Microbial Recovery of Rare Earth Elements from Metallic Wastes and Scrap

by

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To my Parents……..

Abstract

In this thesis the microbial recovery of the rare earth neodymium is being examined using biofilm of the bacterium *Serratia* sp. N14 which has been used previously for the removal of lanthanide elements from liquid solutions. The mechanism used is via the PhoN phosphatase enzyme of the cells which releases phosphate from an organic feedstock and metal cations (M^{2+}) existing in the solution precipitate on the bacterial cell as metal phosphate.

Neodymium was announced in 2013 to be included in the five most critical rare earth elements (REEs) and among the recycling of REEs, this was less than 1%. Therefore, the need to recover neodymium from liquid metallic wastes and scrap leachates would be a crucial step to recycle such metals.

The first aim of this project was to study the ageing properties of the PhoN of *Serratia* sp. N14 cells during one year of storage, with respect to total phosphate release (Pi) and neodymium recovery. A simple predictive mathematical model was produced for the evaluation of metal recovery over various months of storage.

The second goal of this project, was to construct an outflow chamber onto existing equipment for a semi-continuous biofilm preparation as an alternative economic production of biofilm, since the normal ‘’in vessel’’ biofilm production, is not readily scalable. The activity of biofilm from this new method was compared with the biofilm produced in the normal chamber reactor after appropriate times of storage.

The final aim was to establish the potential usefulness of the biofilm with respect to its tolerance to low pH values or to high salt concentrations, since the overall goal is to

recover metals from solid scraps, which may have been leached in strong acid. The robustness of the metal accumulating biofilm is discussed with respect to this envisaged application.

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Edgbaston, October 2013

Dimitrios Vavlekas

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Abbreviations

CFU	Colony-forming Unit
REEs	Rare Earth Elements
HREEs	Heavy Rare Earth Elements
LREEs	Light Rare Earth Elements
(H)	Biofilm produced in 2012 (Culture I) stored in the cold room (4^{o}C) for 3 months prior to examination and examined by Singh (2012)
(J)	Biofilm produced in 2012 (Culture I) stored in the cold room (4^{o}C) for 7 months prior to examination
(K)	Biofilm produced in 2012 (Culture I) stored in the cold room (4^{o}C) for 9 months prior to examination
(L)	Biofilm produced in 2012 (Culture I) stored in the cold room (4^{o}C) for 10 months prior to examination
(M)	Biofilm produced in 2012 (Culture I) stored in the cold room (4^{o}C) for 12 months prior to examination
(Q)	Biofilm produced in 2013 (Culture II), has not stayed in the cold room prior to examination and examined by Singh (2013)
(R)	Biofilm produced in 2013 (Culture II) and stored in the cold room (4^{o}C) for 3 months prior to examination
(S)	Biofilm produced in the outflow chamber in 2013 (Culture II) and stored in the cold room (4^{o}C) for two weeks prior to examination
(V)	Biofilm produced in the outflow chamber in 2013 (Culture II) and stored in the cold room (4^{o}C) for 3 months prior to examination
(5.5 D) or 5.5↓	Descending pH series starting from pH=5.5 and ending at pH=3.5 per 0.5 change in value.
(5.5 A) or 5.5↑	Ascending pH series starting from pH=3.5 and ending at pH=5.5 per 0.5 increments in value
EPS	Extracellular Polymeric Substances

1.0 INTRODUCTION

1.1 The rare earth elements

The rare earth elements (REEs) comprise the 15 lanthanide elements from the periodic table, with atomic numbers from 57 to 71, i.e., from lanthanum to lutetium along with scandium and yttrium (Chen, 2011). They can be subcategorized as light rare earth elements (LREEs) including the ones with atomic numbers 57-63: lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), promethium (Pm), samarium (Sm) and europium (Eu). Heavy rare earth elements (HREEs) are those with atomic numbers ranging from 64-71 up to (Lu):— gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho) , erbium (Er), thulium (Tm), ytterbium (Yb) and lutetium (Lu). Yttrium (Y) is not a rare earth element but is co-located naturally and has similar properties to the heavy REEs. Promethium (Pm) cannot be found in nature and scandium (Sc) is a dispersed element in the lithosphere (Nelson Eby, 1973, Chen, 2011, Henderson *et al.* 2011, Humphries, 2012).

The technical term "lanthanides" derives from the Greek word λανθάνειν (*lanthanein*), which means "to lie hidden" and was probably introduced in 1925 by Victor Goldschmidt (Hakala, 1952). Accordingly, these elements tend to "hide" behind other minerals and although LREEs are not that rare in natural occurrence, the HREEs are less common (Kanazawa *et al*. 2006).

REEs are mainly produced in China, which holds the highest percentage of worldwide production (more than 90%) (Kanazawa *et al*. 2006). In particular according to 2010 U.S. Geological Survey (USGS) data, China holds 97.3% of the mine production, while India has only 2% and Brazil and Malaysia only 0.42% and 0.27% correspondingly (Humphries, 2012).

According to the literature the ion adsorptive clay minerals, which lie over a large area of Southern China, are considered to be the layer which enhanced the formation of REE deposits in that part of the earth. In the Nanling area in China, during the Yinshen age, which occurred 195-130 million years ago, the granitic rocks were considered to be the most hostile environment for ion adsorption deposits. In the subtropical zone, these host rocks underwent chemical and biological weathering, under heat and moist surrounding conditions under which the REEs appeared initially as ions in the upper area of clay minerals, which were then transformed to REE deposits through an ion adsorption type mechanism. The ion-adsorption REE deposits are obtained from the mines by using a method known as open-pit and no milling or/and ore dressing is necessary (Kanazawa *et al*. 2006). The open pit method is usually used to exploit shallow ore bodies, but sometimes pits can reach the depth of 1 km. With a mining rate of more than 20,000 tons of host material per day, these kinds of mining techniques usually result in two main waste streams; a) Rock that has no economic value and has to be wasted in order to reach the orebody, b) Tailings, which are the outcome of a processing plant during a mineral separation process (Dunbar, 2012).

The biggest REE deposit in the world (Kanazawa *et al*. 2006) is located in China, in Bayan Obo. There, in the early middle Proteozoic era, the rift system created a tectonic setting for the Bayan Obo group and the ores deposition (Kanazawa *et al*. 2006). In 2010 according to USGS report (Chen, 2011) the term ‘’deposit’’ is used to describe the rare earth resources while the term ‘’reserve’’ is used to identify the rare earth resources potentially available for industrial production. As far as rare earth deposits are concerned Brazil has the higher rank with 32%, while China follows with 22%. **Figure 1** shows the rare earth distribution in eight of the biggest deposits in the world,

including the Bayan Obo mine in China. The numbers indicated are for million tones of rare earth deposits.

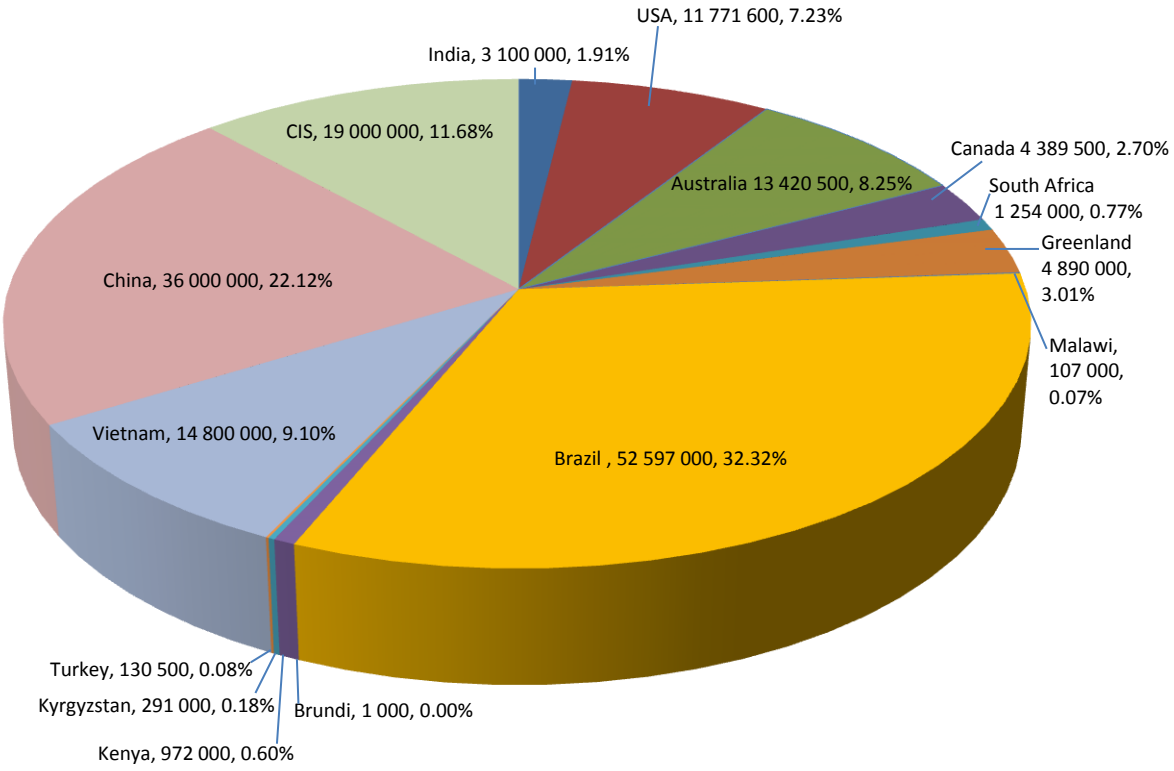
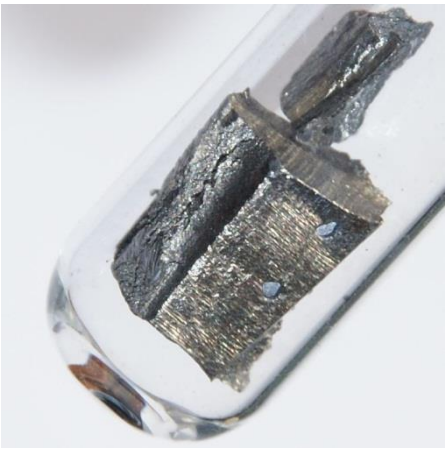


Figure 1: Rare earth deposits 2010. 32.32% Brazil, 22.12% China, 11.68% CIS, 9.11% Vietnam, 8.25% Australia, 7.23% USA, 3.01% Greenland, 2.70% Canada, 1.91%India,0.77% South Africa, 0.60%Kenya, 0.18% Kyrgyzstan, 0.08% Turkey, 0.07% Malawi (Chen, 2011) .

The application of REEs lies in the exploitation of their magnetic properties in 21st century technologies such as electronics, cars and guided missiles. The world demand for REEs is expected to increase from 136,000 tons (estimated in 2010) to 210,000 tons per year by 2015 (Humphries, 2012). The demand for them will increase significantly due to their use in popular consumer goods such as DVDs or incorporation in electronic devices such as mobile phones, computers or digital cameras. In future years hybrid vehicles are expected to increase the demand of REEs even more (Humphries, 2012). In the United States the dominant uses for REEs are mainly for petroleum refining catalysts, automobile

catalysts, medical devices (medical x-ray units) and permanent magnets as well as rechargeable batteries for electric and hybrid vehicles (Humphries, 2012). There are also major defense applications due to their magnetic properties namely guided missiles, communication and satellite systems and aircraft engines (High Pressure Turbine blades) (Majcher, 2011, Humphries, 2012), which makes REEs highly strategic. Furthermore, various electronic and electrical components are using permanent magnets which mainly contain gadolinium, dysprosium, terbium and neodymium (Humphries, 2012).



Neodymium (Nd) is the 4th element in the series of rare earths with atomic number 60. It is a soft silver-yellow metal which is quite malleable (Krebs, 2006).

Figure 2: Figure of rare earth element Nd taken from http://images-of-elements.com/ (March 2013)

Nd was discovered in 1885 by the Austrian chemist Carl Auer Baron van Welsbach who managed to separate the common rare-earth didymium into praseodymium (green didymium) and neodymium (new didymium). Nd is present in significant quantities in the monazite, bastnäsite and allanite ores, (monazite is its main ore); the former primarily consists of Y, Nd, Th, La, Ce as well as small quantities of other REEs. Neodymium is usually extracted from its ores by using sulfuric acid and heat and from the other rare earths by using an ion-exchange method (Krebs, 2006). Although it belongs to the rare earth metals, neodymium is not rare at all. It is the third most abundant of all rare earths and the 27th of all elements on earth (Krebs, 2006).

As well as being vital for the world's strongest permanent magnets (NdFeB) (Humphries, 2012), Nd is also used in sound system components such as speakers, CD-DVD drives or hard disks (Hedrick, 2004). Other applications that Nd can find are in petroleum refining, headphones, hard drives in laptops and auto catalyst and hybrid engines (Humphries, 2012). **Table 1**, shows the latest applications of REEs and their prices according to 2013 data. Prices given for US $/kg.

A) LREEs	Application	Price
Lanthanum (La)[c]	1) Alloys-mischmetals (rechargeable lanthanum NiMH batteries used in electric and hybrid cars, creep resistant magnesium alloys, hydrogen absorption, (2) Laptop computer batteries, (3) High tech digital and video cameras, (4) Fluid cracking catalysts to convert heavy molecules into useful forms(gasoline, diesel, jet fuel), (5) Optical glass(telescopes, binoculars, high-end camera lenses, night vision goggles (containing infrared absorbing glass), fiber optics for transmission rates increase,(6) Lighter flints, phosphors-ray films, lasers,(7)Additive to produce nodular cast iron for automotive rear ends, transmissions and transfer cases,(8)Used in medicine as phosphate binder in patients with kidney abnormality.	$15,0/kg
Cerium (Ce)[c]	1) Cerium oxide is used to polish glass(television faceplates, mirrors, disc drives, silicon microprocessors, optical glass) but also used in glass making industry as a component and in decolorization process, (2)Electronic components(computer chips, transistors), (3) Metal alloys, gemstones and mischmetal,(4)Catalysts and automotive catalytic converters for the CO emission reduction,(5) In steel industries it enhances the reduction of oxides and sulfides,(6) Ceramics, provides the green part of the light spectrum in fluorescent bulbs.	$15,0/kg
Praseodymium (Pr)[c]	1) Primarily when combined with neodymium provides NdFeB world's strongest magnets, improved magnet corrosion resistance, high strength metals,(2)Pigments: It's salts give a vibrant yellow color in ceramics, glass, plastic,(3)It's oxides protect again UV light and yellow flare(used for welder and glass blower goggles production),(4)Photographic filters, airport signal lenses, rechargeable batteries.	$77,0/kg
Neodymium (Nd)[d,c]	1) Manufacture of strongest magnets in the world (NdFeB) for hard drives in laptops, communication devices (headphones), hyrbrid engines, (2)Petroleum refinery and catalysts, (3)Rechargeable batteries, (4) Laser range finders, guidance systems,(5)Glass and ceramic pigments.	$98,0/kg
Samarium (Sm)[c]	1) High temperature «Samarium-Cobalt» permanent magnets, (2) X-Ray lasers, medical uses,(3) Mischmetal, biofuel catalysts, optical glass, nuclear application in reactor control rods,(4)Guided weapons of high accuracy, as well as in developing 'white noise'stealth technology.	$40,0/kg
Europium (Eu)[d,c],	1) Used in phosphors in pilot display screens (televisions, computer screens-Liquid Crystal Displays-for reddish-orange colors), energy efficient fluorescent bulbs and lamps (for blue, reddish-orange colors), (2) Glass additives, (3)Nuclear technology (neutron absorbents).	$2,050.0/kg

B) HREEs	Application	Price
Gadolinium (Gd)[c]	1) Primarily as Magnetic Resonance imaging agent to enhance the clarity of MRI scans through intravenously administration of gadolinium contrasting agents in patients,(2)Gadolinium-Yttrium garnets for microwave applications,(3)Phosphors, glass additives and ceramics,(4) Nuclear application(rods to control fission process due to highest ability to capture thermal neutrons).	$210,0/kg
Terbium (Tb)[d,c]	1) Phosphors for lighting and display in fluorescent bulbs and color TV tubes(yellow-green). Also used in X-Ray intensifying screens and projections televisions as a green emitter of high intensity yellow-green-violet-blue), (2)Permanent magnets in guidance and control systems, targeting and weapon systems, fuel cells(crystal stabilizer).	$2,100.0/kg
Dysprosium (Dy)[d,c]	1) Primarily for the production of NdFeB magnets used in guidance control systems and electric motors, (2)Nuclear applications(neutron absorbents control rods in nuclear reactors), (3) lasers, phosphors, catalysts, ceramics, hybrid engines, (4) Dysprosium-Cadmium chalcogenides are sources of infrared radiation. Also used in radiation badges for radiation exposure detection and monitor, (5) In medicine is used in treatment of rheumatoid arthritis by injection to joints of dysprosium-165.	$1,100.0/kg
Holmium (Ho)[b]	L) Glass coloring (red and yellow pigments in glass and zirconia), (2) High power magnets, (3) Nuclear technology(control rods),(4)Medical and dental technologies, lasers.	-
Erbium (Er)[b]	1) Glassware and ceramic colorant, as an reinforcer in fiber optics, (2)Phosphors, metal alloys, (3)Lasers for medical and dental applications, (4)Nuclear technology in neutron absorbing control rods, (5)Calibration of gamma ray spectrometry.	$275,0/kg
Thulium (Tm)[b]	1) High power magnets, medical X-Ray units, phosphors, lasers, dental technology.	-
Ytterbium (Yb)[b]	1) Laser, steel alloys, (2) Doping of optical materials- Fiber optic technology, (3) Radiation source substitute for X-Ray units,(4)Stress gauges, solar cells.	-
Lutetium (Lu)[b]	1) Catalyst in petroleum refinery (hydrogenation and polymerization process), (2) Single crystal scintillator detector,(3) Specialist X-Ray phosphors.	-
Yttrium (Y)[a,d]	1) Primarily as Yttrium phosphors used in energy efficient bulbs and lamps,(2)Stabilizes zirconium oxide which is used for high temperature applications, such as thermal barrier coating to protect aerospace surfaces of high temperature, (3) Metal alloys, lasers, ceramics, Yttrium-aluminum garnets (jewelry), radar technology, microwave communication for satellite industries, computer monitors, temperature sensors, (4) Catalysts for ethylene polymerization.	$68,0/kg

Table 1 A) and B): Summary of the rare earth elements and their applications (adapted from Sonich-Mulin C.2012, Humphries M.2012, Iamgold REEs, 2012, Rare Earth Elements, 2011). All data for prices were from HEFA rare earths (www.mineralprices.com) as of 26/06/2013 and it's for metal REEs, besides Eu which is for Europium oxide. Prices given for US $/kg. [a]Yttrium is not a rare earth element, but is co-located naturally and has similar properties to the heavy REEs (Henderson *et al.* 2011). [b]The element is substitutable by another REE in some applications. [c]The element is not substitutable in a critical application. [d]The element is on the 'critical list' regarding future supply and price. While some REEs (e.g. the light REEs) are less valuable than others (e.g. critical 'heavy' REEs) in practice it is not possible to 'ignore' these because the REE group elements tend to occur together in ores (bastnäsite and monazite-(Ce)), often with radioactive thorium and uranium (monazite-granitic rocks), which makes the by-products of primary sourcing and refining problematic. (Henderson *et al.* 2011).

REE prices in 2013 are shown in **Table 1**. A sudden price rise has been observed, due to recent restrictions that were made on Chinese exports and shortage of capacity elsewhere, which for the case of neodymium was from $42/kg in April 2010 to $334/kg in

July 2011, while for dysprosium from $250/kg in April 2010 to $2840/kg by July 2011 (Humphries, 2012). It has also been noted that demand for neodymium will be strong (due to permanent magnets-Humphries, 2012), namely 12-16% growth per year and therefore, technology has to be developed from the magnet industry to recycle REEs from scrap magnets (Benecki, 2007). However, according to 2013 data (Binnemans *et al*. 2013), recycling of REEs up to 2011 was less than 1%, a fact attributed to lack of motivation and technological problems as well as inefficient collections. The U.S Department of Energy (DOE) announced that the five most critical REEs are europium, dysprosium, terbium, yttrium and neodymium. In particular, the demand for neodymium and dysprosium over the next 25 years is projected to rise by 700% and 2600% correspondingly (Binnemans *et al*. 2013).

1.2 Applications of neodymium phosphate ($NdPO_4$)

Phosphates in general have been used for various applications such as ceramic materials, food additives, fuel cells, fluorescent materials, detergents, metal surface treatments etc, due their specific properties (hydrophilic, volatile at high temperature, low melting point, corrosiveness)(Onoda *et al.* 2003). In particular the synthesis of rare earth phosphates has received over the years considerable attention since they can be used for applications like powders, dense sintered materials, coatings, laser, heat resistant and ceramic materials, (Zhang *et al.* 2003, Rajesh *et al.* 2007, Sankar *et al.* 2011). They have received an increasing interest and many researchers have focused their efforts in the ceramic materials field, for the progress of thermal protection coatings, laminate composite fiber-matrix interfaces in ceramic matrix compounds (Lucas *et al.* 2003). Rare earth phosphates have also been reported for encapsulation and permanent disposal of

radioactive wastes (U(IV),Th(IV)) (Guan *et al*. 2004) as well as for applications in bioimaging (Zhang *et al.* 2010) fluorescent coatings, due to the bulk properties of lanthanum phosphates (Schuetz *et al.* 2002), as phosphors (Blasse *et al.* 1969) or as catalyst hosts (Kashiwagi *et al.* 2009). Lanthanide orthophosphates, due to their specific properties (very low solubility in water, high thermal stability, high index of refraction and for the case of Nd and Eu, high concentrations of lasing ions) they can be used for applications such as sensors, phosphors, catalysts, heat-resistant materials, ceramic materials and proton conductors (Fang *et al.* 2003).

In particular $NdPO_4$ has been found to have applications in laser physics (Zaldo *et al.* 1998, Guan *et al.* 2004,) on the photoluminescence of Nd^{3+} in potassium titanyl phosphate ($KTiOPO_4$ or KTP) crystals and in ceramic materials (Kaya *et al*. 2002, Guan *et al.* 2004). $NdPO_4$ can be very heat resistant, since its melting point is 1975^{o}C (Kaya *et al.* 2002). Due to its natural layered structure, $NdPO_4$ has also been reported to have tribological properties (Sankar *et al.* 2011). They are also used in biosensing applications (Sheng *et al.* 2009), while catalysts based on $NdPO_4$ have also been found to polymerize styrene (Wu *et al.* 2001).

More generally catalysts based on Nd have been found in the polymerization of diene, acetylene, alkylene and cyclic monomers (Wu *et al*. *2001,* Sankar *et al.* 2011), while in the presence of cerium, neodymium and gadolinium oxide catalysts, the ortho-para hydrogen conversion can be catalyzed (Komarewsky, 1957). All these applications show how interesting material $NdPO_4$ is.

1.3 Bioaccumulation-Biosorption

The environment is burdened every year with heavy metals in many ways e.g. radionuclide wastes from nuclear industries, which result in nuclide migration in the geosphere, iron-rich wastes derived from man's mining of conventional energy sources (coal wastes), outflows from disused mines, wastes from industries that use metal processes or metallic components or even chemical agents applied for a specific purpose. (Macaskie *et al.* 2010). Precious metals (PMs) also occur in substantial amounts in road dusts, due to their shedding from automotive catalytic converters, which contain PM catalysts for atmospheric protection (Macaskie *et al.* 2010).

Each year a considerable amount of industrial waste, produced by various industries, enters wastewater treatment plants (WWTPs). The produced sludge may be contaminated with toxic chemical compounds and therefore it has to be sufficiently treated so that it can be disposed of in an environmentally acceptable way. In order to reduce the number of potentially pathogenic microorganisms or the amount of xenobiotic components, a series of digestion techniques are used to treat the sludge. The final obtained product is potentially a resource rich in nutrients and could be reused for various agricultural purposes as a fertilizer. However, since dangerous heavy metals might persist in the sludge it is considered of major importance to develop eco-friendly methods for their removal (Gao *et al*. 2012).

Recent years have seen an increased demand for the REEs due to their applications in contemporary technologies (**Table 1**). Hence, their imminent shortage refocused the examination of the recovery of REEs as well as heavy metals, thorium and uranium from sludge (Gao *et al*. 2012). The distribution and concentration of heavy metals in sludge has

been widely studied, while the study of REEs in sludge has received only recent interest (Gao *et al.* 2012). Recovery and removal of heavy metals from wastes has received a lot of attention with legislative restrictions on the acceptable levels of either the sequestration level of metal in sludges or industrial runoff releases into the environment (Macaskie *et al*. 1994b).

One method proposed by Deng *et al.* (2009) and Li *et al.* (2010) was the recovery or removal of heavy metals from sludge with the use of ultrasound-assisted acid, a method that was energy consuming and could influence the physicochemical properties of the sludge during metal segregation. Another proposed method (also energy consuming to some extent) was a bioleaching and electro kinetic remediation technology (Peng *et al*. 2011) which can recover and recycle heavy metals in the sludge.

In the last 30 years the biological treatment of industrial wastes which contain heavy metals, especially at low concentrations, has received increased attention compared to conventional methods of metal removal from contaminated wastewaters (Yong *et al*. 1997). Metal removal can take place via coordination, complexation and chelation mechanisms, inorganic microprecipitation or ion exchange adsorption (e.g. Tsezos *et al*. 1981, Kurek *et al*. 1982, Friis *et al.* 1986, Mullen *et al*. 1989, Nourbakhsh *et al*. 1996, Yong *et al*. 1997, Gadd *et al*. 2004) and via enzymatically-assisted mechanisms. The latter is the focus of this study. Metal-accumulation bioprocesses fall into three major categories:

a) Biosorption using non-living, non-growing biomass

b) Bioaccumulation by using live cells

c) Enzymatically-assisted biomineralization

The latter is an example of an intermediate process whereby cells are dead, but robust enzymatic activity can still persist independently of metabolic processes. The use of a single enzymatic step facilitates making a mathematical model when using a non-living biomass since additional complexities are faced when the immobilized cells are live especially when immobilized (Macaskie, 1990, Macaskie *et al*. 2010).

Generally, for toxic or aggressive wastes, non-living biomass is preferred for metal biosorption (Macaskie *et al*. 1997). In 1982 a pioneering study noted high biosorption uptake of thorium from aqueous solutions by using inactive mycelia of *Rhizopus arrhizus* (Tsezos *et al*. 1982). A kinetic mass transfer model for the recovery of metal ions from aqueous solutions using the same inactive cells was developed in 1988 by Tsezos *et al*. and the reaction of the biosorption system in alterations to significant parameters that would disturb the model results, was also examined (Tsezos *et al*. 1992). The engineering parameters of this biomass system have also been previously investigated (Tsezos *et al*. 1990).

Biosorptive heavy metal removal using various species of microorganisms has received much attention for the sequestration of heavy metals from aqueous wastes. Their ability to retain and concentrate heavy metals from liquids relates to their profuse functional groups which can bind metallic elements i.e. hydroxyl, carboxyl, phosphate and sulfhydryl groups. Heavy metal uptake follows a specific mechanism for different metals for each organism and is also influenced by solution parameters such as pH, the presence of complexing ligands and co-ions in the inflow solution (Yong *et al*. 1998, Volesky *et al.* 1995). Ultimately, biosorption is a purely chemical process and the metal loading capacity is dependent on the equilibrium constant of the metal-ligand complex. The biosorptive capacity (typically a few percent of the biomass dry weight) is too low to be economically

useful unless sorbed metal is removed from the equilibrium by further removal into a stable biomineral (Volesky, 1995, Liebenberg *et al*. 2013).

An extensive study has been made of *Serratia* sp. N14, due to its ability to remove heavy metals from wastes. Heavy metal recovery by this organism can take place efficiently, rapidly and can become economically viable for industrial scale purposes (Yong *et al*. 1995 a). It was demonstrated and accurately modeled to take into account the pH and excess SO_4^{2-} ion, using an acidic uranium mine wastewater (Macaskie *et al*. 1997).

1.4 The choice of *Serratia* sp. N14

Bacteria in general are small organisms consisting of usually one cell and their size varies from 1 to few micrometers in length. Bacteria are divided into Gram positive and Gram negative types according to the composition of the outer wall layers.

The Gram negative family of the Bacteria known as Enterobacteriaceae have a rod-like structure and of size between 1-5 micrometers. They are facultative anaerobes having the ability to grow in both anaerobic and aerobic environments. The ubiquitous *Serratia* genus can be found in various sites from plants and soils to the intestines of mammals, depending on the species and genus. *S. marcescens* a common species, can cause hospital infections (Bagheriasl, 2009). *Serratia* sp. N14 is a non-pathogenic strain, which was isolated from soil polluted with lead (Hambling *et al*. 1987) and was described in early literature as *Citrobacter* on the basis of biochemical typing in the pre-molecular biology era. The best conditions for *Serratia* sp. N14 growth are at temperature 30^{o}C and at pH 7.0 (Hambling *et al*. 1987). This specific strain has been found to produce three characteristic enzymes including lipase, gelatinase and deoxyribonuclease (DNAse) (which probably reflect its saprophytic origins) (Hoff G.L. 1984, Lugg *et al*. 2008, Bagheriasl, 2009) as well as an atypical phosphatase, which forms the focus of this study. High molecular

weight substances known as Extracellular Polymeric Substances (EPS), primarily consisting of polysaccharides, have been found to be secreted by the cells and cover their surface. These compounds stay in the outer surface of the cell, (exposed to the environment) and are of major importance in cell adhesion to surfaces, together with adhesive pili (Allan *et al*. 2002), in the formation of a highly cohesive biofilm (Allan *et al.* 2002, Bagheriasl, 2009).

Cells of this bacterium were immobilized as biofilm on a porous matrix and a process for the removal from solution of Cd^{2+} initially (Macaskie *et al*. 1984) and then for various other metals including uranium (Yong *et al*. 1996) and REEs was shown (Singh, 2012). This bioprocess for metal uptake using *Serratia* sp. N14, combines the advantages of bioprocesses (a) and (b) mentioned above in sections 1.3, with none of their disadvantages (Macaskie, 1990*),* such as; a) facing difficulties of producing a mathematical modeling of a system which is non-defined; b) factoring-in toxicity, where a toxic metal could appear at low concentrations (but in this case metal-resistant strains are used); c) growth nutrients are not required, since there is no biomass growth; d) there is no need for unconsumed growth nutrients or metabolic products, which in many cases form complexes with metals and e) there is no need for the metal challenge to be done under permissive conditions (Macaskie, 1990).

1.5 Mechanism of metal cation uptake by *Serratia* sp. N14

The mechanism of metal uptake which is the focus of this study, is that this *Serratia* sp. N14 atypically produces an acid type, heavy metal-resistant phosphatase, which is homologous to the acid phosphatase PhoN characteristic of pathogenic enterobacteria such as *Salmonella typhimurium* (Macaskie *et al*. 1994a, Kier *et al.* 1979, Bonthrone *et al*. 1996); the *phoN* gene was probably acquired via lateral gene transfer in the environment (Macaskie *et al*. 1994a, Mennan, 2010) and was characterized as atypical (Hambling *et al*. 1987). PhoN mediates the hydrolysis of an organic molecule, e.g. glycerol-2-phosphate (G2P), releasing phosphate (HPO_4^{2-}) and therefore a metal cation (M^{2+}) precipitates on the surface of the bacteria as $MHPO_4$ (Macaskie *et al*. 1992a, Jeong *et al*. 1998, Paterson-Beedle *et al*. 2010). A schematic representation of the metal deposition mechanism of *Serratia* sp. N14 cells can be seen in **Figure 3.** The PhoN phosphatase was localized within the periplasmic space (between the two membranes which bound the Gram negative cell) as well as 'tethered' within the EPS, as visualised via immunogold labelling (Jeong *et al*. 1997).

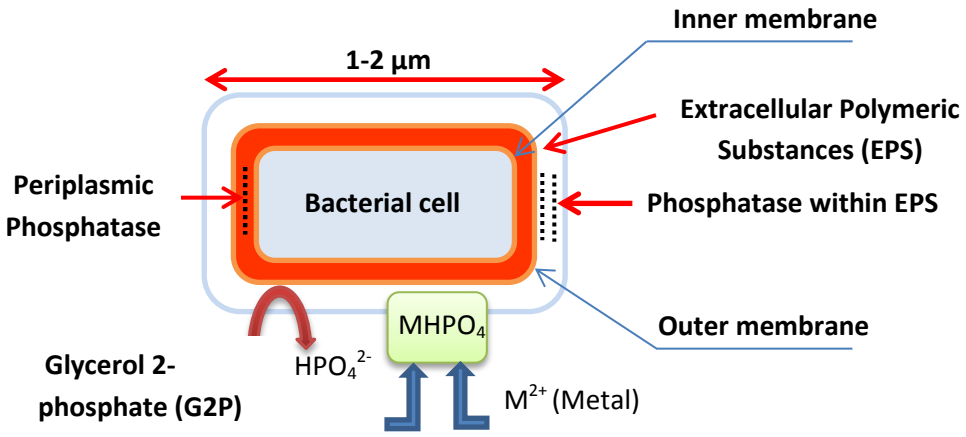


Figure 3. Schematic representation of metal phosphate precipitation around the cell surface as $MHPO_4$. Figure reproduced from Deplanche *et al.* (2011).

During the phosphohydrolytic cleavage of G2P, the phosphate ligand is the product of the reaction. The component reactions occurring during this biocrystallization procedure are the following:

Glycerol-2-phosphate $\xrightarrow[\text{(enzyme)}]{\text{Phosphatase}}$ Glycerol +Phosphate **(1)**

Phosphate + Metal ion $\xrightarrow{\text{Ksp}}$ Metal Phosphate precipitate **(2)**

Where K_{sp} is the conditional solubility product of the metal phosphate, which remains constant for a specific free metal – carrier ionic matrix. The free (available) metal ion concentration in the inflow solution is also influenced by the complexing ligands incorporated in the solution, as well as their concentration (Yong *et al*. 1998). In the case of citrate buffer used in this study (to suppress metal precipitation in solution-Bonthrone *et al.* 1996 and metal hydrolysis –Yong *et al.* 1999) :

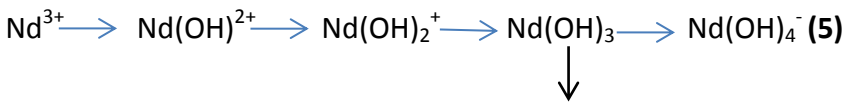
Citrate+Metal ion $\underset{}{\overset{K_1}{\rightleftharpoons}}$ Citrate-Metal **(3)**

Where K_1 is the metal-citrate complex stability constant, which along with K_{sp} are both metal dependent (Yong *et al*. 1998). Hence the ''ease'' by which a metal is precipitated depends on K_1, but the K_{sp} can be exceeded in the presence of additional phosphate, and hence the specific activity of the PhoN phosphatase, which may be dependent on the growth conditions and biofilm age. In the present study:

K_{sp}=[metal][phosphate] i.e for the case studied $NdPO_{4(s)} \longrightarrow Nd^{3+}{}_{(aq)} + PO_4^{3-}{}_{(aq),}$ **(4)** and

$K_{sp} = [Nd^{3+}][PO_4^{3-}]$, so for cases of low free metal K_{sp} remains constant by increasing the concentration of phosphate, i.e a higher enzyme activity.

Other reactions that would take place in the absence of citrate are metal hydrolysis such as:



(5)

Neodymium hydroxide forms at approximately pH 6.5 (Chirkst *et al.* 2010). Once the colloidal insoluble metal hydroxide forms (Gregory *et al*. 2001), it blocks the columns and is not precipitated as phosphate. Hydrolysis is suppressed by metal binding to citrate (citrate buffer solution complexes with lanthanide ions-Akinremi *et al.* 2013), so for this reason citrate is always put in the columns to keep the metal soluble (Bonthrone *et al*. 1996) and pH 5.5 is used to reduce the tendency to hydrolyze, since hydrolysis of the lanthanum ions can be avoided by increasing the acidic conditions of the solution (Sturza *et al.* 2008).

Phosphate release by enzymatic catalysis depends on the pH of the inflow solution (phosphatase activity is high for pH ranged 5.0-7.0) (Tolley *et al*. 1995, Yong *et al.* 1995b), but under acidic conditions the solubility of the precipitate could increase. The phosphate release from G2P by *Serratia* sp. N14 for various values of pH, has showed optimum performance at ~ pH 5.5, but was not different within experimental error between pH 5.0-6.5 (Yong *et al.* 1995b).

In general phosphate is a tribasic ion and in an aqueous solution the equilibria present are the following:

$H_3PO_4 \longrightarrow H_2PO_4^- + H^+ \quad K_A=7.5\ 10^{-3}$ **(6)**

$H_2PO_4^- \longrightarrow HPO_4^{2-} + H^+ \quad K_B=6.2\ 10^{-8}$ **(7)**

$HPO_4^{2-} \longrightarrow PO_4^{3-} + H^+ \quad K_C= 5.0\ 10^{-13}$ **(8)**

For pH values below 4.6 the predominant form of phosphate in an aqueous solution will be as shown in equation 6, for pH values between 4.6-9.7 the as shown in eq.7 and for pH values above 9.7 the predominant form of phosphate as in eq.8. Therefore when reducing the pH of the solution the equilibrium will move from (8) to (6) and at high acidic conditions protons and cationic ligands will ''compete'' for existing phosphate in the solution. At pH values between 5.5-5.0 the forms of phosphate will be $H_2PO_4^-$ and HPO_4^{2-}, while for pH values of the inflow solution 4.5 and below, phosphate will be as H_3PO_4 and $H_2PO_4^-$; hence the concentration of HPO_4^{2-} will also be less, a fact that will retard the metal phosphate formation, even if there was an increase of phosphate production by the bacteria (Yong *et al.* 1995b).

The metal removal process studied in this project, takes place in a flow-through column reactor, where the immobilized cells are supplied with neodymium challenge solution. Within the column a steady state removal of the metal takes place at a fixed flow rate through the phosphate ligand which is being liberated and which precipitates and crystallizes the metal constantly, on the cell surface. The actual metal removal at a given flow rate is dependent on the phosphatase activity in addition to the various chemical factors mentioned earlier, which are superimposed upon an applied enzyme kinetics

model to accurately describe column performance (Macaskie *et al*. 1995a), assuming plug-flow behavior, which has been confirmed using magnetic resonance imaging (Beauregard *et al*. 2010).

1.6 *Serratia* sp. N14 Phosphatase

Generally phosphatases are phosphoesterases that act as catalysts in the hydrolysis of the linkage carbon-oxygen-phosphorus of various phosphate esters. They appear in many types of microorganisms as well as various types of animal and plant tissues. Phosphatases can either be acid or alkaline, depending on their optimum pH. Little is known with regards to the function of the bacterial acid phosphatase *per se* (Mennan, 2010). Production of the PhoN phosphatase is regulated via the PhoP/PhoQ region which is being responsible for some bacterial stress responses (Jeong *et al*. 1999), so it is possible that the enzyme generates phosphate buffer locally in response to a falling pH. In the case of *Serratia* sp. N14 the enzyme has the ability to promote accumulation of metal around the cell, whereby it can confer metal resistance by metal phosphate precipitation away from cellular sensitive sites. It is believed that this enzyme is exported from the inner part of the cell, and coexists out of the cell within the EPS (Mennan, 2010), as shown by immunogold labeling (Jeong *et al*. 1997). Here it cleaves a phosphate group from a substrate source (Mennan, 2010) to form a mineral ''coat'' around the cells.

According to previous studies the acid phosphatase (PhoN) overproduced by *Serratia* sp. N14 consists of two isoforms which were designated as CPI and CPII i.e (CP: *Citrobacter* Phosphatase I & II, (Jeong *et al*. 1992, Jeong *et al*. 1995), but are also known in recent literature as SPI and SPII (from *Serratia* phosphatase-Mennan, 2010). These isoenzymes were designated as such, since they are forms of the same enzyme and are used to

enhance the catalysis of the same chemical reaction, but they have very slightly different amino-acid compositions (Jeong, 1992, Mennan, 2010) as well as other properties (such as kinetic) (Mennan, 2010). Although these isoforms have similar structural and physical properties they were found to be different with respect to their binding properties to cation and anion exchange resins (Jeong *et al*. 1996). CPI (optimum pH 5.75) was preferable for enzyme robustness and stability as well as having a higher resistance in heavy metals, while CPII (optimum pH 6.25) worked more favorably under wider ranging pH conditions (Jeong *et al*. 1998, Mennan, 2010). Furthermore, it was observed that as the pH of growth fell this resulted in loss in phosphatase activity (Tolley *et al*. 1995, Jeong *et al*. 1999). A similar pattern regarding the binding behavior of two acid phosphatases to ion exchange resins, was noted by Kier *et al.* (1977) for *Salmonella typhimurium* (Kier *et al.* 1977, Jeong *et al*. 1998). In 2012 Singh, (2012) confirmed a decrease of the total phosphate liberated and the metal recovered when lowering the pH of a column inflow solution progressively, but that only ~80% of the activity was restored upon return to a favorable pH. This could be attributed to the scenario of the two acid phosphatase isoenzymes described by Jeong (2002). According to this scenario the total enzyme activity is represented as the sum of the activity of the two individual isoenzymes CPI and CPII. It can be hypothesized that at low pH values both of the isoenzymes are influenced (i.e reduced function at lower pH), but only one recovers completely, when resetting the pH to 5.5. However, this was not tested.

1.7 Theoretical model of metal removal in a packed bed system reactor

Previous work (Macaskie *et al*. 1995a) developed and validated a model for the enzymatic reactions which are taking place along with the Michaelis-Menten kinetics (Housecroft *et al*. 2010):

$$E+S \underset{K_2}{\overset{K_1}{\rightleftarrows}} ES \xrightarrow{K_3} E+P \xrightarrow[M]{K_4} MP \quad \textbf{(9)}$$

Where: E: Enzyme(dimensionless), S:Substrate (dimensionless), ES: the Enzyme-Substrate complex (dimensionless), P:Product (dimensionless), M:Metal (dimensionless), MP: the metal phosphate precipitate (dimensionless), K_1,K_2,K_3 the internal kinetic constants of the whole cell phosphatase enzyme(dimensionless). K_4 is an additional kinetic constant for the formation of metal precipitate (dimensionless). This is a ''lumped'' constant taking into account the solution and desolubilization chemistry of a metal in its carrier solution and as such is conditions-specific. In this system the conversion of S to P catalyzed by E is described by the Michaelis-Menten equation (Housecroft *et al*. 2010).

$$-\frac{dS}{dt} = E_oK_3/\left(1+\frac{Km}{S}\right) \quad (1)$$

where: E_o is the total enzyme inside the column (dimensionless), K_m: Michaelis constant (mM), ***S***: the residual substrate concentration in the exit of the column (mM). A modified integrated form of this equation, which describes the metal removal in an immobilized column is the one that follows:

$zY\boldsymbol{S_o}-K_m\ln z(1-zY) = \frac{EoK3}{F}$ (2), where $Y= 1-\frac{\boldsymbol{M}}{\boldsymbol{Mo}}$ (2a) and $X= 1-\frac{\boldsymbol{S}}{\boldsymbol{So}}$ (2b) and

z is the proportionality factor of the fraction: $z= \left(1-\frac{\boldsymbol{S}}{\boldsymbol{So}}\right)/\left(1-\frac{\boldsymbol{M}}{\boldsymbol{Mo}}\right)$ (2c)

F is the flow rate within the column and t is the fluid residence time in the column (t=V/F), where V is the volume occupied by the solid materials in the volume (mL), $\boldsymbol{S_o}$ is

the substrate concentration of the inflow solution (5mM), ***M*** is the metal concentration in the outflow solution and ***Mo*** the metal concentration in the inflow solution. The proportionality factor z describes the excess of phosphate required to achieve metal removal, under a given set of solution conditions (pH, other flow components etc). This integrated form of the Michaelis-Menten equation was developed to describe product release in a flow through column (Fulbrook 1983, cited by Macaskie *et al*. 1995a) was introduced in 1995 by Macaskie *et al.* (Macaskie *et al*. 1995 a). It was further developed to describe metal removal.

Considering V=1 and that z remains constant in the column ($\frac{dz}{dt} = 0$), since phosphate and metal are removed together as 1:1 (Singh,2012)), we get the following equation after differentiating (2c):

$\frac{\mathrm{dX}}{\mathrm{dt}} = \mathrm{z}\frac{\mathrm{dY}}{\mathrm{dt}}$ or $-\frac{1}{\mathrm{So}}\frac{\mathrm{dS}}{\mathrm{dt}} = \mathrm{z}\left(-\frac{1}{\mathrm{Mo}}\frac{\mathrm{dM}}{\mathrm{dt}}\right)$

from (2a): $\frac{\mathrm{dY}}{\mathrm{dt}} = \left(-\frac{1}{\mathrm{Mo}}\right)\frac{\mathrm{dM}}{\mathrm{dt}} = \left(-\frac{1}{\mathrm{zSo}}\right)\frac{\mathrm{dS}}{\mathrm{dt}}$ (2d)

but from (1) : $-\frac{\mathrm{dS}}{\mathrm{dt}} = \mathrm{E_oK_3}/\left(1+\frac{\mathrm{Km}}{\mathrm{S}}\right)$

$\Rightarrow \frac{\mathrm{dY}}{\mathrm{dt}} = \mathrm{E_oK_3}/[\mathrm{z}\mathbf{S_o}(1+\frac{\mathrm{Km}}{\mathrm{S}})]$ (2e)

From (2e) substituting that $\frac{\mathrm{S}}{\mathrm{So}} = 1 - \mathrm{zY}$ we have:

$\frac{\mathrm{dY}}{\mathrm{dt}} = \mathrm{E_oK_3}/[\mathrm{z}\mathbf{S_o}[1+\mathrm{K_m}/\mathbf{S_o}(1-\mathrm{zY})]]$,

which after integration we get equation 2, which is the integrated form of the Michaelis-Menten equation for a system consisting of 1 mM metal solution, 2 mM citrate buffer and 5 mM G2P such as ours (Macaskie *et al*. 1995a).

