collected at the sites furthest from the dam wall, with Mn and Fe concentrations reaching concentrations of 3198.29 mg kg^{-1} and 84660.84 mg kg^{-1}, respectively in the area fed by the Orighstad river (site 5), and 3214.23 mg kg^{-1} and 91487.42 mg kg^{-1}, respectively in the area fed by the Blyderiver (site 3). However, there was not a clear trend with distance closer to the wall, and since we could not obtain sediment samples and related DO profiles for all the sampling sites on all sampling dates, it is not possible to explain the variation, such as the comparatively low values measured at site 4. Thamdrup et al. (1994) indicated that Mn is not only cycled between MnO_2 in aerobic sediment and Mn^{2+} in anaerobic sediment but also between sediments and the overlaying water (Fig. 3.5). Additional sampling, that also include flow in the overlaying water column, would be necessary to consider the potential contribution to variation in these shallow sediments as measured.

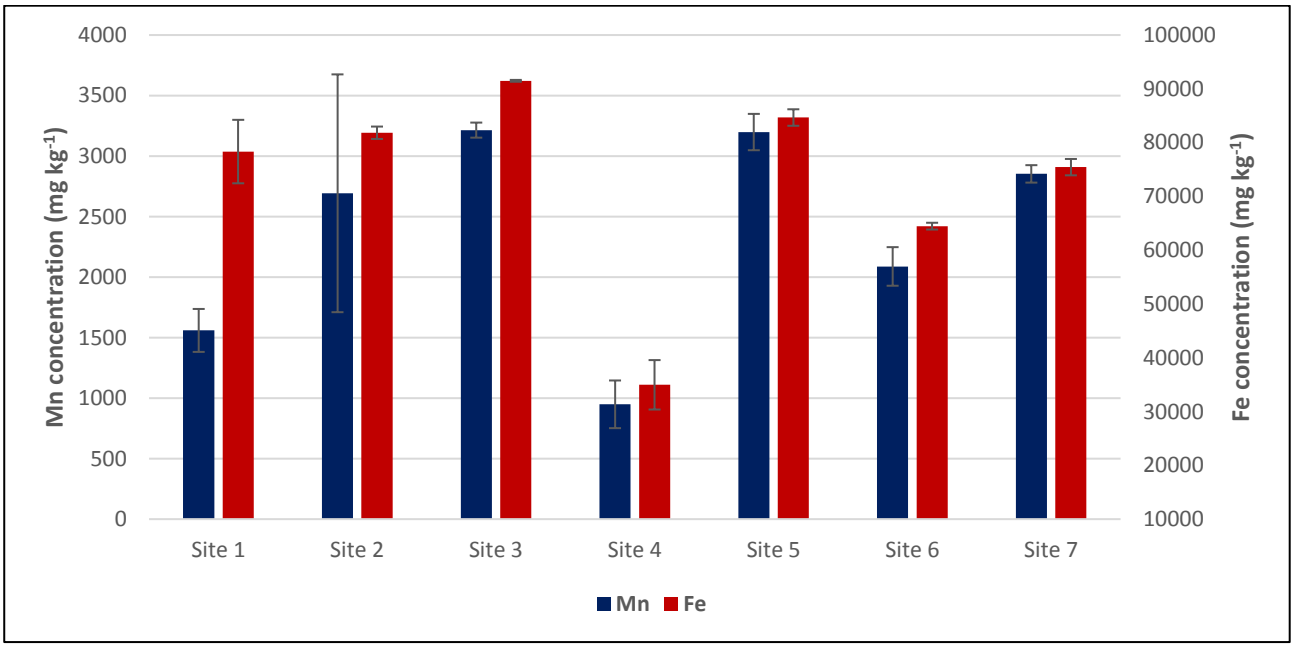

Fig. 3.4 - Sediment samples collected as indicated in Fig. 3.2 on May 2016.

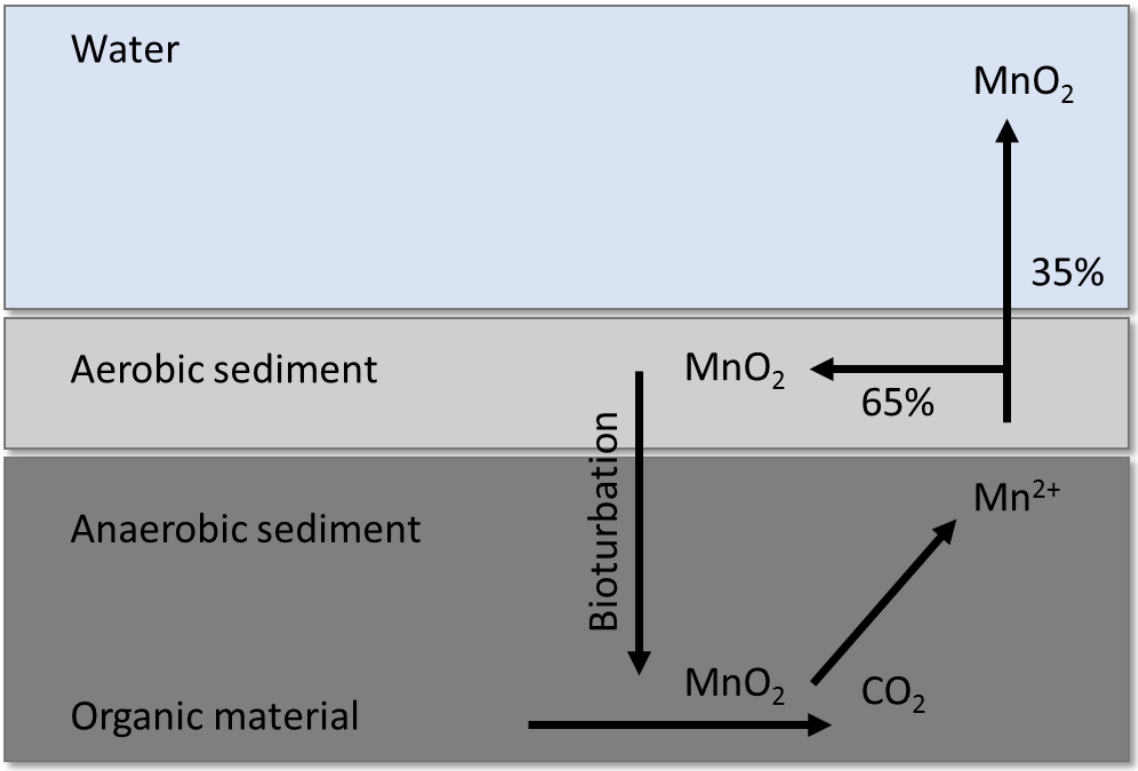

Fig. 3.5 - Cycling between MnO_2 in aerobic sediment and Mn^{2+} in anaerobic sediment, as well as release of a notable fraction back to the aqueous phase through dissimilatory Mn reduction by anaerobic bacteria (Adapted from Thamdrup et al., 1994).

3.3.3. Total Mn concentration in water samples collected downstream along the main LBIS pipelines

Water was collected on two of the sampling dates from the LBIS pipeline network to determine Mn concentrations over distance from the point of extraction. As indicated in Fig. 3.6 and 3.7, total Mn concentration in the water decreased with distance, with the most significant decrease occurring over the first 4.5 kilometres to the LBIS 1 site. During the May 2016 sampling event, total Mn concentrations decreased from 8631 µg l^{-1} in water entering the pipeline to 2756.24 µg l^{-1} at the LBIS 1 sampling point. Total Mn concentrations then further decreased to 134.83 µg l^{-1} in water collected from the sampling point 18.5 km further downstream at LBIS 3. Fe followed the same pattern, albeit at lower concentrations. Mn concentrations did not comply with its target water quality guideline of 0.02 mg l^{-1} for irrigation water (DWAF, 1996).

Similar decreases could be seen in water samples collected during more extensive sampling in February 2017 (Fig. 3.7). Total Mn, Fe and Al concentrations decreased within the first 4.5 km, from 1147.3 µg l^{-1} to 74.05 µg l^{-1}, 6781.31 µg l^{-1} to 81.43 µg l^{-1} and 2224.99 µg l^{-1} to 32.61 µg l^{-1} for Mn,

Fe and Al respectively. However, after the initial decrease, there was a measurable increase for all three elements with distance in all three lines from the LBIS 1 sampling point. Total Mn increased from 74.05 µg l^{-1} at LBIS 1 to 264.32 µg l^{-1} at LBIS 4 downstream on route 1, to 327.02 µg l^{-1} at LBIS 6 on route 2, and 351.65 µg l^{-1} at LBIS 9 on route 3. Fe and Al concentrations followed similar patterns. Once again, the Mn concentrations did not comply with SA irrigation guidelines (DWAF, 1996).

These data indicate notable Mn sequestering from the water over the first few kilometres downstream from the extraction point; ~70% during the May 2016 sampling when intake water contained 8631 µg l^{-1} Mn, and almost 93% during the February 2017 sampling when the intake water contained a lower Mn concentration of 1147.3 µg l^{-1}. It is important to point out that, even when the intake water contained very high Mn (8631 µg l^{-1}), it was further reduced to the same concentration range over the next few km to levels similar to those when the intake water contained 8 times less Mn (1147.3 µg l^{-1}) – suggesting that biofilms play an important role as sink for Mn in the pipe network. Mn oxidizing and reducing microorganisms in the biofilms could potentially be responsible for this decrease by incorporating Mn into the biofilm's extracellular polymeric substances (EPSs). This is achieved though the production of negatively charged EPSs to which the positively charged Mn^{2+} can bind, after which soluble Mn is oxidized enzymatically (Ghiorse, 1984).

Significant quantities of biofilm material collected at the LBIS 1 sampling site, (Fig. 3.8) confirmed that biofilm growth is abundant in the pipeline at this distance, thus it can be assumed that high concentrations of Mn contribute to the growth and maintenance of these biofilms capable of oxidizing and reducing it and incorporating it into the biofilm matrix. The high Mn content shown in Table 3.3 supports this contention. The fact that biofilm Mn concentration decrease with distance (section 3.3.4) suggests that the biofilms may not have reached a steady-state over the full distance of the network, with the possibility that Mn sequestering and overall biofilm-biomass accumulation may continue over time, leading to further reduction in the network's hydraulic capacity. The demonstrated variability in Mn concentration in the intake water as shown in Fig. 3.3A – D, considered in view of the further reduction beyond 4.5 km when the intake Mn was high, and which was comparable to when the intake Mn was low, thus warrants further investigation to better understand Mn cycling between biofilms and the aqueous phase in the pipes. Of particular interest would be to determine i) whether intake of water with low Mn concentration will ultimately lead to washout of Mn from the system, and ii) at what threshold Mn concentration this will happen.

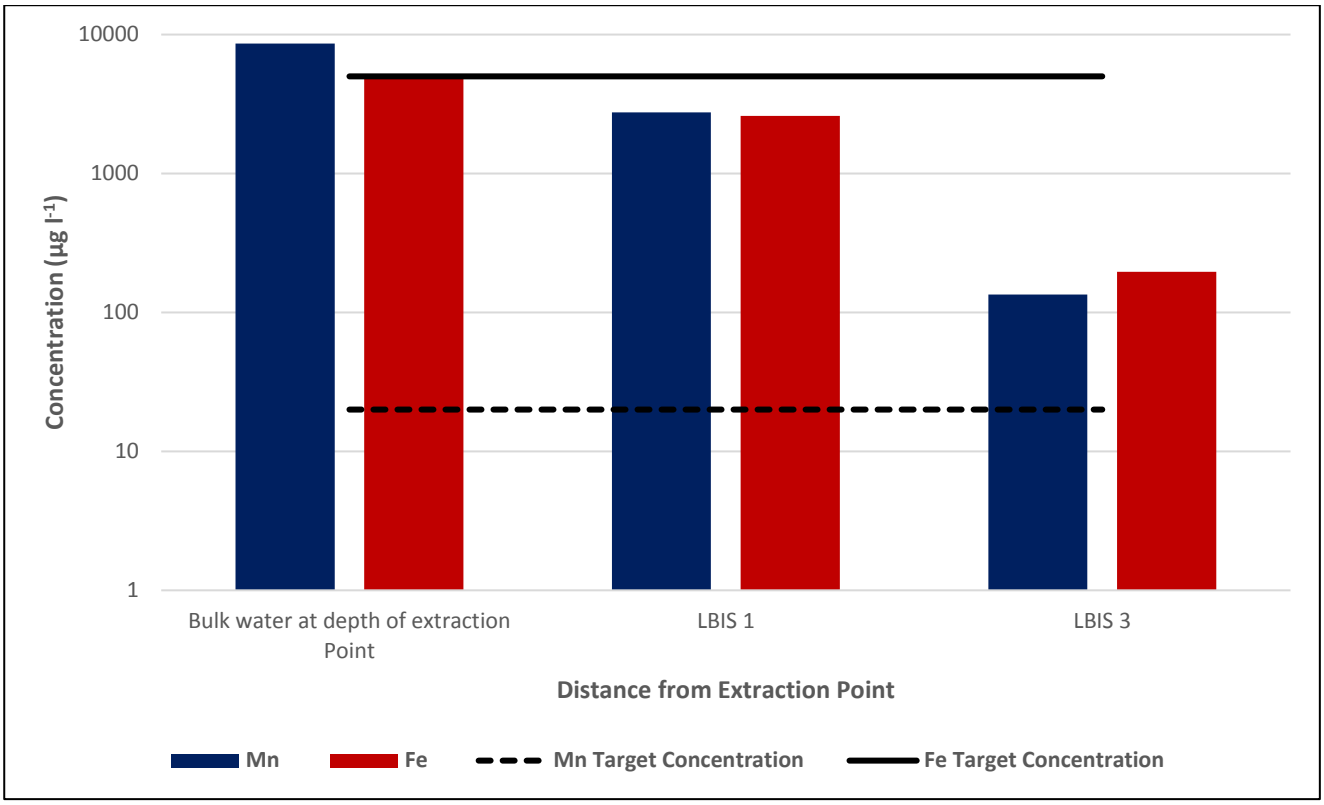

Fig. 3.6 - Total Mn and Fe concentrations in water sampled at the LBIS 1 and LBIS 3 extraction points at 4.5 km and 23 km downstream from the dam wall, respectively. The dotted and solid lines indicate South African target water quality guidelines in irrigation water for Mn and Fe, respectively.

The water higher up in the column with high DO and low Mn concentrations may not sustain biofilms such as those that have developed under low DO and high Mn concentrations. It is speculated that Mn cycling between these biofilms and the overlying water occurs in a similar manner as that described in Fig. 3.5, where Mn is cycled between sediment and overlying water. Soluble Mn entering the pipeline can be oxidized by Mn oxidizing bacteria in the aerobic upper layers of the biofilms in the presence of adequate DO in the bulk aqueous phase, depending on the DO concentrations in the intake water or introduced through turbulence. Mn reducers in the deeper, hypoxic layers of the biofilm will reduce oxidized Mn back to the soluble state, where after it can be oxidized again by the Mn oxidizing bacteria in the cycle, releasing a portion of the oxidized Mn back into the overlying water. During periods when DO concentrations in the intake water are lower, such as the 1.65 mg l^{-1} experienced at 44 m depth during May 2016, it is speculated that Mn reducing bacteria may be dominant, reducing MnO_2 in the overlaying water inside the pipeline and releasing soluble Mn back into the overlaying water. As the constant presence of high concentrations of Mn may select for the presence of Mn oxidizing and reducing microorganisms, extracting water from

shallower depths where Mn concentrations are lower may potentially lead to washout of these biofilms.

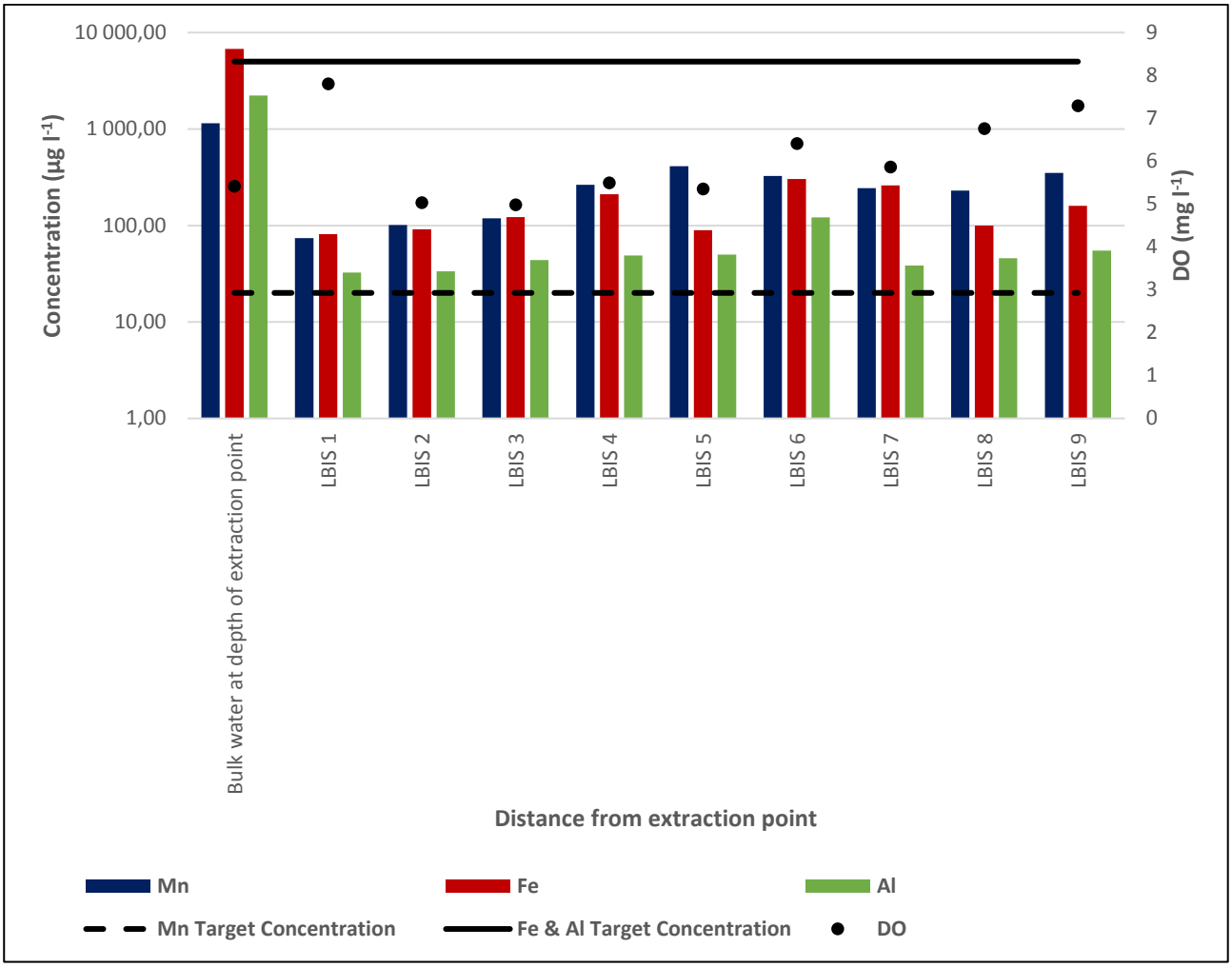

Fig. 3.7 - Total Mn, Fe, Al and DO concentrations in water sampled at the extraction point and downstream on routes 1, 2 and 3. The dotted and solid lines indicate South African target water quality range in irrigation water for Mn, Fe and Al, respectively.

A **B**

Fig. 3.8 - Example of biofilm removed from an experimental bypass, 4.5 km downstream from the dam wall at LBIS 1 showing A), the dark brown colour indicative of Mn and Fe, and B) the amount of biofilm removed from a 2000 mm long, 81.9 mm inner diameter pipe section.

3.3.4. Biofilm Mn content in the LBIS network with an increase in distance from the extraction point

Biofilm samples were collected at three locations at sampling points LBIS 1, LBIS 10 and LBIS 6; the only sampling points that allowed sufficient access to allow representative biofilms. Total biofilm Mn concentration decreased with an increase in distance from the extraction point, with biofilm samples collected at LBIS 1 showing the highest concentration of Mn at 30105.4 mg kg^{-1}, decreasing to 23501.9 mg kg^{-1} at LBIS 10 and 13727.7 mg kg^{-1} at LBIS 6. Comparing data to that of Fig. 3.7, it appears that, upstream from the LBIS 1 mark, more Mn is oxidized and precipitated from the aqueous phase and get incorporated into the biofilm than the fraction being released back into the aqueous phase by reductive dissolution. Fig. 3.7 shows there is not a decrease in DO with distance, thus there may not be a higher rate of reductive dissolution downstream, in which case the biofilm will keep on incorporating Mn and grow (thus the point where more Mn are oxidized and precipitate from the aqueous phase and get incorporated into biofilm than the fraction being released back into the aqueous phase by reductive dissolution will gradually migrate downstream).

3.3.5. Elemental composition of biofilms

Biofilm samples collected from the LBIS pipeline were dried and subjected to SEM/EDS to observe the biofilm structure and determine elemental composition, as well as its estimated contribution

toward total weight percentage. Elemental analysis indicated that Mn and Fe were not uniformly distributed through the biofilm matrix, which is not surprising in view of the frequent mention of biofilm heterogeneity in literature (Donlan, 2002; Jahn et al., 1999). Elemental analysis of biofilm samples collected from October 2015 to February 2017 all indicated the presence of Mn, Fe and Al as some of the most abundant metals, with Mn reaching concentrations as high as 33% biofilm dry weight concentration for samples collected in October 2015.

Interestingly, an abundance of diatoms of various shapes could be observed in these samples. The occurrence of diatoms in biofilms associated with the oxidation and reduction of Mn has previously been reported by Kielemoes et al. (2002). In their research, it was found that diatoms might adhere to biofilms during periods when flow conditions are stagnant. However, SEM/EDS imaging (Fig. 3.9) indicated the presence of diatoms in biofilm samples subjected to continuous high-flow conditions, indicating that diatoms may also adhere to biofilms under fast flowing conditions.

Table 3.3 - Elements present in biofilm samples obtained 4.5 km downstream at the LBIS 1 sampling point in October 2015, May 2016 and February 2017 as identified through SEM/EDS.

Elements	**October 2015**	**May 2016**	**February 2017**
	Weight %		
Mn	33.3	21.7	9.3
Fe	6.1	20.7	29.9
Al	5.5	10.6	1.6

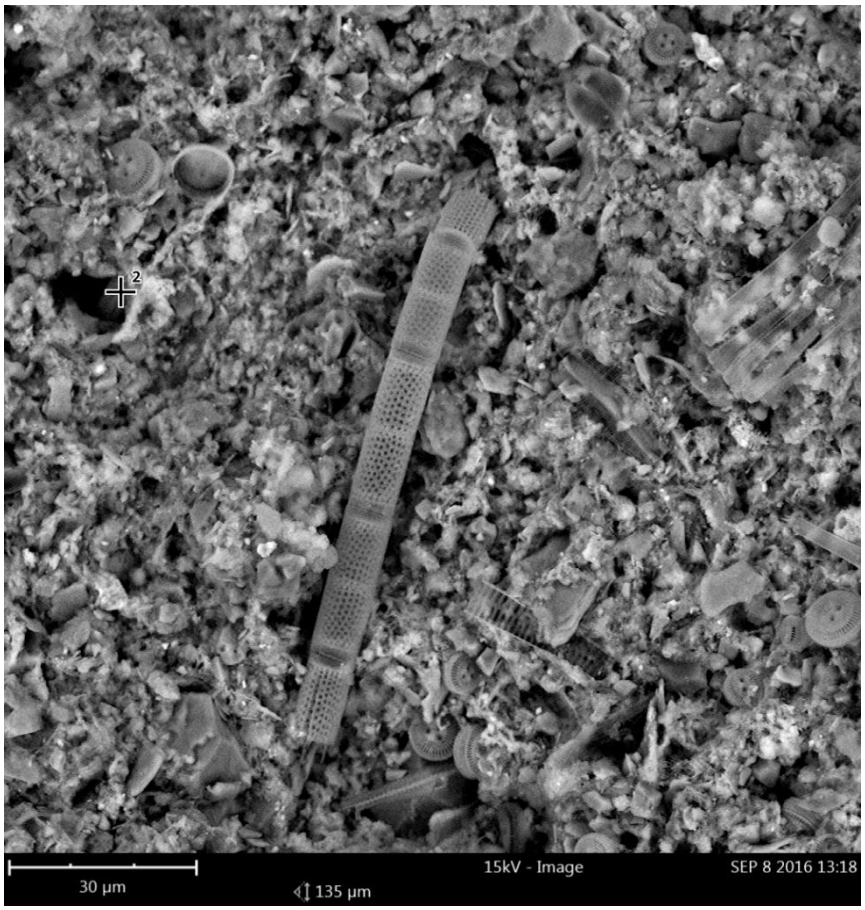

Fig. 3.9 - SEM image of a biofilm sample collected at the LBIS 1 sampling point showing diatoms imbedded in the biofilm matrix.

3.4. Conclusions

This study showed that biofilms with a Mn content of up to 30,000 mg kg^{-1} biofilm dry weight formed in the LBIS, which offers a possible explanation for the reduction in hydraulic capacity experienced in the system. The reservoir's water column showed a characteristic profile, with DO concentration above 7 mg l^{-1} in the upper regions, followed by a rapid decline to as low as 1.65 mg l^{-1} in the deeper zones, with total Mn concentrations having a strong inverse relationship with the measured DO profile, ranging from <5 µg l^{-1} near the surface to >8000 µg l^{-1} near the water-sediment interface. Mn concentration in both the aqueous phase and biofilms in the pipeline decreased from point of extraction, suggesting continued Mn attenuation and increase in biofilm biomass that may lead to further loss in hydraulic capacity over time.

Since water for the main 1500 mm diameter pipeline of the irrigation system is extracted from this low DO – high Mn zone, it is recommended that experimental pipe rigs be installed and supplied with water extracted at various depths to monitor biofilm development under higher DO concentrations

and lower total Mn concentrations experienced at these respective depths, and also to assess whether switching to water with higher DO, extracted from the upper zones, would ultimately lead to washing-out of Mn released by reductive dissolution from the existing biofilms. These measures may ultimately offer a solution to the current situation where concentrations of Mn and Fe in the irrigation water do not comply with national irrigation guidelines.

Acknowledgements

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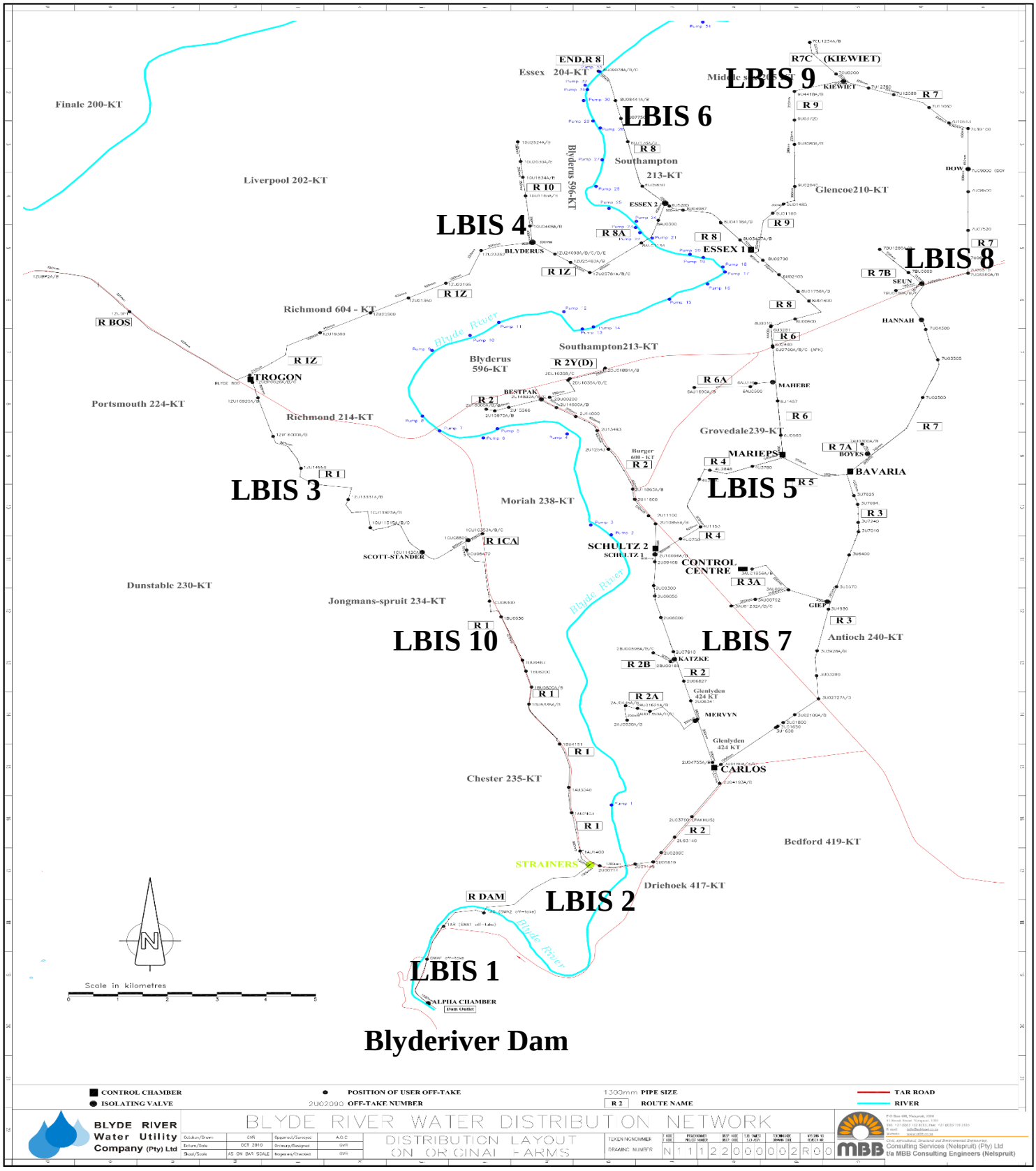
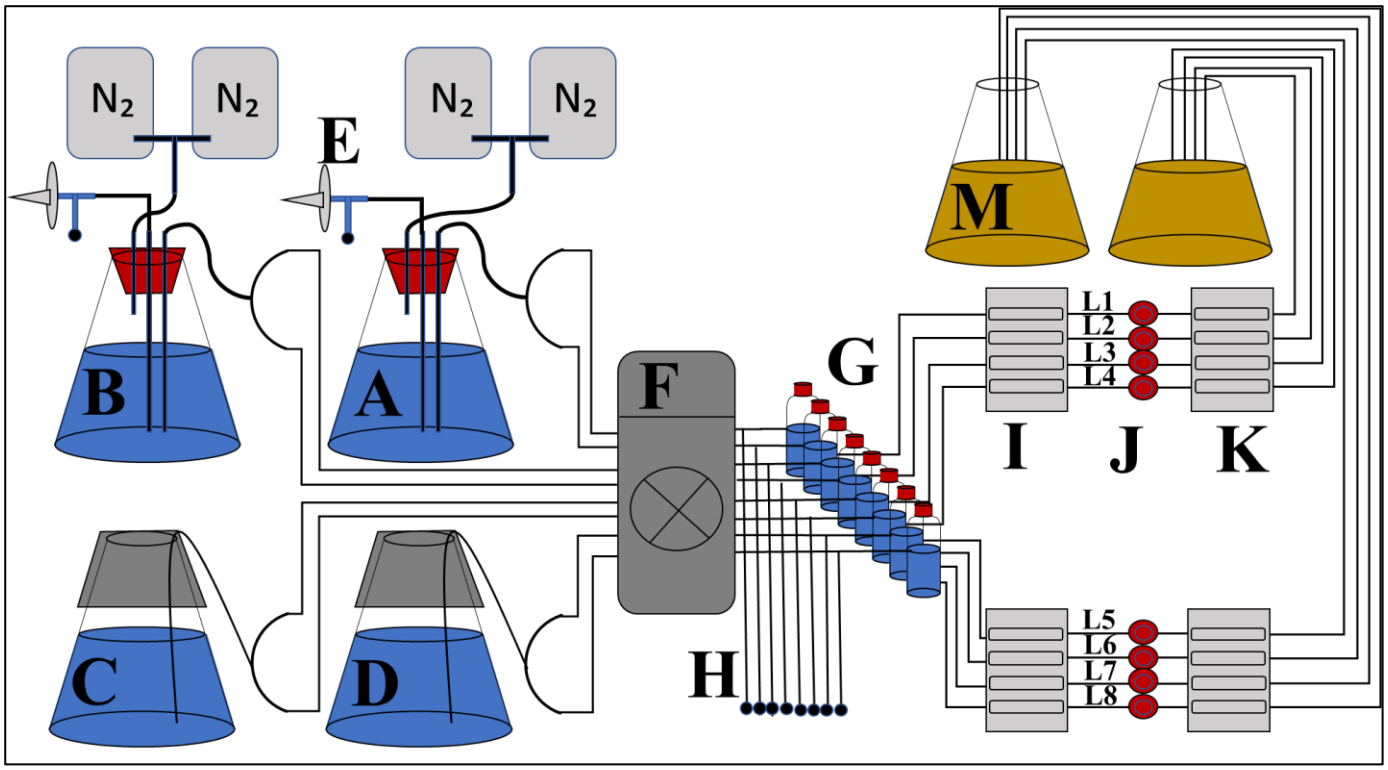
Supplementary Material



Appendix A - Schematic layout of the Lower Blyde Irrigation System (LBIS) located downstream from the Blyderiver dam in the Mpumalanga and Limpopo provinces, South Africa. LBIS 1 – LBIS 10 indicate sampling sites (Courtesy of Mr Org van Rensburg, MBB Consulting Engineers).



