ATTENUATION OF NITRATE FROM SIMULATED AGRICULTURAL WASTEWATER USING AN IMMOBILIZED ANAEROBIC BIOFILM

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MASTER OF SCIENCE

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ABSTRACT

A number of methods are currently in use for attenuating nitrates from wastewater with varying degrees of efficiency. Bioremediation using bacteria may be an efficient and cost effective method. In an anaerobic bioremediation system, nitrate can replace carbon dioxide as an electron acceptor and aids in nitrate attenuation by assimilatory reduction. The purpose of this study was to investigate nitrate attenuation in a hyperfiltration system using a pure culture of strictly anaerobic, facultative *Methanobrevibacter ruminantium* bacteria. Filtration experiments were conducted using amalgamated Na- montmorillonite clay-glass beads compacted at 500 psi differential hydraulic pressure with or without a biofilm. A simulated agricultural wastewater of 3.105×10^{-4} moles/L of NO_3^- was bioremediated. The use of bacteria in attenuating nitrates offers promising results on a bench-scale.

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J_v	advective solution flux	8
D	diffusion coefficient of the solute	8
x	position of interest	8
$C_{(x,t)}$	transient state concentration of solute at any point x in moles·cm^{-3}	9
C_i	initial concentration of dissolved anion	9
C_0	concentration adjacent to the dimensionless matrix	9
R_{mf}	microbial retardation factor	9
M_m	total microbial mass	9
$C_{(e,t)}$	transient concentration of solute exiting membrane (moles·cm^{-3})	10
ΔP	transmembrane pressure	11
L_P	hydraulic permeability of a membrane	11
σ	reflection coefficient or membrane efficiency for solute	11
R_T	total resistance due to membrane and adsorbed solutes	11
C_x	concentration at a distance x from the membrane	12
J_s	solute advection toward the membrane by hydraulic forces	12
q_s	mass transfer coefficient for molecular diffusive process (cm·s^{-1})	13

Δx	membrane thickness (cm)	13
D_s	effective solute diffusion coefficient	13
η	membrane porosity	13
M_C	mass of homoionic geomembrane (g)	13
A	cross sectional area of the membrane (cm^2)	13
θ	volumetric water content	13
R_s	solute retardation factor due to sorption	13
ρ_b	bulk density ($g \cdot cm^{-3}$)	13
K_{mD}	linear partitioning coefficient between the biofilm and the solute	14
D_m	substrate effective diffusion coefficient ($cm^2 \cdot s^{-1}$)	14

CHAPTER 1. INTRODUCTION

This chapter addresses the following sections: 1) Nitrate pollution and its environmental impacts, 2) Nitrate removal technologies, 3) Hyperfiltration systems, 4) Hypothesis, 5) Objectives, 6) Scope and 7) Organization of thesis.

1.1. Nitrate pollution and its environmental impacts

Nitrate pollution of water bodies around the world is a great environmental concern and has a direct impact on the economic sustainability of many countries like the USA (Hudak, 2000; Nolan *et al.*, 1997), UK, Denmark, Belgium, France (Fried, 1991; Strebel *et al.*, 1989), and India (Agrawal *et al.*, 1999). Groundwater contamination in farming and rural districts worldwide has shown increasing levels of highly mobile nitrate nitrogen (NO_3–N) at concentrations higher than the US Environmental Protection Agency (EPA) and World Health Organization's (WHO) regulatory limits (World Health Organization, 2004). Groundwater nitrate pollution from anthropogenic sources has increased significantly within the last ten years due to an increase of agricultural activities giving rise to human health risks (Barbash and Roberts, 1996; Hirata *et al.*, 1992; National Research Council, 1993; Trauth & Xanthpopoulos, 1997; Westrick *et al.*, 1984). Previous studies have indicated that agricultural uses of nitrogenous fertilizer play a great role in introducing nitrate into groundwater bodies (Fetter, 1999; Hudak, 1999). Elevated concentration of nitrates in drinking water is detrimental to human health causing illnesses such as *methemoglobinemia* (blue baby syndrome) in infants and stomach cancer in adults (Hajhamad and Almasri, 2009; Mirvish, 1991; Winton *et al.*, 1971). Apart from effects on human, nitrates

have also had an astounding effect on test animals causing heart and behavioral problems (Shuval and Gruener, 1972). Even a nitrate concentration much lower than that of the maximum contaminant level for drinking water contribute to increased rates of eutrophication in surface waters (Cole 1983).

Sources of nitrates contamination of groundwater can be broadly categorized as point and non-point sources. Non-point sources of NO_3-N include agricultural application of nitrogenous fertilizers and manure, leguminous crops, dissolved nitrogen in precipitation, irrigation return-flows, and dry deposition (Leterme *et al.*, 2006; Manassaram *et al.*, 2006; Mattern *et al.*, 2009; Nolan and Stoner, 2006). Point sources of NO_3-N include livestock and waste lagoons, spills and septic systems. Cesspits can also a play major role in nitrate pollution (Hajhamad and Almasri, 2009; Joosten *et al.*, 1998; Mitchell *et al.*, 2003; Stournaras, 1998; Tait *et al.*, 2008; Wolf *et al.*, 2003)**.**

The background levels of nitrates tend to generally increase due to several factors such as: agricultural and refuse dump runoffs during high precipitation, low infiltration events for surface water, high infiltration and low runoff events for groundwater, or even natural cataclysmic events like faulting through a waste lagoon or sewer system (Liu *et al.*, 2005; Mattern *et al.*, 2009; Mitchell *et al.*, 2003; Stournaras, 1998; Tait *et al.*, 2008; Wolf *et al.*, 2003). The increase in nitrate concentrations in groundwater worldwide has been attributed to a great demand for nitrogenous fertilizers as well as domestic and industrial wastewater discharge (Kross *et al.*, 1993). In fact, most countries have reported having levels of nitrates above 50 mg/L of NO_3-N (Billen and Garnier, 1999; Billen *et al.*, 2001; Billen *et al.*, 2005; Cinnirella *et al.*, 2005; Garnier *et al.*, 2005; McLay *et al.*, 2001; Vinten and Dunn, 2001). In the United States Great Plains region, low Nitrogen Use Efficiency (NUE) and nitrate leaching under irrigated and

dry land crop production systems have contributed to significant groundwater degradation. The United States Geological Survey (USGS) estimates that about thirty percent of residential wells in North Dakota contain NO_3-N at levels of 14 mg/L (Nolan *et al.*, 1998).

1.2. Nitrate removal technologies

Nitrate removal from water bodies is difficult but active research continues in developing various physicochemical and biological processes to attenuate it. The processes include distillation, reverse osmosis, ion exchange, biofilm-electrode reactor, electro-dialysis, and treatments that focus on biological methods (Clifford and Liu, 1993; Dhab, 1987; Dries *et al.*, 1988; Lin *et al.*, 2011; Park *et al.*, 2006; Van Der Hoek *et al.*, 1988; Wang and Qu, 2003). Biological methods of remediation are usually known as bioremediation.

1.2.1. Bioremediation

Bioremediation can be defined as the process whereby pollutants and organic wastes are biologically remediated or degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities (Davis *et al.*, 1994; Glazer and Nikaido, 1995; Holden and Firestone, 1997; Mallavarapu *et al.*, 2011). Because bioremediation uses relatively low-cost, low-technology, which generally have a high public acceptance and can often be carried out on site, it is becoming a popular technique of choice for the remediation of polluted soils and groundwater (Vidali, 2001). Bioremediation uses various natural components including microbes in considerable amounts to clean the environment where factors such as temperature, nutrients and oxygen are adequate for bioremediation to occur

accurately and properly (Allard and Neilson, 1997; Margesin, 2000; Sparrow and Sparrow, 1988).

Microorganisms have shown tremendous success at remediation of aquifers that have become contaminated, yet much more work still needs to be done (Caterina *et al.*, 2010; La Mantia *et al.*, 2008). Some of these microbes have been responsible for engulfing harmful pollutants such as, gasoline, chlorinated solvents and oil that have been introduced into the environment (Holliger *et al*., 1993; Melanie and Joseph, 1996). Metal reducing bacteria in single well push-pull experiments have effectively reduced nitrate concentrations from groundwater (North *et al.*, 2004). Denitrifying bacteria have been efficacious in attenuation of nitrates in enhanced *in-situ* biological denitrification (EISBD) (Calderer *et al.*, 2010).

Denitrifying microorganisms can use nitrate as a terminal electron acceptor for respiration. There are about 45 genera of bacteria and fungi which can reduce nitrate dissimilatively to nitrite (Payne, 1973). This reduction of nitrate to nitrite does not result in a loss of fixed inorganic nitrogen. Of interest are the denitrifying bacteria that are capable of reducing nitrate to the gaseous forms of molecular nitrogen (N_2) and nitrous oxide (N_2O), which may be easily lost to a surrounding ecosystem (NRC, 1993). Facultative denitrifying microorganisms in the absence of oxygen replace aerobic respiration with anaerobic respiration which means that oxygen is replaced by an alternative electron acceptor such as nitrate (Sabina, 2002; Kluber and Conrad, 1998).

1.2.2. Bioremediation techniques

Depending on the degree of saturation and aeration of an area, different techniques of bioremediation are employed to clean variety of pollutants introduced into the environment. In

the case of *in situ* remediation, microorganisms are applied to soil and groundwater at the site with minimal disturbance whereas *ex situ* techniques are those that are applied to either excavated soil or pumped groundwater (Lens *et al.*, 2005; Mathur *et al.*, 2011; Vidali, 2001).

One of the most common *in situ* treatment techniques, bioventing, involves supplying air and nutrients through wells to contaminated soil to stimulate the indigenous bacteria (Lynch and Moffat, 2005; USEPA, 2006; Vidali, 2001). Evans and Trute (2006) used gaseous electron donor injection technology (GEDIT), an anaerobic variation of petroleum hydrocarbon bioventing, to stimulate *in situ* biodegradation of groundwater nitrate and perchlorate.

Biosparging (which involves injection of air under pressure below the water table to increase oxygen concentrations to enhance aerobic degradation of contaminants such as nitrates) and bioaugmentation are other in situ techniques of bioremediation which can also be used for nitrate attenuation on site (Dobson et al., 2004; USEPA, 2006; Vidali, 2001). Desired rates of bioremediation depend on the effective oxygen diffusion rates. Thus *in situ* bioremediation depends on the depth of the soil and can be treated effectively up to a certain depth (Dobson *et al.*, 2004; Lynch and Moffat, 2005; Maliyekkal *et al.*, 2004; Vidali, 2001).

There are two types of *ex situ* bioremediation processes, the slurry phase and the solid phase (Lens *et al.*, 2005; Mathur *et al.*, 2011; USEPA, 2006; Vidali, 2001). The slurry phase involves combining water and the contaminated soil. After combining them both, they are further degraded in a bioreactor. The solid phase involves placing the contaminated soil in nutrient rich oxygen and moist environment where decomposition occurs (USEPA, 1996). Bioremediation can also be affected by immobilizing microbes on a conservative matrix or in a biofilm (Flemming, 1995).

1.2.3. Biofilms

Biofilms can be defined as a collection of microbial cells in nature that adhere onto surfaces (Sutherland, 2001). They can occur in almost every moist environment where flow of sufficient nutrient is available and where surface attachment can be achieved. Biofilms are generally formed by many species of bacteria, fungi, algae and protozoa (Liang *et al*., 2011; Singh *et al*., 2006; Sutherland, 2001). They can also be formed by single species of bacteria (Costerton *et al.*, 1994; Liang *et al*., 2011; Mary and O'toole, 2000). Approximately 97% of the biofilm matrix is either water, which is bound to the capsules of microbial cells, or solvent, the physical properties of which (such as viscosity) are determined by the solutes dissolved in it (Singh *et al.*, 2006). Biofilm formation can be described as a complex multifactorial process because the microorganisms whether species of bacteria, fungi, algae or protozoa, grow on different surfaces producing extra cellular polymers (Singh *et al.*, 2006; Sutherland, 2001). They are known to affect other compounds because of the physiological responses that they exhibit during water, inorganic and organic solute absorption that prove to be beneficial especially for remediation practices (Flemming, 1995). Biofilm-mediated bioremediation provides a safer and proficient alternative to bioremediation with microorganisms because cells in the biofilms have better chance of adaptation and survival as they are protected within the matrix (Singh *et al.*, 2006). A biofilm can exist in a hyperfiltration system as a component of an amalgamated membrane system (Flemming and Schaule, 1988; Schneider *et al*., 2005).

1.3. Hyperfiltration systems

Hyperfiltration membranes have been used for both desalination as well as industrial water production practices (Baltasar and Lourdes, 2012; Malaeb and Ayoub, 2011; Wiesner and Aptel, 1996). Hyperfiltration has been a highly effective process in the removal of organic and inorganic compounds from water bodies using natural and synthetic membranes (Huang *et al.*, 1998). Hyperfiltration is a process in which the applications of hydraulic gradient results in a solute concentration increase on the high pressure side of the membrane and in a more dilute solution issuing out on the downstream side (Fritz, 1986; Fritz and Eady, 1985; Fritz and Marine, 1983; Fritz and Whitworth, 1994; Oduor and Whitworth, 2005). When the applied hydraulic pressure gradient across the membrane, ΔP, exceeds the osmotic pressure gradient ($\Delta\pi$), hyperfiltration occurs (Fritz, 1986). This phenomenon is possible due to inherent membrane imperfections stemming from unbalanced surficial charges on the clay (Oduor *et al.*, 2006). Further, micron sized platelets with preferential orientation through compaction, and attendant small pores arising from compaction aid in providing sufficient conditions for chemical osmosis, thermo osmosis, and electro osmosis (Engelhardt and Gaida, 1963; McKelvey and Milne, 1960; Oduor *et al.*, 2006; Young and Low, 1965). Hyperfiltration process relies specifically on maintaining sufficient flux rates for the membranes. The long term flux decline may contribute to membrane fouling brought about as a result of a combination of factors like influent rejected by (i) type of membrane, (ii) soluble inorganic compounds, (iii) colloidal or particulate matter, or even by (iv) the attachment and growth of microorganisms on the membrane surface also called foulant (Speth *et al.*, 2000). The foulant layer including biofilms are capable of creating compact cakes or film layer which serves as an extra barrier for filtration purposes (Gabelich *et al.*, 2002; Speth *et al*., 2000). In hyperfiltration some synthetic membranes have a great variety of bacteria

that they can harbor, for example, *Mycobacterium* spp., *Flavobacterium* spp., and *Pseudomonas* spp. (Schneider *et al.*, 2005).

1.3.1. Theory

To analyze any solute depletion by a biofilm, an amalgamated membrane approach is required. Such amalgamated membranes can be comprised of compacted Na-montmorillonite clay with an immobilized biofilm. For simplicity, considering that the biofilm cements itself on the surface of the clay platelets then the whole system can be considered as an integral unit. A disturbance (unbalanced surficial charge, attendant small pores etc.) in the integral system will result in significant attenuation of the first mobile ions creating a pseudo-equilibrium or non-excited state (Oduor *et al.*, 2009). Diffusive flux can be adequately defined by Fick's first law since the variation of the flux depends on distance and the concentration gradient varies with time (Semprini *et al.*, 1990). When the solution is advected through the membrane, the solute builds up at the high-pressure side and the concentration changes with time is a function of the advective solution flux (J_v), the diffusion coefficient of the solute (D), and the position of interest (x) within the high-pressure interface where $x = 0$ (Fritz and Marine, 1983; Fritz and Whitworth, 1994). This modified advective–diffusive differential equation can be expressed as (for example Fritz and Marine, 1983; Fritz and Whitworth, 1994; Kedem and Katchalsky, 1958):

$$\left(\frac{\partial C}{\partial t}\right) = -\left(\frac{J_v}{R}\right)\left(\frac{\partial C}{\partial x}\right) - \left(\frac{D}{R}\right)\left(\frac{\partial^2 C}{\partial x^2}\right) \qquad (1)$$

where R is the retardation factor which is a function of concentration per unit area (Monod's concentration, $r_c = \frac{\mu_{\max} C_D}{K_D + C_D} C_C$, where microbial biofilm is present (Kim and Corapcioglu, 1997)). In the Monod's equation, C_D denotes the contaminant mass concentration in the aqueous phase, μ_{max} is the maximum specific growth rate achievable when $K_D << C_D$, and K_D is the Monod half-saturation constant, which is the value of the concentration of the substrate where the specific growth rate has half of its maximum value. For uniform water content, $\partial C/\partial x\big|_{x=\infty} = 0$ conditions with an insignificantly invariant flux of dissolved anion of concentration C_0 adjacent to the dimensionless matrix and an initial concentration of dissolved anion, C_i the approximate solution for the Eq. (1) can be (Anar *et al., 2012*):

$$C_{(x,t)} = \left[\frac{C_0 - C_i}{2}\right]\left\{\text{erfc}\left(\frac{R_{mf}\,x - J_v t}{2\left(DR_{mf}\,t\right)^{1/2}}\right) + \text{erfc}\left(\frac{R_{mf}\,x + J_v t}{2\left(DR_{mf}\,t\right)^{1/2}}\right)\right\} + C_i \qquad (2)$$

where R_{mf} is a dimensionless microbial retardation factor. This factor can be evaluated as a function of concentration per unit area (C/A), total mass of the microbes (M_m), diffusion coefficient of the solutes (D), and solution advective flux (J_v); that is, $R_{mf} = F\left(C/A, M_m, D, J\right)$(Anar *et al., 2012*). Solute concentration adjacent to the amalgamated membrane on the influent side, C_0, is usually higher than the influent concentration, C_i, and C_0 can be determined by using Dirichlet or Type 1 boundary conditions (Oduor and Whitworth, 2004):

$$C_0 = \begin{cases} C_i & \text{for} \quad t = 0 \\ \left[\frac{C_i}{2}\right]\left\{\left[1 + \text{erf}\left(\frac{J_v{}^2 t}{4D}\right)\right]\right\} + C_i & \text{for} \quad t > 0 \end{cases} \tag{3}$$

Diffusion in porous media occurs less rapidly than that in the free solution because of the tortuosity effects (Marshall, 1948; Wyllie, 1948). Thus the term effective diffusion coefficient is used for porous media and is usually represented by D^* (Marshall, 1948). This effective diffusion coefficient can be defined by the relation $D^* = wD$, where w is the tortuosity coefficient, a unitless constant determined empirically from laboratory experiments (Marshall, 1948). Tortuosity coefficient typically ranges between 0.01 and 0.5 (Freeze and Cherry, 1979). The value of the tortuosity coefficient depends on the sediment type and the coarser the sediments the higher the tortuosity coefficient and vice versa (Freeze and Cherry, 1979).

To model the time varying effluent concentration, Equation (1) can be solved considering the boundary conditions $C_{(0,0)} = C_i$, $C_{(x,t)} = C_{e(t)}$ when $x = \Delta x$ and $\left.\frac{\partial C}{\partial x}\right|_{x=\infty} = 0$ (Oduor and Whitworth, 2004):

$$C_{e(t)} = C_i - \left[C_{(0,t)} \left(1 - \exp\left(\frac{J_v \Delta x}{2D}\right)\left(1 - \sqrt{\left(1 + \frac{8D}{(J_v)^2 t}\right)}\right)\right)\right] \tag{4}$$

According to the principle of mass conservation, the rate at which mass density increases at any point in the system must be equal to the rate at which matter flows toward that point (Fritz and Whitworth, 1994; Fritz and Marine, 1983; Oduor and Whitworth, 2005). For a system containing n solutes (Ogata and Banks, 1961; Oduor and Whitworth, 2004):

$$J_v = \frac{L_P}{R_T}\left(\Delta P - \sum_{i=1}^{n}\sigma_i \Delta\Pi_i\right) \quad (5)$$

where ΔP is the transmembrane pressure, L_p is the hydraulic permeability of a membrane, and σ_i is the reflection coefficient or membrane efficiency for solute *i*, $\sigma \approx (C_0 - C_e)/(C_0 + C_e)$ (Fritz and Marine, 1983). The theoretical osmotic pressure existing across the membrane as generated by solute *i* is denoted by $\Delta\Pi_i$ and is calculated from Van't Hoff equation (Alexander, 1990; Benzel and Graf, 1984; Bernstein, 1960; Fritz and Eady, 1985; Fritz and Marine, 1983; Fritz and Whitworth, 1994; Kemper, 1960; Kharaka and Smalley, 1976; Marshall, 1948; Mckelvey and Milne, 1962; Whitworth and Fritz, 1994; Wyllie, 1948). For most dilute organic solutions, the osmotic pressure tends to go to zero unless an ionic solute component is a part of the transformation byproduct (Fritz and Marine, 1983). R_T is the total resistance due to membrane and adsorbed solutes on the surface of the membrane. For most of the ionic solutes, $R_T = 1$ (Anar *et al*., *2012*). But for solutes that react with membrane matrix, this value is less than 1 and for solutes that are greatly sorbed, the value is greater than 1 (Anar *et al*., *2012*).

Because of the rejection of solute at the high-pressure side of the membrane, concentrations of the solutes increase adjacent to the membrane (Fig 1.1), and this buildup of solute concentration immediately adjacent to the membrane on the influent side is denoted by C_0. This concentration is higher than the influent concentration, C_i. The effluent concentration, C_e, increases with time until steady-state is attained after time, *t*, and at this point $C_e \approx C_i$. The distance $x = \Delta x$ is equivalent to membrane thickness in cm. The length $x = \delta$ corresponds to the distance where there is no effect of concentration buildup on the influent side. C_0 also gives rise

to a back diffusive flux, $J_d = -D \cdot \partial C / \partial x$, on the influent side (Alexander, 1990; Benzel and Graf, 1984; Fritz and Eady, 1985; Fritz and Marine, 1983; Fritz and Whitworth, 1994). The solution flux, J_v exits through the membrane whereas, $J_s = C_x J_v$, where C_x denotes the concentration at a distance x from the membrane, and J_s is the solute advection toward the membrane by hydraulic forces (Oduor *et al.*, 2006).