From this equation the apparent K_m constant can be calculated (by trial and error method since both, K_3 and K_m are unknown), which is a measure of the affinity of the enzyme in the column, to a degree a change of the pH has an effect on the apparent K_m, (substrate-dependent) (Tolley *et al*. 1995). That will occur, since the enzyme will carry a pH-dependent surface charge influencing interactions with charged substrates, while the substrate will also become protonated at low pH. The K_m for a particular E and S is constant (Tolley *et al*. 1995). The affinity between them is independent of time and flow rate as well as of substrate concentration. However, anything that modifies a substrate (for example if G2P formed a complex with the metal) would effectively change the chemical nature of the substrate. Also, pH could also have that effect as at low pH:

$G2P + H^+ \overset{pka}{\rightleftharpoons}$ G2P protonated **(10).**

As the negative charges get neutralized by adding protons the K_m will also change. Functional groups on the enzyme (amines, carboxyls), will also become protonated. The reason why the flow rate is important is because the chemical speciation above takes time to establish equilibrium, as do the other chemical reactions. At high speeds the flow may pass the enzyme before a new equilibrium is achieved. The exact chemical situation will be different at each place in the column.

In the case of the system used in this project the total phosphate release was given from the equation $X=\frac{S}{So}$ so therefore substituting this form to the integrated form of Michaelis-Menten equation (2) we have(for the same $Y=(1-\frac{M}{Mo})$:

$\frac{dX}{dt} = z\frac{dY}{dt}$ or $-\frac{1}{So}\frac{dS}{dt} = z\left(-\frac{1}{Mo}\frac{dM}{dt}\right)$

from (2a): $\frac{dY}{dt} = \left(-\frac{1}{Mo}\right)\frac{dM}{dt} = \left(\frac{1}{zSo}\right)\frac{dS}{dt}$

and since from (1): $-\frac{dS}{dt} = E_oK_3/\left(1+\frac{Km}{S}\right)$

$\Rightarrow \frac{dY}{dt} = \left(-\frac{1}{zSo}\right)E_oK_3/\left(1+\frac{Km}{S}\right)$

and since we have for our system $\frac{S}{So} = zY$ we get the differential equation:

$\frac{dY}{dt} = \left(-\frac{1}{zSo}\right)E_oK_3/\left(1+\frac{Km}{So\ zY}\right)$ or $-zSo\left(1+\frac{Km}{So\ zY}\right)dY = E_oK_3dt$ or

$-zS_odY - \frac{Km}{Y}dY = E_oK_3dt$ and after integration the new integrated form received is:

$zYS_o + K_m lnY = -E_oK_3t$ and for $t = \frac{1}{F}$ we get:

$$zYS_o + K_m lnY = -E_oK_3/F \quad (3)$$

This equation describes the metal removal in the immobilized column as used in this study. As a working system the shape of the flow rate activity relationship forms a shallow sigmoidal curve on a plot of metal removal activity versus ln(flow rate).

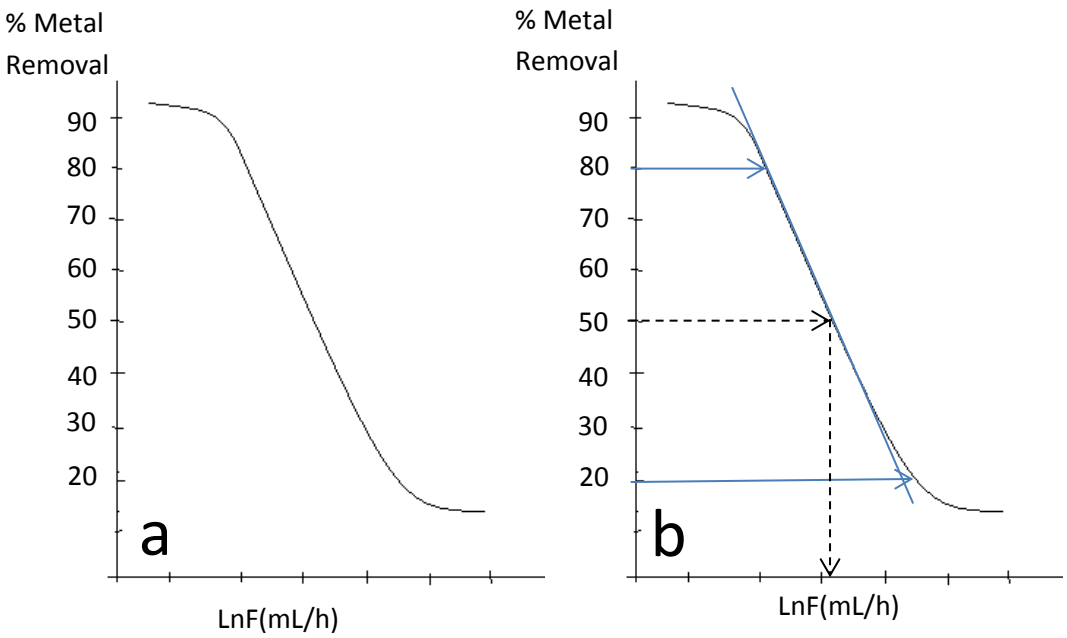


Figure 4: a) Shallow sigmoidal curve obtained for metal recovery when plotting metal removal activity versus ln(flow rate), **b)** Part of the curve between 20-80% metal removal is within experimental error of a straight line (correlation coefficient is typically>0.9) and as such, the flow rate giving half maximum activity can be measured.

For simplicity, since the deviation from a straight line for the part of the curve between approximately 20% to 80% metal removal is well within experimental error, a straight line can be drawn (Macaskie *et al.* 1995a, Roig *et al.* 1995, Tolley *et al.* 1995, Yong *et al.* 1995b, Yong *et al.* 1996, Finlay *et al*. 1999) and hence best fit lines can be used. Next, by determining that flow rate giving 50% removal of metal a definition of column activity is obtained ($FA_{1/2}$) that encompasses all of the enzyme, kinetic and chemical factors within the column as a single parameter with which to compare different columns and conditions. This is defined as the flow rate (mL/h) at which 50% of the column inflow metal solution is removed (Yong *et al*. 1997) or the flow rate which gives 50% of cleavage of the substrate into final product for metal-unsupplemented columns (Macaskie *et al*. 1995 b). Note that these are not necessarily the same, since full substrate cleavage is neither assumed, neither is full incorporation of phosphate into $NdPO_4$ precipitate. Formation of $NdPO_4$ (1:1 ratio of Nd:P) was confirmed by Singh (2012). Also since the $FA_{1/2}$ value is an observed 'lumped' parameter no information is conveyed about whether a

change in this value is attributable to enzymatic, solution chemistry or metal desolubilization factors, or components of all three.

Phosphate precipitation reactions such as the ones examined in this study visualized as the $FA_{1/2}$ value, may be influenced by various interrelated physicochemical factors such as pH, metal complexing agents, ionic matrix composition and also substrate concentration. The speciation of heavy metal cations in solution can be modified by common anions such as chloride, nitrate and sulfate (Yong *et al*. 1999), by complex formation (or inhibiting the release of phosphate by the cells at high concentration of these anions), reducing therefore the production of the metal precipitate and resulting in a slower flow rate to give $FA_{1/2}$. The effects of excess NO_3^- and SO_4^{2-} were modelled precisely using the above system (Yong *et al.* 1997, Yong *et al.* 1999), where it can be shown to what extent the original $FA_{1/2}$ can be restored by slowing the flow rate i.e an invaluable tool for process design as it enables calculation of the column volume required to treat a given target flow.

In industrial treatments of solid waste, nitric acid (or *aqua regia*) is used in metal dissolution (Yong *et al*. 1999, Deplanche *et al*. 2012). The acids can be neutralized with NaOH, but the high concentration of anions persists. Removal of this by ion-exchange is too costly for large scale use. According to previous tests, both chloride and nitrate were inhibitory to phosphatase activity. For example previous studies in the presence of nitrate ion at a concentration 0.3 mol/L noted a decrease in phosphatase activity of approximately 15-20% at a fixed flow rate (Yong *et al*. 1995 b). In addition, it was reported that chloride inhibition was attributed to its concentration (Smyth *et al*. 1992). Sulfate inhibition was overcome by simply slowing the flow rate and the amount of this slowing

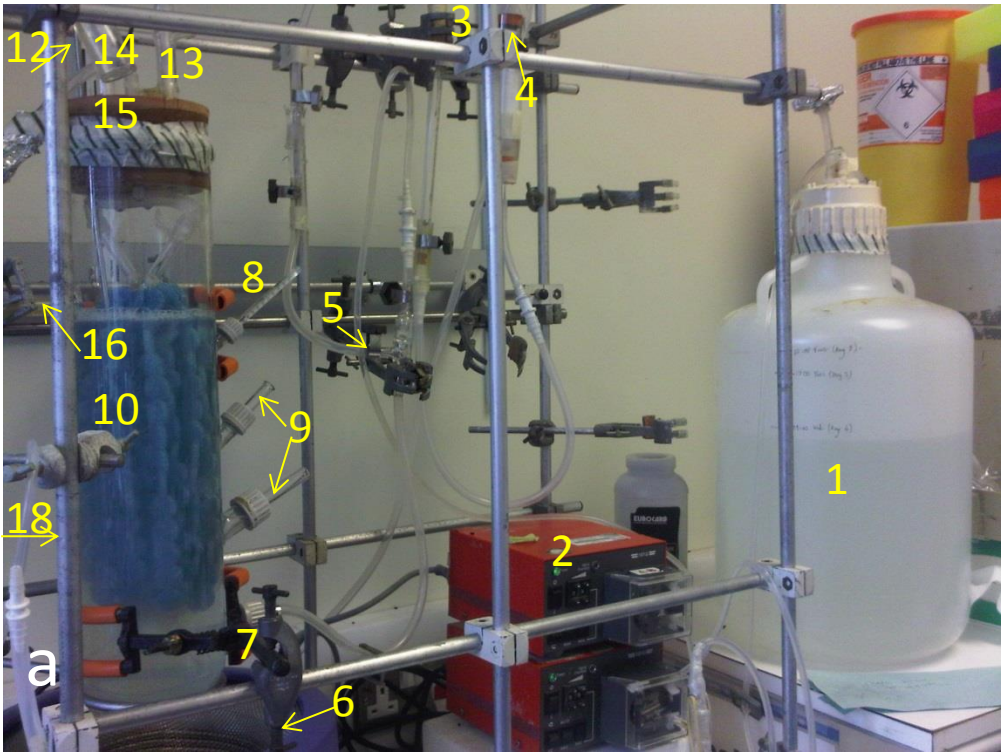
was informed by application of the model described above (Yong *et al.* 1999). The effects of combined anions (e.g NO_3^- +Cl^-) has not been tested.

1.8 Immobilizing the biomass for application in metal removal in flow through bioreactors

During a continuous industrial process, the microbial biomass has to preserve its ability to recover metals throughout the process without loss. Hence, the use of appropriate immobilization techniques offers advantages such as minimum blocking in continuous flow systems, better reusability and higher biomass loading (Holan *et al.* 1994, Das *et al.* 2008), as well as easy separation of biomass and product.

Depending on the uptake capacity, kinetics, physical characteristics or the process mechanism of the biosorbents, various types of bioreactors have been proposed for continuous and batch mode. Typical reactors were proposed in the past such as: a) continuous flow or batch stirred tank reactors, b) pulsating, fixed packed or fluidized bed contactors and c) multiple bed contact arrangements (Das *et al.* 2008). A very simple system is an immobilized cell packed bed flow through reactor, which was adopted in this work. In order to immobilize *Serratia sp.* N14 as a self-adhered biofilm ready for subsequent use in a packed bed column, an air lift fermentor was used as a chemostat (D=0.1) for simultaneous production of *Serratia sp.* N14 planktonic cells (which can be immobilized by chemical coupling techniques) (Finlay *et al*. 1999) and self-immobilization of biomass (biofilm) (Paterson-Beedle, Macaskie 2005, Finlay *et al*. 1999). The adhesion and cohesion strength of biofilm is high (Paterson-Beedle *et al.* 2005, Myszka *et al*. 2012) and resistance of the biofilm to detachment in use (causing column blocking) was discounted using magnetic resonance imaging (Graf von der Schulenburg D.A. *et al*. 2007,

Beauregard *et al*. 2010). This fermentor was used to produce the biofilm used in the current project (**Figure 5a**). However; this approach is not applicable to a large scale manufacturing process. The turnaround time for the fermentor is ~2 weeks. A modified system was used previously (P. Clarke G. Sharma, personal communication L.E. Macaskie) whereby biofilms were generated using the main vessel run-off in an ''off-chamber''. Hence batches of immobilized cells can be made using cells continuously generated using the main vessel chemostat. However, 'exit' cells are nutrient-depleted since the chemostat is operated under carbon-restriction to upregulate PhoN activity and adhesive pili production (Allan *et al*. 2002). Biofilm made in this way was used to remove Mn^{2+}as $MnHPO_4$ (P. Clarke and G. Sharma, personal communication to L.E. Macaskie), but the efficacy of the 'main chamber' and 'off chamber' biofilm was not compared. The outflow chambers (2) can be seen in **Figure 5c)**.



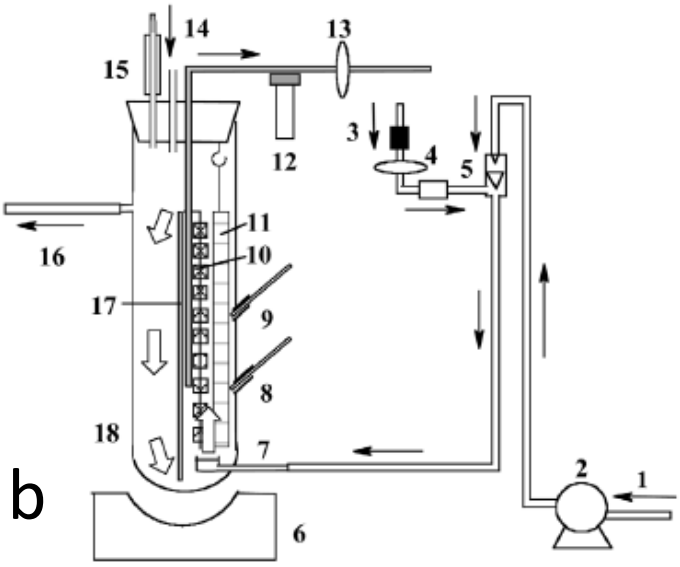


Figure 5: a)and **b)** Air-lift fermentor (main vessel) for simultaneous production of *Serratia* sp. N14 cells and biofilm. (1) Sterile medium, (2) peristaltic pump, (3) air inflow, (4) charcoal filter, air filter, (5) air and medium mixing chamber, (6) heating mantle for steady temperature (30^{o}C -Main chamber was held at 30^{o}C, while off chambers were both run at room temperature 22^{o}C. Therefore enzyme activity will be less at 22^{o}C than at 30^{o}C), (7) double sparger for inlet port and air providing and distribution, (8) thermometer port, (9) extra port, (10) strands of polyurethane foam discs, *(11) cage with ceramic Raschig rings-omitted in 2013's run, since this is an optional cage for being able to evaluate alternative supports such as glass or plastic discs* (Paterson-Beedle,2005), (12) vessel for collecting test samples, (13) filter for air, (14) port for culture entry, (15) air filter exit, (16) outflow port, which will be the inlet for outflow chambers, (17) glass central partition *(*18)

fermentor body (main vessel).(Picture b and description taken by Patterson-Beedle and Macaskie 2005).**c)** Outflow chamber compartments for simultaneous production of biofilm. In the above figures one can see the main vessel used in the project ran in May 2013 by Dr. Angela Murray and Sarah Singh (**5a**), which is being compared with the one proposed by Paterson-Beedle M. and Macaskie L.E. in 2005 (**5b**).

1.9 Preliminary work using immobilized biofilm of *Serratia* sp. N14 for REE recovery, using flow-through column reactor

Biomass produced and immobilized on foam discs was used to recover neodymium and europium from flows supplemented with Nd^{3+} or Eu^{3+} (as nitrate-Deplanche *et al*. 2011). Here, the immobilized cells were challenged with 1mM neodymium solution, 2 mM citrate buffer and 5 mM glycerol-2-phosphate at pH 5.5 and a slow flow rate (5 mL/h). The metal removal was found to be approximately 85% for the case of Eu and more than 90% for the case of Nd. A control reactor was also used, which contained heat treated biofilm and this showed that enzyme activity had to be present in order to be able to recover metal, since little metal was removed in this case (Deplance *et al*. 2011).

In 2012 a previous researcher (Singh, 2012) examined immobilized biofilm 3 months after being harvested for Nd and Eu recovery at different flow rates and for decreasing pH values. The results of that study (Singh, 2012) showed that, in general the *Serratia* sp. N14 PhoN phosphatase is a robust enzyme that has the ability to remove Nd^{3+} and Eu^{3+} even at high flow rates and under acidic conditions down to pH 3.5. A reduced recovery at pH 4.0 or 3.5 was primarily attributed to the chemistry of the test solution rather than to the enzymatic activity in acidic conditions; pH values less than 3.5 inactivated the enzyme. The identity of the crystalline component of the accumulated material was confirmed to be $NdPO_4$ using X-Ray powder diffraction (Singh, 2012). However, the earlier study was done using a single biofilm preparation only and for commercial development an indicator of

the inter-batch variation is required, as well as insight into the behavior of the biofilm under more prolonged storage. A similar biofilm made in the same way using the related organism *Escherichia Coli* detached from the support with only mild shaking and was not visible after ~1 week, accompanied by an odor of decay (C. Mennan, unpublished) but immobilized biofilm of the *Serratia* has been used successfully for uranium recovery after 5-6 months (Macaskie *et al.* 1992b) and for continuous metal removal over extended period (Macaskie, 1990), but no rigorous study of the effect of storage on biofilm activity has been made previously.

1.10 AIMS AND OBJECTIVES

Studies described in this thesis use reactors packed with microorganisms, which can be described as nano-factories. These have various advantages such as being less expensive than chemical methods of metal removal or being environmentally friendly (Deplanche *et al*. 2012) and for a valuable metal like Nd, produce a mineral solid suitable for onward refining or even in direct catalytic applications. For the immobilized nanofactory biofilm loaded foam discs are produced in a fermentor vessel with a turnaround time for each fermentor preparation of approximately 10-14 days, which is unacceptable for bulk manufacture for an industrial process. Therefore the first objective of this study was to construct and evaluate a modular system, whereby a biofilm-forming chamber was inserted into the continuous biomass outflow from the main chamber of the chemostat vessel. This would enable a semi-continuous process for biofilm preparation by switching between two identical ‘’off chambers’’ retained for a certain time in the flow (a cartridge-type of preparation). Alternatively, for research purposes biofilm enhancers or inhibitors could be added to off-chamber one, whilst retaining off chamber two

unsupplemented as a control. In the system described here an example of biofilm inhibitor was added to one of the ''off chamber'' vessels, and the effect of this was evaluated by other workers.

A previous study has suggested that biofilm grown in this way removed $MnHPO_4$ acceptably (P. Clark and G. Sharma University of Loughborough, unpublished work), but the activity of biofilms grown in the main chamber and those in the ''off-chamber'' has not been compared directly previously. The first objective of this study was:

- Design the ''off chamber'' and evaluate the biofilm produced by this new method versus the normal method of in vessel biofilm production, for potentially economic production of biofilm in a scalable way. The stimulatory or adverse effects of ''main chamber'' biofilm versus ''off chamber'' biofilm will be compared by the criteria of phosphate release from glycerol-2-phosphate and removal of Nd^{3+} using biofilms taken at a comparable time 'post-harvest'.

The second question that should be answered is if the produced biofilm can be stored and for how long the biomass can remain active. This relates to the ''shelf-life'' of the final product and is a key factor for a commercial bioprocess. Hence, the second objective was to:

- Evaluate the effect of ageing of stored biofilm over a year at $4^{O}C$ as compared to freshly harvested biofilm.

Since the overall goal is to recover metals from solid scraps, which may have been solubilized in strong acid, it was essential to establish the potential limits of usefulness of the biofilm in real life conditions, specifically to establish the tolerance of the system to

low pH values or high salt concentration (as a result of pH neutralization) and the ability to recover from such injury. Hence, the third objective was to:

- Establish the durability of the biomass to low pH and its operation in diluted *aqua regia*. By using these two parameters it will be possible to calculate to what extent the flow rate needs to be slowed to maintain to a constant level of metal removal.
- The limit of tolerance of the system to adverse conditions will also be established as the final objective**.**

2.0 MATERIALS AND METHODS

All reagents were of analytical grade and the metal uptake experiments were done at room temperature (22^{o}C ± 5^{o}C), since preliminary studies have shown that temperature influences the rate of the reaction (Macaskie *et al*. 1997). All glassware was washed, rinsed 3 times with 100 mM HCl and then rinsed again 3 times with distilled water, in order to ensure phosphate and metal free glassware.

I. GENERAL PROCEDURES

2.1 Cell preparation, coating the foam discs with biofilm and biofilm storage

Biomass for the bulk of this study was grown in 2012 (Murray, A.J., 2012 personal communication) and provided from storage at 4 ^{o}C by S. Singh and A.J. Murray as part of a larger waste remediation project. The method for growth is as follows: *Serratia* sp. N14 was used by permission of Isis Innovation Oxford and was cultured in minimal medium, which contained (g/L): Tris hydroxy methyl-aminomethane, 12.0; KCl, 0.62; (pH to 7.0 with a 3000 mM HCl to avoid precipitation in the next stage) and then was added $(NH_4)_2HPO_4$, 0.96; $MgSO_4.7H_2O$, 0.063; $FeSO_4.7H_2O$, 0.00032; (minimal salts medium). The pH was then adjusted to 7.24.

The cultures were transferred from nutrient agar plates and were pregrown in either 50 or 100 mL of the minimal medium (above), under non carbon limiting conditions (via the addition of 2.13 g/L- lactose in aerobic environment shaken at 30^{o}C) (Yong *et al*. 1997). The carbon source was lactose throughout and for carbon limiting culture phase the lactose concentration was 0.6 g/L added aseptically as a concentrated solution after autoclaving. Following pre-growth in non-carbon limiting and then carbon limiting batch

culture, the inoculum (100 mL) was transferred into a 2.5 L air-lift fermentor (the dimensions of the main vessel were: length = 45 cm, internal diameter = 10 cm, for a working volume of 2.5 L as described at Paterson-Beedle *et al*. 2005) in order to create a carbon restricted aerobic condition to stress the bacteria and upregulate production of the desired enzyme PhoN and pili for biofilm production (Alan *et al*. 2002). 280 threaded polyurethane foam discs (already autoclaved, each one with 2cm diameter and 0.5 cm depth- a gift from from Recticel, Belgium) were tied into the chemostat fermentation vessel from a glass rod, to be coated with *Serratia* sp. N14 **Fig. 6a)**. The glass rod was secured in the middle of the reactor. The culture was grown batch-wise for 24 h and then switched to continuous mode for 6 days (Finlay *et al*. 1999) via peristaltic addition of fresh media, at a flow rate F. After the 6th day at steady state continuous culture the foam discs were visibly coated with biofilm (dilution rate D=0.1 h^{-1}, since for Vol=2.5 L and F= 0.24 L/h, D=0.1 h^{-1} (Macaskie *et al*. 2005). They were taken from the fermentor and were stored at 4^{o}C (cold room) in a humid environment suspended above isotonic saline (8.5% g/L NaCl), prior to examination (Finlay *et al*. 1999). As shown in **Fig. 6b)** the strings were tied from a glass rod which was secured at the top of a cylindrical vessel (in the middle of it). The isotonic saline was at the bottom of the cylinder and was not coming in contact with the foam discs. The whole procedure with the fermentor was repeated in this study in May 2013 (to obtain fresh biofilm) to compare with stored biofilms in two independent preparations made one year apart. In summary the experimental materials were for preparation (I): Biofilm from culture (I), (H)-tested by Singh, (2012) and (J), (K), (L) and (M), plus a second preparation (culture(II)) prepared for this study (Q)-tested by Singh (2013) and (R) as a comparator.

Biofilm tested	Culture (I)	Culture(II)	
Months	Main vessel	Main vessel	Off chamber
0	NT	**(Q)**	**(S)**
3	**(H)**	**(R)**	**(V)**
7	**(J)**	NT	NT
9	**(K)**	NT	NT
10	**(L)**	NT	NT
12	**(M)**	NT	NT

Table 2: Biofilms used in this study. In order to compare the two independent preparations biofilms from cultures (I) and (II) were compared at the three month stage (H)-(R), in order to provide two independent results under identical conditions of growth storage and evaluation. NT: not tested

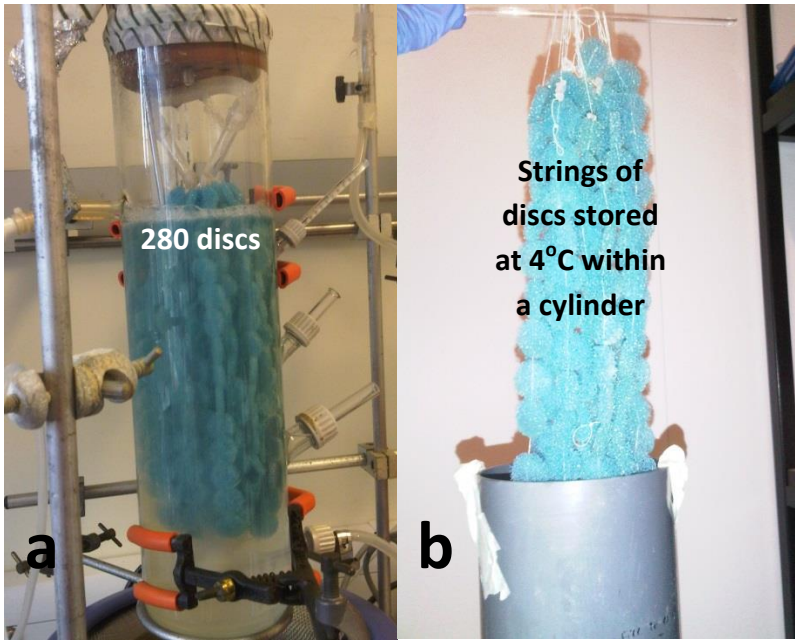


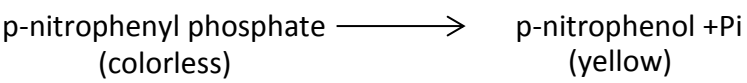
Figure 6: a) Photo of the fermenter and foam discs (280 discs) **b)** biofilm stored in cold room at 4°C via suspension over isotonic saline.

Biofilms from the main chamber were used as shown in **Table 2**. For comparisons between the main chamber biofilm and the off chamber biofilm the biofilms were compared as freshly harvested and after 3 months from culture (II) only.

2.2 Spectrophotometric methods

2.2.1 Measurement of phosphatase specific activity

In order to measure the phosphate liberation (established assay) the *p-nitrophenyl phosphate* (pNPP) assay was used. The pNPP assay is quick and easy, but the rate of Pi



release from pNPP is not necessarily the same as that from glycerol-2-phosphate (G2P); hence pNPP is used to assay enzyme activity routinely, but for phosphate (Pi) release experiments Pi analysis was done via a more accurate assay directly from column outflows. For the pNPP assay from colorless pNPP, yellow p-nitrophenol is produced, which relates to enzyme activity of the sample. The definition of phosphatase activity (units) is the nmol of p-nitrophenol (pNP) liberated per min per mg of bacterial protein. The optical density of the cells at 600nm (OD_{600}) is converted to bacterial protein by using a conversion factor obtained by the method of Lowry assay (at OD_{600}=1, path length=1cm and 0.278 mg protein mL^{-1}) (Macaskie *et al*. 2005). For phosphatase assay in a water bath set to 30^{o}C, 1.8 mL of 20 mM MOPS-NaOH buffer (3-[N-morpholino]-propanesulfonic acid, at pH 7) was inserted into two tubes along with 0.2 mL of the sample (or calibration standard, p-nitrophenol). The system was left to equilibrate for 15 min and then 5 mL of 200 mM NaOH was added to one of the tubes to inhibit the phosphatase activity (for the background). The reaction was initiated by the addition of 0.4 mL pNPP solution (which was stored in the dark -12 mg/mL) to both the tubes and after 2-3 minutes the development of a yellow color confirmed the presence of p-nitrophenol and therefore indicated enzyme activity. The reaction was quenched by timed addition of NaOH (5mL).

The liberated p-nitrophenol from pNPP was expressed as phosphatase specific activity (nmol PNP/min/mg protein) and was estimated by the yellow color at 410 nm (Ultrospec 3300 pro UV/Visible Spectrophotometer, Amersham Biosciences). The cell density (for protein estimation) was checked by adding 1.8 mL MOPS buffer (20 mM pH 7) in a separate tube along with 0.2 mL cells and the OD at 600 nm was determined using as blank distilled water (Yong *et al*. 1997). OD_{600} was converted to dry wt/mL and protein/ml via previously determined conversion factors by other researchers (Jeong, 1992, Mennan, 2010). The equation used for calculation of phosphate specific activity is:

Specific Activity=$[OD_{410}\ 10^9/(t\ 18472)]\ [7.4/10^3\ 0.552\ OD_{600})]$ nmol/min/mg protein (4)

(Jeong, 1992), where t=the time required for the reaction to take place from addition of pNPP, until addition of NaOH, which terminates the reaction, 18.472= is the molar extinction coefficient (mM^{-1}, cm^{-1}) and 0.552 is the protein conversion factor (Jeong, 1992, Mennan, 2010).

2.2.2 Assay of inorganic phosphate

Inorganic phosphate in the outflow solution of columns was determined by adding 0.97 mL of distilled water to a 2 mL cuvette, along with 0.6 mL of 2.5% (w/v) sodium molybdate in 1670 mM H_2SO_4 , 0.03 mL of the sample and 0.4 mL of freshly made (on a daily basis from the stock) stannous chloride ($SnCl_2$). (0.25mL of stock solution in 100 mL 1000 mM HCl; Stock solution: 3 g $SnCl_2$ in 5 mL concentrated HCl). The phosphate was visualized via a color change in the solution when adding the 0.4 mL $SnCl_2$ and the blue complex was estimated at A=720 nm 20 min after mixing the reagents (Yong *et al*. 1997)

and was compared to a calibration curve which was prepared using standards with phosphate concentrations from 0.5-5 mM and assayed in the same way. (Appendix I p.1)

The phosphate assay needs to be done immediately after the samples are collected from the column outflow, since phosphate concentration may change over time (i.e by adsorption onto the vessel walls) in case of being refrigerated or frozen and analyzed at a later time. The Spectrophotometer used was a Ultrospec 3300 pro UV/Visible Spectrophotometer, Amersham Biosciences.

2.2.3 Estimation of heavy metals in solution

Arsenazo III (2,2'-[1,8-dihydroxy-3,6-disulpho-2,7-naphthalene-bis(azo)] dibenzen-arsonic acid, is used for the spectrophotometric determination of neodymium where only one metal is present (otherwise interference can occur) and allows the analysis of a large number of samples, without specialized equipment (Yong *et al*. 1996). Nd^{3+} (or Eu^{3+}) in the outflow solution was determined by adding 0.03 mL of the column outflow solution to 1.97 mL of assay solution (1000 mM ammonium acetate buffer (pH 3.3) (Deplanche *et al*. 2011, Singh, 2012)). The metal in the solution was determined by adding 0.1 mL of 0.15% (w/v aq) arsenazo III. To make a stock solution of arsenazo III the solution was allowed to stand for an hour and then filtered to remove the solids; it is stable for 3 months in the dark). The purple complex was estimated at A=652 nm using an Ultrospec 3300 pro UV/Visible Spectrophotometer, Amersham Biosciences. The calibration curve, was prepared in the same way (Yong *et al*. 1997) (Appendix I page 2). A new calibration curve was prepared each time a new arsenazo III solution was made. Although the arsenazo III solution can stay stable for months in the absence of light, all the experiments that follow were conducted with arsenazo III solutions less than 3 months old. Calibration curves from

Nd^{3+} solution prepared in June 2012 by previous researcher (Singh, 2012) and Nd^{3+} solution prepared in December 2012 by this researcher, were compared and gave identical results.

For all columns the outflow solution was determined against the inflow solution (1mM metal) as a calibration within the assay. Metal removal was expressed as % of the input metal removal unless stated otherwise.

2.3 Pump speed calibration

Prior to challenging columns with test solution a pump calibration was performed using an external peristaltic pump (Watson Marlow 323s) using water in order to check the flow rate of the pump for aqueous solutions. The head of the pump had 5 channels and initially the calibration was done only for one channel (3), but two months later when two columns were tested in parallel, another calibration was done for channels (3) and (4). Although the results were the same for channels (3) and (4), (which were going to be used for testing the 9 months old biofilm), the flow rate appeared to be quite different compared to the calibration which was performed for 7 month old biofilm at channel (3). The results from both the calibrations can be seen in Appendix I p.3.

Due to these differences the actual flow rate was measured in each experiment (by collection of column outflow over a set time). Although the tubings used during the project were the same, the difference in the flow rate might be attributed to the pump and precise set up of tubings in the pump head.The points that were tested during the experiments can be seen in Appendix I p.4

2.4 Column description

The biofilm coated foam discs from a previous study (Singh, 2012), prepared by Dr. Murray, (Murray A.J., 2012 personal communication) were initially tested after 3 months of storage by Singh (2012) and subsequently were kept at 4^{o}C for several months in order to check in the present study the ageing of the biofilm with respect to phosphate release and metal recovery. For testing 8 foam discs coated with *Serratia* sp. N14 biofilm were stacked in a glass column in order to be challenged with the inflow solution.

The column comprised a packed-bed system reactor which consisted of a cylindrical glass tube of 5 cm height and internal diameter 2 cm. Inside the reactor were placed 8 reticulated polyurethane foam discs. Each of the foam discs had been coated with *Serratia* sp. N14 biofilm and after being packed in the reactor the column was challenged with the inflow solution (upward flow **Fig.7**) (Deplanche *et al*. 2011), which comprised 1 mM neodymium solution (Neodymium (III) nitrate hexahydrate 99.9%, Aldrich), 2 mM citrate buffer (100 mM stock pH 6.0; stock: approximately 88 mL of citric acid (100mM) in 900mL of sodium citrate (100 mM), adjust the pH at 6.0 and make to 1 L) and 5 mM glycerol-2-phosphate, (0.1 mM stock, β-Glycerophosphate Disodium salt Pentahydrate, Calbiochem).

Figure 7: Column packed with 8 foam discs.

The pH range for the citrate buffer was (3.0-6.2) (Ruzin, 1999, for a pKa_1 =3.06 pKa_2= 4.76 pKa_3= 5.4). The inflow solution at pH 5.5 is referred to as **standard solution**. For the inflow solution: Inside an 1000 mL volumetric flask, 900 mL of distilled water were added along with 10mL from 100 mM metal solution (1 mM), 20mL from 100 mM citrate buffer (2 mM) and 50 mL from 100 mM G2P (5 mM). The approximate pH of this solution was 7.2, which was adjusted to the desired value each time (5.5 or below) with 100 mM HCl. The rest of the flask was filled in with distilled water and the pH was rechecked. The column was challenged with inflow standard solution at a range of pHs (usually the limit is one pH unit either side of the pKa) in order to check the robustness of the Bacteria in acidic environment.

For expressing the column efficiency the factor $FA_{1/2}$ is used (see introduction paragraph 1.7). A minimum of two column volumes (approximately 16 mL) for each new sample that was taken at the outflow at a specific flow rate, was allowed to pass through the column in order to ensure that the old solution has been replaced by the new one. Thus the test sample represents the outflow solution at the current value of pH or flow rate (Macaskie *et al*. 1995 a, Singh, 2012). Insufficient material was available to use a fresh column at each pH to be tested. Hence, the pH was reduced step-wise (0.5 change with the use of 100 mM HCl) from 5.5 to pH 3.5 with 2 column volumes of wash at each flow rate (Descending pH series-(D)). To check for retention of column activity for subsequent experiments the pH was increased stepwise to the original pH (Ascending pH series-(A)) in 0.5 increments.

2.5 Use of *aqua regia* in the column

In order to check the robustness of the *Serratia* sp. N14 PhoN phosphatase in high salt concentration and separate the effects of pH from the effects of chloride and nitrate, the column was challenged with partially neutralized *aqua regia* solution (original solution: 3 parts HCl 11960 mM with 1 part HNO_3 15600 mM). The neutralization of *aqua regia* was performed in a fume hood using either NaOH pellets or 10000 mM NaOH solution to various pH values from 5.0-1.0. It was very unlikely that the columns would function under such conditions. A gradually increasing amount step-wise of NO_3^- and Cl^- was examined at the lowest pH that supported enzyme activity with full recovery upon return to pH 5.5 (i.e no permanent pH damage). The challenge solution was supplemented to various concentrations (10-50% v/v) *aqua regia*. The column was challenged for 4 column volumes with nitrate/chloride (approximately 32 mL) [32 mL/h: The $FA_{1/2}$ value was compared to that obtained using the initial standard inflow solution at pH 5.5]. After that the standard inflow solution was set to flow through the column for an hour of ''rescue'' (rescue: to check that the enzyme is still functioning correctly). The phosphate concentration and metal recovery were then calculated at the outflow solution to check the activity of the column ''rescue'' as a deviation from the original $FA_{1/2}$ value (without *aqua regia*) or as the % of metal removal at the original $FA_{1/2}$ in the presence of *aqua regia.* A check for column recovery was done prior to each new experiment to ensure the enzyme was still functioning correctly.

II. METHOD DEVELOPMENT

2.6 Designing the outflow chamber fermenter (off chamber)

Preliminary tests for designing the outflow chambers were done in plastic bottles previously used for soft drinks (in conjunction with Dr. Angela Murray). Once the tests were done, the final plans were given to a glass blower (Chemistry department, The University of Birmingham), to produce the outflow chamber vessels of appropriate size out of glass.

The dimensions were chosen to accommodate strings of 8, 2cm foams (16 cm per string-discs end-on; **Fig.9b**). At the end of each string was an extra piece of thread to connect the ensemble with a weight (consisting of plastic tube with glass beads inserted), to maintain the line/string taut within the reactor. The upper ends of all 12 strings were tied together at the lid of the reactor (**Figure 9a).** The total height of the system was estimated to be around 26 cm. A double sparger was also inserted at the bottom approximately in the middle of each reactor that would allow distribution of air throughout the two identical ''off-chambers'' ensuring good mixing **(Figure 9b-c).**

The diameter of the vessel was chosen to be 10cm so that the 12 main pieces would not be squeezed or touch during biofilm formation. Moreover, it was also taken into account the double sparger that was inserted into the vessel, as well as the extra weight that also had to be inserted under the double sparger.

The lid of each vessel was a plastic piece taken from a bottle of soft drinks and inverted. Twelve holes were made in each plastic lid, at exactly the same level, so that the inflow solution would be flow into each hole, and be equally distributed over each string

(Figure 9d). The whole system can be seen at **Figures 9e)** and **f).**The dimensions of the vessel can be seen in **Figure 8** that follows.

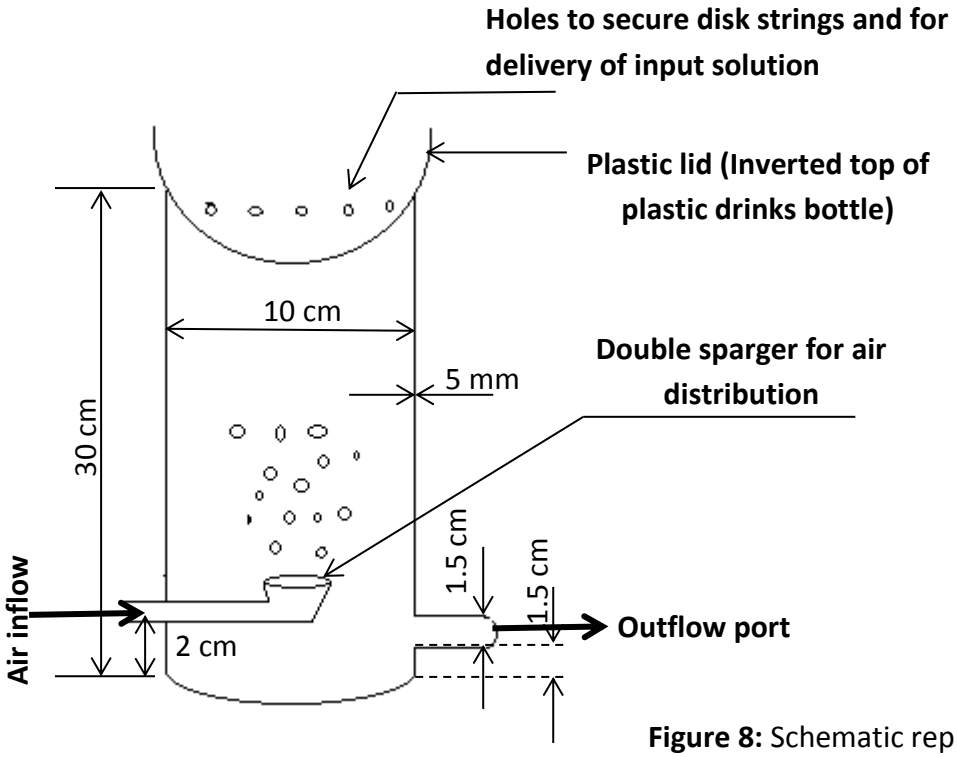


Figure 8: Schematic representation of the outflow vessel and its dimensions

The outflow part of the reactor was fitted with a plastic tube and the outflow was controlled with an adjustable screw valve. Given the approximately 120 mL/h inflow from the main vessel the outflow valve was adjusted approximately to that flow rate, using a measuring cylinder and a timer. The chamber inflow comprised the outflow from the main fermenter (carbon restricted chemostat at D=0.1 h^{-1}). Note that the flow was delivered at a flow rate of 240 mL/h. The main vessel outflow was divided via a Y piece into 2 off vessels each receiving 120 mL/h. With a fluid volume per reactor of 2000 mL this would have been D=0.06 h^{-1} for each vessel. No attempt was made to impose the same dilution rate onto the main vessel and outflow chambers. The solution into the off-chambers was

carbon-depleted (due to its consumption in the main chamber) so quantitative comparisons are not possible. In addition, the main chamber was maintained at 30^{o}C while no heating was supplied to the off-chambers. No further bacterial growth was assumed, due to carbon depletion; but the effect of the lower temperature as a factor influencing development and activity of the ''off chamber'' biofilm was not tested.

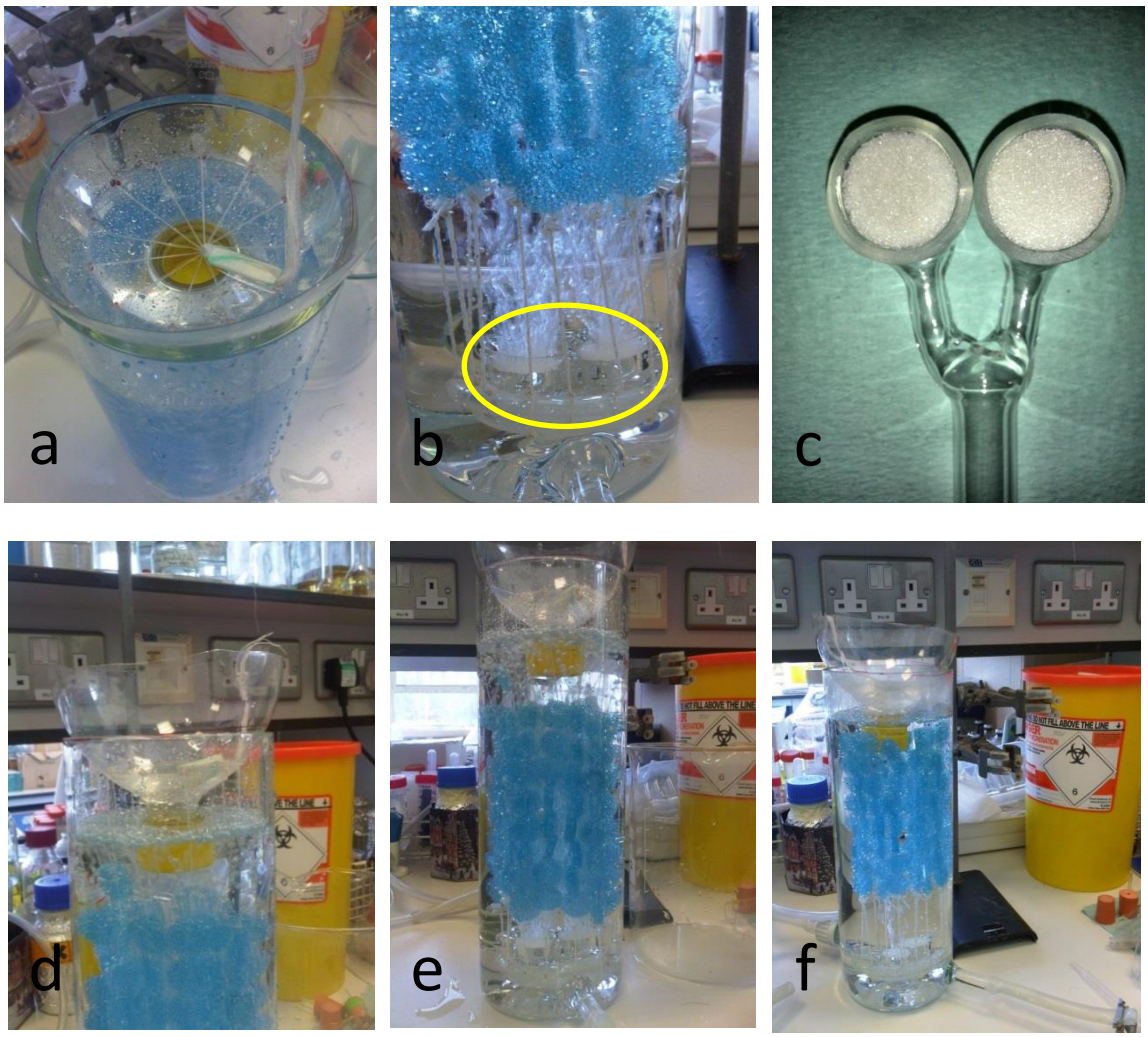


Figure 9: Components of the off chamber reactor: **a)** 12 strings tied in the lid of the reactor, **b), c)** Double sparger (yellow circled) inserted at the bottom of the reactor, in the middle of each vessel that would allow equally distributed air in each vessel, **d)** Lid of the reactor made from inverted plastic soft drinks bottle, **e)** and **f)** General image of the outflow chamber.