Appendix B – Laboratory scale flow system with four different growth conditions for the cultivation of Mn oxidizing and reducing biofilms. A: Growth medium for lines 1 and 2. B: Growth medium for lines 3 and 4. C: Growth medium for lines 5 and 6. D: Growth medium for lines 7 and 8. E: 0.22 µm filters through which nitrogen gas was sparged. F: Watson Marlow 205S peristaltic pump. G: Glass bubble traps. H: In line diversions for DO measurements. I: Upstream flowcell channels connected to downstream flowcell channels (both inoculated) (K) via 4.5 m Tygon® tubing with in line bubbletraps (J). L 1-8: Respective lines representing different DO and Mn concentrations. M: Waste effluent collected from the flow system.

Chapter 4

Laboratory simulations

4.1. Introduction

Mn is a persisting contaminant in water distribution systems, and internationally this problem has been reported several times (Bryant et al., 2011; Cerrato et al., 2010; Gantzer at al., 2009; Mouchet, 1992; Stauffer, 1986). This often leads to poor water quality as high concentrations of Mn can cause discolouration, staining and unpleasant smells in water (Cerrato et al., 2010; Gantzer et al., 2009). Mn oxidizing and reducing bacteria play important roles in the redox processes of Mn, with Mn oxidizing bacteria capable of accelerating the oxidation of Mn^{2+} to MnO_2 compared to chemical oxidation (Tebo et al., 2005; Webb et al., 2005). Although the importance of Mn oxidation has been questioned previously, several benefits are associated with this process, such as the protection against ROS and UV radiation (Brouwers et al., 2000). Furthermore, Mn oxidizing biofilms have shown faster rates of biomass accumulation in the presence of Mn oxides (Parikh et al., 2005).

As shown in Table 3.3, biofilms collected during sampling from the inside of the LBIS pipelines contained high concentrations of Mn. However, the Mn concentrations in these biofilms decreased with an increase in distance from the LBIS extraction point in the dam wall of the Blyderiver dam. Similarly, Mn concentrations in the water decreased with distance from the extraction point (Fig. 3.6 & 3.7). As these biofilms lead to a decrease in water delivery, their growth and development needs to be understood in order to control them.

This requires intricate knowledge regarding factors such as DO concentration and pH and how it affects Mn oxidation. Previous studies evaluating biofiltration facilities worldwide have established that the optimal conditions for biological oxidation of Mn requires an environment with a pH level between 7.5 and 8.3 and DO concentration between 5 and 6 mg l^{-1} (Du Toit et al., 2015). However, although these conditions are optimal for biological oxidation and can be maintained in a carefully controlled treatment facility, changing environmental conditions caused by rainfall and temperature differences do not always allow for these optimal conditions in real-world situations. As a result, biofilms inside the LBIS pipeline network are subjected to a range of DO and Mn concentrations in the water flowing through this pipeline network throughout the year.

The experiments carried out in this chapter aimed to simulate the environmental conditions inside the LBIS network in terms of DO and Mn concentrations and to study the effect thereof on the growth and development of biofilms. As limited literature is available on studies of Mn biofilms in the laboratory, all growth conditions were simulated based on water analyses of the Blyderiver dam performed during the duration of this study. Simulating the entire range of conditions that these

biofilms are subjected to throughout the year was not possible and as a result, a selected range of DO and Mn concentrations was chosen based on measurements reported in chapter 3 for different depths in the Blyderiver dam water body.

4.2. Materials and Methods

4.2.1. Culturing conditions

Growth medium simulating water from the Blyderiver dam was supplied to biofilms in flow systems (Fig. 4.1) at a flow rate of approximately 12 ml h^{-1} per channel using a Watson Marlow 205S peristaltic pump. The growth medium/synthetic ground water (SGW) was prepared according to a protocol representing the concentrations of elements in the Blyderiver dam in October 2015 and consisted of 0.0854 g l^{-1} $Ca(NO_3)_2.4H_2O$ (Univar, Saarchem), 0.0009 g l^{-1} KOH (Emsure®), 0.0941 g l^{-1} $MgSO_4.7H_2O$ (Sigma-Aldrich), 0.00002 g l^{-1} $H_2NaPO_4.2H_2O$ (Sigma-Aldrich), 0.0185 g l^{-1} $MnSO_4.H_2O$ (Sigma-Aldrich), and 0.0085 g l^{-1} Na_2CO_3 (Merck). TSB (Biolab) was also added at a concentration of 0.25 g l^{-1} to encourage growth and simulate the presence of algal exudates as carbon source. The SGW was adjusted to a pH of 8.0 and autoclaved. All subsequent flow systems were supplied with SGW prepared according to this protocol, unless stated otherwise.

4.2.2. Validation of biofilm growth in flow systems

Flow systems were constructed using silicone tubing with an inner diameter of 1.6 mm connected to flowcells at both ends of the flow system as illustrated in Fig. 4.1. These upstream and downstream flowcells were separated by 4.5 m of silicone tubing to allow for depletion of nutrients and accumulation of metabolites over distance. Prior to inoculation, all lines in the system were sterilized with a 10% bleach solution, where after it was flushed with sterile distilled water overnight/15 h. SGW was then flushed through the system prior to inoculation, where after flow was stopped. The flow system was then inoculated with 200 µl per channel with sample collected from the Mn treatment filter at the Preekstoel water treatment facility (see chapter 2) to determine the time needed for visible biofilm growth to occur. Both upstream and downstream flowcell channels were inoculated. All inoculated flow cell channels were left under stagnant conditions for 30 min before flow was initiated at approximately 12 ml h^{-1}. Following inoculation, biofilms were subjected to light microscopy every 48 h to visualise biofilm growth and development. The sterilization and inoculation protocol used in this section was followed for all subsequent flowcell experiments.

4.2.3. Preparation of a standardized inoculum

To study the behaviour of manganese oxidizing – and reducing biofilms in laboratory scale experimental systems and to increase reproducibility, a standard inoculum was prepared from which all subsequent experimental systems were inoculated. Biofilm samples were collected from the inside of the LBIS pipeline network during sampling in October 2016 and mixed in a 2:1 ratio with biofilm and water samples collected from the Mn treatment well at the Preekstoel water treatment facility in Hermanus to select for a spectrum of Mn oxidizing and reducing bacteria. A linear flow system consisting of eight lines was connected to eight flowcell channels in which biofilms could be cultured. Two hundred microliters of the biofilm and water mixture were used to inoculate each of the eight flowcell channels, respectively. Syringes containing glass beads with a diameter of approximately 2 mm were connected downstream from the flowcells to collect any detached biofilm material as well as planktonic cells released from biofilms. Biofilms were cultured for 2 weeks, where after the flow system was terminated. Freezer stocks were prepared by collecting glass beads from the top third in each syringe, as well as liquid contained within the syringe and preserving it in 30% glycerol (uniLAB®) at -80°C.

4.2.4. Determining the suitability of a standard inoculum

Four Erlenmeyer flasks containing 50 mL SGW adjusted to a pH of 8.0 were each inoculated with the contents of one freezer stock respectively and incubated on a shaker at 120 rpm for 48 h at 26°C. After 48 h, the optical density (OD) of the culture was measured at an absorbance of 600 nm, where after it was diluted to an OD of 0.1. Serial dilutions were prepared and plated out onto two sets of selective Mn oxidation agar adjusted to pH 7.4 and 8.0 respectively as described by Cerrato et al. (2010), as well as Tryptic Soy agar (TSA) adjusted to the same respective pH levels. The drop plate method (Herigstad et al., 2001) was used to plate out serial dilutions onto these plates to determine average colony forming units (CFU) per millilitre. Both selective Mn oxidation and TSA plates were incubated at 26°C for 48 h, where after colonies were counted to determine average CFU ml^{-1}.

4.2.5. Mn partitioning in an open oxygen system

A flow system similar to that illustrated in Fig. 4.1 and consisting of six lines was constructed. All medium influent reservoirs were only covered with aluminium foil, allowing oxygen to enter the system. Four standard flow cell channels were inoculated with 200 µl of a standardized culture diluted to an OD of 0.1 as described in section 4.2.4. The system consisted of the same upstream and

downstream flowcell configuration as described in section 4.2.2. Both upstream and downstream flowcells were inoculated. Lines one and two were supplied with SGW containing 6 mg l^{-1} of soluble Mn (0.0185 g l^{-1} $MnSO_4$), while lines three and four were supplied with SGW containing no Mn. Lines five and six served as sterile control lines and were also supplied with SGW containing 6 mg l^{-1} Mn. Biofilms were grown for 504 h/21 days with effluent samples collected during the first 24 h and every 48 h thereafter. Serial dilutions were prepared from collected effluent and plated out onto selective Mn oxidation agar and TSA using the drop plate method. Both selective Mn oxidation agar and TSA were adjusted to a pH value of 8.0. All agar plates were incubated at 26°C for 48 h. Following serial dilutions, all samples originally collected from the flow system channels were treated with 65% nitric acid prior to Mn analysis. Mn concentrations at the end of each line were measured using the Spectroquant® Manganese Cell Test (1.00816.0001, Merck). This test was used in combination with the Spectroquant® Pharo 300 (Merck) to determine Mn concentrations present in the effluent samples.

4.2.6. Simulating the LBIS network and the physico-chemical conditions experienced inside this pipeline

Laboratory scale linear, closed flow systems were developed in which DO concentration could be adjusted and maintained to replicate environmental conditions present at each of the possible extraction depths (Fig. 4.1). Four growth conditions were selected as suitable to be tested in the DO system and were each maintained in duplicate in the flow system (Table 4.1). These conditions were as follows:

Table 4.1 - Growth conditions present in lines 1 – 8 for the comparison of biofilm growth and development under different DO and Mn concentrations.

Line	**DO (mg l^{-1})**	**Mn (mg l^{-1})**
1 + 2	Between 1 – 2	6
3 + 4	Between 5 – 6	6
5 + 6	Between 7 – 9	6
7 + 8	Between 7 – 9	0.019

Flow systems consisted entirely of non-permeable Tygon® tubing with an inner diameter of 1.6 mm to limit oxygen entering the flow system to that present in the influent reservoirs. Influent reservoirs containing SGW for lines one and two were prepared as described in section 4.2.1 and allowed to cool down following autoclaving, where after it was sparged with high purity nitrogen gas through 0.22 µm filters (Merck) for 30 min. To maintain this DO concentration throughout the duration of the experiment, influent reservoir flasks were sealed with rubber stoppers through which SGW samples could be extracted by means of sealed connections with 1 mL glass pipettes. These flasks were connected to impermeable, sterile wine bags filled to maximum capacity with high purity nitrogen gas passing through 0.22 µm filters to allow filling of headspace with O_2-deplete gas, as well as to maintain low DO concentrations during SGW extraction.

The same procedure was followed for influent reservoirs containing SGW for lines three and four with DO concentrations of 5 – 6 mg l^{-1}, where sparging was only performed for 5 min. Influent reservoirs for lines five to eight with DO concentrations between 7 and 9 mg l^{-1} were cooled down and only covered with aluminium foil. SGW for lines 7 and 8 were adjusted accordingly to contain only 0.019 mg l^{-1} Mn. To prevent shear-damage or loss of biofilms cultivated in this system, impermeable glass bubble traps with sealable lids were connected in-line, upstream from both inoculation points (Fig. 4.1 G and J) to collect air bubbles introduced into the system during media changes approximately every 8 days.

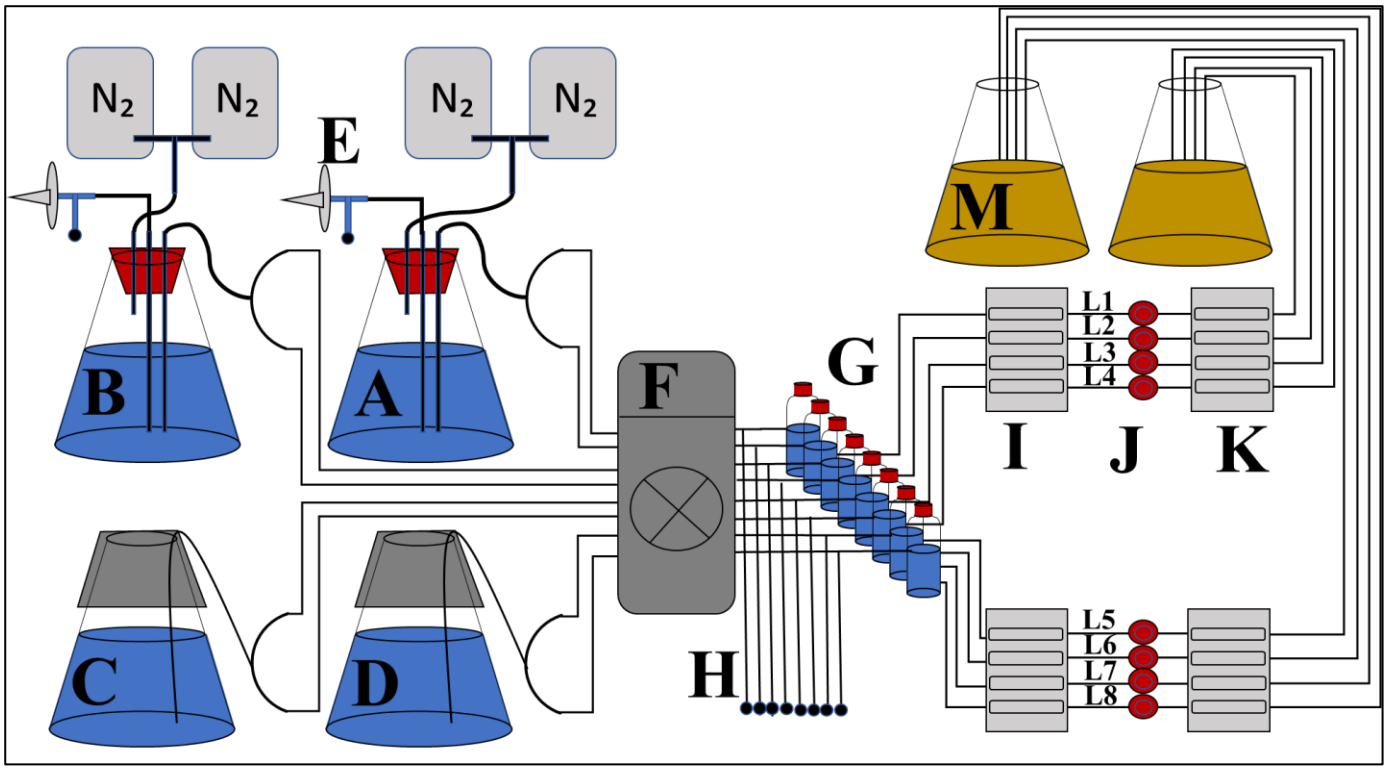


Fig. 4.1 – Laboratory scale flow system with four different growth conditions for the cultivation of Mn oxidizing and reducing biofilms. A: Growth medium for lines 1 and 2. B: Growth medium for lines 3 and 4. C: Growth medium for lines 5 and 6. D: Growth medium for lines 7 and 8. E: 0.22 µm filters through which nitrogen gas was sparged. F: Watson Marlow 205S peristaltic pump. G: Glass bubble traps. H: In line diversions for DO measurements. I: Upstream flowcell channels connected to downstream flowcell channels (K) via 4.5 m Tygon® tubing with in line bubbletraps (J). L 1-8: Respective lines representing different DO and Mn concentrations. M: Waste effluent collected from the flow system.

In-line diversions were built into the flow system to redirect flow to a glass manifold where DO concentration could be measured using a YSI Professional Series DO meter. DO measurements were conducted every 48 h or as frequent as possible, especially after media changes. Thick sections of Tygon® tubing with an inner diameter of approximately 3 - 4 mm and a length of 8 cm were used for biofilm cultivation rather than flowcells in the flow system of this specific section as it provided a larger surface for biofilm growth than standard flow cells as well as more robust connections, allowing for easier access during effluent collection without causing leaks. One section of tubing was connected just downstream from the first bubble trap (Fig. 4.1 G) in each of the eight lines, as well as further downstream after a second bubble trap (Fig. 4.1 J). Each line thus contained two inoculation points separated by 4.5 m Tygon® tubing. Biofilms were cultivated by inoculating these sections of tubing with 200 µl of the standardized inoculum diluted to an OD of 0.1 as described in section 4.2.4. This experiment was performed twice under identical conditions. In both experiments,

biofilms were cultured for 28 days and influent media flasks replaced every 8 days or a frequently as needed.

4.2.6.1. Enumeration of growth on TSA, selective Mn oxidation agar and selective Mn reduction agar.

Effluent samples were collected from both upstream and downstream inoculation points in each line every four days/96 h. Serial dilutions were prepared from the original collected samples and plated out onto TSA, selective Mn oxidation agar and selective Mn reduction agar using the drop plate method (Cerrato et al., 2010; Herigstad et al., 2001) and incubated at 26°C for 48 h. Selective Mn reduction agar plates were incubated for 168 h/7 days under anaerobic conditions using anaerobic gas packs (AnaeroPacks, Davies Diagnostics). All three agar types were adjusted to pH 8.0. After the respective incubation periods, growth was evaluated to determine average CFU ml^{-1}.

4.2.6.2. Mn analysis of collected effluent

After serial dilutions were prepared and DNA had been extracted (section 4.2.6.4), the remainder of collected effluent samples were treated with 65% nitric acid where after it was subjected to Mn analysis using the Spectroquant® Manganese Test (1.01846.0001-Merck). This test was used in combination with the Spectroquant® Pharo 300 to determine Mn concentrations present in the effluent samples.

4.2.6.3. Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM/EDS)

Following termination of the biofilm flow systems after 28 days, biofilms were removed from the 4.5 m Tygon® tubing sections connecting the upstream inoculation points to the downstream inoculation points. These biofilms were dried for 72 h at 26°C before being subjected to SEM/EDS using the Zeiss Merlin scanning electron microscope equipped with an energy dispersive X-ray spectrometer at CAF. All samples were coated in gold prior to analysis.

4.2.6.4. DNA extraction and polymerase chain reaction (PCR) for Automated Ribosomal Intergenic Spacer Analysis (ARISA)

DNA was extracted from effluent samples collected on days 8, 16, 24 and 28 using the Zymo *Quick* – DNA™ Fecal/Soil Microbe Miniprep Kit. Two millilitres of collected sample were centrifuged at 10000 RCF for 10 min where after the supernatant was removed. This step was replicated three times, where after the Zymo *Quick* – DNA™ Fecal/Soil Microbe Miniprep Kit's protocol was followed. After 28 days, media flow was terminated and biofilms inside the thick sections of tubing were also sacrificed for DNA extraction.

Following successful DNA extraction, samples were subjected to gene amplification of the bacterial ITS region using fluorescently labelled primers. PCR's were performed (Table 4.2) in triplicate for all samples to account for PCR bias. Bacterial community analysis was performed by amplifying the hypervariable lengths of the ITS regions between the 16S and 23S rDNA genes with ITS primers.

Table 4.2 - Bacterial primer set used for genetic amplification using PCR for ARISA of bacterial communities (Lawson, 2015 as modified from Slabbert et al., 2010).

Primer set	Primer name	Primer sequence	Fluorescent Label
Bacterial	ITSF-FAM	'5-GTCGTAACAAGGTAGCCGTA-3'	6-carboxy-fluorescein
	ITSReub	'5-GCCAAGGCATCCACC -3'	

The reaction mixture contained 4.1 µl MilliQ water, 5.0 µl Readymix (Kapa Biosystems), 0.2 µl Forward Primer, 0.2 µl Reverse Primer and 0.5 µl extracted DNA.

The following PCR conditions were used for ARISA of bacterial communities:

- Initial denaturing step at 95°C for 5 minutes
- 40 cycles of denaturation at 95°C for 45 seconds, annealing at 56°C for 50 seconds and elongation at 72°C for 1:10 minutes
- Final elongation step 72°C for 7 minutes
- Hold at 4°C

After amplification, all PCR products were visualised on a 1% agarose gel stained with Ethidium Bromide under UV light. Triplicate samples were pooled and sent for analysis at CAF where it was subjected to capillary electrophoresis on the automated ABI 3500XL Genetic Analyser (Applied Biosystems). The LIZ® 1200 size standard was used for all samples (Slabbert et al., 2010). Following capillary electrophoresis, a fluorescence electropherogram was received and analysed on the GeneMapper™ 5 software to convert data that represented operational taxonomic units (OTUs) into peaks that would represent fluorescence intensity and to obtain a genotypes table for further analysis. A bin size of 3 was used in GeneMapper™ 5 to compensate for overestimation. Prior to further analysis, the electropherogram was trimmed from 100 bp to 1200 bp to cut out primer dimers and other DNA artifacts. Further analysis was carried out using the Vegan package (Oksanen, 2018) in R (Version 3.3.3).

4.2.7. Confocal laser scanning microscopy (CLSM)

A flow system identical to that described in Fig 4.1 was constructed for culturing biofilms to be subjected to CLSM after 32 days. This experiment was replicated twice. The thick Tygon® tubing sections used for biofilm cultivation in the described experimental systems in section 4.2.6 were replaced with standard Perspex flow cells with sealed connections for biofilm visualization with CLSM. The substratum for biofilm growth consisted of a microscope glass cover slip. After 32 days, flow was terminated, and biofilms were stained with 200 µl of a 1 µl ml^{-1} working stock of Syto 9™ (LifeTechnologies™), where after it was incubated in the dark for 40 min to allow for binding of the stain to the biofilms. After the incubation period, flow was resumed at approximately 12 ml h^{-1} per channel for 15 min to wash out any residual stain not bound to biofilms. Flowcells were then disconnected from the flow system and sealed, where after microscopic observations and imaging of the biofilms were conducted using a Carl Zeiss LSM 780 confocal microscope at CAF. Imaging conditions similar to that set out by Heydorn et al. (2000) were followed.

Biofilm images were obtained using a 40× objective and acquired at random along a transect down the middle of each flowcell channel. Each biofilm image stack was comprised of images acquired at 1.68 µm intervals through the biofilm of each selected imaging location. Image scanning was carried out with a 488 nm laser. Three image stacks were collected per flow channel to obtain an average of the biofilm parameters to be analysed in each channel. Using the COMSTAT version 1 package (Heydorn et al., 2000) in MATLAB (TheMathWorks) version 8.3.0.532 (R2014a), average biomass, surface to biovolume ratio and biofilm maximum thickness in each flow channel were calculated and compared between growth conditions (Heydorn et al., 2000). Additionally, effluent was collected for Mn analysis from the end of each line after 28 days during the second replicate and filtered through 0.22 µm syringe filters, where after both filtered and unfiltered aliquots were treated with 65% nitric acid prior to Mn analysis.

4.2.8. Sterile flow system

A sterile/uninoculated flow system was constructed and maintained under identical conditions as described in Fig 4.1. This system was monitored for 5 days, where after effluent samples were collected from each line at the end of the flow system and filtered through 0.22 µm syringe filters. Both filtered and unfiltered aliquots were treated with 65% nitric acid prior to Mn analysis using the Spectroquant® Manganese Test in combination with the Spectroquant® Pharo 300.

4.3. Results and Discussion

4.3.1. Validation of biofilm growth in flow systems

Biofilm formation in initial flow systems were evaluated using light microscopy and indicated that thick visible biofilm formation was present after approximately 96 h. Biofilm growth was visibly less in flowcells connected downstream compared to those connected upstream. It was assumed that this was likely due to nutrient depletion as biofilms growing upstream from the downstream inoculation point was utilizing the nutrients in the SGW before it reached biofilms in the downstream flowcell. It was decided to incorporate this upstream and downstream arrangement in subsequent flow systems as it simulated anticipated nutrient depletion with distance in the LBIS pipeline network.

4.3.2. Determining the suitability of a standard inoculum

Colony growth on TSA and selective Mn oxidation agar was evaluated after 48 h of incubation at 26°C to determine average CFU ml^{-1}. After the growth data in Fig. 4.2 showed differences between inocula used, all data was subjected to two-way analysis of variance (ANOVA) with replication performed at a significance level of 0.05 to determine whether any differences observed were significant. The outcome of this test showed a P-value < 0.05 for variance between inocula, indicating that there is sufficient evidence at a 95% confidence interval to infer that the inoculum used has an effect on average CFU ml^{-1}. However, P-values > 0.05 showed insufficient evidence at a 95% confidence interval to infer that pH has an effect on average CFU ml^{-1}, or that there is any significant interaction between inoculum used and the pH of the growth media. It was therefore decided that all subsequent experiments would be carried out using growth media adjusted to a pH level of 8.0, which most accurately represents optimal conditions for Mn oxidation as well as the pH levels observed at the Blyderiver dam. Although the differences observed between inocula were statistically significant, the observed differences in average CFU ml^{-1} were not in orders of magnitude and thus it was concluded that different freezer stocks would not affect biofilm development drastically in subsequent experiments.

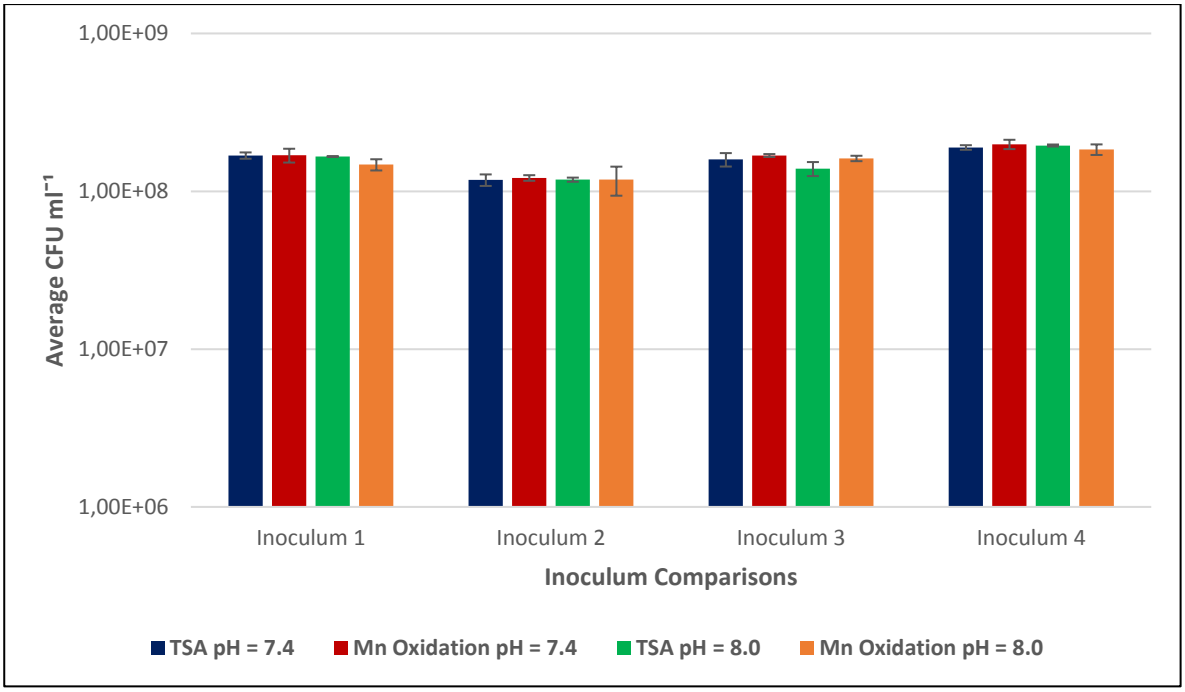


Fig. 4.2 - Average CFU ml^{-1} of four freeze cultures plated out onto TSA and selective Mn oxidation plates adjusted to pH 7.4 and 8.0 respectively.