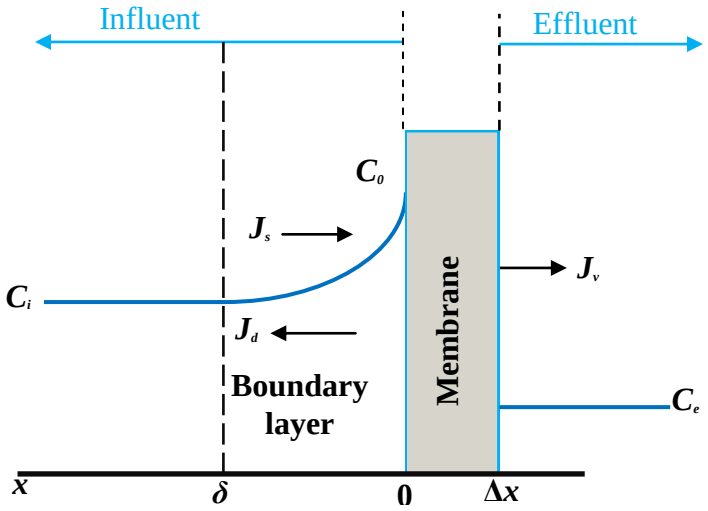

Fig 1.1 Illustration of concentration profile layer (from Oduor *et al.*, 2006).

Mass balance across the membrane can be determined considering a closed system (Fritz and Eady, 1985; Fritz and Marine, 1983; Fritz and Whitworth, 1994; Oduor *et al.*, 2006). If we consider the efficiency of the system less than 100% as typical of geomembranes (Alexander, 1990; Fritz and Eady, 1985; Fritz and Marine, 1983; Fritz and Whitworth, 1994; Whitworth and Fritz, 1994) then:

$$C_x J_v + D\frac{dC}{dx} = C_e J_v \qquad (6)$$

If δ is the thickness of the boundary layer cm, then applying the conditions of, $C_x = C_o$ (m*M*) at $x = 0$ and $C_x = C_i$ at $x = \delta$ (cm), in steady state, integration of Equation (6) gives a relation for mass transfer (Fritz and Marine, 1983; Oduor *et al*., 2005):

$$q_s = J_v \ln\left[\frac{C_i - C_e}{C_0 - C_e}\right] \qquad (7)$$

where q_s (cm·s^{-1}) is the mass transfer coefficient for molecular diffusive process and can be derived from $q_s = (D/\delta)$, where, D (cm^2·s^{-1}) is the effective diffusion coefficient. Equation (7) is known as the 'film-model' relationship (Oduor *et al*., 2009).

Determination of effective diffusion coefficient in the presence of a biofilm

Biofilms can act as cements or glues to bind clay platelets (Boley and Overcamp, 1998; Cheshire *et al.*, 1983; Hozalski and Bouwer, 1998; Payne, 1988; Tisdall and Oades, 1982). Interactions between montmorillonite and biofilm will affect the cation exchange capacity and viscosity and hence the effectiveness of montmorillonite as a membrane (Anar *et al*., *2012*). To take the presence of microbial populations into account a solute transport equation can be applied based on the change in viscosity and effluent concentration especially for anaerobic degradation (Chambon *et al.*, 2010). For describing transport of solutes in the presence of a biofilm, the basic advection-diffusion transport equation (Eq. 1) needs to be modified. If we consider that there is no biotransformation for the initial phase of solute transport, then (Clapp *et al.*, 1999;

Domenico and Schwartz, 1998; Fetter, 1988; Haws and Rao, 1994; Olesen *et al.*, 2001; Salvage, 1998; Yeh *et al.*, 1998):

$$\left(\frac{\partial C}{\partial t}\right) = -\left(\frac{J_v}{R_{mf}}\right)\left(\frac{\partial C}{\partial x}\right) - \left(\frac{D}{R_{mf}}\right)\left(\frac{\partial^2 C}{\partial x^2}\right) \tag{8}$$

the R_{mf} coefficient is evaluated as a function of porosity, η, θ Henry's constant K_H (cm^3 montmorillonite water per cm^{-3} montmorillonite air), and the linear partitioning coefficient between the biofilm and the solute, K_{mD} (cm^3 solution g^{-1} biofilm) (Anar *et al.*, *2012*; Olesen *et al.*, 2001):

$$R_{mf} = \left(\frac{\eta K_H}{\theta}\right) + \left(\frac{\rho_b K_H K_{mD}}{\theta}\right) \tag{9}$$

In this case, the total effective diffusion coefficient can be calculated from the relation (Anar *et al.*, *2012*):

$$D = \left(\frac{D_m}{\theta R_{mf}}\right) + \left(\frac{D_s}{\eta R_s}\right) \tag{10}$$

where D_m and D_s, are substrate and solute effective diffusion coefficients in the microbial biofilm and membrane media respectively. R_{mf} and R_s are microbial and membrane retardation factors.

1.4. Hypothesis

Methanobrevibacter ruminantium can be effective in reducing NO_3^- in influent solution hyperfiltrated through compacted 80% glass beads and 20% Na-montmorillonite clay.

1.5. Objectives

The objective of the research was to assess the efficacy of immobilized *Methanobrevibacter ruminantium* in attenuating nitrate.

1.6. Scope

The study concentrated on utilization of compacted clay and glass beads in the presence of nitrate contaminated water and a particular strain of bacteria. The examination was based on quantitative data collected over a period of two years, 2010 to 2012.

1.7. Organization of thesis

This thesis presents the findings of the study carried to see the effectiveness of the bacterial strain in reducing nitrate level concentrations in wastewater. This document begins with an introduction which is Chapter 1, and literature review in Chapter 2. These two are followed by a methodology section adopted for the experiment in Chapter 3. Results are presented and discussed in Chapter 4. Chapter 5 contains the study findings in the form of a conclusion. References and appendices are also included following Chapter 5.

CHAPTER 2. LITERATURE REVIEW

This chapter addresses four sections: 1) Clay and clay minerals 2) Interactions between bacteria and clay soils, 3) Methanogenic bacteria in bioremediation, and 4) Membrane bioreactor and isolation.

2.1. Clay and clay minerals

Clay minerals are hydrous aluminosilicates and are the finest fraction of the inorganic component of soils (Carter *et al.*, 1986; Zhang, 2010). Clay minerals vary in their particle size, chemical composition, surface charge properties, cation exchange capacity, and water retention properties (Carter *et al.*, 1986). The structure of clays consists of tetrahedron and octahedron sheets (Birkeland, 1999). The geometric arrangement of the particular cations and anions namely silicon, aluminum, magnesium, iron oxygen and hydroxide respectively make these two types of structure (Fig 2.1). The principal clay minerals found in soils are members of the kaolinite, montmorillonite, and illite groups (Birkeland, 1999). The difference between clay and clay mineral is that clays are made of one type of mineral whereas clay minerals are made of more than just one type of minerals (Bergaya and Lagaly, 2006).

2.1.1. Smectite

The smectite group of clay mineral consists of a large variety of minerals having a 2:1 geometric layer structure (Birkeland, 1999). There are three known forms of smectites found to exist in soils namely Montmorillonite, Beidellite and Nontronite (Birkeland, 1999). Na-montmorillonite is a mono-cationic form of smectites where the Na^+ ions form of

montmorillonite varies the amounts of interlayer water and has a high cation exchange capacity: and therefore high surface charges (Guichet, 2008). The cation exchange capacity is defined as the ability to absorb cations nutrients or the total number of positive charges absorbed at a given pH (Favre *et al.*, 2006; Stucki *et al.*, 1997).

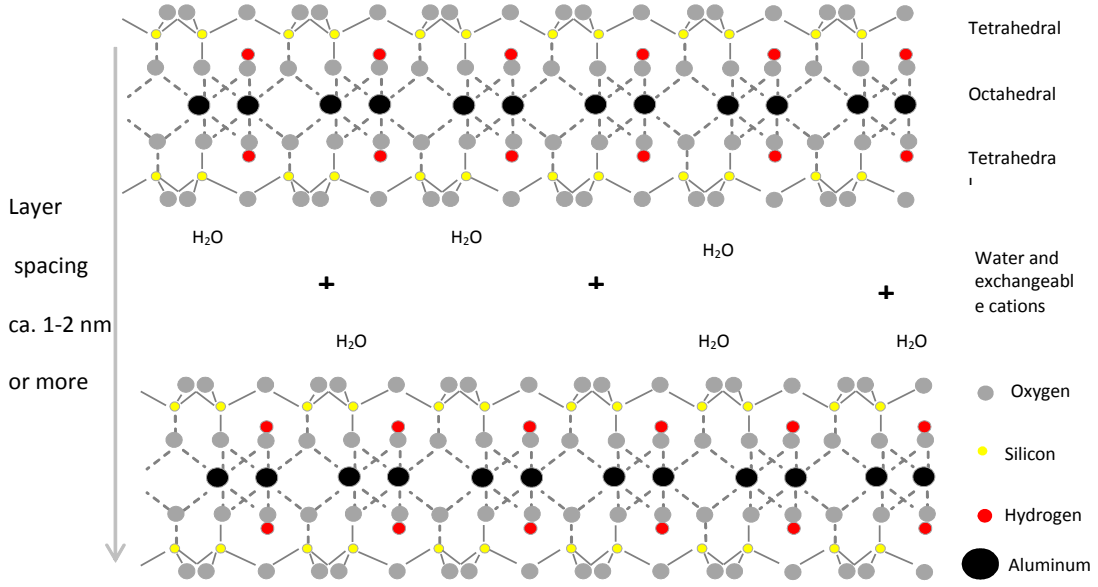

Fig 2.1 Structure of montmorillonite (redrawn from Schmidt *et al.*, 2005).

2.2. Interactions between clay and bacteria

Clay minerals, organic matter and microorganisms are closely associated and interact in various environmental processes in soils (Bollag, 2008; Filip, 1973). These interactions are especially important in areas like the sediment-water interface and the soil rhizosphere where microbial activities are intense and low-molecular weight biochemicals are abundant (Bollag, 2008).

In his studies, Filip (1973) confirmed the significance of clay minerals as a factor influencing the biochemical activity of soil microorganisms. The soil microflora is influenced directly by the effect of clays on the microbial cells, and indirectly, by their effect on the environment. The direct effects are projected into fundamental processes of the biogenic element cycle, including humification processes. Filip (1973) also mentioned that character and mechanism of the effect depend on the microorganism species, quantity and quality of the mineral sorbents present in the soil and on other ecological factors.

Microbial activities play significant role in bioremediation (Lin *et al.*, 2011; Park *et al.*, 2005; Vasiliadou *et al.*, 2007). Formation of biofilms as a result of bacterial colonization shows astounding effects not only on bacteria itself as a newly formed colony but it also greatly alters the matrix or surfaces where they are attached (Bishop, 2007; Kim *et al.*, 2002; Prakash *et al.*, 2003). Because of their diversity and abundance within the environment, bacteria tend to be a highly regarded candidate for bioremediation (Flemming, 1995; Kim *et al.*, 2002; Prakash *et al.*, 2003). Due to the relatively small size of the bacteria and taking into account its high surface area in relation to volume, it can be concluded that these two features determine the fate of contaminants that are interacting and present along with the bacteria in the soil (Filip, 1973). Bacteria, as suggested by Korber *et al*. (1995) are almost always found attached onto surfaces. Understanding the principle existing between the microbe and soil interaction requires the thorough examination of the microbe while it is still attached onto the clay's surface (Filip, 1973). Scanning electron microscopes (SEM) (Rogers *et al*, 1998) and atomic force microscopy (AFM) (Grantham and Dove, 1996) are quite common techniques used to examine the microbial-soil interaction.

The activity of molecular nitrogen fixing microorganisms is likewise influenced by clay minerals (Filip, 1973). Non-symbiotic microorganisms which fix nitrogen under anaerobic conditions obtain the necessary energy by fermentation of carbohydrates by a process which is influenced by the sorbents (Gupta and Roper, 2010). The metabolism of aerobic nitrogen fixers have long been known to be influenced by mineral colloids and nitrogen fixation depends quantitatively on the type of clay mineral and physical and chemical properties of the soil (Gupta and Roper, 2010). In agreement with this it was found that the colloid particles of montmorillonite, which increase the total active surface of the culture, influenced the fixation process more than coarser particles (Filip, 1973). Macura and Pavel (1959) when added bacteria from *azotobacter* genus to montmorillonite, observed that the amount of nitrogen fixed per utilized glucose unit increased, as well the absolute amount of nitrogen compared with the control. The addition of sorbents to sand cultures of clover inoculated with a rhizobe culture stimulated nodulation and nitrogen fixation (Filip, 1973; Turner, 1955).

2.3. Methanogenic bacteria in bioremediation

Methanogenic bacteria belong to the domain of *Archaea*, and fall within the kingdom *Euryarchaeota* (Woese *et al.*, 1990). These obligate anaerobes can unambiguously be differentiated from other microorganisms since they all can produce methane as a major catabolic product (Bergey, 1994). Methanogens from ruminants have also the distinction of increasing atmospheric methane, a greenhouse gas; cattle typically lose 6 % of ingested energy as methane (Johnson and Johnson, 1995). A number of methanogens have been isolated from ruminants, but only a few have been consistently found in high numbers (Stewart *et al.*, 1997). It

is likely that major species of rumen methanogens are yet to be identified (Raskin *et al.,* 1994; Wolin *et al.,* 1997). The most common species of methanogens that have been isolated from the rumen are strains of *Methanobrevibacter, Methanomicrobium, Methanobacterium*, and *Methanosarcina* (Wolin *et al.*, 1997).

Though methanogens are metabolically restricted, they exhibit extreme habitat diversity. Species have been isolated from virtually every habitat in which anaerobic biodegradation of organic compounds occurs, including freshwater and marine sediments, digestive and intestinal tracts of animals, and anaerobic waste digesters (William *et al.,* 1987). They exist in different shapes, sizes, forms, and physiologically can be represented by extremely thermophilic, moderately thermophilic, and many mesophilic isolates (William *et al.,* 1987). Strictly anaerobic methanogenic bacteria derive their energy from conversion of simple substrates such as H_2-CO_2, formate, acetate, and methanol to methane (Daniels *et al.*, 1984; Negash *et al.,* 1990). They are also known to grow in areas where electron acceptors, for example, O_2, NO_3^-, Fe^{3+} and SO_4^{2-} are limited (Jones *et al.*, 1987). These bacteria are very sensitive to changes within their immediate environment and perform well within a pH range of 6.0 to 8.0 (Balch and Wolfe, 1976).

Methanobrevibacter ruminantium was proposed as a new species in a methanogenic group by Smith and Hungate (1958). They are short, gram-positive coccobacilli. All isolates can use H_2 and CO_2 as substrates for growth and methanogenesis. They use formate poorly, and do not use acetate, methanol, and trimethylamine as substrates (Miller *et al.,* 1986). This activity will be lost unless strictly anaerobic conditions are maintained throughout the isolation and incubation procedures. $^3H_2$, but not ^3H_2O, was readily incorporated into methane (Frank *et al.,* 1979).

The cell envelope of *M. ruminantium* has a distinctive triple-layered appearance (Zeikus, 1977). The cell wall consists of an inner electron-dense layer, closely adjoined to the plasma membrane, followed by a thicker, more electron-transparent middle layer, and a rough, irregular outer layer (Miller *et al.*, 1986; Zeikus, 1977). Cells of *M. ruminantium* appear to be undergoing constant cell division; before one cross wall is completed, a second division is initiated (Zeikus, 1977). Cells are nonmotile to poorly motile with an optimal growth range of 37 to 39° C. DNA base composition range can be expected to include 27.5 to 32 mol% G + C. *M. ruminantium* (Smith and Hungate, 1958). Balch and Wolfe (1958) designated the type species for the genus *Methanobrevibacter*, as it was the first species characterized (Balch et al., 1979).

Nitrate can replace carbon dioxide as an electron acceptor with the generation of another reduced product - in this case, ammonia, i.e. nitrate is reduced to nitrite and then to ammonia (Leng, 2008; Van Nevel and Demeyer, 1996). Bacteria that reduce nitrate to ammonia are more active in the rumen when substantial amounts of nitrate have been included in the diet for extended periods (Leng, 2008). Ammonia is usually generated from degradation of dietary protein or by supplementation with urea (Leng, 2008).

2.4. Membrane bioreactors and isolation

A number of experiments have been performed involving biofilm aiming at attenuation of nitrates (Lin *et al.* 2011; North *et al.* 2004; Park *et al.* 2006; Park *et al.* 2005; Vasiliadou *et al.* 2007). Lin *et al.* (2011) investigated the effectiveness of various rumen microbial fractions in reducing nitrates and assessed the effect of nitrate on *in vitro* fermentation characteristics. Using

both physical and chemical methods they differentiated the rumen microbial population into Whole Rumen Fluid (WRF), protozoa (Pr), bacteria (Ba), and fungi (Fu) and ran an incubation studies for 24 h. During incubation they observed that WRF, Pr and Ba fractions had an ability to reduce nitrate, and the rate of nitrate disappearance for the Pr fraction was similar to the WRF fraction, while the Ba fraction needed an adaptation period of 12 h before rapid nitrate disappearance. Methane (CH_4) production was highest in the WRF fraction and the Pr fraction had the highest prevailing H_2 concentration ($p<0.05$) and acetate to propionate ratio ($p<0.05$). The Pr fraction as well as the Ba fraction appears to have important roles in nitrate reduction. Figure 2.2 (from Lin et al. 2011) below depicts the nitrate depletion and nitrite accumulation for 6, 12 and 24 h *in vitro* incubation by different ruminal microbes fractions from cattle not adapted to exogenous dietary nitrate.

In their studies North *et al.* (2004) demonstrated that metal-reducing microorganisms can effectively promote the precipitation and removal of nitrate from contaminated groundwater. Stimulating microbial communities in the acidic subsurface by pH neutralization and adding electron donor to wells, they conducted single-well push-pull tests at a number of treated sites and found that nitrate, Fe(III), and uranium were extensively reduced and electron donors (glucose, ethanol) were consumed. Their examination of sediment chemistry in cores sampled immediately adjacent to treated wells 3.5 months after treatment showed that sediment pH increased substantially (by 1 to 2 pH units) while nitrate was largely depleted. They observed a large diversity of 16S rRNA gene sequences in the subsurface sediments, including species from the α, β, δ, and ϒ subdivisions of the class Proteobacteria, as well as low and high-G+C gram-positive species. Following *in situ* biostimulation of microbial communities within contaminated sediments, sequences related to previously cultured metal-reducing δ-Proteobacteria increased

from 5% to nearly 40% of the clone libraries (Fig 2.3). During the later stages of successive push-pull treatment, utilization of electron acceptors and the electron donor occurred simultaneously in a parallel, linear relationship (North *et al.* 2004).

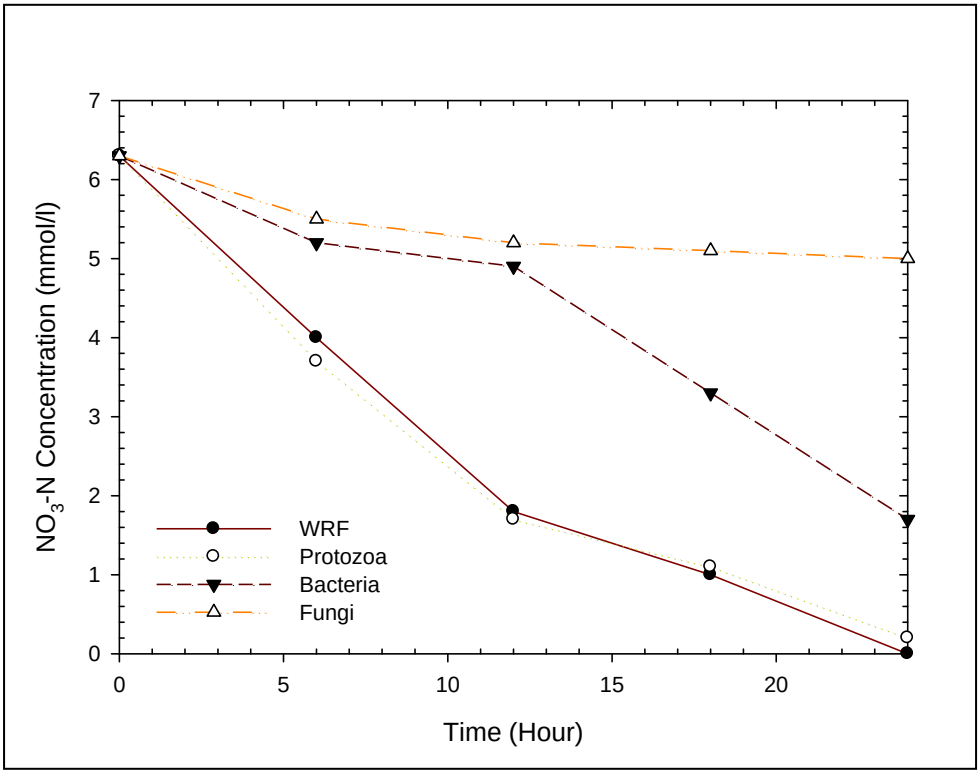

Fig 2.2 The concentration of nitrate and nitrite in ruminal microbe fractions at 6, 12 and 24 h *in vitro* incubation. Solid line represents nitrate concentration during incubation periods; concentration of nitrite is shown as a short dashed line on the right y-axis; WRF (■), whole ruminal fluid; Pr (▲), protozoa; Ba (●), bacteria; Fu (♦), fungi (redrawn from Lin *et al.*, 2011).

The progress of biological phenol and nitrate removal in batch assays of mixed bacteria culture were examined by Vasiliadou (Vasiliadou *et al.* 2007). They aimed to study the performance of the culture under aerobic conditions for biological phenol and nitrate removal in synthetic wastewater containing phenol and nitrates at various concentrations. They observed maximal removal rates of 1.07 mg NO_3^--N l^{-1} h^{-1} for feed concentrations 20 mg NO_3^--N l^{-1} in a

draw-fill experiment (Fig 2.4). The rates of biological nitrate removal were found to be inhibited beyond 40 mg NO3--N l-1. Vasiliadou et al. (2007) also developed a kinetic model and determined its kinetic parameters. Their proposed model is capable of describing accurately enough, cellular growth, nitrate and nitrite utilization, in the presence of various concentrations of nitrate (20–100mg NO3--N l-1).