Two identical outflow vessels were connected with the outflow of the main vessel. The connection was made with a Y glass piece and plastic tubes were used for extensions. For this project they were used 2 outflow chambers instead a large one, as they were being used for separate experiments. The complete experimental set up is shown **Figure 10**.

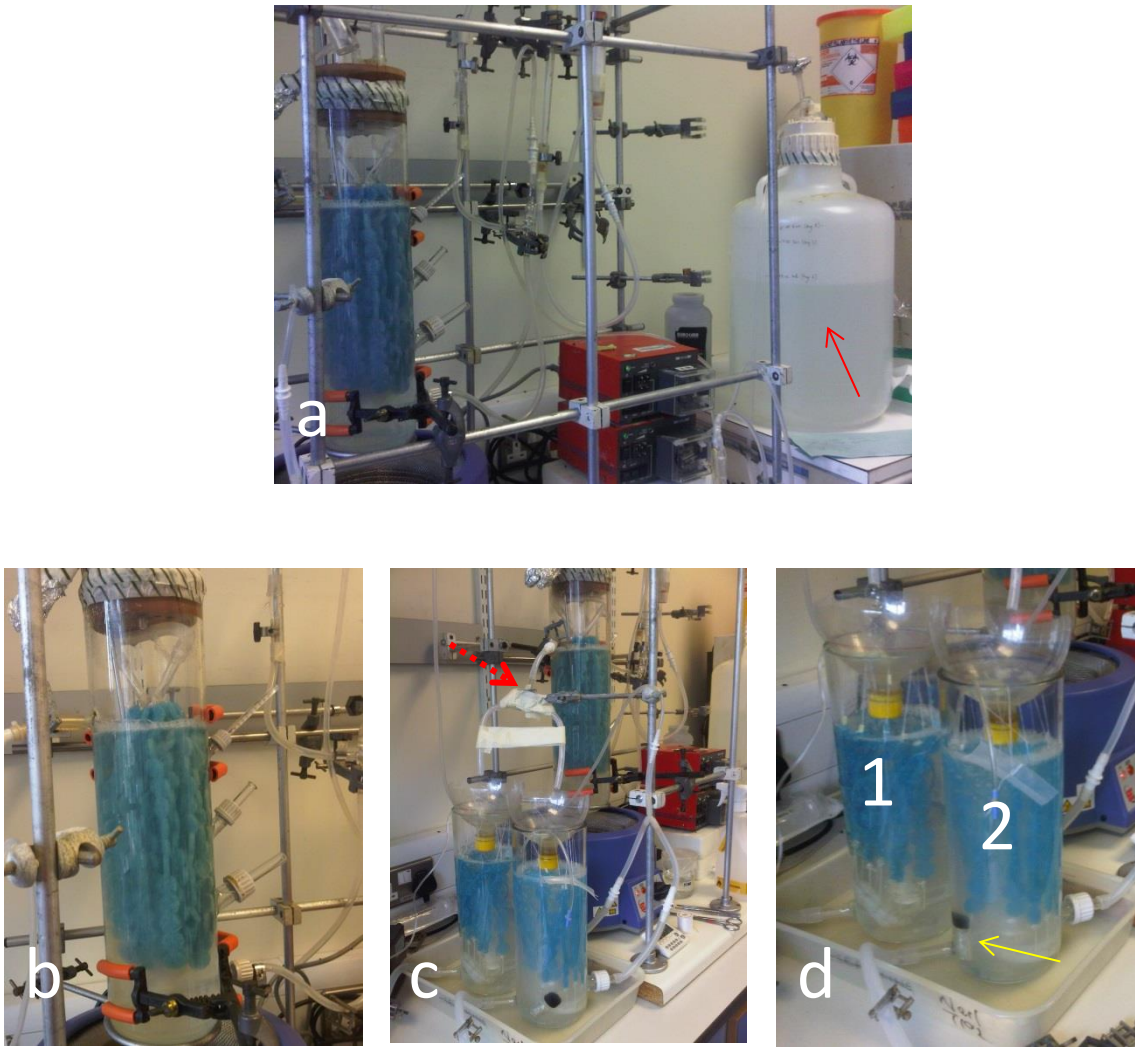


Figure 10: a) Main fermentation vessel with carbon source (red solid arrow). Fermentation conducted by Dr. Angela Murray as part of wider remediation project (picture taken by Dr. Jimmy Roussel), **b)** Main vessel, **c)** Main vessel connected with outflow chambers via a glass Y piece (red doted arrow) and extension tubes, **d)** Outflow vessels **1)** Dr. Mark Webber's test (including an inhibitor), **2)** Present study, without an inhibitor including the control sample for vessel 1 which was inserted in vessel 2 and maintained in there attached to a glass vial filled with glass beads, so that the one edge of this string will also remain in the bottom of the reactor (yellow arrow).

The main vessel was set up and operated as described in section E. After 24 hours when the main vessel was switched to continuous mode the two outflow chambers were connected to the outflow of the main vessel as seen in **Figures 10c)** and **d)**. Vessel number 1 was used for the experiments of Dr. Mark Webber from the University of Birmingham, School of Biosciences, who was evaluating the impact of continuous exposure to the efflux pump inhibitor, chlorpromazine on bacterial biofilm formation on a foam substrate in a the outflow chamber fermenter system. The inhibitor was incorporated in vessel 1 with an external peristaltic pump with a flow rate of 64 mL/h. The control was vessel number two, (no inhibitor). No external heating was provided in either of the outflow chamber vessels and the experiment was done at room temperature (22^{o}C). When the two vessels were filled with the inflow solution to a level that would cover completely the entire of the eight discs, the outflow from both the vessels was opened through the adjustable screws. Considering that the outflow of the main vessel was separated equally in the Y rod (240 mL/h =120 mL/h +120 mL/h), each outflow chamber was powered with 120 mL/h from the main vessel. For vessel number two the outflow was adjusted to that value through a measuring cylinder and a timer, while for vessel number one the outflow was adjusted to approximately 184 mL/h, since the inflow of the inhibitor solution was 64 mL/h. The schematic representation of the whole procedure can be seen in the following **Figure 11**.

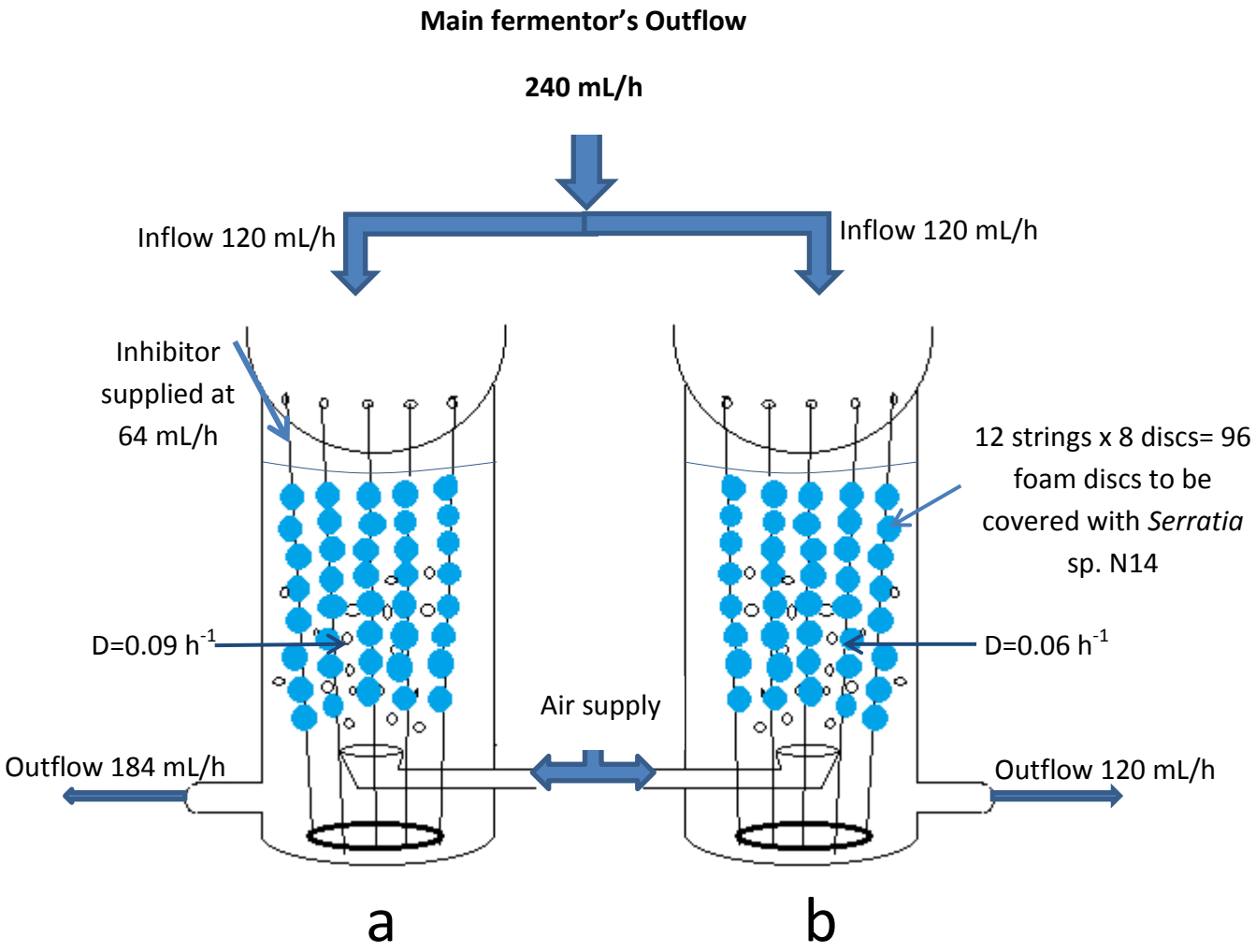


Figure 11: Schematic representation of the two outflow chambers, **a)** Using the inhibitor chlorpromazine for Dr. Mark Webber's experiments and **b)** Without an inhibitor, for testing the activity of the *Serratia* sp. N14 on the foam discs. From that figure the two chambers received the same number of cells but the cells in the inhibited reactor had 30% less time to adhere and form biofilm. Therefore the two off chamber reactors were not strictly comparable. However, this was a prototype experiment to test the off chamber method.

In **Figure 11** one can see the two separate reactors that were fed from the overflow of a master chamber with equal flow rates. To chamber a) a peristaltic pump introduced chlorpromazine continually to maintain a constant concentration of 64 mg/L (an initial volume of chlorpromazine was added at the beginning of the experiment to establish a baseline concentration of the drug), while reactor b) acted as a drug free control.

At this point it has to be noticed that, the total flow into the control was approximately 2/3 of the flow of the vessel treated with the inhibitor. The cells cannot grow in the off chambers, as the carbon source has been all used up in the main vessel, before the cells reach both the outflow vessels. Therefore for someone to estimate the cell count in the free cells it was required to increase by calculation the cell number/ml in the treated vessel by ~ 30% in order to make the two sets comparable. As seen from **Figure 11,** in each reactor, foam disks were suspended on weighted lines to allow media to perfuse within the matrix of each disk freely.

At this point it worths mentioning the fact that in the main vessel the Bacteria can be divided into the ones that were immoblized on the foam discs and the ones that for some reason (perhaps weak cells), did not manage to stay on the foam discs. On the other hand, in the outflow chamber, the Bacteria that were present, were the ones that did not form biofilm in the main vessel. Now combined with the fact that the temperature function of the outflow chamber was room temperature, it can be imposed that the Bacteria immobilized in the outflow chamber was a different phenotype than *Serratia* sp. N14 and therefore in that case again the two sets of biomass produced (from main vessel and outflow chamber) cannot be readily comparable. A check of the phenotype of the Bacteria immobilized in the outflow chamber was beyond the remit of this study.

III. DESCRIPTION OF BIOFILM TESTS

2.7 Description of column tests using biofilms of various ages

	Culture (I)	Culture(II)	
Sample tested on month:	**Main vessel**	**Main vessel**	**Off chamber**
0	NT	**(Q)**	**(S)**
3	**(H)**	**(R)**	**(V)**
7	**(J)**	NT	NT
9	**(K)**	NT	NT
10	**(L)**	NT	NT
12	**(M)**	NT	NT

Table 3: Synoptic table for the conducted experiments. NT: not tested.

2.7.1. Experiment description for (J) sample:

Using the 7 month old biofilm the experimental procedure was divided into two subparts. Initially the response of the biofilm was determined in phosphate release and metal recovery when dropping the pH (with 100 mM HCl) directly from 5.5 to 3.5 and then bringing it immediately back to 5.5 (biofilm-pH ''shock''). This first column was initially tested for Eu^{3+} removal for one day, then changed into Nd^{3+} removal the next day.

The experiment was repeated using another column with step-wise decrease from 5.5 to 3.5 and then back to 5.5 with 0.5 of a pH unit change in pH value at each time (gradual pH). The phosphate release and metal recovery were compared with results obtained from a 3 month old biofilm tested by a previous MSc researcher (Singh, 2012). The column at pH 5.5 ''collapsed'' (collapsed: in metal removal even for slow flow rates there was little recovery from the biofilms) in the descending series of tests due to an unknown reason and only for that pH value and so the results for this pH value were not taken into account.

Immediately after returning to pH 5.5 in the ascending series, the column was challenged for 4 column volumes (1 column volume = 8 mL) with 10% *aqua regia* solution held at pH 5.0. Following this the concentration of *aqua regia* was increased from 10% to 50%, with 10% increments, (being 100% : 3 parts HCl, 11960 mM with 1 part HNO_3, 15600 mM)) and then by lowering the pH value (with 100 mM HCl) in 0.5 pH unit steps each time, for the same increasing concentrations of *aqua regia* i.e. the next challenging solution was *aqua regia* at pH 4.5 for 10%-20%-30%-40%-50% concentrated solutions. After each *aqua regia* solution the column was ''rescued'' by being flushed again for 2 column volumes with the standard solution (pH 5.5) in order to check the phosphate release and the metal recovery of the column (''recovery''), before the next *aqua regia* addition.

2.7.2. Experiment description for (K) sample:

At 9 months two strings of biomass loaded foam discs, were taken from storage at $4^{o}C$ and were tested in order to:

- Check the activity of (K) in phosphate release and metal recovery with gradual pH alterations,
- Compare the results between them in order to provide an estimation of reproducibility within the biofilm with respect to its response to low pH, as it ages,
- Compare the results with (J), conducted 2 months previously as well as with the (H) (Singh, 2012).

Two different columns were run in parallel with identical experimental conditions. During the experiment the same 16 flow rate values as for (J), were tested for both the

columns. The columns were assayed for phosphate release and metal recovery starting from pH 5.5 dropping down to pH 3.5 step-wise (gradual pH test at (J)) and then again gradually resetting back to pH 5.5. [the two separate columns had both identical initial weights (8 foam discs loaded with biofilm), for column 3, W_1: 2.712 g and for column 4, W_2: 2.5089 g). Assays were done in triplicate and the repeatability was within 5% with the exception of three cases which were less than 10%. Both the columns after that were challenged again with *aqua regia* solutions altering the pH value and the *aqua regia* concentration in the same way previously described for (J).

2.7.3. Experiment description for (L) sample:

The aim of this experiment was to develop a simple mathematical predictive model to describe metal removal. This was done by finding a correlation in the equations that describe the metal removal vs the flow rate for gradual pH test, for all 3 samples of biomass. Since gradual pH test has been performed again for 7 and 9 months old biofilm, the experiment for (L) in pH descending series (5.5-3.5), was not done for all the pH values, but only for pH 5.5-4.5-3.5. The flow rate was checked and was set to the same as for the one imposed at the (K) experiment.

At pH 5.5 all the flow rate points were tested again after two column volumes using (L) sample and the results were compared with the ones received for (J) and (K). Since the pump calibration for (J) test gave different results from the one at 9 and 10 months old, the flow rate and metal removal were compared for actual flow rates for all sets of biomass.

For the pH tests: 5.0-4.0-3.5 the column was challenged for four column volumes with the inflow solution at three different flow rates a)One at slow flow rate, which would give high metal removal approximately 70% (15rpm-16.8 mL/h), b) One at medium flow rate, which would give a metal removal close to 30% (50rpm-57 mL/h) and c) one at high flow rate (95 rpm-106.5 mL/h) for 10% removal. The choice of four column volumes for equilibration was made due to a blocked column (blocked tubing) from the (K) tests that was observed at a slow flow rate (16.8 mL/h) and for which it was noticed that 2 column volumes was not enough to bring the column to the expected metal removal and phosphate release. Using 4 column volumes (~32 mL) the column was brought back to a comparable phosphate release and metal removal with the repeat column. The results from this 4 column volume equilibration experiment were characterized as experimental results, which were compared with the theoretical results obtained from the linear part of the lines for metal removal vs ln(flow rate) for either 8 (n=8) or 11 points (n=11), the latter to provide more robust data. From the total of 16 points that were giving the sigmoidal curve, the ones that were omitted in all of the graphs, were the ones that were not belonging to the linear part of the sigmoidal curve i.e. the first one from very slow flow rates 5.68 mL/h and the last four ones starting from 132-287 mL/h. Through these lines a simple predictive model was found for the metal removal (see Appendix V page 11-Method development of a simple mathematical predictive model). The $FA_{1/2}$ value was estimated from the plot of activity vs LnF for the three flow rates tested.

When doing the pH ascending test from 3.5 to 5.5 (in gradual pH using (L) sample), the experiment was done in exactly the same way that was done in previous experiments, with the only difference being that the column after each pH test was left in the standard

solution overnight (pH 5.5)-''rescue''. That was primarily done because it was noticed that the biomass at low pH values was ''shocked'' and so that was a good way of observing the results when ''rescuing'' the column from the acidic conditions overnight and for comparing the results with the previously conducted experiments.

2.7.4 Experiment description to compare sample (M) with biofilm produced in the outflow chamber at 0 months (S).

The production of new biofilm (preparation (II)) in the off chamber (fermentation II) was compared with the biofilm made in preparation (I) and had been stored at $4^{O}C$ for 12 months. The two columns were tested in parallel at comparable flow rates for the gradually descending-ascending pH experiment as described for (K). At the end of the experiment both the columns were challenged again with *aqua regia* solution of the same pH range and concentrations, as previously described.

2.7.5 Experiment description using samples (R) and (V)

Three months after the new fermenter's run (May 2013), the biomass produced in the main fermenter vessel (R) and the one produced in the outflow chamber (V) were tested again in phosphate release and metal recovery. Two columns were ran in parallel again for the case of the (V) in the same gradual descending-ascending pH experiment, while two weeks later 1 column was tested from the main vessel fermenter for the same experiment, in order to compare the results of this year's fermentor's run, with the ones produced in 2012 by Dr. Angela Murray (2012, personal communication) and assayed by previous MSc student (Singh, 2012). Here, generation of results using (V) sample allows a direct comparison with main chamber biofilm produced after storage at 3 months. This

key experiment was designed to show the reproducibility between two independent biofilm preparations made in consecutive years.

2.8 Scanning Electron Microscopy (SEM) and Energy-Dispersive X-Ray spectroscopy (EDX) Analysis

Biofilms coated with metal, which was produced for the initial experiment of this project (J) (column used for pH ''shock''), along with (L) (control sample) and also the biofilm produced from the outflow chamber (V), were squeezed from foam discs, dried and crushed into powder and placed on insulating sticky tape and then attached to SEM-stubs. Initially when using (J) sample, the column had been challenged for one day with inflow solution using as metal europium at pH 5.5, but switched the next day to neodymium and thereafter. All three samples were analyzed using a JEOL JSM-6060 LV Scanning Electron Microscope. An INCAx-sight (Oxford Instruments) Energy-Dispersive X-Ray detector was also attached to the same equipment. All samples were gold coated (with a sputter coater) prior to analysis. All SEM images were acquired by Dr. Angela Murray.

2.9 X-Ray powder diffraction-XRD Analysis

Powder from the same samples described in section 2.8 were all analyzed by XRD at the Chemistry Department University of Birmingham by Dr. Jackie Deans on a Bruker AXS D8 diffractometer. All samples were stored in the fridge prior to analysis for several months. Three days before the analysis they were left overnight in 20mM MOPS-NaOH (pH 7.0) and the next day they were rinsed with MOPS-NaOH and three times with distilled water. They were then left to dry out at room temperature prior to analysis

(Singh, 2012). All samples were examined between 5-60 degrees 2θ, 0.02 degrees per step with a time per step of 1.1 seconds, for a total scan period of 53 min for each sample. Acquired powder patterns were compared to a standard database (Kirik *et al*. 1998), obtained from Chemistry department, The University of Birmingham, to assign the peaks for identification of the formed material.

2.10 Data presentation

In the tables that follow the ones with the pink background shows the results from previous work (Singh, 2012), which are compared with the blue background tables, which depict the results of phosphate release and metal recovery for the coated discs with 7,9,10 and 12 months old *Serratia* sp. N14 biofilm of this study. Preparation I (Culture (I)), uses biofilms produced by Murray (2012, personal communication) that were stored for use in this study.

Preparation I: (J), (K), (L), (M) are compared with (H) from (Singh, 2012).

In order to produce a complete picture of the effect of ageing on biofilm activity, a fresh set of biofilms (Preparation II –Culture (II)) were prepared and tested immediately (0 months) and after 3 months. Testing of both sets of biofilm at the 3 month stage enabled pooling the data.

Preparation II: The second biofilm preparation, which was produced in the main fermentor vessel (Q)- analysed by Singh (2013), (R), (work done by Murray (Dr. Angela J. Murray , 2012, personal communication) and the biomass that was produced in the outflow chamber fermenter (S) and (V).

Normalization of data

The use of $FA_{1/2}$ value (see Introduction, paragraph 1.7), allows normalization of data in two ways:

a) The flow rate required to achieve 50% total phosphate release or 50% metal removal,

b) The Pi release on metal removal at the flow rate of the control column or conditions for each experiment, this control being afforded a value of 100%. Hence given two experiments even if the actual numerical data are different (for example by using two differently aged biofilms to test the effects of an imposed parameter) the use of 100% for the control activity in each case will remove the effect due to ageing.

Summary of the ageing study:

Culture (I)			**Culture(II)**					
Main vessel			**Main vessel**			**Outflow chamber**		
Sample tested at month:	**Source of data**	**Code**	**Sample tested at month:**	**Source of data**	**Code**	**Sample tested at month:**	**Source of data**	**Code**
0	NT	-	0	Singh, 2013	**(Q)**	0	This study	**(S)**
3	Singh, 2012	**(H)**	3	This study	**(R)**	3	This study	**(V)**
7	This study	**(J)**	NT	NT	-	NT	NT	-
9	This study	**(K)**	NT	NT	-	NT	NT	-
10	This study	**(L)**	NT	NT	-	NT	NT	-
12	This study	**(M)**	NT	NT	-	NT	NT	-

Table 4: Source of data, biofilm Preparation (I) and (II). NT: Not tested.

From this table it can be seen that two independent preparations were tested at the 3 month stage. Hence, the reproducibility was established between two independent biofilm preparations.

3.0 RESULTS AND DISCUSSION

SECTION A.

3.1 Reproducibility of phosphate release and metal removal by two independent biofilm preparations tested after 3 months at 4^{o}C

3.1.1 Comparison of (H) and (R) with respect to (Pi) release

In order to compare columns, use was made of that flow rate giving a comparable extent of phosphate release or metal removal and other data compared to this reference value (normalized data). This value was that flow rate giving half maximal phosphate release or metal removal, unless stated otherwise. According to previous research done (Singh, 2012), the results from the phosphate (Pi) assay can be linked with the results from the metal assay as follows: the metal recovered inside the column (by difference in the inflow and outflow metal concentrations) is equal to the phosphate concentration in the precipitate (molar ratio of metal: phosphate in the precipitate is 1:1 i.e. $NdPO_4$ as confirmed by X-Ray diffraction (Singh, 2012). Therefore, the total phosphate liberated (Pi) will be the sum of the outflow concentration calculated by assay plus the phosphate accountable within the phosphate-metal precipitate in the column estimated by Nd removal (Deplanche *et al*. 2011, Singh, 2012)**.**

In **Figure 12** one can see the comparison between total phosphate released when using the biomass produced in the main vessel in 2012 and tested after three months of storage in the cold room by Singh (Singh 2012) and the total phosphate released when testing the biomass produced in May 2013 after three months of storage at 4 ^{o}C. Considering 100% the 5 mM G2P, we get:

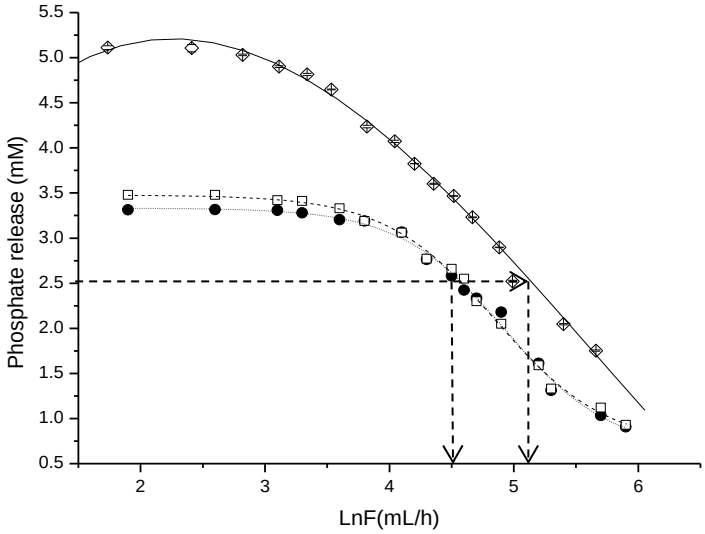


Figure 12: The total amount of liberated phosphate (Pi) using *Serratia sp.*N14 to recover Nd (●) and Eu (□) at pH 5.5 when using 3 months old biomass for increasing flow rates from 6.8mL/h to 374 mL/h. $FA_{1/2}$=~90 mL/h. Data taken by Singh (2012)**.** Data for **(◊)** showing the total amount of liberated phosphate for Nd to be recovered, at pH 5.5 when using (R) at pH 5.5 for increasing flow rates from 5.68 mL/h to 286.98 mL/h. $FA_{1/2}$=176.28 mL/h (Error bars are shown only for the hollow diamonds).

Both experiments were done directly upon bringing the biomass out from the cold room and filling the column with the inflow solution at pH 5.5. Hence, with respect to phosphate liberation the second preparation was nearly twice as effective as the first preparation after storage for three months. At this point it has to be mentioned that the phosphate release data of Singh (2012) are here questionable since, unlike biofilm from culture (II) complete G2P cleavage appeared not to occur even at slow flow rates. However, in each case the Pi release was to excess over the metal concentration.

3.1.2 Comparison of (H) and (R) with respect to metal removal

The same comparison as above was made for the case of Nd removal. The results can be seen and compared in the following figure**:**

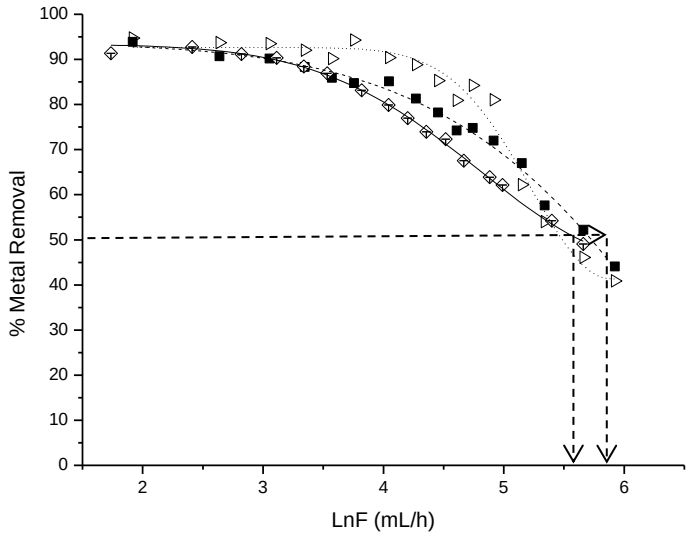


Figure 13: Total amount of europium (■) and neodymium (▷) removal at pH 5.5 when using 3 month old biomass for increasing flow rates from 6.8 mL/h to 374 mL/h. $FA_{1/2}$=273.14 and $FA_{0.6}$= 200 mL/h. Data taken from Singh's work (2012)**.** Data for (◊) are for total neodymium removal at pH 5.5 when using (R), for increasing flow rates from 5.68 mL/h to 286.98 mL/h. Error bars are shown only for the hollow diamonds. $FA_{1/2}$=274.89 mL/h and $FA_{0.6}$=163.11 mL/h.

Isolating now the linear data from all three sigmoidal curves shown above, the following graph is produced, that shows a) identical Nd/Eu removal and b) reproducibility of the results for neodymium removal.

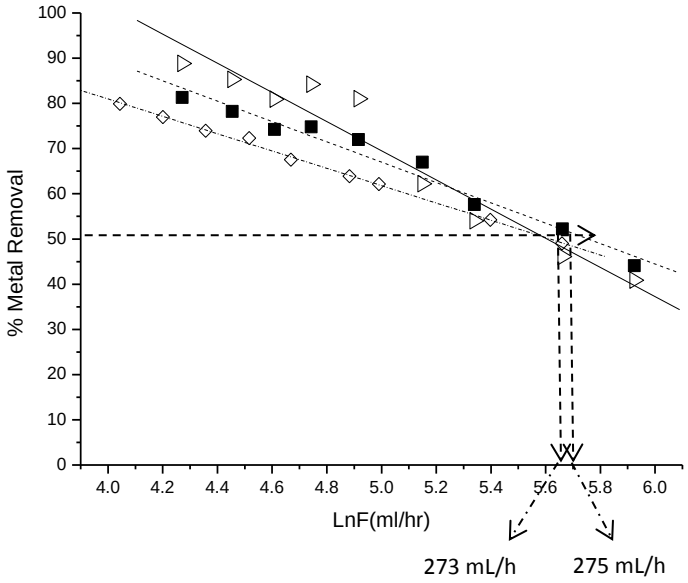


Figure 14: Hollow symbols: Nd removal by preparation (I) (▷) and (II) (◊). (■): Eu removal (preparation (I)). Data are pooled for Nd and Eu. Preparation (I) $FA_{1/2}$=273mL/h. Preparation (II) $FA_{1/2}$=275 mL/h. Data for (▷) and (■) were given by Singh (2012). From this figure it is concluded that two independent biofilm preparations gave results reproducible within 1%. That is a very important conclusion that shows interbatch reproducibility.

Here it is concluded that although preparation (II) gave nearly twice the phosphate release as preparation (I), $FA_{1/2}$ in metal removal by the two preparations was identical within 1 %. The reason for the discrepancy in the phosphate release data is not apparent, but may relate to aspects of metal desolubilization (see later in discussion).

SECTION B.

3.2 Effect of ageing on biofilm activity

3.2.1 Effect of ageing on phosphate release (Pi) by the biofilms

Separate preparation of biofilms gave almost identical results with respect to metal removal at the 3 month stage (section A). The following figures show the results for phosphate release when using (H), which are compared with (K), (L), (M) samples. The initial activity of fresh biofilm on harvest was not done (Singh, 2012). For the second preparation the initial activity was measured by S. Singh (unpublished), against which the activity obtained at 3 months was compared. Since the two preparations give nearly identical $FA_{1/2}$ values at the 3 month stage (section A), it was assumed that their initial activities would have been, similarly, identical.

Due to the fact that only (K) sample was done in parallel columns (i.e made from the same culture), all the data that will follow in this section will be compared with the results obtained from (K), since the reproducibility of that experiment was within 5%. Therefore, for more robust data all experiments are compared with data obtained for (K) in total phosphate release (Pi) and metal recovery (see Appendix VII-repeatability results for (K) page 27).

F**igure 15b)** shows for (K) (arrowed), (L) and (M) the $FA_{1/2}$ value in total phosphate release of 15.3 mL/h, 9.4 mL/h and 2.5 mL/h respectively. Compared with the $FA_{1/2}$ value found for (R) sample, which was 176.28 mL/h, the $FA_{1/2}$ falls to 15.3 mL/h for (K) and 2.5 mL/h for (M). Hence, the time required for the phosphatase to cleave G2P and produce phosphate increases with the age of the biofilm, since the ability of the biomass to react

with 50% of the G2P inserted in the inflow solution drops from a high flow rate (176.28 mL/h) to a very slow flow rate (2.5 mL/h), within a period of 9 months of extra storage in the cold room. Experiment (J) was excluded from these calculations as the column contents had collapsed as previously explained, due to an unknown error**.**

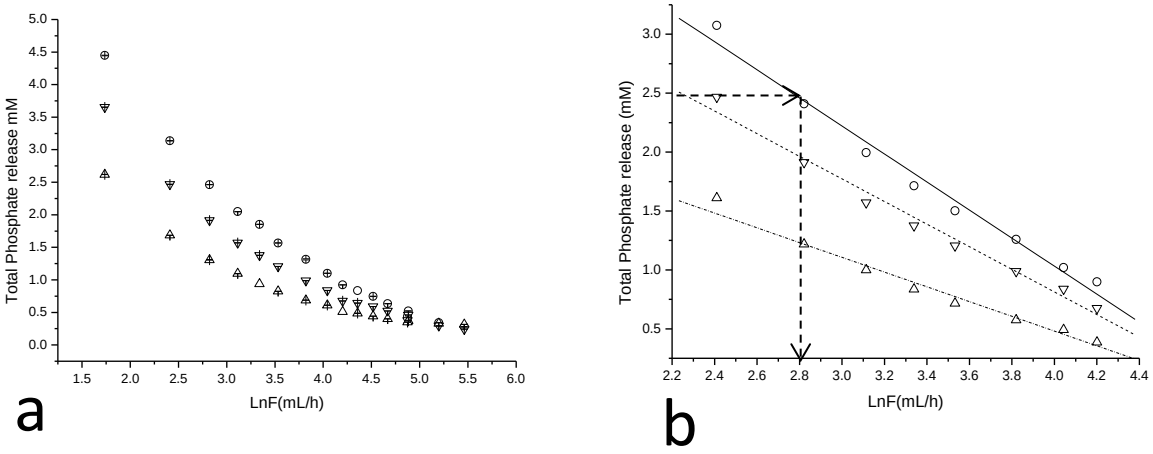


Figure 15: a) Total amount of liberated phosphate at pH 5.5 when using the results from the experiments (K) shown as (**O**), (L) shown as (▽) and (M) shown as (△) , for increasing flow rates from 5.68 mL/h to 286.98 mL/h. Error bars are shown calculated as standard deviation. **b)** Selected lines from the linear parts of the sigmoidal curves shown in a), used to calculate the $FA_{1/2}$ values for total phosphate release in (K), (L) and (M). Value for (M) was extrapolated as seen from the graph, since the actual result of that experiment fell outside the linear part of the graph.

Effect of biofilm ageing on Pi release			
Biofilm age	**$FA_{1/2}$ (mL/h)**	**% activity compared to 3 month old biofilm samples**	**Source of data**
(R)	176.28	100	Fig.13
(K)	15.9	9.0	Fig.15b)
(L)	9.4	5.3	Fig.15b)
(M)	2.5	1.4	Fig.15b)

Table 5: Comparison for $FA_{1/2}$ values in Pi. Value for 3 months was derived from culture (II), while for 9, 10 and 12 from culture (I). Considering the value for 3 months at (II) as 100% activity, the values for 9, 10 and 12 were expressed relative to that value.

By plotting the $\ln(FA_{1/2})$ values against biofilm age the activity was shown to fall in a linear fashion over 12 months (**Figure 16**) i.e activity was lost exponentially during storage.

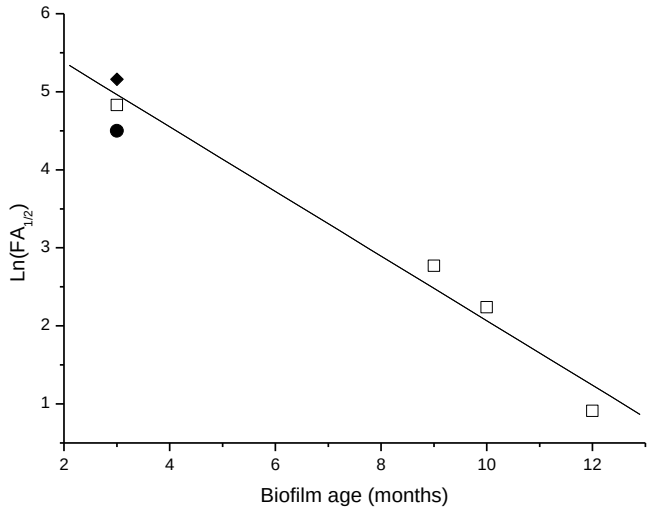


Figure 16: Effect of biofilm ageing on phosphate release. Values taken from section A. (●): ln($FA_{1/2}$ of (H) sample (value from Singh(2012)=4.5. (♦): ln($FA_{1/2}$ of (R) (value from this study)= 5.17. (□): ln($FA_{1/2}$ of Pi).Value for 3 months is the average of the above (4.84), while for 9,10,12, directly from this study and culture (I) (2.77,2.24 and 0.91 correspondingly).

Plotting now the log of the second column of **Table 5** versus the age in months, we get the following figure.

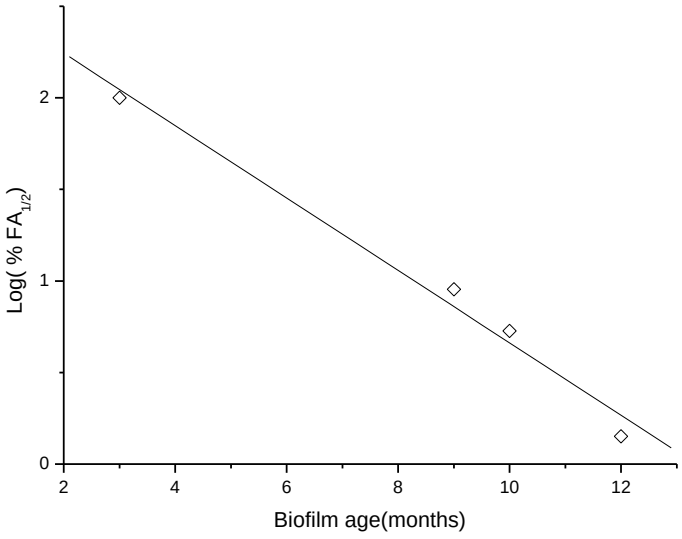


Figure 17: Effect of biofilm ageing on phosphate release. (◊**)**: log (% $FA_{1/2}$ of Pi). Plotting the log values of **Table 5** (second column). Data for 3 months taken from culture (II), while for 9,10,12 from culture (I), both from this study.

3.2.2. Effect of ageing in metal recovery

Figure 12 for (O) and (■) from culture (I), shows that full cleavage of G2P (5mM) was not achieved even at very slow flow rates. Hence, in order to achieve sufficient Pi in the columns the concentration of G2P was always 5mM (i.e. present in excess) and that of challenge metal 1 mM. **Figures 16** and **17** show phosphate release by the same columns after various extents of ageing. The same comparison as above was made for the case of Nd removal. The results can be seen and compared in the following figures**:**

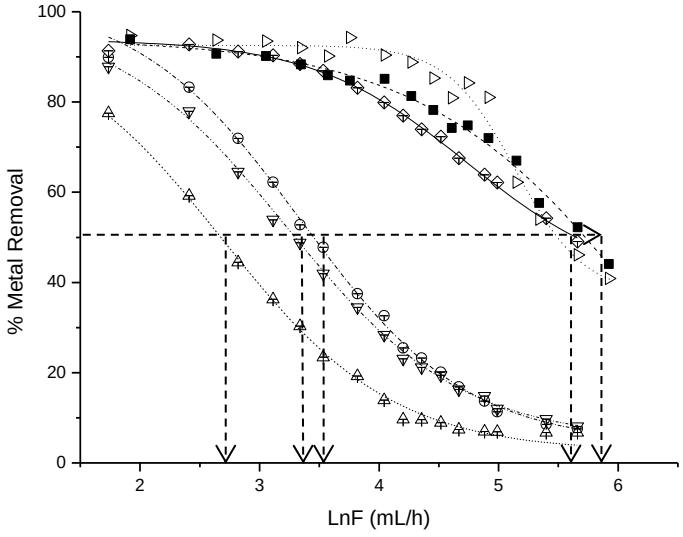


Figure 18: Recovery of neodymium (▷) and europium (■)(pH 5.5) for (H) sample. Taken from (Singh,2012). Data for (◊) shows the results for (R), for (O) the results for (K), for (▽) the results for (L) and for (△) the results for (M). Comparison of Nd removal for 3, 9,10 and 12 months old biomass at pH 5.5. Error bars (assay replicates) are shown only for the experiments conducted in this project (Error bars for results from Singh, 2012 are excluded).

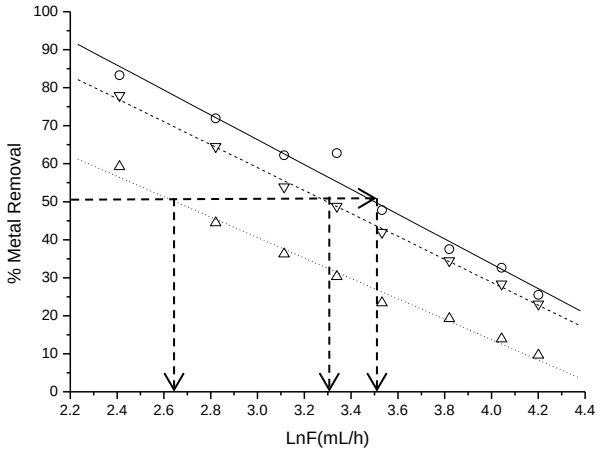


Figure 19: Lines obtained from the linear part of the sigmoidal curves shown above for the experiments (K) shown as (**O**), the results for (L) shown as (▽) and the results for (M) shown as (△), used for calculation of the $FA_{1/2}$ values.

According to Singh (2012) the $FA_{1/2}$ value for Nd removal at 3 months was ln(5.61)=273.14 mL/h. Keeping the same biomass at 4^{o}C for an additional 6-9 month period reduces its ability to recover metal, from $FA_{1/2}$ of 273.14 mL/h to 33.1mL/h for (K) to 26.99 mL/h for (L) and to 14.1 mL/h for (M). **Table 6** that follows illustrates this.

a

mL/h	% Nd recovery (3 months)-(H)
6.8	94.7
14.0	93.7
21.2	93.5
28.4	92.0
35.6	90.2
42.8	94.3
57.2	90.4
71.6	88.8
86.0	85.3
100.4	80.9
114.8	84.2
136.4	81.0
172.4	62.2
208.4	54.0
287.6	46.1
374.0	40.9

b

mL/h	% Nd Recovery (9 months)-(K)	% Nd Recovery (9 months repeat)-(K)	% Nd Recovery (10 months)-(L)	% Nd Recovery (12 months)-(M)
5.68	89.9	90.3	87.8	77,5
11.14	83.2	82.3	77.9	59,2
16.8	71.9	70.9	64.5	44,5
22.5	62.2	60.0	53.9	36,3
28.2	54.3	51.7	48.8	30,3
34.2	47.8	45.3	42.0	23,4
45.6	37.5	35.2	34.5	19,2
57.0	32.6	29.6	28.4	13,9
66.75	25.5	25.6	23.1	9,7
78.0	23.3	22.3	21.2	9,6
91.5	20.1	19.3	19.4	9,0
106.5	16.9	16.0	16.3	7,4
132.0	13.7	12.4	14.8	7,0
160.4	11.4	9.8	12.0	6,9
220.94	8.5	7.3	9.7	6,7
286.98	7.6	5.6	8.1	6,7

Table 6: a) Effect on Nd recovery for the standard solution (pH 5.5) at increasing flow rates for the 3 months old *Serratia* sp. N14. Taken from Singh, (2012) **b)** Effect on Nd recovery for the standard solution (pH 5.5) at increasing flow rates for (K),(L),(M) samples. ''Repeat'' refers to two independent columns from the same preparation. Highlighted: approximate 60% removal points. Corresponding flow rates are shown.

From **Tables 6a)** and **b)** it can be concluded that the effect of ageing means that the flow rate needs to be decreased by 10-fold to maintain $FA_{0.6}$ i.e from ~170 mL/h with 3

months old biofilm to ~18 mL/h with 9, 10 and 12 month old biofilm. Less than one column volume per hour can be processed if >80% metal recovery is to be achieved with 12 months old biomass.

In an effort to further quantify the relationship between ageing of bacteria and metal removal the $FA_{1/2}$ values for all the experiments were calculated from their corresponding equations. The equations used were from the pH descending series (before nucleation-see section C) and are the following:

$Y(H)=-0.3226X+2.3084$ $R^2=0.9376$ (Singh's data) (4)

$Y(R)=-0.1916X+1.5759$ $R^2=0.9976$ (5)

$Y(K)=-0.3266X+1.643$ $R^2=0.9756$ (6)

$Y(L)=-0.3016X+1.494$ $R^2=0.9975$ (7)

$Y(M)=-0.2685X+1.2113$ $R^2=0.9877$ (8)

The following table shows the results from the comparison of the calculated $FA_{1/2}$ values at pH 5.5 for the experiments (Q), (H), (R), (K), (L), (M).