4.3.3. Mn partitioning in an open oxygen system

The aim of this experiment was to evaluate whether the presence of Mn would have an influence on the growth of Mn oxidizing and reducing biofilms without any adjustments to DO concentrations. With all SGW influent reservoirs only covered with sterile aluminium foil, DO concentrations in the influent media were in the range of 7 – 9 mg l^{-1}. The second aim was to determine whether the presence of Mn oxidizing and reducing biofilms would facilitate oxidation of Mn supplied in the growth medium, followed by incorporation into the biofilm structure and ultimately leading to a decrease in Mn concentration in the effluent. Growth was evaluated by serial dilution and plating out onto TSA and selective Mn oxidation agar, followed by incubation at 26°C for 48 h.

Average CFU ml^{-1} between the different growth conditions differed drastically after the first 24 h, with biofilms from the upstream inoculation points supplied with SGW containing Mn showing the least growth. Both downstream biofilms supplied with SGW with and without Mn showed higher growth than upstream biofilms during the first 24 h. This was observed on both TSA (Fig 4.4) and selective Mn oxidation plates (Fig. 4.5). However, after 72 h, growth started to stabilize and average CFU ml^{-1} remained similar overall for all conditions, with no significant differences observed between any of the conditions tested, suggesting that cell attachment equalled cell detachment once the carrying capacity of the system was reached. The same pattern was again observed on both growth media types used. One-way ANOVA was used to determine whether differences observed over the total period of 21 days on both growth medium types were significant. Growth measured from upstream and downstream biofilms were subjected to separate statistical analysis as the goal of the statistical tests were not to determine whether differences between upstream and downstream biofilms were significant, but rather to determine the significance of differences observed between test conditions both upstream and downstream.

One-way ANOVA analysis of differences in average CFU ml^{-1} from upstream biofilms showed a P-value > 0.05 on both TSA and selective Mn oxidation agar, indicating that the differences observed between test conditions upstream were not significant. Differences in average CFU ml^{-1} from downstream biofilms produced the same result. It was concluded that in this laboratory scale flow system with biofilms subjected to DO concentrations of 7 – 9 mg l^{-1}, neither the presence of Mn nor the absence thereof had any significant effect on planktonic cell yield by these biofilms. Considered from an ecological perspective, this is an interesting result as it demonstrates the role of biofilms as a mode of proliferation, as opposed to the conventional view of biofilms as a survival strategy.

Mn concentrations measured in the effluent at the same sampling intervals described above also showed little difference over the time period of the experiment (Fig. 4.3). Mn concentration in lines one and two containing biofilms were evaluated against the two sterile control lines to determine whether the presence of biofilms potentially capable of oxidizing and reducing Mn would lead to a decrease in Mn concentration in the effluent, compared to that of the sterile control.

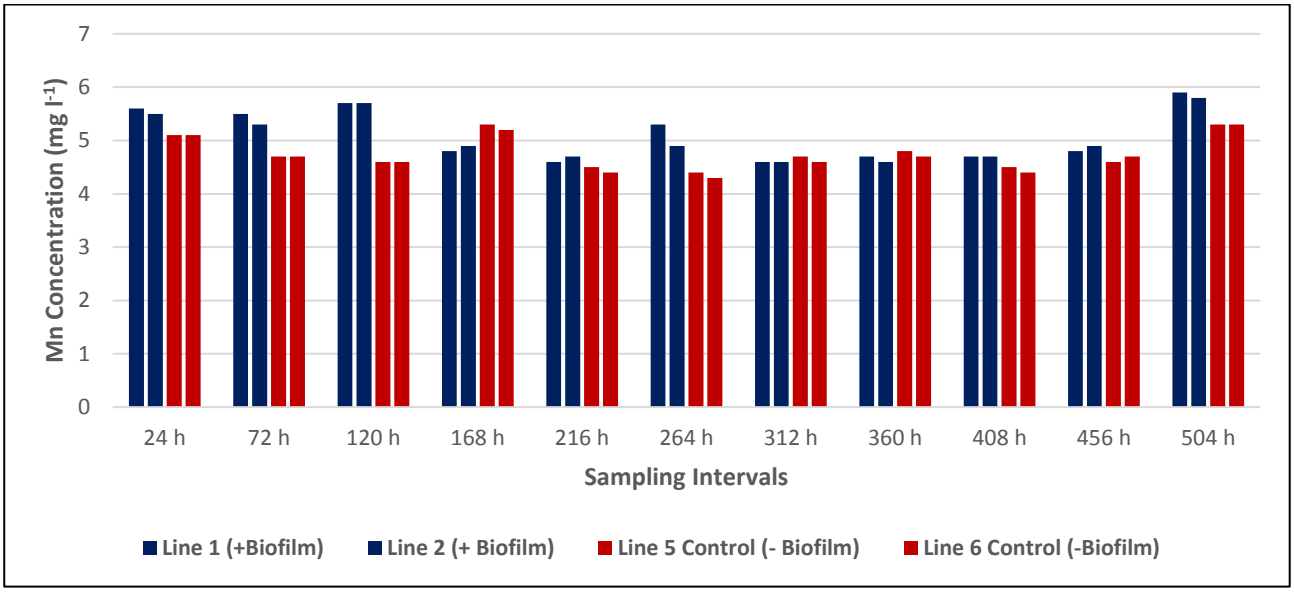


Fig. 4.3 - Mn concentrations measured at the end of all four lines supplied with SGW containing 6 mg l^{-1} Mn.

One-way ANOVA indicated that the differences in Mn concentration observed between lines containing biofilm growth compared to the sterile control were not significant, with a P-value > 0.05. However, a clear pattern observed throughout the duration of the experiment was the lower Mn concentrations in the effluent of the control channels compared to that of the effluent containing biofilm growth. Although this difference was not significant, it is noticeable and unexpected. As the reservoir for this system was only covered with sterile aluminium foil, allowing oxygen to enter and mix with the water, it was speculated that in the absence of biofilms some of the Mn could be oxidized, causing it to precipitate and collect at the bottom of the tubes. Due to the low flow, the precipitated Mn would not leave the system along with the effluent. In the presence of biofilms, microbial activity would lead to a decrease in DO concentration within the flow system, maintaining Mn in the soluble state and causing any precipitated Mn to undergo reduction, thereby keeping it in the aqueous phase leaving the system. The inability of biofilms to oxidize Mn and incorporate it into the biofilm structure was thought to be due to the high DO concentrations of 7 – 9 mg l^{-1}, as optimal Mn oxidation occurs at DO concentrations of 5 – 6 mg l^{-1} with pH levels between 7.5 – 8.3 (Du Toit et al.,2015). This was taken into account for the next experimental flow system.

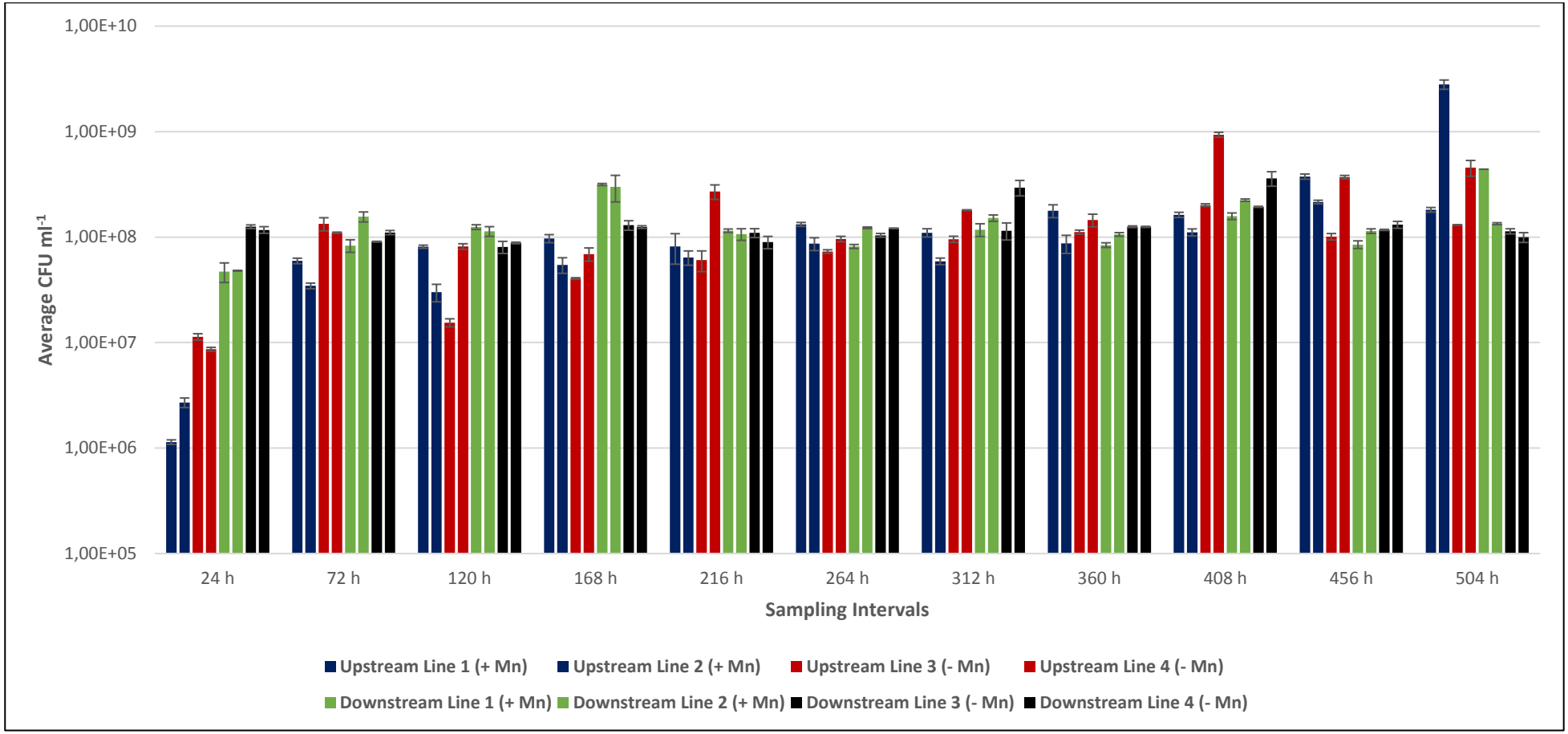


Fig. 4.4 - Enumeration of culturable bacteria (CFU ml^{-1}) on TSA obtained from effluent collected from the upstream and downstream inoculation points.

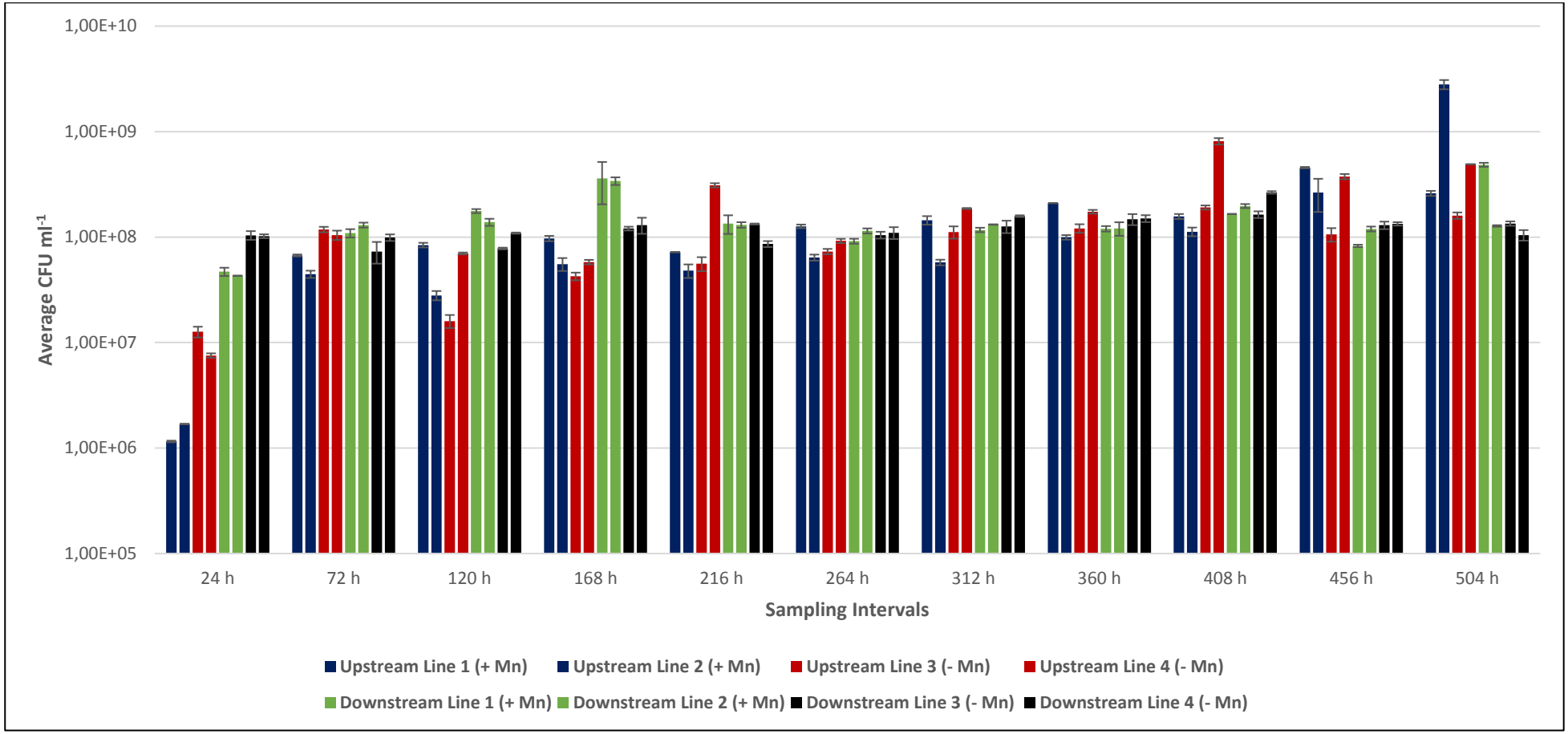


Fig. 4.5 – Enumeration of culturable bacteria (CFU ml^{-1}) on selective Mn oxidation agar obtained from effluent collected from the upstream and downstream inoculation points.

4.3.4. Simulating the LBIS network and the physico-chemical conditions experienced inside this pipeline

This linear flow system testing the effects of various DO and Mn concentrations on the development of Mn oxidizing and reducing biofilms was used to determine whether significant differences could be measured between biofilms grown in SGW with low DO and high total Mn concentrations compared to those grown in SGW with high DO and low total Mn. The overall goal of this experiment was to test the hypothesis that biofilm formation would be less significant when the LBIS extraction point in the Blyderiver dam wall is shifted from deeper depths with low DO and high total Mn concentrations to a shallower depth with high DO and low total Mn concentrations.

4.3.4.1. Enumeration of culturable bacteria – Replicate 1

After growth on TSA, selective Mn oxidation agar and selective Mn reduction agar had been evaluated to determine average CFU ml^{-1}, little difference could be seen in biofilm productivity as measured in planktonic cell yield, grown under these different conditions. Initially, it was expected that biofilms grown under DO concentrations of 1 – 2 mg l^{-1} and 5 – 6 mg l^{-1} might show higher growth, due to the optimal parameters present for Mn oxidation. In contrast, biofilms grown in channels with DO concentrations of 7 – 9 mg l^{-1} in lines five to eight were expected to show less growth, especially due to the low Mn concentration of 0.019 mg l^{-1} in lines seven and eight. One-way ANOVA analysis was used to determine whether the differences in growth observed between the different conditions were significant at a confidence level of 95%.

Growth measured on TSA plates (Fig. 4.6) from effluent collected from the upstream and downstream inoculation points were subjected to separate respective one-way ANOVA tests as differences between growth under the tested conditions were mainly compared between groups at the respective positions upstream and downstream. Differences observed between growth at upstream and downstream biofilms within a line are discussed in section 4.3.4.2. One-way ANOVA indicated that differences observed between groups from upstream and downstream inoculation points respectively were not significant, resulting in a P-value > 0.05. This was true for growth on all three medium types. Considering the high degree of functional redundancy and diversity in heterogeneous microbial communities, the results on TSA plates could have been expected. However, this was also true for average CFU ml^{-1} counts on selective Mn oxidation agar (Fig. 4.7) as well as Mn reduction agar (Fig. 4.8), which was not expected. These results indicate that although the Mn oxidation agar and Mn reduction agar used allows for the growth of Mn oxidizing and reducing microorganisms

respectively, it does not select for these microorganisms exclusively. From this first trial on a laboratory scale level, it was thus concluded that the concentration of DO and Mn did not have any significant effect on the productivity of the biofilms (measured as planktonic cell yield).

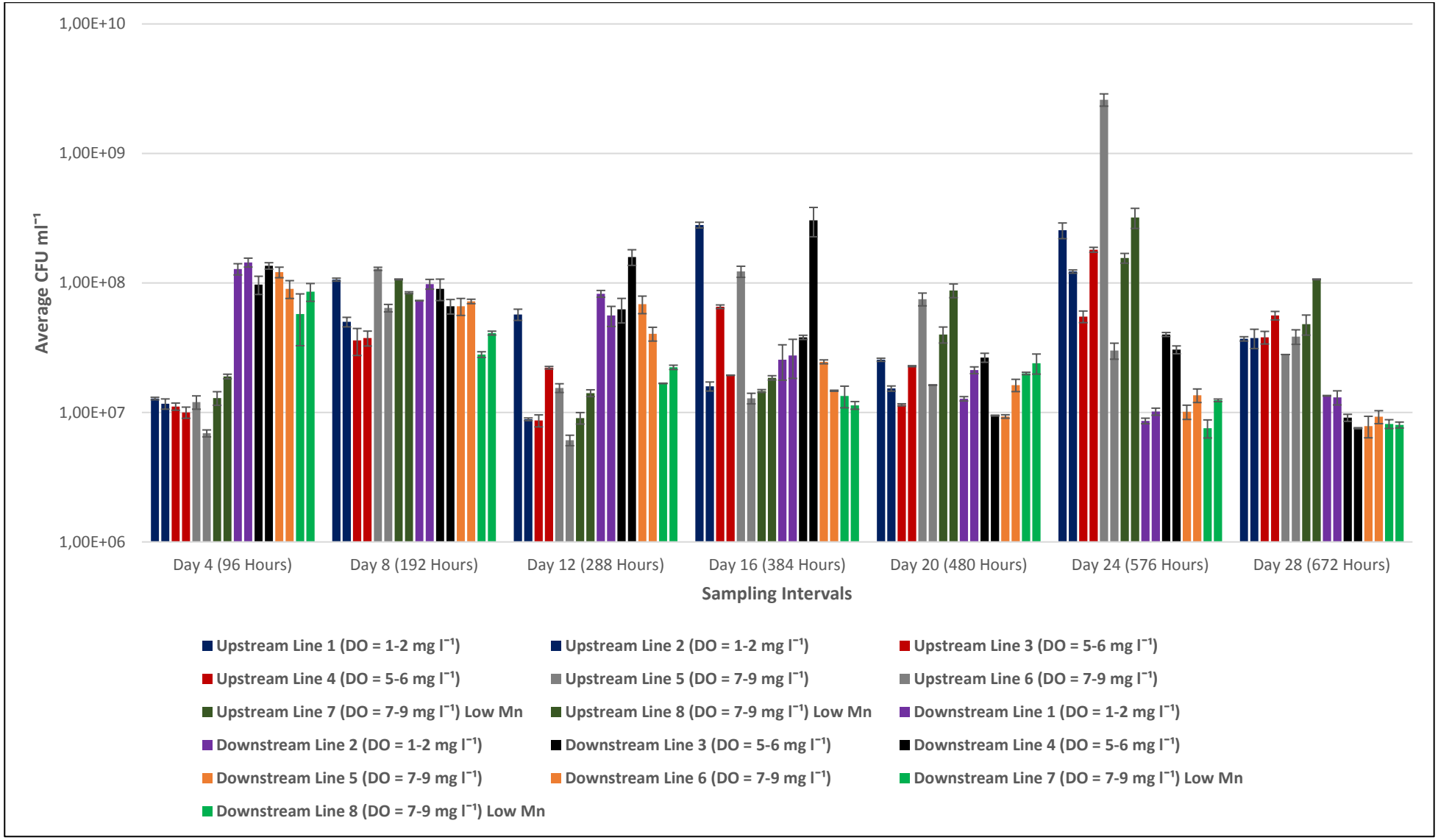


Fig. 4.6 - Enumeration of culturable bacteria (CFU ml^{-1}) on TSA obtained from effluent collected from the upstream and downstream inoculation points over a period of 28 days.

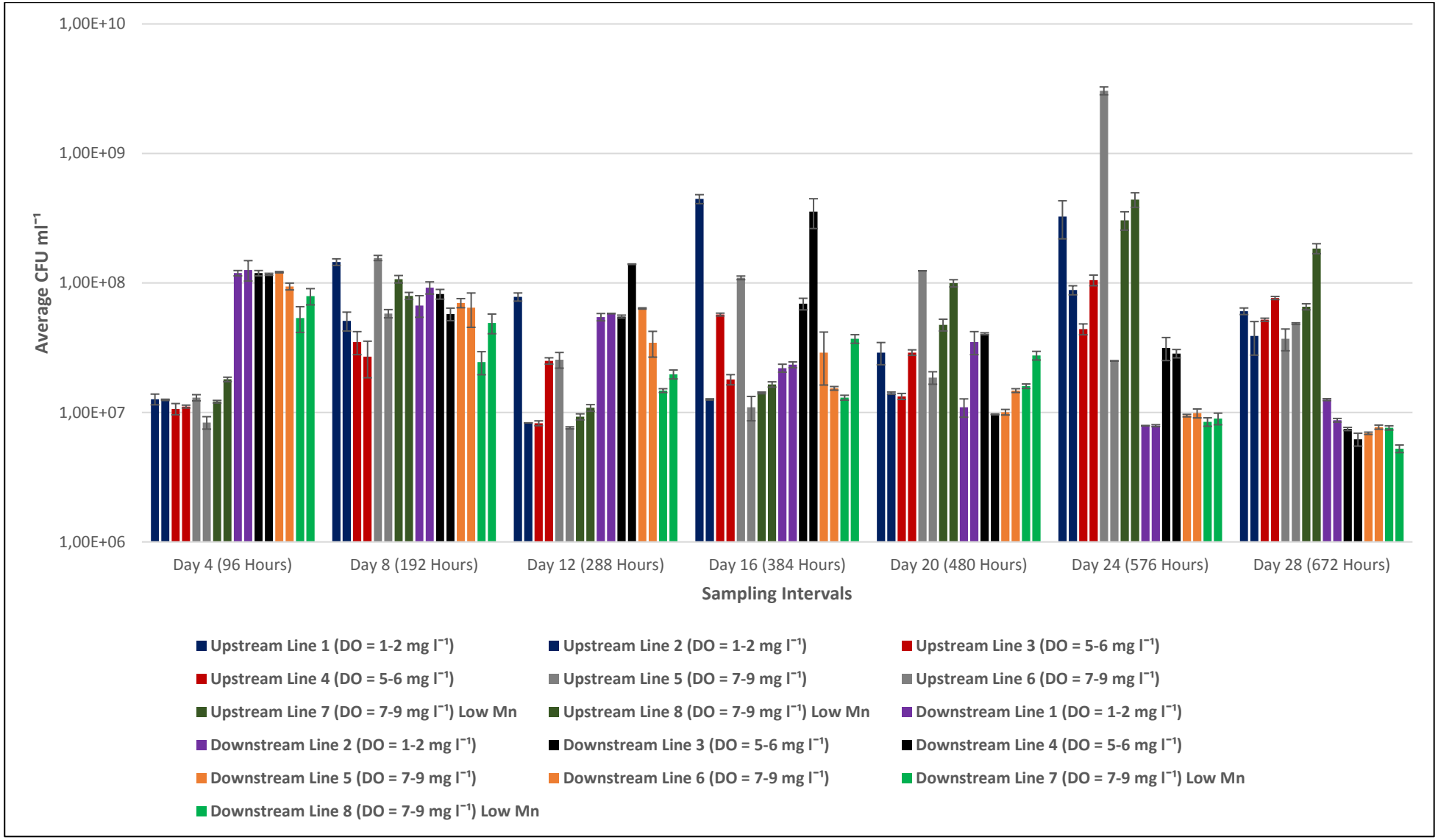


Fig. 4.7 - Enumeration of culturable bacteria (CFU ml^{-1}) on selective Mn oxidation agar obtained from effluent collected from the upstream and downstream inoculation points over a period of 28 days.

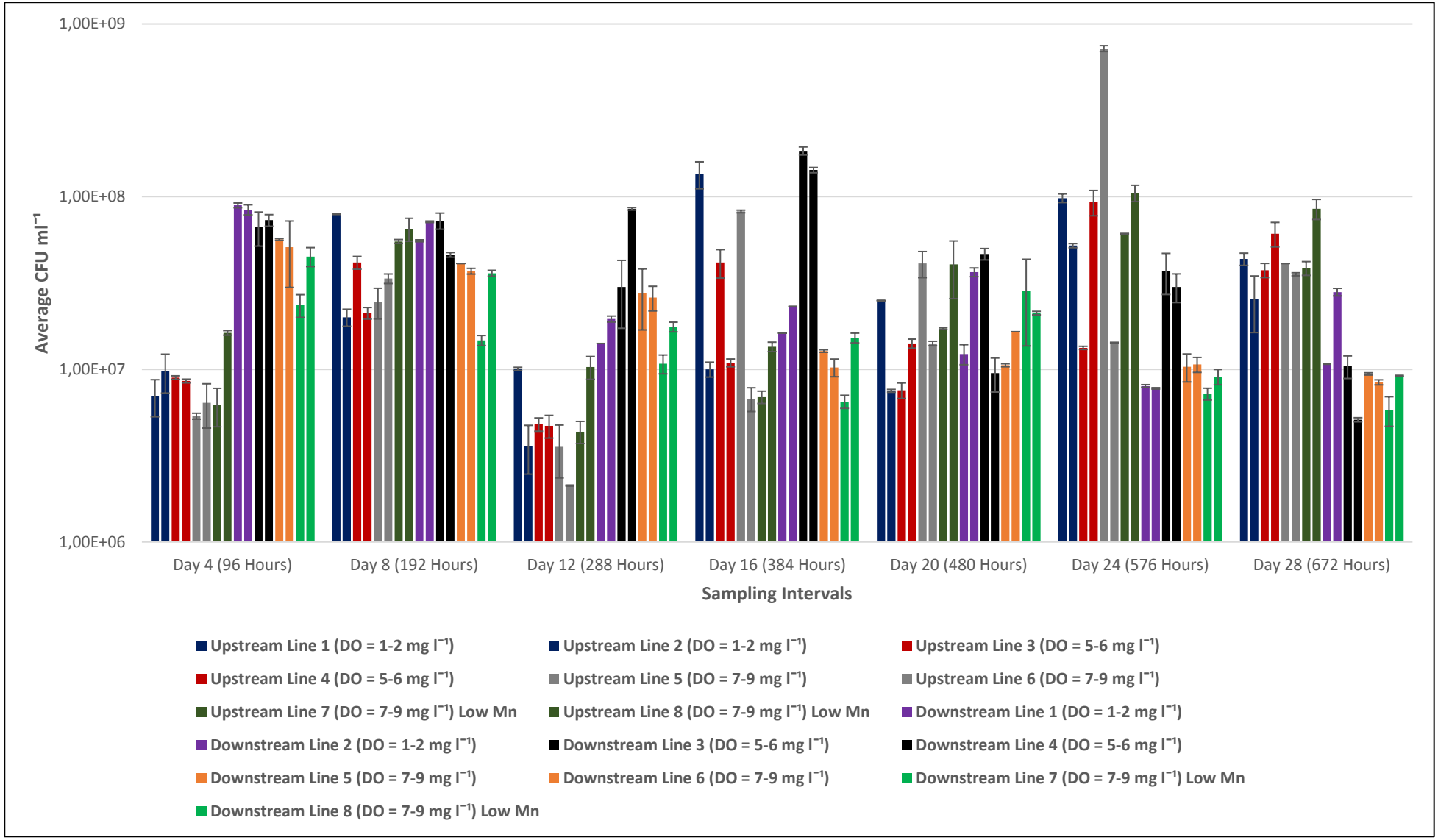


Fig. 4.8 - Enumeration of culturable bacteria (CFU ml^{-1}) on selective Mn reduction agar obtained from effluent collected from the upstream and downstream inoculation points over a period of 28 days.

4.3.4.2. Enumeration of culturable bacteria – Replicate 2

A second replicate simulating the LBIS network conditions experienced inside the pipeline was carried out in order to confirm the results obtained during the first replicate, as this system has multiple factors affecting the overall activity of the biofilms cultured inside the flow channels. Although growth conditions were maintained to be identical to that of the first experiment, clear differences in activity could be observed between the two experiments when comparing Fig. 4.6 – 4.8 to Fig. 4.9 – 4.11. Whereas the activity between biofilms from the upstream and downstream inoculation points underwent a clear shift over the duration of the first replicate of this experiment, this pattern could not be observed during the second replicate experiment. After four days of growth, a difference in average CFU ml^{-1} is visible between upstream and downstream biofilms, with upstream biofilms showing higher average activity overall in terms of cell yield. This pattern gets more prominent after day 8 of the experiment, carried on throughout and could be observed on all three medium types (Fig 4.9 – Fig. 4.11). Although this growth pattern differs to some degree from that observed in the first replicate of this experiment where the planktonic cell yield was initially higher at the downstream inoculation point after 4 days and underwent a clear shift during the remaining 24 days of the experiment, both replicates show higher biofilm activity for upstream biofilms compared to downstream biofilms after approximately 24 days.