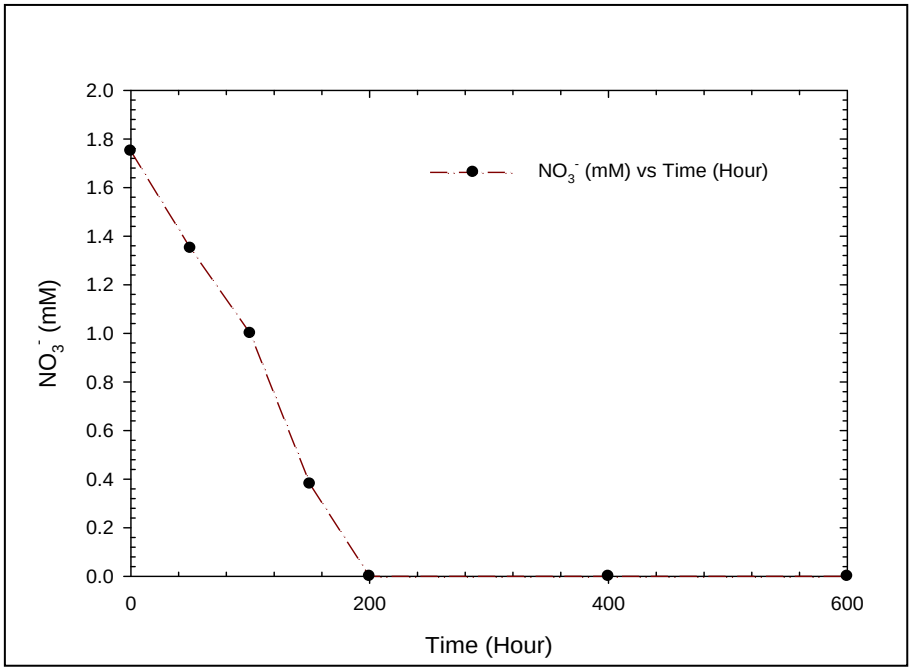

Fig 2.3 Rates of nitrate reduction in contaminated well FB34 (redrawn from North *et al.*, 2004).

Some researchers also used electrode in bioremediation techniques as direct electron donor to the microorganisms to reduce nitrates. For example, Park et al., 2006 investigated nitrate reduction using an electrode as a direct electron donor in a biofilm-electrode reactor to treat an aqueous solution containing high concentration of nitrate. In the biofilm-electrode reactor, nitrate was reduced in the absence of organic substances by accepting electrons from the electrode. As nitrate concentration decreased, oxidation-reduction potential (ORP) gradually decreased from −80 to −260mV. From the biofilm electrode, they detected 10 bands out of

which 4 bands (EB4, EB6, EB7, and EB10) were dominant. In gene analysis of 16S rDNA, the researchers observed that the major populations are α-proteobacteria, β-proteobacteria, ϒ-proteobacteria and flavobacteria.

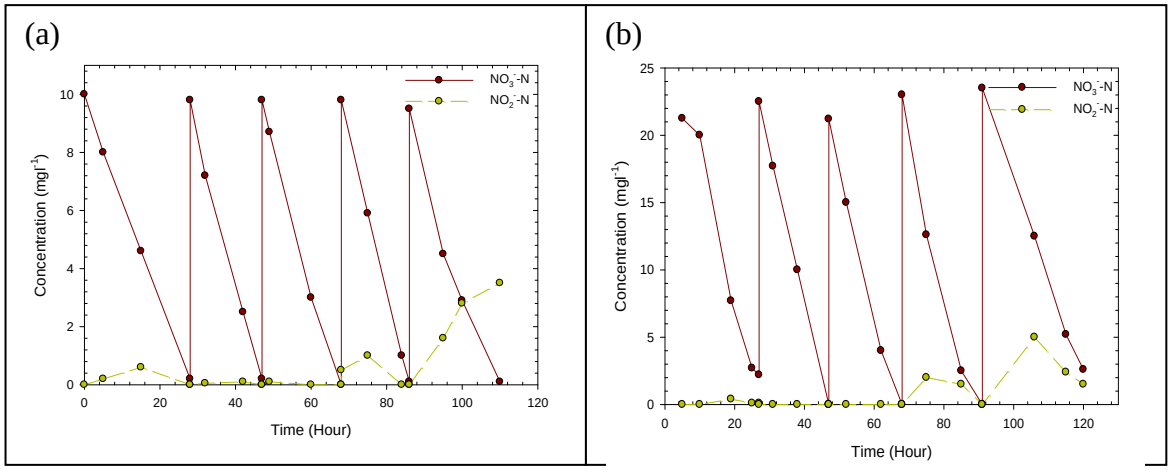

Fig 2.4 Nitrate-, nitrite-nitrogen concentration vs. time for the draw-fill experiments: (a) 10mg NO_3^--N l^{-1} and (b) 20mg NO_3^--N l^{-1} (redrawn from Vasiliadou *et al.*, 2007).

In their experiment, Park *et al*, (2006) observed that 70 mg of NO_3^--N l^{-1} was rapidly reduced to nitrogen with consumption of electrons from the electrode (Fig 2.5). The pH increased slightly to 7.4 as the nitrate reduction proceeded. The dissimilatory reduction of NO_3^- to N_2 produces a strong base. The release of alkalinity occurs when nitrite ($NO_2^{-)}$ is reduced to nitric oxide (NO) resulting in an increase in pH in this system.

In order to estimate the effect of initial concentration on nitrate reduction rate, they varied initial nitrate concentration from 20 to 492 mgNO3--N l-1 (Fig 2.6). As the initial nitrate concentration increased, the nitrate reduction rate increased and nitrite was accumulated during nitrate reduction. However, the nitrite accumulated in the medium did not affect the nitrate reduction rate.

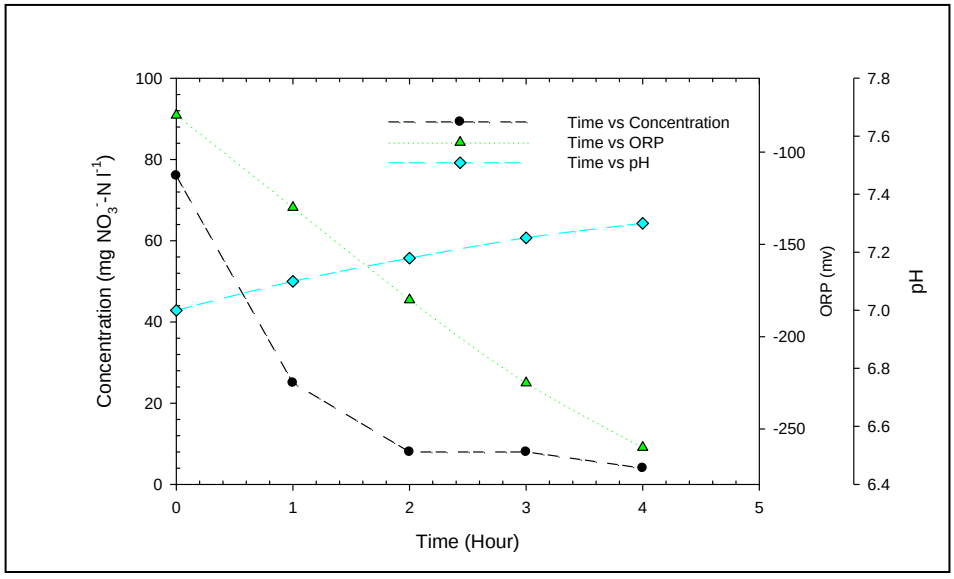

Fig 2.5 Biological nitrate reduction using electrons from cathode in a biofilm electrode reactor. The electric current was maintained at 200 mA. Temperature was maintained at 30°C (redrawn from Park *et al.*, 2006).

Biofilm-electrode reactor (BER) is a reactor which employs autotrophic denitrifying microorganisms immobilized on the surface of the cathode (Gregory *et al.*, 2004; Park *et al.*, 2005; Park *et al.*, 2006). In the BER, hydrogen produced from electrolysis of water can be used as an electron donor. However, Gregory *et al.* (2004) reported that the hydrogen production rate from the electrolysis of water was 6×10^{-7} mmol h^{-1}, which is equivalent to a current of 2.6×10^{-5} mA with a sterile electrode after a period of flushing with N_2/CO_2. This rate of hydrogen production was 10,000-fold slower than the average rate of current consumption by nitrate-reducing enrichments. Park *et al.* (2006) reported that the maximum nitrate reduction rate to nitrogen gas under the condition of 200mA of current was 434.78 mg NO_3^- −N l^{-1} h^{-1}. For reduced nitrate of 434.78 mg NO_3^- −N needed 2.16×10^{-5} mol h^{-1} (Fig 2.6). But, at an applied current of 200 mA, the electrode produced hydrogen at a maximal rate of 1.38×10^{-7} mol h^{-1}. This rate of electrochemical hydrogen production was 100-fold lower than the rates of nitrate

reduction. These results demonstrated that the enrichment culture reduced nitrates by using the electron donor from the electrode.

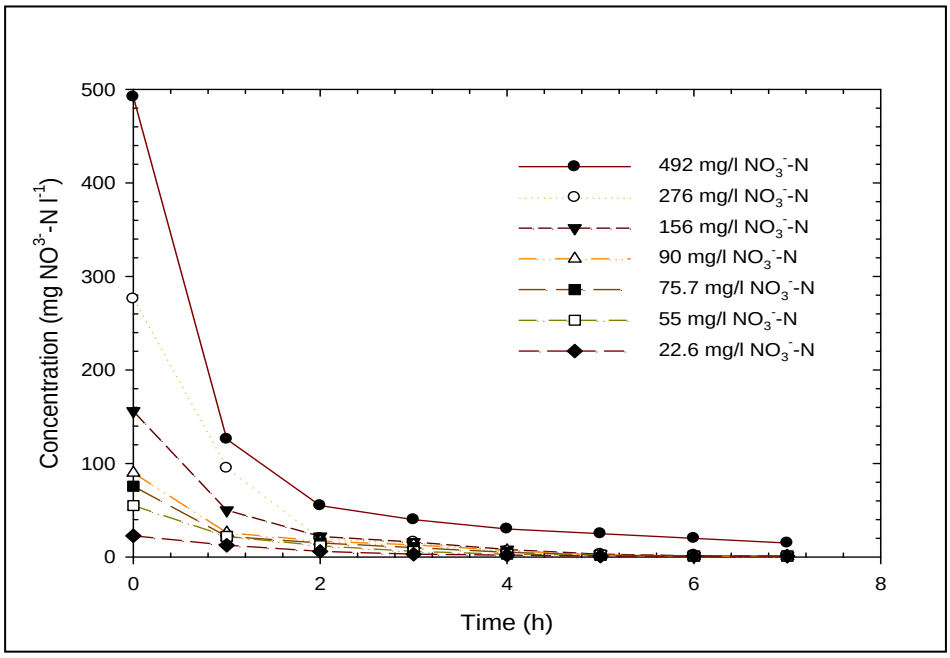

Fig 2.6 Biological nitrate reduction using electrons from cathode in a biofilm-electrode reactor (redrawn from Park et al., 2006).

Park *et al.*, (2005) performed a series of experiments in which nitrate was reduced by using electrodes as direct electron donors in a biofilm-electrode reactor (Fig 2.7.) that showed that 70 mg NO_3 -N/l was rapidly reduced to nitrogen with consumption of electrical current. The nitrate was actually reduced when the micro-organisms consumed electrons from the electrodes- in the absence of organic substances. They concluded that the nitrate was reduced as a result of using electrons from the cathode once organic substances were not present. For example, experimental results proved that 25 mg NO_3 -N/l was reduced to 5mg NO_3 -N/l in 10 minutes.

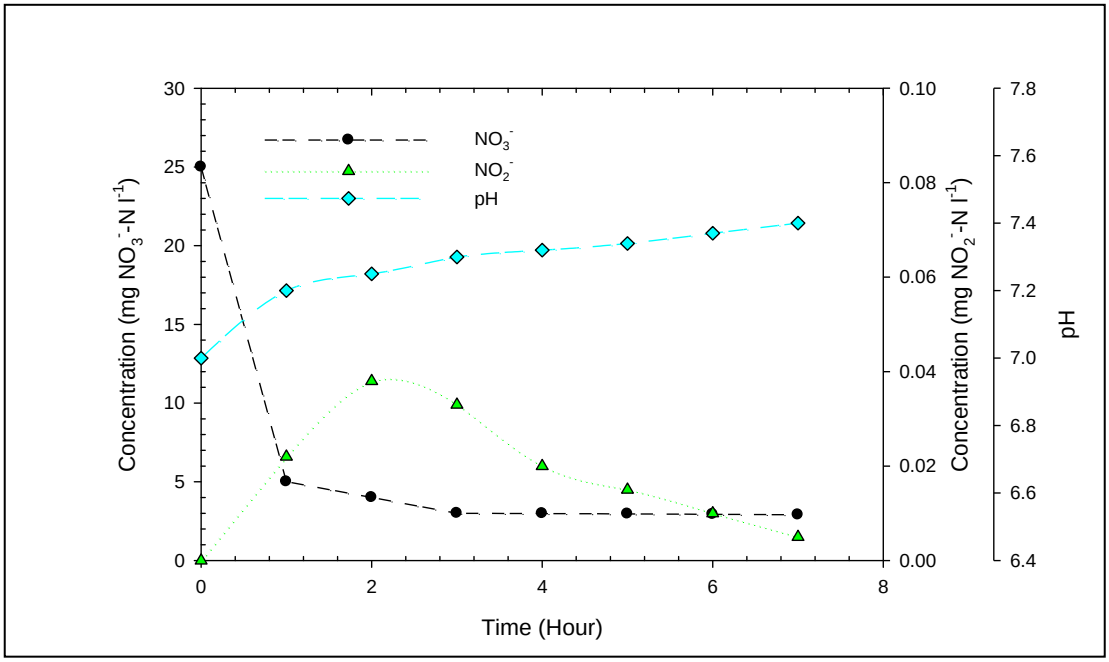

Fig 2.7 Biological nitrate reduction using electrons from cathode in a bio-film-electrode reactor (redrawn from Park *et al*., 2005).

CHAPTER 3. METHODOLOGY

This chapter is divided into four sections: 1) *Methanobrevibacter ruminantium* growth, 2) simulated wastewater, 3) experimental method, and 4) chemical analysis.

3.1. *Methanobrevibacter ruminantium* growth

The *Methanobrevibacter ruminantium* was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The strain was grown in a nitrate mineral salt medium with 0.1% sterile methanol following the procedure provided by RIKEN BRC (Appendix A, page 86). The medium as supplied by RIKEN BRC was prepared by dissolving 1.0 g $MgSO_4.7H_2O$, 0.20 g $CaCl_2.6H_2O$, 2.0 ml chelated iron solution, 1.0 g KNO_3, 0.5 ml trace element solution, 0.272 g KH_2PO_4, 0.717 g $Na_2HPO_4.12H_2O$, and 12.5 g purified agar in 1 liter of deionized water. After adjusting the pH to 6.8 the solution was autoclaved at 121^0 C for 15 minutes. 1.0 ml of filter-sterilized methanol was added to prepare the growth medium. Chelated iron solution was prepared by adding 0.5 g of Ferric (III) chloride, 0.2 g EDTA, sodium salt, 0.3 ml HCl (concentrated) into 100 ml of deionized water. Trace element solution was prepared by dissolving 500.0 mg of EDTA, 200.0 mg of $FeSO_4.7H_2O$, 10.0 mg $ZnSO_4.7H_2O$, 3.0 mg $MnCl_2.4H_2O$, 20.0 mg H_3BO_3, 20.0 mg $CoCl_2.6H_2O$, 1.0 mg $CaCl_2.2H_2O$, 2.0 mg $NiCl_2.6H_2O$, and 3.0 mg $Na_2MoO_4.2H_2O$ in 1.0 liter deionized water.

Methanobrevibacter ruminantium strains were then incubated at 37°C for 48-72 hours in an anaerobic chamber (Whitley Workstation DG 250) with an anaerobic gas mixture of 80% Nitrogen, 10% Carbon-dioxide and 10% Hydrogen using the Balch and Wolfe method (Balch

and Wolfe, 1976). The bacteria along with the growth medium were then transferred to the experimental cell inside the workstation area of the anaerobic chamber (Fig 3.1).

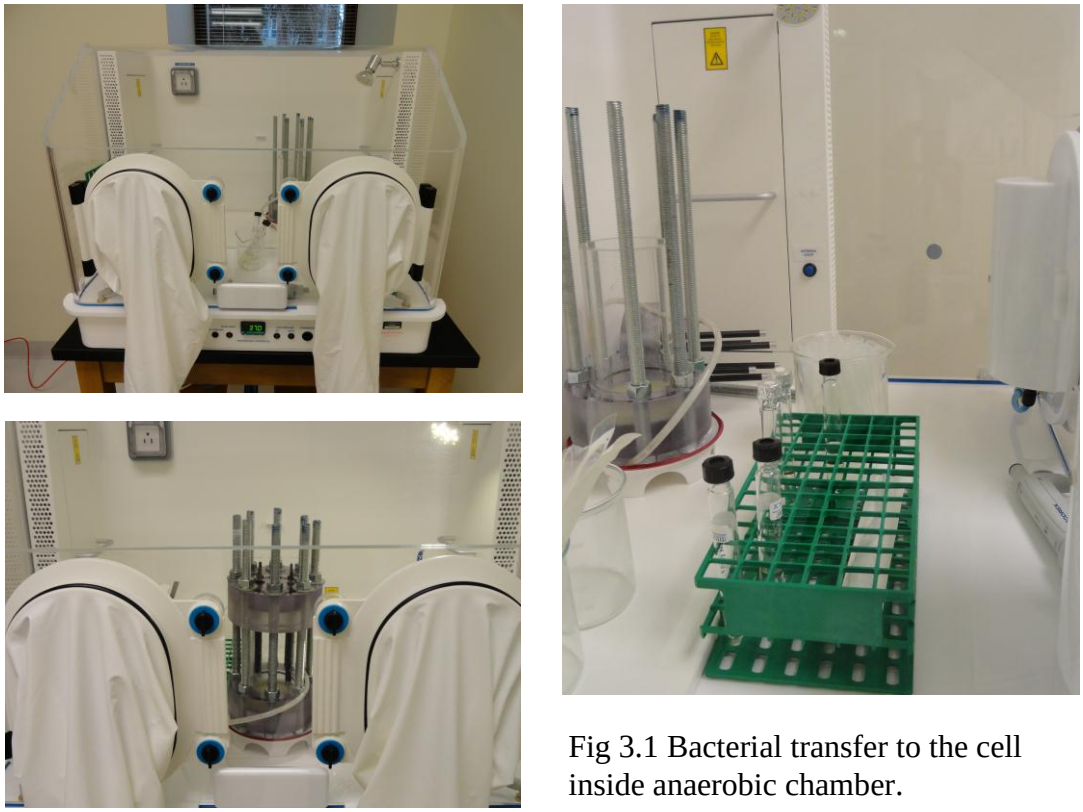

Fig 3.1 Bacterial transfer to the cell inside anaerobic chamber.

3.1.1. SEM of *Methanobrevibacter ruminantium* bacteria

Bacteria samples on moist clay surface were treated by applying 2.5% glutaraldehyde in sodium phosphate buffer (pH 7.4, Tousimis, Rockville MD, USA) with a pipette (Mussati *et al.*, 2005). A portion of about 1 cm^2 of the clay on the filter-paper substrate was cut out with a razor blade and allowed to air dry at room temperature overnight. The dried section was adhered to a cylindrical aluminum mount with silver paste. A conductive gold-palladium layer was applied to the surface using a sputter coater (SCD 030, Balzers, Liechtenstein). Specimens were observed

and imaged at 15 kV with a JEOL JSM-6490LV scanning electron microscope (JEOL USA, Peabody MA).

3.2. Simulated wastewater

0.055 g Sodium nitrate (Alfa Aesar, analytic grade 98%) salt was added to 2 L DI water and dissolved thoroughly. The nitrate solution was then degassed using the Schlenk Flask method (Fig 3.2). One end of the Schlenk flask was connected to the nitrate solution reservoir and the other end was connected to a vacuum pump with plastic tubing. Degassing was then done at an interval of 10 minutes until no bubbles were observed.

Fig 3.2 Degassing nitrate solution using Schlenk Flask.

3.3. Experimental method

Two grams of Wyoming (Crook County) bentonite, whose X-ray diffraction indicated as Na-montmorillonite, and 8 g of glass beads (Ferro Corporation, Sample ID 2332.5) were mixed together with 50 ml of deionized water and stirred for half an hour to prevent flocculation. The

mixture was poured into an acrylic tube (6.0″ in length) that was held in place by aluminum rods and custom made plastic fittings with one end closed by filter paper, one 0.2 micron and one 0.1 micron Milipore® Nylon membrane (Fig 3.3). The top plastic fitting had 3 pairs of electrodes (4.5″, 5.0″ and 5.5″ of length) to determine the conductivity inside the cell at three different depths (Fig 3.4). These electrodes were coated with 1/1000ths Teflon® film (PCI #425013, Precision Coatings Inc., St. Paul, Minnesota, USA) for corrosion resistance with an exposed conductive tip of 0.5″. Conductivity inside the experimental cell was measured using a Keithley Picoammeter (Model no 6485). The end caps were fitted with Swagelok® stainless-steel fittings for influent and effluent intake and flow through, respectively. The influent line was further connected to a Swagelok® gage (0– 500 psi) with one end attached to a Mighty Mini® HPLC pump (runs at a fixed pressure of 500 psi) and solution delivery module (Fig 3.5).