Months kept in 4°C	Ln($FA_{1/2}$)	$FA_{1/2}$ mL/h
(Q)	6.22	505
(H)	5.61	273.1
(R)	5.62	274.6
(K)	3.50	33.1
(L)	3.30	27.0
(M)	2.65	14.1

Table 7: Comparison of the $FA_{1/2}$ values for (Q), (H), (R), (K), (L), (M) data. The values for (H) was from Singh (2012), while (Q) was determined by S. Singh for this study.

Freshly harvested biomass (Q) analyzed by S. Singh in 2013 gave an $FA_{1/2}$ value of 505 ml/hr. After 3 months this was halved to 273 ml/hr. This suggests that half of the activity was lost in a 3 month storage period, i.e. an exponential decay with time.

Gathering and plotting all the $FA_{1/2}$ values from the data obtained, we receive the following **Table 8** .

Code	(Q)	(H)	(R)	(K)	(L)	(M)
Months (culture)	0(II)	3(I)	3(II)	9(I)	10(I)	12(I)
% Cells activity (mL/h)	100	54.07	54.37	6.55	5.34	2.79
Log(% cells activity)	2.00	1.73	1.74	0.82	0.73	0.45

Table 8: Reduction of cells activity through time. Considering highly active cells as 100% at 0 months ($FA_{1/2}$ was 505mL/h) the $FA_{1/2}$ value for (H) was given at ln(5.61)=273.14 mL/h. Both values for (Q) and (H), were given by S. Singh (Singh, 2012).

A more detailed assessment was done by importing data from Singh (2012), who also analyzed in 2013 a new preparation of freshly harvested biomass. The $FA_{1/2}$ value for fresh biofilm was taken as 100% and the $FA_{1/2}$ for the other biofilms were normalized relative to this (**Fig. 20).** A logarithmic plot Log (% $FA_{1/2}$) value versus the age of the biofilm gives a straight line (R^2=0.9941), as seen in **Fig. 21**. It is concluded that the activity of the biofilms with respect to Nd recovery decreases exponentially during storage at 4^{o}C.

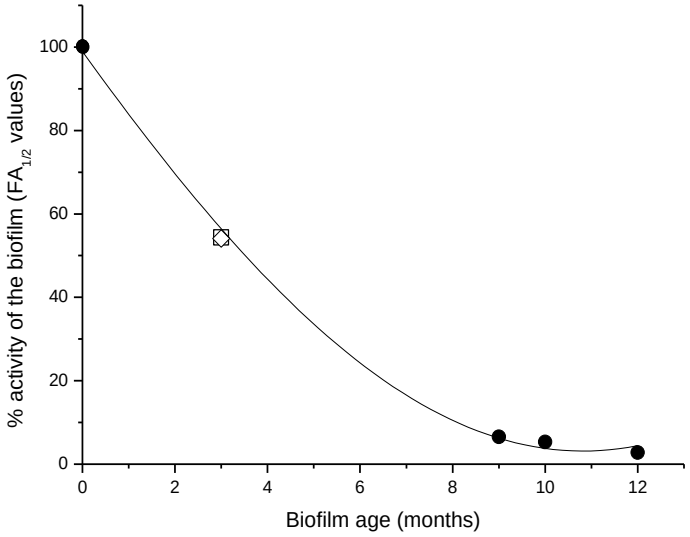


Figure 20: Relationship between biofilm (months) and the respective $FA_{1/2}$ values (●). The value for 0 months old biomass was taken by Singh (2013). Value for 3 months was the average from two independent cultures ((H):(□) and (R):(◊)). Values for 9, 10 and 12 months given from culture (I) of this study. All data are shown in **Table 8**.

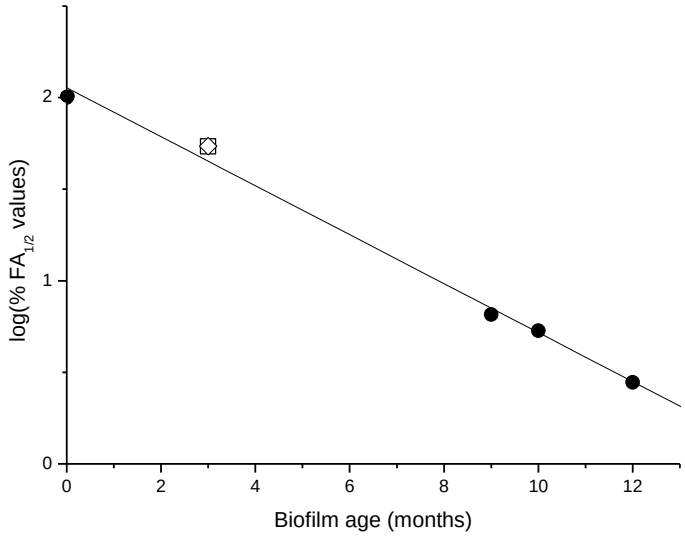


Figure 21: log values of **Table 8** ($FA_{1/2}$ values normalized as shown in **Figure 21**) versus biofilm age (months)(●). Value for 0 months given by Singh (2013) from (Q). Value from 3 months is the mean from two independent three month preparations ((H): (□) analyzed by Singh(2012) and (R): (◊) from the current study). Values for 9, 10 and 12 months taken from data of this study and are all from culture (I).

SECTION C.

3.3 Column ''rescue'' by pre-nucleation with $NdPO_4$

The experiment was initially conducted directly via inserting the inflow solution in the column and measurement of Nd^{3+} in the outflow at various flow rates and was repeated the next day at the same pH value, after leaving the column in the standard solution overnight to promote potential additional nucleation of $NdPO_4$. The results before and after the overnight static period can be seen in **Figure 22**.

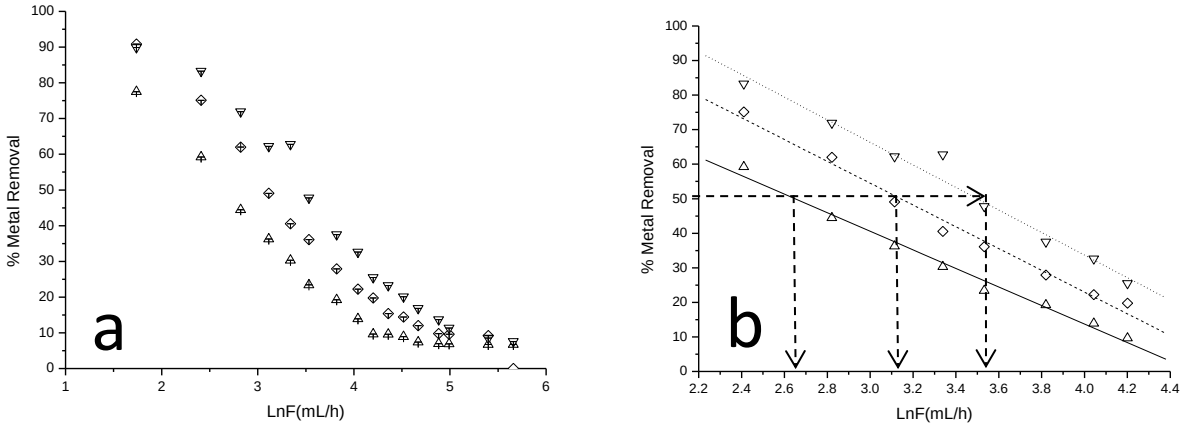


Figure 22: a) Comparison of the results obtained for metal removal of (M) sample at pH 5.5 without (△) and with pre-nucleation (◊) shown with results from (K) sample (▽). Error bars are shown only for the data for a) graph. **b)** Lines obtained from a) to calculate $FA_{1/2}$ before nucleation of (M)= 14.1 mL/h, $FA_{1/2}$ after nucleation of (M)= 23.16 mL/h. For (K) experiment $FA_{1/2}$= 33.16 mL/h.

The above results show that after 12 months of storage the biofilm retain only ~ 5% of the activity of the fresh biofilm. The $FA_{1/2}$ value of a fresh column containing (M) sample was 14.1 mL/h**.** In comparison the $FA_{1/2}$ value for (K) was 33.2 mL/h. The postulated additional nucleation via exposing the column in the standard solution overnight resulted in a consequent increase in the $FA_{1/2}$ value to 23.2 mL/h after testing at various flow rates. It was concluded that the $FA_{1/2}$ value was increased by 39.2%, by pre-nucleation in biofilms stored for 12 months. There was insufficient material to repeat this experiment

with additional samples. However, in the rest of this thesis, columns that had been used for multiple samples may have shown higher activity than freshly challenged columns and in future work an overnight static period would be recommended (Section 6.0).

SECTION D.

3.4 The effect of pH and *aqua regia* in the column

3.4.1 Effect of pH shock from pH 5.5 to pH 3.5

In an effort to mimic the acidic conditions of industrial wastes an initial experiment was done only for the (J) sample, in which a fresh column was 'shocked' by being challenged on the first day with the standard solution at pH 5.5, the second day with an inflow solution at pH 3.5 and the third day the pH of the inflow solution was recovered to pH 5.5. The results of this experiment can be seen in the following tables. The outflow column is the total phosphate released measured from the outflow of the reactor, while the precipitate column shows the result from the phosphate release inside the column, which is 1:1 with the metal recovered inside the column (molar ratio) (Singh, 2012). The total Pi is the sum of the outflow and the precipitate column.

Effect of ''pH shock'' in Pi for (J) experiment				
pH	**Column volumes (wash prior to sampling)**	**Outflow (mM)**	**Precipitate (mM)**	**Total Pi liberated (mM) $FA_{1/2}$ in metal removal (33 mL/h)**
5.5	2	1.02	0.53	**1.55**
3.5	2	0.18	0.20	**0.38**
5.5	2	0.50	0.50	**0.99**

Table 9: Effect on total Pi liberated when reducing the pH directly from 5.5 to 3.5 and then resetting back to 5.5 with no intermediate pH values at $FA_{0.5}$ (in metal recovery).

Table 9 confirms that when a younger column (H) was used the column activity did not return to normal with respect to phosphate release, after pH 'shock'. Throughout the

data it is confirmed the same notice that low pH columns do not ever recover full activity in phosphate release.

Effect of pH ''shock'' on Nd recovery for (J)		
pH	**Column volumes (wash prior to sampling)**	**% Nd Recovery at $FA_{1/2}$ 33 mL/h**
5.5	2	52.8%
3.5	2	19.5%
5.5	2	50%

Table 10: Effect on metal recovery when reducing the pH directly from 5.5 to 3.5 and then resetting back to 5.5

The experiment started at pH 5.5 and was restored to the same value after being reduced to 3.5 (''pH shock''). **Table 10** shows that the column does return to almost 100% activity after ''shock''. That notice goes against with **Table 9** and the experiments that follow. The results for pH ''shock'' in phosphate release are in accordance with the results of gradual shift for phosphate release (paragraph 3.4.2.) as well as the two isoezymes theory described by Jeong (2002), (mentioned in paragraph 1.6) since from 31.2% the recovery drops down to 7.6 % and then recovers back to 19.8 %. This suggests that after resetting the pH from high acidic conditions, the more pH sensitive isoenzyme CPII does not recover fully and therefore the restoration of the system to the initial conditions does not bring the initial phosphate released back to 31.2%. This pattern reflects the pattern of incomplete recovery of phosphate release discussed in section D. In an attempt to reduce this loss of activity the pH was taken down gradually 0.5 pH units at a time (Descending series(D)), then brought back gradually to pH 5.5 (Ascending series (A)).

3.4.2. Effect of gradual pH decrease to avoid shock in an attempt to retain phosphatase activity.

3.4.2.1 Effect of varying values of pH on (K) in total phosphate release (Pi)

Figure 23 shows the data for (K) sample with respect to the liberated phosphate concentration from the initial standard solution (pH 5.5) followed by progressively exposure to pH 3.5 and progressive recovery to pH 5.5 (in 0.5 increments).

Two columns run in parallel gave very similar results (repeatibility <5%-see Appendix VII). The initial value for total Pi liberated was close to 4.5 mM and decreased after that point. From the data obtained 4.42 mM of G2P reacts in the column and only 0.58 mM leaves from the column unreacted. That amount of G2P that has not reacted might be attributed to the low activity of the biomass, which had been stored up to this point in the cold room for 9 months and had lost 93 % of its initial activity (data obtained from **Table 8**).

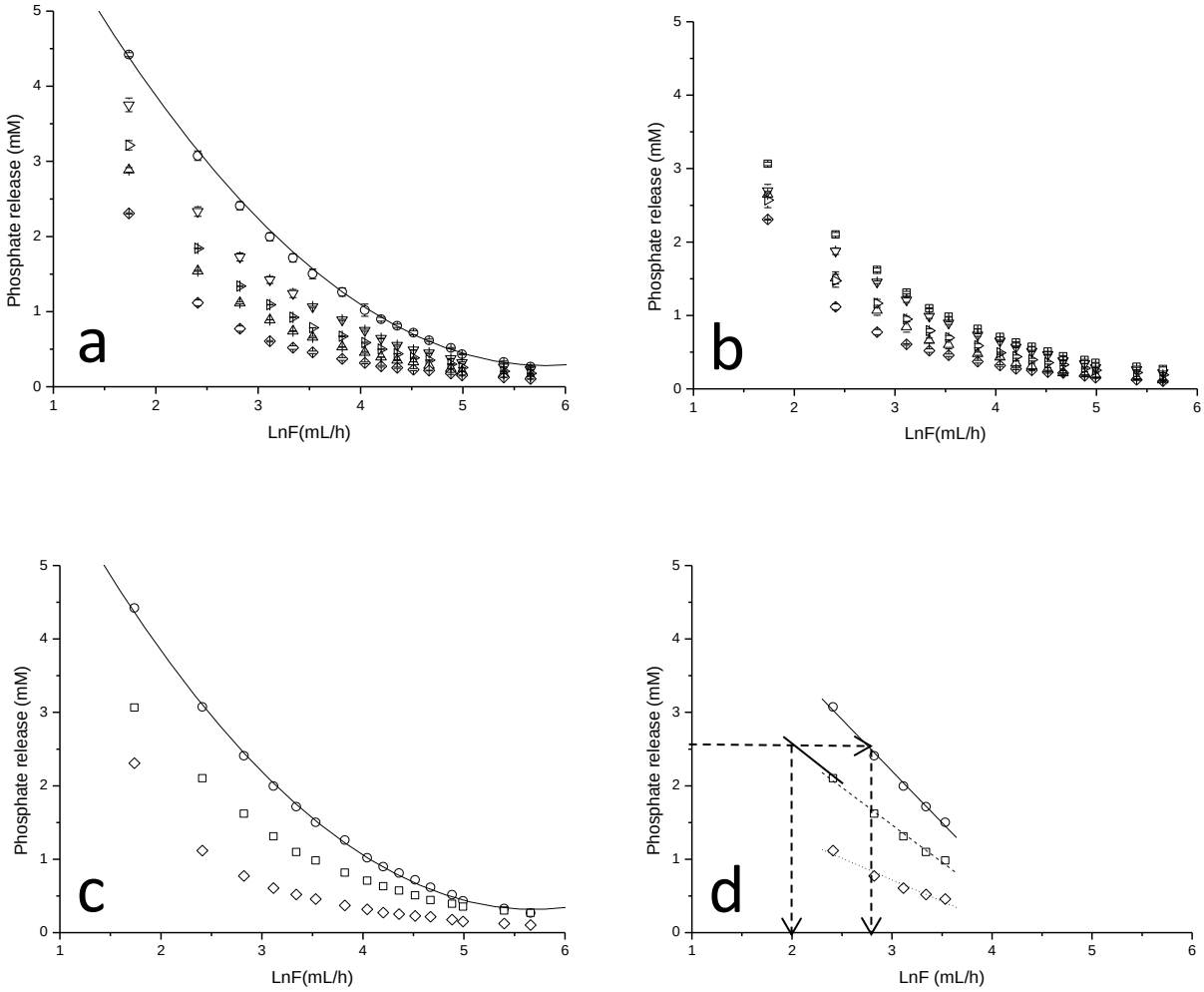


Figure 23: Effect of gradual pH change in the column activity for (K) sample. a) The total amount of liberated phosphate for decreasing gradually the pH from 5.5 to 3.5-Descending series (D) and increasing flow rates at each pH from 5.68 mL/h to 286.98 mL/h. Symbols used: pH 5.5 in the descending (D) series(O), pH 5.0 (▽), pH 4.5 (▷), pH 4.0 (△) and pH 3.5 (◊). **b)** The total amount of liberated phosphate for increasing gradually again the pH from 3.5 to 5.5-Ascending series (A) and increasing flow rates at each pH from 5.68 mL/h to 286.98 mL/h. Symbols used: pH 3.5 (◊), pH 4.0 (△), pH 4.5 (▷), pH 5.0 (▽) and pH 5.5 in the ascending (A) series (□). Error bars are shown only for graphs a) and b). Note the higher activity at pH 5.5 was lost when dropping the pH to 3.5 and then raising it again. **c)** Selected curves from graphs **a)** and **b)** of pH 5.5 in the descending series (D) (O), in the pH 3.5 (◊) and in the pH 5.5 in the ascending series (A) (□). **d)** The $FA_{1/2}$ values in Pi given from the lines obtained from the curves given in **c)** and are calculated only for the hollow circles (O - pH 5.5(D)) and squares (□-pH 5.5(A)). $FA_{1/2}$ in the descending series 16.2 mL/h, while that from the ascending series 7.3 mL/h. 54.9% of the column activity was non-recoverable when bringing the pH down to 3.5 from 5.5 and resetting it back again to 5.5.

Note: From this graph it is clear that the decrease in the pH causes decrease in phosphate release. An important note that has to be mentioned at this point is that the ionic strength of the solution changes with the extra Cl^- added and therefore we end up having two variables coupled, when increasing the acidic conditions, i) ionic strength and ii) change of the pH, which they can both be elucidated. A valid control could have been done to elucidate those two variables. That control would consist of two experiments a) Keeping steady the ionic strength and change the pH. Theoretically that could have been achieved by calculating for the lowest pH, what's the maximium amount of Cl^- added as HCl i.e. chloride buffers (using NaCl) could have been prepared for different pHs and for experiment b) By keeping pH the same (i.e 5.5) and change the ionic strength by using free acid this time and not buffer solution. Had the amount of 100 mM HCl added to increase the acidic conditions been measured, it could have been ploted as **Figure 23f)** the Cl^- concentration versus pH.

Almost 55% of the column activity was lost after this experiment. It was not possible to know which isoenzyme had aged more. Note, however that Jeong (1999) found that the ratio of CPI/CPII varied according to the specific activity. He made a detailed study of the growth conditions that promote production of one isoenzyme (CPI) over the other (CPII). The proportion of (CPI/CPII) found by Jeong was (4:1) for a total specific activity ranging from 650-750 U, while for higher activity cells (1500 U) the proportion was approaching unity (Jeong *et al*. 1999).

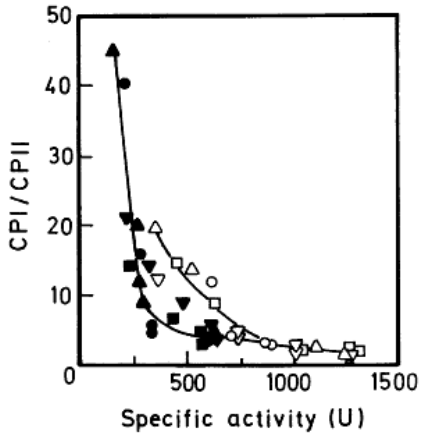


Figure 24: With filled symbols one can see the ratio of CPI over CPII in batch cultures of *Serratia* sp. N14), while in open symbol the phosphatase overproducing mutant dc5c (not related to this project). The *Serratia sp.* N14 cells were disrupted and CPI and CPII were extracted and separated by ion-exchange chromatography. Data from four batches are shown with solid symbols. Figure and data taken from Jeong's *et al.* data 1999.

Extending the results from the graph above it can be concluded that the molar ratio CPI/CPII for values greater than 1000 Units goes even closer to unity. From **Figure 22**, for slow flow rates (12 mL/h) the decrease observed in total phosphate release was from 3.07 mM in the descending series of pH 5.5 to 2.10 mM in the ascending series of the same pH value. Keeping in mind that at (K) experiment, the specific activity of the cells was approximately 2000 Units, the molar ratio of CPI/CPII is found to be 3.07/2.10=1.46.

Note, however, that the growth conditions used in each experiment were not exactly the same i.e. the growth conditions used by Jeong (Jeong *et al*. 1999) was batch culture (non carbon-limiting) whereas the present study used carbon limiting continuous culture. Other tests of Jeong *et al*. (1999) used glycerol as carbon source in continuous culture with dilution rate D=0.125 h^{-1} (V=3L vessel), while the growth conditions in this experiment used lactose as a carbon source, with dilution rate D=0.1 h^{-1} (V=2.5 L vessel). It can be speculated that the non-recoverable portion of enzyme activity following pH 'shock' is that of the more sensitive isoenzyme but fractionation tests were not performed to confirm this.

From **Tables 11** and **12** that follow it can be concluded that the effectiveness on phosphate release and therefore metal recovery of the *Serratia sp.* N14 species is reduced over a 12 month period, prompting the more detailed study of biofilm ageing (Section C).

The total liberated phosphate in standard solution was compared with previous research done when using the (H) sample. In the following tables blue color shows the results for (J), (K), (L) and (M) (this study), compared to (H)(Singh,2012) (pink color). The value $FA_{0.6}$ was used by Singh (2012) and therefore the same value was used in the next two tables in order to compare the data between them.

pH	Column volume	Pi liberated for (H) (mM)($FA_{0.6}$) (200.3 mL/h) (100%)	Pi liberated for (J) (mM) ($FA_{0.6}$) (32 mL/h) (16%)	Pi liberated for (K)/repeat (mM) ($FA_{0.6}$) (22 mL/h) (11%)	Pi liberated for (L) (mM) ($FA_{0.6}$) (18 mL/h) (9%)	Pi liberated for (M) (mM) ($FA_{0.6}$) (11 mL/h) (5.5%)
5.5	2	1.88	-	2.04/1.94	1.57	1.61
5.0	2	1.66	1.37	1.46/1.38	-	1.31
4.5	2	1.17	0.76	1.10/1.09	0.88	1.21
4.0	2	1.12	0.76	0.89/0.89	-	1.25
3.5	2	1.01	0.82	0.60/0.62	0.58	1.20
4.0	2	-	0.84	0.77/0.92	0.83	1.17
4.5	2	-	0.93	0.91/0.99	1.12	1.41
5.0	2	-	1.02	1.18/1.23	1.19	1.47
5.5	2	-	1.06	1.29/1.33	1.20	1.51

Table 11: Effect on Pi liberated when dropping down the pH gradually from 5.5 to 3.5 and then resetting to 5.5 with intermediate pH values at $FA_{0.6}$ (in metal removal). By setting the flow rate to a reference value for each experiment in each case the activities can be normalized. In this table the $FA_{0.6}$ is used in all experiments, since $FA_{0.6}$ for metal removal was used for (H) (Singh, 2012). The experiment performed by Singh (2012) was done only for the pH descending series. From the table it can be seen that the pattern followed is the same: an initial value which decreases when dropping the pH to 3.5 and then increases back to a value lower than the initial. Where two numbers are shown there are duplicate experiments.

Figure 25 shows for the (K) experiment the total phosphate release for all pH values at a specific flow rate of 34 mL/h ($FA_{1/2}$ of metal removal).

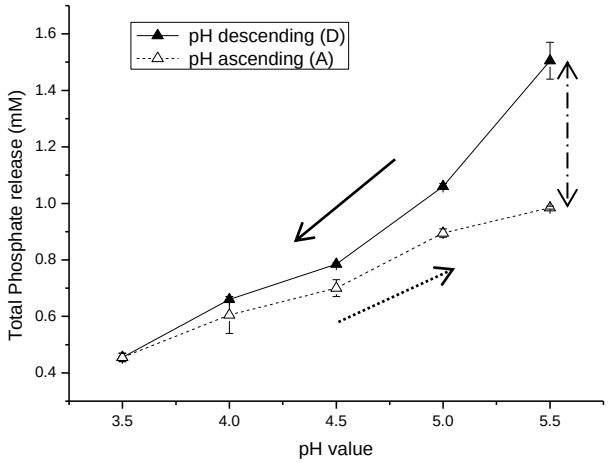


Figure 25: Total phosphate (Pi) release for changing values of pH for 9 months old *Serratia* sp. N14 coated foam discs at a flow rate of 34 mL/h, which was the $FA_{1/2}$ value at pH 5.5 for metal removal (see next section). Data from two columns are averaged.

Figure 25 shows that there is a non-recoverable portion of activity (~40%), which might be attributed to the hypothesis of the two isoenzymes. The total phosphate release does not reach its initial values, due to the drop of the inflow solution to pH 3.5, which may make the more sensitive isoenzyme CPII inactive and not capable of recovery when resetting the pH back to 5.5.

3.4.2.2 Effect of varying values of pH using (K) sample in metal recovery

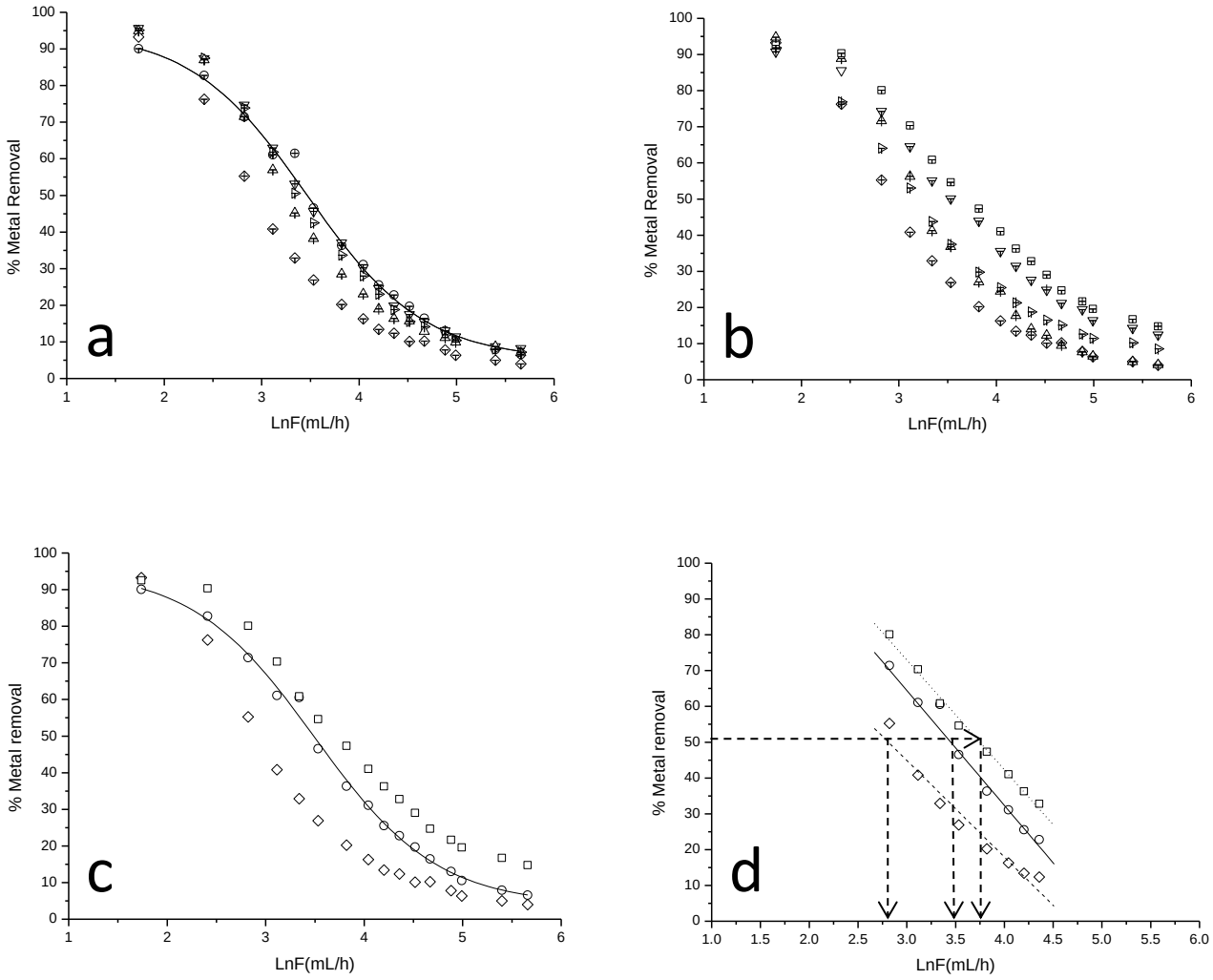


Figure 26: a) Effect on Nd recovery of gradually decreasing the pH from 5.5 to 3.5 for increasing flow rates at each pH from 5.68 mL/h to 286.98 mL/h. Symbols used: pH 5.5 in the descending (D) series(O), pH 5.0 (▽), pH 4.5 (▷), pH 4.0 (△) and pH 3.5 (◊). **b)** Effect on Nd recovery for increasing gradually again the pH from 3.5 to 5.5 for increasing flow rates at each pH from 5.68 mL/h (5rpm) to 286.98 mL/h (260 rpm). Symbols used: pH 3.5 (◊), pH 4.0 (△), pH 4.5 (▷), pH 5.0 (▽) and pH 5.5 in the ascending (A) series (□). Error bars are shown only for graphs a) and b). **c)**Selected curves from graphs **a)** and **b)** for pH 5.5 in the descending series(D) (O), pH 3.5 (◊) and pH 5.5 in the ascending series(A) (□). **d)** The $FA_{1/2}$ values in metal removal given from the lines obtained from the curves given in **c)** and are calculated only for the open circles and diamonds (O-pH 5.5(D), ◊-pH 3.5) and open squares (□-pH 5.5(A)). $FA_{1/2}$=33.1 mL/h at pH 5.5 in the descending series, $FA_{1/2}$=16.6 mL/h at pH 3.5 and $FA_{1/2}$= 42.4 mL/h at pH 5.5 again in the ascending series.The activity of the cells drops to 49.8% of its initial value when lowering the pH to 3.5.

The same **Note** made on page 76 paragraph 3.4.2.1 applies for these results as well. In the tables that follow it can be seen the effect of changing the pH in more acidic conditions down to 3.5, on the metal recovered in the column, with increasing flow rates.

T**able 12** shows that for 60% metal removal, all the results seem to be cohesive, (pH 5.5 for (J) was not taken in account, since as already mentioned the column collapsed due to an unknown error). The same pattern is followed as expected in the descending/ascending experiment that shows that pH ''shock'' was not the problem.

pH	Column volume	% Nd recovery at 200.3mL/h ($FA_{0.6}$) (H) (100%)	% Nd Recovery at 32mL/h ($FA_{0.6}$) (J) (16%)	% Nd recovery at at 22mL/h ($FA_{0.6}$) (K/repeat) (11%)	% Nd recovery at 18mL/h ($FA_{0.6}$) (L) (9%)	% Nd recovery at 11mL/h ($FA_{0.6}$) (M) (5.5%)
5.5	2	51.73%	-	62.2/60.0%	53.9%	59.2%
5.0	2	52.73%	64.0%	64.1/61.8%	-	73.3%
4.5	2	48.03%	50.9%	61.8/61.9%	60.3%	74.8%
4.0	2	50.62%	52.9%	57.2/56.8%	-	85.9%
3.5	2	47.89%	58.7%	40.7/40.9%	49.2%	81.1%
4.0	2	-	55.2%	53.0/59.0%	60.5%	75.3%
4.5	2	-	62.9%	51.2/54.9%	75.0%	81.7%
5.0	2	-	64.7%	64.5/64.6%	68.4%	83.5%
5.5	2	-	62.1%	71.2/69.5%	76.8%	86.4%

Table 12: Effect on metal recovery when reducing the pH gradually from 5.5 to 3.5 and then resetting back to 5.5 using (H), (J), (K), (L) and (M) biofilms.

From **Table 12** the $FA_{0.6}$ for (K) was observed at 22mL/h, while for (H) it was observed at 200mL/h. Moreover, from the whole experiment it was noticed that the more the biomass had stayed in the cold room, the longer it required to recover in the standard solution. **Figure 27** shows the results for the $FA_{1/2}$ values in metal removal, for all the pH values at a flow rate set at 34 mL/h using cells from the (K) sample.

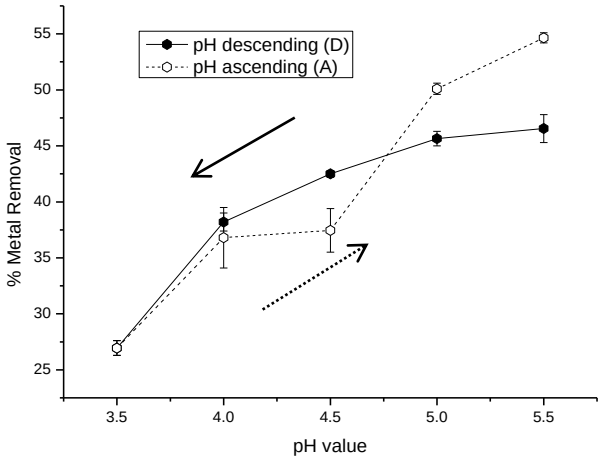


Figure 27: Metal removal for changing values of pH at $FA_{1/2}$ (34 mL/h) using (K) (averaged data from two experiments). In this experiment the inverse was shown to the phosphate release in **Figure 25.** The reason for this is not clear, but will be re-examined in subsequent sections. Possible explanation for that might be the column conditioning noted earlier (Section C) and suggested as additional nucleation.

3.4.2.3 Effect of varying values of pH using (R) sample, in (Pi) release

Figure 28 shows the pH experiments that were repeated for 3 month old biofilms from main culture (II) vessel. The **Note** of paragraph 3.4.2.1 applies also for these results.

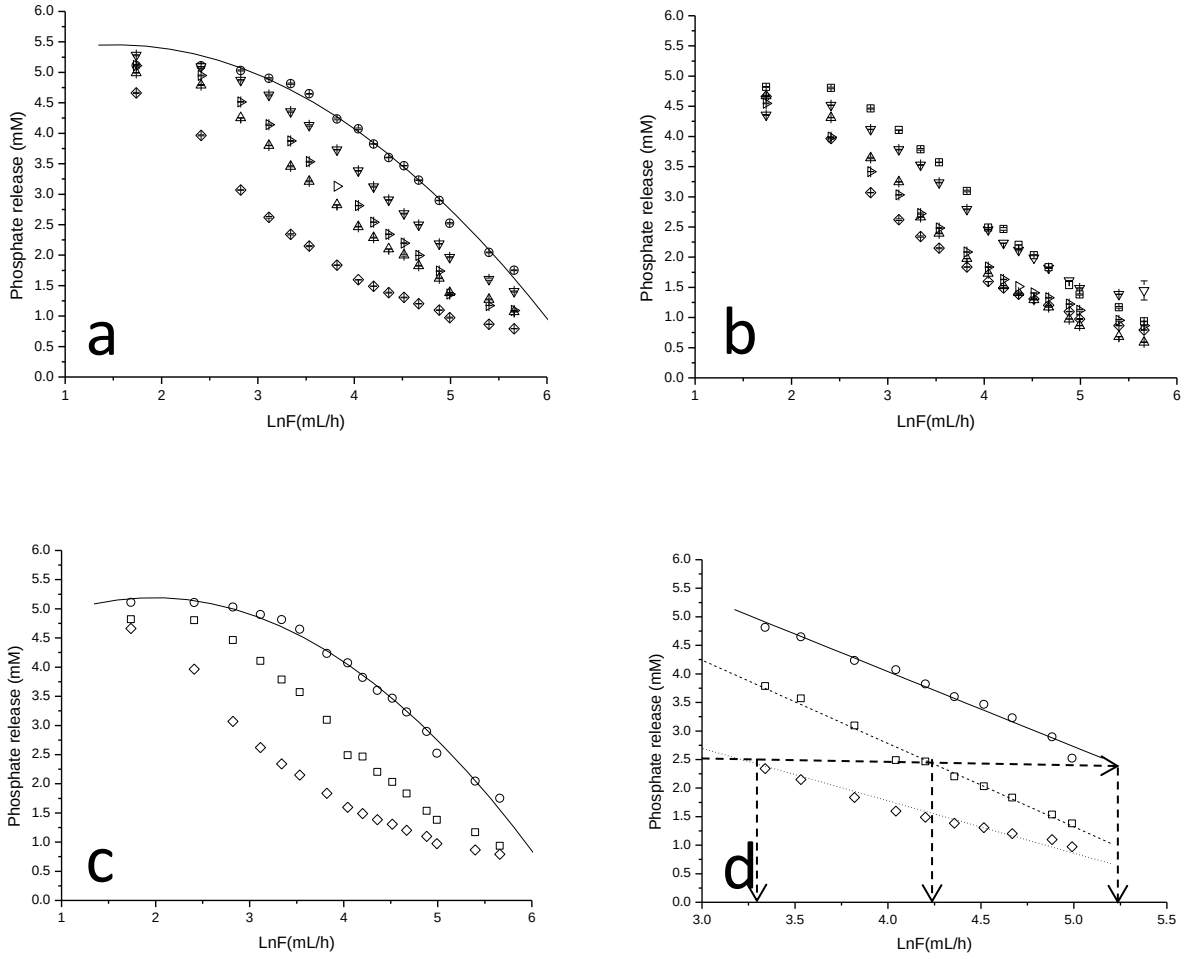


Figure 28: a) Phosphate liberation with respect to flow rate at pH reduced stepwise from 5.5 to 3.5 and increasing flow rates (from 5.68 mL/h to 286.98 mL/h. Symbols used:pH 5.5 in the descending (D) series (o), pH 5.0 (▽), pH 4.5 (▷), pH 4.0 (△) and pH 3.5 (◊). **b)** by increasing the pH step-wise from 3.5 to 5.5 and increasing flow rates (from 5.68 ml/hr to 286.98 ml/hr). Symbols used: pH 3.5 (◊), pH 4.0 (△), pH 4.5 (▷), pH 5.0 (▽) and pH 5.5 in the ascending (A) series (□). Error bars are shown only for graphs a) and b). (triplicate assays). **c)** Selected curves from graphs **a)** and **b)** for pH 5.5 in the descending series (D)- (o), pH 3.5 (◊) and pH 5.5 in the ascending series (A) (□) . **d)** The $FA_{1/2}$ values in Pi release given from the lines obtained from the curves given in c) and are calculated only for the open circles (at pH 5.5(D), open diamonds at pH 3.5) and open squares(□-pH 5.5(A)).

From **Figure 28** $FA_{1/2}$ in the descending series is 176.28 mL/h, while that from the ascending series is 66.28 mL/h. Almost 62.4% of the activity was lost when bringing the pH down to 3.5 from 5.5 and resetting it back again to 5.5.

3.4.2.4 Effect of pH using (R) sample in metal removal

Metal removal was examined at pH 5.5-3.5 (descending step-wise) and at pH 3.5-5.5 (ascending step-wise) as shown in **Figure 29**.

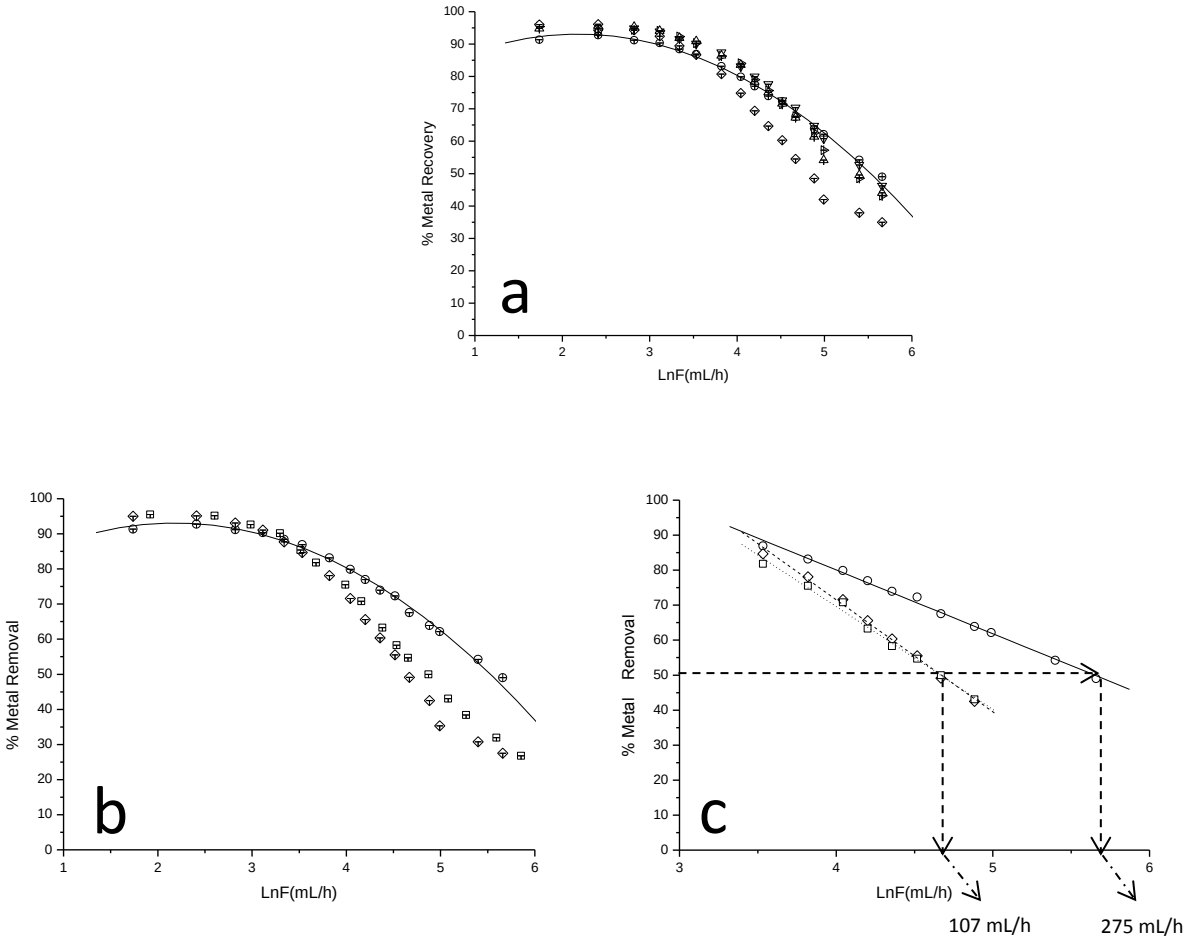


Figure 29: a) Nd recovery with respect to flow rate from pH 5.5 to 3.5. Symbols used: pH 5.5 in the descending (D) series (O), pH 5.0 (▽), pH 4.5 (▷), pH 4.0 (△) and pH 3.5 (◊). **b)** Selected curves from **a)** for pH 5.5 in the descending series(D)- (O), pH 3.5-(◊) (pH 5.5 in the ascending series (A)(□). **c)** The $FA_{1/2}$ values in metal removal given from the lines obtained from b) and are calculated only for the open circles and diamonds (O -pH 5.5 and ◊-pH 3.5).

The same Note made on page 76 applies also in this experiment. From **Figure 29** the $FA_{1/2}$ values found were: $FA_{1/2}$= 274.89 mL/h at pH 5.5 in the descending series, $FA_{1/2}$= 106.81 mL/h at pH 3.5 and $FA_{1/2}$= 106.23 mL/h at pH 5.5 in the ascending series.The activity of the cells is reduced by 61% of its initial value when lowering the pH to 3.5.

At this experiment it was noticed that although the biomass initially had the same behaviour compared with the biofilm described by Singh, (2012) i.e culture (I) biofilm tested at three months, (the $FA_{1/2}$ value was identical as shown in section A), it showed a different behaviour when doing the ascending pH experiment. More specifically in the descending series the biomass behaved as was expected and reached a low value in acidic conditions at pH 3.5. However, after that point the biofilm did not recover. A possible explanation is the change of the flow rate, probably due to the contact of the tubing with the pump head. More possible explanations for that and the figure produced when plotting the data can be seen in Appendix III page 6.

In contrast when the same experiment was done previously (Singh,2012) the biofilm recovered completely in metal removal, when resetting the pH from 3.5 to 5.5. However, since the previous experiments in this study using aged biofilm from fermenter (I) all showed (partial) recovery upon return to pH 5.5 and the present experiment was not repeated, this result was considered to be eroneous. The question of biofilm recovery following exposure to pH 3.5 will require re-examination in future work. It is possible that pH 3.5 represents a sharp ‘’tipping point’’ below which the enzyme cannot recover. Further studies are beyond the scope of this study.

A summary table from the results seen so far in the Descending-Ascending series of tests follows.

% activity at $FA_{1/2}$(or $FA_{0.6}$) value				
	% activity before pH descent (pH 5.5)		% activity after pH descent (pH 5.5)	
	Pi release	Metal recovered	Pi release	Metal recovered
Cuture(I)				
(K) ($FA_{0.6}$)	100	100	36.8	114.4
(K) (repeat) ($FA_{0.6}$)	100	100	31.4	115.8
(L) ($FA_{0.6}$)	100	100	23.6	142.4
(M) ($FA_{0.6}$)	100	100	6.3	145.9
Culure(II)				
(R) ($FA_{1/2}$)	100	100	61.8	61.35

Table 13: Summary of the results seen so far for the step-wise change of pH value.

For Culture (I) Pi release it can be noticed that the longer the biofilm remained in the cold room, the less% activity it was lost after the descending-ascending pH test. The inverse is noticed for metal removal of the same culture. The greater than 100% in the last column, can be justified from the pattern shown in **Figure 27**, where for all the columns of culture (I) the $FA_{0.6}$ value after the pH test was higher than the initial value before the test. Possible explanation for that is that the columns at pH 5.5 (D) was not nucleated, but at pH 5.5 (A), it was. As for culture (II) the results seem to be identical.