These findings confirm initial expectations and observations made during initial validation experiments in section 4.3.1. From these observations, it was expected that average CFU ml^{-1} would be lower for biofilms at the downstream inoculation point throughout the experiment, as initial observations of biofilms using light microscopy indicated that biofilm accumulation was visibly less in the downstream inoculation point compared to that of the upstream inoculation point in that system. Due to the complex branching and length of the LBIS pipeline network, it was decided that this would be replicated in the final laboratory scale flow systems of section 4.3.4. It is suggested that the lower growth observed in downstream biofilms is a result of nutrient depletion. Biofilms growing at the first inoculation point upstream and downstream toward the second inoculation point are speculated to use most of the nutrients and energy sources in the SGW. This observation is discussed further at the end of this section. Although it could be expected that the high concentration of total Mn in the SGW at this point could serve as an energy source, there is no evidence to suggest that this happened (Tebo et al., 2005). The reason for the differences in activity observed between the two respective replicate experiments is not clear. However, due to the stochastic nature of biofilm development, independent rounds of biofilm cultivation rarely result in identical outcomes (Heydorn et al., 2000).

Growth data from all three medium types (Fig. 4.9 – Fig. 4.11) were subjected to one-way ANOVA to investigate whether any observed differences in activity for the upstream inoculation point were significant at a confidence level of 95%. Statistical analysis indicated that differences observed between average cell yield on TSA plates (Fig. 4.9) were significant, with a P-value < 0.05. Further statistical analysis was carried out using the Real Statistics Resource Pack software (Zaiontz, 2013) and subjecting the data to the Tukey Honestly Significant Difference (HSD) test which indicated that significant differences could be observed when comparing average CFU ml^{-1} of lines 7 and 8, both containing DO concentrations between 7 – 9 mg l^{-1} and Mn concentration of 0.019 mg l^{-1}, with that of almost all other conditions tested in the other 6 lines. Average CFU ml^{-1} from line 8 did not differ significantly from that of line 3. Statistical analysis of differences between average CFU ml^{-1} between downstream biofilms showed no significant difference, with a P-value > 0.05.

Statistical analysis of differences between average CFU ml^{-1} counted on selective Mn oxidation plates (Fig. 4.10) for upstream biofilms showed a P-value < 0.05, indicating that these differences were significant. Further analysis using the Tukey HSD test indicated that average CFU ml^{-1} from lines 7 and 8 differed significantly from that of lines 2 and 5, containing SGW with DO concentrations of 1 – 2 mg l^{-1} and 7 – 9 mg l^{-1} respectively. Interestingly, as lines with the same conditions were supplied with SGW from the same reservoir, it was expected that significant differences in activity between conditions would be seen between growth in both lines per condition, i.e. growth in lines 7 and 8 would differ significantly from growth in both lines 5 and 6. However, this was not the case, as growth in lines 7 and 8 only differed significantly from that of lines 2 and 5 but not from lines 3 and 6. Differences between activity in biofilms in the downstream inoculation point plated out onto selective Mn oxidation agar was also significant, with further analysis showing significant differences between growth in lines 7 and 6.

Lastly, evaluating growth on selective Mn reduction agar (Fig. 4.11), no statistically significant differences could be observed between average CFU ml^{-1} for the different biofilms at the upstream inoculation point. The same was true for growth measured for biofilms at the downstream inoculation point. After analysing growth data for upstream and downstream biofilms from both experimental replicates on all growth media types, it was concluded that none of the conditions had an overall significant effect on the productivity of these biofilms. Although statistically significant differences in average CFU ml^{-1} could be observed on TSA and selective Mn oxidation media types over the duration of the second experimental replicate, the lack of statistically significant differences between average CFU ml^{-1} on all growth medium types respectively in the first replicate suggest that none of the conditions tested had a statistically significant overall effect. This finding is further supported by

the lack of statistically significant differences of measured growth on selective Mn reduction media during the second replicate. Lastly, the few significant differences in growth that were observed were only observed between both lines of a condition on TSA and selective Mn oxidation plates for upstream biofilms during the second replicate. However, the conditions that did differ significantly did not correspond between these two medium types. Significant differences observed between growth on selective Mn oxidation plates for downstream biofilms could only be measured between single lines of a condition.

Although it was observed that average CFU ml^{-1} was lower for biofilms at the downstream inoculation point compared to that of the upstream inoculation point for all three medium types during both replicates, further investigation points to some important findings. It was initially expected that average CFU ml^{-1} would be less at the downstream inoculation point due to the depletion of nutrients by biofilms growing upstream. However, although differences can be seen in Fig. 4.6 – Fig. 4.8, these differences were not found to be significantly different after subjecting the results to one-way ANOVA (i.e. differences measured within each line between upstream and downstream inoculation points). Statistically significant differences could however be observed between average CFU ml^{-1} of biofilms at upstream and downstream inoculation points on all three medium types for the second replicate (Fig. 4.9 – Fig. 4.11). Investigating the results of all three medium types for both replicates shows a clear pattern. Although some statistically significant differences exist when comparing cell yield between inoculation points for the second replicate, the differences overall between average CFU ml^{-1} at the upstream inoculation points and downstream inoculation points are not in orders of magnitude. This was surprising as it was expected that upstream biofilms would utilize most of the nutrients, leading to starvation conditions further downstream. Similar results have been reported previously by Bester et al. (2009) where they reported that biofilms supplied with different concentrations of carbon produced planktonic cell yields which eventually stabilized at similar levels. It is therefore presumed that the upstream biofilms will not continue to develop, utilizing all available nutrients and produce higher cell yield indefinitely, but rather that this phenomenon will occur during the early stages of biofilm development and that cell yield from these biofilms will level off with biofilm age and stabilize at levels similar to downstream biofilms.

The hypothesis that this experiment set out to prove was that biofilm activity in terms of cell yield in SGW containing high DO concentrations between 7 – 9 mg l^{-1} and low Mn concentrations of approximately 0.019 mg l^{-1}, such as that present in the water of the Blyderiver dam at 20 m depth, would be significantly less than biofilms grown in SGW containing a Mn concentration of 6 mg l^{-1} and a DO concentration between 1 – 2 mg l^{-1} such as that present at the current extraction depth.

Based on data obtained through both replicates of this experimental flow system, this hypothesis was disproved under the conditions of these laboratory-scale systems. However, other factors need to be taken into account, such as the duration of the experiment (which was relatively short compared to the time that e.g. the Preekstoel biofilters for Mn/Fe removal at Hermanus took to stabilize), and the labile nutrients added to the SGW in the flow system.

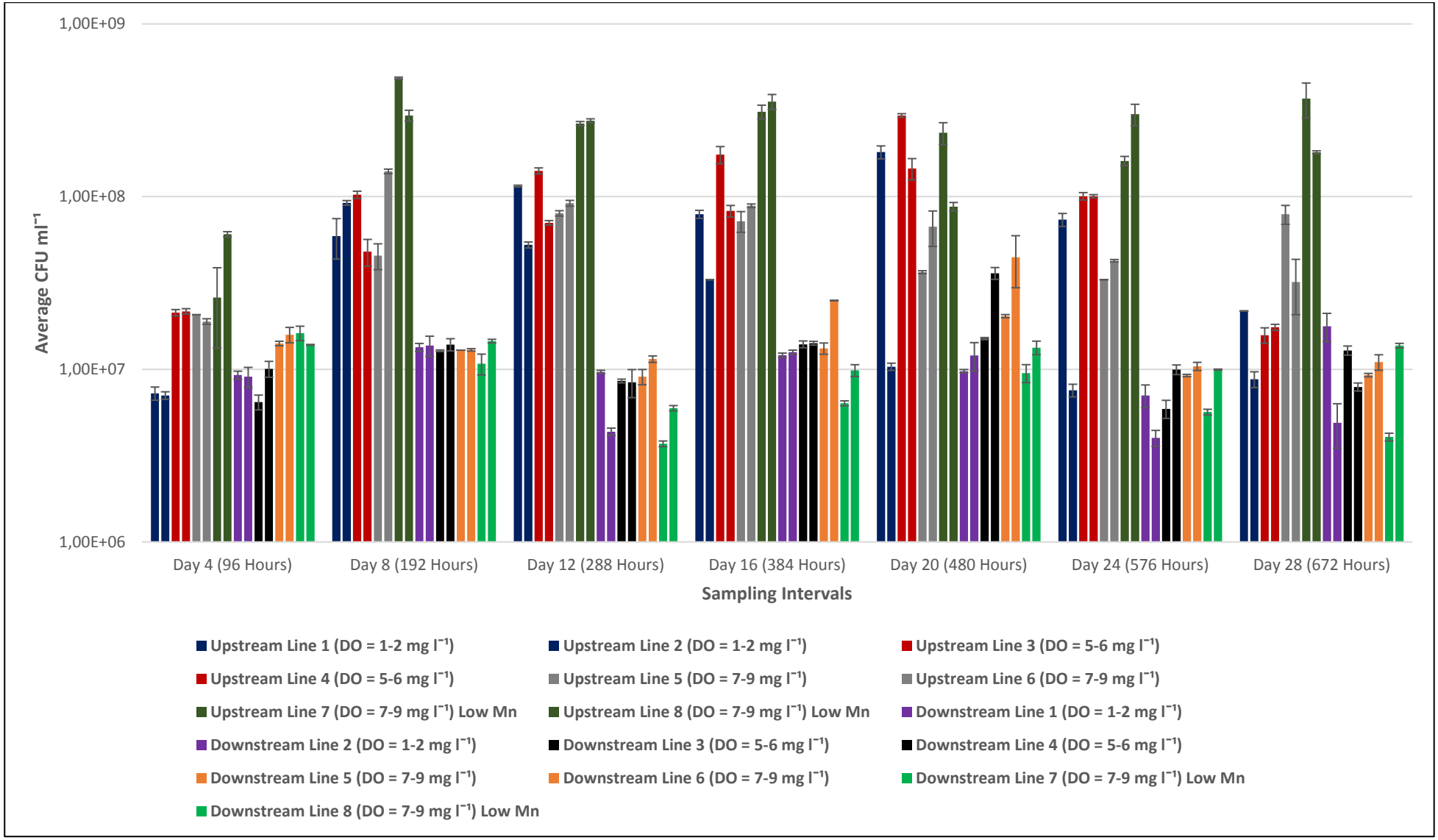


Fig. 4.9 - Enumeration of culturable bacteria (CFU ml^{-1}) on TSA obtained from effluent collected from the upstream and downstream inoculation points over a period of 28 days.

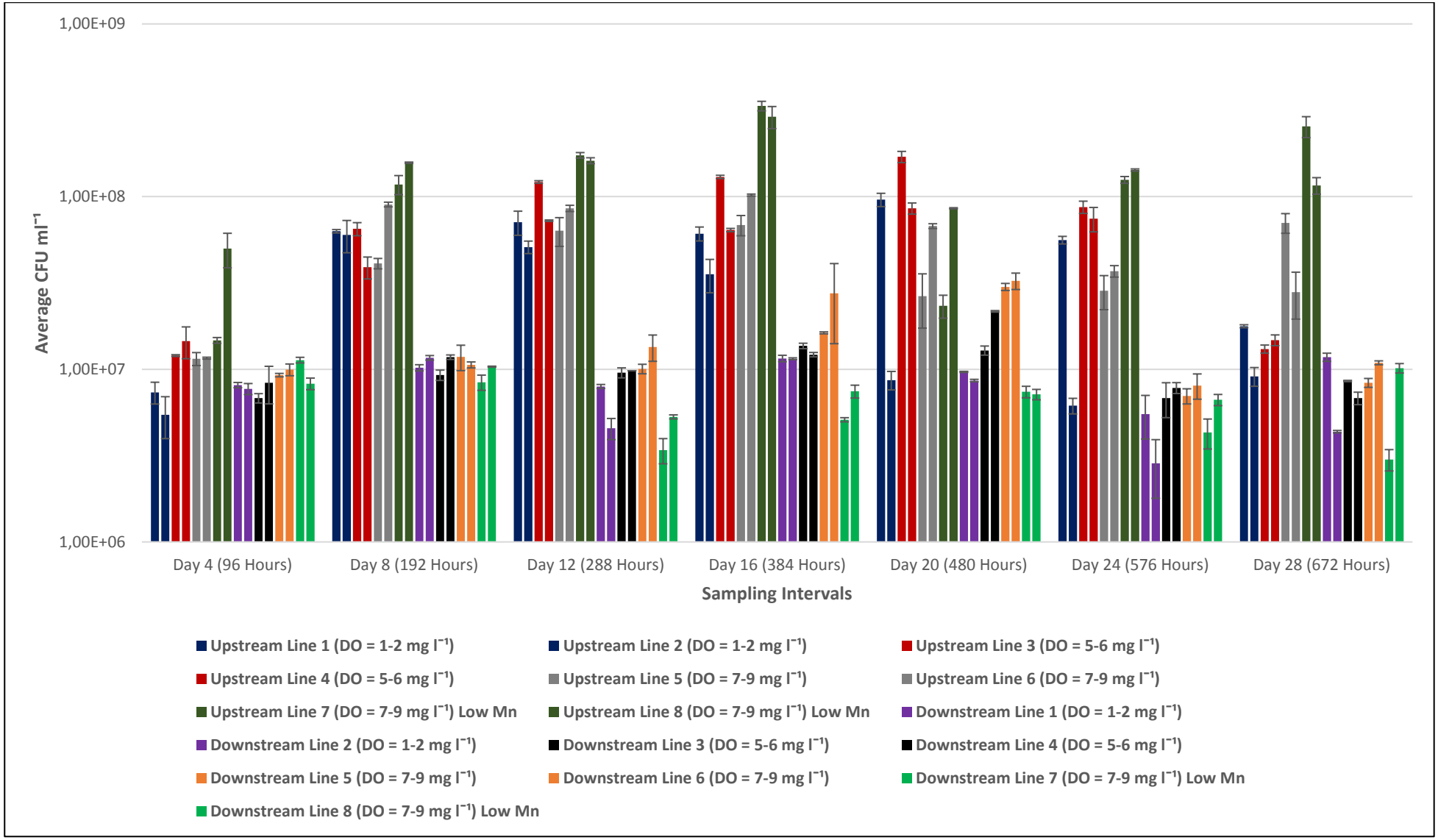


Fig. 4.10 - Enumeration of culturable bacteria (CFU ml^{-1}) on selective Mn oxidation agar obtained from effluent collected from the upstream and downstream inoculation points over a period of 28 days.

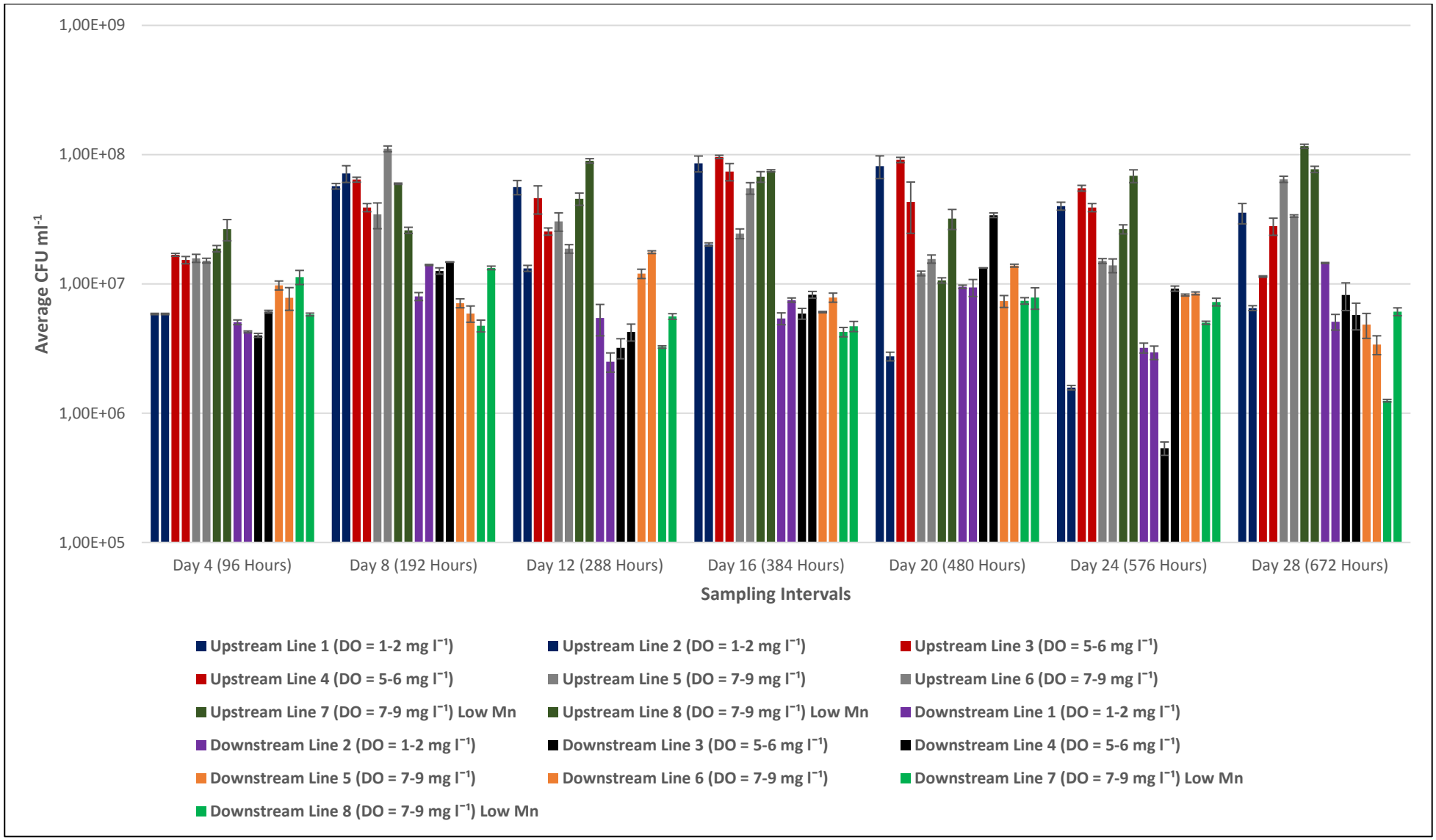


Fig. 4.11 - Enumeration of culturable bacteria (CFU ml^{-1}) on selective Mn reduction agar obtained from effluent collected from the upstream and downstream inoculation points over a period of 28 days.

4.3.4.3. Mn concentration in effluent – Replicate 1

Mn concentrations were measured to investigate whether it was removed from the aqueous phase possibly due to incorporation into the biofilms (Fig. 4.12). Due to the high concentrations of Mn that were present in biofilm material collected from the inside of the LBIS pipeline network (Table 3.3), it was expected that biofilms grown in lines with DO concentrations of 1 – 2 mg l^{-1} and 5 – 6 mg l^{-1} would be capable of oxidizing and incorporating Mn. Under favourable conditions, these microorganisms are capable of oxidizing Mn and coating themselves in the resultant Mn oxides as a means of protection (Brouwers et al., 2000; Du Toit et al., 2015). In order to test this hypothesis, Mn was measured in the effluent collected from both upstream and downstream inoculation points respectively to determine whether any decrease in total Mn concentration had occurred for any growth condition during the first replicate of this experiment. Effluent from lines seven and eight were not subjected to Mn analysis as the influent Mn concentration of 0.019 mg l^{-1} in these lines was below the detection limit of the equipment available in the laboratory.

All data collected from Mn measurements were subjected to one-way ANOVA at a 95% confidence interval. Comparisons between Mn measurements collected from the upstream inoculation point effluent showed no statistical differences. The same was true for comparisons between Mn measurements of downstream inoculation point effluent. As the goal of these experiments were to determine whether Mn would be incorporated into the biofilms structure, statistical analysis was also performed on differences seen between upstream and downstream measurements within each line. No statistically significant difference was observed at a confidence interval of 95%. Three findings were made after analysis of the collected Mn data: (1) Mn concentrations in effluent collected in upstream reactors showed no clear decrease compared to the Mn concentration of approximately 6 mg l^{-1} in the reservoir supply SGW. (2) No statistically significant difference at a confidence level of 95% could be observed between Mn concentrations in effluent collected from channels with different DO concentrations. (3) No statistically significant differences could be observed between Mn measurements in effluent collected from the upstream inoculation points compared to that of the downstream inoculation points.

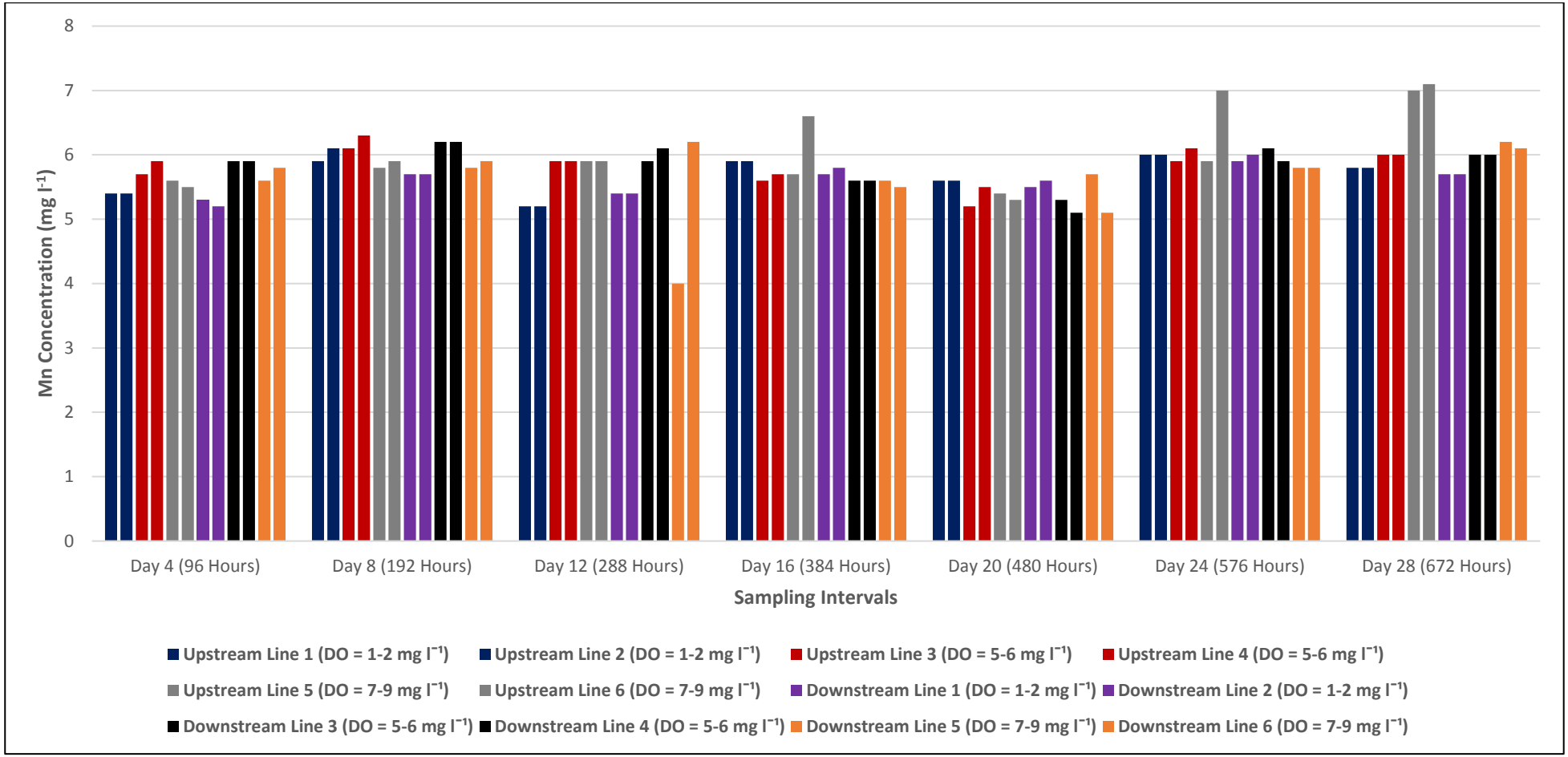


Fig. 4.12 - Mn concentration measured in the effluent at both upstream and downstream inoculation points over 28 days.

4.3.4.4. Mn concentration in effluent – Replicate 2

As with the first replicate of this experiment, Mn concentrations were measured every four days to investigate whether Mn concentration in the effluent was decreasing, pointing to oxidation and possible incorporation into the biofilm structure. Mn was again measured in the effluent collected from both upstream and downstream inoculation points (Fig. 4.13) and all measurements were subjected to one-way ANOVA. Statistical analysis indicated that no statistically significant differences could be seen between Mn measurements collected from upstream biofilms with $P > 0.05$. However, statistical analysis of Mn measurements in effluent from the downstream inoculation point did show significant differences, with further analysis indicating that Mn concentration in effluent collected from line 6 differed significantly from that of lines 1 and 2.

Differences seen in the Mn concentration between effluent collected from upstream and downstream inoculation points were also subjected to one-way ANOVA and revealed that no Mn measurement in effluent collected from any downstream inoculation point differed significantly from the Mn concentration in effluent collected from its corresponding upstream inoculation point. The same three conclusions could thus be made as in replicate 1 regarding Mn concentration: (1) Mn concentrations measured throughout the flow system showed no clear decrease compared to the Mn concentration of 6 mg l^{-1} in the reservoir of the supply SGW. (2) Very little statistically significant differences can be observed in Mn concentration between lines subjected to different growth conditions. These statistically significant differences were not as a result of growth conditions. (3) No statistically significant decreases in Mn concentration can be observed within lines between the upstream and downstream inoculation points. It was thus ultimately concluded that growth conditions had little effect on Mn oxidation and that very little Mn was oxidized and incorporated into the biofilm structure, corresponding to the conclusions made from the first replicate.

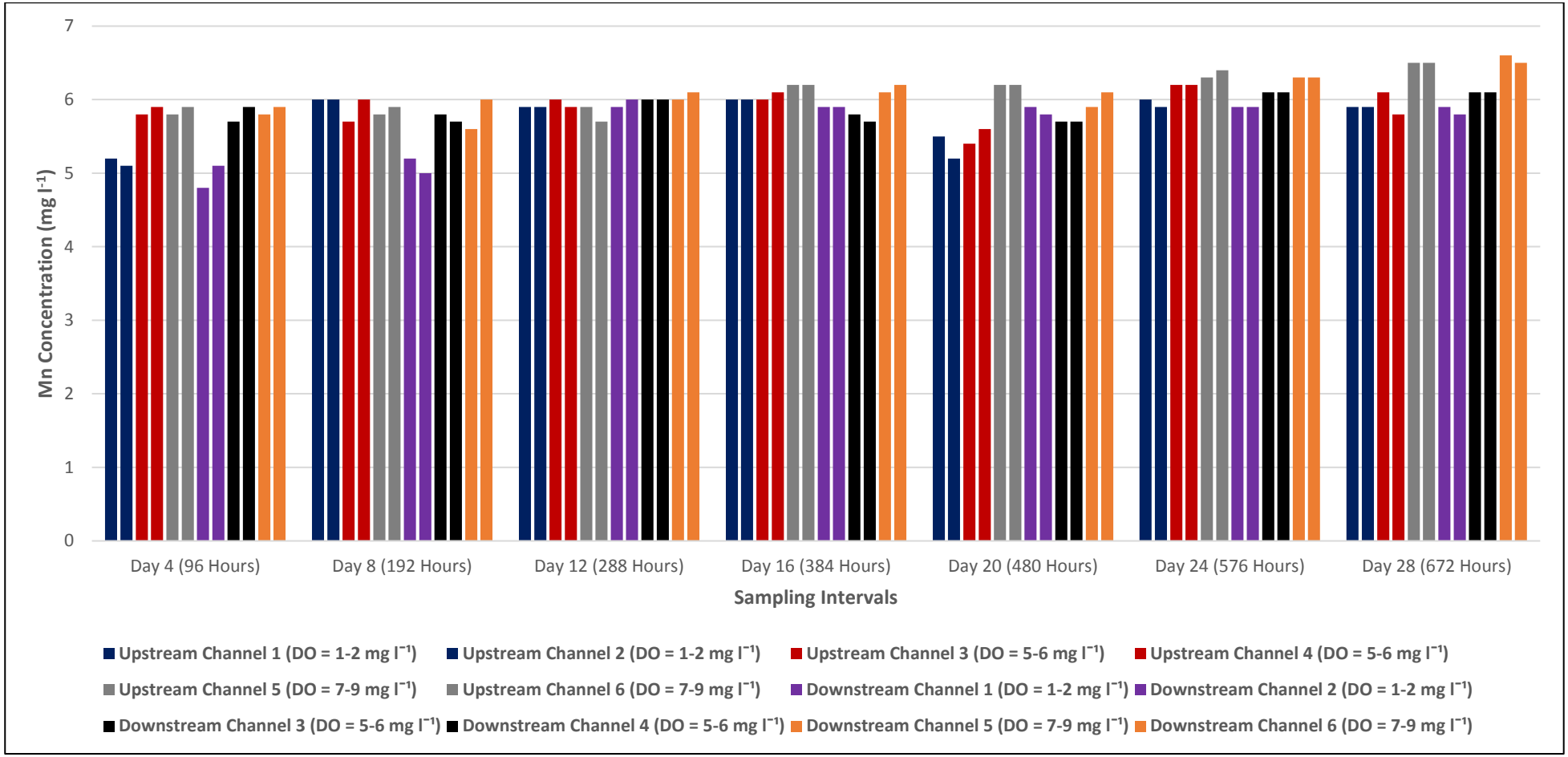


Fig. 4.13 - Mn concentration measured in the effluent at both upstream and downstream inoculation points over 28 days.