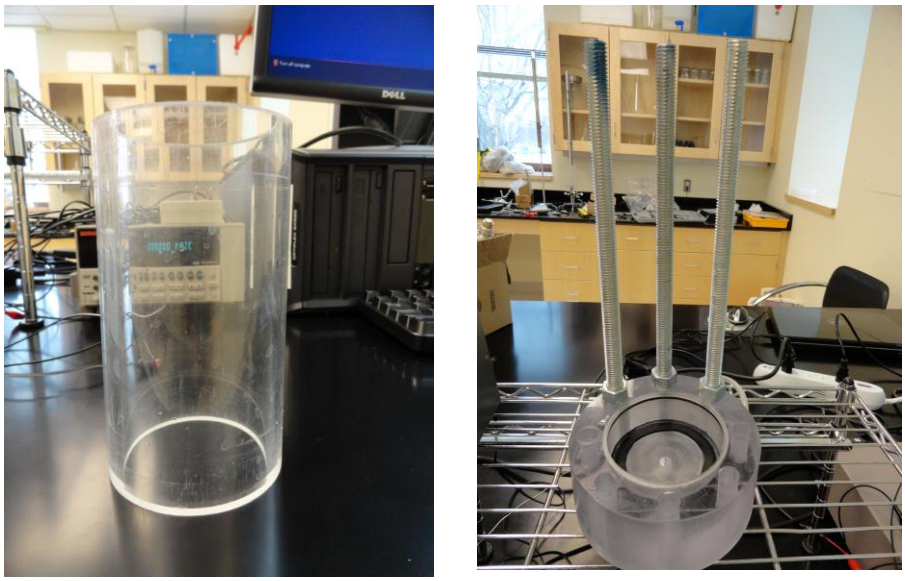

Fig 3.3 Acrylic tube and plastic fittings.

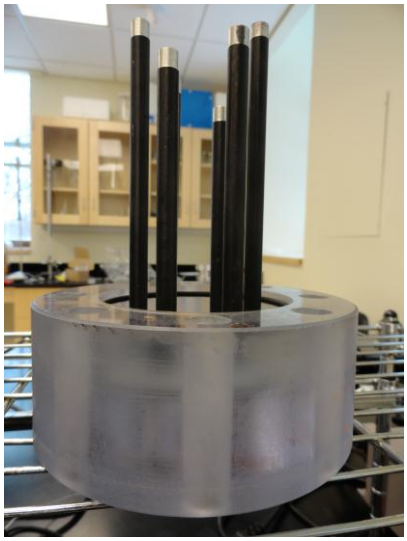

Fig 3.4 Top plastic fittings with electrodes.

Fig 3.5 Mighty mini pump.

After the entire compaction process, the deionized water was replaced by a nitrate solution. The nitrate solution was then hyperfiltrated through both control and main experiment setups. For the controls, $NaNO_3$ solution was hyperfiltrated for 18 days until a steady state was attained. The effluent was periodically collected and the solution flux was determined at each sample collection time. The pump had a secondary pressure gage with auto-off settings for selective pressure settings and a flow meter that was calibrated in ml/min. Solution delivery was accomplished through stainless steel tubing from Swagelok® that could withstand fluid pressures of more than 500 psi. The clay was compacted by setting the pump flow rate to 3 ml/min at 500 psi. At the end of 12 hours, the flow rate was reduced to 1.5 ml/min with same pump setting of 500 psi. After the compaction, the pump flow setting was further reduced to a flow rate value of 0.1 ml/min at 500 psi. The picture of the complete experimental setup is shown in Figure 3.6 and a schematic diagram of the setup is given in Figure 3.7.

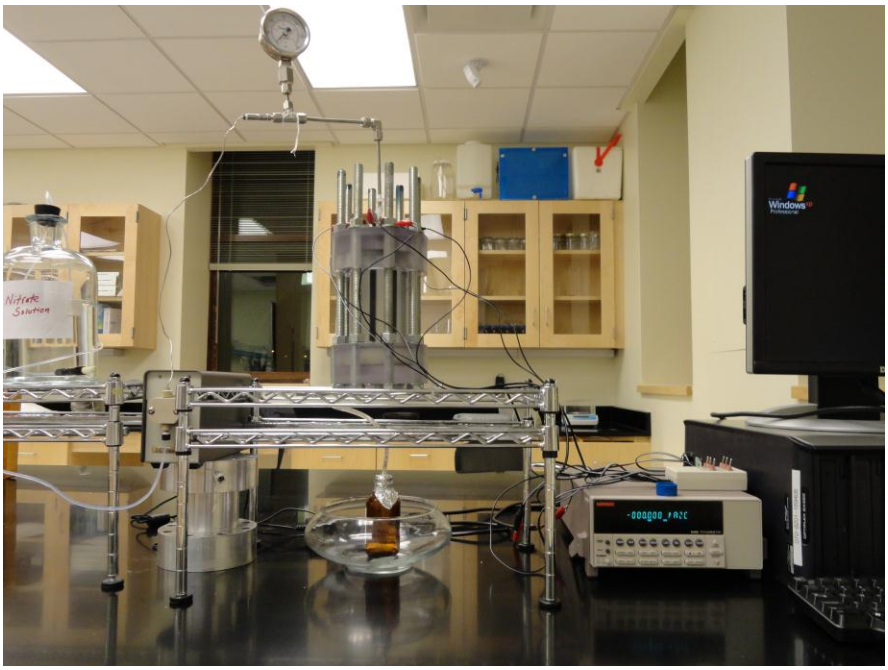

Fig 3.6 Complete experimental setup.

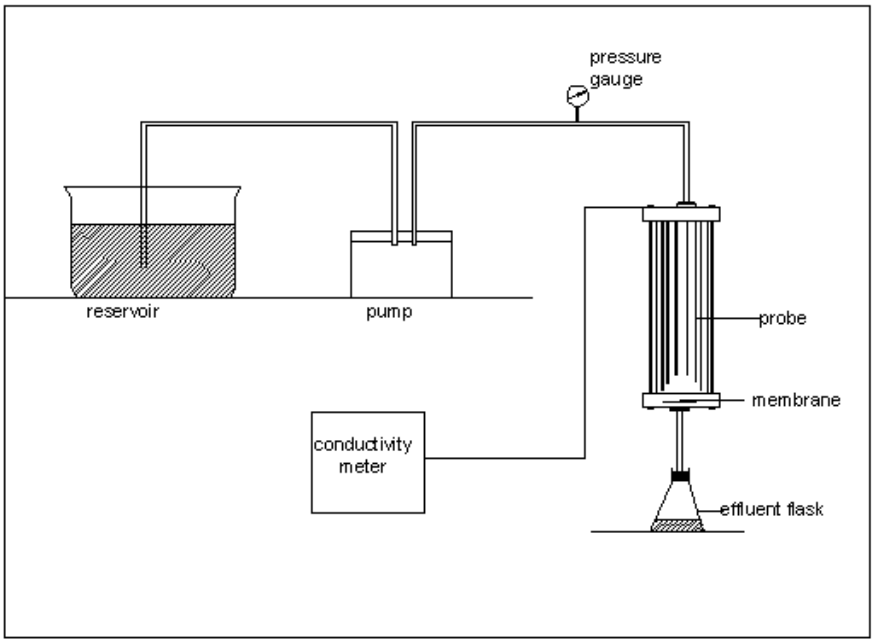

Fig 3.7 Schematic diagram of the experimental setup.

For the bacterial system, *M. ruminantium* bacteria along with the growth medium were injected into the experimental cells in suspension inside the anaerobic chamber. The bacterial components later became inoculated onto the clay membrane. Degassed nitrate solutions were then added to the system and hyperfiltrated through the biomembrane. Effluent samples were then collected at different time intervals for 24 days (13 days for repeated experiment) until a steady state was attained. In the initial run, the pump stopped working several times because of the back pressure generated on the reservoir tank, and the stop time intervals were unaccounted. The samples were collected in 125 ml glass bottles and their contents weighed. The effluent weight was determined, collection time noted, and temperature values recorded in ºC. The pressure setting recorded by the pump and pressure gage was also noted. The samples collected were then stored in a refrigerator for further chemical analysis. After the sampling was completed, the membrane was extracted in order to measure membrane thickness and for taking SEM images.

3.4. Chemical analysis

The chemical analysis involved testing Ultra-high purity water, deionized water, and various parts per million standard concentrations of $NaNO_3$ along with the effluent samples. This was accomplished using a Dionex Ion Chromatograph 2000 (ICS 2000) System. The ICS 2000 system requires initial calibration, and thereafter priming of the pump for 15 to 20 minutes followed by the creation of a program, method, and sequence setup which involves particular settings for the analysis of the samples. The ICS 2000 system has an EluGen KOH cartridge of which the created eluent concentration was set to 20.0 mM KOH (lower than norm value), an

operating temperature of 30° C, an injection volume of 20 µl and an anion atlas electrolytic suppressor that detects the analytes. The samples were all individually transferred to 5 ml vials with filter caps up to their respective fill lines and placed into cassettes, holding six vials each. Filter caps were used to prevent inadvertent evaporation, contamination, and spillage prior to analysis. Samples were automatically injected into the ICS2000 (Fig 3.8) from the AS40 autosampler based on a programed sequence. The first three samples in the analysis were buffers of MilliQ water (Ultrahigh purity water), which were followed by three vials containing deionized water. Ultrahigh purity water was used: (a) to flush out detrital ions in the ICS 2000 and (b) as a buffer since the first few samples usually drift. Subsequent vials included four standards consisting of sodium nitrate solutions, and 16 – 20 effluent samples. All the samples were loaded automatically and injected through an AS40 autosampler. Ultrahigh purity helium gas was used to stabilize the system background pressure. All samples were analyzed in triplicate. Nitrate peak specification window was set ± 0.535 minutes, and the peak ranged between 9.715 minutes and 10.785 minutes (Cheshire *et al.*, 1983).

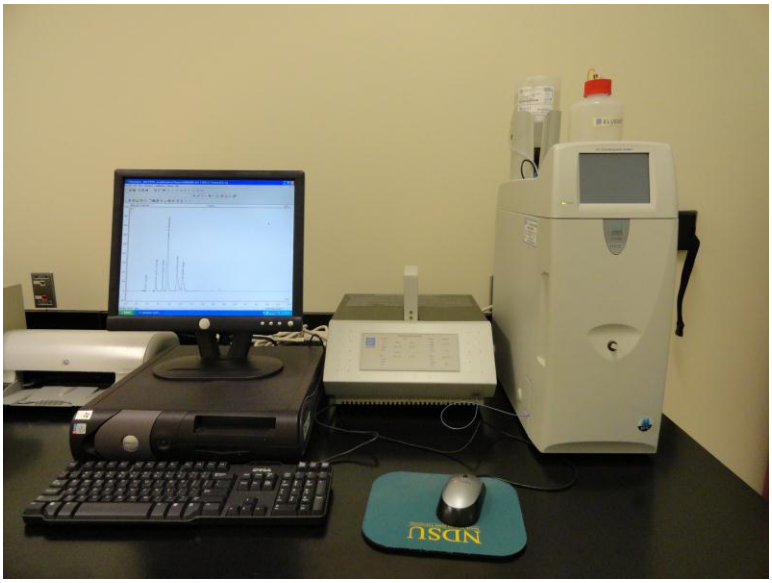

Fig 3.8 Dionex ICS 2000 Ion Chromatograph.

CHAPTER 4. RESULTS AND DISCUSSIONS

This chapter includes the following sections: 1) SEM images of *Methanobrevibacter ruminantium* and glass beads-clay mixture, and 2) Solution flux and attenuation of nitrate.

4.1. SEM Images of *Methanobrevibacter ruminantium* and glass beads-clay mixture

Figure 4.1 shows the SEM image of the bacteria while Figures 4.2 and 4.3 show the SEM images of the glass beads and glass beads-clay mixture, respectively. The shape of the bacteria in SEM image is found to be little bit distorted because of the air drying method used.

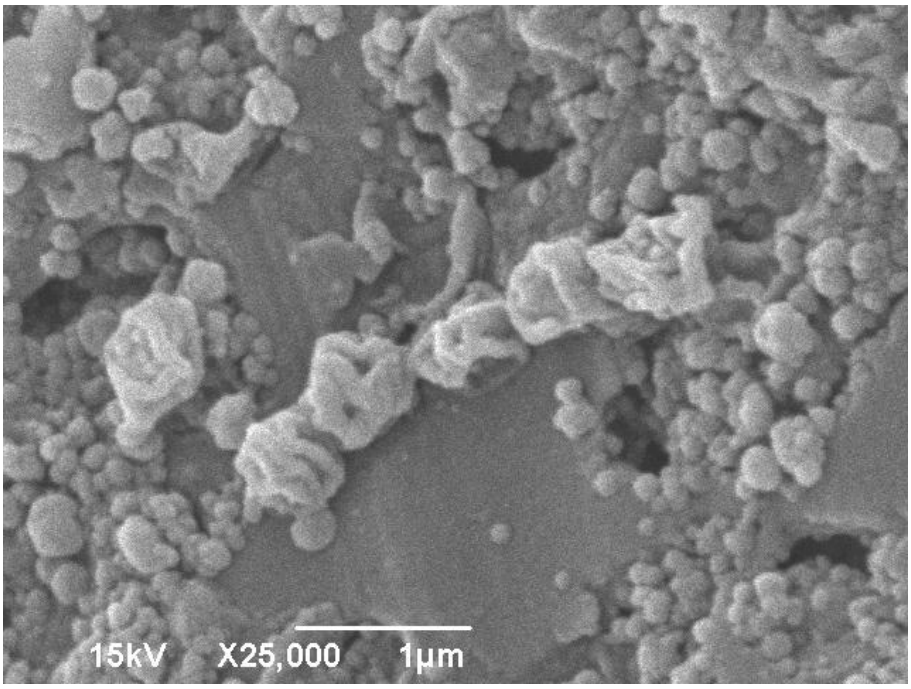

Fig 4.1 SEM image of *Methanobrevibacter ruminantium* bacteria.

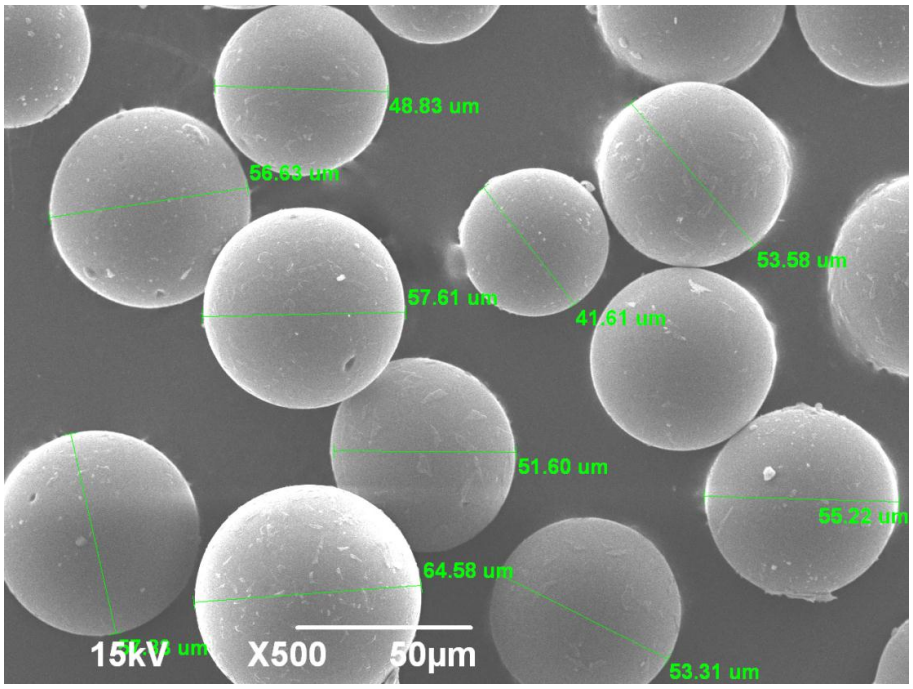

Fig 4.2 SEM image of glass beads used.

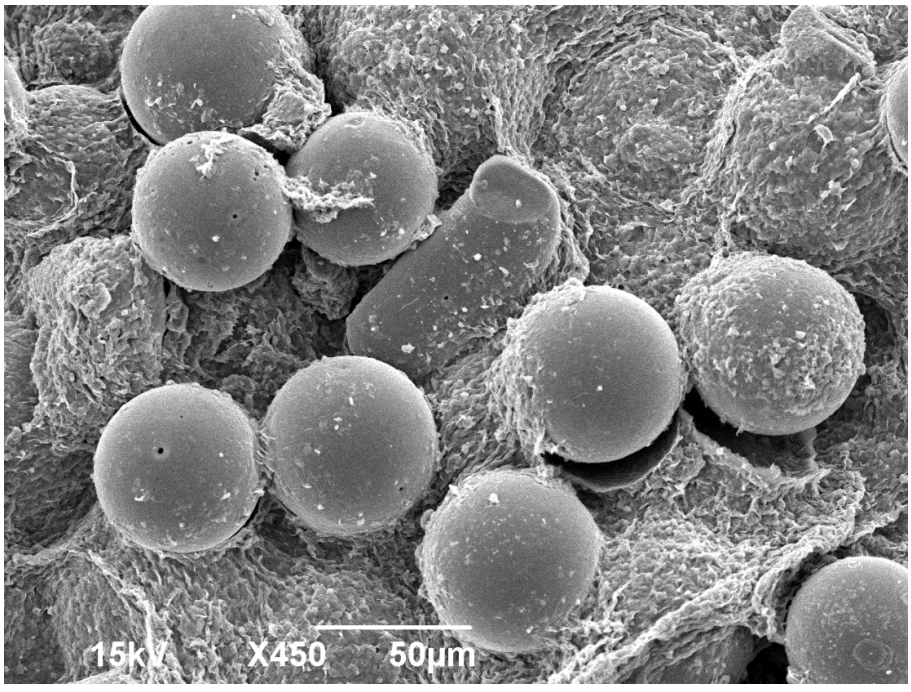

Fig 4.3 SEM image of glass beads-Na montmorillonite mixture.

A biofilm of *Methanobrevibacter ruminantium* on the glass beads-clay surface is shown in Figure 4.4.

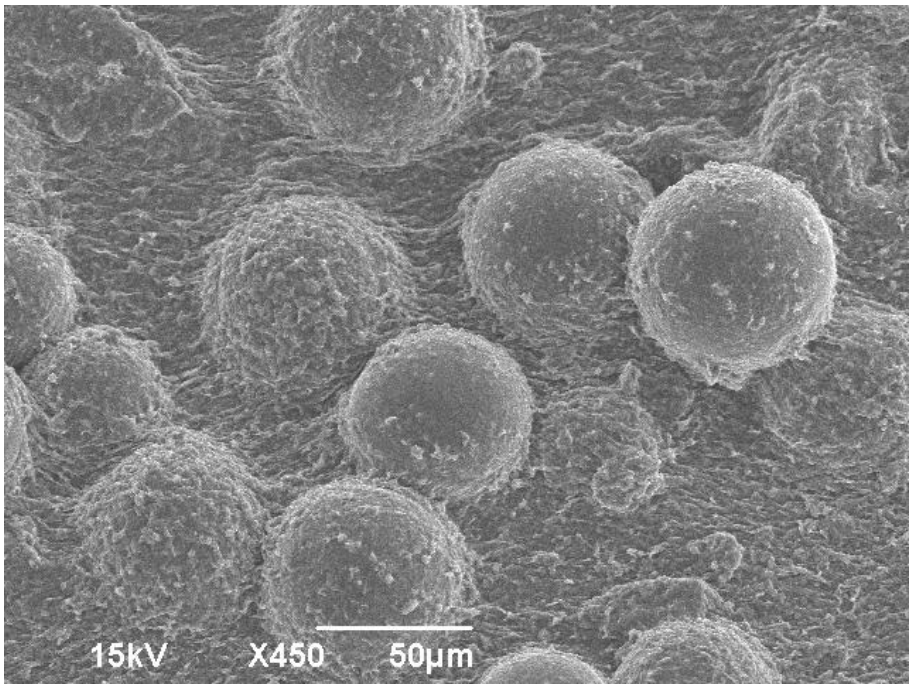

Fig 4.4 SEM image of the biofilm on glass beads-Na montmorrilonite surface.

4.2. Solution flux and attenuation of nitrate

Variation of the solution flux, J_v, with time, t, for the control system is depicted in Figure 4.5. The solution flux, J_v, variation with time, t, for bacterial system is presented in Figure 4.6. Time variation was measured as cumulative time passed after each successive sample was collected. The characteristic decay-curve variation is typical of a membrane system with initial rapid decline state followed by quasi-steady and steady-state variations. For the control system, the initial flux value of the blank experiment was $J_v = 1.455\times10^{-6}$ m/s which is higher than the experimental J_v value. At steady state the value of solution flux decreased to 0.065×10^{-6} m/s.

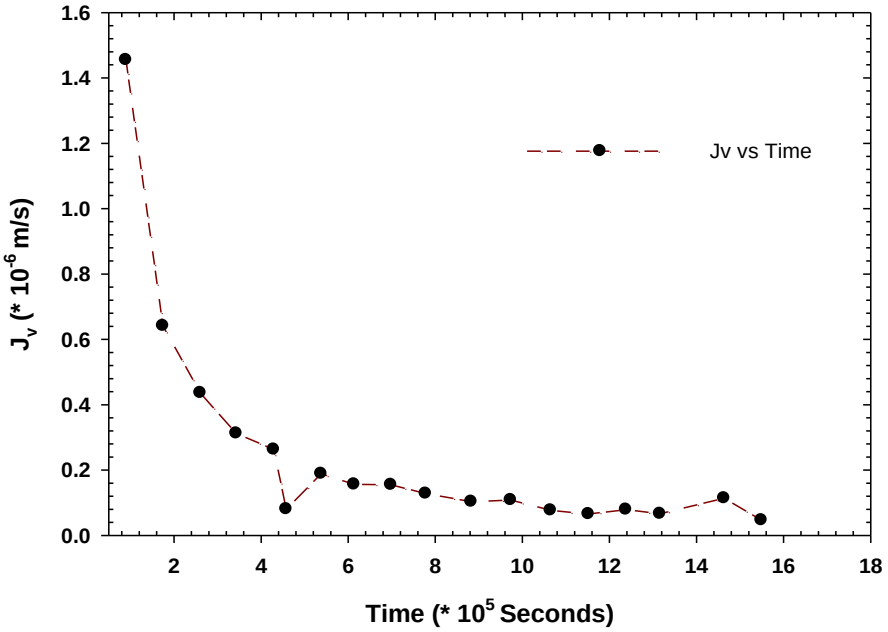

Fig 4.5 Solute flux variation with time for control.

The initial flux value for the bacterial system was $J_v = 1.598\times10^{-7}$ m/s and at onset of steady state the value decreased to 0.115×10^{-7} m/s. This indicates that the presence of biofilm reduced the solution flux through the membrane. The lower steady-state values might be the result of increased osmotic pressure with significant solute retentions, development of biofilm, and possible fouling which led to retardation of nitrates passing through the compacted glass beads-Na montmorillonite bio-membrane.