3.4.3 Effect of *aqua regia* in the column (Initial study to determine effect of chloride and nitrate decoupled from pH effects)

This study shows that the effect of low pH (shock or gradual change) was to inhibit ~ 24 % of the column activity in Pi release and 29 % in metal removal, but the residual column activity was stable. This column was designated as ''conditioned'' for subsequent studies to impose additional stresses. The conditioned column was challenged with *aqua regia* solution to check its durability in high salt concentration. **Table 14** shows the change in phosphate concentration and metal recovery before and after challenging the column with various concentrations of *aqua regia* (pH 5.0). A small increase in activity in 20% *aqua regia* solution can be attributed to the fact that the column had been ''rescued'' in

the standard solution overnight. The experiments for 30% and 40% were conducted on the same day with recovery steps, so therefore the column did not have the same opportunity to recover in the standard solution.

(J) sample pH value 5.0

aqua regia concentration	Metal Removal at $FA_{1/2}$ value	Total Pi Phosphate release (mM) – ($FA_{1/2}$ in metal removal)
0%	49.6%	1.704
10%	49.8%	0.686
20%	68.5%	0.921
30%	51.1%	0.575
40%	47.2%	0.534

Table 14: (J) Sample. Effect of neutralized *aqua regia* (pH 5.0) on total Pi liberated and metal recovery, at various concentrations (%v/v).

The conclusion from this table is that the column is stable at pH 5.0 in the presence of 40% neutralised *aqua regia* with respect to metal removal, but phosphate release is reduced by 2/3rds at 30% and 40% *aqua regia* salt concentration. However, since phosphate is present to excess over the metal removal the metal precipitation is little affected. Importantly, this test shows that excess NO^-_3 and Cl^- at pH 5.0 have little effect on metal removal. Other tests at lower pH values/concentrations of *aqua regia* showed a permanent inactivation at pH 2.0 and 50% *aqua regia.*

A study was carried out at pH values of 2.0 and 1.0 below the pH inhibition ''cut off'' of the enzyme (**Table 15** below). However, the results of **Table 15** appear to contradict the pH study of the previous section, since metal removal appears to be identical at pH 5.5 and 2.0, despite that previous work (Singh, 2012) and current study have shown that pH 3.5 is the lowest practical operational pH. Note, however that the final pH of the column and exit solution was not checked.

Table 15 that follows shows the results of the final experiment when leaving the column at pH 1.0 and 50% *aqua regia* for two days and calculating phosphate release and metal recovery on the third day. For very high acidic conditions such as for this case, the experiment was performed in the fume hood and only for the 50% concentrated *aqua regia*. At this point it has to be mentioned that the golden color produced when mixing HCl and HNO_3, was due to the production of nitrosyl chloride (NOCl). NOCl would be present in higher concentrations for low pH solutions, but not for high (pH 5.0).

pH	***aqua regia* concentration**	**Metal Removal ($FA_{1/2}$)**	**Total Pi (mM) – ($FA_{1/2}$ in metal removal)**
5.0	**0%**	55.1%	0.978
2.0	**10%**	59.3%	0.778
2.0	**20%**	52.1%	0.692
2.0	**30%**	65.0%	0.792
2.0	**40%**	65.6%	0.795
2.0	**50%**	58.5%	0.703
1.0	**50%**	22.2%	0.592

Table 15: (K) sample. Effect of neutralized *aqua regia* (at various concentrations v/v%) at pH 2.0 and 1.0 on metal recovery. Total metal recovery loss 30% at $FA_{1/2}$ in metal recovery (Standard solution=pH 5.5), **b)** Effect of 50% neutralized *aqua regia* at pH 2.0 and 1.0 on total phosphate release. Comparison with $FA_{1/2}$ values in metal removal and total Pi, obtained for (K) after the pH descending-ascending experiment, before the use of *aqua regia*.

Due to strong acid-strong base equilibrium in the neutralization of *aqua regia* with NaOH, the pH value of the *aqua regia* solution would change very easily even with small quantities of NaOH added. Therefore, since the pH of the column was nominally at 5.5, the inflow solution may not preserve the pH value indicated from the pH-meter, once mixed with the column solution (note the citrate buffer concentration was only 2mM). It is also possible that 50% *aqua regia* at acidic pH caused spontaneous chemical hydrolysis of G2P and phosphate release. Although that was not tested, an easy control experiment

that could have been done, was the addition of G2P in 50% *aqua regia,* to check if phosphate was produced.

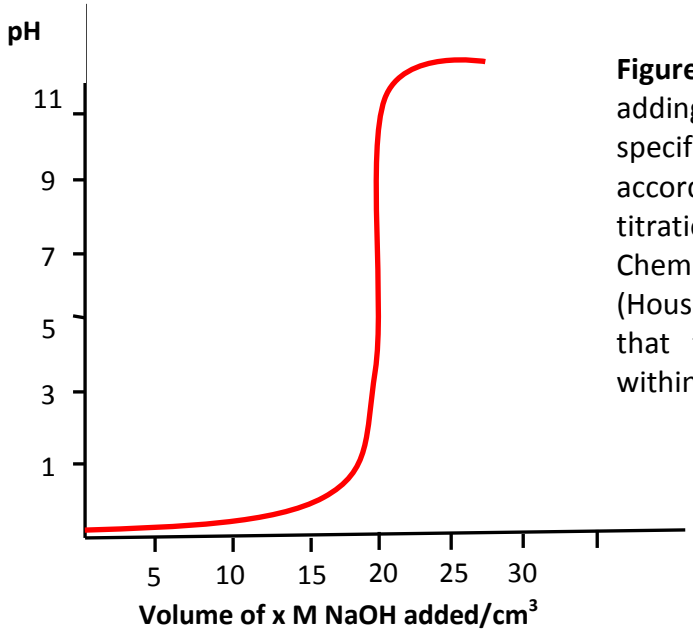


Figure 30: The variation in pH when adding an amount of x M NaOH to a specific amount of aqueous HCl according to strong acid-strong base titration. Figure reproduced from Chemistry Housecroft and Constable (Housecroft C.E. *et al*. 2010). This shows that the persistence of the set pH within the column cannot be assumed.

Due to this potential problem the tests to determine tolerance of the columns to *aqua regia* are uninterpretable in the absence of rigorous pH control. To increase the concentration of citrate buffer is impractical as this would form metal complexes. Further experiments were not possible due to limited supply of biofilm material.

After the pH descending –ascending experiment the purpose of this section was the use of *aqua regia* to kill the column. The overall goal was to establish the usefulness of the biofilm in real life i.e how tolerant was the biomass to low pH values or high salt concentration, since the recovery of metals from solid scraps, would require leaching in strong acid.

The overall conclusion is that the anion concentration of *aqua regia* does not affect column activity, but the effect of reduced pH cannot be readily estimated without a more rigorous means of pH control.

SECTION E

3.5 Outflow chamber biomass

3.5.1 Phosphatase activity of the biofilm

The phosphatase specific activity of the cells was tested from culture (I) initially for (J) sample, by squeezing off biofilm from the foam discs; fermentor (II) outflow was collected and the activity of the free cells in the outflow chamber was tested on the 2nd, 5th, 6th and 7th day for two different samples taken from the outflow chamber liquid (outflow stream) and was estimated through the equation:

Activity=[OD_{410} 10^9/(t 18472)] [7.4/10^3 0.552 OD_{600})] (Jeong, 1992, Mennan, 2010)

The total specific activity was calculated initially for (J) biofilm cells and for the fresh biomass from the outflow chamber reactor **(Table 20)**.

Specific activity (U)	Sample (J) (U)	Free cells from outflow chamber liquid (U)			
		Day 2	Day 5	Day 6	Day 7
Sample 1	1713	-	1881	1723	1263
Sample 2	2074	2576	2177	2015	1451
mean	1894	2576	2029	1871	1357

Table 16: Specific activity of the cells for two samples from (J) and from outflow chamber biofilm for days: 2-5-6-7.U: units(nmol product/min/mg protein).

Although the conditions were not the same, in both cases we compare phosphatase activity of the *Serratia* sp. N14 cells, which are intended to be used for Nd recovery. The

average values from the outflow chamber were compared to the activity of the cells taken from the foam discs (J) and they were found similar up to the 6th day of the continuous flow. The specific activity of the cells on harvest from (J) was on average 1894 U (activity units) and the specific activity of the free cells of the outflow chamber on the 6th day become comparable to the (J) cells (1869 U).

3.5.2 Examining the biofilm formed in the outflow chamber

Figure 31 shows that although the biofilm coating deposited in the outflow vessel is hardly visible (foam disc no 2) , it can be compared with the coating made in the main fermentor vessel (in terms of activity of the biomass) and which has been kept in the cold room for 1 year (foam disc no 3).

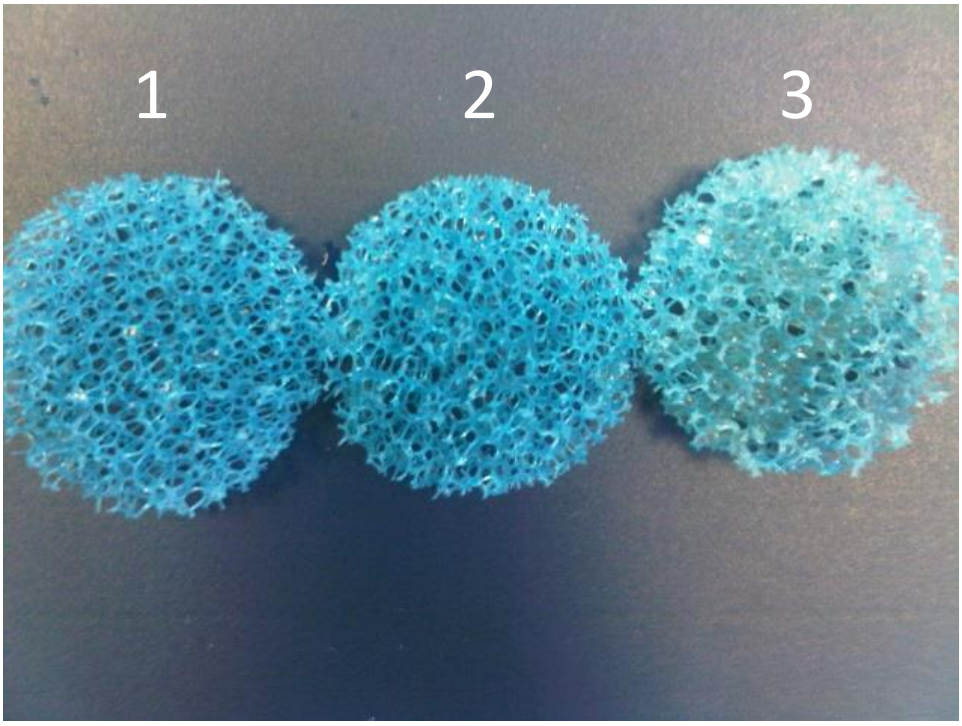


Figure 31: 1) foam disc with no coating at all, 2) foam disc coated with *Serratia* sp. N14 from outflow chamber, 3) foam disc coated with *Serratia* sp. N14 from main fermentor vessel kept in the cold room for 1 year.

On the second day after switching to continuous flow the coating of the biofilm on the foam discs started to be visible in the main vessel reactor, but biofilm was not yet visible in the outflow chamber foam discs. After the 3rd day biofilm started to be visible on foam in both the outflow chambers, and it was also noticed that the suspension in the vessel that was receiving the inhibitor was more transparent than the control vessel, due to dilution by the inhibitor dose stream (~30% dilution-see II. METHOD DEVELOPMENT, paragraph 2.6) (**Figure 32a).** However, the total cells dosed into both off-chambers were the same and no growth could occur due to carbon depletion within the main vessel mainstream.

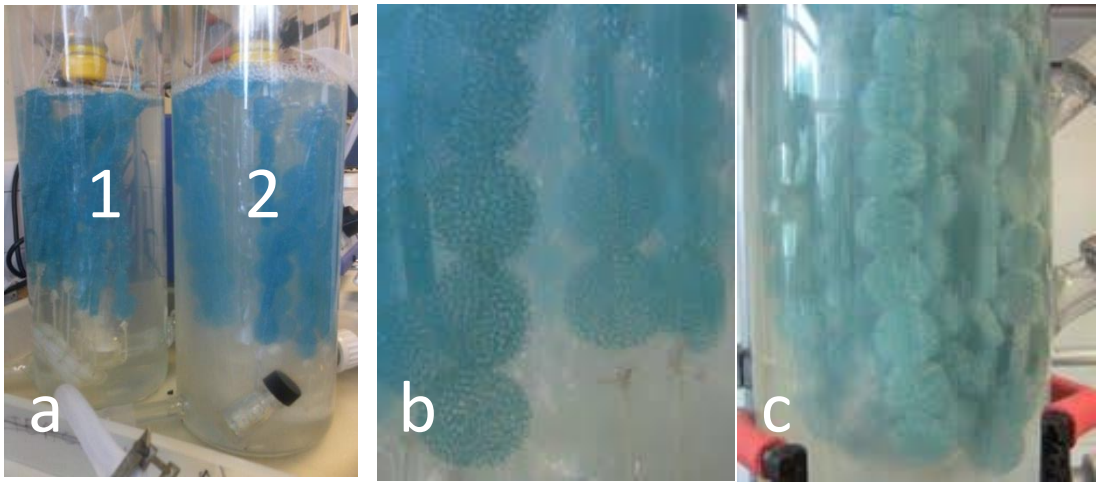


Figure 32: a) the two outflow vessels **1)** Including the inhibitor (more transparent solution), **2)** without the inhibitor, **b)** Foam discs inserted in the outflow vessel of this study **(a2)**, on the third day when the first signs of biofilm started to appear on foam discs and **c)** Visible *Serratia* sp. N14 coated on the foam discs of the main vessel at the same time (picture **c** taken by Dr. Jimmy Roussel). Fermentation in main vessel conducted by Dr. A. Murray.

On the 7th day the system was disconnected and the biomass produced on the foam discs (main chamber and off-chambers) was drained and placed in plastic containers, above isotonic saline (8.5%w/v, Paterson-Beedle & Macaskie, 2005), covered with aluminum foil, sealed in polyethylene bags and stored at 4^{o}C prior to testing.

3.5.3 Examination of columns prepared using outflow chamber biofilms

3.5.3.1 Metal recovery using (S) sample

One week after storage at 4^{o}C, 8 foam discs were weighed and placed in the column reactor in order to be examined. The difference in biofilm on the foam discs taken out of the main vessel was much higher (weight of 8 foam discs 2.2181 g), compared with the biofilm produced in the outflow chamber (0.9418 g), indicating a thicker biofilm deposition in the main chamber.

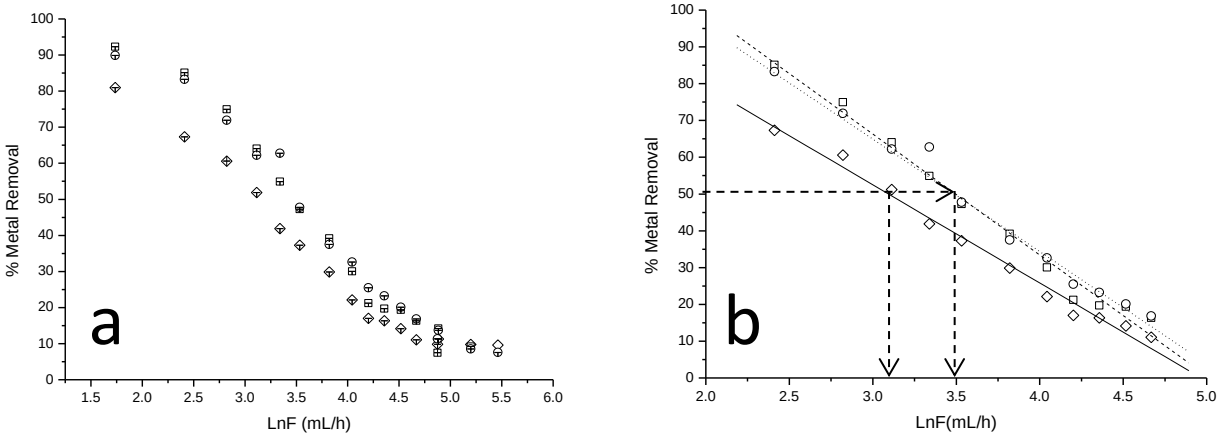


Figure 33: a) Comparison of the metal removal using (S) sample at pH 5.5 before (open diamonds) and after (open squares) nucleation (see section C) compared with a (K) column (open circles). Error bars are shown only for graph a), **b)** lines obtained from a) to calculate the $FA_{1/2}$ before nucleation for (S) =22.57 mL/h, $FA_{1/2}$ after nucleation for (S) =32.96 mL/h. For (K) $FA_{1/2}$=33.68 mL/h (before nucleation).

Figure 33 shows that when using the biofilm from the outflow chamber following nucleation (24 hours), the columns remove metal comparably to main chamber biofilms stood for 9 months. At 12 months the biomass had lost more of its ability to recover neodymium (sections B and D), but by leaving the column for 24 hours to nucleate (section C), the results are comparable as when using the biomass directly from the outflow chamber before nucleation.

The results are summarized in the following table:

Summary		
Use the biomass from outflow chamber before nucleation $FA_{1/2}$=22.57 mL/h (Fig.33b)	**Or**	**Use the biomass from main vessel after nucleation for 24 hours, which had been stored in cold room for 12 months $FA_{1/2}$= 23.16 mL/h (Fig.22b)**
Use the biomass from outflow chamber after nucleation for 24 hours $FA_{1/2}$=32.96 mL/h (Fig.33b)	**or**	**Use the biomass from main vessel before nucleation, which has been stored in cold room for 9 months $FA_{1/2}$= 33.68 mL/h (Fig.33b)**

Table 17: Comparison of biomass used from outflow chamber and main fermentor vessel. Results for nucleation can be seen in section C.

An interesting observation was that since the biofilm obtained on the foams in the outflow vessel is hardly visible, surface of the biofilm was still sufficient to interact with the metal. The better results after nucleation (nucleation enhanced activity as seen in section C) obtained, are probably attributed to diffusion of the substrate into deeper layers of the biofilm and therefore a higher amount of substrate reacting with the cells, which means more phosphate released and therefore more metal recovered.

The outflow nucleation observation could again form the basis for future experiments, where the main vessel fermentor does not have to be run 7 days for biofilm formation, but less than that, since biomass was visible coated on foam discs, by the 3rd day of the fermentation.

3.5.3.2 Pi release using (S) sample

On the other hand for the case of total phosphate released (Pi) from (S), the biofilm behaved in a similar way as if storing the biofilm from the main vessel at 4^{o}C for 10 months **(Figure 34)**.

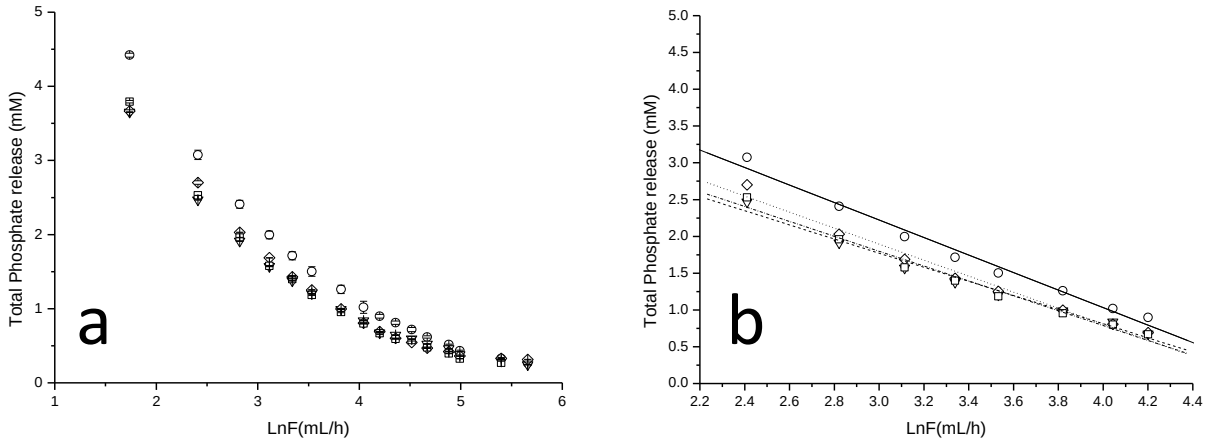


Figure 34: a) Comparison of total Pi released using (S) sample at pH 5.5 before (open diamonds) and after (open squares) nucleation, shown with (K) (open circles) and (L) (open triangles) samples. Error bars are shown only for graph a), **b)** lines obtained from a) to calculate the $FA_{1/2}$ (in total Pi) before nucleation of (S) =11.52 mL/h, $FA_{1/2}$ after nucleation of (S) =10.03 mL/h. For (K) $FA_{1/2}$=15.9 mL/h and for (L) $FA_{1/2}$=9.4 mL/h (before nucleation).

Figure 34 shows that the biomass from the outflow chamber can be compared with the (L) experiment with respect to Pi release. In contrast to metal removal, the addition of a pre-nucleation step did not influence the ability of the columns to produce phosphate. This proves that the enhanced acitivity was due to nucleation and not to any enhanced substrate penetration into the biofilm.

SECTION F.

3.6 Comparison of biofilm grown in the main chamber and outflow chamber. Effect of storage and pH on outflow chamber biofilm.

The biofilm produced in the main vessel from Culture (II) was compared with the biofilm produced in the outflow chamber at 0 months (before storage at 4^{o}C) and 3 months after being stored in the cold room. The $FA_{1/2}$ values are shown and compared in the following table.

Culture(II)	Main fermenter $FA_{1/2}$	Outflow chamber $FA_{1/2}$
0 months	505 mL/h	22.5 mL/h
3 months	275 mL/h	2.49 mL/h

Table 18: Comparison of $FA_{1/2}$ values for 0 and 3 months from the main and outflow vessel. Value 505 mL/h taken by Singh's work (2012). Value 275 mL/h shown in sections A and B. Value 22.5 mL/h is taken from section E, while value 2.49 mL/h is an extrapolated value shown in detail in the following figure.

Hence, from **Table 18** it can be seen that using main chamber biofilm the activity approximately halves during 3 months of storage, whereas that from the outflow falls ~ 10 fold. It is concluded that while the new method produces biofilm of acceptable activity, the long term storage prognosis is poor and this method would have little potential as an alternative method of biofilm growth for real commercial application. The reason for the short life of the off chamber biofilm was not investigated.

Three months after storage at 4^{o}C the outflow chamber biofilm was tested with respect to phosphate release and metal removal. The results for metal removal were compared with (H) sample produced from culture (I) and (R) sample from culture (II), both from the main vessels. The results are shown in the following figure.

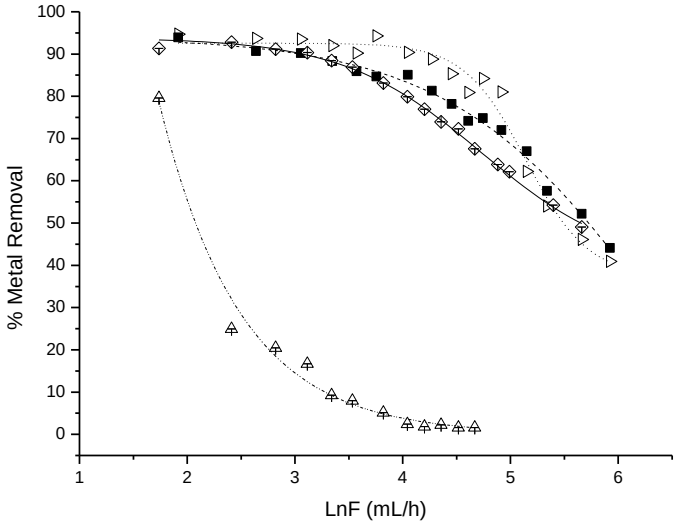


Figure 35: Metal removal using (H) sample, (▷):Nd, (■):Eu, (R) sample, (◊):Nd and (V) sample (△):Nd. Error bars are shown only for (R) and (V) samples. Data for (▷):Nd, (■):Eu were taken by Singh's work (2012).

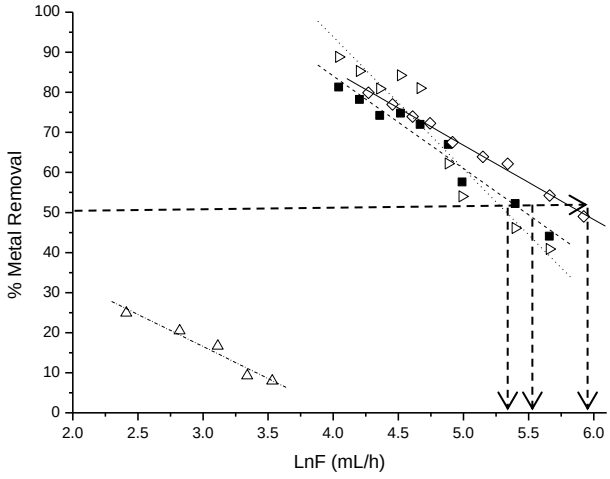


Figure 36: Lines obtained from the above figure for calculation of $FA_{1/2}$ values. Data (▷):Nd, (■):Eu were taken by Singh's work (2012). For Nd:(▷) from (H), $FA_{1/2}$ =273 mL/h, for Nd:(◊) from (R), $FA_{1/2}$ =275 mL/h and from (V), $FA_{1/2}$ =2.49 mL/h.

From this figure it can be seen that after 3 months of storage the biofilm produced in the main vessel is more effective (100 fold stronger to recover Nd). From the **Table 18** and **Figure 35** it is concluded that biofilm produced in the outflow chamber is not only already

weak, but also more sensitive, since it cannot be stored ($FA_{1/2}$ found from figure **36)** is not a good estimate). A simple pNPP test was performed 4 months after keeping the biofilm from the outflow chamber at 4°C and the yellow color produced (explained in section I. GENERAL PROCEDURES) could hardly be visible.

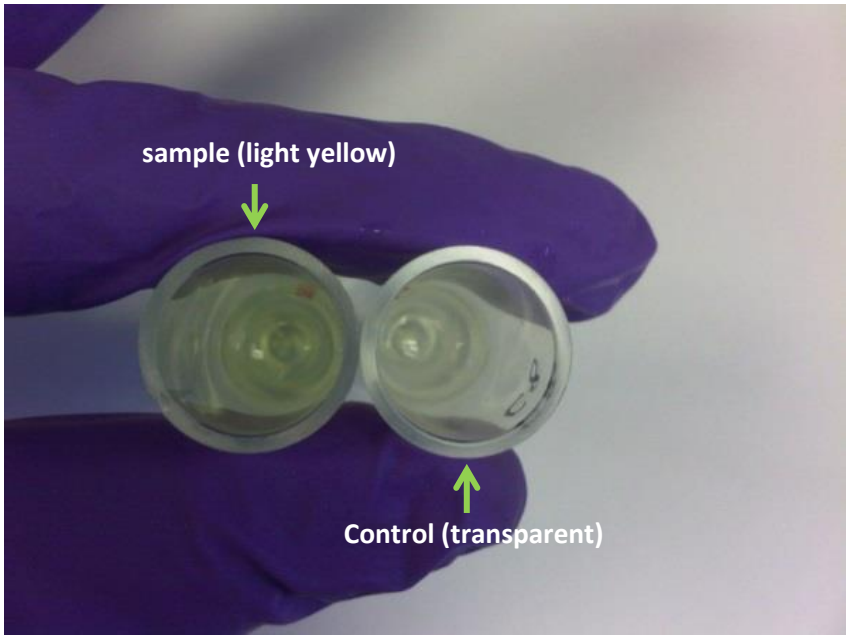


Figure 37: pNPP test for biofilm produced in the outflow chamber and stored at 4°C for 4 months. The difference in color was only obvious when focusing the camera from the top of the tubes and the specific activity was found to be at a very low value of 157.8 Units (in comparison value using (J) sample, which was ~1900 U and the Total Phosphate release for that was found to be 2.4 mM at 16 mL/h).

This confirms that the loss of activity in stored biofilm of the outflow chamber was attributable to loss of activity in the outflow chamber biofilm as compared to main chamber biofilm. The reasons underlying the enzyme stabilization are unclear.

However, the cells in the main chamber outflow have been ‘‘selected’’ as the less adhesive cells in the population. Since much of the enzyme is held tethered supported within the extracellular polymer, it may be that the polymer of this sub population is less

well defined and less ''supportive'' to the enzyme, but further tests were beyond the remit of this study.

3.6.1 Effect of pH in outflow chamber biofilm.

The pH descending-ascending experiment (step-wise) was performed for sample (V). The $FA_{1/2}$ in total Pi has dropped from 11.52 mL/h (pH 5.5 (D)) to 1.4 mL/h (pH 5.5 (A)). In the next paragraph it is shown that at very slow flow rates there is the possibility of diffusion that could make the latter value (1.4 mL/h) questionable. The reverse again was noted for metal recovery where the $FA_{1/2}$=22.1 mL/h in the descending series (pH 5.5(D)), while for the ascending series it was found 25.54 mL/h (pH 5.5(A)). **Figure 37** shows that the step-wise decrease and increase of pH was performed for (V) sample as well.

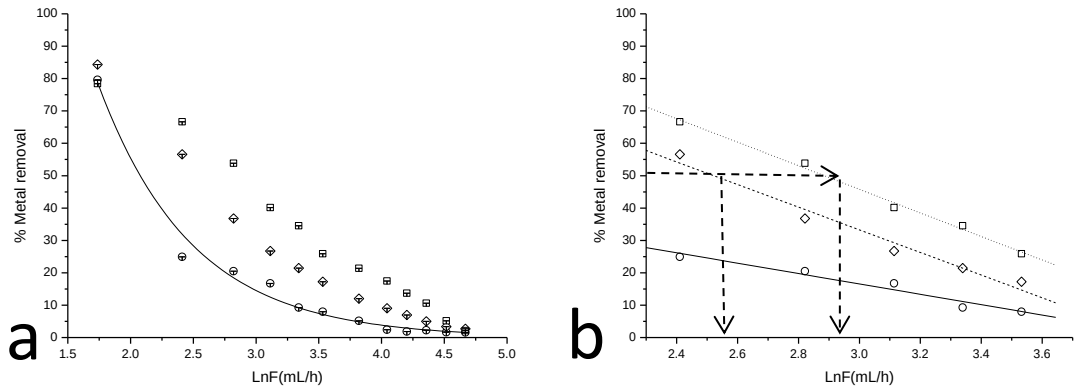


Figure 38: a) Metal removal for (V) sample plotted for three pH values: (**O**): 5.5 (D), (◊): 3.5, (□): 5.5(A), **b)** The lines used to calculate the $FA_{1/2}$ values from **a).**

F**igure 38a)** shows that as the pH decreases, (pH 5.5-open circles to 3.5-open diamonds) the $FA_{1/2}$ values increase as well. In the descending series (pH 3.5-open diamond to 5.5-open squares) the $FA_{1/2}$ values increase even more. An important point here is that the columns apparently recover continuously no matter what the pH of the

inflow solution is. Possible explanation for that is the effect of nucleation. The column at pH 5.5(D) had not yet been nucleated, while at pH 3.5 and 5.5(A), it had been nucleated.

3.6.3 Testing the column volumes required for the outflow chamber biofilm to achieve steady metal removal

Whereas a wash volume of 2 column volumes (8 mL = 1 column volume) was normally sufficient to displace ''old'' solution (Singh, 2012 and current study for (R) sample), given the possibility of an increased impact of diffusional processes at low flow rates, a series of tests were done to check that two column volumes was an appropriate ''displacement volume'' at the slower flow rates required for experiments with outflow chambers.

Biomass from the outflow chamber stored at 4 ^{o}C for 0 ((S) sample) and 2 months was tested in metal recovery (fresh column before nucleation) finding at flow rates of 5.68 mL/h and 11.14 mL/h 33.8% and 28.7% metal removal respectively. The column was left for three days in the standard solution (i.e for nucleation) and was re-evaluated at 5.68 mL/h (less than 2 column volumes of wash) with an observed metal removal of 97%. The column was then tested again at 16 flow rates with 2 column volumes of wash between them. The metal recovery fell from 85.86% (5.68 mL/h) to 48.64% (11.14 mL/h). It was expected that the column would have flushed out the non-precipitated Nd^{3+} and non-precipitated phosphate during a 2 column volumes wash. An experiment was designed to test, whether using outflow chamber biofilm, 2 column volumes of wash between samples was sufficient. A repeat column (column 2) was re-tested on the same experiment and the results found are shown in **Table 19**.

	F= 5.7 mL/h			F= 11.1 mL/h		
	Before nucleation	**After nucleation**	**% Increase**	**Before nucleation**	**After nucleation**	**% Increase**
Column 1	33.8	85.9	254.1	28.7	48.7	169.7
Column 2	17.5	90.8	518.9	14.0	38.1	272.1

Table 19: Metal removal of biofilm from the outflow chamber stored in the cold room for 2 months, before and after nucleation and two column volume wash at the new flow rate between samples.

Table 19 shows the need of the column for nucleation in order to have higher metal removal. The results were re-examined in order to check whether the reduction in metal removal (from 85.9 % for 5.68 mL/h to 48.7 % for 11.14 mL/h) after nucleation was due to mixing the solution being retained in the column for three days with the inflow solution, or due to reduced capability of the columns to recover metal at a slow flow rate. For that reason an experiment was performed in which the metal recovery of both the columns was tested at a constant flow rate as shown in **Fig.39:**

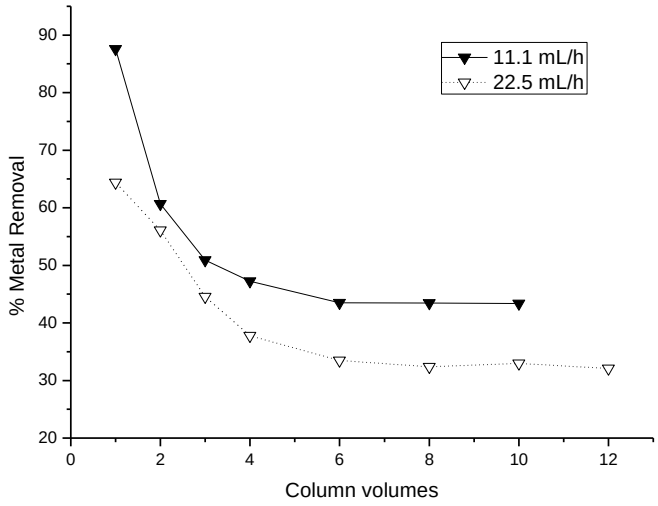


Figure 39: Nd removal at two different flow rates tested for various values of column volumes (''wash volumes''). Each flow rate was examined on a different day and all samples for each flow rate were taken on the same day.

F**igure 39** shows that after 4-6 column volumes the metal removal achieves a steady value. However, the difference is small between 4 and 6 column volumes, meaning that

for 4 column volumes the metal removal drops from 47.26% to 43.51%. The behaviour was the same for column 2.

Due to time restrictions the experiment of testing the number of column volumes required to get a steady metal removal for the slowest flow rate (6.8 mL/h- see Appendix I page 3 for new flow rate points) was left for the end of this project by which time the columns had been stored for 4 months at 4^{o}C. Initially the biofilms were left for two days in the standard solution and tested on the third day, but the 4 month old biofilms showed as expected much reduced activity compared to the recovery obtained when the biomass was stored for 2 months. Hence, in contrast to the main chamber biofilms (**Fig. 20** activity half-life in 3 months), the outflow chamber biofilms lost activity by 88.9 % during only 3 months of storage. Hence, outflow chamber biofilms were less resilient to ageing (this accelerated loss was noted earlier in this section). The column was left for one more day (extended nucleation) with the same results as the column measured after 2 months of storage (nucleated for three days). The results for both the columns can be seen in the following figure.

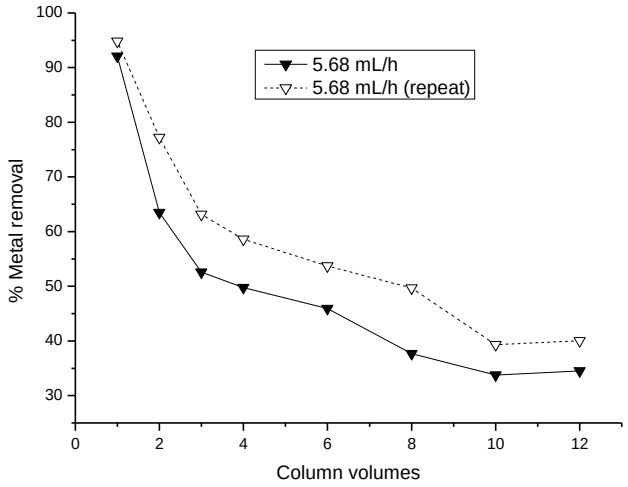


Figure 40: Column volumes required to reach a steady metal removal for biofilm immobilized in the outflow chamber and which had been stored for 4 months. The biomass had stayed for nucleation in the standard solution for 3 days prior to analysis.

Figure 40 clearly shows that for both columns the metal removal stabilized after 10 column volumes and that the metal recovered was 35.5% (column 1) and 40.0% (repeat column). It is concluded that as biofilm from the outflow chamber ages (i.e. activity is reduced so a slower flow rate is needed), more displacement volume is required in order to attain steady state metal removal.

One possible reason for this is the very slow flow rate required at the low column activities shown by the outflow chamber biofilms. Possible break down of plug flow at very slow flow rates and possible diffusion rates exceeding flow rates, so the columns self-mix by diffusing at very long residence times.

Preliminary research by Claire Mennan (see Deplanche *et al*. 2011) has shown that neodymium along with europium were both recovered by fresh biofilm (at pH 5.5 inflow solution) and the recovery has remained constant throughout the research, which lasted for 54.5 hours at an even slower flow rate of 5 mL/h.

In order to check this observation, another experiment was done using highly active biofilm from the main vessel fermenter which had been stored for 3 months (sample (R)). The column was challenged for 2-4 and 6 column volumes at a slow flow rate 11.1 mL/h and showed an identical steady metal removal of 95% and 98% respectfully i.e. an extended wash was not required for relatively fresh biofilms from the main vessel and directly comparable to the outflow chamber biofilm.

This is in accordance with previous work (Singh, 2012) where both metal removal and total phosphate release Pi (from culture (I)), were tested in neodymiun and europium recovery in increasing acidity conditions at a fixed flow rate (200 mL/h). The column was tested after 2-4 and 6 column volumes of wash. Both total Pi released and metal removal were the same independent of the wash volume.

In an effort to explain in terms of mixing/diffusion/back mixing in plug flow systems such as the one examined (at different flow rates), it is suggested that at faster flow rate we get less back-mixing due to diffusion, while at slow flow rate we have more back mixing again due to diffusion (i.e. where the diffusion rate is much less than the flow rate, unwanted mixing would not be an issue).

The conclusion about the outflow chamber biofilms is that they are unsuitable, because low activity of the biofilm needs a slow flow rate, which increases back the activity into the column due to diffusion and therefore more column wash is needed for steady metal removal.

3.6.4 Diffusion study for the aged biomass

Using biomass which was kept at 4^{o}C, for 0-3 months, current and previous studies (Singh, 2012, Mennan, 2010) showed that for highly active biomass the biofilm's speed was high in interacting with G2P (high reaction rate due to a large amount of enzyme) and therefore the diffusion rate was high. Considering a modification of Fick's Law for diffusion through a cell membrane, where the rate of diffusion is given by the expression:

$dn/dt = P*A*(dC/dx)$

where P is the permeability constant (the ease of a molecule entering a cell), A is the membrane area and the concentration gradient, dC/dx, represents the molecule concentration in and out of the cell over a cell membrane with width dx. Therefore, the rate of diffusion, dn/dt, represents the difference in the number of diffusing molecules through a cell membrane over time (Darnell *et al*. 1986, LeFevre, 1961). For highly active cells the interaction time with G2P is short (even for high flow rates) and the diffusion rate is high (but the residence time per voxel is short). On the other hand, for aged cells (7 months old and beyond) or even more for cells from the outflow chamber (even weaker biomass), the interaction time increases (slow flow rate and prolonged period of nucleation), in order to achieve again a high diffusion.

For the case of the outflow chamber biofilm the column was left for nucleation for 2-3 days (aged three months or more) to recover from storing. During this period the concentration within the column of the phosphate released was increasing (steady state). When the pump was on, even for slow flow rates the concentration of phosphate was decreasing since new inflow solution was entering the column and the diffusion rate was

decreasing again, therefore more column volumes were required to achieve a steady metal removal. In future studies this could form the basis of how the project could proceed for more robust data, developing a concept/model through fluid dynamics whereby checking if the diffusion rate is greater than the flow rate or the opposite, one could predict the behavior of the column.

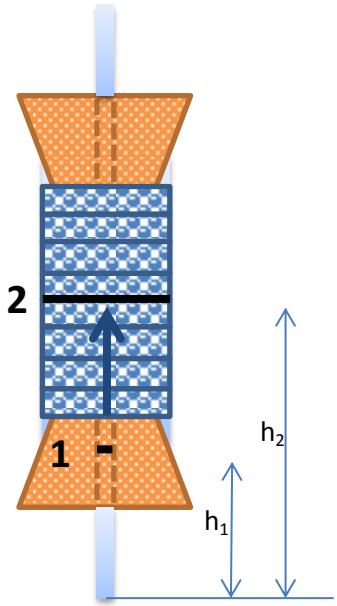


Figure 41: Schematic representation of a column

An idea for future studies would be to see the project from an engineering point where one could consider the column as a closed cylindrical tank, where the Bernoulli equation could be applied to calculate the velocity or pressure within a specific part of the column such as:

$$p_1 + ½\ \rho u_1^2 + \rho g h_1 = p_2 + 1/2 \rho u_2^2 + \rho g h_2$$

where p_1, u_1 are the pressure and velocity at point 1, p_2, u_2 are the pressure and velocity at point 2, ρ is the density of the fluid and $g=9.81 m/sec^2$. From there and keeping in mind the dimensions of the column it will be possible to determine the number of column volumes wash for steady metal removal, having already known the diffusion rate for aged biomass.

For a fresh column it is assumed that pressure is not an issue. However, in the case of non-homogenous precipitate deposition (e.g. a high deposition at the input end of high activity column at slow flow rate) back-pressure will become an issue, since the pressure term in the Bernoulli equation will not be constant. Hence, the distribution of metal precipitate in the column is important.

Previous research has shown (Singh, 2012) that using a highly active column (H) an early block of the column (i.e in the bottom disc –end right) as shown in **Fig. 42a)** was observed. In **Figures 42b)** and **c)** one can see a more equally distributed of $NdPO_4$ throughout the column. That could be explained since, the same rpm points used for (H) (Singh, 2012), gave higher flow rates (even for slow flow rates), fact that resulted in blocking the column, since the base of the column mostly reacted with the inflow solution, while leaving the top of the column almost empty from Nd deposition **(Fig.42a).** On the other hand, the slower flow rates used in this project resulted in more equally distributed $NdPO_4$ throughout the column (flow rates seen in Appendix I page 4), since for this flow rate, less reaction occurred in the initial discs and hence more substrate was available to react later in the column.

This is explained in terms of the high activity column (at slow flow rates) exhausting the supply of Nd^{3+} at an early stage in the column whereas the less active columns (at the same flow rate) showed less activity (Nd deposition) in the first segments, because the phosphate release was slower and hence more soluble Nd^{3+} passed to distal parts of the column.

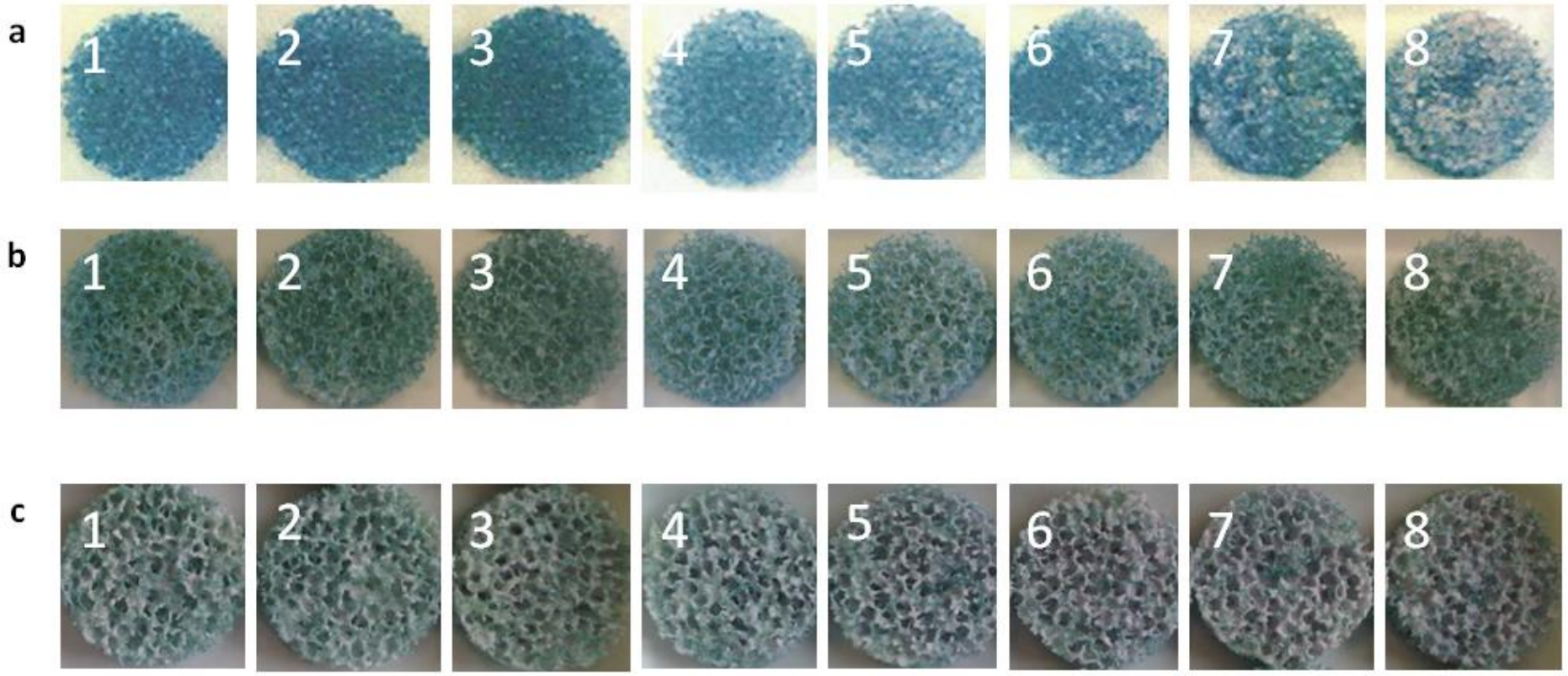


Figure 42 : Image showing accumulation of Nd phosphate deposit on biofilm coated discs starting from the top (left) towards the bottom of the column (end right), **a)** Column using (H) sample (Singh, 2012) shows most of the $NdPO_4$ at the bottom of the column (end right), **b)** Column using (L) sample and **c)** Column using (R) sample .The last two show a distribution of Nd deposit throughout the length of the column compared to sample (H).