4.3.4.5. SEM/EDS analysis on biofilms – Replicate 1

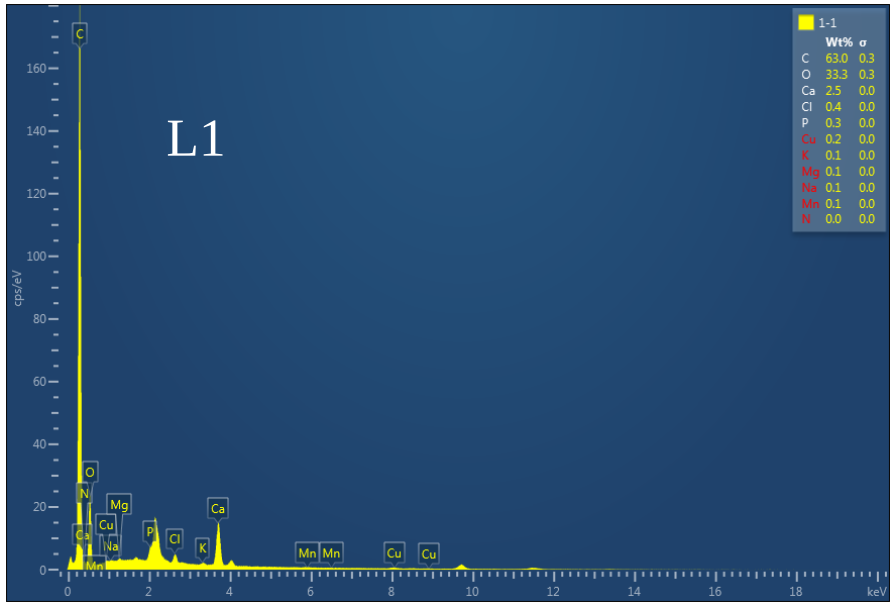
The results presented in Fig. 4.12, showing no statistically significant difference between Mn in the effluents collected from upstream and downstream inoculation points, nor between systems with low and high DO concentrations, were not expected. This could have been due to the fact that there was not sufficient biofilm biomass to attenuate the high concentration from the aqueous phase to the extent that the difference would be significant. Direct measurement of Mn in the biofilm biomass with SEM/EDS was thus subsequently applied to test that possibility. Although SEM/EDS analysis indicated the presence of Mn in these samples, concentrations were very low in biofilms from all eight lines, all ranging between weight percentages of 0 and 0.2%, as indicated in Fig. 4.14. The results from SEM/EDS thus corresponded with Mn measurements collected from effluent in section 4.3.4.3, indicating that the little decrease in total Mn concentration seen in Mn measurements is due to the absence of any significant incorporation of Mn into the biofilm structure. A second round of experimentation was subsequently performed to confirm this finding.

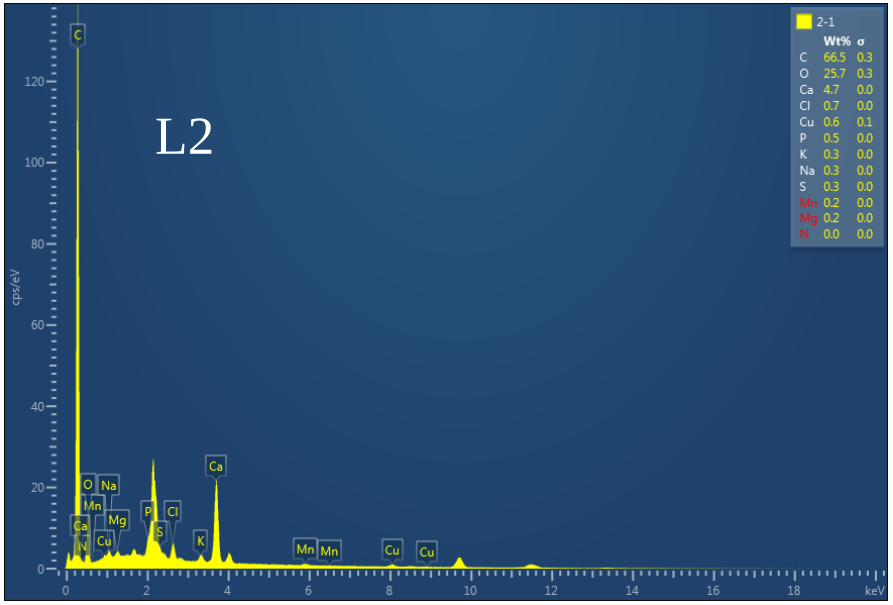
4.3.4.6. SEM/EDS analysis on biofilms – Replicate 2

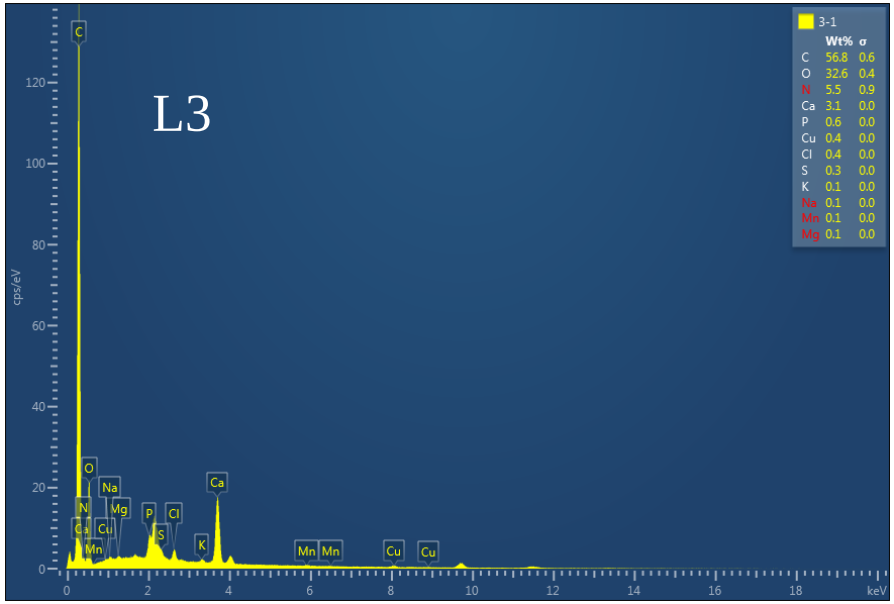
Results obtained for replicate 2 were similar to that obtained in replicate 1. SEM/EDS analysis on biofilm samples from all eight channels showed very little to no incorporation of Mn in the biofilm structure, with weight percentages all ranging between 0 and 0.1% (Fig. 4.15). These results also corresponded with results from Mn measurements in section 4.3.4.4, where very little decrease could be seen between influent and effluent Mn concentrations. SEM/EDS analysis confirmed that in this case, as with replicate 1, this was due to a lack of significant incorporation of Mn into the biofilm structure.

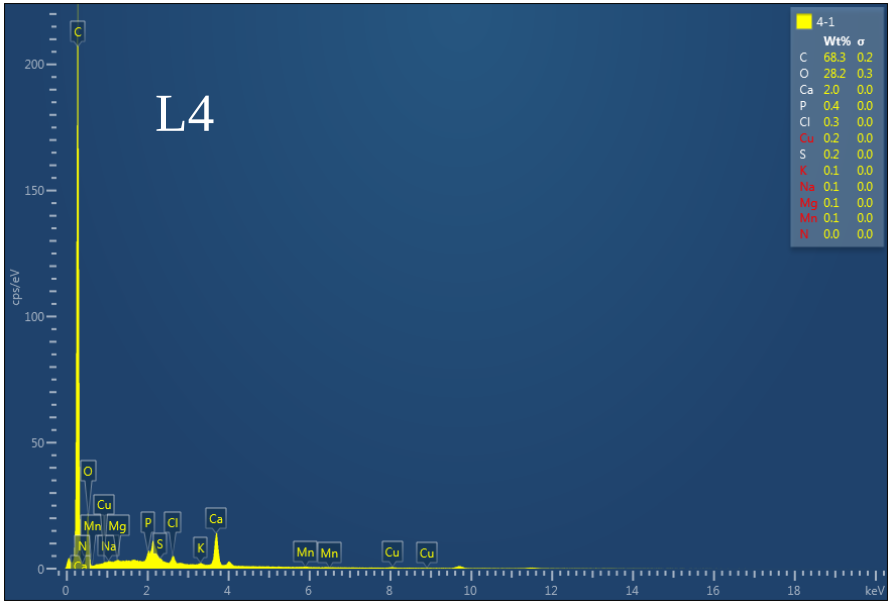
Based on water (Fig. 3.6) and biofilm samples collected from the LBIS pipeline network, as well as SEM/EDS analysis performed on biofilm samples in section 3.3.5, it was initially expected that laboratory scale flow systems would show incorporation of Mn into the biofilm structure, leading to a decrease in Mn concentration in the effluent collected in sections 4.3.4.3 and 4.3.4.4. However, as biofilms cultivated in laboratory scale flow systems did not show significant Mn incorporation into the biofilm structure, it is accepted that the conditions inside these channels were not optimal for Mn oxidation and incorporation of Mn oxides into the biofilm structure. As previously mentioned, the duration of the experiment might be a contributing factor, as these systems were only operated for 28 days at a time, whereas previous studies have reported that 3 months were necessary for these microbial communities to become sufficiently established for Mn oxidation to occur (Du Toit et al.,

2015; Thompson et al., Unpublished technical reports). It has also been found that the dynamic interactions observed in nature together with a constantly changing environment is near impossible to simulate in a laboratory setup (Kielemoes et al., 2002). Furthermore, it is possible that the biofilms were not thick enough, (typically ranging between 10 and 70 µm (Fig 4.28), compared to those in the LBIS that were cm's thick) to establish sufficient gradients to maintain the majority of accumulated Mn as is the case in sediments (Fig. 3.4 & 3.5). Additionally, biofilms were supplied with growth medium containing Mn at a concentration of 6 mg l^{-1} at approximately 12 ml h^{-1} per channel. At this flow rate, biofilms are supplied with 6 mg Mn approximately every 83 hours. However, based on the growth data represented in Fig. 4.5, biofilms seem to have reached a steady state after approximately 72 hours, after which they most likely did not accumulate biomass much further. It is speculated that the total biofilm inside the flow channel weighed less than the total mass of Mn added during the duration of the experiment and that the sorption capacity of the biofilms was exceeded. This would have made any further incorporation of Mn unlikely, causing it to leave the system in the aqueous phase.









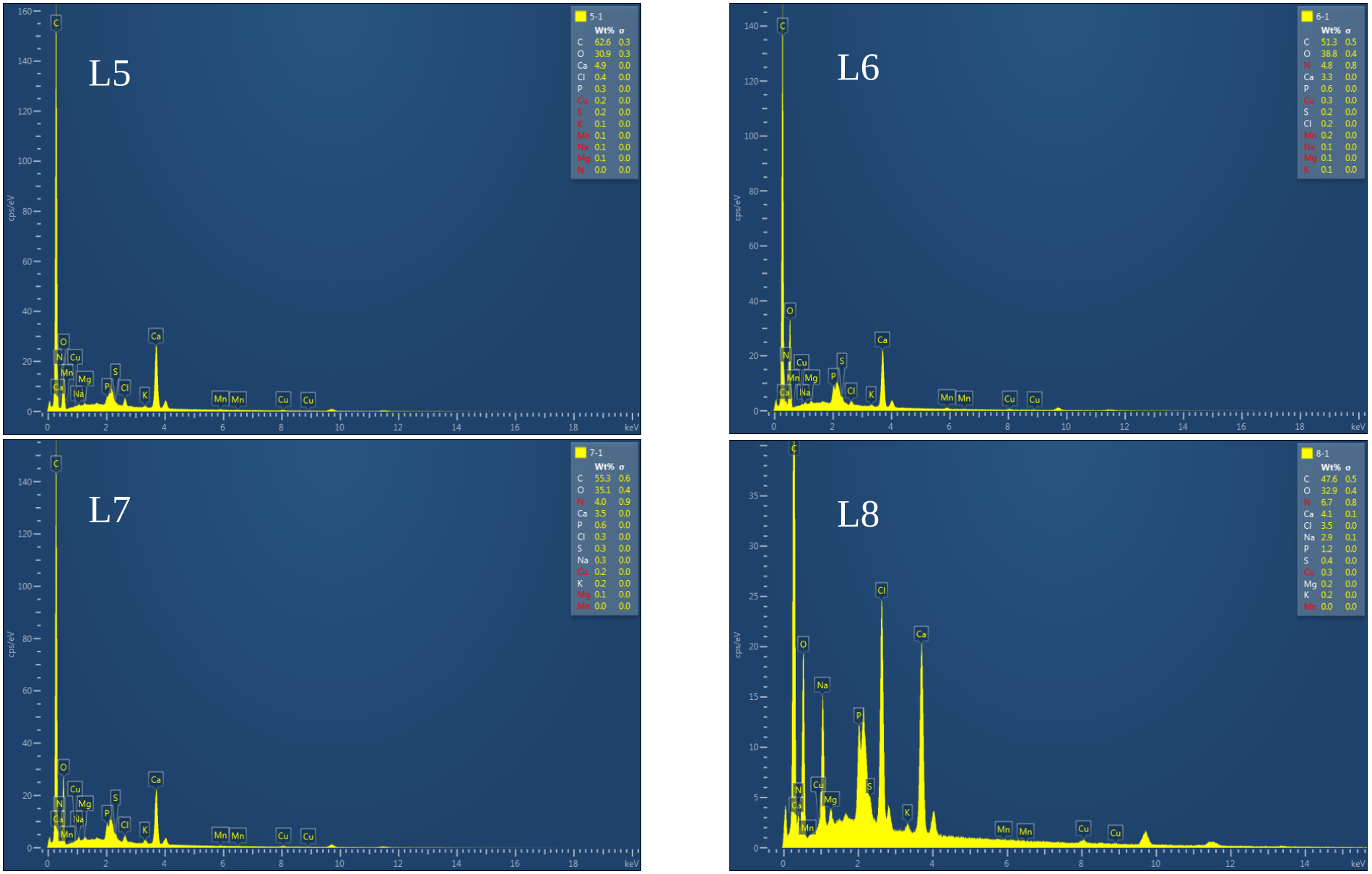
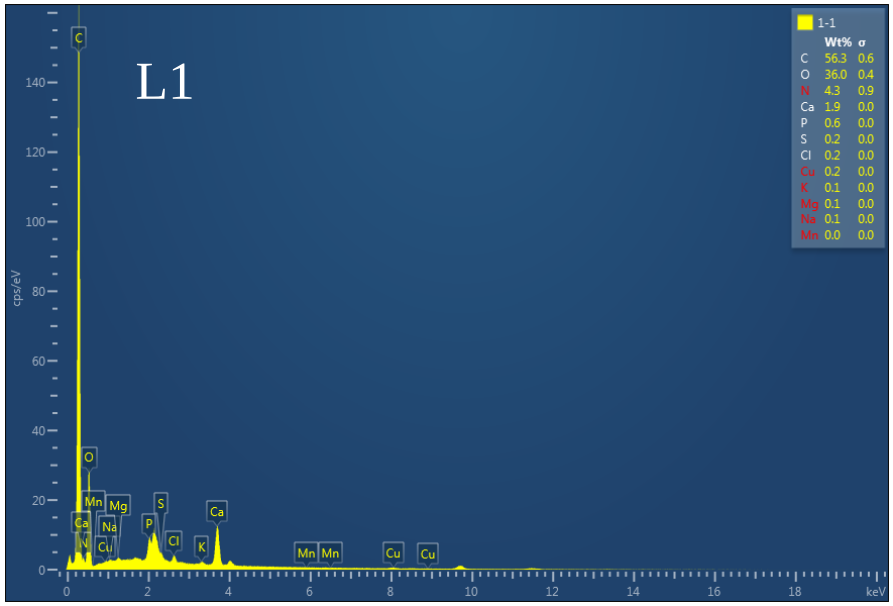
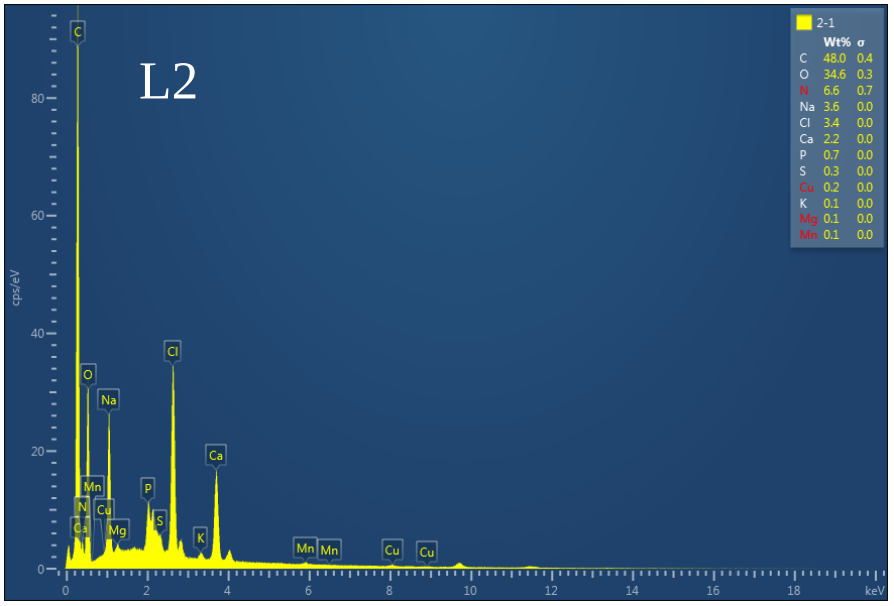
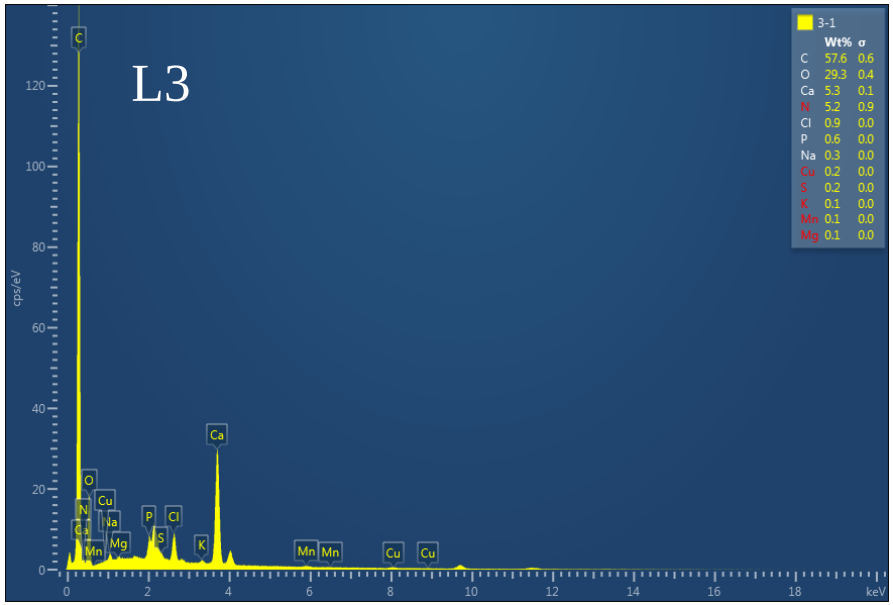
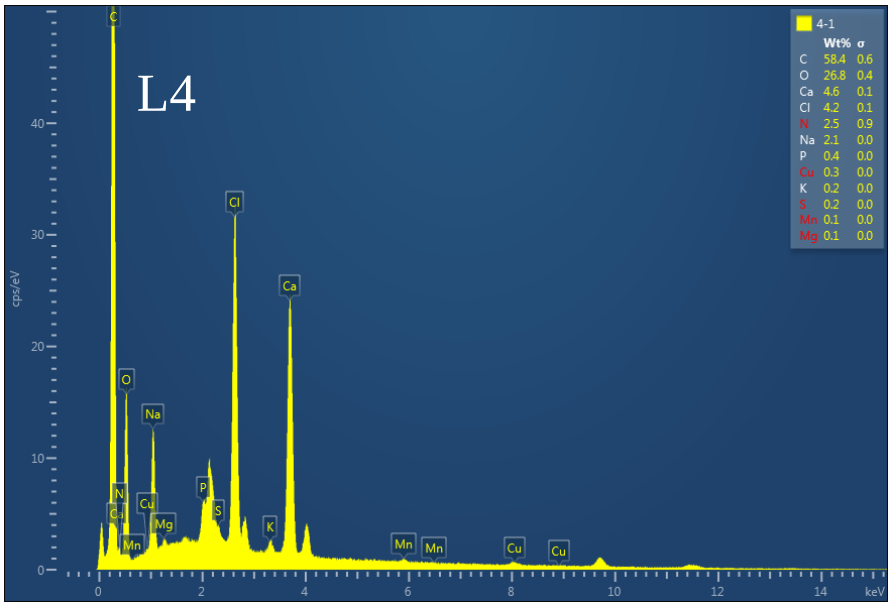


Fig. 4.14 - SEM/EDS analysis of biofilms collected from lines 1 – 8 of replicate 1. Symbols represent lines, eg. L1 = Line 1.









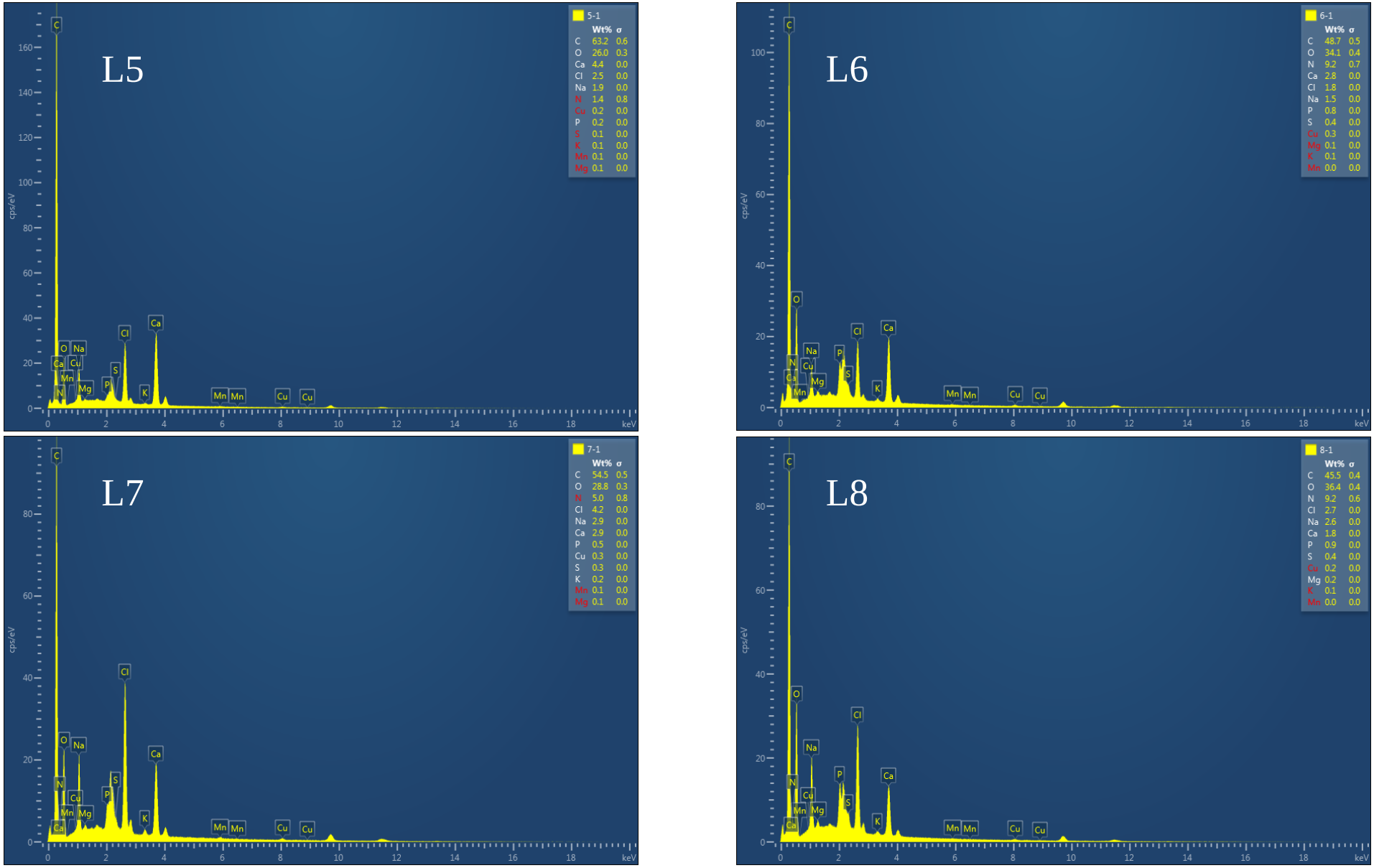


Fig. 4.15 - SEM/EDS analysis of biofilms collected from lines 1 – 8 of replicate 2. Symbols represent lines, eg. L1 = Line 1.

4.3.4.7. ARISA analysis – Replicate 1

Effluent collected from the flow systems every eight days at both the upstream and downstream inoculation points were subjected to ARISA following DNA extraction. ARISA was used as a means of providing an estimation of community composition and diversity in the different biofilms present under the different growth conditions. ARISA was also performed on biofilm material following termination of the experiment on day 28 to evaluate whether significant differences exist between the communities in the effluent collected compared to that of the biofilms. ARISA was used as this molecular method allows for the estimation of microbial diversity without the need to culture microbes, which cannot account for all microorganisms present in the sample (Richaume et al., 1993; Slabbert et al., 2010). Additionally, ARISA can be used to estimate the community diversity without being biased toward fast growing and dominant microorganisms present in the sample. Although both bacterial and fungal ARISA can be performed, only bacterial ARISA was performed on these samples as this study did not focus on fungi. For bacterial ARISA, the intergenic spacer region between the 16S and the 23S subunits of the rDNA genes are targeted in the rRNA operon, which is responsible for displaying size and sequence heterogeneity between species (Slabbert et al., 2010).

4.3.4.7.1. Operational Taxonomic Unit (OTU) Frequency

OTU frequency was averaged for each growth condition and measured as an indication of relative species abundance (Fig. 4.16) in samples and generally refers to clusters of sequences that have high similarity and are widely used as basic diversity units for large-scale characterization of microbial communities (Schmidt et al., 2014). Although differences in OTUs could be observed between treatment conditions, none of the samples showed significant increases or decreases in OTUs compared to the other conditions. Statistical analysis using one-way ANOVA indicated that the observed differences in OTU numbers between samples were not statistically significant at a confidence level of 95%. It was thus concluded that species richness remained very similar under all growth conditions throughout the duration of the experiment, with DO and Mn concentrations having no significant effect on the development and community composition of the biofilms. Samples collected from the upstream inoculation points of lines 7 and 8 at 672 h were excluded during analysis due to poor DNA quality.

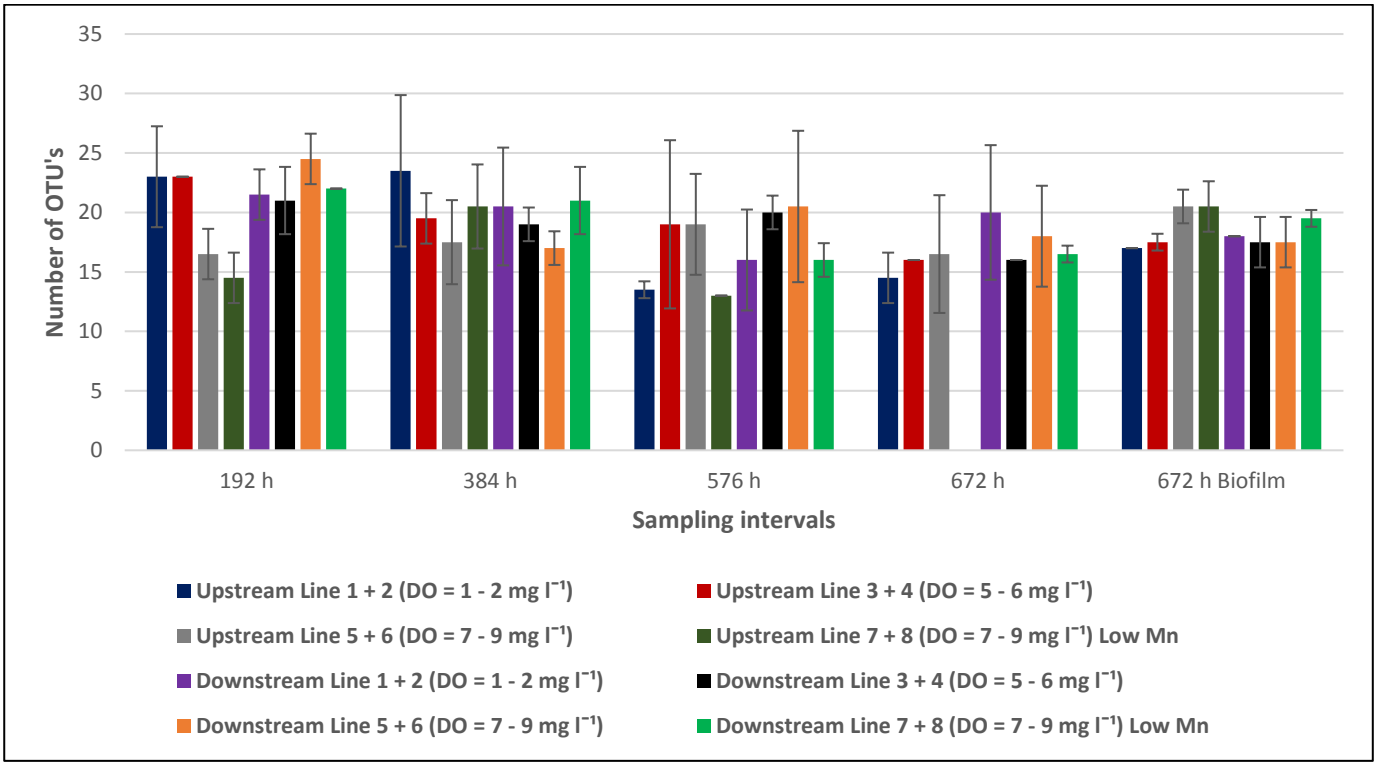


Fig. 4.16 - OTU frequency averaged for each growth condition in effluent samples collected from the upstream and downstream inoculation points over 28 days. OTU frequency in biofilms samples is reported at 672 h

4.3.4.7.2. Frequency of Sizes

Fragment sizes ranging predominantly between 610 and 970 bp showed a high occurrence, indicating the presence of Gram-negative bacteria (Fig. 4.17). However, shorter fragment sizes between 400 and 490 bp, indicating the presence of Gram-positive bacteria, were also detected (Ciccazzo et al., 2014; Fisher & Triplett, 1999; Ranjard et al., 2000). It can thus be assumed that communities in these samples were dominated by Gram negative bacteria. Mn oxidizing bacteria are not limited to either group but can be either Gram- positive or Gram-negative depending on the genus. Mn oxidizing bacteria such as those from the *Pedomicrobium* genus are known to be Gram-negative, whereas *Bacillus* capable of oxidizing Mn are Gram-positive (Gebers, 1981;Willey et al., 2011).