The concentrations of the solution after being filtrated through compacted glass beads-clay-bacteria and glass beads-clay (control) is depicted in Figure 4.7(a). The glass-beads and Na-montmorillonite mixture (control) showed minimal membrane properties and effluent nitrate concentrations were found to be very close to the reservoir nitrate concentrations.

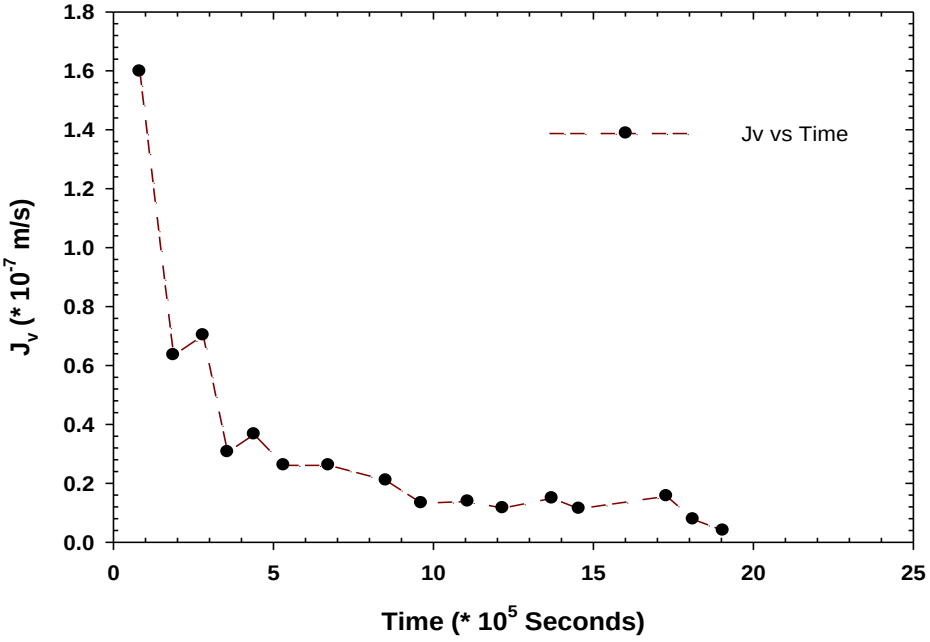

Fig 4.6 Solute flux variation with time for bacterial system.

When filtered through glass-beads and Na-montmorillonite, influent nitrate concentration of 3.105×10^{-4} moles/L was reduced to 2.826×10^{-4} moles/L, which is around 8.99 % reduction. Saindon and Whitworth (2006) investigated the membrane properties of five different mixtures of clay and glass beads at low compaction pressure and found that the higher the percentage of glass beads, the lower the membrane properties. They observed a reflection coefficient of 0.07 using a mixture of 12 % clay and 88 % glass beads. The presence of Methanobrevibacter ruminantium played a significant role in reducing nitrate concentrations of the reservoir solution filtered through the system. For the first run with the bacteria, after the 11th day of the experiment effluent nitrate concentration reached as low as 2.50 × 10-7 moles/L, which is about 99.9 % reduction in the concentrations (Fig 4.7 (a) and 4.8 (a)).

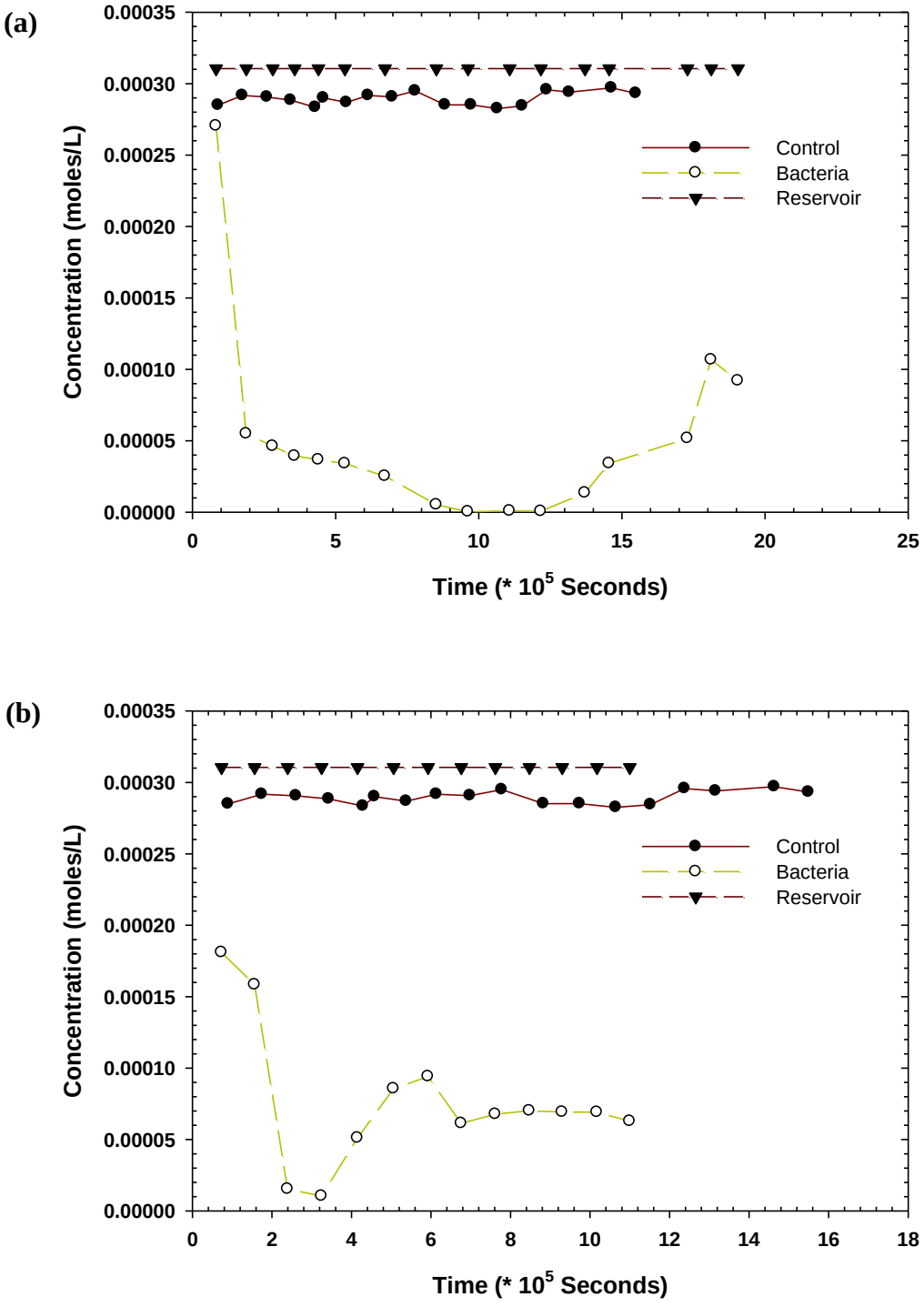

Fig 4.7 (a) Effluent nitrate concentrations filtered through the various system, (b) values for repeated experiment with the bacteria.

When the experiment was repeated, NO_3^- effluent concentration reached as low as 1.03×10^{-5} moles/L after the 4th day of the experiment, which is around 96% reduction in influent nitrate concentration (Fig 4.7 (b) and 4.8 (b)). The control system showed a maximal value of 9% nitrate reduction (Fig 4.7 (a)), which indicates that, the nitrate reduction in the bacterial system results can probably be attributed to the presence of the biofilm. For the first set of experiment, after around 72 hours the rate of nitrate reduction slows down and showed a steady state reduction of nitrate (Fig 4.8 (a)). After the 11th day, the effluent nitrate concentration again started to increase although the effluent nitrate concentrations were below the influent nitrate concentration throughout the experiment. Initially the effluent nitrate concentrations showed a decreasing trend. This might be because of the development of the biofilm. *Methanobrevibacter ruminantium* usually takes 2-3 days to appear after incubation depending on the number of colonies and energy sources (Miller et al., 1986).

Methanogenic bacteria are capable of utilizing nitrates as an alternative energy source and could potentially replace urea in diets to provide nitrogen for microbial protein production and growth (Belay *et al.*, 1990; Guo *et al.*, 2009; Leng, 2008). In a different experimental setup, Guo *et al.* (2009) observed that NO_3-N disappeared to background level and was not detectable to microbial cells after 24 hours of incubation.

After some time the effluent nitrate concentrations started to increase. This might be due to bacterial depletion. The bacteria might have started depleting due to (a) water level decrease in the reservoir which possibly increased infusion of dissolved oxygen into the feed solution, and (b) an increase in pressure gradient across the membrane that might have affected the microbial biophysical integrity.

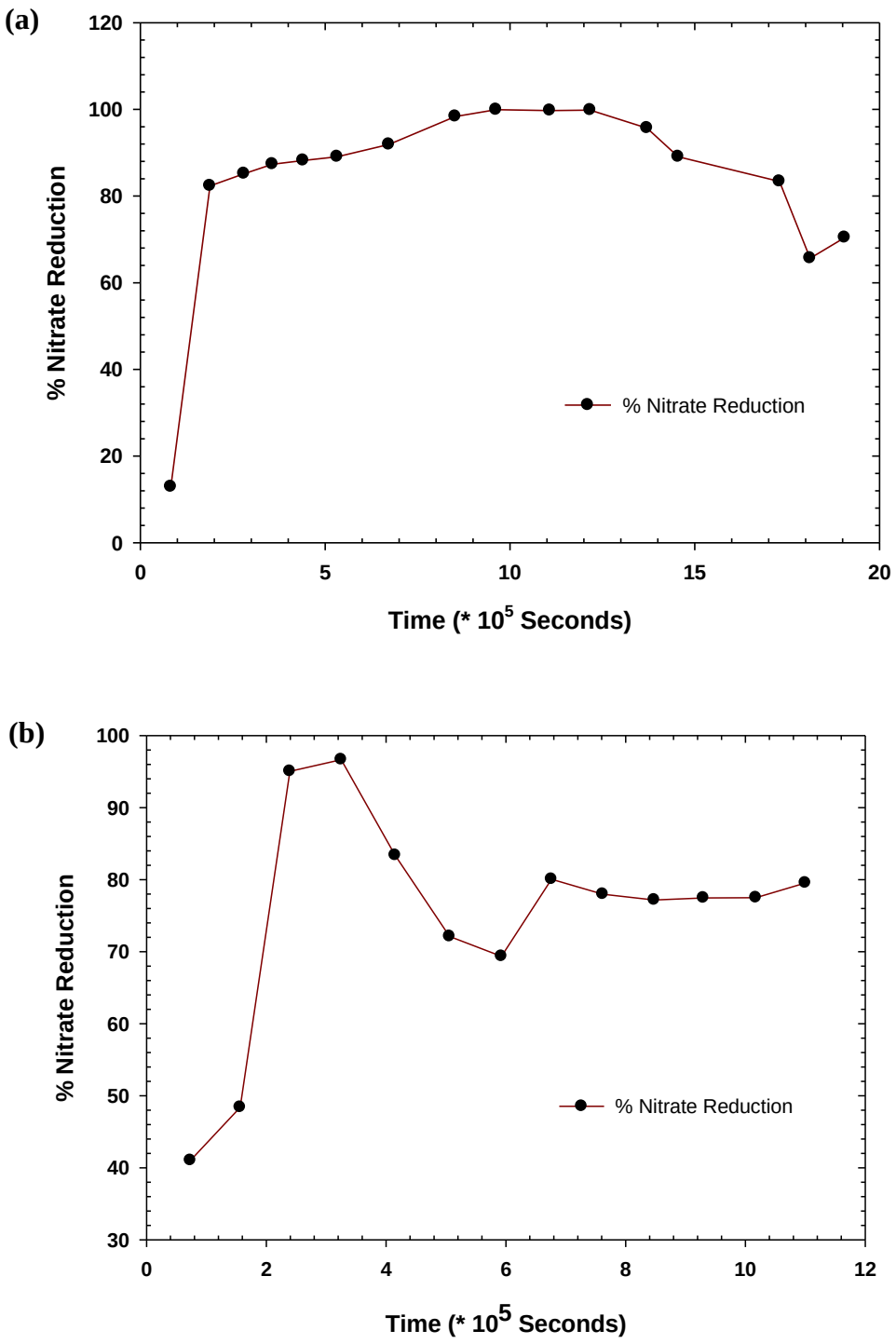

Fig 4.8 (a) Percent nitrate reduction with time for bacterial systems, (b) Percent nitrate reduction for the repeated experiment.

The results of the experiments indicate that the *Methanobrevibacter ruminantium* is capable of attenuating nitrates from the simulated wastewater though the mechanism of nitrate depletion in the experiment with bacteria is not well understood. But the chromatographs for the bacterial system showed both nitrate and nitrite peaks (see Appendix C). It is worthwhile to note that various studies have indicated that biofilms constantly change in composition, thus may create variables which may include gradients of organic nutrients and inorganic materials such as oxygen, nitrogen and phosphates that are pertinent for many biological processes (Zheng and Bennett, 2002). Other nitrate reduction studies with other strains of bacteria were successful because those bacteria used nitrates in their anaerobic respiration processes (Casey *et al.,* 1998; Kalinowski *et al.,* 2002).

Conductivity inside the cell was measured at three different depths (4.5″, 5.0″ and 5.5″) using three pairs of probes. The distance between the tip of the probes and the surface of the membrane were 0.35, 0.85 and 1.35 inches. The conductivity values at these depths for the control system are depicted in Figure 4.9. For this system, higher conductivity values were observed for the probes farthest from the membrane surface and lower values for the closest. This might be because of the fact that, the probes farthest from the surface of the membrane covers more area and volume hence more ions of solution than that for probes closest to the surface. The conductivity values for the bacterial system are presented in Figure 4.10. The values for the bacterial system are much lower than those of the control system. This is because of the less ionic activity in the bacterial system resulting from the microbial utilization of the nitrate ion. For the bacterial system, higher conductivity values were observed at the middle depth. The conductivity values observed for the systems did not follow the conceptual trends.

More research needs to be carried out to confirm the trends and reasons behind the observed trends.

The surface-fit robust plane feature of the Table Curve® 3D was used to ideally fit the outliers to a plane. This robust model was used to lessen the impact of outliers upon the overall surface fits. This model uses a simple plane equation with four minimization criteria available within the Table Curve® 3D's non-linear fitting algorithm. These models were then ranked based on their R2 value. The difference between the best fits of the bacterial system and control system will be useful in finding out the microbial retardation factor. Comparisons between control and bacterial system for all the four equations based on rank are shown in Figure 4.11, 4.12, 4.13 and 4.14 respectively.

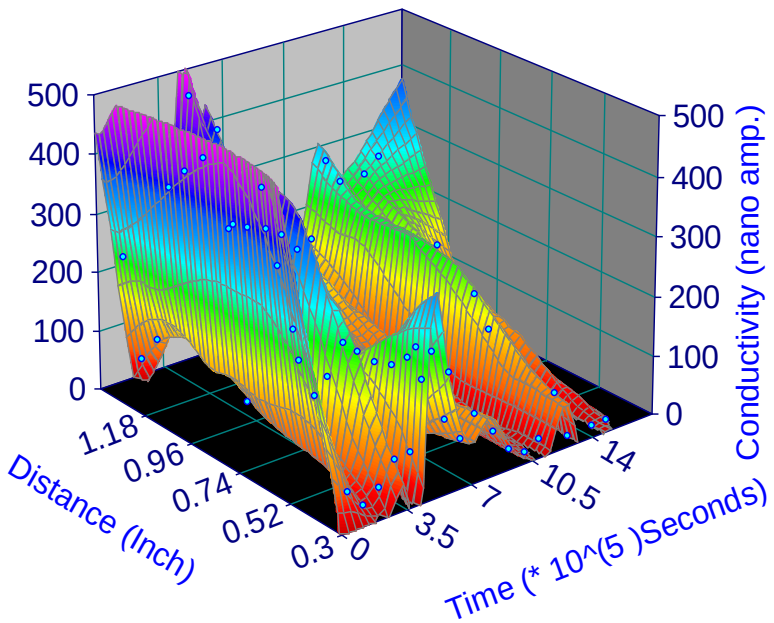

Fig 4.9 Conductivity at different depths inside the cell for control systems.

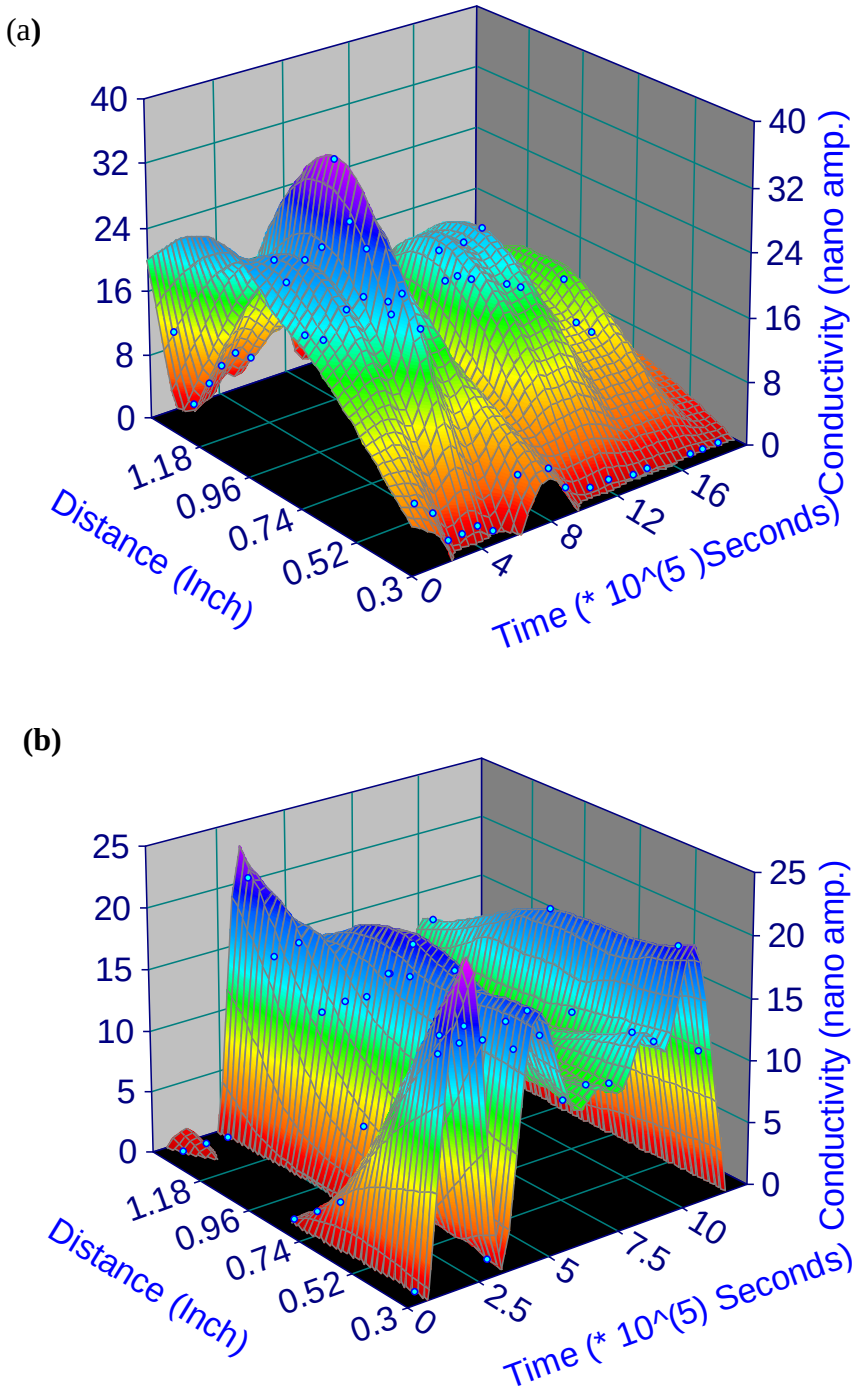

Fig 4.10 (a) Conductivity at different depths inside the cell for bacterial systems (b) Values for the repeated experiment.

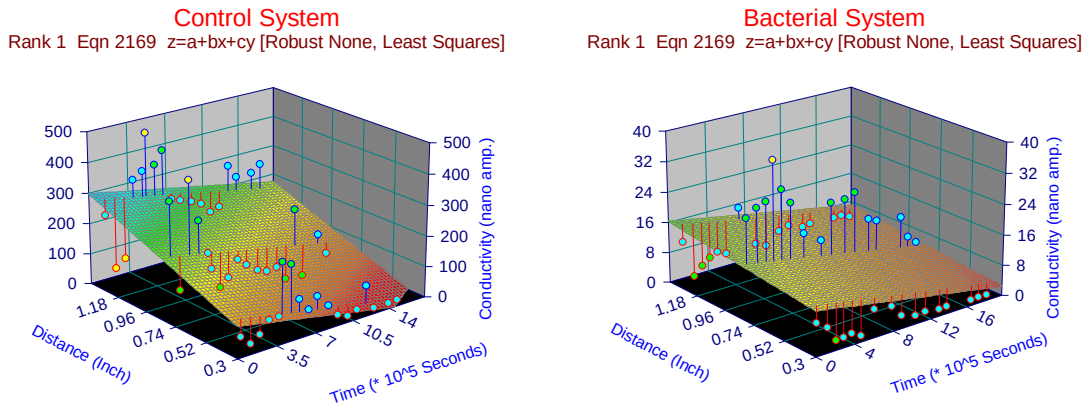

Fig 4.11 Comparison between the systems using surface-fit robust plane rank 1.