SECTION G.

3.7 Analysis of metal precipitate formed on columns.

3.7.1 SEM analysis

Foam discs with metal precipitate were removed from columns after completing experiments of lowering the pH gradually from 5.5 to 3.5 and then resetting to 5.5 (per 0.5 increments) and were stored in the fridge prior to analysis to dry out. Before the analysis powder from metal precipitate was disrupted from foam discs and was placed on SEM stubs as described in III. DESCRIPTION OF BIOFILM TESTS.

In order to rule out flow rate phenomena (discussed in previous section) an aged biofilm from Culture (I) (L)-sample, was selected so as to have similar activity at similar flow rate. Powder from disrupted foam discs from the columns for (J), (L) and (V) were examined using SEM and EDX analysis and were compared with each other.

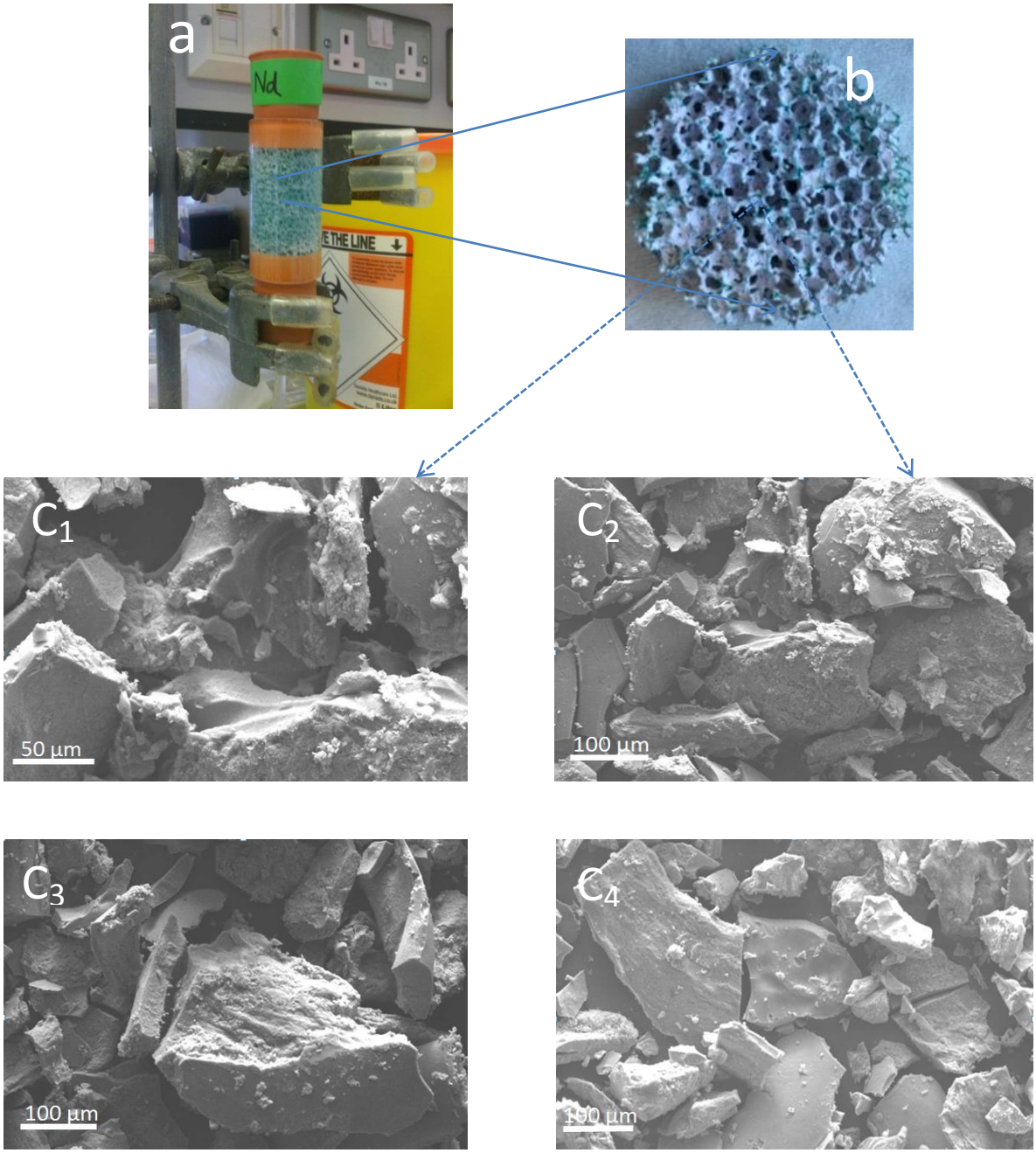


Figure 43: a) Column filled with foam discs after the pH descending-ascending series experiment. One can notice the almost equally distributed $NdPO_4$ throughout the column. **b)** Foam disc with purple precipitate of $NdPO_4$. Dried powder of $NdPO_4$ was disrupted from foam disc and was analyzed using SEM. **c)** Four areas of precipitate were taken from the disc to produce the images. All SEM images were taken by Dr. Angela Murray. All views from the 4 images were the same. Image **C_1** is repeated for better visualization in **Figure 44** as **a).**

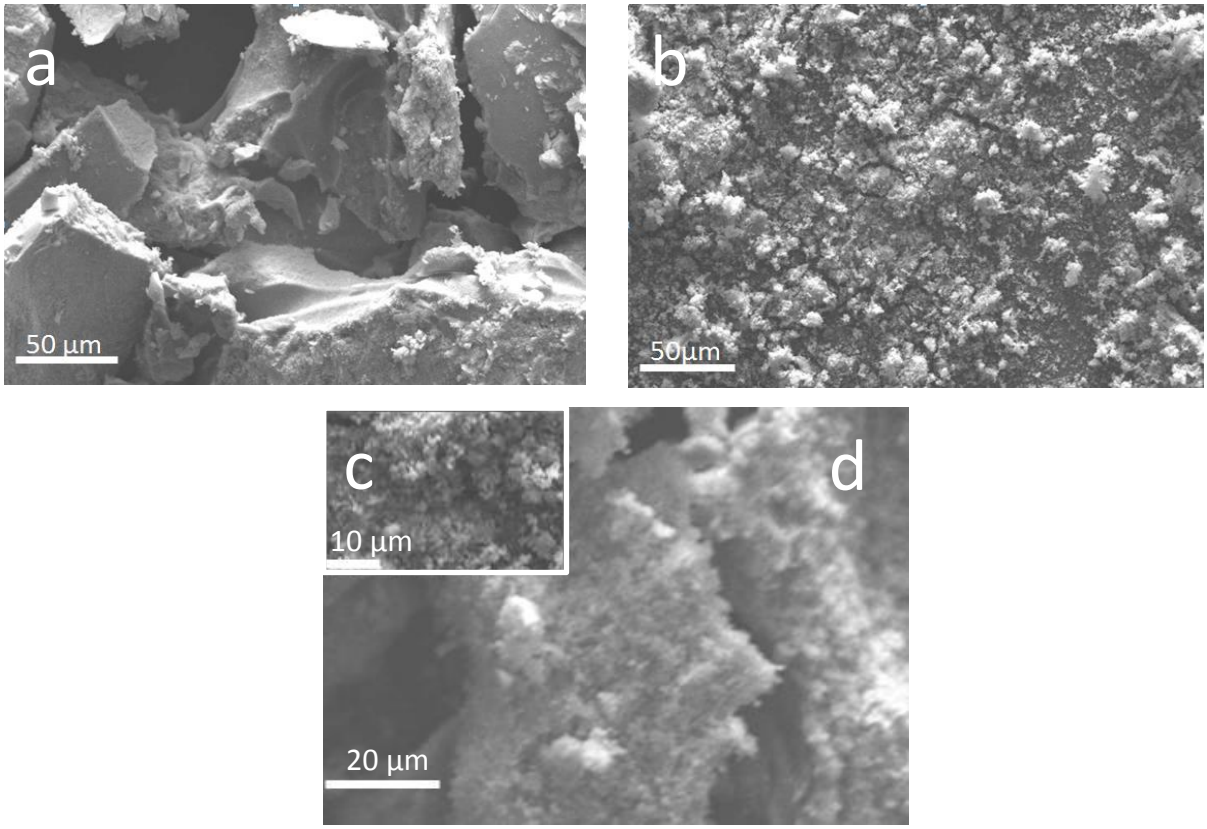


Figure 44: Examination of Nd-loaded biofilm by SEM **a)** (J), **b)** and **c)** (V) and **d)** (L). All four images show powder disrupted from foam discs. Images taken by Dr. Angela Murray.

The powder obtained from the main vessel (**Figures 44a)** and **d))** showed clear large crystal regions, whereas the corresponding material from the outflow chamber biofilm (**Figures 44 b)** and **c))**, shows a finer powder. Since the phosphatase activity of the two preparations was comparable (Section E) it may be assumed that a lighter biofilm loading (seen in following **Table 20**) from outflow chamber biofilms, has resulted in a less cohesive material as shown in **b)** and **c)**. Alternatively, since during storage the biofilm EPS may have been degraded, any structural role for this in maintaining crystallite dispersion, may have been lost.

	(J)	(L)	(V) (1)	(V) (2)
Weight of biofilm (g) before experiment	1.9391	1.9898	0.6628	0.6771
Weight of biofilm and loading (g) after experiment	3.3325	5.1545	4.168	3.9276
Total loading (g)	1.3934	3.1647	3.5052	3.2505

Table 20: Estimation of total loading on biofilms for (L) and (V) (1)and (2)(where 2 is the repeat column). The foam discs were weighed before and after performing the pH experiment (5.5-3.5-5.5 per 0.5 increments) and was subtracted the weight of 8 dry foam discs which was found to be on average 0.279 g. Total loading was calculated by subtracting for each column the first row from the second.

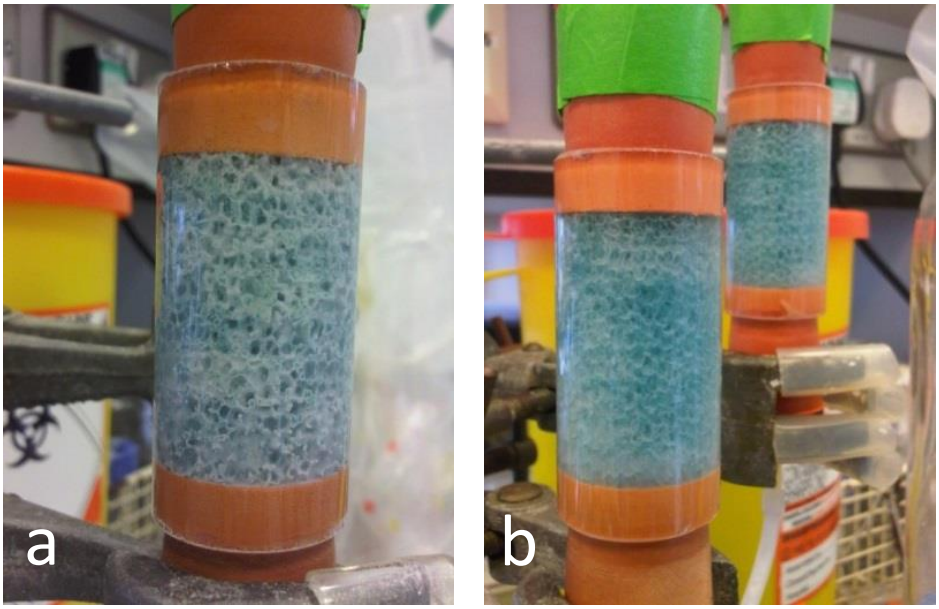


Figure 45: Comparison of two columns, one from main vessel **(a)** and one from outflow chamber **(b)** after the pH descending-ascending experiment. The difference in the quality of the powder is obvious even before dessication (the latter gives a finer powder).

3.7.2 Energy-Dispersive X-Ray spectroscopy (EDX) analysis

Fig. 46 shows EDX analyses for the same three samples (a-(J), b-(L) and c-(V)). The area of interest for each sample can be seen in the Appendix IV at pages 8-10, where one can see the SEM picture along with the EDX analyses (analyses done by Dr. Angela Murray). Most of the EDX spectra were identical, thus the most representative are shown in **Fig. 46.**

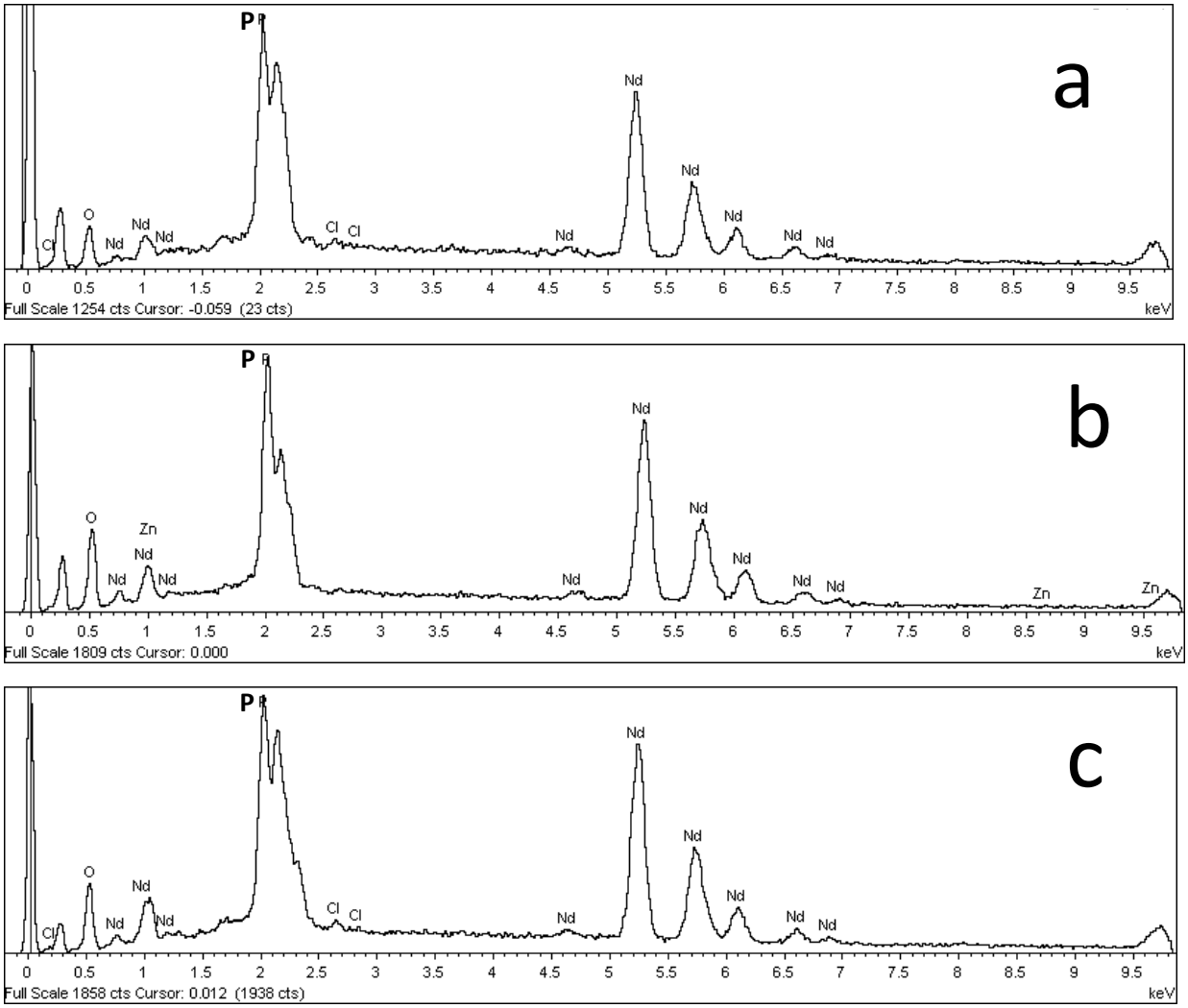


Figure 46: a) Analysis for (J): 12.21%(O),19.33%(P),0.84%(Cl),67.63%(Nd) **b) Analysis for (L):** 18.94%(O),17.36%(P),-0.48%(Zn),64.18%(Nd) **c) Analysis for (V):** 15.66%(O),15.32%(P), 0.83%(Cl), 68.18%(Nd). All three spectra show the peaks generated by X-Rays emitted for each sample.

The various peaks observed for Nd are attributed to the fact that Nd has in general odd properties and very strange optical behaviour. It has electrons in weird places and therefore when the Nd atom is being hit by the high speed electron of the instrument, it excites in various wavelengths, that's why we see multiple energies from one metal.

The % weight and atomic analysis for each sample is shown in the following table (two sites of interest for each sample).

	(J)		(L)		(V)	
Element	**Weight %**	**Atomic %**	**Weight %**	**Atomic %**	**Weight %**	**Atomic %**
O	12.21/11.24	40.61/39.64	7.26/18.94	34.21/54.26	12.94/15.66	46.73/49.7
P	19.33/16.71	33.19/30.44	9.04/17.36	22.01/25.68	12.00/15.32	22.37/25.11
Cl	0.84/1.10	1.26/1.75	-	-	0.69/0.83	1.12/1.19
Nd	67.63/70.54	24.94/27.59	83.71/64.18	43.78/20.40	74.37/68.18	29.78/24.00

Table 21: % Analysis for the three powder samples. The two values in each column indicate the two sites of interest for each sample. Data taken from Appendix IV.

In order to calculate the ratio of P:Nd from the table above we get (from the atomic weight%):

Element	**Atomic %**		**Atomic %**		**Atomic %**	
P	33.2	30.4	22.0	25.7	22.4	25.1
Nd	24.9	27.6	43.8	20.40	29.78	24.00
Ratio	1.33	1.10	0.50	1.26	0.75	1.05

Table 22: Calculation of the ratio P:Nd. The average of the ratio values is 1.0, which is in agreement with preliminary studies by Singh (2012).

Analysis by Energy-Dispersive X-Ray spectroscopy indicated the presence of elements in the formed powder. The analysis for all the samples confirmed the existence of Nd in greatest amount, which was followed by the presence of oxygen and phosphorus, as expected from the formation of $NdPO_4$ powder. Small amounts of Cl and Ca were also identifiable, from either the biofilm component or background contamination. A small amount of Eu was expected to be seen in spectrum a), since the column with (J) sample

was challenged for one day with europium solution, but that was not observed, possibly being below the detection limit.

3.7.3 XRD analysis

The samples that were examined for the XRD analysis were initially the same as for the case of SEM-EDX ((J), (L) and (V). However, the results for (J) and (V) suggested that there was no crystalline structure. The reason for that for (J), might be the fact that the experiment was terminated prematurely. Produced powder taken from the foam discs was predominantly cellular material and had relatively little bio mineral. On the other hand for the case of the outflow chamber again no crystal structure could be seen probably due to very small amount of biomass present to produce $NdPO_4$ and hence very low loading of the latter.

For that reason another sample was tested, again from the main vessel and culture (II), (sample (R)) and was compared with the tested sample (L) from culture (I), as well as with the sample which was from culture (I) and was tested by a previous MSc student (Singh, 2012).

The XRD data confirm that the material accumulated by all three biofilms has low crystallinity. From the results seen in **Figure 47**, one can notice that low crystal structure can be seen for all samples ((H), (R) and (L)) and the closest match on the peaks was found for neodymium phosphate hydrate ($NdPO_4.2H_2O$). Specifically the peaks obtained from the 3 months old biomass for cultures (I) and (II), showed almost identical, fact that shows once again the good reproducibility results for both cultures. Another notable notice is that the crystallinity of the material is not affected when using an old biofilm, as seen

when comparing (L) and (H),(R). The pattern shows low resolution which means that the crystalline structure is in the size of nanoparticles (although **Figure 44** showed that the deposits appeared to be large crystals).

These findings were confirmed when calculating the crystallite size from the Scherrer equation (Jaboyedoff *et al*. 1999), for all the samples tested as seen below.

$$L= (K*\lambda)/\beta_{\Delta 2\theta}*\cos\theta$$

Where K is the Scherrer constant the value of which depends from the shape of the particle, the size distribution as well as the way the width is determined (usually ranging between 0.89-0.94), λ is the X-ray wavelength, $\beta_{\Delta 2\theta}$ is the full width of the peak (radians units) and θ is the diffraction angle. From this equation one can see that the broader a peak gets (increasing $\beta_{\Delta 2\theta}$), the smaller the crystallite size gets (decreasing L) (Jaboyedoff *et al*. 1999, Speakman, 2013). The results obtained were 14.8 nm for sample (H), 14.5 nm for sample (R) and 11.9 nm for sample (L). These (H) and (R) results once again confirm the good reproducibility (<1%) of the results between the two cultures, when tested at the 3 months stage.

Comparison of the results using (H), (R) and (L) samples

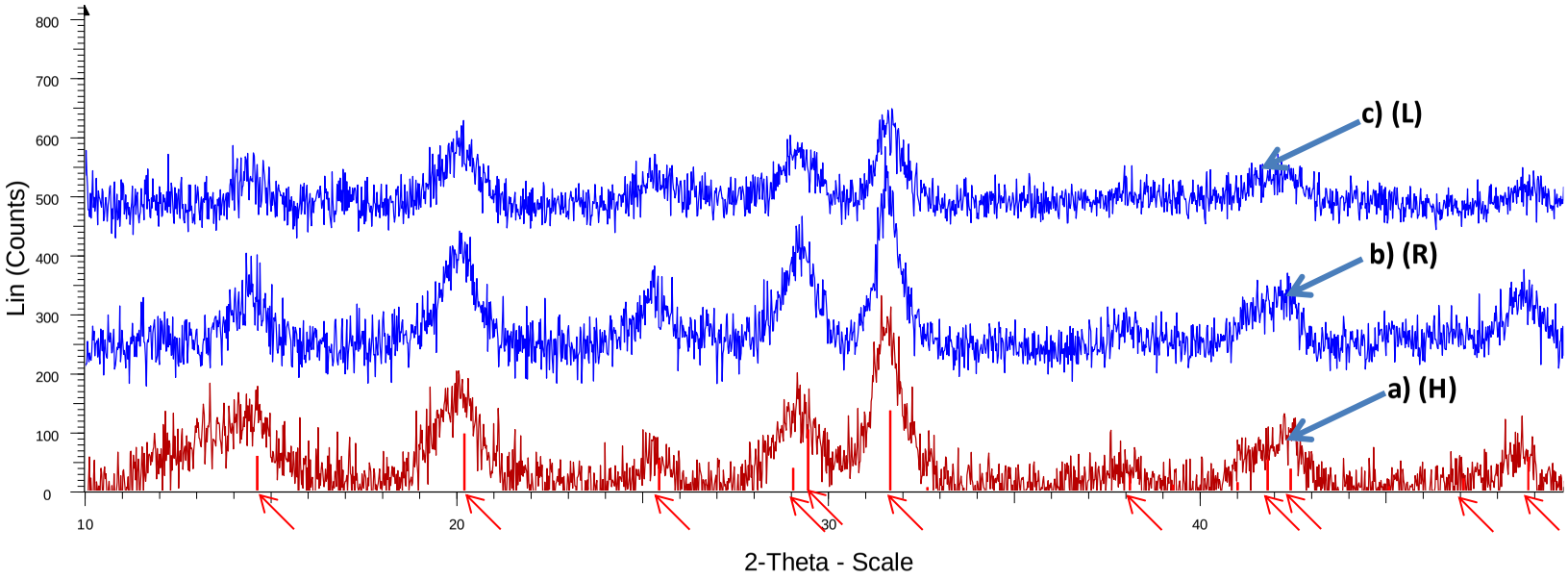


Figure 47: Comparison of a) (H) (red-data from Singh,2012), b) (R) (blue) and c) (L) (blue). Data were acquired by Dr. Jackie Deans. Reference sample $NdPO_4.2H2O$ (Red arrowed lines- sample used from Chemistry department-The University of Birmingham; Data based on Kirik *et al*. 1998)

4.0 CONCLUSIONS

Many important findings were revealed from this project, forming a basis for future research projects for the biorecovery not only in neodymium from waste solutions, but also from elements with similar chemical properties, by *Serratia* sp. N14 cells. Previous studies (Macaskie *et al*. 1994b, Tolley *et al*. 1995, Singh, 2012) have shown that increasing flow rates in the column generally leads to reduction in metal recovery, a pattern that was expected and noticed also in this project. That decrease in total Pi release at high flow rates, is attributed to the fact that at slow flow rates more substrate reacts with the enzyme, within each time unit and therefore more inorganic phosphate is produced for the Nd recovery. On the other hand, increasing the flow rate means that less substrate reacts with the enzyme to produce Pi and therefore there is less time to create nucleation foci and precipitate at the surface of the cells, in order for Nd metal to be recovered as $NdPO_4$ (Macaskie *et al*. 1994b).

Previous studies (Singh, 2012) based on a single experiment only have indicated that not all the substrate reacts with the enzyme even at very slow flow rates (6.8 mL/h), a fact that was not observed during more extended examination, during this project, when testing the (R) sample. In 2012 it was observed that only 3.5 mM of the total 5 mM G2P was reacting with the cells and 1.5 mM was remaining unreacted and was flowing out in the outflow pool. A possible explanation for that phenomenon was attributed to transphosphorylation activity of the enzyme in which phosphate returns back to the substrate by the enzyme (Jeong *et al*. 1996). Another possible explanation for that was that the extracellular polymeric substances of the *Serratia sp.* N14 biofilm contains free phosphate, besides bound (Jeong *et al*. 1996). No conclusion could be made at that point

after that first experiment and further analysis was proposed for that result. Current study showed that all substrate was reacting with the cells when testing the new set of biomass produced in 2013 and being kept in the cold room for 3 months. However, the results for Nd recovery were identical for both biofilm preparations and liberated phosphate was to excess over Nd in both studies.

When doing the experiment using (L) sample, the column was left overnight in the standard solution after each pH change, but since it was noticed that there was not much of a difference in the obtained results, the rest of the experiments were done as initially arranged i.e. expose the column overnight in the inflow solution of each pH change (after the pH 5.5 experiment (D), which was tested for the before and after nucleation experiment) .

Generally it is concluded that the ageing factor influences the effectiveness of the column in phosphate release and therefore in metal recovery too. From the data the post 7 months old biofilms have comparable activity and future experiments can test how a fresh biofilm loses activity at 0-3 months. Leaving the cells for 9 months or more in the cold room, it reduces their ability to recover neodymium by 94 %. Therefore, future studies can give an insight why the biomass behaves like that between 0-9 months of storage. Results have shown that after the 7th month up until the 12th the reduction lies in a comparable level, so most of the loss occurs within the initial months of storage. In particular almost half of the biomass's activity (46 %) is being lost during the first three months of storage.

As far as the effect of varying the values of pH in the recovery process, again previous research has shown lowering the pH in more acidic conditions is followed by a reduction

in Pi liberation (Tolley *et al*. 1995, Singh, 2012). Although that was noticed again in the current research for both phosphate release and metal recovery, when bringing the column back to pH 5.5 per 0.5 pH change only the results for phosphate release agree with the results obtained from Singh (2012). More specifically the total Pi liberated was reduced when bringing the pH to acidic conditions from 5.5 to 3.5 and in the ascending series the total Pi liberation was increasing again, but to a lower level than the one started in the pH descending series. A possible scenario was considered as an explanation of this phenomenon, which although has not been proved, is still supported in this project. In this scenario the two acid phosphatase isoenzymes (CPI and CPII) of a new column, form the total enzyme activity, being represented as the sum of the activities of the two isoenzymes, but they differ slightly in terms of optimum pH, activity and sensitivity to metal. Increasing the acidity of the solution, both isoenzymes would get affected in the acidic environment, but on recovering back to pH 5.5 it is suggested that only one isoenzyme fully recovers (CPI, which although less active than CPII, is more resistant to metal anions and has a lower optimal pH). Therefore the total enzyme activity after recovery, might reflect the activity of the sole remaining isoenzyme. On the other hand, in terms of metal recovery, the trend followed in the pH descending series was the one expected, since metal removal decreases for increasing acidity of the solution, but in the pH ascending series the inverse was noticed compared to total Pi liberated. As long as there is excess phosphate over neodymium the loss of one isoenzyme may not impact upon metal removal. Previous research (Singh, 2012) has shown that the cells fully recover in metal removal, when resetting the pH back from pH 3.5 acidic conditions (in that case the recovery was done directly from 3.5 to 5.5 without 0.5 pH changes), but the current project has shown that in the pH ascending series per 0.5 pH change, the metal removal at

pH 5.5 graph reaches higher values than the pH 5.5 graph in the descending series. A possible explanation for that might be the fact that biomass has stayed in the inflow solution for longer time.

A very important note was indicated in page 76 in which it is mentioned that the extra addition of chloride in the solution, did not change only the pH, but the ionic strength as well. Therefore, the decrease in the total phosphate release and metal removal was attributed to two variables coupled, i) ionic strength and ii) change of the pH. These can both be elucidated in a future project, by doing two valid control experiments, already mentioned on the same page. The main idea behind this is that in a solution with ionic strength (high salt solution), the water will tend to go from higher concentration solution to lower, which means that the water will come out of the cell (dehydration of the cell), fact that will reduce the activity of the enzyme.

Aqua regia tests have shown the robustness of the enzyme in high salt concentration at pH 5.0 (or less) is high, since even for 50% (v/v) *aqua regia* the column is still active. These experiments are not ideal, since to conserve materials, the *aqua regia* experiments, used columns already used from previous experiments. It would be notable to test a fresh column directly on *aqua regia* solution, as metal phosphate could act as a protection barrier for the cells.

Challenging the columns with increasing concentrations of *aqua regia* resulted in inactivation of the columns when using the rescue solution at pH 5.5 at very low pH values (pH 1.0 of the *aqua regia* solution). The pH of the solution in the column or the outflow was not checked. *Serratia* sp. N14 cells have proved to be highly robust bacteria, both in high salt and acidic conditions.

Previous research (Tolley *et al*. 1995, Yong & Macaskie, 1995 b), has shown that metal phosphate at lower pH becomes more soluble for the cases of lanthanum and uranyl phosphate. The steady metal removal throughout the whole *aqua regia* tests, confirms that once $NdPO_4$ has been formed it remains securely on the foam discs and exposure of the biomass to highly acidic solutions, does not influence this retention.

The foam discs on the main vessel were visibly coated with biofilm, but the run of the main fermantor continued for up to 7 days. By day 3 the immobilized cells on the foam discs, start to age. Their population though is still growing, since they have a supply of carbon (lactose). During the first days of the run within the main vessel, there is lots of lactose in the inflow solution plus lots of glycerol phosphate. During these days in the outflow solution there is a fair amound of glycerol phosphate, which means that only lactose is being used by the cells and glycerol phosphate flows through the reactor. As the population grows even bigger (and older), in the outflow solution of the main vessel the lactose is still not existent as in the initial days of the run, but the glycerol phosphate concentration decreases as well, since it is being used as a carbon source by the cells. An extended growth period takes place between 3-7 days and the point of that period is to starve the Bacteria. The point of this starvation is to stress the cells and make them produce the PhoN enzyme, with which they can metabolise organic phosphates (glycerol phosphate) into inorganic phosphorus and glycerol (alcohol) (Macaskie *et al*. 1992a, Jeong *et al*. 1998, Patterson-Beedle *et al*. 2010). That in an industrial scale is expensive, since feeding a young culture (less than 3 days) can be tolerable from an economic point of view, but feeding an old culture and a big population of cells (beyond 7 days) for the production of the PhoN is expensive.

For the case of the outflow chamber reactor again an interesting notice is mentioned in page 49. The outflow chambers were powered with cells that were not immobilized in the main vessel (weak cells) and: 1) there was no carbon source, 2) the temperature was room temperature and 3) the cells might have been genetically different. The reason for 3) can be justified by the fact that in previous research done (de Visser *et al.* 2006) using E.Coli, different beneficial mutation in the majority of the population was noticed by 300 generations. Keeping the cells for 7 days, that exceeded the 300 generations, that is why it is believed that the cells might have been genetically different. No experiment though was conducted to prove this. Moreover, it worths mention the fact that the vessels (main and outflow), they both had different dilution rates. They were chemostat vessels connected in series such as a gradostat reactor (Lovitt *et al*. 1981) and they had different dilution rates. One with a higher dilution rate and another with a lower one. This observation can lead to the conclussion that the vessel with the higher dilution rate can lead to faster growing strains, while the one with the lower dilution rate can produce slower growing strains. This again could be even more interesting to manage to produce fast growing strains from slow growing strains, but enhances even more the not proven conclusion stated above, that the two vessels (main and outflow), contained different strains and therefore a direct comparison could not be achieved.

A very interesting finding was revealed when comparing the (S), (K) and (M) samples, since leaving the column from the outflow chamber for nucleation for 24 hours, compensates for 3 months of storage of the biomass. The two outflow chambers could either be used the way they were used in the current project, by testing the influence of an inhibitor in the biomass or for biofilm production, by switching the vessels during the 7

day production of biofilms in the main vessel, since in an industrial scale a 7 day run would be unacceptable (semi-continuous process for biofilm preparation). The ageing effect on the other hand of the outflow chamber biomass cannot be compared with the one that was produced in the main vessel. Biomass from outflow chamber lost very quickly its activity and within 4 months of storage the phosphatase activity dropped from 1700-2000 to 157 Units (Section F), where metal removal was stabilized at 35-40% after 3 days of nucleation at a flow rate of 6.8 mL/h. A possible explanation for that is that outmost layers of a highly loaded biomass act as protecting layers to the biomass that remains attached directly on the foam discs. Consequently, a lightly loaded biomass does not have a protection layer and remains exposed directly to the surrounding conditions.

Moreover, in nature the extracellular polymeric substances are protecting the cells from desiccation, from various predators such as biocides, toxins and protozoans, but also control the diffusional mass transport which takes place in various densities (Costerton *et al*. 1995), one could suggest that the outmost layers of the heavy loaded biomass, protects the innermost layers and therefore that could bring an insight, of when testing the biomass directly in *aqua regia* solution (without being covered with metal phosphate). Durability of the biomass might have been increased due to this highly packed structure of the biofilm, so therefore one interesting further experiment would be to test the exposure of various loadings of biomass on foam discs (lightly-medium-heavily loaded), in various concentrations of *aqua regia,* in order to test how robust the bacteria are, when exposed directly to *aqua regia* solutions. Columns that were tested before nucleation showed that 2 column volumes might be enough for each set of flow rate points, but exposing the biomass in the standard solution for 24 hours or more showed that good measurements

for metal recovery can be taken after 4-6 column volumes, since deviation from 4-6 column volumes in metal recovery was < 5%. Furthermore, it could also be hypothesized that since the extracellular polymeric substances provide protection on the cells from various predators, this could also mean that the metal loaded on the surface of the cells, does not reach the enzyme.

A very interesting control experiment that could also have been done, was to use a killed biofilm to check the amount of binding made on dead cells. That was going to be tested since EPS are very sticky for metals and since we are dealing with sticky metals it would have been very interesting to see if from the amount recovered, how much percentage of the recovery was due to interaction of sticky surfaces (EPS-metals, tubing-metals).

This research could form the basis for next experiments, where seeing the column from an engineering point of view (calculation of tubing and column dimensions), would give an insight of knowing the exact number of column volumes required for the inflow solution to pass through the column in order to have a representative sample of each set flow rate point. However, the activity of the biomass seems to be a limiting factor in this research, but this can also be overcome with further studies. Therefore, the study of the rate of the reaction between substrate and biomass for various months of age of the biofilm would enhance even more the future study.

Reduced activity of the biomass turned out to be a good opportunity for studying the column volumes required for steady metal removal. Below a certain flow rate, data would not be robust due to loss of plug flow. In case that it will be proved that for 6.8 mL/h are required 10 column volumes to get the first measurement for any biomass age, then

studying the effect of increasing flow rates of the biomass to produce phosphate or recover metal, should start from the previous day (since for 10 column volumes steady metal removal, it is required more than 15 hours of continuous flow of the pump). Furthermore, it should also be examined how many column volumes are required after each change of flow rate on a continuous flow , namely after 6.8 mL/h check directly 13.5 mL/h and wait for a steady measurement, after that the next flow rate, until the last point, since in this project it was tested each flow rate per day.

XRD and EDX analyses both gave identical results with the ones presented in a previous project (Singh, 2012), which again proves the consistency of the method used. Indeed at the end of each experiment, the biofilm along with the metal phosphate, was left to dry out. After that it was easily removed from the foam discs, just by rubbing the foams on collection media. This reliable analytical method, could give 100% recovery from the produced metal phosphate.

Comparing now the results when using (H) sample, given by Singh (2012), with the (R) sample observed in this project, one would conclude in general that biomass if of the same quality for both cultures, behaved better for the given flow rates. Slower flow rates again gave more time for the substrate to react with the cells and therefore no blocking of the columns was observed as well as equally distributed metal phosphate was found throughout the project **(Fig. 42)**. The number of column volumes has been found to be more than at least four in order to have a steady metal removal for each flow rate at slow flow rates, but that was not tested on a continuous flow rate change. For each pH test in all the graphs in phosphate release or metal recovered produced, the lines that were used to calculate $FA_{1/2}$ or the simple mathematical predictive model, used as initial point the

one after 4 column volumes so therefore the experimental error should be small. Very interesting was also found the fact that the whole amount of G2P was used from the cells for phosphate release (in contrast with previous studies (Singh, 2012) where 1.5 mM was remaining unreacted) and therefore the total amount of Pi liberated was 173.61 mL/h rather than 90mL/h (for (H)), both at $FA_{1/2}$ values in phosphate release. On the other hand, both columns behaved quite identically in terms of metal recovery, since $FA_{1/2}$ was found in culture (I) 273 mL/h, and in culture (II), 275 mL/h (reproducibility between researchers <1%).The $FA_{0.6}$ though was found with a difference of 18% (200 mL/h-2012 and 163.11 mL/h-2013). Therefore the conclusions up to this point can be the summarized:

- Reproducibility between cultures (I) and (II) was within 1% in metal removal,
- Over 50% of ageing effect on biofilm occurs during the first three months of storage,
- The ability of the column to withstand and recover from pH stress is unaffected by ageing as observed by previous research (Singh,2012) and current experiments (summarized in **Table 13** section D),
- 50% *aqua regia* is tolerated at pH 5.0. Lower pH values could also be considered, but a more reliable pH control is required,
- The biofilm from the outflow chamber can be compared with biomass that was produced in the main vessel and has been stored for up to a year. However, a faster ageing occurs, which requires extended nucleation for the biofilm to recover from storing as well as more column volumes of wash to attain steady metal removal,
- EDX analysis confirmed again the ratio Nd:P=1:1, which was shown by previous studies (Singh, 2012),

- XRD analyses confirms again low crystallinity of $NdPO_4$ (Singh, 2012) and the results from two independent cultures give both very close results in crystallite size (<1%),
- A simple predictive model through the 10 months old biomass was created and developed (see Appendix V –Method development for a simple predictive model). Although in the beginning when using only the (K) results the equations seemed to have been different for each pH change, the results from the (L) sample proved that 7 and 10 months old results are closer to the experimental values obtained for 3 different flow rate points (slow-medium-fast).

5.0 FUTURE WORK

Serratia sp. N14 has proved to have a robust phosphatase enzyme that can not only remove metal under acidic conditions, but also remains active at high salt concentration at pH 3.5. A factor that is limiting the industrial scale of this project is the expense of the substrate glycerol 2-phosphate (G2P), which has been considered as unattractive from an economic point of view. That issue could be overcome by using phytic acid (Inositol phosphate-IP_6) rather than G2P, a material produced on a large scale as a fuel ethanol production waste. This waste disposal issue can be tackled by adding phytase (Paterson-Beedle *et al*. 2010). Therefore, within the metal recovery technology, a more environmental approach could be the utilization of disposed phytic acid, which focuses in the biologically available substrate from biodiesel wastes (Paterson-Beedle *et al*. 2010). On the one hand an advantage of using phytic-acid is that it possess 6 mol of phosphate/mol rather than 1 mol of phosphate /mol, which is found in the case of G2P (Mennan, 2010). Earlier studies (Paterson-Beedle *et al*. 2010) have used phytic acid to recover uranium from aqueous solutions. On the other hand, phytic acid has been found

to irreversibly bind every heavy metal (metals never debind) (Duffin, 1989), so an option in our case would be to have the metal irreversibly chelated on the phytic acid and so there will be no phosphate production in the system. Although Inositol (produced from hydrolysis of IP_6 or fermentation) has commercial potential (in the treatment of panic and obsessive compulsive disorder or in depression), it is not yet clarified to what extend the value for the purification of the waste inositol from metal precipitation can be compared with the value of commercially sold inositol (Fux *et al*. 1996, Paterson-Beedle *et al*. 2010). Tributyl Phosphate (TBP) is another cheap alternative for G2P substrate, which has been proposed again for industrial scale use (Yong *et al*. 1999). Previous research (Michel *et al*. 1986) showed that Tri-n-butyl Phosphate has proved to be the best phosphate donor compared to other alkyl phosphates whereas the removal of Cd^{2+} for this substrate (5 mM) was 68.4%, rather than ~97% shown for the case of G2P (5mM).

As far as ageing of the cells is concerned, one idea in order to increase the efficiency in phosphate release or metal removal would be to find a way to better preserve their activity. One possible solution would be to freeze the cells in lower temperatures (perhaps with the use of liquid nitrogen), since enzyme can remain active and cell viability is preserved in liquid nitrogen (according to Deplanche *et al.* 2011, only live cells have the ability to recover metals). Also freeze-thaw may disrupt the adhesive quality of the film, since this depends on heavily hydrated sticky extra cellular polymers. Generally, cryoprotectants are common, but would mean an additional cost. Freeze-drying is a very common way to preserve both whole cells and enzymes.

The use of a more sophisticated predictive mathematical model would also be of great interest for the continuation of this study. The differential equation (3) already

proposed: ($zYS_o+K_m lnY=-E_oK_3/F$), could be the initiation of a project in order to calculate the specific parameters of this system i.e (apparent K_m- product of several parameters). In the same way it could also be found a simple mathematical predictive model like the one produced in this project, either for highly active biomass or for the case of phosphate release. Predicting phosphate release has been proved to be more complicated than the case of metal removal due to the participation of two isoenzymes with slightly different properties and therefore due to time restrictions that was not accomplished.

Initially the experiments started with europium but due to problems with Eu supply further work was focused on neodymium. Eu(III) is very useful, because one can use its fluorescence as an analytical method. It would have been interesting to find out if $EuPO_4$ is fluorescent and, if so, at what excitation and emission wavelength.In that case it would have been easy to be able to do direct measurement of metal accumulation, by 'looking inside' a column loaded with $EuPO_4$ and actually see a gradual appearance of fluorescence from the bottom (heavy loading) to the top (light loading) instead of just relying on purple colour by eye for the case of neodymium. If a number of biofilm cubes were suspended in a flask containing $Eu(NO_3)_3$ and challenged with some G2P and $Eu(NO_3)_3$ solution it might be possible to get an estimation of how different one disc is from another. That would give the possibility to be seen if the columns are homogeneous or if there are regions of high metal phosphate deposition and other regions of empty space where the flow could not reach. Moreover, since the lanthanide elements are paramagnetic (i.e. gadolinium), they are widely used as contrast agents for MRI. MRI has been used to develop column models (Creber *et al*. 2010) and imaging of lanthanum, copper and chromium distribution has been done (Nott *et al.* 2005, Beauregard *et al.* 2010). A very easy experiment could

also have been if cutting a loaded foam disc with $NdPO_4$ in half or quarters to check the amount of loading to various parts of the discs (externally or internally).

Since preliminary studies have shown that temperature influences the rate of the reaction (Macaskie *et al*. 1997) that means that *Serratia* sp. N14 behave differently at different temperatures. An interesting thought would also be for the case of the outflow chamber to change the temperature in which the biomass was immobilized. The experiment with the outflow chamber was performed at room temperature (22^oC), while it is known from literature that *Serratia* sp. N14 growth occurs in aerobic shaken environment at (30^oC) (Yong *et al*. 1997). That should be tested in subsequent tests by keeping the temperature of the outflow chamber at 30^oC and if that scenario will be proved, then it should be taken into consideration, when organizing the procedure for an industrial scale, since avoiding usage of external heating would reduce even more the cost of the process .