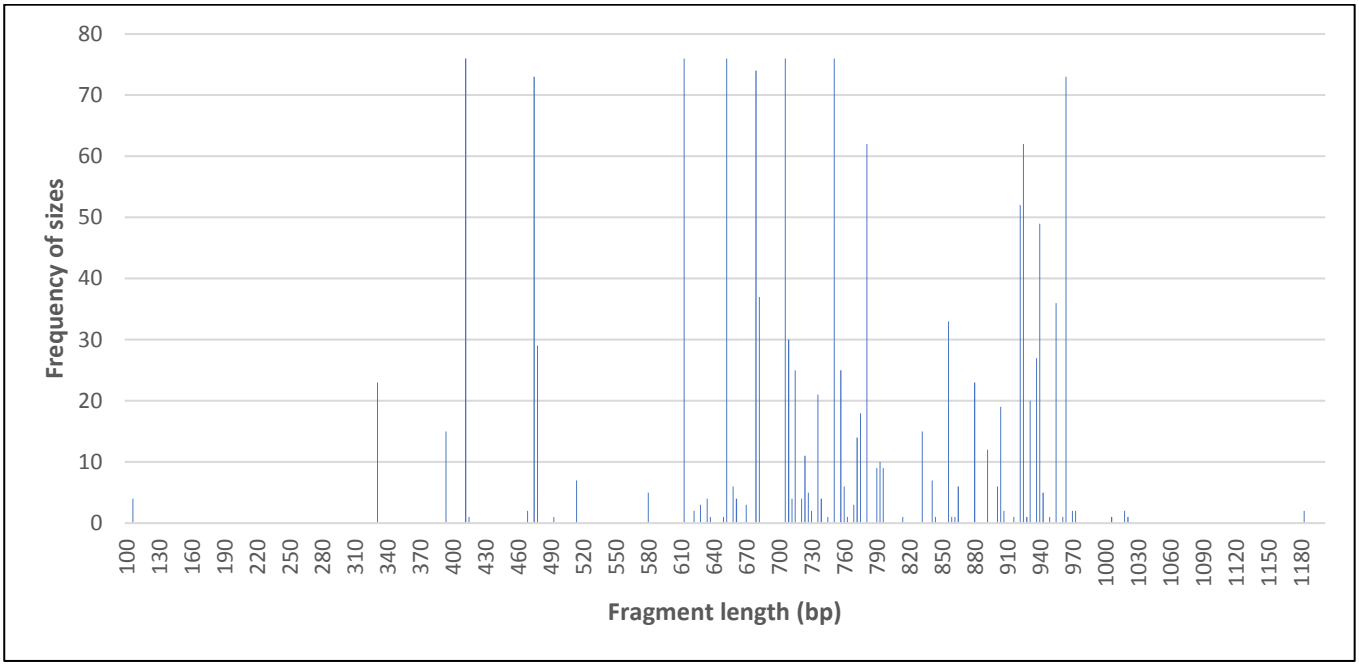


Fig. 4.17 - Frequency of fragment lengths showed a higher occurrence of fragment lengths between 610 and 970 bp, indicating the presence of Gram-negative microorganisms.

4.3.4.7.3. Alpha diversity

Species diversity can be separated into two components: (1) the species richness referring to the number of species present and (2) the relative abundance or dominance of those species. Biodiversity within each sample can be better explained using the Shannon-Weaver and the Simpson indices (Kerkhoff, 2010). Data from each growth condition was averaged for duplicate lines for analysis.

4.3.4.7.3.1. Shannon-Weaver Index

The Shannon-Weaver index typically generates values between 1.5 and 3.0 and is rarely greater than 4. Increases in the richness and evenness of a community typically sees an increase in the Shannon index (Kerkhoff, 2010). The data represented in the Shannon index in Fig. 4.18 shows values mostly between 2 and 3 and does not point to clear differences in alpha diversity for each growth condition between samples. Statistical analysis indicated that no statistically significant differences exist in alpha diversity between samples (including both upstream and downstream effluent and biofilm samples), leading to the conclusion that diversity within samples were very similar for all growth conditions tested. The Shannon-Weaver index uses the following formula (Hill, 1973):

$$H = -\sum_{i=1}^{S} p_i \log_b p_i$$

Since the Shannon index gives an indication of both the species richness and the dominance in a community, it is difficult to compare communities when they differ greatly in species richness. For this reason, the Simpson index (Fig. 4.19) can be used as it gives a more direct estimate of species richness with a better indication of dominance (Kerkhoff, 2010).

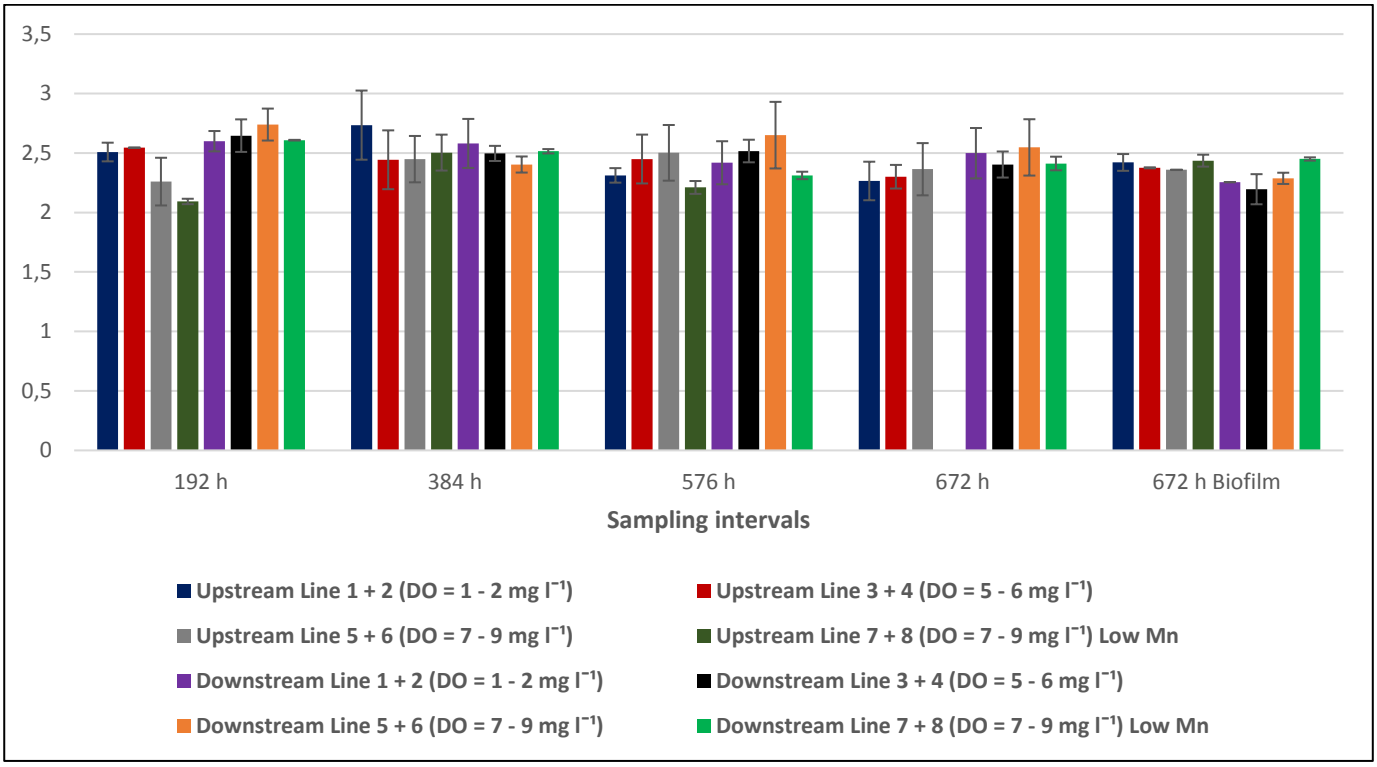


Fig. 4.18 – Shannon diversity index representing averaged alpha diversity for each growth condition over a period of 28 days. Shannon diversity in biofilm samples is reported at 672 hours.

4.3.4.7.3.2. Simpson's diversity index

The Simpson index uses the following formula (Hill, 1973):

$$D = 1 - \sum_{i=1}^{S} p_i^2$$

The Simpson index is used as a measure to more accurately determine the differences within a community, especially when the inverse (1/D) of the Simpson index is used. Whereas the Shannon index incorporates both species richness and dominance, increasing as both the richness and the evenness increases, the Simpson index (Fig. 4.19) accounts for these values separately. Using the Simpson index, D is a measure of dominance, with diversity decreasing as dominance increases (Kerkhoff, 2010). Statistical analysis using one-way ANOVA indicated that differences in alpha diversity observed between samples were not statistically significant with P-value > 0.05, indicating that the alpha diversity of the different growth conditions were very similar.

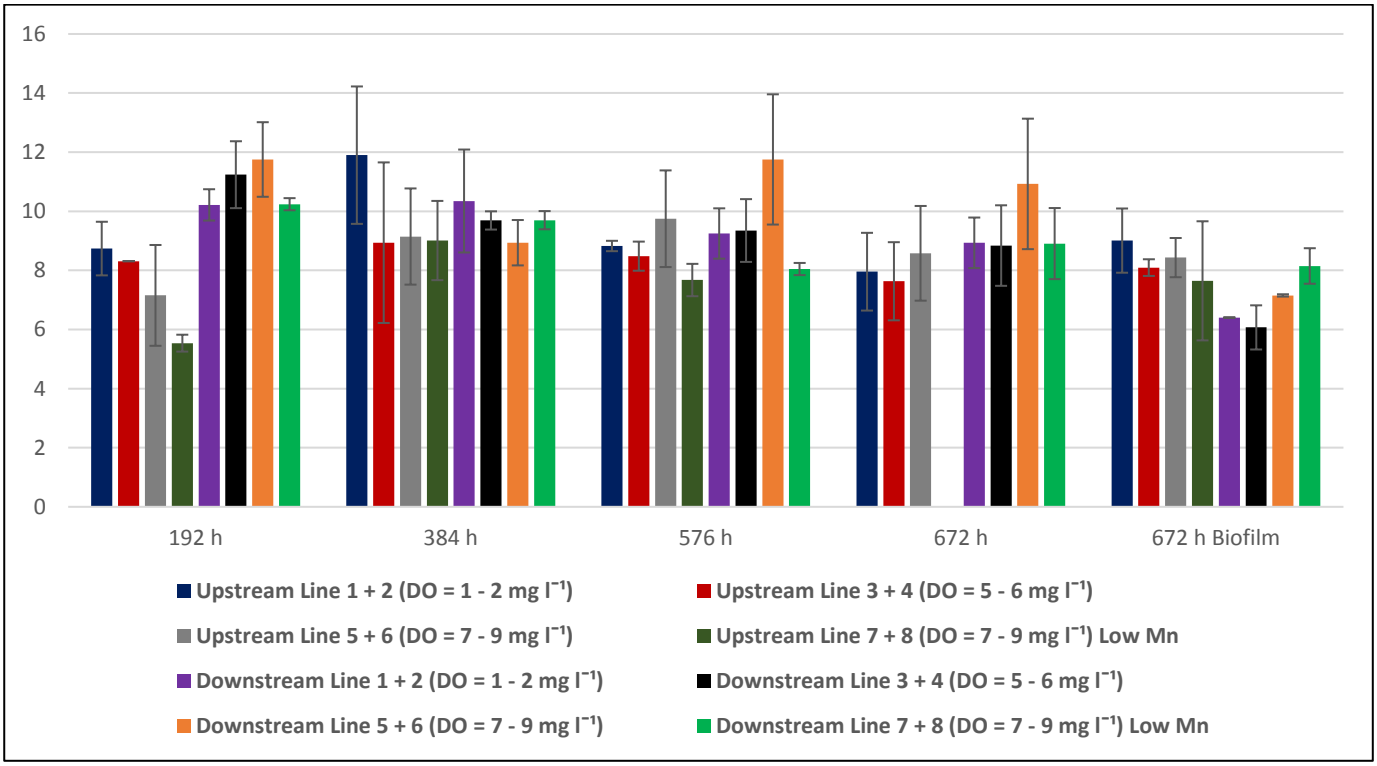


Fig. 4.19 – Inverse Simpson's diversity index representing averaged alpha diversity for each growth condition over a period of 28 days. Inverse Simpson's diversity in biofilm samples is reported at 672 hours.

4.3.4.7.4. Beta diversity

While alpha diversity using Shannon and Simpson indices typically refers to the variation in species within a site or sample, beta diversity refers to the variation in species between sites/samples. Although many different measures of beta diversity have been described previously, no consensus has been reached about which ones are the most appropriate. For this study, the Bray Curtis dissimilarity index was used (Anderson et al., 2011; Bray & Curtis, 1957) and can be described using the following formula:

$$b_{ii'} = \frac{\sum_{j=1}^{J} |n_{ij} - n_{i'j}|}{n_{i+} + n_{i'+}}$$

Non-metric multidimensional scaling (NMDS) was used for multivariate analysis of community fingerprints from collected effluent and biofilm samples from the different growth conditions over time and to provide a graphical ordination of the samples based on their similarity to other samples (Fig. 4.20). The NMDS plot indicated that no distinct groups of communities formed over the 28-day time period of the experiment, with all the communities clustering close together. Analysis of similarities (ANOSIM) was used to assess the statistical differences between groups (Wood et al., 2008). ANOSIM analysis showed a P-value < 0.05, indicating that statistically significant differences did exist at a confidence interval of 95%. However, as seen in section 4.3.4.2, these differences, although significant, are not as a result of the growth conditions experienced by these communities. In the event where growth conditions would have a direct effect on community structure, it is expected that communities would associate in distinct clusters according to their growth conditions experienced, showing small differences between time periods as these communities change over time. However, in this case, no distinct clusters could be observed, leading to the conclusion that any significant differences are as a result of outliers and the stochastic nature of biofilm growth, and cannot be attributed to the influences of specific growth conditions experienced by these communities. The lack of distinct clusters also serves as indication that the community profiles did not differ significantly between collected effluent samples and biofilms collected at the end of the experiment.

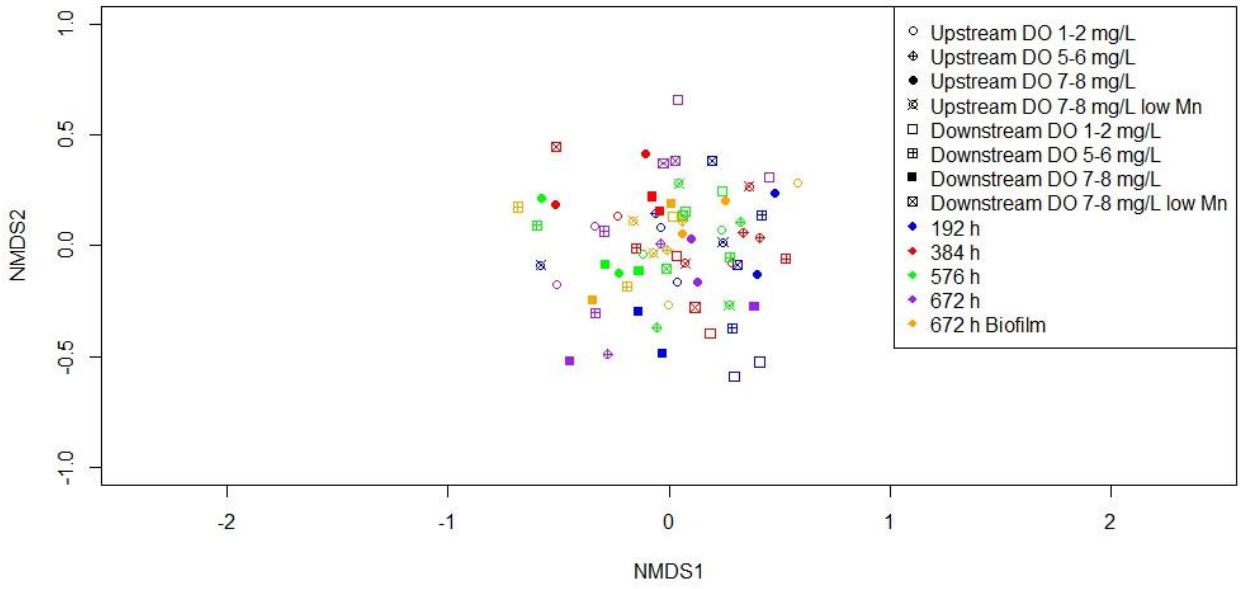


Fig. 4.20 - NMDS plot of ARISA fingerprints from all samples collected over the 28-day duration of the experiment showed no clear differences in community structure due to the influences of growth conditions. Growth conditions are indicated by symbols, each corresponding to different colours based on sampling intervals.

4.3.4.8. ARISA analysis – Replicate 2

ARISA was also performed on the second replicate of the experiment. All samples were subjected to bacterial ARISA.

4.3.4.8.1. OTU Frequency

OTU frequency was again measured as an indication of relative species abundance per sample (Fig. 4.21). Differences could be observed between the OTU's per treatment condition, but no differences were found to be statistically significant at a confidence level of 95%. It was concluded that, as found in the first replicate, species richness remained very similar under all growth conditions and that the concentration of DO and Mn had no significant effect on the development and community composition of these biofilms.

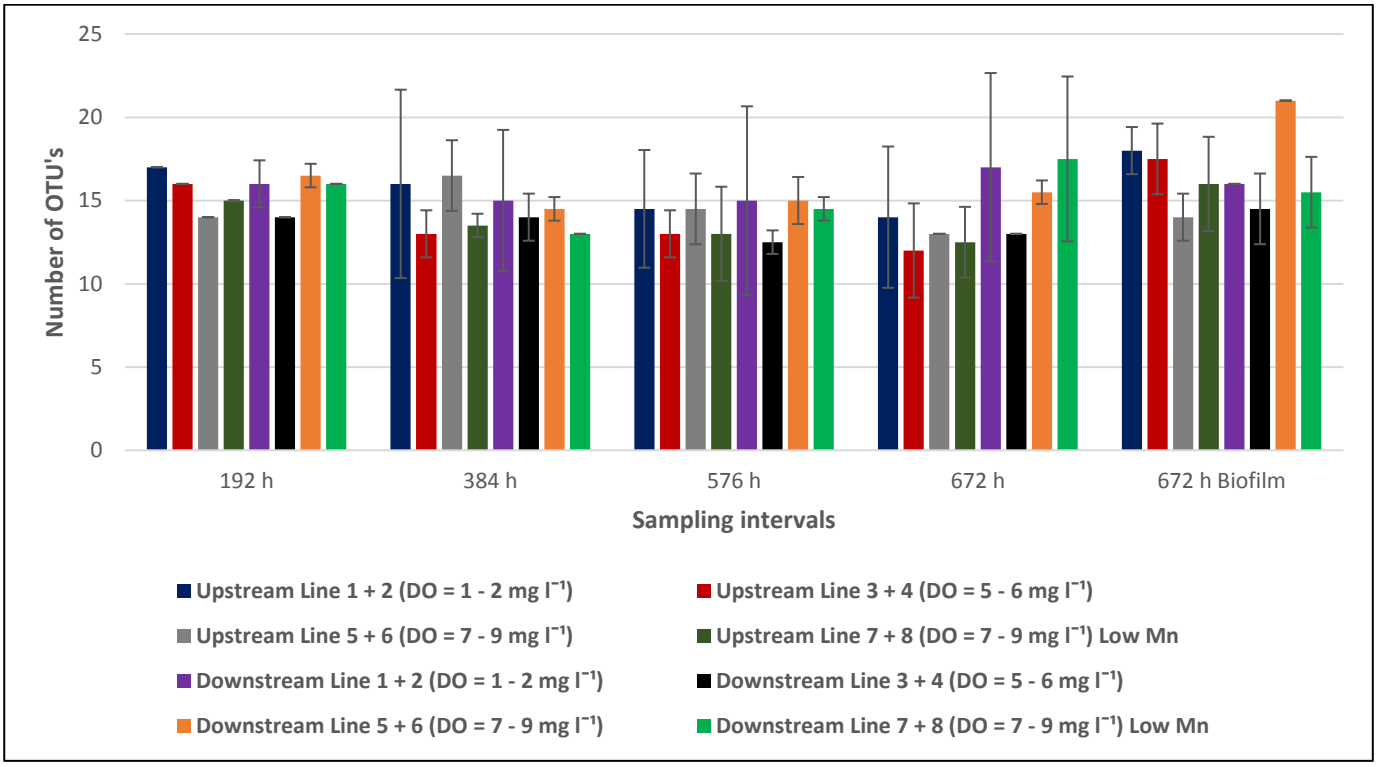


Fig. 4.21 - OTU frequency in effluent samples collected from the upstream and downstream inoculation points over 28 days. OTU frequency in biofilm samples is reported at 672 h.

4.3.4.8.2. Frequency of Sizes

Very similar to that found in the first replicate, fragment sizes ranging predominantly between 610 and 970 bp showed the highest occurrence (Fig. 4.22), pointing to the presence of Gram-negative microbes (Ciccazzo et al., 2014; Fisher & Triplett, 1999; Ranjard et al., 2000). However, shorter fragments sizes between 400 and 490 bp were also detected, pointing to the presence of some Gram-positive organisms. It was concluded that, as for replicate one, the communities in these samples were predominantly comprised of, but not limited to, Gram-negatives, with the presence of some Gram-positives.

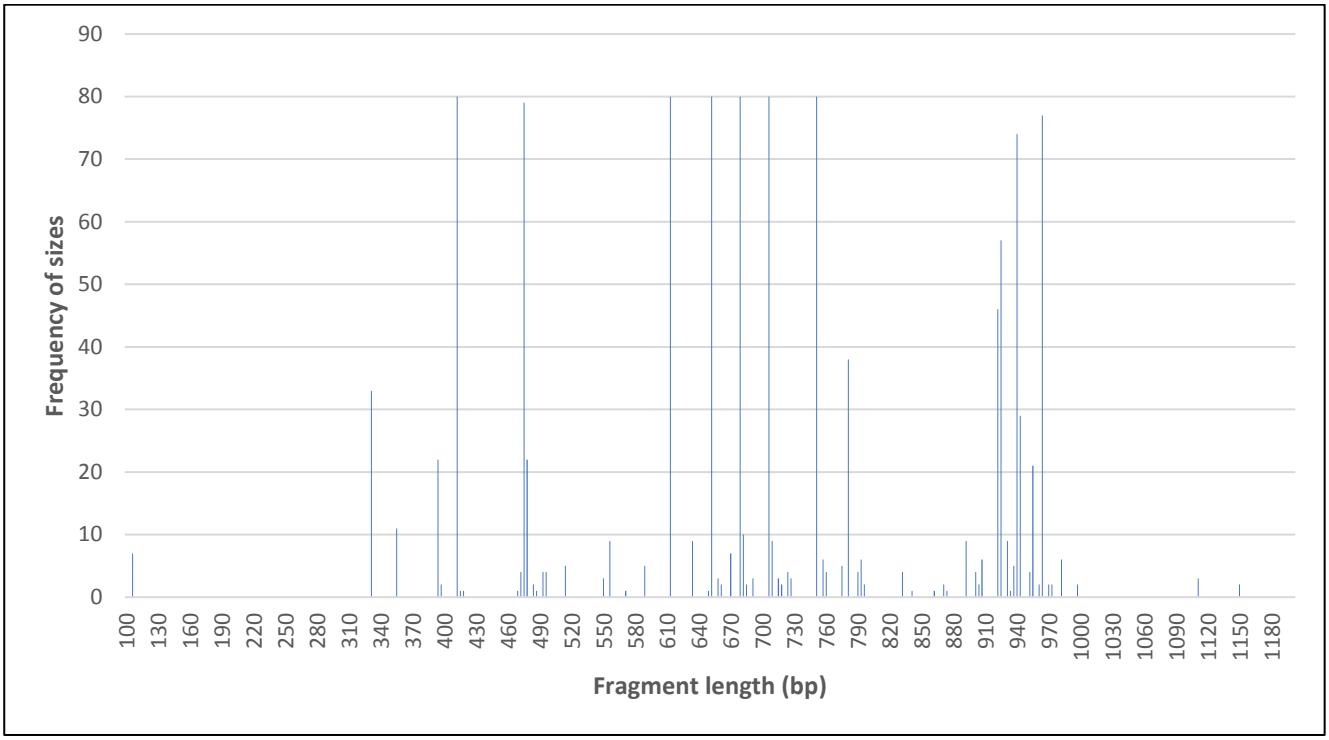


Fig. 4.22 - Frequency of fragment lengths indicated a higher occurrence of fragment lengths between 610 and 970 bp, indicating the presence of Gram-negative microorganisms.

4.3.4.8.3. Alpha diversity

4.3.4.8.3.1. Shannon-Weaver index

The Shannon-Weaver index, (Fig. 4.23) indicated no clear differences in alpha diversity between samples. However, statistical analysis showed a significant difference between the alpha diversity in the effluent collected from the upstream inoculation point in lines 7 and 8 compared to effluent collected from the downstream inoculation point in lines 1 and 2. As stated before, with regards to alpha diversity, data from each growth condition was averaged for duplicate lines for analysis. However, these statistically significant differences were not considered as they did not represent or point to a clear pattern or difference between community composition caused by different growth conditions.

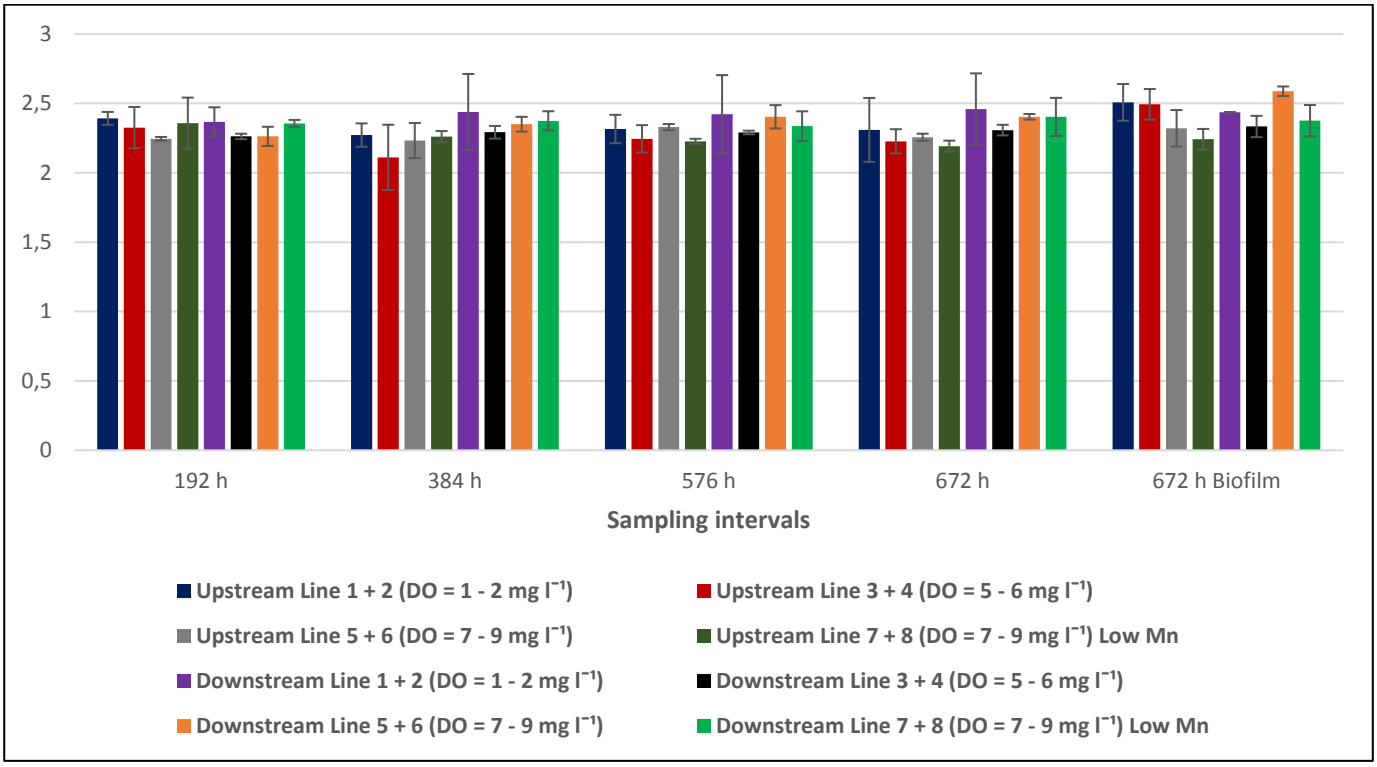


Fig. 4.23 - Shannon diversity index representing averaged alpha diversity for each growth condition over a period of 28 days. Shannon diversity in biofilm samples is reported at 672 hours.

4.3.4.8.3.2. Simpson's diversity index

The inverse Simpson's diversity index for replicate 2 (Fig. 4.24) indicated that statistically significant differences in alpha diversity could be seen between samples with P-value < 0.05. Further analysis corresponded with the results from the Shannon index, indicating that the differences between samples collected from the upstream inoculation point in lines 7 and 8 differed close to statistically significant from that of samples collected from the downstream inoculation points in channels 1 and 2 with P = 0.05. However, similar to results from the Shannon index, the Inverse Simpson's index indicated that although significant differences were observed, these differences in community composition were not as a result of growth conditions tested.