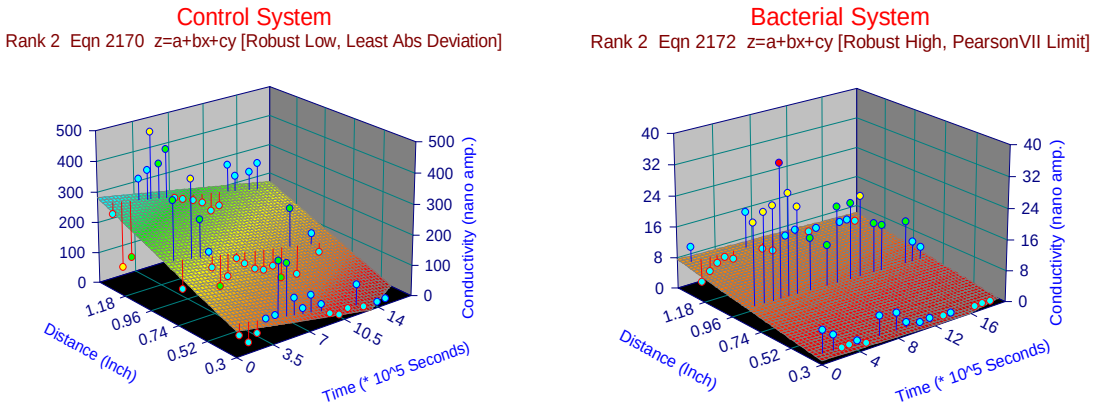

Fig 4.12 Comparison between the systems using surface-fit robust plane rank 2.

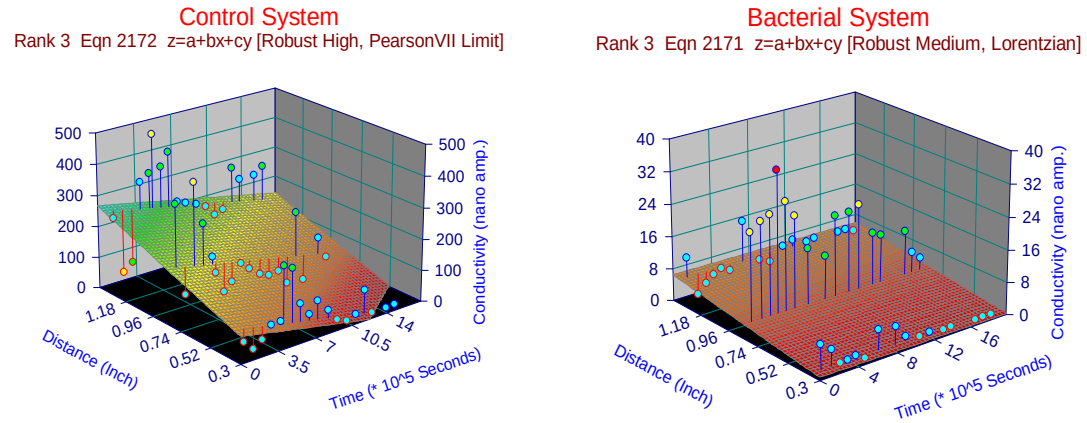

Fig 4.13 Comparison between the systems using surface-fit robust plane rank 3.

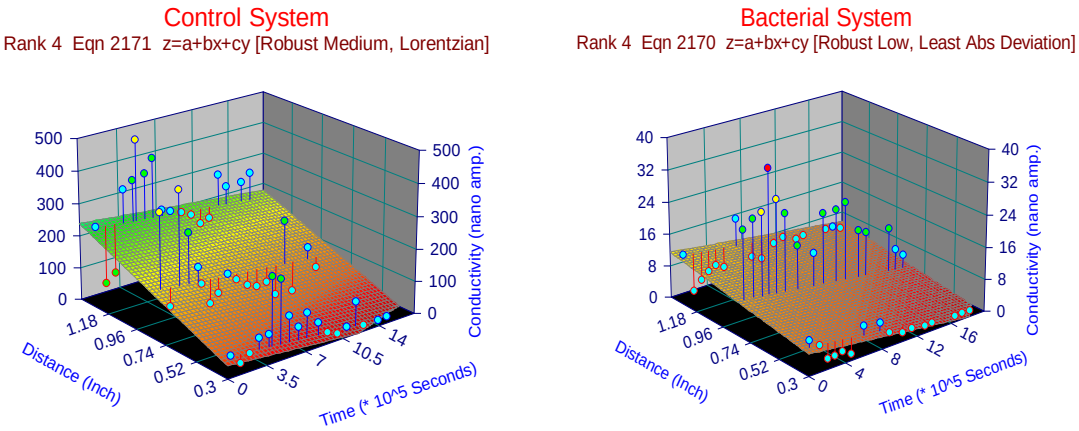

Fig 4.14 Comparison between the systems using surface-fit robust plane rank 4.

CHAPTER 5. CONCLUSIONS

Groundwater contamination by nitrates is a major issue worldwide. The continuous development and expansion of the agricultural sector presents remarkable challenges to agencies dealing with environmental issues that are tasked with implementing more stringent policies to ensure balance between anthropogenic impacts on water quality. It is imperative that future work continue to evaluate remediation techniques that would enhance remediation processes and minimize amount or reduce toxicity of pollutants in the environment. Bioremediation offers one possible solution in minimizing nitrate pollution. Studies have indicated that microbes are capable of consuming various natural and artificial pollutants.

This study harnessed biostimulated remediation of simulated wastewater. An experimental analysis was executed in an attempt to determine if *Methanobrevibacter ruminantium* was capable of reducing nitrate levels in waste water. The experimental setup involved the assemblage of a static cell encased in an acrylic tube held together by aluminium fittings. The *Methanobrevibacter ruminantium* bacterium was then introduced into separate experimental cells for analysis and these in turn ingested nitrates in the ambient simulated wastewater. Microbial activity of *Methanobrevibacter ruminantium* played a significant role in the attenuation of nitrate.

If we intend to model the effluent concentration and the evolution of the transient concentrations at a distance from the high pressure side of compacted montmorillonite, glass beads and *Methanobrevibacter ruminantium* membranes to a reasonable degree, the solution to advective–diffusive differential equation needs to be modified to factor in the microbial retardation. This factor will be helpful in providing the general correctness for the modified bio-

membranes. The general trend difference observed between the conductivities of the control and microbial system might be useful in finding out the microbial retardation factor. A fouling correction may also need to be incorporated into the models. The development of models depends highly on accurate and representative diffusion coefficient values and constant temperature conditions.

The experiment was successful in this laboratory setup; however, it is not yet conclusively known if such a bench-scale system could be applied in real-life scenarios. The probable applications of these models may be to provide an insight in defining the fate and transport of contaminants through barriers with high microbial activities under anaerobic conditions.

There are a lot of research opportunities in this field. Research work can be carried out to calculate the microbial retardation factor and then the developed microbial retardation factor can be applied to the ion transport in the presence of a biofilm. Research work can also be carried out to find out the Concentration Profile Layer development on the high pressure side of the membrane using conductivity parameters. In this experiment only one influent concentration value was used to gauge the effectiveness of *M. ruminantium* in attenuating nitrate. In the future, research can be carried out to determine the effective range of concentrations *M. ruminantium* can reasonably attenuate.

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APPENDIX A. *METHANOBREVIBACTER RUMINANTIUM*

MATERIAL TRANSFER AGREEMENT

(Form M-11-1)

RIKEN BRC

MATERIAL TRANSFER AGREEMENT
(For Distribution to a Not-For-Profit Organization)

RECIPIENT

Recipient Scientist: Peter Oduor

Recipient Organization: North Dakota State University

Address: Stevens Hall Room No. 227

1340 Bolley Drive, Fargo, ND- 58102, USA

This Material Transfer Agreement sets forth the terms and conditions under which RIKEN BioResource Center (hereinafter referred to as 'RIKEN BRC') will provide with the RECIPIENT, and the RECIPIENT will receive, the biological material specified as Methanobrevibacter Ruminantium (JCM13430)

and its derivatives (hereinafter referred to as the 'BIOLOGICAL RESOURCE') in response to the RECIPIENT's request, and with which the RECIPIENT scientist and organization agree before the RECIPIENT receives the BIOLOGICAL RESOURCE:

1. The RIKEN BRC, a non-profit public organization financed by the Japanese Government, is engaged in collection, maintenance, storage, propagation, quality control and distribution the biological resources, in order to contribute to the Japanese and international scientific community in the field of life sciences.
2. (a) The RECIPIENT shall use the BIOLOGICAL RESOURCE for the following specific purpose: Graduate Research Project titled "Biostimulated remediation of wastewater"

   (b) The RECIPIENT shall obtain a written prior permission from the RIKEN BRC for the usage of the BIOLOGICAL RESOURCE for any other purposes than the purpose specified above.
3. The RECIPIENT shall not use the BIOLOGICAL RESOURCE for diagnosis or treatment of humans or other direct applications to human bodies or as food source for humans.
4. The RECIPIENT agrees to use the BIOLOGICAL RESOURCE complying with the following terms and conditions requested by the DEPOSITOR, which are specified in the RIKEN BRC Catalog or Website: No restriction

In the case requested by the DEPOSITOR, the RECIPIENT should obtain an approval from the DEPOSITOR using the APPROVAL FORM prior to entering the AGREEMENT with the RIKEN BRC.

(10.07.01)

5. The RECIPIENT agrees to expressly describe that "the BIOLOGICAL RESOURCE (the resource name) was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan" in Materials and Methods, the Acknowledgement or any other appropriate section in any publication reporting the use thereof. The RECIPIENT also agrees to send information regarding such publication to the RIKEN BRC. The RIKEN BRC may request the RECIPIENT to report on progress and/or results obtained through the use of the BIOLOGICAL RESOURCE, and the RECIPIENT shall respond truthfully to such a request by the RIKEN BRC. The RIKEN BRC may disclose publicly such information to increase the value of the BIOLOGICAL RESOURCE, and to demonstrate the contribution of the RIKEN BRC.
6. The RECIPIENT shall bear the cost of shipping, handling, part of production and other expenses necessary for preparation or distribution of the BIOLOGICAL RESOURCE for the RECIPIENT.
7. The access to the BIOLOGICAL RESOURCE is limited to the RECIPIENT and the RECIPIENT's co-workers and students who work for the purpose specified in Section 2(a) under the direct supervision and full responsibility of the RECIPIENT. The RECIPIENT shall not distribute, resell or otherwise dispose of the BIOLOGICAL RESOURCE to any third party. The disposition hereunder shall include any acts to transfer all or any part of the intellectual property or grant a license thereunder with respect to the BIOLOGICAL RESOURCE.
8. Nothing in this AGREEMENT shall be interpreted that the RIKEN BRC grants the RECIPIENT any rights under any patents or other intellectual property, or licenses thereunder with respect to the BIOLOGICAL RESOURCE.
9. The RECIPIENT shall assume all liability for claims against the RECIPIENT and the RIKEN BRC by third parties relating to alleged infringement of any patent, copyright, trademark or other intellectual property rights, which may arise from the use, storage or disposal by the RECIPIENT of the BIOLOGICAL RESOURCE.
10. The RECIPIENT acknowledges that the BIOLOGICAL RESOURCE delivered pursuant to this AGREEMENT may have defective, hazardous or faulty properties and may not necessarily fit for a particular purpose and that the RECIPIENT assumes all liability for any consequences resulting from the use by the RECIPIENT of the BIOLOGICAL RESOURCE.
11. The RECIPIENT agrees that any handling or other activities of the BIOLOGICAL RESOURCE in its laboratory shall be conducted in compliance with *all applicable* laws, regulations and guidelines. The RECIPIENT shall, if necessary, take all steps or procedures to comply with legal requirements for handling of the BIOLOGICAL RESOURCE.
12. Both parties shall discuss to enable amicable resolution of any accidents during shipment of the BIOLOGICAL RESOURCE.
13. In case the RECIPIENT is in breach of this AGREEMENT, the RIKEN BRC may request the RECIPIENT to cease its subsequent use of the BIOLOGICAL RESOURCE and other resources of the RIKEN BRC.
14. Both parties shall discuss in good faith to enable the amicable resolution of matters, arising in connection with the interpretation or performance hereof as well as the matters which are not expressly set forth in this AGREEMENT.
15. Any matter or dispute which cannot be settled through said amicable discussion shall be subject to the exclusive jurisdiction of Tokyo District Court, Japan. This AGREEMENT shall be

governed in accordance with the laws of Japan.

The RECIPIENT and the RIKEN BRC do hereby sign two original copies of this AGREEMENT and each party holds one signed copy.

RIKEN BioResource Center
3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan
Director
Yuichi Obata, Ph.D.
Signature: Y. Obata
Date: Jan 13. 2012

RECIPIENT

Organization: North Dakota State University
Address: 228, Stevens Hall, 1340 Bolley Drive, NDSU, Fargo, ND 58108, USA
Name of Authorized Representative: Kevin D. McCaul
Job Title: Dean of the College of Science and Mathematics
Signature: Kevin D. McCaul
Date: 12-23-11

Name of Scientist: Peter G. Oduor
Job Title: Associate Professor
Signature: Peter Oduor
Date: 12/23/11

JAPAN COLLECTION OF MICROORGANISMS

Microbe Division, RIKEN BioResource Center
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E-mail : inquiry@jcm.riken.jp

REF NO. 110619
Date: 25/01/2012

DELIVERY SLIP

Dr. Peter G. Oduor
North Dakota State University

Dear Sir/Madam:

The microbial culture(s) that you ordered is/are enclosed. Please verify the contents and return the enclosed receipt. Damaged or missing culture(s) must be reported within 1 month of the receipt. The culture(s) is/are nonreturnable.

For data on culture conditions, such as media formulations and cultivation temperature, and for other information, please visit our online catalog of strains at http://www.jcm.riken.jp/JCM/catalogue.shtml. Inquiries regarding methods for cultivation should be addressed to inquiry@jcm.riken.jp.

If the research performed using this/these culture(s) is published, it would be a valuable reference for our catalog database. You are required to send us a reprint of your published article or other information on your research.

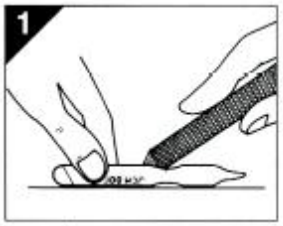
Please note that you MUST NOT transfer these cultures to a third party.

Your order number:
Request for payment of this order will be sent separately.

Microorganisms MD: (JCM medium number)	TM: cultivation temperature	JCM no.	Qty
Methanobrevibacter ruminantium MD: 530 ★ Anaerobic ★	TM: 37C	13430	4
	Total:	1 strains	4 tubes

HOW TO REVIVE LYOPHILIZED OR L-DRIED CULTURES

1) Keep the ampoule on a flat place, and make a file-cut at the neck.

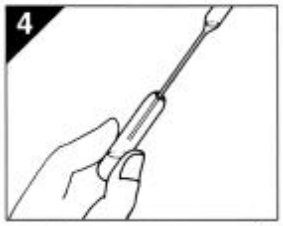


2) Wipe the ampoule with cotton wool containing 70% alcohol.

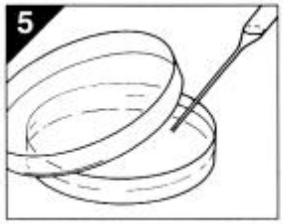
3) Cover the ampoule with a sterile cotton sheet, and cut it carefully at the neck. Do not use a cotton sheet containing alcohol.

4) Using a sterile Pasteur pipette, add 0.3 to 0.5 ml of a suitable rehydration fluid* into the ampoule.

*See the JCM On-line Catalogue (http://www.jcm.riken.jp/JCM/catalogue.html).



5) Spread the sample on a suitable plate and incubate it under the directed condition. The subculture should be established in fresh media only after confirming its purity. A careless single-colony isolation may lead to picking up an unusual strain. It is recommended to revive the strain simultaneously using liquid culture.



Japan Collection of Microorganisms, RIKEN BioResource Center

APPENDIX B. LABORATORY NOTES

Project No. _____

TITLE _____ Book No. _____

From Page No.

2 gms of Na-Montmorillonite (Source Clay minerals Repository, Dept of geology, University of Missouri) and 8 gms of Glass beads (Ferro Corporation, Sample ID. 2932-5) were mixed, 50 ml of DI water was added and stirred the Coir for 30 minutes. It was then poured to the experimental cell having 0.1 µm Nylon membrane.

Initially the DI water was passed through at a flow rate of 2 ml/min for 6 hrs and then the flow rate was reduced to 1 ml/min till the cell is filled up with DI. The DI was continued to pass through and reading was recorded.

START TIME: 5:02 PM 05.24.11
INITIAL TEMP: 20.5 °C
PUMP PRESSURE: 500 PSI

Sample No	Date	Time	Temp °C	Pressure Pump	Pressure Meter	Mass M+L	Mass M+L+S	Comments
MB 1	05.25.11	8:26am	20.9°C	500	0	22.720	132.525	Flow rate 0.2 ml/min
MB2	05.26.11	1:41 PM	20.5	500	0	22.375	204.215	
MB3	05.27.11	12:54PM	19.6°C	500	0	26.060	120.860	Flow rate
MB 4	05.28.11	12:04 PM	19.6°C	500	0	22.965	133.465	reduced to
MB 5	05.29.11	11:14 AM	19.2°C	500	0	22.580	126.885	0.1 ml/min
MB 6	05.30.11	10:45 am	19.3°C	500	0	22.450	133.425	
MB 7	05.31.11	10:47	19.6°C	500	0	22.415	135.810	

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0.110 gm of $NaNO_3$ is added to 4L of DI water and mixed thoroughly. The 50^{th} was allowed to pass through the cell and following reading was recorded.

START TIME: 06.03.2011 @ 10.16 am

INITIAL TEMP: 21.1°C

PUMP PRESSURE: 500

Salt Conductivity: 86.6 mS/cm

Sample ID	Date	Time	Temp °C	Pressure Pump	Pressure Meter	Mass B+L	Mass B+L+S	Comments Flow rate
MJB001	06.04.11	11:10 am	20.7°	500	0	22.700	179.62	0.1 ml/min
MJB002	06.05.11	10:52 am	20.6°C	500	0	22.585	157.559	
MJB003	06.06.11	10:42 am	21.2°C	500	0	22.340	199.048	
MJB004	06.07.11	9:35 am	21.3°C	500	0	22.615		
MJB005	06.08.11	9:37 am	20.7°C	500	0	22.975	158.579	
MJB006	06.09.11	5:32 pm	20.7°C	500	0	22.125	66.840	
MJB007	06.09.11	3:46 pm	20.6°C	500	0	22.765	144.750	
MJB008	06.10.11	12:46 pm	20.2°C	500	0	22.530	132.765	
MJB009	06.11.11	12:19 pm	20.4°C	500	0	22.665	152.032	
MJB010	06.12.11	10:30 am	19.7°C	500	0	22.125	141.840	
MJB011	06.13.11	03:36 pm	21.0	500	0	22.180	139.710	
MJB012	06.14.11	04:40 pm	21.3	500	0	22.160	146.032	
MJB013	06.15.11	06:15 pm	20.4°C	500	0	22.690	120.605	
MJB014	06.16.11	06:23 pm	21.0°C	500	0	22.135	112.365	
MJB015	06.17.11	06:16 pm	20.7°C	500	0	22.185	139.970	
MJB016	06.18.11	04:05 pm	20.7°C	500	0	22.520	126.545	
MJB017	06.20.11	08:57 am	20.7°C	500	0	22.465	222.860	
MJB018	06.21.11	08:40 am	20.4°C	500	0	22.760	109.850	

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Conductivity			Effluent
1	2	3	conductivity
47.86 N	44.64 N	0.140 µ	0.0010 N
42.81 N	33.11 N	0.1289 µ	0.0012 N
23.08 N	50.15 N	0.1322 µ	7.448 N
2.40 N	0.160 µ	0.245 µ	0.58 µ
09.04 N	0.097 µ	0.182 µ	12.90 N
05.56 N	0.0923 µ	0.161 µ	14.27 N
05.40 N	0.105 µ	0.153 µ	55.86 N
16.32 N	0.076 µ	0.127 µ	7.420 N
10.35 N	56.65 N	198.60 N	102.145 N
5.06 N	118.54 N	196.321 N	12.601 N
7.64 N	61.24 N	173.50 N	147.24 N
2.15 N	91.20 N	0.214 µ	68.04 N
21.4 N	118.8 N	0.212 µ	72.4 N
32.7 N	121.1 N	0.216 µ	81.3 N
19.79 N	125.6 N	156.1 N	87.7 N
43.0 N	144.0 N	149.2 N	0.0052 µ
44.43 N	134.4 N	142.7 N	128.1 N
53.6 N	133.8 N	148.1	

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2 gms of Na-Montmorillonite and 8 gms of Glass beads (Ferro Corporation, Sample ID 2332·5) were mixed in 50 ml of DI water. The mixture was stirred for 30 minutes. The mixture was then transferred to the experimental cell which was carefully placed in the anaerobic chamber. The mixture was allowed to settle and then Methanobrevibacter Ruminantium was added to the cell and allowed to immobilize to the cell.

0.055 gms of $NaNO_3$ was added to 2L of DI water and mixed throughly. The solution was degassed and then allowed to pass through the membrane. Initially the flow rate was set 5 ml/min which was then reduced to 0.1 ml/min. The following readings were then recorded.