Moreover, the biofilm immobilized in the main vessel could be visible in foam discs on the third day. Had the temperature of the outflow vessel been kept at 30^oC, the biofilm could have been produced much sooner and therefore could have been used faster rather than waiting for the whole procedure to end up in 7 days. The experiment with the outflow chamber proved that lightly loaded biomass can be compared with highly loaded ones, so therefore only the surface layer of the biomass is interacting with the metal. That could be an initial step to calculate the amount of biomass required to recover specific amounts of metal.

The efficiency of the column for metal removal was found to have higher values when tested the biomass from the outflow chamber. That again could form the basis for

future projects, in order for someone to investigate the minimum amount of biomass that could have, not only the highest metal removal (and phosphate release), but also the highest efficiency of the columns. That could be achieved, in the case of the main vessel run, when limiting the number of days to less than seven; since it was observed the biofilm could be visible on foam discs on the third day after switching to continuous flow.

Last but not least the chemistry of the inflow solution could bring more light in the recovery of metal in highly acidic conditions. Michel *et al*. 1986 and Yong & Macaskie in 1995a found that metal phosphate formation is being mediated by phosphate release. *Aqua regia* results and preliminary experiments in high acidic conditions (Singh, 2012) have shown that *Serratia* sp*.* N14 can withstand conditions at low pH and that reduced recovery is mainly attributed to the chemistry of the solution. That finding was proved earlier by Yong *et al*. 1995b, where it was mentioned that for pH values of the inflow solution less than 4.5, the concentration of HPO_4^{2-} decreases, fact that reduces the metal phosphate formation, even if there was an increase of phosphate production by *Serratia* sp. N14 (Yong *et al.* 1995b). In 1995 Tolley *et al.* noticed that for the recovery of lanthanum at low pH more phosphate is necessary, while when using (V) sample, it was noticed that leaving the biomass for nucleation even at pH 3.5 was more essential for the biomass to recover rather than testing it directly from the cold room, fact that shows the need of the biomass for nucleation (Macaskie *et al*. 1994b). All these conclusions could focus the research more in studying the chemistry of the solution used to challenge the columns, since chemical factors have been established in previous studies (Macaskie *et al*. 1994b) to influence the whole process.

Overall *Serratia* sp. N14 has proved to be a very robust bacterium that can withstand exposure to very high acidic conditions and high salt concentration. The new technology introduced with the outflow chamber reactors also has promising industrial applications. Both experiments are in early stage research and can form the basis for future more sophisticated applications, like challenging industrial scale columns for the recovery of various rare earths from solutions of real metallic wastes and scrap.

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APPENDIX I

Calibration Curves

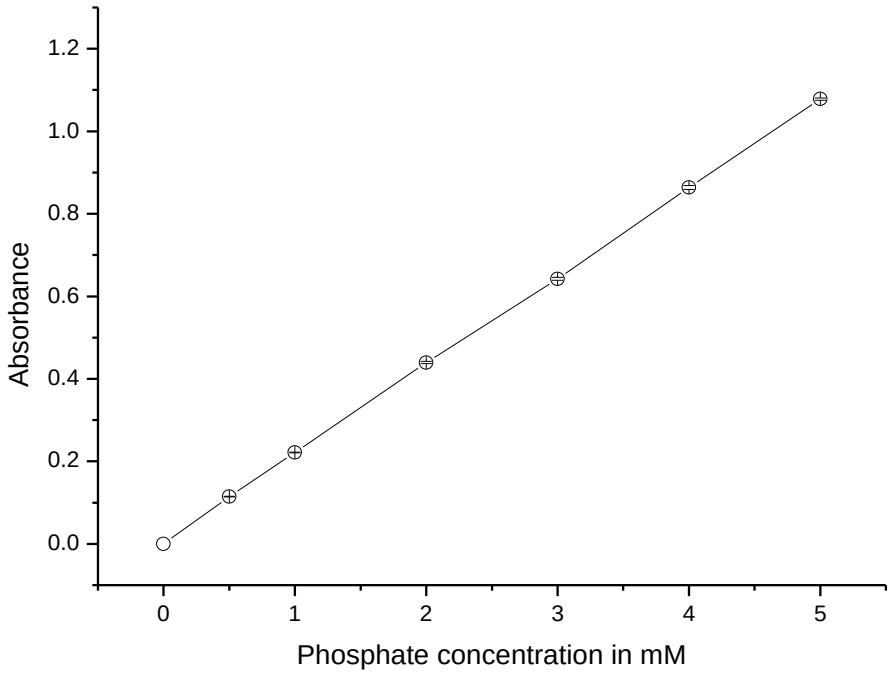


Figure 1: Calibration curve for phosphate concentration in the assay for inorganic phosphate (section 2.2.2).

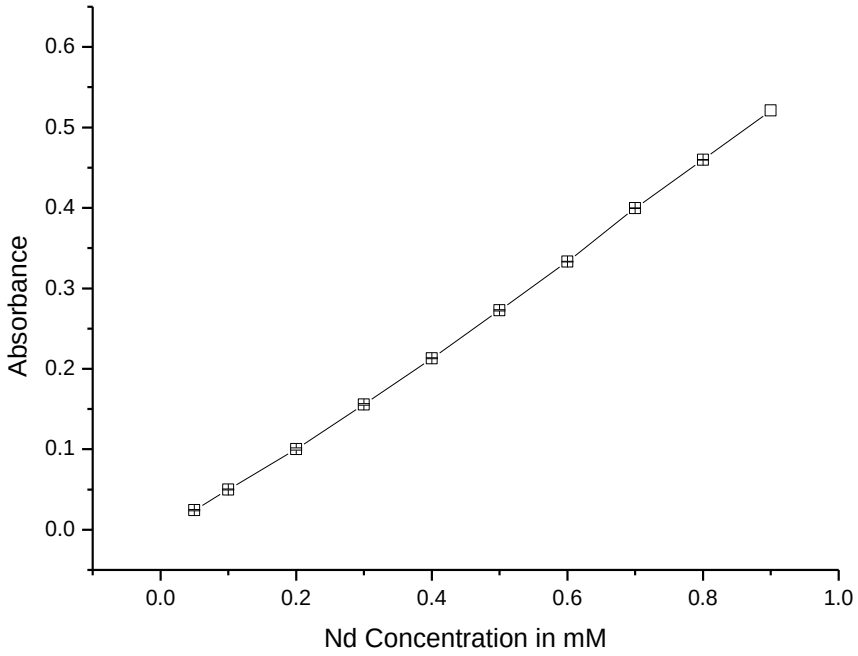


Figure 2: Standard curve for neodymium concentration in the heavy metal assay (section 2.2.3).

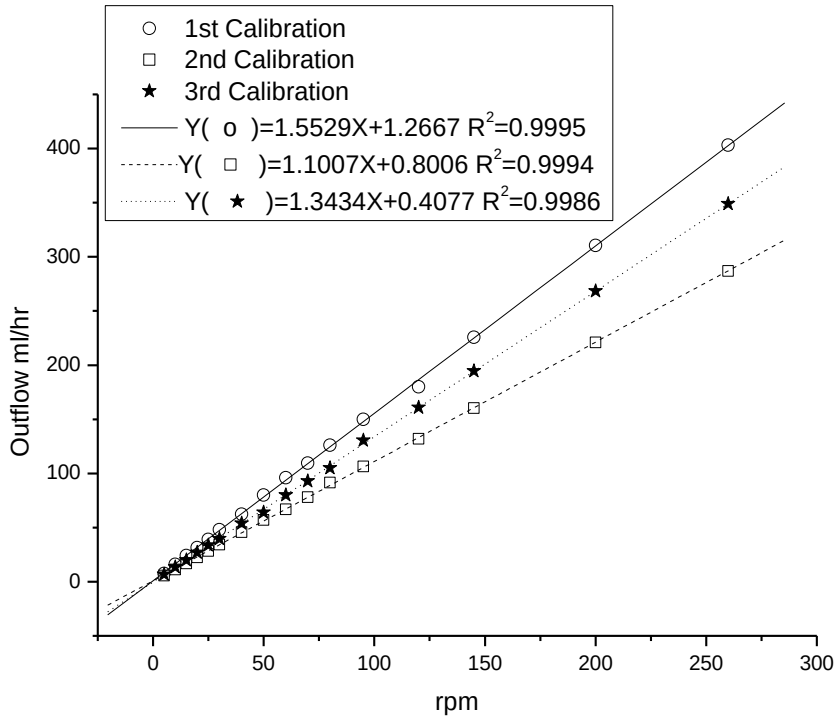


Figure 3: Pump speed calibration: The 1st pump speed calibration was performed for channel (3) and was used only for the results of the 7 months old biomass, while the second one was performed for two channels (3) and (4), which they both gave identical outflow results and were used for testing the 9, 10,12 months old biomass, as well as the biomass prepared from the outflow chamber and the new set of biomass prepared in May 2013 from the main vessel.The third one was used only for the ascending series of (R) experiment and the last experiment of the testing the column volumes at 5rpm for steady metal removal.

In the following table can be seen the three different flow rates given for three different pump speed calibrations (same pump and tubing used).The first calibration was used for the experiments done with (J) sample, the second one for almost all the experiments done during this project and the last calibration was done after the pH ascending experiment at (R) starting from pH 4.0 to pH 5.5.

rpm	mL/h (1st calibration)	mL/h (2nd calibration)	mL/h (3rd calibration)
5	7.6	5.7	6.8
10	15.9	11.1	13.5
15	24.0	16.8	19.8
20	31.5	22.5	27.0
25	39.0	28.2	33.6
30	48.0	34.2	39.6
40	62.4	45.6	54.0
50	80.0	57.0	64.0
60	96.0	66.8	80.0
70	109.5	78.0	93.0
80	126.0	91.5	105.0
95	150.0	106.5	130.5
120	180.0	132.0	160.8
145	225.6	160.4	194.4
200	310.5	220.9	268.3
260	403.2	286.9	348.9

The actual flow rate for each experiment was measured volumetrically for a fixed time, using a measuring cylinder and a timer.

APPENDIX II

Serratia sp. N14 growth in batch culture

Serratia sp. N14 cells were inoculated into the minimal solution described in paragraph 2.1 (p. 32) and incubated at 30 C^{o} in 50 and 100 ml conical flasks. During the incubation period no nutrients were added or waste products were removed and therefore the growth in such a closed system is a batch culture. A typical growth curve has a sigmoidal shape and consists of 1) the lag period (the period between inoculation and onset of exponential growth division rate. That depends from factors such as the age of the culture, the suitability and composition of the nutrient medium as well as the previous cultural history), 2) the exponential phase, when cells are dividing (doubling) at a constant rate and 3) the stationary phase in which the cells can no longer reproduce due to nutrient depletion or buildup of toxic waste products and the death phase (bacterial death in the nutrient media) (Schlegel H.G.1992).

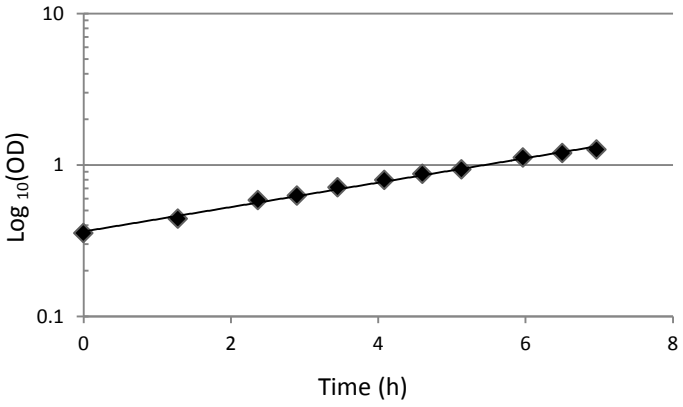


Figure 1: The logarithmic phase of *Serratia* sp. N14 cells after 5-6 hours of inoculation. The OD was checked every 30-60 min at 600nm using an Ultrospec 3300 pro UV/Visible Spectrophotometer, Amersham Biosciences

APPENDIX III

Possible explanations for obscure results for (R) sample

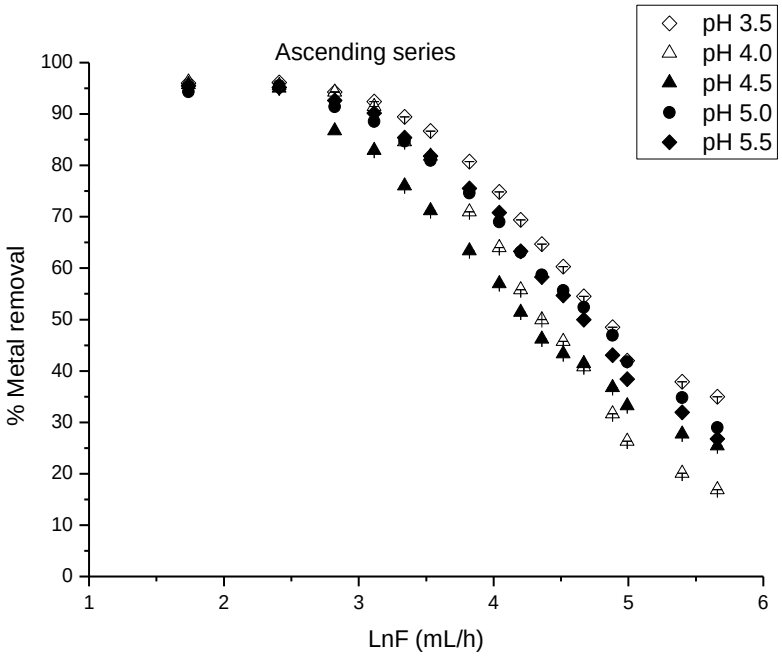


Figure 1: Effect on Nd recovery for increasing gradually again the pH from 3.5 to 5.5 for increasing flow rates at each pH from 6.8 mL/h (5rpm) to 348.9 mL/h (260 rpm)

Figure produced from plotting the data when analyzing (R) sample. Three possible explanation might be attributed to that phenomenon, described in paragraph 3.4.2.4.(curves at pH 3.5 and 5.5 almost overlap):

- When the biomass was produced in May 2013 in the main fermentor vessel, the charcoal filter was exploded from the glass cylinder which it was kept, due to pressure issues and that might influence the durability of the biomass produced in 2013. If that is the case then the results of this phenomenon might have influenced the durability of the biomass and that will be seen in subsequent tests i.e. in the pH descending –ascending series when the biomass might be retested at 7 months after being kept in the cold room or even if new *aqua regia*

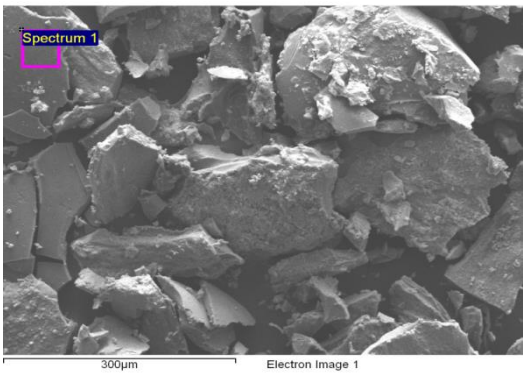
experiments might be performed, where for that scenario the biomass might appear lower durability in acidic conditions or high salt concentration.

- The arsenazo (III) solution has ended when reaching pH 3.5 and so a new solution had to be made and therefore another calibration curve had to be prepared for that. Although the two calibration curves gave again identical results, comparing the pH descending experiments with a specific calibration curve and the pH ascending pH series with another calibration curve, might have been the reason for giving this odd graph.
- A last explanation might be the fact that at pH 4.0 in the ascending series the flow rate of the pump at each rpm point changed again. Perhaps this has to do with the contact of the tubing with the rotating system of the pump. Another calibration was performed for that reason, which gave intermediate results obtained from the previous two calibrations (results seen in page 4).

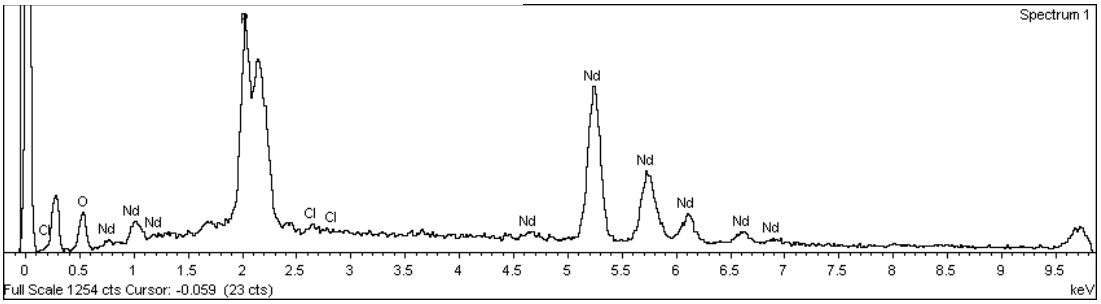
APPENDIX IV

SEM images and EDX data

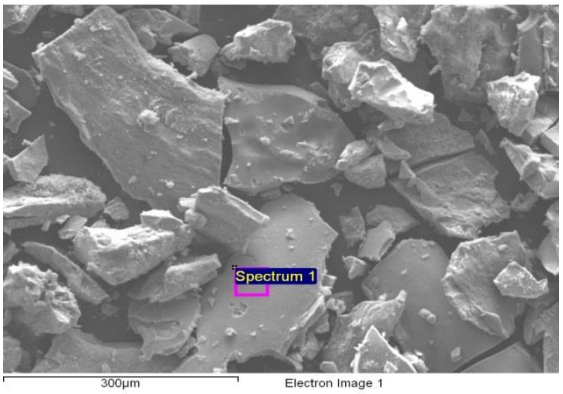
(J) Sample, Site of interest 2



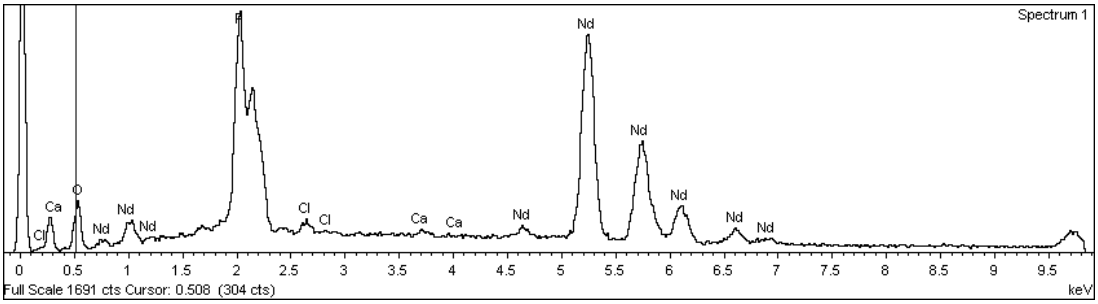
Element	Weight%	Atomic%
O	12.21	40.61
P	19.33	33.19
Cl	0.84	1.26
Nd	67.63	24.94
Totals	100.00	



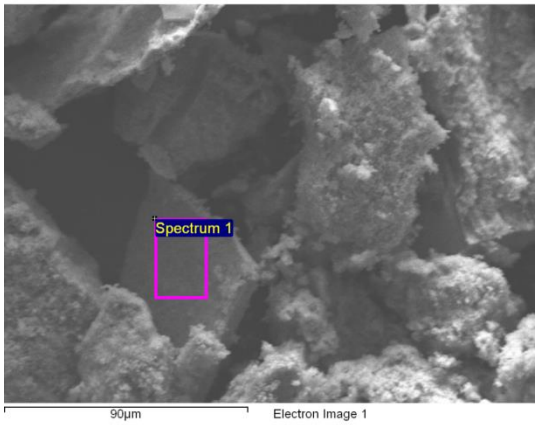
(J) Sample, Site of interest 3



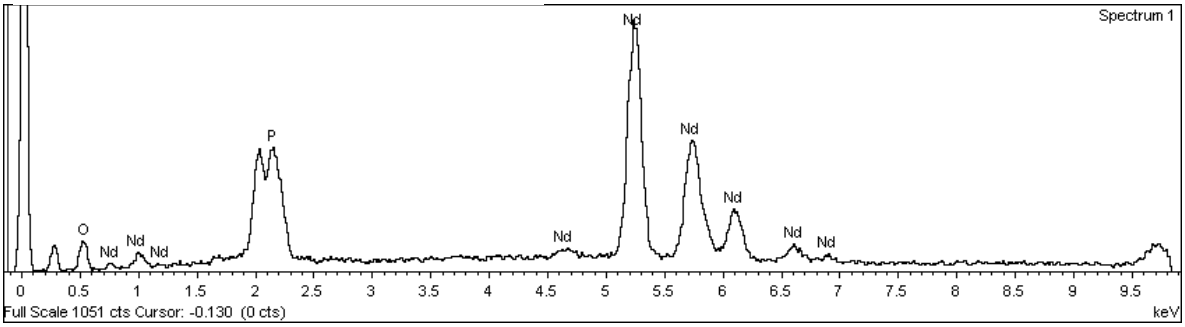
Element	Weight%	Atomic%
O	11.24	39.64
P	16.71	30.44
Cl	1.10	1.75
Ca	0.41	0.58
Nd	70.54	27.59
Totals	100.00	



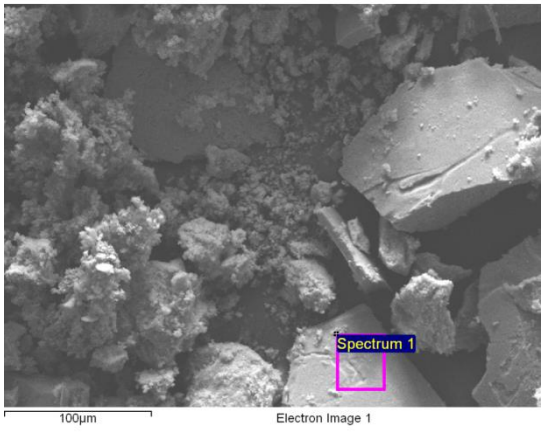
(L) Sample, Site of interest 1



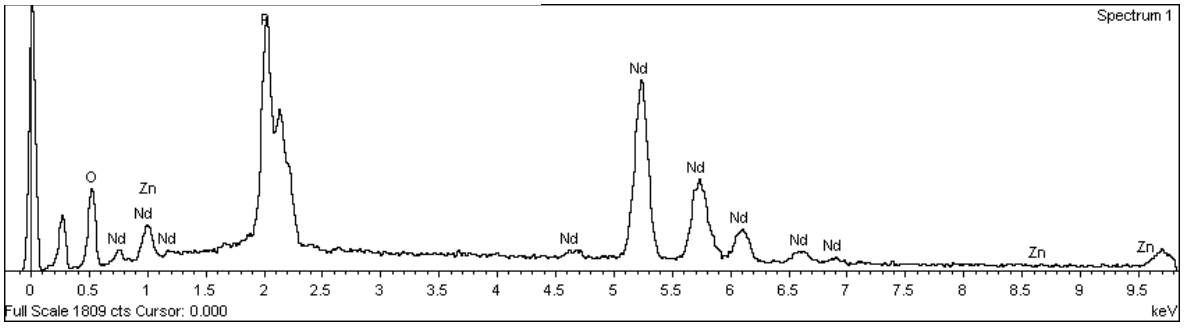
Element	Weight%	Atomic%
O	7.26	34.21
P	9.04	22.01
Nd	83.71	43.78



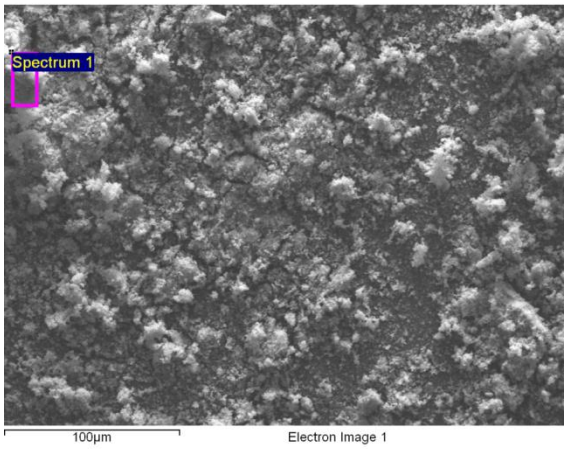
(L) Sample, Site of interest 2



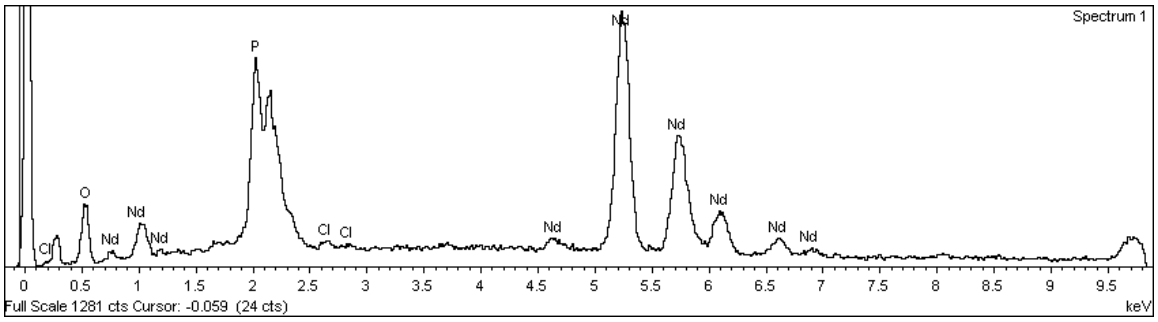
Element	Weight%	Atomic%
O	18.94	54.26
P	17.36	25.68
Zn	-0.48	-0.33
Nd	64.18	20.40
Totals	100.00	



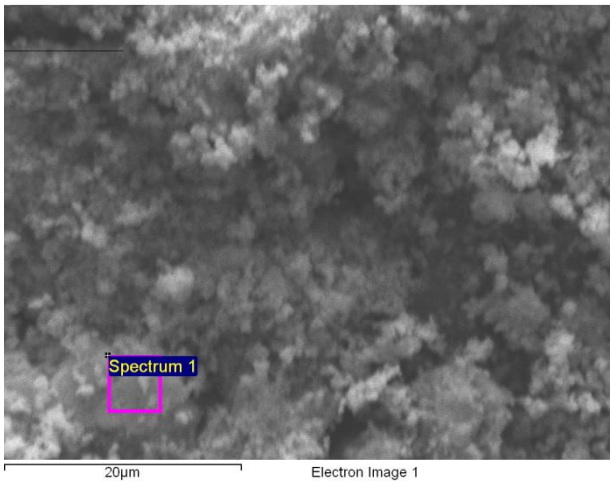
(V) Sample, Site of interest 1



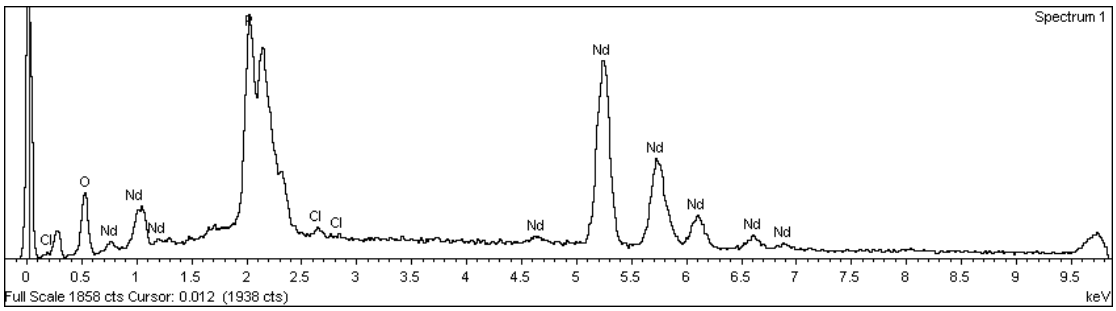
Element	Weight%	Atomic%
O	12.94	46.73
P	12.00	22.37
Cl	0.69	1.12
Nd	74.37	29.78
Totals	100.00	



(V) Sample, Site of interest 2



Element	Weight%	Atomic%
O	15.66	49.70
P	15.32	25.11
Cl	0.83	1.19
Nd	68.18	24.00
Totals	100.00	

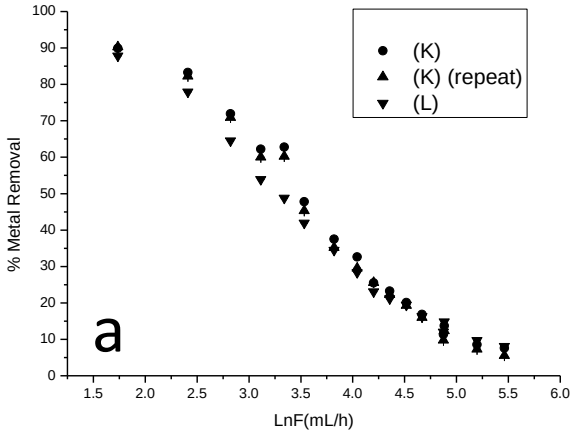


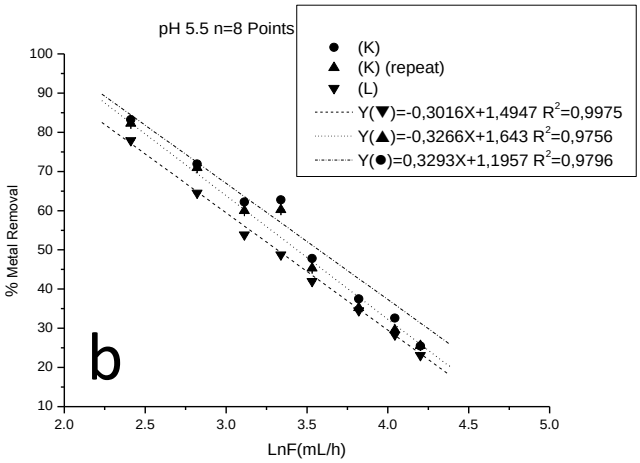
APPENDIX V

Development of method to production of simple predictive mathematical model to describe metal removal using samples (J),(K) and (L)

As previously explained in paragraph 2.7.3 using (L) sample, the gradual pH test in the descending series was done only for pH values 5.5-4.5-3.5. On the other hand, starting from 3.5 in pH ascending the column was left overnight at the standard solution at pH 5.5 after each of the pH change. That was done primarily, since it was noticed when using (K) sample that at pH 4.0 and 4.5 the biomass was not fully recovered after the pH was brought down to 3.5 only for two column volumes and rather doing the experiment for more column volumes it was decided to leave the column exposed to standard solution (pH 5.5) overnight after each pH test (''recovery period"). At this point it is also important to note that the effect of pH has two effects: a) on the activity of the enzyme in the catalytic reaction, but recoverable on return to permissive conditions and b) permanent enzyme damage and inactivation. This experiment is looking at a) only, since enzyme damage was not proved. Therefore, the aim of this experiment is to create a simple mathematical model to predict metal removal, using biofilm from culture (I) tested at 7,9 and 10 months after storage at 4 ^{o}C.

The following figures show the equations derived for each pH test (pH series descending-ascending). In all the figures that follow the sample (J) is shown as (■), sample (K) as (●), sample (K) repeat as (▲), sample (L) as (▼).





In all the figures that follow the n=8 or n=11 points that were used, were the ones that were giving the best possible straight lines. The choice for n=8 or n=11 was for exactly the same points in all the equations derived. The choice of 8 or 11 points was done in order to receive the linear part of the sigmoidal curve.The latter was chosen for more robust data.

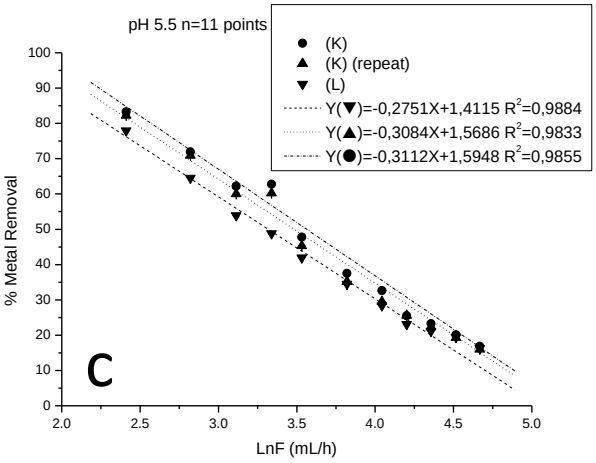


Figure 1: a) Metal removal for (K) sample at pH 5.5 (Descending series). Sample The equations derived for the metal removal for the two sets of data (9 and 10 months) can be seen in the following figures**: b)** Metal removal equations for 9 and 10 months old biomass at pH 5.5 (8 points), **c**) Metal removal equations for 9 and 10 months old biomass at pH 5.5 (11 points). Graph and equations for (J) sample, were omitted for this pH value(5.5).

Following the experiment at pH 5.0 sample (L), there were compared (actual metal removal versus that produced by equation) 3 points one at a slow flow rate (approximate metal removal 70%), one at medium flow rate (approximate metal removal 30%) and a third at high flow rate (approximate metal removal 10%). Due to the blocked column that occurred at the 9 months old experiment at a slow flow rate (17 mL/h), 2 column volumes was insufficient to bring the column to an expected metal removal and phosphate release and 4 column volumes (~32 ml) was used to bring the column back to a comparable with

the repeat column, phosphate release and metal removal. Therefore, for the pH 5.0 experiment (L), the inflow solution was left for 4 column volumes for each flow rate and it was observed that the metal removal was identical with the results taken from the equation Y=-0.3123X+1.6086 which was derived from the (K) data. The same procedure was done for pH 4.0 and 3.5 again for 4 column volumes and the results obtained were compared with the theoretical value received from the (K) data.

% Nd Removal (L) pH=5.0			
mL/h	**Theoretical value**	**Experimental value**	**Deviation from predicted value**
17	72.7%	67.7%	5%
57	34.5%	37.4%	2.9%
107	15%	21.4%	6.4%

% Nd Removal (L) pH=4.0			
mL/h	**Theoretical value**	**Experimental value**	**Deviation from predicted value**
17	69.2%	64.7%	4.5%
57	25.2%	33.2%	8%
107	6%	12.3%	6.3%

% Nd Removal (L) pH=3.5			
mL/h	**Theoretical value**	**Experimental value**	**Deviation from predicted value**
17	56.3%	71.1%	14.8%
57	13.9%	31.9%	18%
107	8%	20.4%	12.4%

Table 1: Comparison of theoretical and experimental value for three flow rates at pH 5.0, pH 4.0 and pH 3.5 (from the pH descending series) using (L) sample.

For pH 3.5 the experimental results obtained were for 17 mL/h: 71.1%, for 57 mL/h: 31.9% and for 107 mL/h: 20.4%. Initially the results seemed not to follow the predictive model from the equations derived when using the (K) sample. However, a check for all the points at this pH value proved, that the equations derived when using the (J) and (L)

samples, results were closer than the ones for (K) and therefore the equation chosen, was derived according to (L) sample data.

Before dropping the pH to 3.5 another experiment was performed for all the 16 flow rate points at pH 4.5 and then down to pH 3.5.The results obtained for the pH 4.5 experiment can be seen in **Figure 2** that follows.

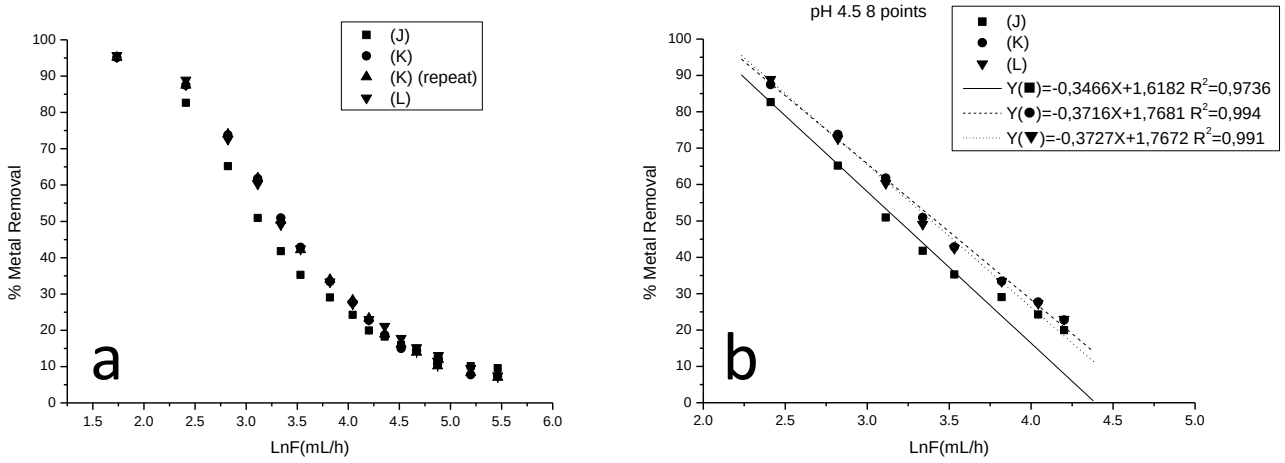


Figure 2: a) Metal removal for (J), (K) and (L) samples at pH 4.5 **b)** Metal removal at pH 4.5 for 7- 9 and 10 months old *Serratia* sp. N14 . The lines received for the data at pH 4.5 .

After this experiment the column was left overnight in to the standard solution and the next test was for pH =3.5. Starting from this pH value in the pH ascending test all the flow rate points were once again tested, in order to compare the new equations for (L) sample. So starting from pH 3.5 the results obtained were the following:

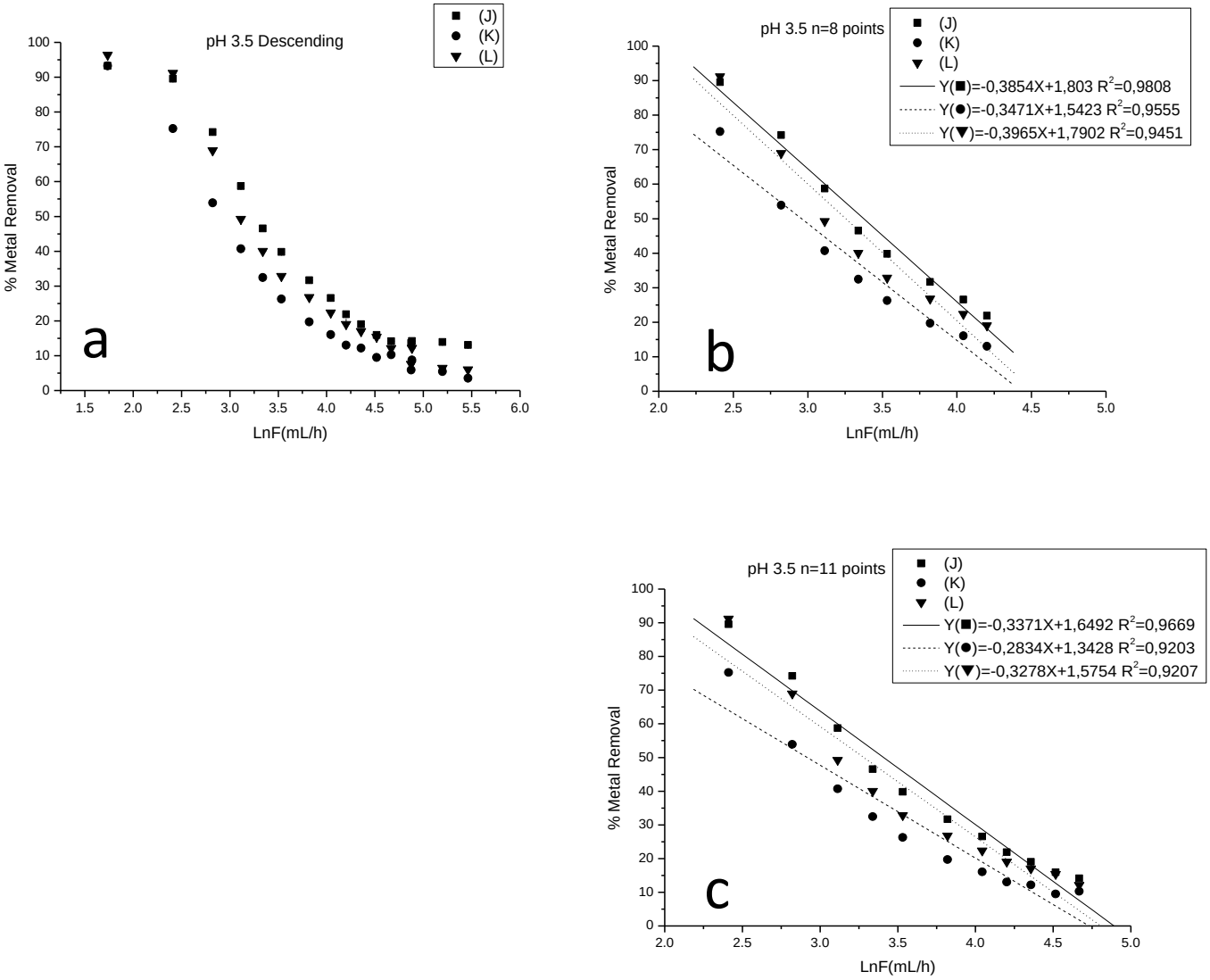


Figure 3: a) Comparison of (J),(K) and (L) in metal removal at pH 3.5. **b)** Comparison of n=8 points of (J),(K) and (L) in metal removal at pH 3.5.**c)** Comparison of n=11 points of (J), (K) and (L) in metal removal at pH 3.5.

% Nd Removal						
pH 3.5	**Experimental values**			**Proposed Theoretical values**		
mL/h	**(J)**	**(K)**	**(L)**	**(J)**	**(K)**	**(L)**
17	74.2%	56.7%	69%	71.3%	56.3%	67.15%
57	26.5%	16.5%	22.4%	24.2%	13.9%	18.7%
107	14.2%	10.2%	12.1%	<1%	8%	6%

Table 2: Comparison of experimental with proposed theoretical values for three points of flow rates at pH 3.5. Note: the proposed theoretical values were derived from equations produced from plotting either 8 or 11 consecutive points from the experimental values.

For 8 points:

(J): Y=-0.3854X+1.8003 (1)

(K): Y=-0.3471X+1.5423 (2)

(L):Y=-0.3965X+1.7902 (3)

For 11 points:

(J): Y=-0.3371X+1.6492 (4)

(K): Y=-0.2834+1.3428 (5)

(L):Y=-0.3278X+1.5754 (6)

Comparing now the experimental values earned earlier when doing the 3 flow rate point experiment with the values earned from these equations, it is concluded that the best line that would describe the metal removal in this pH value is the 7 month-11 point equation namely: Y=-0.3371X+1.6492 (3.5) (17)

By doing exactly the same procedure for pH 4.0 in the ascending experiment we get the data shown in **Figures 4 a), b), c).**

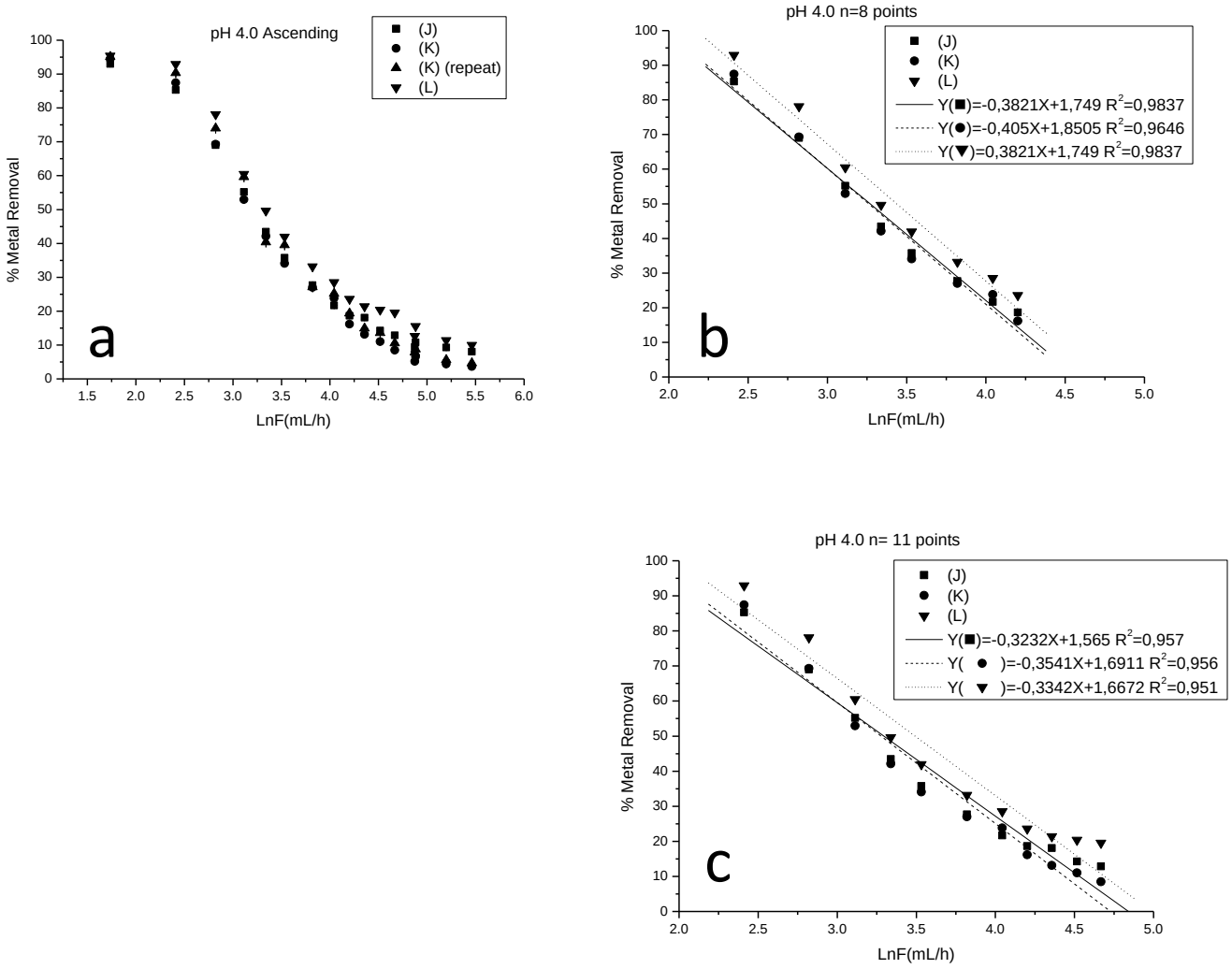


Figure 4: a) Comparison of 7-9 and 10 months old *Serratia* sp. N14 coated foam discs in metal removal at pH 4.0. **b)** Comparison of 8 points of 7-9 and 10 months old *Serratia* sp. N14 coated foam discs in metal removal at pH 4.0. **c)** Comparison of 11 points of 7-9 and 10 months old *Serratia* sp. N14 coated foam discs in metal removal at pH 4.0 (ascending).