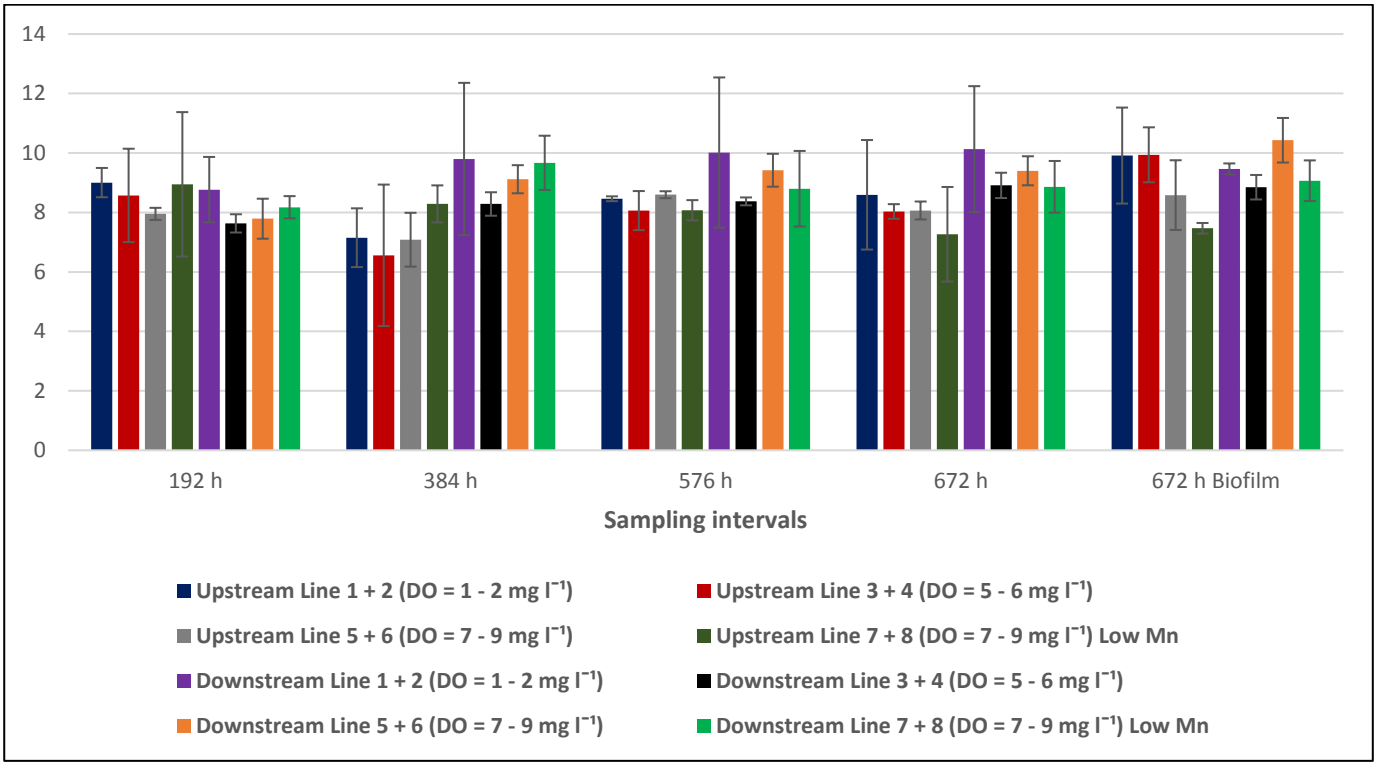


Fig. 4.24 - Inverse Simpson's diversity index representing averaged alpha diversity for each growth condition over a period of 28 days. Inverse Simpson's diversity in biofilm samples is reported at 672 hours.

4.3.4.8.4. Beta diversity

Results obtained from the Bray-Curtis dissimilarity index corresponded with that obtained from replicate 1, indicating that no distinct groups of communities formed over the duration of the experiment, with all the communities in all samples clustering close together. ANOSIM produced a P-value < 0.05, indicating that statistically significant differences could be observed between the community composition between samples. However, due to the absence of distinct cluster formation for the communities of respective growth conditions, it can be concluded that these statistically significant differences are not as a result of the influences of the growth conditions, but that these differences should be regarded as outliers. As discussed for ARISA of replicate 1, significant influences of growth conditions on community development would have been indicated as the presence of separately clustered communities, possibly showing limited shifts over time. This was however not the case, with the NMDS plot (Fig. 4.25) indicating one overlapping cluster, not easily distinguishable between growth conditions. Additionally, corresponding to the result of replicate 1, the lack of clearly distinguishable communities further indicates that no significant difference exists in the community composition of collected effluent samples compared to that of biofilm samples collected from the system after 28 days.

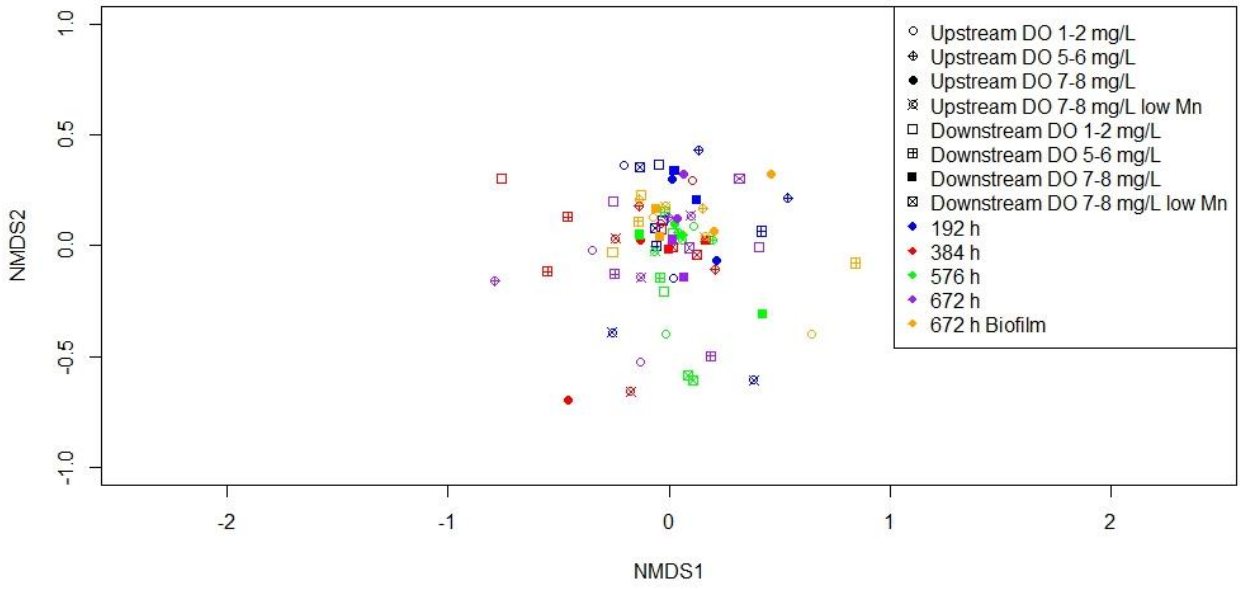


Fig. 4.25 - NMDS plot of ARISA fingerprints from all samples collected over the 28-day duration of the experiment showed no clear differences in community structure due to the influences of growth conditions. Growth conditions are indicated by symbols, each corresponding to different colours based on sampling intervals.

4.3.5. CLSM assisted analysis of biofilms

Two separate replicates of flow systems with identical conditions to that described in section 4.2.6 and Table 4.1 were used for microscopic analysis of biofilm structure. Thick Tygon® tubing sections were replaced with Perspex flow cells covered with a glass cover slip in order to visualize biofilm structure. Although the COMSTAT package (Heydorn et al., 2000) used for analysis in Matlab has various analysis functions available, it was decided that biomass, surface to biovolume ratio and biofilm maximum thickness would be the functions most relevant to this study. The overall goal of this experiment was to investigate whether any comparisons could be made between biofilm planktonic cell yield in the flow systems of sections 4.3.4.1 and 4.3.4.2 with biofilm growth through microscopic analysis using CLSM.

4.3.5.1. Evaluation of biofilm biomass, surface to biovolume ratio and biofilm maximum thickness – Replicate 1

Analysis of data obtained through confocal microscopy showed no clear differences or patterns in total biomass accumulation in channels under the different conditions tested (Fig. 4.26). Upstream

flowcell channels 2 and 3 showed visibly higher amounts of biomass compared to the other channels. However, the duplicate channels for these conditions, i.e. upstream flowcells channels 1 and 4 showed much lower amounts of biomass, averaging out to biomass amounts similar to that observed in other channels. One-way ANOVA analysis of observed differences showed a P-value > 0.05 for upstream channels, indicating that no statistically significant differences could be observed between accumulated biomass in the upstream flowcell channels at a confidence level of 95%. Statistical analysis of differences in accumulated biomass observed between channels for downstream biofilms did however show statistically significant differences. Further analysis using the Tukey HSD test revealed that accumulated biomass in downstream channel 6 differed significantly from that of downstream channels 2, 4, 5 and 7. This result was interesting, as it indicated that accumulated biomass in channel 6 differed significantly from accumulated biomass in one channel of every other condition tested. However, it also differed significantly from channel 5, representing identical conditions to that in channel 6. This result should thus be seen as an anomaly that does not accurately reflect the outcome of the experiment.

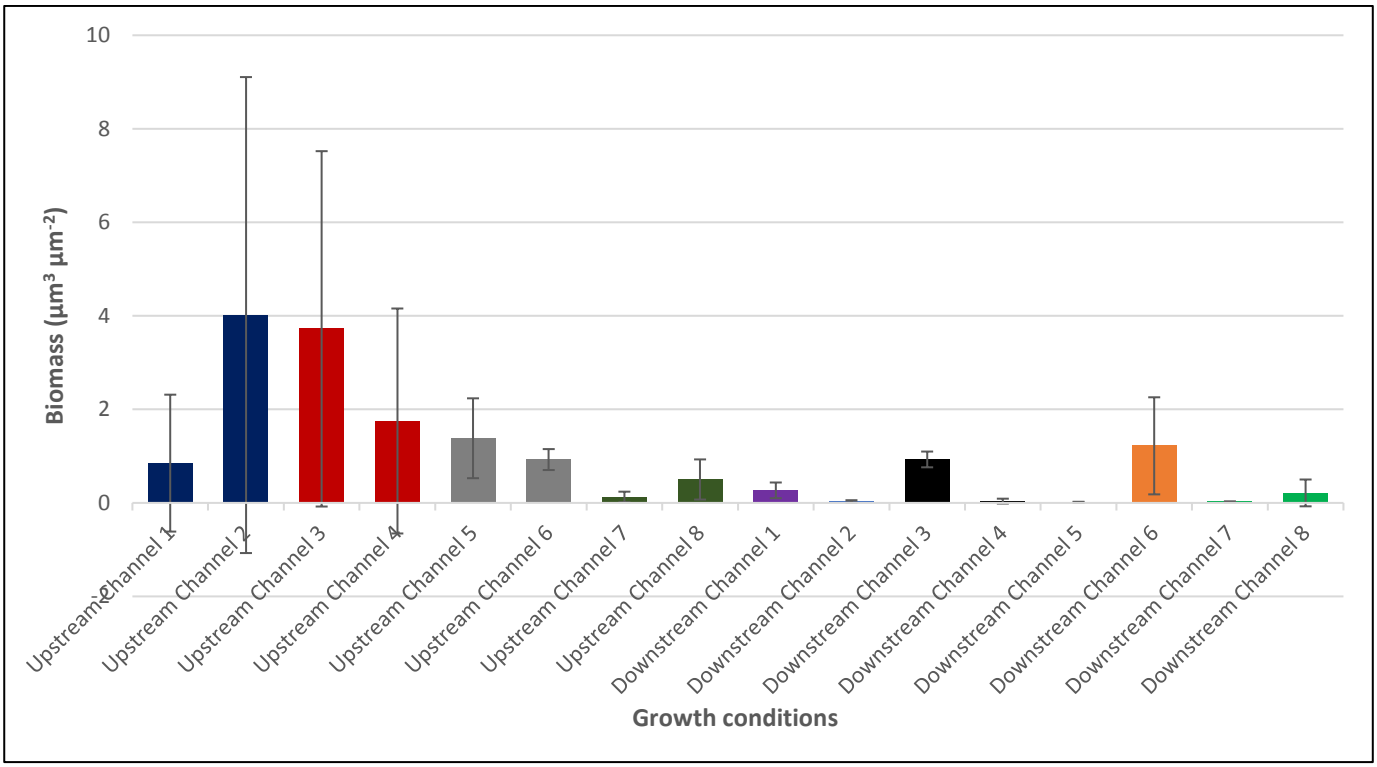


Fig. 4.26 – Biomass measured by CLSM analysis for different flowcell channels representing different growth conditions.

Surface to biovolume ratio showed no clear pattern for differences between surface to biovolume ratio for biofilms in upstream flowcell channels (Fig. 4.27). One-way ANOVA analysis indicated that the differences observed were not statistically significant. However, comparing differences in surface to biovolume ratio for downstream biofilms showed interesting and significant results. Surface to biovolume ratio in channel 3 differed significantly from that of channels 2, 4 and 5. Interestingly, surface to biovolume ratio for both channels 7 and 8 (with identical growth conditions) were significantly less than that of channels 2, 4 and 5. Lastly, surface to biovolume ratio in channel 5 also differed significantly from that of channels 1 and 6. From these results it is clear that the surface to biovolume ratio in channel 5 can be seen as an outlier, especially since it differs significantly from that in channel 6, which had identical conditions to that of channel 5. Although the surface to biovolume ratio in channel 7 and 8, both representing identical conditions, were significantly less than that of one channel of every other condition, it cannot be conclusively stated that the conditions present in channels 7 and 8 result in a smaller surface to biovolume ratio, especially since these differences could only be observed between one channel of each condition.

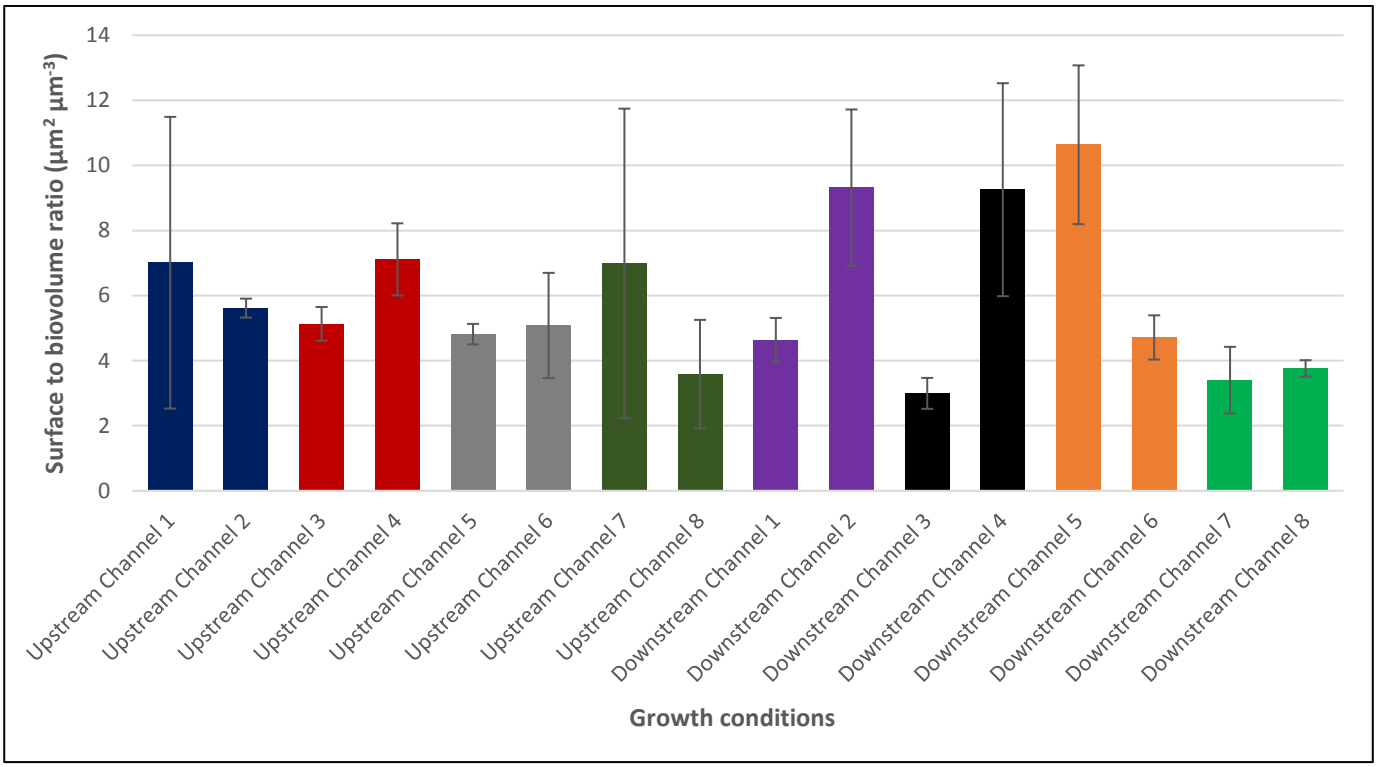


Fig. 4.27 – Surface to biovolume ratio measured by CLSM for different flowcell channels representing different growth conditions.

Biofilm maximum thickness was measured as this is a very common method of evaluating biofilm development (Heydorn et al., 2000), but also as this potentially plays a role in affecting water flow in pipelines. By comparing the maximum thickness of biofilms grown upstream (Fig. 4.28) and subjecting the results to one-way ANOVA and the Tukey HSD test, some statistically significant differences can be observed, with biofilms in channels 3 and 5 displaying larger maximum thicknesses than that observed for other upstream biofilms. Statistical analysis indicated that maximum thickness of biofilms in channel 3 were significantly more than that of channels 1 and 7, while maximum thickness of biofilms in channel 5 were significantly more than that of channels 1, 7 and 8. Evaluation of maximum thickness of biofilms in downstream channels shows the maximum thickness of biofilms grown in channel 6 differs significantly from that of biofilms in channels 1, 2, 4, 5, 7 and 8. Due to the significant differences seen between channel 5 and 6 with identical growth conditions, as well as the lack of any other significant differences in biofilm maximum thickness in downstream channels, it can be assumed that the maximum biofilm thickness observed in channel 6 is an outlier.

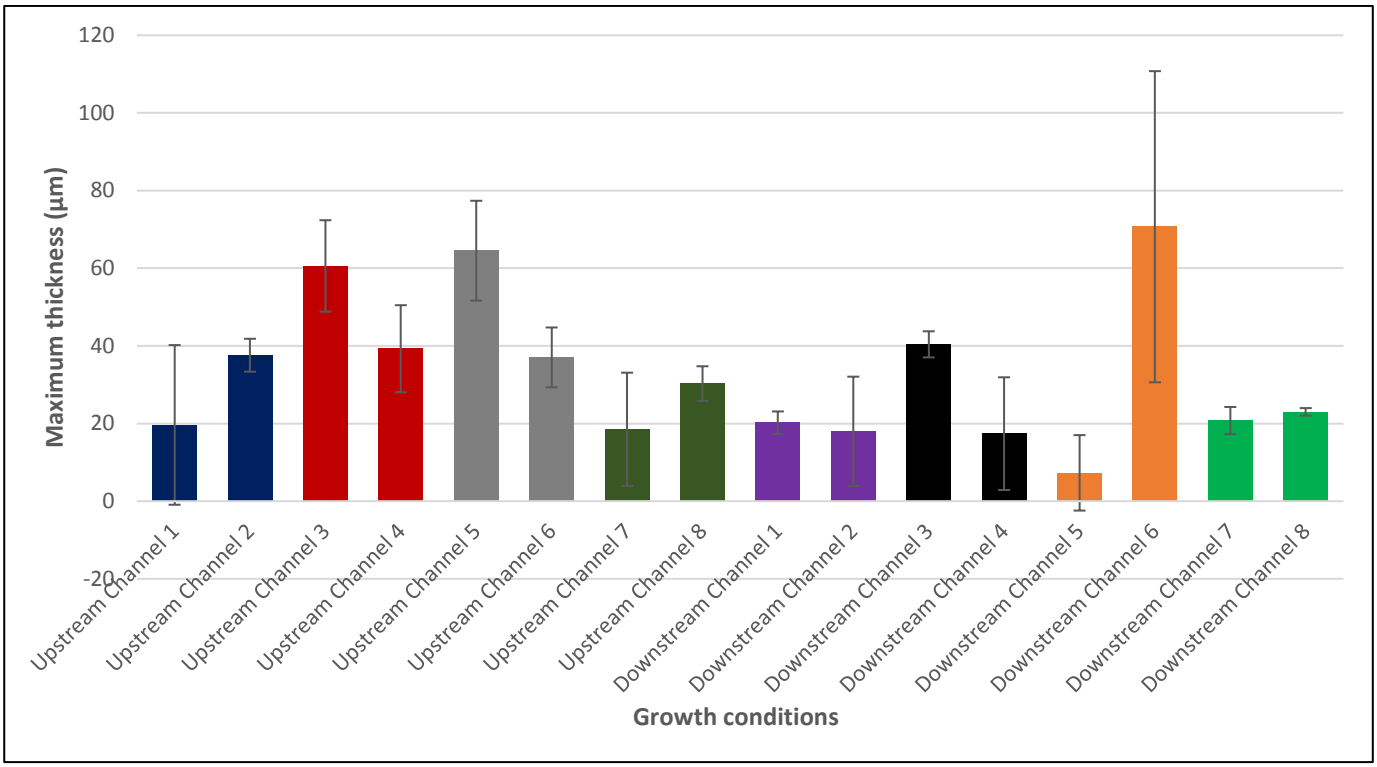


Fig. 4.28 – Biofilm maximum thickness as measured by CLSM for different flowcell channels representing different growth conditions.

Comparing the results from measurements for biomass, surface to biovolume ratio and biofilm maximum thickness, it is clear that no growth condition had any significantly different effect on biofilm development. Although statistically significant differences could be observed between

biofilms cultivated under different DO and Mn concentrations, these differences were often only present for one channel of a specific condition. In order to conclusively state that one test condition is more favourable for biofilm formation compared to the other conditions, statistically significant differences needed to be observed between test conditions consistently, where biofilms from both channels in a given growth condition differ significantly from biofilms in both channels of the compared condition. This was not the case for this flow system. However, as previously mentioned, due to the stochastic nature of biofilm development, it is not always possible to replicate biofilm growth, even under identical conditions (Heydorn et al., 2000). Additionally, the period of 32 days during which these biofilms were cultivated might not have been sufficient for the growth conditions to influence biofilm biomass, surface to biovolume ratio and maximum thickness significantly, leading to the unclear differences observed in Fig. 4.26 – 4.28. For these reasons, a second replicate of this experiment was performed for comparison.

4.3.5.2. Evaluation of biofilm biomass, surface to biovolume ratio and biofilm maximum thickness – Replicate 2

A second replicate of the flow system was constructed for microscopic analysis. Identical conditions to that used for the first replicate were used. For comparison, biofilm biomass, surface to biovolume ratio and maximum biofilm thickness were evaluated.

Initial observations of measured biomass in Fig. 4.29 indicated that accumulated biomass in upstream channels 3 and 4 were more than that observed in other upstream channels. However, statistical analysis indicated that there were no statistically significant differences between accumulated biomass at the upstream inoculation point. The same was true for differences observed in accumulated biomass at the downstream inoculation point. It was concluded that the concentrations of DO and Mn had no significant effect on the accumulated biomass in the different flowcell channels. Although the accumulated biomass measurements differed for the two replicates, in both cases it was concluded that this was not as a result of the concentrations of DO or Mn that the biofilms were subjected to. It is therefore possible that the physical conditions in the flow system, e.g. shear, flux or boundary conditions exerted a stronger influence on biofilm structure than DO, and that the supplied Mn concentrations greatly exceeded the sorption capacity of the resulting biofilms.

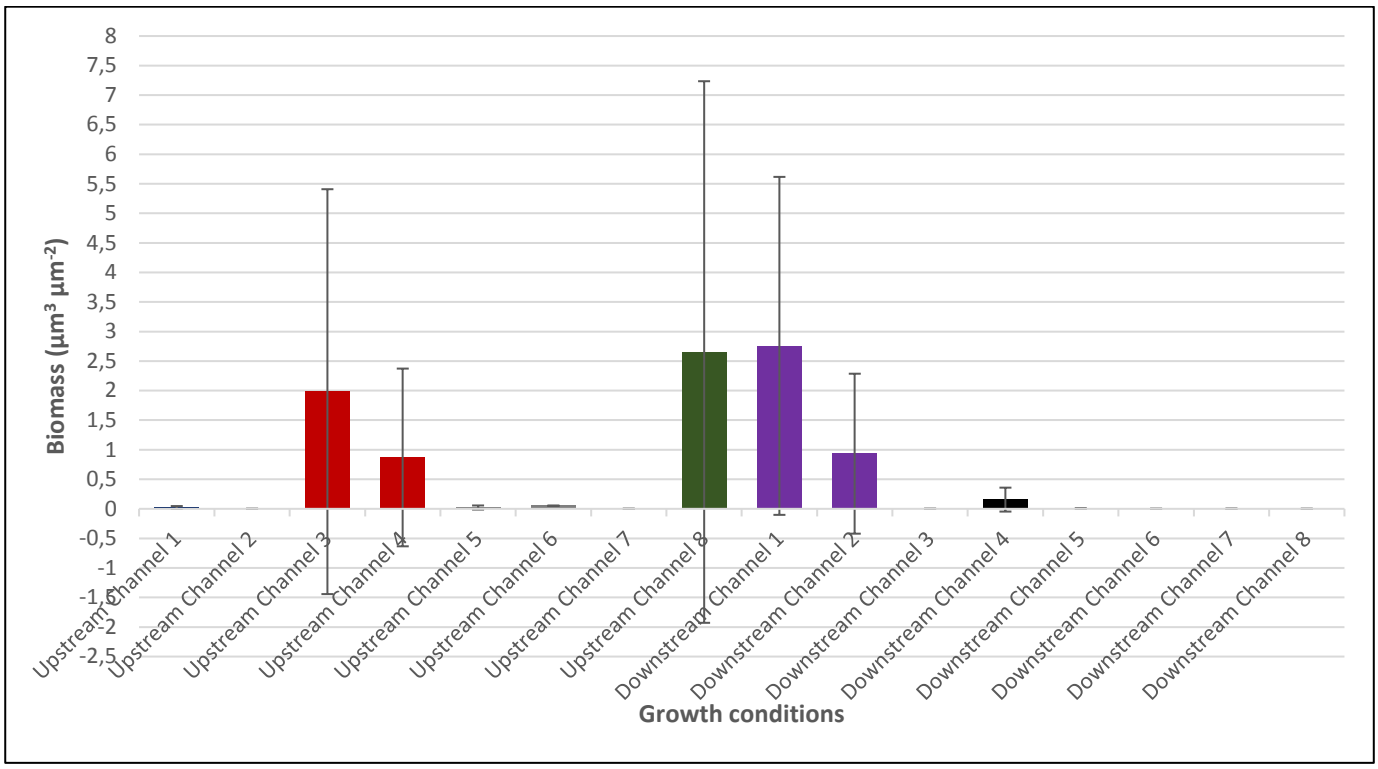


Fig. 4.29 - Biomass measured using CLSM analysis for different flowcell channels representing different growth conditions.

Surface to biovolume ratio for biofilms at the upstream inoculation points were very similar (Fig. 4.30), without many clear differences. Statistical analysis using one-way ANOVA indicated that the differences observed were not statistically relevant. However, the same was not true for biofilms at the downstream inoculation points, with surface to biovolume ratio for biofilms in both channels 1 and 2 being significantly less than that of channels 3, 5, 6, 7 and 8. The results in terms of surface to biovolume ratio did not correspond with that of replicate one. Whereas the surface to biovolume ratio of biofilms in channels 7 and 8 were significantly less than that of the biofilms in channels 2, 4 and 5, this was not the case in replicate 2, where surface to biovolume ratio in channels 1 and 2 were the only channels to differ significantly from the others. This indicated that the significant differences observed could not be conclusively attributed to the growth conditions in which these biofilms were cultivated.

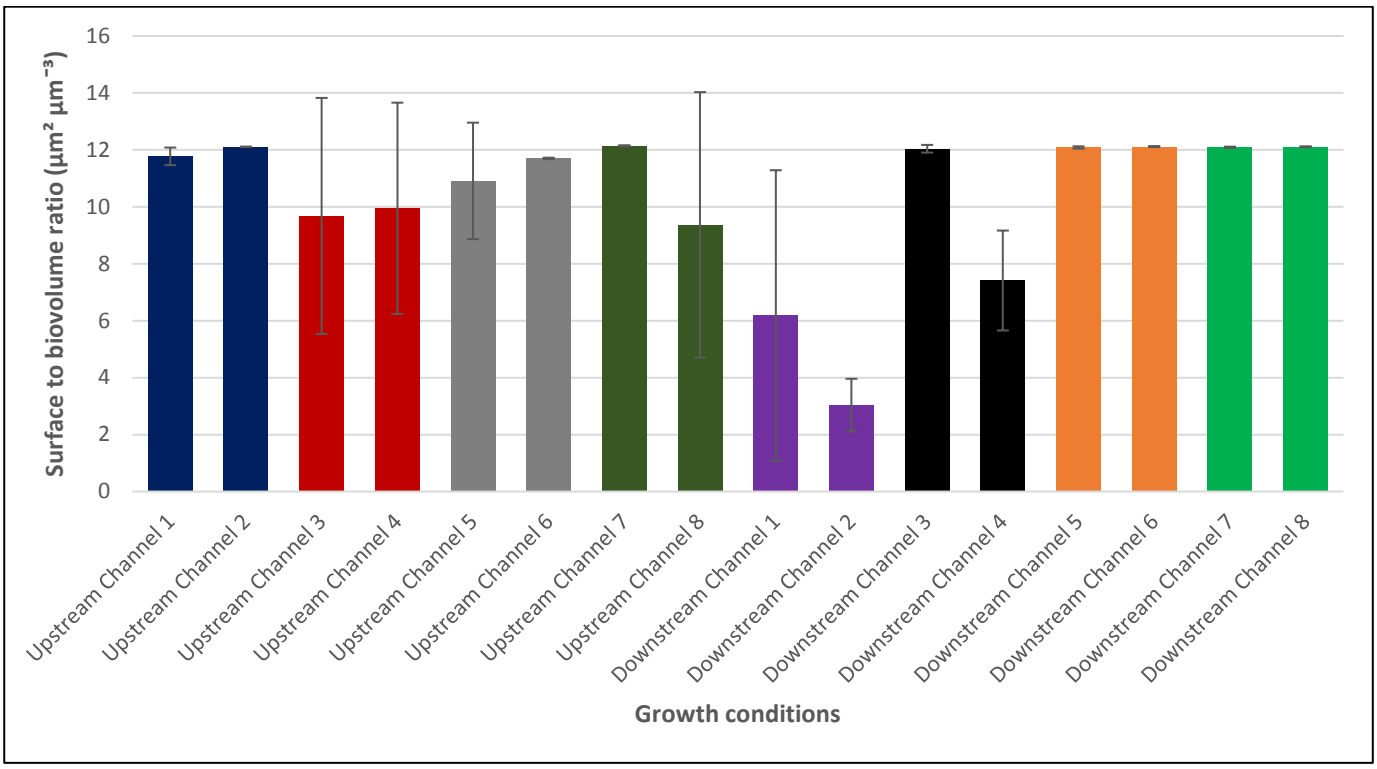


Fig. 4.30 - Surface to biovolume ratio measured by CLSM for different flowcell channels representing different growth conditions.