Start time: 7:45 pm 28-11-2011

Initial Temp: 23.6°C

Initial Pressure: 500 PSI

Sample	Date	Time	Temp °C	Pressure Pump	Pressure Meter	Mass B+L	Mass B+L+S	Comments
MRB1	24·11·2011	6:42 PM	23·5°	500	0	99·160	160·0	
MRB2	26·11·2011	10:05 AM	24·9°c	500	0	98·960	154·1	Pump stopped slowing into
MRB3	27·11·2011	11:44 AM	24·5°c	500	0	99·130	190·0	night
MRB4	28·11·2011	5:01 PM	24·4°c	500	0	99·505	149·885	pump stopped
MRB5	29·11·2011	4:08 PM	24·6°c	500	0	99·720	174·0	
MRB6	30·11·2011	5:48 PM	28·4°c	500	0	99·865	164·0	
MRB7	02·12·2011	8:41 AM	18·1°c	500	0	99·005	180·0	
MRB8	04·12·2011	10:28 AM	20·7°c	500	0	99·625	182·0	
MRB9	05·12·2011	5:14 PM	19·2°c	500	0	99·285	158·0	
MRB10	07·12·2011	9:42 AM	21·0°c	500	0	99·40	170·0	
MRB11	08·12·2011	3:59 PM	20·6°	500	0	99·260	164·0	
MRB12	10·12·2011	10:40 AM	21·1°c	500	0	100·005	194·0	
MRB13	11·12·2011	10:10 AM	21·6°c	500	0	99·875	176·0	
MRB14	13·12·2011	10:10 AM	19·8°c	500	0	99·485	224·0	Pump stopped working for 24
MRB15	16·12·2011	9:00 AM	18·2°c	500	0	99·535	164·0	
MRB16	17·12·2011	11:12 AM	21·0°c	500	0	99·640	174·940	

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R

Conductivity

1	2	3	Effluent
0.007M	0.025M	0.011M	
0.005M	0.027M	0.001M	
0.001M	0.029M	3.1N	
1.1N	0.038M	4.7N	
1.5N	0.304	5.7N	
0.1N	0.036M	4.5N	
5.8N	0.017M	0.016M	
5.2N	0.014M	5.0N	
1.9N	0.035M	3.7N	
0.7N	0.023M	6.7N	
0.9N	0.029M	7.5N	
0.9N	0.016M	5.9N	
0.4N	0.015M	6.5N	
0.02N	0.014M	6.0N	
0.01N	0.008M	6.1N	
0.01N	0.006M	5.2N	

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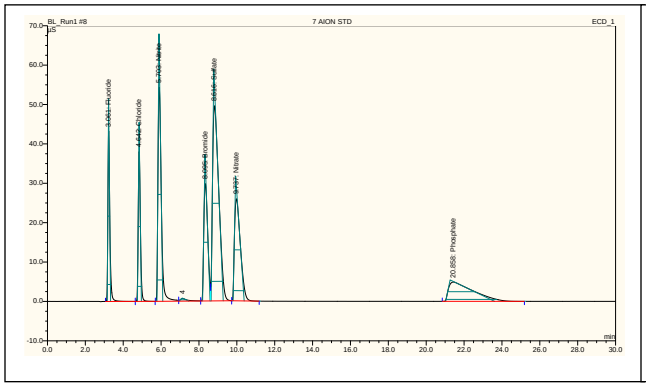
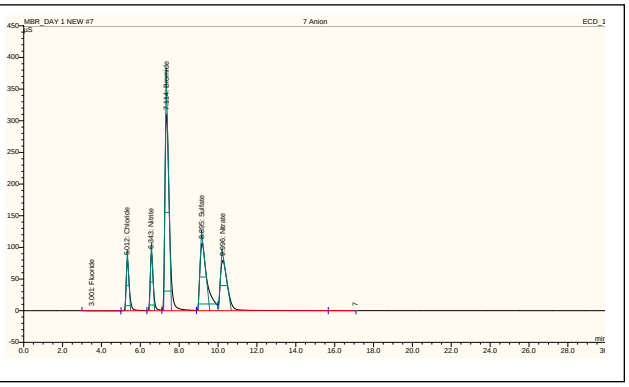
APPENDIX C. CHROMATOGRAPH