% Nd Removal						
pH 4.0	**Experimental values**			**Proposed Theoretical values**		
mL/h	**(J)**	**(K)**	**(L)**	**(J)**	**(K)**	**(L)**
17	69%	74%	78.1%	65.3%	69.2%	72.4%
57	21.7%	25.2%	28.5%	25.8%	25.9%	31.6%
107	12.9%	10.6%	19.5%	5%	4%	10.7%

Table 3: Comparison of experimental with proposed theoretical values for three points of flow rates at pH 4.0. Note: the proposed theoretical values were derived from equations produced from plotting the points from the experimental values.

For 8 points:

(J): Y=-0.3821X+1.749 (8)

(K): Y=-0.405X+1.8505 (9)

(L):Y=-0.3961X+1.8614 (10)

For 11 points:

(J): Y=-0.3232X+1.565 (11)

(K): Y=-0.3541X+1.6911 (12)

(L):Y=-0.3342X+1.6672 (13)

Again now when comparing the experimental values earned earlier when doing the 3 flow rate point experiment for 4 column volumes, with the values earned from these equations, it can be noticed that the best line that would describe the metal removal in this pH value is the (L)-n=11 point equation namely:

Y=-0.3342X+1.6672 (4.0) (14)

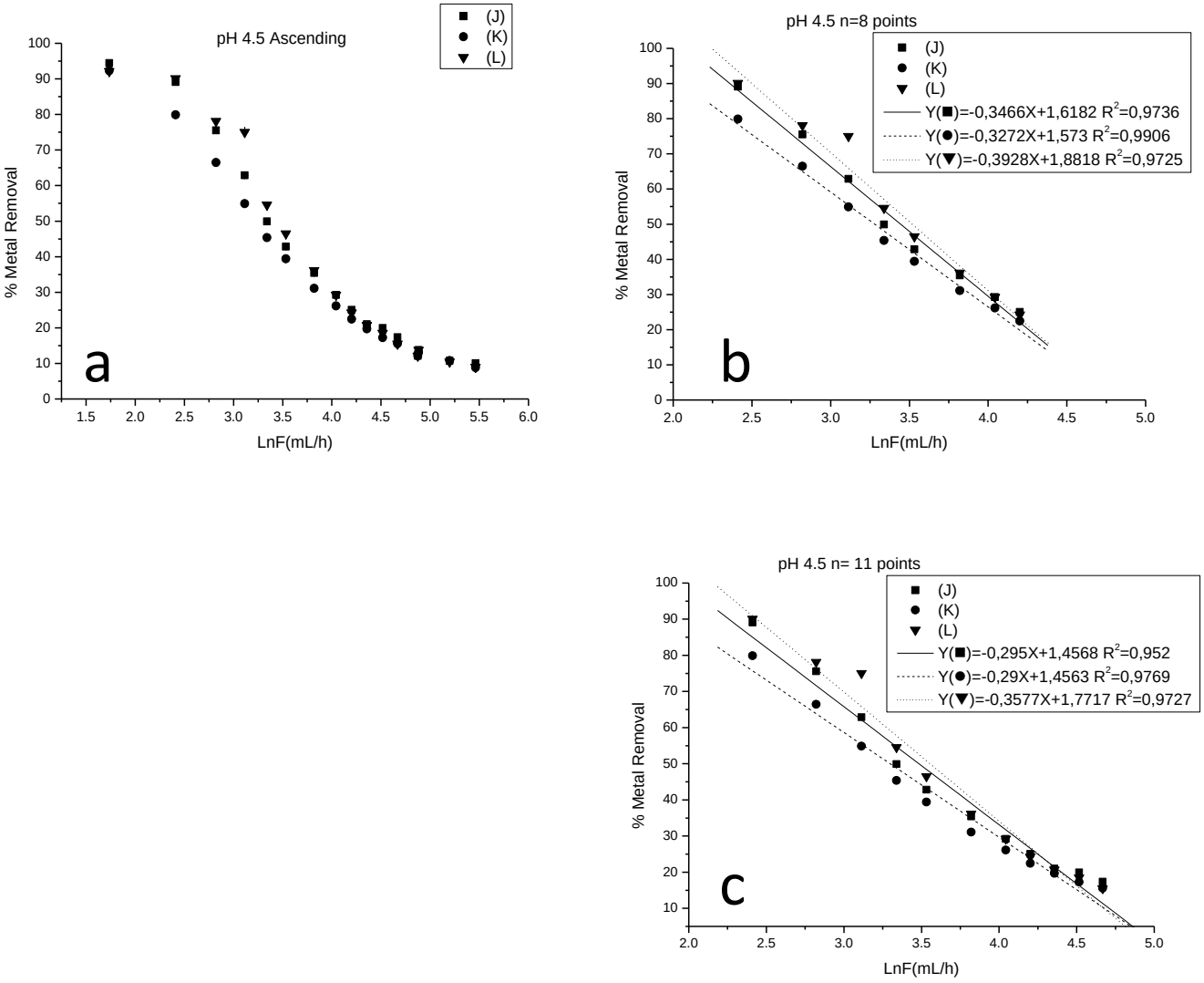


Figure 5: a) Comparison of samples (J),(K),(L) in metal removal at pH 4.5 (ascending). **b)** Comparison of 8 points of (J),(K),(L) in metal removal at pH 4.5. **c)** Comparison of 11 points (J),(K),(L) in metal removal at pH 4.5. Metal removal is increased especially for slow rates for 10-25%.

% Nd Removal						
pH 4.5	**Experimental values**			**Proposed Theoretical values**		
mL/h	**(J)**	**(K)**	**(L)**	**(J)**	**(K)**	**(L)**
17	65.2%	66.5%	78.1%	65.3%	69.2%	72.4%
57	24.3%	26.1%	29.2%	25.8%	25.9%	31.6%
107	14.9%	15.5%	15.5%	5%	4%	10.7%

Table 4: Comparison of experimental with proposed theoretical values for three points of flow rates at pH 4.5. Note: the proposed theoretical values were derived from equations produced from plotting the points from the experimental values.

For 8 points:

(J): Y=-0.3466X+1.6182 (15)

(K): Y=-0.3272X+1.573 (16)

(L):Y=-0.3928X+1.8818 (17)

For 11 points:

(J): Y=-0.295X+1.4568 (18)

(K): Y=-0.29X+1.4563 (19)

(L): Y=-0.3577X+1.7717 (20)

These equations were compared with the ones received for the pH 4.5 (pH descending) and after comparing all the results obtained it was noticed that the most suitable equation would be for the (L)-n=11 point:

Y=-0.3383X+1.6632 (4.5) (21)

For the case of pH 5.0 experiment we had the following figures:

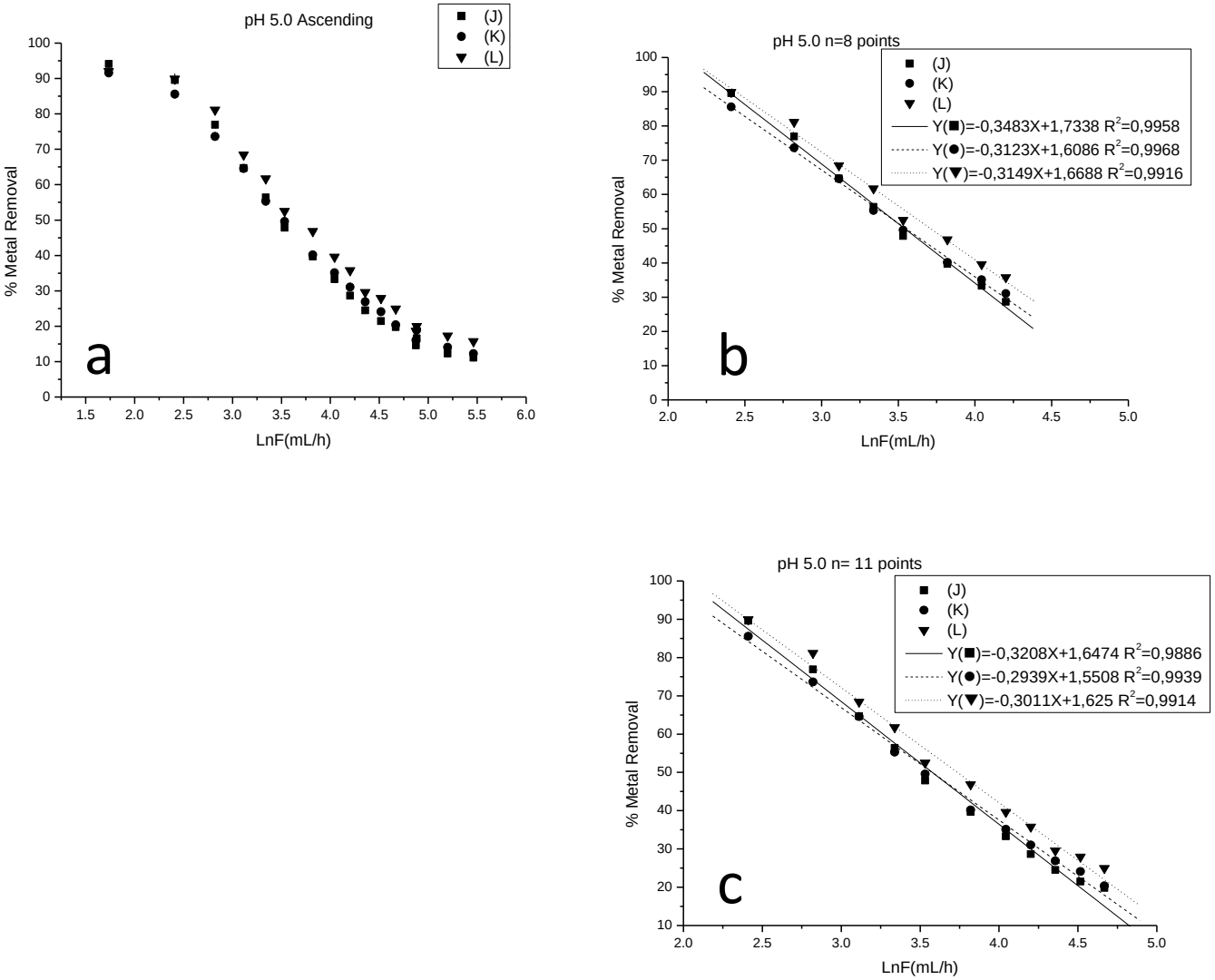


Figure 6: a) Comparison of samples (J),(K),(L) in metal removal at pH 5.0 (ascending). **b)** Comparison of (J),(K),(L) in metal removal at pH 5.0. **c)** Comparison of 11 points of (J),(K),(L) in metal removal at pH 5.0.

% Nd removal						
pH 5.0	**Experimental values**			**Proposed Theoretical values**		
mL/h	**(J)**	**(K)**	**(L)**	**(J)**	**(K)**	**(L)**
17	76.9%	73.6%	81.2%	75%	72.7%	75.1%
57	33.3%	35.1%	39.6%	32.6%	34.6%	34.6%
107	19.8%	20.4%	24.9%	10.8%	15.1%	10.8%

Table 5: Comparison of experimental with proposed theoretical values for three points of flow rates at pH 5.0. Note: the proposed theoretical values were derived from equations produced from plotting the points from the experimental values.

For 8 points:

(J): Y=-0.3483X+1.7338 (22)

(K): Y=-0.3123X+1.6086 (23)

(L): Y=-0.3483X+1.7338 (24)

For 11 points:

(J): Y=-0.3208X+1.6474 (25)

(K): Y=-0.2939X+1.5508 (26)

(L):Y=-0.3011X+1.625 (27)

Once again when comparing the experimental values earned earlier when doing the 3 flow rate point experiment with the values earned from these equations, it can be noticed that the best line that would describe the metal removal in this pH value is the (K)-n=8 point equation namely: Y=-0.3123X+1.6086 (5.0) (28)

Finally for the case of pH 5.5 it was noticed that when starting from 5.5 in pH descending series, the results were different from the ones in pH ascending series at the same pH value (5.5). Both the experiments for (K) and (L) were done immediately when getting the biomass out of the cold room and testing it directly, namely there was no nucleation of the column in the standard solution. Had the column been left overnight in the standard solution once brought out of the cold room, the results might have been more coherent and there might have been only one equation describing our system. Therefore, in this case the results were:

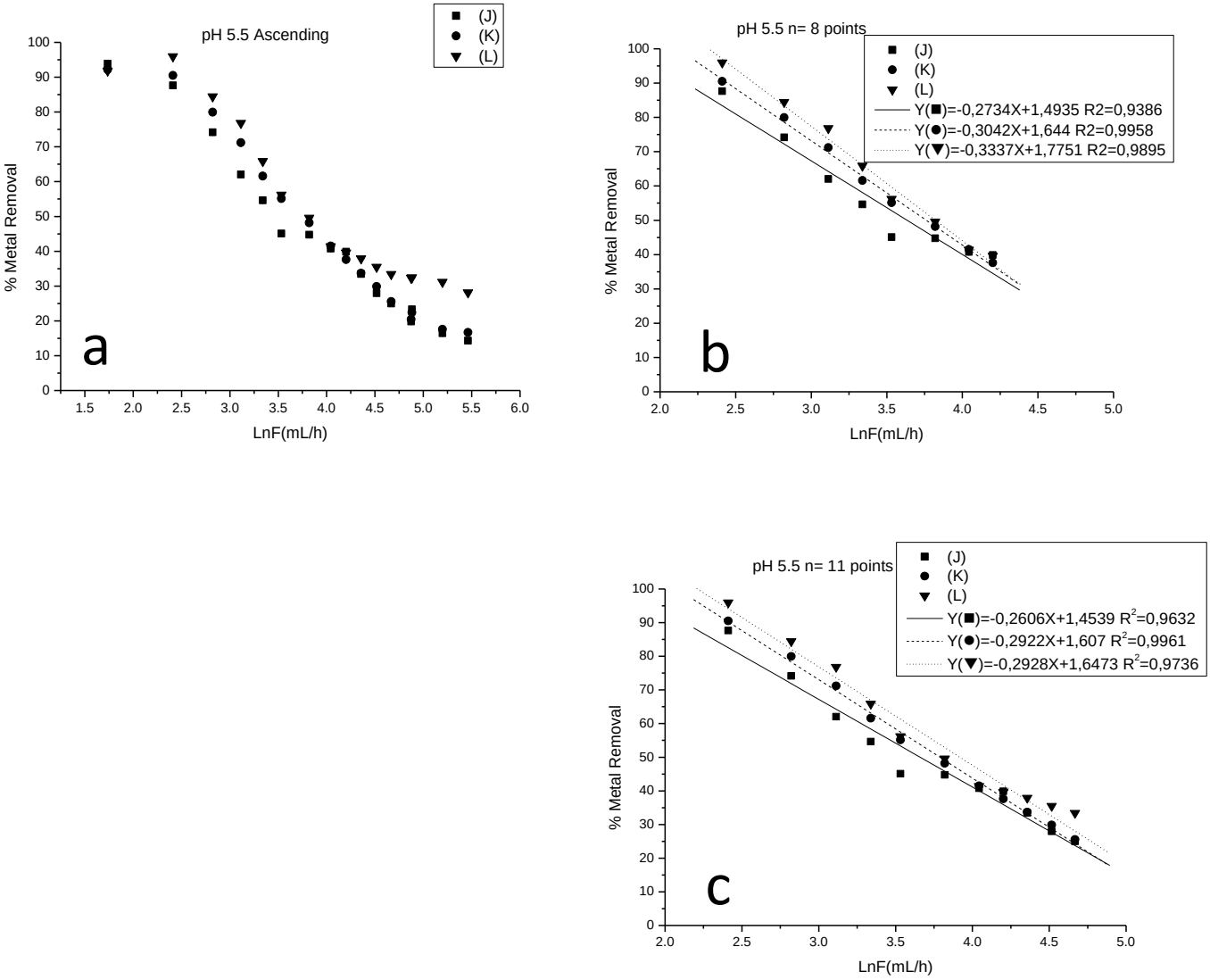


Figure 7: a) Comparison of samples (J),(K) and (L) in metal removal at pH 5.5 (ascending). **b)** Comparison of 8 points of (J),(K),(L) in metal removal at pH 5.5. **c)** Comparison of 11 points of (J),(K),(L) in metal removal at pH 5.5.

%Nd removal						
pH 5.5	**Experimental values**			**Proposed Theoretical values**		
mL/h	**(J)**	**(K)**	**(L)**	**(J)**	**(K)**	**(L)**
17	74.2%	80%	84.4%	72.2%	78.6%	83.4%
57	40.8%	41.5%	41.4%	38.8%	41.3%	42.6%
107	25%	25%	33%	21.7%	22.4%	21.7%

Table 6: Comparison of experimental with proposed theoretical values for three points of flow rates at pH 5.5. Note: the proposed theoretical values were derived from equations produced from plotting the points from the experimental values:

For 8 points:

(J): Y=-0.2734X+1.4935 (29)

(K): Y=-0.3042X+1.6443 (30)

(L):Y=-0.3337X+1.7751 (31)

For 11 points:

(J): Y=-0.2606X+1.4539 (32)

(K): Y=-0.2922X+1.607 (33)

(L):Y=-0.2928X+1.6473 (34)

From the given equations and from the results obtained so far from the previously conducted experiments it was noticed that the most suitable equation for the pH 5.5in the descending series experiment was Y=-0.3084X+1.5686 (5.5↓) (35) with 69.8%, 32.2% and 12.9% recovery, while for pH 5.5 recovering from pH 3.5 was : Y=-0.2922X+1.607 (5.5↑) (36) with 78.3%, 42.6% and 24.3% recovery for the specific three flow rate values shown in all tables.

APPENDIX VI

Illustration of efficacy of the new biofilm-growth method for evaluation of a biofilm inhibitor.

Experiments and results in this Appendix were from Dr. Mark Webber who carried out this evaluation, using biofilms prepared by the author, A.J.Murray and S.Singh

The experimental description that follows was given by Dr. Mark Webber. At defined time points two foam disks were removed from reactor a) using a sterilised surgical clamp and scalpel and placed in a sterile universal tube. At the same time two 1mL aliquots of planktonic culture were removed from the reactor. The foam disks were washed briefly in phosphate buffered saline (PBS-purchased pre-made at 1X concentration from Fisher Scientific), then added to 500 µL of PBS in a fresh universal tube and vortex- mixed repeatedly (5 x 30s) to remove biofilm cells into solution. These samples and the corresponding planktonic samples were then serially diluted and used to inoculate LB agar plates, which were incubated and then enumerated (colony forming units/mL (CFU)). Calculation of viable numbers in the original samples from control and inhibitor-closed chambers were then made by Dr. Mark Webber.

In the control reactor the planktonic cells reached concentrations of 10^8 CFU/mL after 24h and maintained this level throughout the experiment. The addition of chlorpromazine to the second chamber resulted in a decrease in viable cell numbers of approximately 30% less than ten-fold (according to the notice made in page 43) at 24 and 72h, but not at 48h. The number of biofilm associated cells present on the foams (these are not affected by the dilution factor), was reduced to a greater extent by the presence of chlorpromazine (100 fold by 72h) and this was statistically different to the control reactor.

Conclusions: The reactor offshoot vessels were able to maintain large populations of cells and these were able to form biofilms. The addition of chlorpromazine decreased biofilm formation, but also reduced planktonic numbers of cells (a slight inhibitory effect on viability in the conditions studied). The effect of chlorpromazine was to reduce by more than 10-fold the number of biofilm-cells after 24 and 48 h and by ~ 100-fold after 72 h. Hence the utility of using the experimental design developed as a methodological tool in this study is proved.

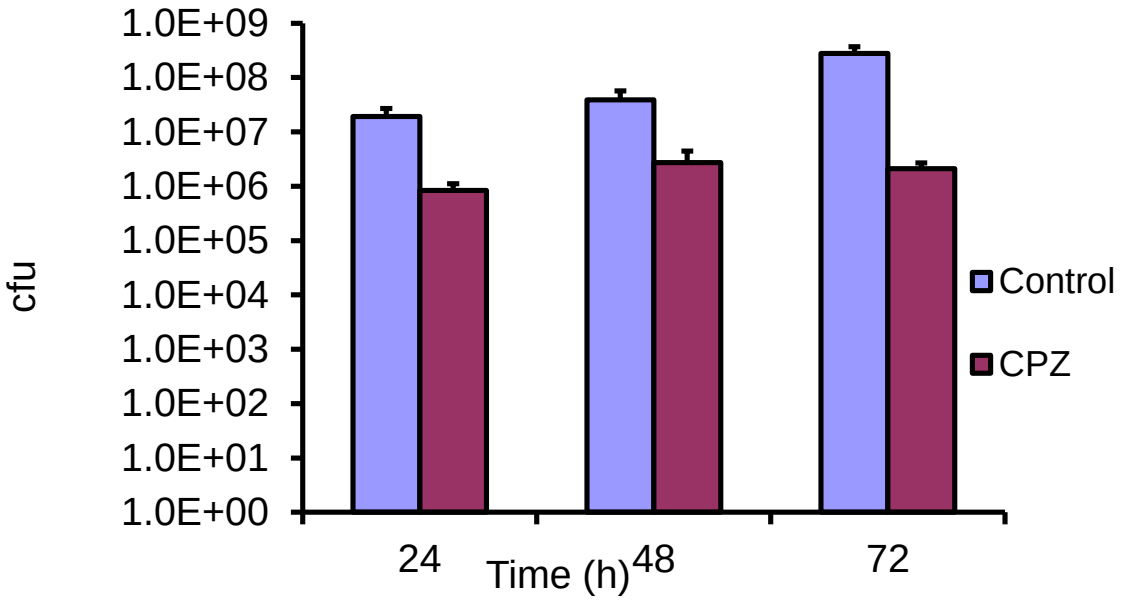


Figure 1: Colony forming units after 24, 48 and 72 hours from samples (suspended cells) taken without an inhibitor (control sample-blue color) and when using chlorpromazine (CPZ) as an inhibitor (purple color).

APPENDIX VII

Repeatability results for (K) sample

pH 5.5

Rpm	LnF (mL/h)	Total Phosphate (Pi)	Repeat (Pi)	% Difference	Metal Recovery	Repeat	% Difference
5	1.736699	4.447876	4.394374	1.1%	89,9%	90,3%	0,4%
10	2.410798	3.137363	3.012781	2.5%	83.2%	82.3%	1.0%
15	2.821379	2.463232	2.355736	2.1%	71.9%	70.9%	1.0%
20	3.113515	2.047855	1.941865	2.1%	62.2%	60.0%	2.2%
25	3.339322	1.853152	1.745313	2.2%	62.8%	60.2%	2.6%
30	3.532226	1.566778	1.436208	2.6%	47.8%	45.3%	2.5%
40	3.819908	1.316024	1.203255	2.3%	37.5%	35.2%	2.3%
50	4.043051	1.101063	0.938417	3.3%	32.6%	29.6%	3.1%
60	4.200954	0.922446	0.873875	1.0%	25.5%	25.6%	0.1%
70	4.356709	0.835096	0.789731	0.9%	23.3%	22.3%	1.0%
80	4.516339	0.747426	0.689783	1.2%	20.1%	19.3%	0.8%
95	4.668145	0.634337	0.602533	0.6%	16.9%	16.0%	0.9%
120	4.882802	0.523369	0.507221	0.3%	13.7%	12.4%	1.3%
145	4.99	0.441098	0.423669	0.3%	11.4%	9.8%	1.6%
200	5.397891	0.341297	0.313858	0.5%	8.5%	7.3%	1.2%
260	5.659413	0.281954	0.251236	0.6%	7.6%	5.6%	2.0%

pH 5.0

Rpm	LnF (mL/h)	Total Phosphate (Pi)	Repeat (Pi)	% Difference	Metal Recovery	Repeat	% Difference
5	1.736699	3.843741	3.659864	3.7%	95.9%	95.3%	0.7%
10	2.410798	2.395358	2.268509	2.5%	88.0%	86.8%	1.2%
15	2.821379	1.779637	1.672413	2.1%	75.3%	74.1%	1.2%
20	3.113515	1.46912	1.376963	1.8%	64.1%	61.8%	2.4%
25	3.339322	1.293663	1.186935	2.1%	54.7%	51.6%	3.1%
30	3.532226	1.073627	1.053806	0.4%	46.3%	45.0%	1.4%
40	3.819908	0.898588	0.882512	0.3%	36.8%	37.3%	0.6%
50	4.043051	0.762648	0.738906	0.5%	30.5%	29.9%	0.5%
60	4.200954	0.640121	0.646653	0.1%	24.1%	25.1%	1.0%
70	4.356709	0.562734	0.540249	0.4%	20.3%	19.4%	0.9%
80	4.516339	0.492446	0.485246	0.1%	17.4%	17.5%	0.1%
95	4.668145	0.458769	0.439835	0.4%	15.6%	15.3%	0.3%
120	4.882802	0.37322	0.37524	0.0%	12.5%	13.6%	1.1%
145	4.99	0.333107	0.307911	0.5%	12.2%	10.6%	1.6%
200	5.397891	0.258678	0.262671	0.1%	8.2%	9.2%	1.0%
260	5.659413	0.226922	0.220537	0.1%	8.6%	7.8%	0.8%

pH 4.5

rpm	LnF (mL/h)	Total Phosphate (Pi)	Repeat (Pi)	% Difference	Metal Recovery	Repeat	% Difference
5	1.736699	3.27761	3.152361	2.5%	95.1%	95.2%	0.1%
10	2.410798	1.839409	1.844069	0.1%	87.5%	87.5%	0.0%
15	2.821379	1.33407	1.345658	0.2%	73.8%	74.0%	0.2%
20	3.113515	1.095819	1.089754	0.1%	61.8%	61.9%	0.2%
25	3.339322	0.920733	0.927909	0.1%	50.9%	50.2%	0.7%
30	3.532226	0.783736	0.786819	0.1%	42.8%	42.2%	0.6%
40	3.819908	0.666316	0.678199	0.2%	33.4%	34.0%	0.6%
50	4.043051	0.579538	0.593541	0.3%	27.7%	28.3%	0.6%
60	4.200954	0.496038	0.503261	0.1%	22.7%	23.3%	0.6%
70	4.356709	0.440249	0.437857	0.0%	18.7%	18.9%	0.2%
80	4.516339	0.376422	0.391435	0.3%	15.0%	16.2%	1.2%
95	4.668145	0.354652	0.354776	0.0%	14.4%	13.9%	0.5%
120	4.882802	0.306973	0.295113	0.2%	11.9%	12.0%	0.1%
145	4.99	0.256653	0.25367	0.1%	10.6%	10.2%	0.5%
200	5.397891	0.202884	0.207001	0.1%	7.7%	8.3%	0.6%
260	5.659413	0.17463	0.180548	0.1%	7.4%	7.0%	0.3%

pH 4.0

Rpm	LnF (mL/h)	Total Phosphate (Pi)	Repeat (Pi)	% Difference	Metal Recovery	Repeat	% Difference
5	1.736699	2.917102	2.855537	1.2%	94.9%	95.0%	0.1%
10	2.410798	1.534376	1.549217	0.3%	86.8%	87.2%	0.4%
15	2.821379	1.103523	1.130939	0.5%	71.2%	71.8%	0.6%
20	3.113515	0.893012	0.889043	0.1%	57.2%	56.8%	0.4%
25	3.339322	0.734817	0.745147	0.2%	44.9%	45.5%	0.6%
30	3.532226	0.647548	0.67119	0.5%	37.4%	39.0%	1.6%
40	3.819908	0.534393	0.526184	0.2%	28.8%	28.2%	0.5%
50	4.043051	0.445471	0.469532	0.5%	22.4%	23.8%	1.5%
60	4.200954	0.392664	0.3972	0.1%	18.8%	19.2%	0.5%
70	4.356709	0.349497	0.358692	0.2%	16.2%	16.6%	0.5%
80	4.516339	0.33182	0.330143	0.0%	15.5%	15.9%	0.5%
95	4.668145	0.283993	0.283993	0.0%	12.9%	12.9%	0.0%
120	4.882802	0.236756	0.245385	0.2%	11.0%	11.4%	0.4%
145	4.99	0.208478	0.202684	0.1%	10.0%	9.9%	0.1%
200	5.397891	0.173272	0.176231	0.1%	8.8%	8.6%	0.2%
260	5.659413	0.144131	0.137747	0.1%	7.4%	6.6%	0.8%

pH 3.5

rpm	LnF (mL/h)	Total Phosphate (Pi)	Repeat (Pi)	% Difference	Metal Recovery	Repeat	% Difference
5	1.736699	2.297987	2.315073	0.3%	93.3%	93.3%	0.0%
10	2.410798	1.076989	1.155858	1.6%	75.3%	77.2%	2.0%
15	2.821379	0.737418	0.808692	1.4%	53.9%	56.7%	2.8%
20	3.113515	0.596557	0.615911	0.4%	40.7%	40.9%	0.2%
25	3.339322	0.49669	0.543608	0.9%	32.4%	33.4%	1.0%
30	3.532226	0.439548	0.474335	0.7%	26.3%	27.6%	1.3%
40	3.819908	0.352031	0.388643	0.7%	19.7%	20.7%	1.0%
50	4.043051	0.306424	0.328046	0.4%	16.1%	16.5%	0.5%
60	4.200954	0.262394	0.283178	0.4%	13.1%	13.7%	0.7%
70	4.356709	0.243017	0.261951	0.4%	12.2%	12.5%	0.3%
80	4.516339	0.214248	0.240701	0.5%	9.5%	10.7%	1.2%
95	4.668145	0.211313	0.218512	0.1%	10.3%	10.2%	0.1%
120	4.882802	0.191344	0.163314	0.6%	8.7%	6.9%	1.9%
145	4.99	0.142802	0.158087	0.3%	5.9%	6.8%	0.9%
200	5.397891	0.12584	0.122981	0.1%	5.5%	4.5%	0.9%
260	5.659413	0.098796	0.109421	0.2%	3.5%	4.4%	0.9%

pH 4.0

Rpm	LnF (mL/h)	Total Phosphate (Pi)	Repeat (Pi)	% Difference	Metal Recovery	Repeat	% Difference
5	1.736699	2.613769	2.688917	1.5%	94.4%	95.1%	0.7%
10	2.410798	1.428776	1.594381	3.3%	87.4%	90.3%	2.9%
15	2.821379	1.001922	1.137519	2.7%	69.3%	74.0%	4.7%
20	3.113515	0.771838	0.917393	2.9%	53.0%	59.6%	6.6%
25	3.339322	0.627817	0.689458	1.2%	42.1%	40.4%	1.8%
30	3.532226	0.544198	0.66542	2.4%	34.1%	39.5%	5.4%
40	3.819908	0.457792	0.500864	0.9%	27.0%	27.1%	0.1%
50	4.043051	0.402741	0.461394	1.2%	23.8%	25.2%	1.4%
60	4.200954	0.316878	0.381248	1.3%	16.2%	19.3%	3.2%
70	4.356709	0.271295	0.347896	1.5%	13.2%	14.9%	1.8%
80	4.516339	0.252856	0.286137	0.7%	11.0%	13.6%	2.6%
95	4.668145	0.193169	0.246766	1.1%	8.5%	10.6%	2.1%
120	4.882802	0.183778	0.231729	1.0%	6.6%	8.7%	2.2%
145	4.99	0.160284	0.213338	1.1%	5.2%	7.8%	2.7%
200	5.397891	0.136813	0.169479	0.7%	4.4%	5.6%	1.2%
260	5.659413	0.117016	0.127222	0.2%	3.6%	4.7%	1.0%

pH 4.5

rpm	LnF (mL/h)	Total Phosphate (Pi)	Repeat (Pi)	% Difference	Metal Recovery	Repeat	% Difference
5	1.736699	2.467223	2.673561	4.1%	91.4%	92.3%	0.9%
10	2.410798	1.384342	1.559953	3.5%	73.8%	79.9%	6.1%
15	2.821379	1.108665	1.21899	2.2%	61.6%	66.5%	4.8%
20	3.113515	0.912695	0.986828	1.5%	51.2%	54.9%	3.7%
25	3.339322	0.765073	0.820123	1.1%	42.2%	45.4%	3.2%
30	3.532226	0.665549	0.726417	1.2%	35.5%	39.4%	3.9%
40	3.819908	0.559517	0.616664	1.1%	28.5%	31.1%	2.6%
50	4.043051	0.482845	0.498992	0.3%	24.8%	26.1%	1.3%
60	4.200954	0.415592	0.460584	0.9%	20.1%	22.5%	2.3%
70	4.356709	0.378366	0.415716	0.7%	17.8%	19.7%	1.9%
80	4.516339	0.337761	0.374249	0.7%	15.8%	17.2%	1.5%
95	4.668145	0.315549	0.34326	0.6%	14.6%	15.5%	0.9%
120	4.882802	0.269842	0.293608	0.5%	12.1%	13.2%	1.1%
145	4.99	0.239715	0.267302	0.6%	10.8%	12.1%	1.4%
200	5.397891	0.209736	0.235055	0.5%	9.7%	10.8%	1.1%
260	5.659413	0.183135	0.196571	0.3%	8.2%	8.8%	0.6%

pH 5.0

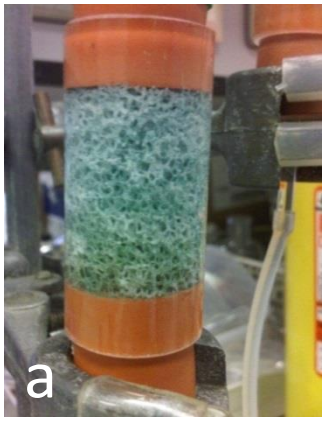
rpm	LnF (mL/h)	Total Phosphate (Pi)	Repeat (Pi)	% Difference	Metal Recovery	Repeat	% Difference
5	1.736699	2.609362	2.785793	3.5%	89.9%	91.6%	1.6%
10	2.410798	1.831004	1.919541	1.8%	85.6%	85.6%	0.0%
15	2.821379	1.433947	1.472164	0.8%	74.9%	73.6%	1.3%
20	3.113515	1.178953	1.239111	1.2%	64.5%	64.6%	0.1%
25	3.339322	0.958869	1.018756	1.2%	54.9%	55.3%	0.4%
30	3.532226	0.880053	0.911786	0.6%	50.6%	49.6%	1.0%
40	3.819908	0.749606	0.716702	0.7%	47.6%	40.2%	7.5%
50	4.043051	0.639587	0.66158	0.4%	36.0%	35.1%	0.9%
60	4.200954	0.557811	0.575712	0.4%	31.9%	31.0%	0.9%
70	4.356709	0.509098	0.511023	0.0%	28.2%	26.9%	1.4%
80	4.516339	0.45915	0.470814	0.2%	25.6%	24.1%	1.5%
95	4.668145	0.377817	0.383688	0.1%	22.0%	20.4%	1.6%
120	4.882802	0.342859	0.343107	0.0%	19.9%	18.9%	0.9%
145	4.99	0.286255	0.289338	0.1%	16.7%	16.1%	0.6%
200	5.397891	0.258497	0.26116	0.1%	14.5%	14.0%	0.5%
260	5.659413	0.219026	0.218731	0.0%	12.6%	12.3%	0.3%

pH 5.5

rpm	LnF (mL/h)	Total Phosphate (Pi)	Repeat (Pi)	% Difference	Metal Recovery	Repeat	% Difference
5	1.736699	3.040554	3.089296	1.0%	92.2%	92.9%	0.7%
10	2.410798	2.077598	2.130681	1.1%	90.5%	90.2%	0.3%
15	2.821379	1.594692	1.644125	1.0%	80.0%	80.2%	0.3%
20	3.113515	1.294009	1.327272	0.7%	71.2%	69.5%	1.6%
25	3.339322	1.092565	1.104229	0.2%	61.6%	60.1%	1.5%
30	3.532226	0.978223	0.985671	0.1%	55.1%	54.2%	1.0%
40	3.819908	0.818394	0.812824	0.1%	48.1%	46.5%	1.6%
50	4.043051	0.711671	0.704152	0.2%	41.5%	40.6%	0.9%
60	4.200954	0.641483	0.622601	0.4%	37.6%	34.9%	2.7%
70	4.356709	0.579061	0.568684	0.2%	33.7%	31.9%	1.8%
80	4.516339	0.511561	0.503871	0.2%	29.9%	28.2%	1.7%
95	4.668145	0.450816	0.435927	0.3%	25.5%	23.9%	1.6%
120	4.882802	0.400993	0.383711	0.3%	22.4%	21.0%	1.4%
145	4.99	0.359402	0.347052	0.2%	20.4%	18.7%	1.7%
200	5.397891	0.309307	0.290744	0.4%	17.6%	15.9%	1.7%
260	5.659413	0.297128	0.240501	1.1%	16.7%	12.9%	3.8%

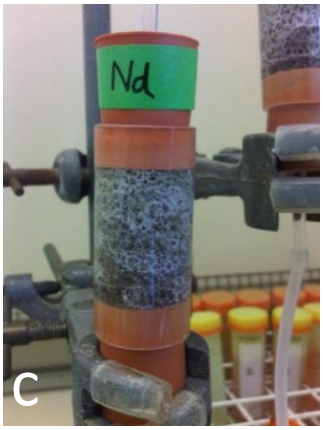
APPENDIX VIII

Figures for *aqua regia* experiments

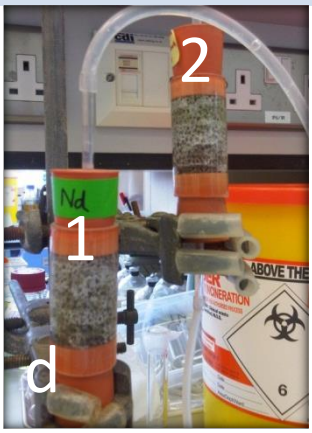


a: 50% *aqua regia* pH 2.5

b: 50% *aqua regia* pH 2.0

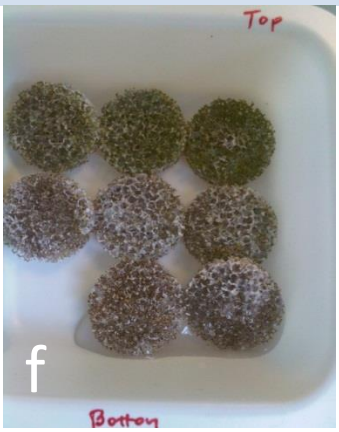


c: 50% *aqua regia* pH 1.0



d:1) pH 1.0 column
2) Control column

e: pH 1.0 treated: column was inactive



f: pH 2.5 treated: column was still active

Figure 1: a) and **b):** *Aqua regia* experiment when the first signs for denaturing the biomass started to appear **c)** *Aqua regia* experiment at pH 1.0,**d)** showing both the columns at the end of the experiment **1)** Test column found inactive and **2)** Control column at pH 2.5 was still showing metal removal **e)** After the *aqua regia* experiment the inactive column was discarded **f)** The column left at pH 2.5 was still active despite the brown color visible in both e) and f).

Aqua regia experiment using (K) sample. The columns were challenged initially with 10% *aqua regia* solution at pH 5.0. The concentration *of aqua regia* was increased up to

50% (step-wise).The activity of the column was checked with the use of the standard solution at pH 5.5, immediately after challenging the column with the 10% *aqua regia* solution at 16.8 mL/h for four column volumes. Upon showing that the column was still active at pH 5.5, (no sign of reduced metal recovery), the pH was dropped 0.5 per time. For lower than pH 2.5 values the experiment was conducted in the fume hood only for the 50% concentration *aqua regia* again for dropping down the pH value per 0.5. At that point it was noticed that the loaded *Serratia* sp. N14 discs were becoming brown. Hence, the experiment was continued only for one column, while the other column was left as a control.

At pH 1.0 the column was inactive; there was no phosphate release and negligible metal recovery in the test column. The corresponding activity of the control column was still 80%. **Figure 1** shows the inactivated column compared to the control that was not progressed to pH 1.0, but was left at pH 2.5 for 4 days. The results from the *aqua regia* experiments are shown in the following tables.

a

pH	*aqua regia* concentration	% Metal Removal for (K) (23 mL/h)	% Metal Removal for (K) (repeat) (23 mL/h)
5.5	**0%**	71.2	69.5
5.0	**10%**	76.8	74.9
	20%	70.2	70.3
	30%	76.3	77.5
	40%	74.2	71.2
	50%	69.4	68.4
4.5	**10%**	67.5	65.3
	20%	61.1	64.1
	30%	64.3	67.0
	40%	73.3	72.4
	50%	65.2	68.4
4.0	**10%**	56.2	57.8
	20%	63.9	66.3
	30%	59.9	63.9
	40%	61.2	60.1
	50%	59.8	57.0
3.5	**10%**	51.4	52.5
	20%	65.6	66.1
	30%	68.3	66.5
	`40%	63.8	68.4
	50%	58.8	59.6
3.0	**10%**	50.4	49.2
	20%	49.8	54.2
	30%	50.3	53.7
	40%	55.2	56.9
	50%	57.2	55.7

b

pH	*aqua regia* concentration	Phosphate release (23 mL/h) for (K) sample	Phosphate release (23 mL/h) for (K) (repeat)
5.5	**0%**	1.294	1.327
5.0	**10%**	0.934	0.907
	20%	0.787	0.784
	30%	0.911	0.893
	40%	0.829	0.774
	50%	0.770	0.751
4.5	**10%**	0.821	0.779
	20%	0.759	0.784
	30%	0.745	0.762
	40%	0.889	0.888
	50%	0.742	0.810
4.0	**10%**	0.658	0.678
	20%	0.826	0.868
	30%	0.716	0.744
	40%	0.692	0.666
	50%	0.697	0.655
3.5	**10%**	0.608	0.628
	20%	0.881	0.834
	30%	0.798	0.778
	`40%	0.748	0.784
	50%	0.662	0.737
3.0	**10%**	0.605	0.595
	20%	0.575	0.629
	30%	0.624	0.618
	40%	0.636	0.653
	50%	0.640	0.623

Table: (K) sample a) Effect of increasing concentrations of neutralized *aqua regia* at pH 5.0-3.0 on metal recovery. **b)** Effect of increasing concentrations of neutralized *aqua regia* at pH 5.0-3.0 on total phosphate release. The choice for 23 mL/h was done since the initial experiment with *aqua regia* tested at 7 months (J), was tested at that flow rate which was the $FA_{1/2}$ value for (J) sample. Moreover, the none treated with *aqua regia* values were from the columns at pH 5.5, after the gradual descending- ascending pH experiment.

In the following experiment the only difference made is that after each pH change the column was tested for phosphate release and metal recovery with a solution with a pH value equal with the *aqua regia* tested, rather than the standard solution (pH 5.5), which was used to preliminary experiments as ''rescue'' solution.

a

pH	*aqua regia* concentration	% Metal Removal (23 mL/h) for (S) sample	% Metal Removal (23 mL/h) for (M) sample
5.5	**0%**	52.6	62.3
5.0	**10%**	74.1	71.1
	20%	69.8	66.4
	30%	72.6	71.1
	40%	74.7	70.0
	50%	67.2	68.4
4.5	**10%**	59.3	58.8
	20%	71.8	68.2
	30%	68.5	66.2
	40%	63.4	61.2
	50%	84.4	85.6
4.0	**10%**	85.2	87.7
	20%	76.0	76.3
	30%	69.6	73.2
	40%	72.1	66.1
	50%	65.0	64.7
3.5	**10%**	74.4	73.5
	20%	65.7	70.1
	30%	67.7	69.1
	`40%	70.6	70.8
	50%	68.7	70.7
3.0	**10%**	67.1	63.1
	20%	67.2	68.4
	30%	68.5	66.5
	40%	63.8	63.1
	50%	74.0	76.2

b

pH	*aqua regia* concentration	Total Pi(mM) –($FA_{1/2}$ in metal removal) for (S)	Total Pi(mM) –($FA_{1/2}$ in metal removal) for (M)
5.5	**0%**	1.397	0.941
5.0	**10%**	1.036	0.917
	20%	0.843	0.791
	30%	0.823	0.794
	40%	0.796	0.752
	50%	0.711	0.713
4.5	**10%**	0.771	0.767
	20%	0.930	0.888
	30%	0.826	0.794
	40%	0.758	0.760
	50%	1.154	1.156
4.0	**10%**	1.059	1.088
	20%	0.938	0.943
	30%	0.894	0.908
	40%	0.839	0.815
	50%	0.771	0.795
3.5	**10%**	0.989	1.012
	20%	0.933	0.979
	30%	0.876	0.948
	40%	0.877	0.852
	50%	0.770	0.808
3.0	**10%**	0.905	0.906
	20%	0.870	0.896
	30%	0.915	0.865
	40%	0.797	0.764
	50%	0.951	0.934

Table: Samples (S) and (M) a) Effect of increasing concentrations of neutralized *aqua regia* at pH 5.0-3.0 on metal recovery. **b)** Effect of increasing concentrations of neutralized *aqua regia* at pH 5.0-3.0 on total phosphate release. The choice for 23 mL/h was done since the initial experiment with *aqua regia* tested at 7 months (J), was tested at that flow rate which was the $FA_{1/2}$ value for (J). Moreover, the none treated with *aqua regia* values were from the columns at pH 5.5, after the gradual descending- ascending pH experiment