No significant differences could be observed between biofilm maximum thickness at the upstream inoculation point (Fig. 4.31). However, significant differences could be observed at the downstream inoculation point, with biofilm maximum thickness in channel 1 being significantly more than that of channels 3, 5, 6, 7 and 8. Measurements collected for maximum thickness in replicate 2 did not correspond to that of replicate 1. Although the maximum thickness of biofilms in channel 1 at the downstream inoculation point was significantly more than that of other channels in replicate 2, this significant difference could only be observed for one channel of the low DO and high Mn concentration channel. Biofilms in channel 2 showed no significant difference to that of the other downstream channels, indicating that the higher measured maximum thickness of biofilms in channel 1 could not conclusively be attributed to the growth conditions.

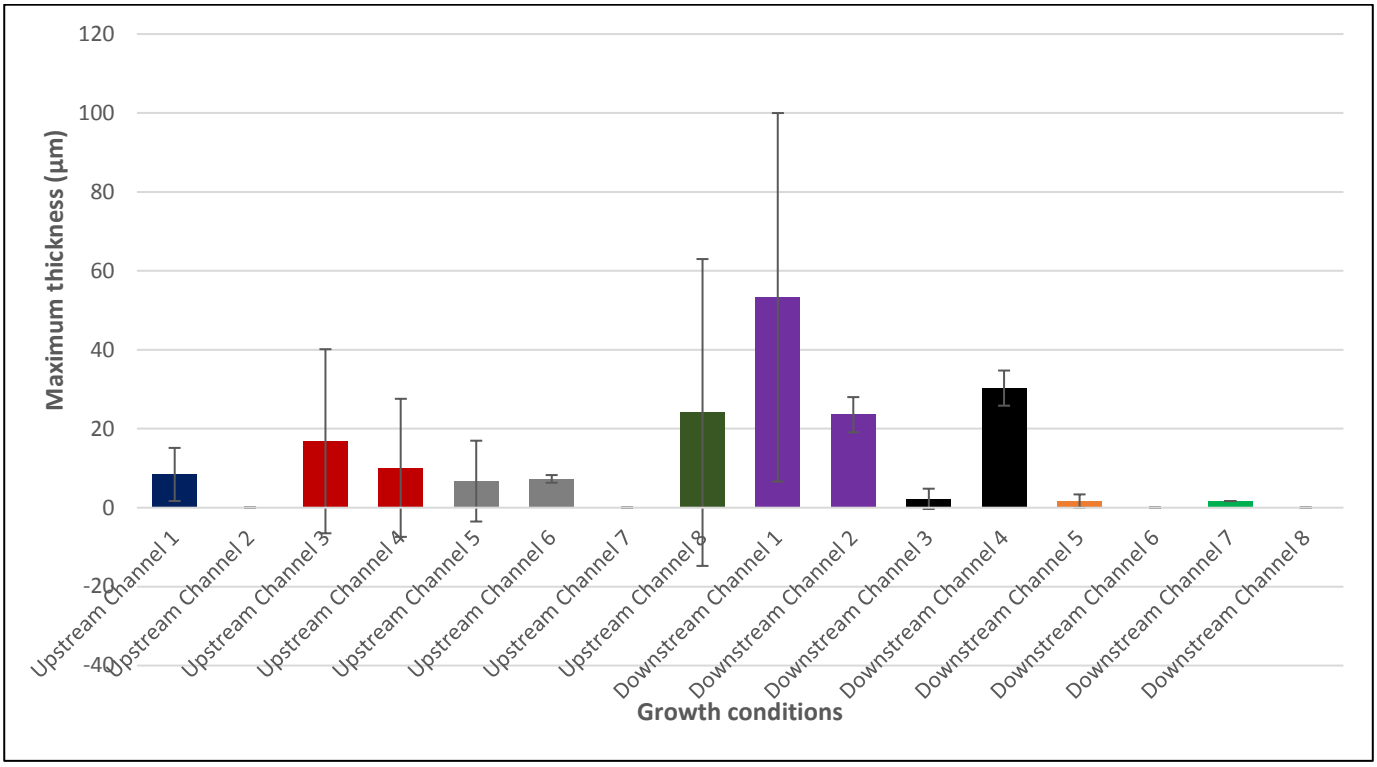


Fig. 4.31 - Biofilm maximum thickness as measured by CLSM for different flowcell channels representing different growth conditions.

From the analyzed data obtained from CLSM for both replicates of the flow system, it was concluded that, for these two laboratory scale flow systems, the different DO and Mn concentrations had no significant effect on the development and growth of Mn oxidizing and reducing biofilms. Although significant differences could in some cases be observed between biofilms grown under these different growth conditions, the differences were rarely present in biofilms of both channels of that specific growth condition. The significant differences observed further did not correspond between biofilms of upstream and downstream inoculation points, i.e. significant differences seen at the upstream inoculation point did not necessarily correspond to that seen at the downstream inoculation point. Lastly, significant differences observed did not correspond between biofilms of the two replicate experimental systems. Although it could be expected that not all significant differences would correspond between the replicates as a result of the stochastic nature of biofilm growth and development (Heydorn et al., 2000), it is expected that significant differences overall would correspond between these replicates, as well as form a general trend due to the different growth conditions that these biofilms were subjected to, which was not the case for these replicates. As explained for the first replicate, the duration of the experiment may have been insufficient for the growth conditions to have had a statistically significant effect on the biomass, maximum thickness and surface to biovolume ratio. These results may have differed if these experimental systems were operated for the three-month period as described in section 2.4.1 to allow for sufficient establishment

of Mn oxidizing biofilms, such as observed in the full-scale Preekstoel biofilter used to remove Mn from groundwater before blending with surface water (Du Toit et al., 2015).

4.3.5.3. Oxidation test

The lack of any significant decrease in Mn concentration in the effluent of the flow systems in sections 4.3.4.3 and 4.3.4.4 led to speculation that Mn could potentially be oxidized but not incorporated into the biofilm structure. As the test used for Mn measurements only measures total Mn concentrations, previous measurements may have included oxidized Mn. To capture any Mn oxides, effluent was filtered through 0.22 µm syringe filters and compared to unfiltered effluent as indicated in Fig. 4.32. Mn measurements from lines 7 and 8 were excluded as concentrations in the effluent was too low to be accurately measured by the equipment in the laboratory.

Results from the Mn measurements indicated that some Mn oxidation took place, but that the percentage of Mn oxidized was extremely low, with line 5 showing the highest percentage oxidation at 6.5%, indicating that the biofilms did not cause significant oxidation of Mn. This possibly indicates that Mn oxidizing microorganisms only comprised a minor component of the biofilm community or that the duration of the experiment might be a factor. Thus, as was concluded from the previous flow systems, this result would potentially be different if these biofilms were given more time to become established and thus be more capable of oxidizing Mn. For comparison, a sterile flow system was constructed and is discussed in section 4.3.6.

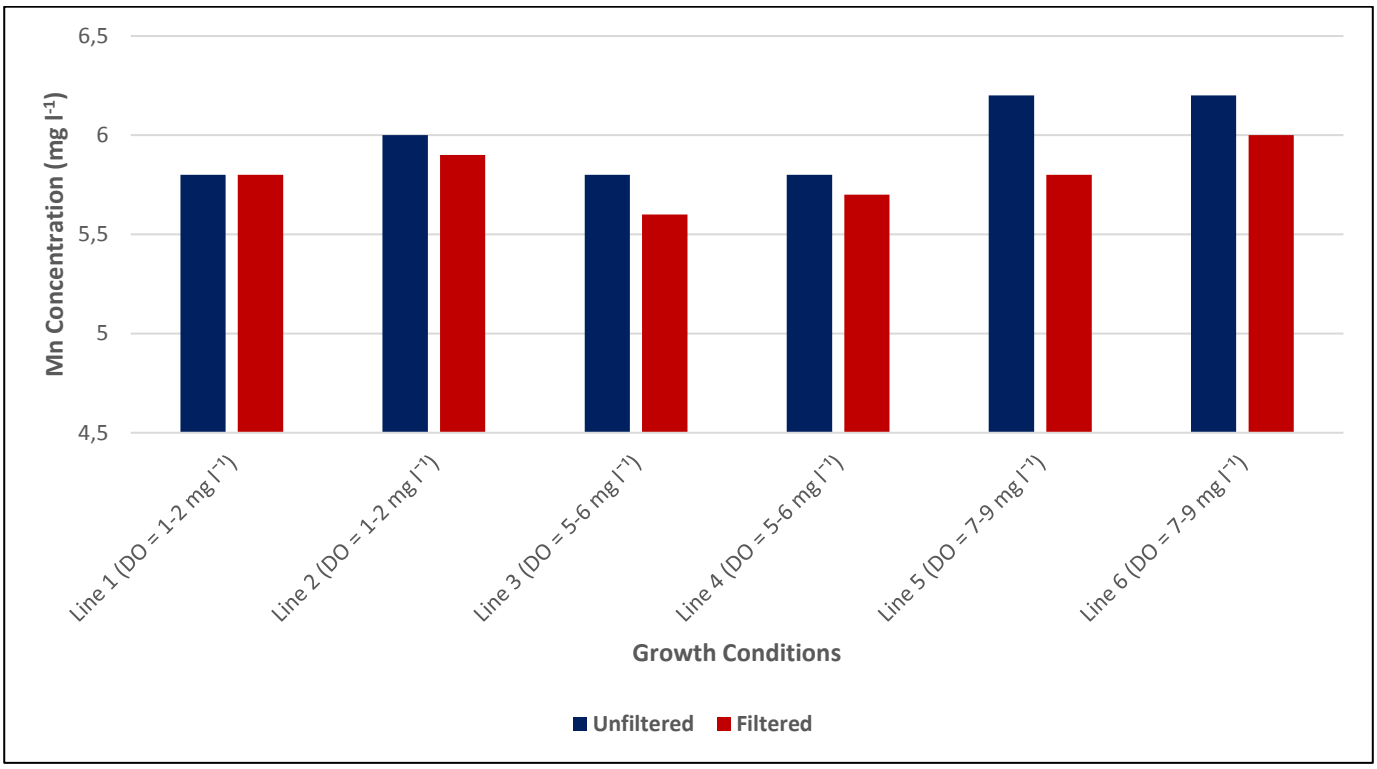


Fig. 4.32 – Mn measurements comparing the Mn concentration of filtered and unfiltered effluent collected from the second replicate flow system for microscopic analysis after 28 days.

4.3.6. Sterile flow system

A sterile flow system was constructed to serve as a control for every growth condition tested and to determine whether any changes observed in Mn measurements were as a result of Mn oxidation by microorganisms or physico-chemical conditions. This system was maintained under identical conditions to that used for systems described in section 4.2.6 for 5 days, after which Mn concentrations in the effluent was measured and filtered to determine which percentage of total Mn was oxidized.

Mn concentrations in the effluent remained very similar between conditions (Fig. 4.33), with no noticeable decrease for any of the growth conditions. All filtered samples had lower Mn concentrations than that observed in unfiltered samples, pointing to Mn oxidation and resulting in the capturing of Mn oxides in the filters. Both lines one and two showed oxidation percentages between 12 and 13%. Lines 3 and 4 showed higher oxidation percentages between 18 and 21% and lastly lines 5 and 6 had oxidation percentages between 14 and 15%. These results indicate that Mn can be oxidized to a degree under physico-chemical conditions alone without the aid of Mn oxidizing bacteria. Interestingly, Mn concentration in unfiltered samples collected from the sterile system (Fig. 4.33) were lower than that of both unfiltered and filtered samples collected from the flow system

containing biofilms in Fig. 4.32. This indicates capturing of Mn in the sterile system between the influent and the effluent points. Similar to that observed in section 4.3.3, it is speculated that due to the limited degree of oxidation of Mn under physico-chemical conditions alone, oxidized Mn particles will precipitate in sterile flow systems and collect in the bottom of the tubes where it will not be washed out with the effluent due to the low flow rate. In the presence of biofilms however, the decrease in DO concentration caused by microbial activity may lead to a reduction of oxidized Mn particles, preventing it from precipitating and thereby keeping it in the aqueous phase leaving the system. This also illustrates the role of biofilms in creating anaerobic conditions where Mn reducing bacteria have a notable effect on the overall Mn cycle.

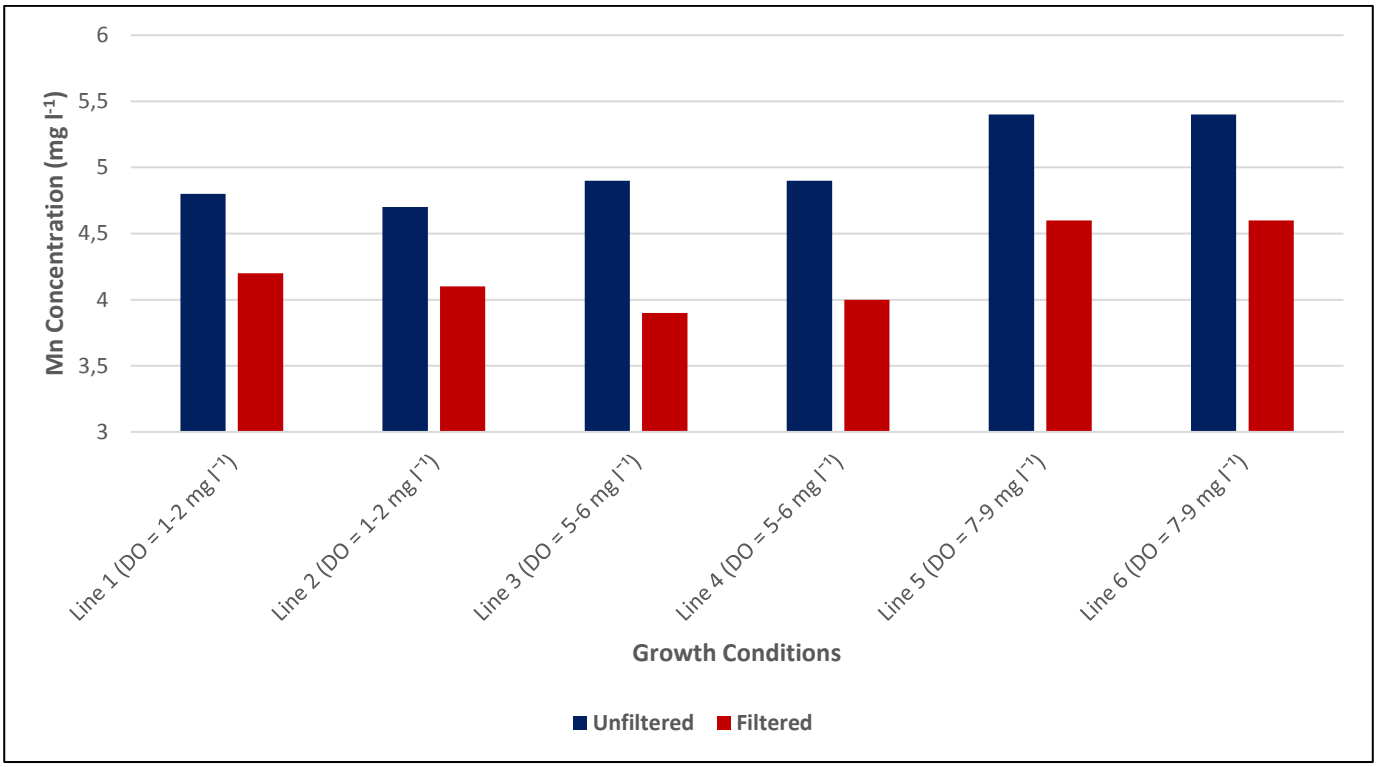


Fig. 4.33 - Mn concentrations of both filtered and unfiltered effluent collected from a sterile flow system. All lines were supplied with SGW with a Mn concentration of 6 mg l^{-1}.

The inability of biofilms to oxidize Mn and incorporate it into the biofilm structure in section 4.3.3 was thought to be due to the high DO concentrations of the SGW entering the system. However, the system tested in this section as well as in 4.3.5.3 were both supplied with SGW with various concentrations of DO. It would thus be reasonable to assume that Mn concentrations in the effluent would be lower for lines with DO concentrations of 5 – 6 mg l^{-1} firstly in Fig. 4.32, as this would allow for optimal biological oxidation conditions allowing incorporation into the biofilm structure and secondly in Fig. 4.33, as these conditions would allow for limited Mn oxidation under physico-chemical conditions, allowing particles to precipitate. However, this was not the case in either Fig.

4.32 or Fig. 4.33 and can possibly be explained by the inadequate duration of the experiment, as well as the less than expected Mn oxidation under physico-chemical conditions alone.

4.3.7. Conclusion

The data obtained in this chapter differed from that which was initially expected. Results in sections 4.3.4.1 and 4.3.4.2 indicated that the different growth conditions tested had no significant effect on the cell yield of these mixed community biofilms. This observation was confirmed by microscopic analysis using CLSM in section 4.3.5, where no clear differences could be seen in biomass, surface to biovolume ratio or biofilm maximum thickness for any of the conditions tested. In cases where statistically significant differences were observed, these differences were concluded to be outliers or the result of physical forces such as shear or flux and not as a result of the different growth conditions that these biofilms were subjected to.

No significant decrease could be observed between Mn concentrations in effluent collected at the downstream inoculations point compared to the upstream inoculation points in sections 4.3.4.3 and 4.3.4.4, indicating that very little or no Mn was incorporated into the biofilm structure and subsequently removed from the water. This finding was confirmed with SEM/EDS analysis on biofilms collected from the inside of the flow lines between the upstream and downstream inoculation points. SEM/EDS analysis indicated that very little Mn was present in the biofilm structure and could not result in a significant decrease in Mn concentration measured in the effluent.

Finally, ARISA in sections 4.3.4.7 and 4.3.4.8 indicated that the alpha diversity within samples did not differ significantly overall from one growth condition to the next but in instances where statistically significant differences did exist, these differences could not be attributed to the growth conditions. Although statistically significant differences could be observed for beta diversity, these differences also could not be attributed to the growth conditions in which these communities developed. However, even though the data differed from initial expectations, all the results from this chapter correspond to one another. Growth data corresponded with that of CLSM analysis, with both showing that none of the growth conditions tested had any significant impact on the growth and development of these biofilms. Finally, this observation was also confirmed by ARISA. Results from Mn measurements was confirmed by SEM/EDS. Interestingly, although very little to no Mn was incorporated into the biofilm structure, these results serve as indication that soluble Mn is not incorporated in biofilm structure unless conditions are optimal, with Mn oxidizing bacteria actively oxidizing it and incorporating it into the structure.

Several factors could have attributed to the unexpected results obtained. Firstly, although 28 days was enough for steady state biofilms to form in these systems, it is possible that these biofilms have not yet reached a steady state composition. Research at water treatment facilities employing biological oxidation have reported colonization periods between two to three months before biofilms were capable of adequately oxidizing Mn (Du Toit et al., 2015; Thompson et al., Unpublished technical reports). It is therefore possible that the biofilms cultivated in this laboratory scale system required a longer establishment period before showing significant Mn oxidation. However, these time periods were unrealistic due to the limitations on time to perform these experiments.

Another factor which may have led to the observed results was the addition of TSB in the SGW. Although TSB was included as a carbon source to mimic the presence of algal products in the Blyderiver dam, this may have accelerated growth of other heterotrophs capable of outcompeting the Mn oxidizing bacteria. Although this was not proven, biofilm development with and without the addition of TSB in the SGW needs to be investigated.

It is recommended for future studies that Mn oxidizing - and reducing biofilms are cultured in flow systems identical to those used in this chapter, but with the inoculation points substituted with microfluidic channels to more accurately simulate the shear and flow conditions experienced inside the LBIS pipeline network. Microfluidic channels should be constructed based on the calculated Reynolds numbers and flow conditions inside the LBIS pipeline, which is not possible with conventional flow cells. Experiments investigating the effects on biofilms when flow of nutrients is stopped, followed by high flow should be conducted. The rationale behind this experiment is to evaluate the effect on biofilms when they are deprived of nutrients and especially in this case, Mn. It is hypothesized that nutrient deprivation may lead to biofilm washout after being exposed to nutrient deprived environments for a specific period of time. The effects on biofilms can be evaluated with CLSM. The growth and oxidizing capabilities of these biofilms should be examined over a longer period of up to three months under the same growth conditions tested in this chapter. Organisms known to be capable of oxidizing Mn should also be isolated from these mixed community biofilms to obtain pure cultures, where after it should be used as inoculum to obtain pure culture biofilms capable of oxidizing Mn for comparison with mixed community biofilms.

Chapter 5

General conclusions and recommendations

5.1. General conclusions

It was found that the water of the Blyderiver dam has a characteristic profile, with an inverse relationship between DO and Mn concentrations. DO concentrations above 7 mg l^{-1} were measured in the upper regions of the water, followed by a rapid decline to below 2 mg l^{-1} in the deeper regions. Total Mn concentration had an inverse relationship with measured DO concentrations, ranging between 10 and 100 µg l^{-1} near the surface to higher than 8000 µg l^{-1} near the sediment water interface. Due to water currently being extracted from the lowest extraction point near the sediment water interface, this indicates that very high concentrations of Mn are entering the LBIS pipeline. The Mn rich water may select for microorganisms that derive energy from the Mn cycle. These microorganisms may potentially dominate in biofilms in the pipeline where it serves as a catalyst for the build-up of a matrix composed of Mn and the extracellular polymeric matrix produced by these biofilms. Biofilm formation in the LBIS pipeline is estimated to cause a reduction in hydraulic capacity of up to 20%. Mn concentration in both the aqueous phase and biofilms in the pipeline decreased downstream from the point of extraction, suggesting that Mn is incorporated into the biofilm structure, leading to an increase in biomass. This may lead to further loss in hydraulic capacity over time.

Interestingly, the water profiles from October 2015 and May 2016 differed from those of October 2016 and February 2017, with the last two profiles only showing a rapid decrease in DO mirrored by a rapid increase in Mn close to the deepest regions where samples were collected. Additionally, water levels differed significantly during these two sampling events, with the dam only being 40% full in October 2016 and 100% during February 2017. For October 2016 this could be explained by low rainfall, resulting in higher volumes of water extracted compared to inflow into the dam. This allowed for a large portion of the hypoxic deeper region to be extracted and DO rich water from shallower depths being drawn down, aerating the deeper water and ultimately increasing the DO concentration throughout most of the water column. The higher DO concentration seen throughout most of the water profile during February 2017 could potentially be attributed to heavy rainfall during the few weeks prior to sampling, leading to the high inflow of DO rich water and possibly leading to sufficient mixing to result in a temporary high DO throughout most of the water column.

Despite the distinct profiles followed by Mn and DO concentrations with increasing depth, no clear decrease could be observed in the temperature profile with increasing depths, thus leading to the conclusion that the water column in this dam shows distinct DO and Mn profiles with depth without being thermally stratified. This was unexpected and indicates that factors such as rainfall, climate

and water extraction rate may affect these concentration and temperature profiles. It was further speculated that the presence of algal exudates and dead algal cells settling out to the deeper water may be responsible for lower DO concentrations at greater depths, where bacteria and other microorganisms present at these depths use oxygen for their metabolic processes during biodegradation of algal biomass, resulting in hypoxic conditions which may ultimately contribute to Mn reduction. The data obtained from sampling at the Blyderiver dam, as well as knowledge gained from literature indicated that the processes involved in Mn oxidation and reduction, especially when biological oxidation is involved, are complex and not yet fully understood. For this reason, these biofilms and their role in Mn oxidation needed to be studied in laboratory experiments.

Biofilms were cultured in laboratory simulated flow systems in different concentrations of DO and Mn based on measurements taken at specific depths in the Blyderiver dam. Although growth data from selective Mn oxidation agar, selective Mn reduction agar and TSA showed statistically significant differences in average CFU ml^{-1} between growth conditions in some cases, these differences could not be conclusively attributed to the effects of the different DO and Mn concentrations. Significant differences did not occur consistently on all growth media types over the experimental time period of 28 days, nor did they occur for both lines of one condition compared to both lines of another condition (eg. both lines with DO 1 – 2 mg l^{-1} compared to both lines of DO 5 – 6 mg l^{-1}). The statistically significant differences that did occur could also not be correlated between the two full replicate experiments of 28 days each. Based on all these factors, it was concluded that different concentrations of DO and Mn were not the cause of the differences seen in growth of the laboratory biofilms but that these differences could rather be attributed to physical forces such as shear and flux, as well as to the stochastic nature of biofilms, making it difficult to replicate biofilm growth between two experiments (Heydorn et al., 2000).

These results corresponded to that of data obtained through CLSM analysis. Here the biomass, surface to biovolume ratio and maximum thickness of biofilms grown under each condition were studied to evaluate whether DO and Mn concentrations had any significant effect on the growth and overall structure of these biofilms. Statistically significant differences could be seen in some cases; however, these differences could not be conclusively attributed to the effects of DO and Mn concentrations and were also regarded as outliers and attributed to the stochastic nature of biofilm development and the effect of physical forces.

No significant decrease was measured between Mn concentration in the effluent collected from the upstream inoculation point compared to that of the downstream inoculation point for any of the

growth conditions tested. Further, almost no statistically significant differences in the Mn concentrations could be detected in the effluent collected between different growth conditions. In the case where these differences were significant, it was concluded that these differences were not as a result of different DO and Mn concentrations. These results were further investigated through the use of SEM/EDS to determine whether any Mn was incorporated into the biofilm structure and if so, whether the concentrations incorporated were significantly different between growth conditions. In contrast to Mn being concentrated up to levels of 30105.4 mg kg^{-1} in biofilms in the irrigation pipeline, SEM/EDS analysis indicated that very little to no Mn was incorporated into the biofilms cultivated in the laboratory, and that none of the growth conditions, regardless of DO or Mn concentration, had any significant impact on this process.

ARISA showed no statistically significant differences in the OTU numbers between samples for both replicate experiments, indicating that species richness remained very similar under all conditions over the 28-day duration of the experiment. Some statistically significant differences were observed in the alpha diversity of samples. The same was true for the beta diversity, however, these differences could not be attributed to growth conditions. The similarity measured in biofilm function (Mn accumulation) and form (surface-to-volume ratio and thickness) were thus also evident in biofilm community composition.

Interestingly, results from the sterile flow system showed better Mn oxidation than that found in the non-sterile system. Lines in which DO concentration ranged between 5 – 6 mg l^{-1} showed the highest oxidation between 18 and 21%. This corresponded with literature stating that 5 – 6 mg l^{-1} is the optimal DO range for biological oxidation of Mn. The oxidation of Mn in the absence of any biofilms indicated that Mn can be oxidized under physico-chemical conditions alone, although biological oxidation is typically seen to increase the rate of this process significantly (Webb et al., 2005). The higher oxidation percentages observed in sterile conditions compared to that of unsterile conditions was unexpected. Although Mn measurements in systems containing biofilms showed that a lower percentage of Mn was oxidized, it was expected that the percentage oxidized Mn in the sterile system would be similar or lower, but not higher, with both systems subjected to identical growth conditions. However, this difference indicates that the presence of biofilms possibly leads to changes in DO concentration and pH levels, changing the physico-chemical conditions to less than optimal in this case. It is also possible that the activity of Mn reducing bacteria was higher relative to the Mn oxidizing bacteria.

Ultimately, the results obtained from laboratory scale flow systems did not correspond with what has been observed on the inside of the LBIS pipeline. Very little to no Mn was incorporated into the structure of biofilms cultivated in the laboratory, whereas biofilms with Mn content of up to 30105.4 mg kg^{-1} were collected from the inside of LBIS. Also, no significant or noteworthy decrease in Mn concentration in the aqueous phase were measured between upstream and downstream inoculation points in the laboratory scale flow system, whereas these decreases were observed in all three routes in the LBIS from which water samples were collected. The differences between results obtained from environmental data and laboratory experimental data can be explained by a number of factors. Firstly, the duration allowed for biofilm cultivation in the laboratory scale flow systems may have been insufficient and should be increased. The concentration of TSB added to the SGW may have been too high, allowing for heterotrophs, and Mn reducers to outcompete the Mn oxidizers, resulting in biofilms consisting predominantly of microbes unable to oxidize Mn. Lastly, the elimination of other elements present in the Blyderiver water such as Fe and Al from the SGW may have contributed to a different community composition in laboratory cultured biofilms compared to what is found inside the LBIS, as the interaction between Mn, Fe, Al and the respective microbes participating in their redox reactions are not yet fully understood.

5.2. Future studies

A number of suggestions are recommended for future research. Experimental pipe rigs could be installed and supplied with water extracted from various depths in the Blyderiver dam to monitor biofilm development under various DO and Mn concentrations present at respective depths. Effective methods for eliminating the existing biofilms from the inside of the LBIS pipeline (e.g. scouring or fluctuating flow rates) should be investigated to monitor the in-situ development of new biofilms under higher DO and lower Mn conditions. Biofilm development in these rigs should be monitored over time with regular sampling intervals, preferably once every second week, and subjected to ICP-MS analysis to measure Mn and Fe content in biofilms.

Future laboratory scale experiments should focus on longer cultivation periods for Mn oxidizing and reducing biofilms, allowing up to three months of growth under the range of growth conditions tested in chapter 4. These experiments should further focus on the isolation of Mn oxidizing and reducing microbes in pure culture, followed by biofilm cultivation with these microorganisms to study their ability to oxidize and potentially incorporate Mn into the biofilm structure. Attention should also be given to microfluidic flow chambers in which biofilms can be cultivated under flow conditions with shear more similar to that experienced in the LBIS pipeline. The growth and development, as well

as changes in biofilm structure under fluctuating flow conditions in this device should be studied using time-lapse microscopy.

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