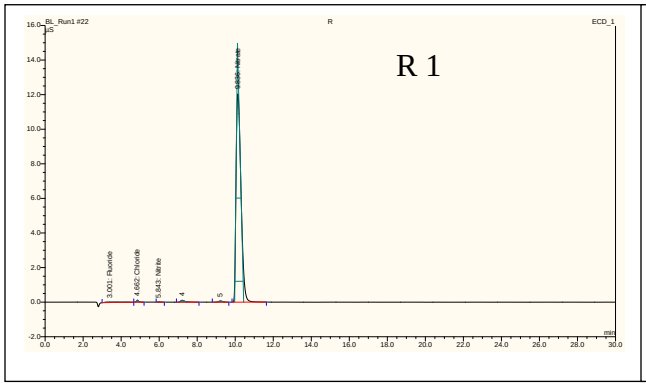
Control System	Bacterial System

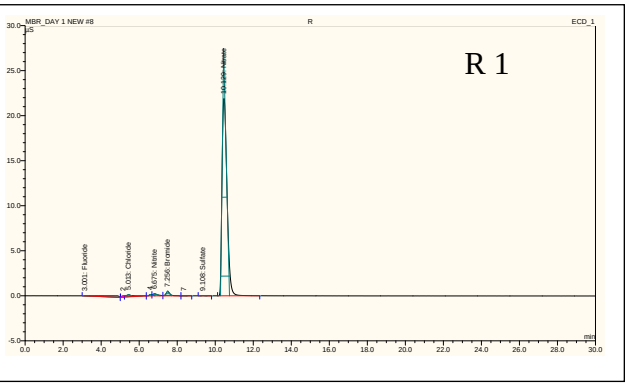
Seven anion standards

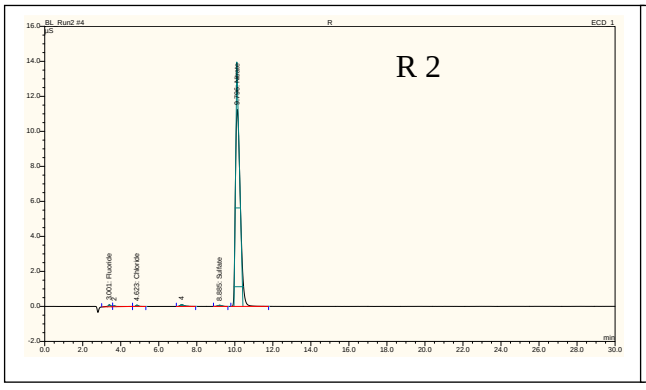


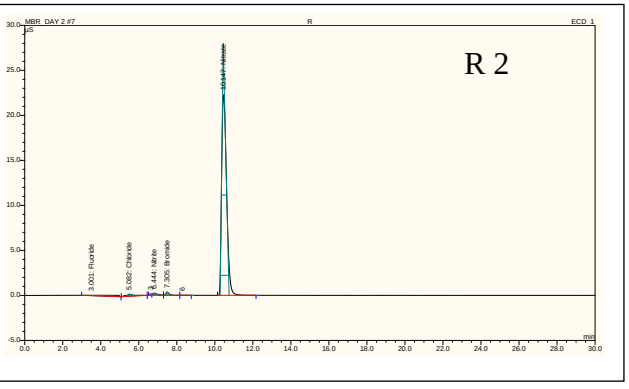


Reservoir solution



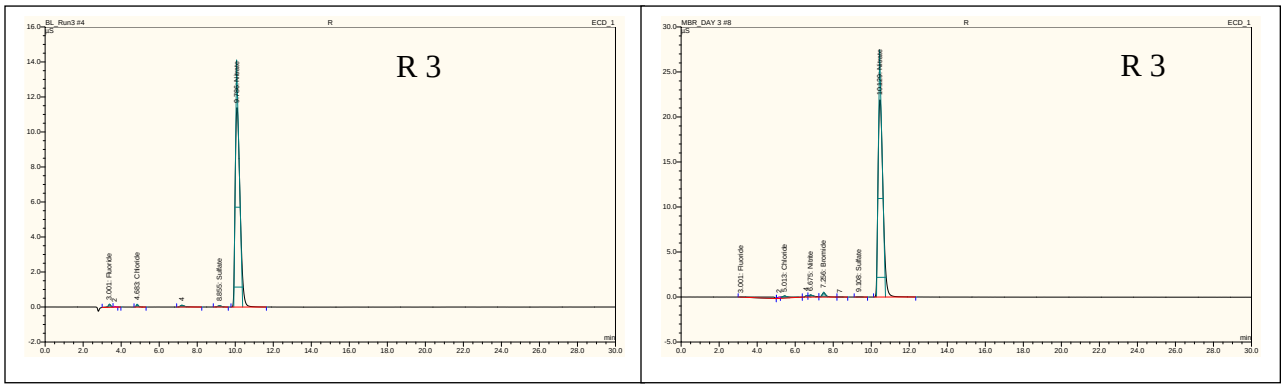




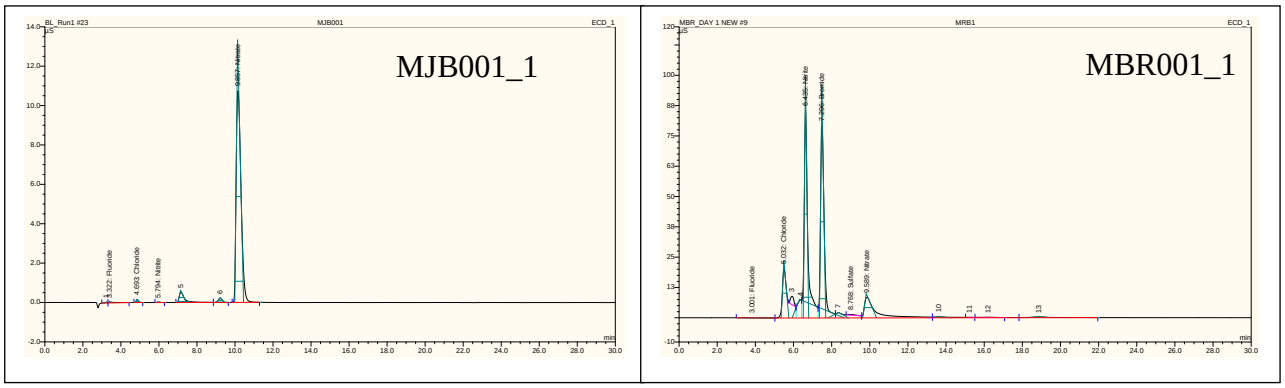


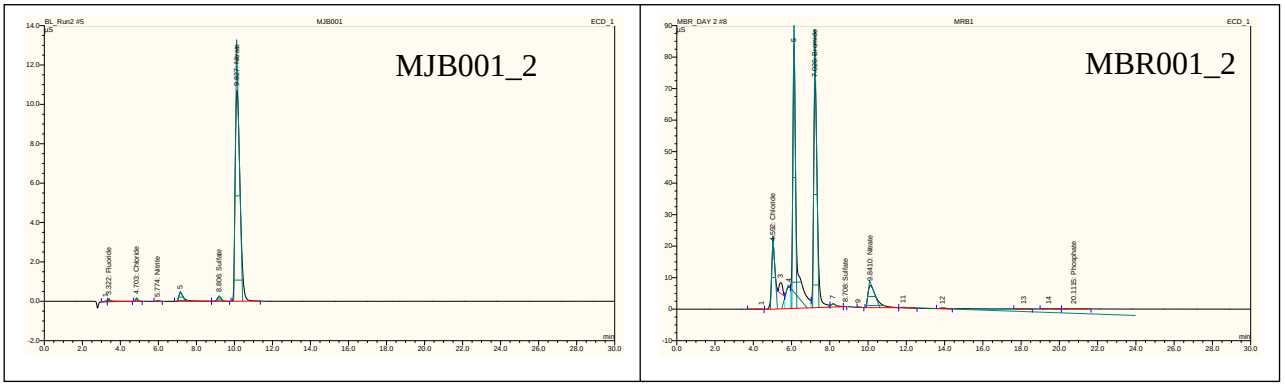
Control System	Bacterial System

Reservoir solution



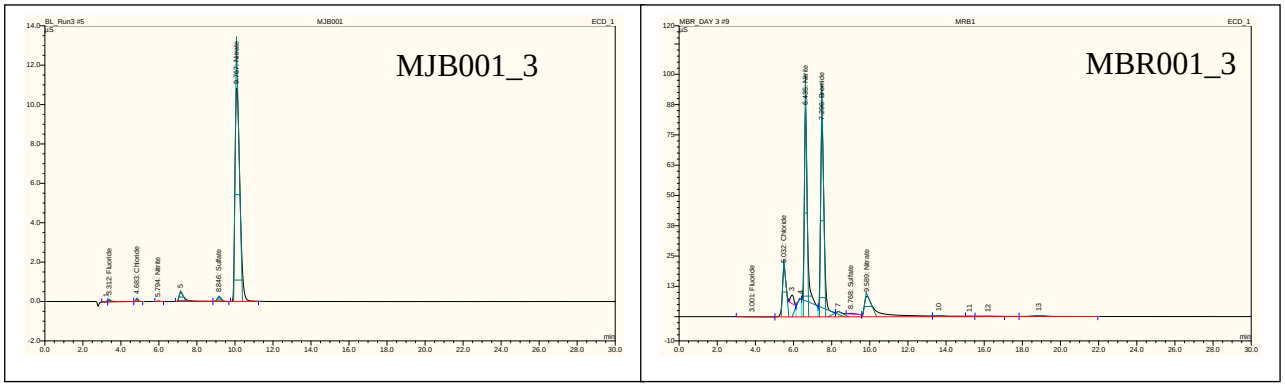
Effluent solution: Sample 1



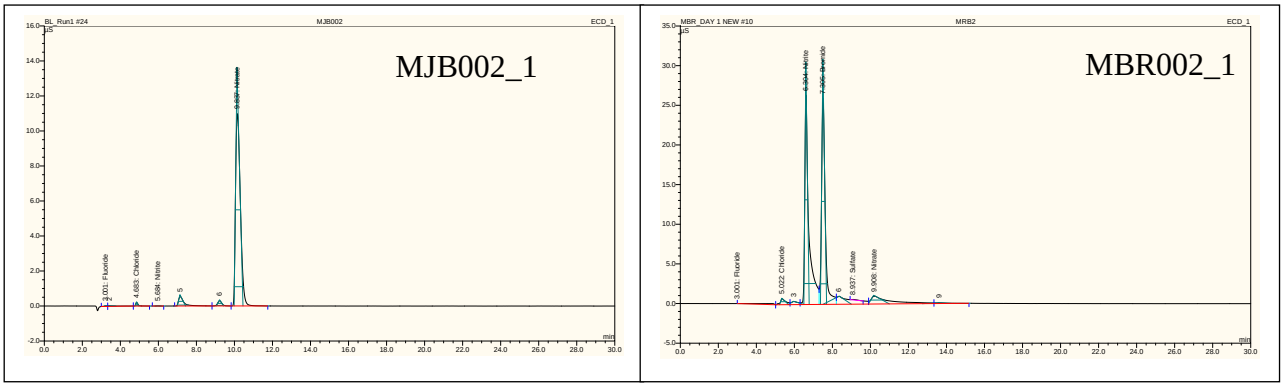


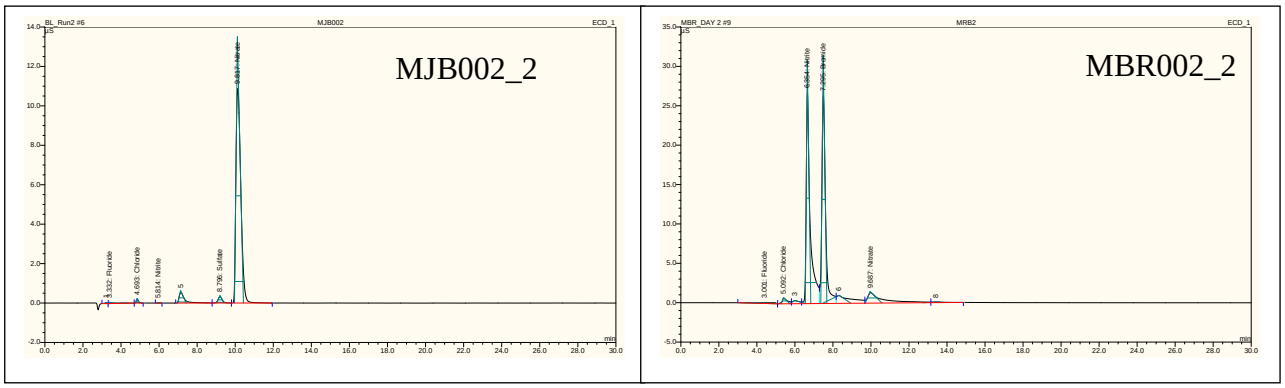
Control System	Bacterial System

Sample 1



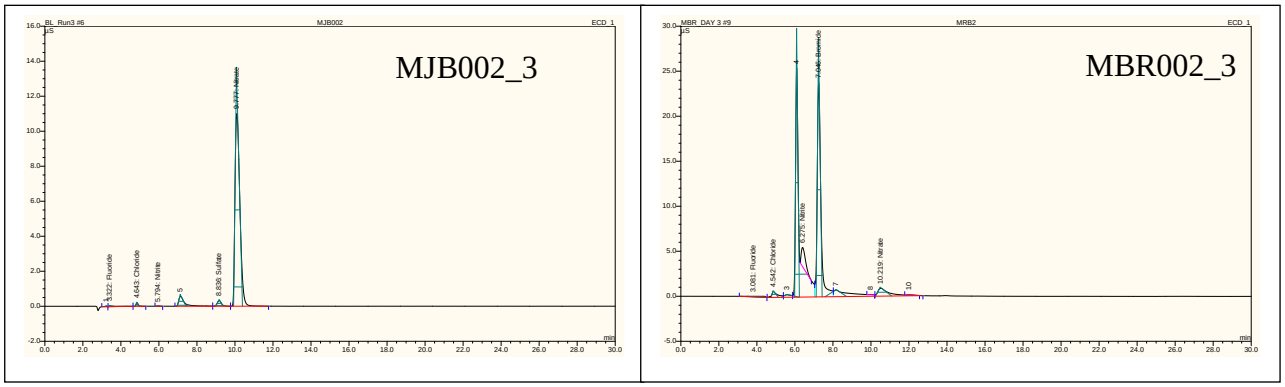
Sample 2



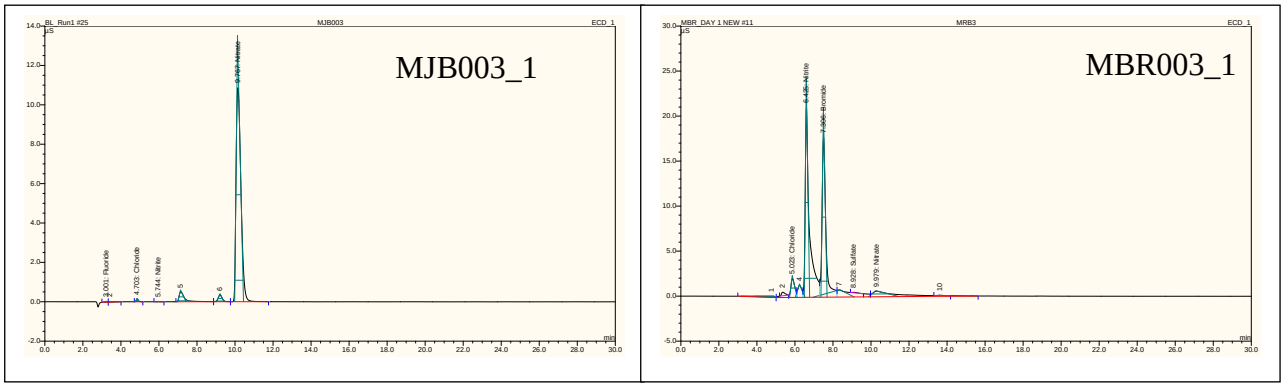


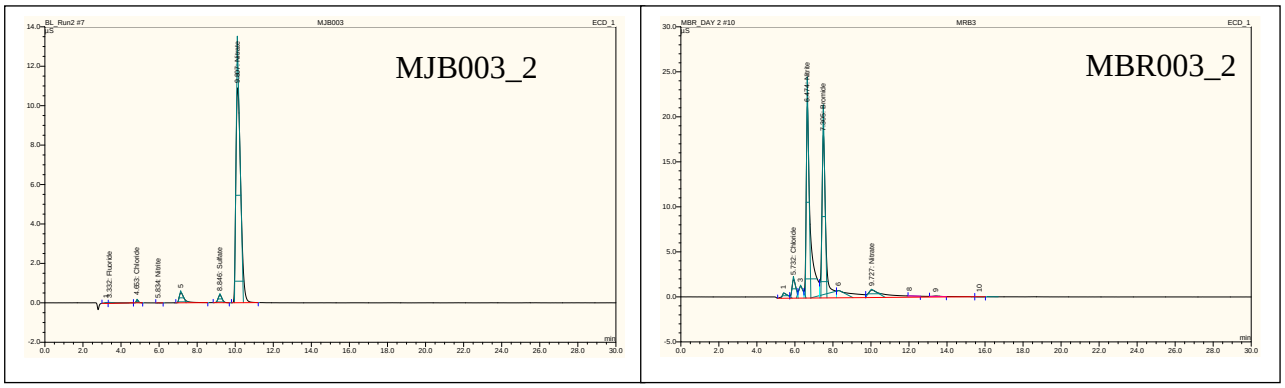
Control System	Bacterial System

Sample 2



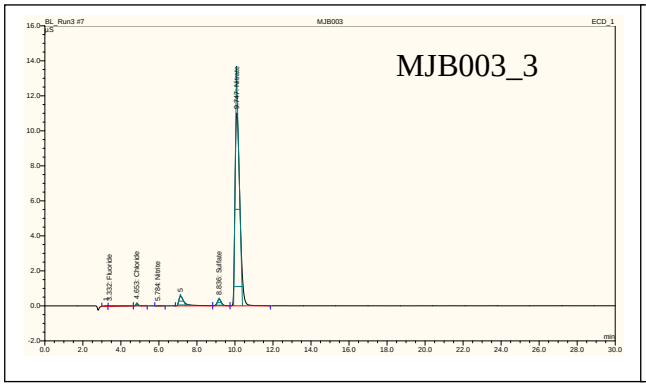
Sample 3

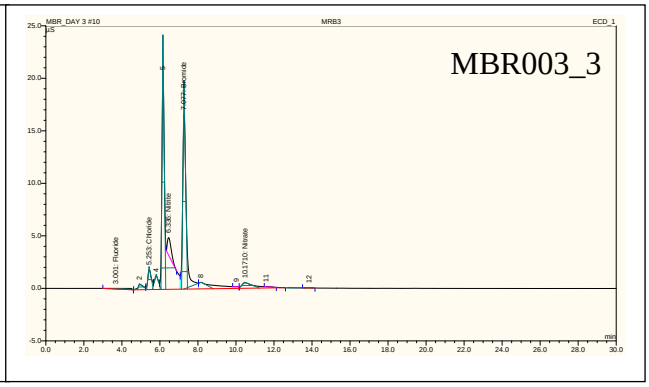




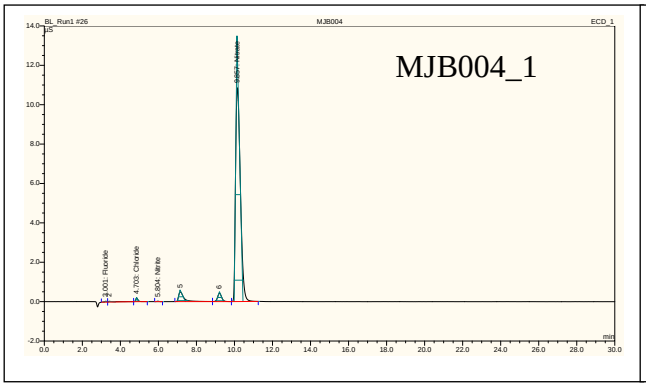
Control System	Bacterial System

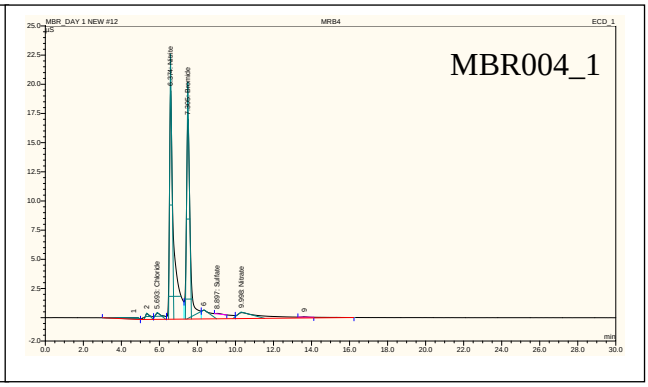
Sample 3

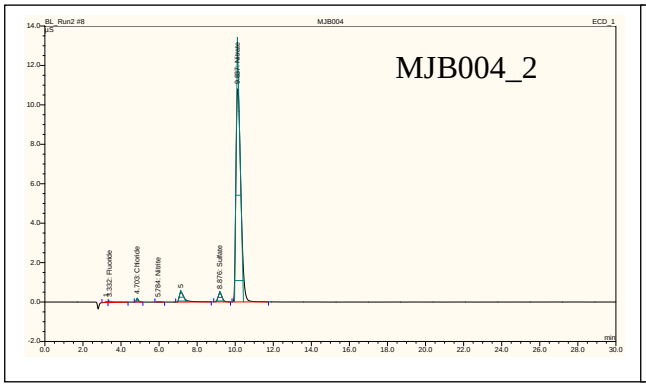


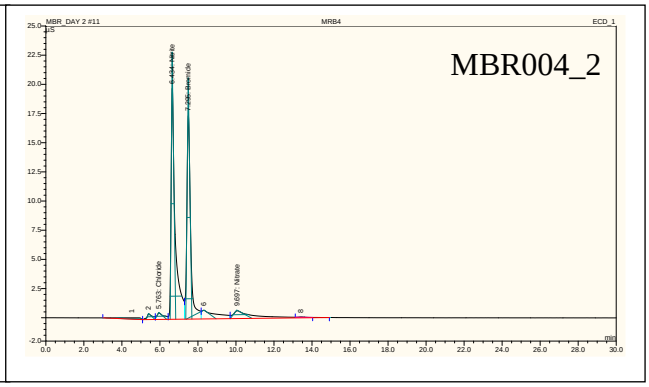


Sample 4



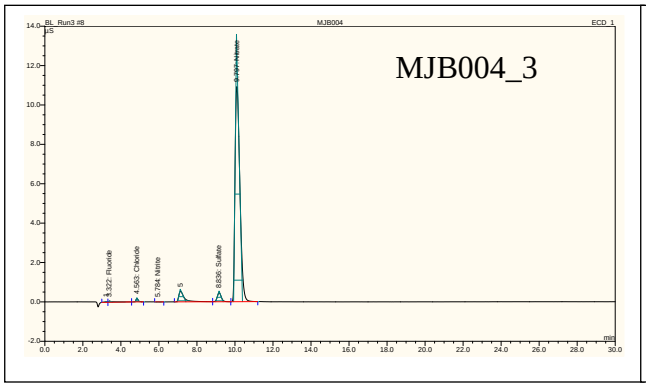


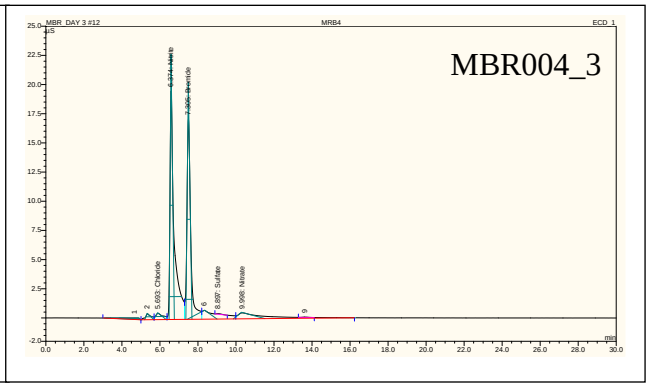




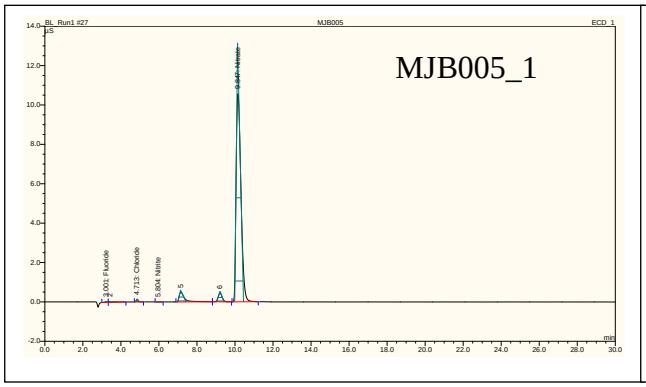
Control System	Bacterial System

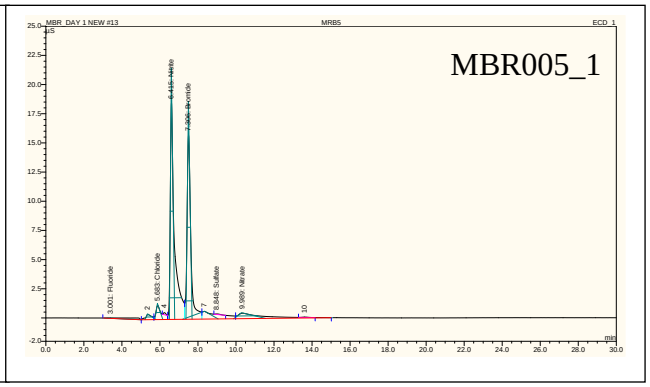
Sample 4

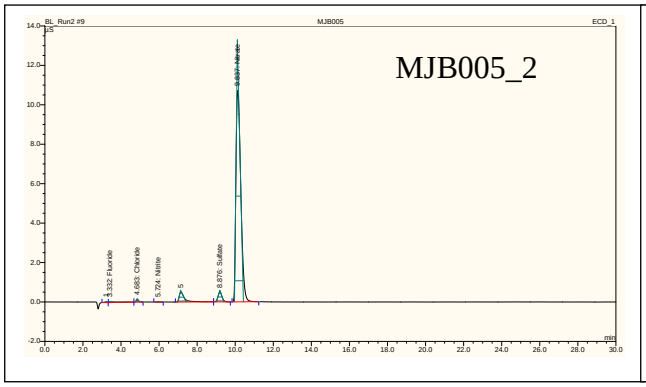


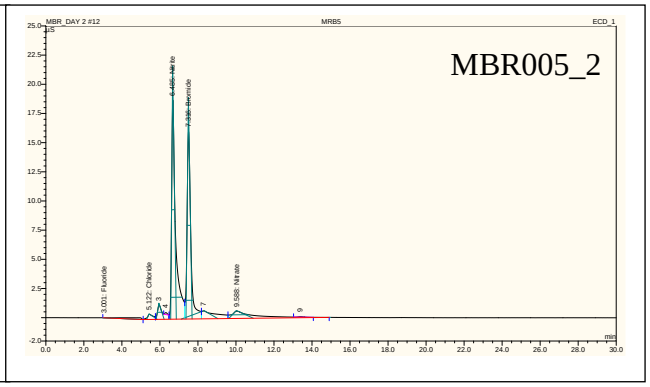


Sample 5



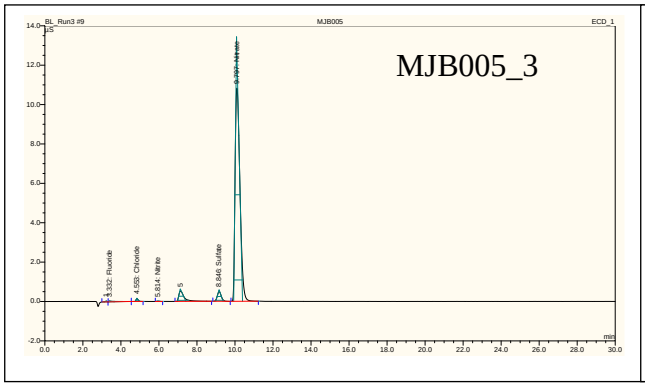


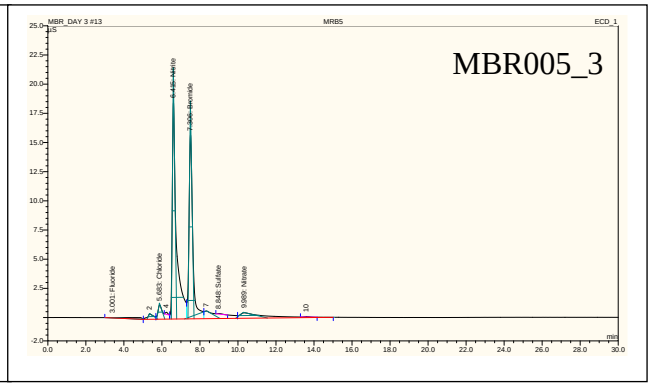




Control System	Bacterial System

Sample 5





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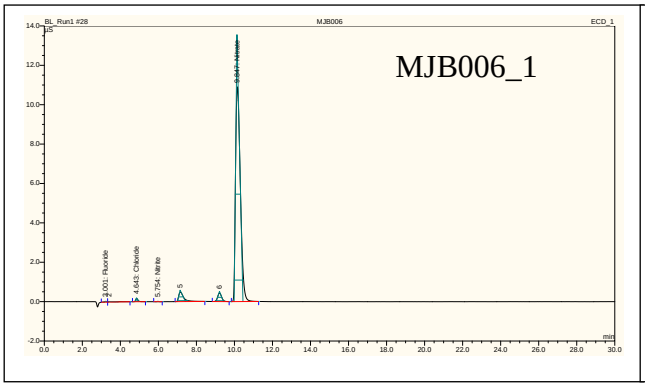
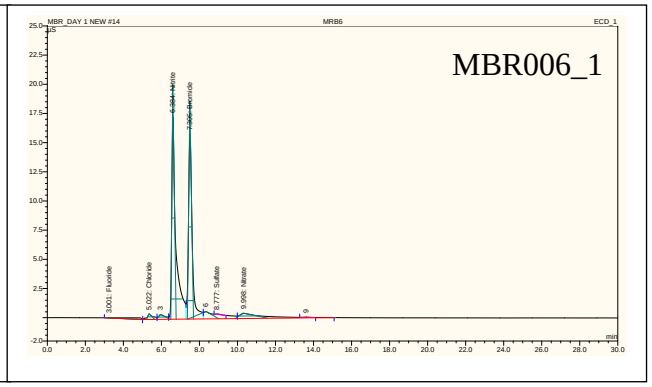
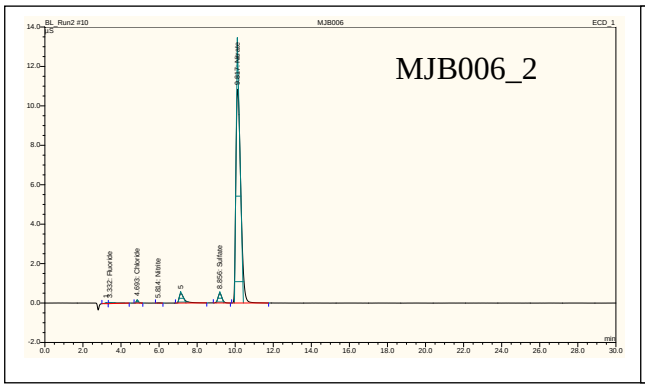
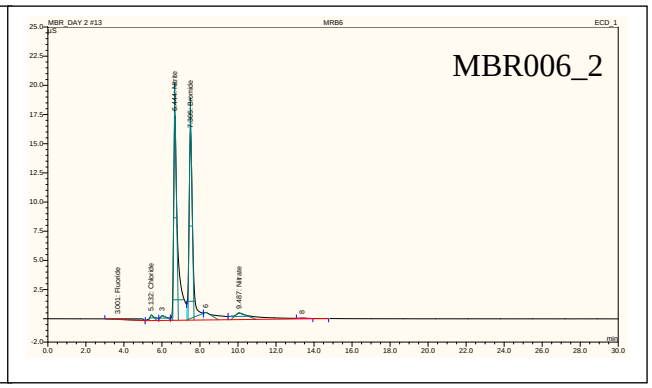


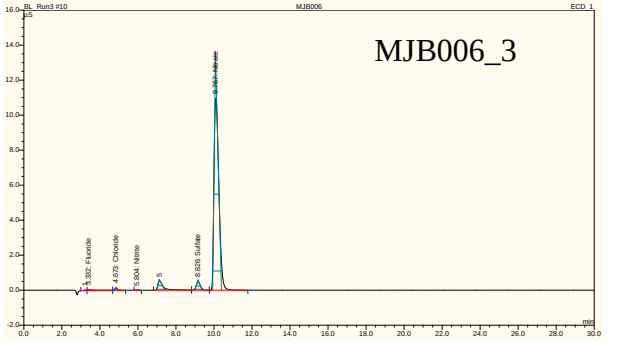


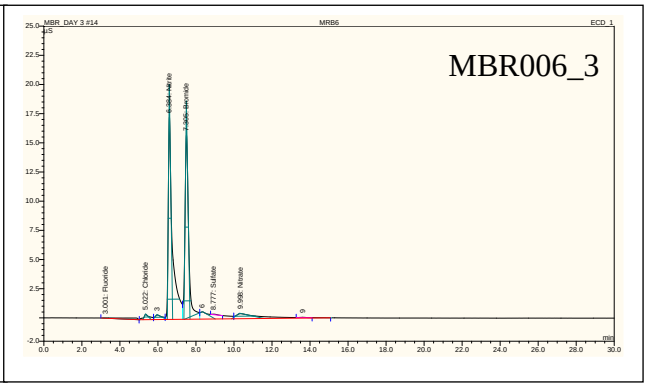




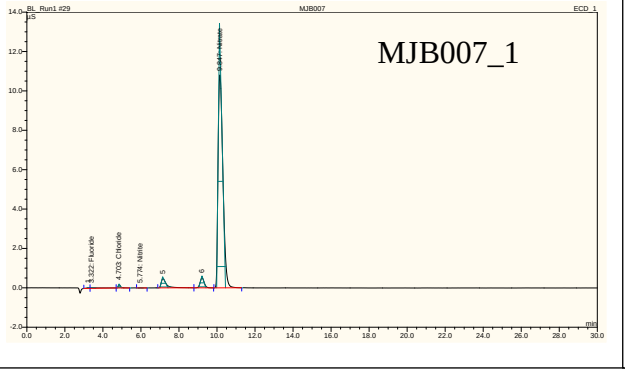
Control System	Bacterial System

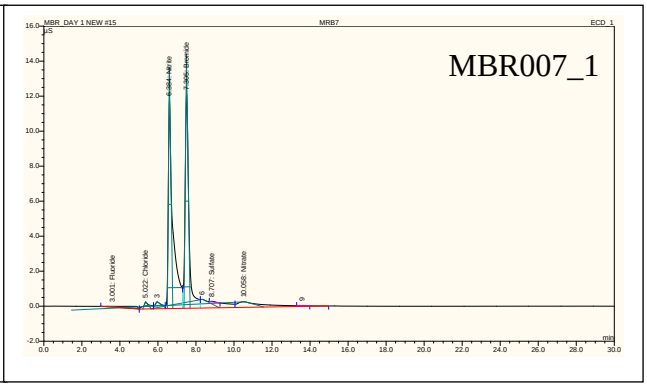
Sample 6

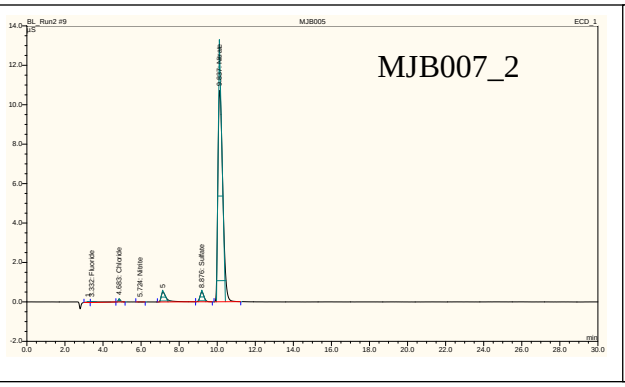


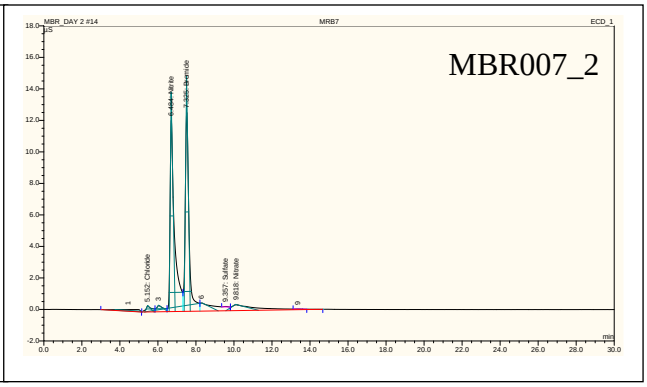


Sample 7



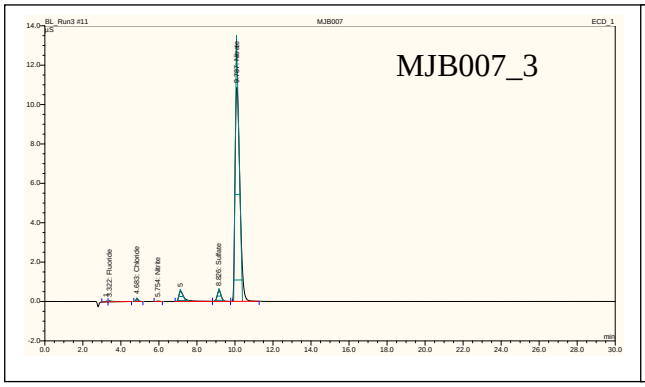


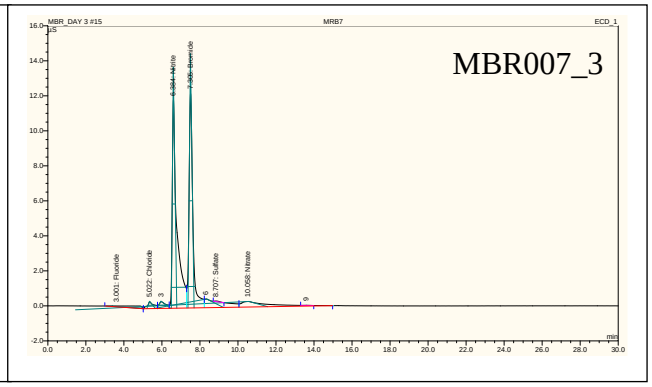




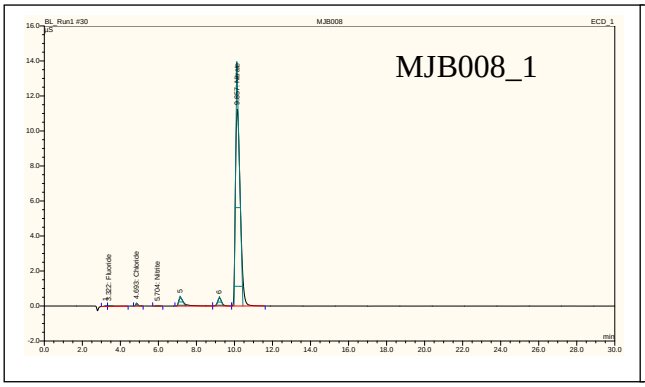
Control System	Bacterial System

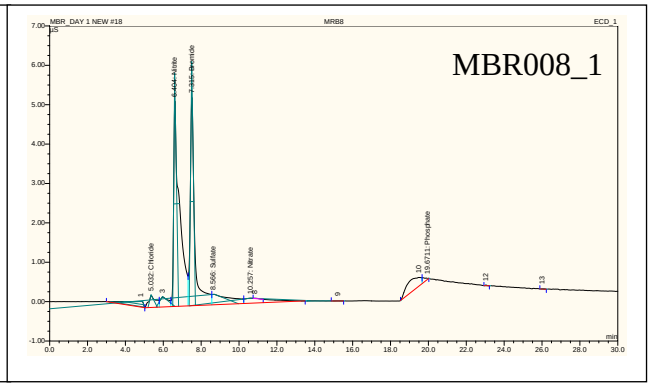
Sample 7

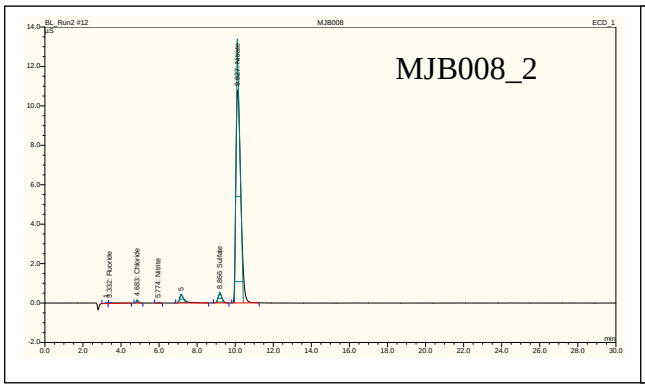


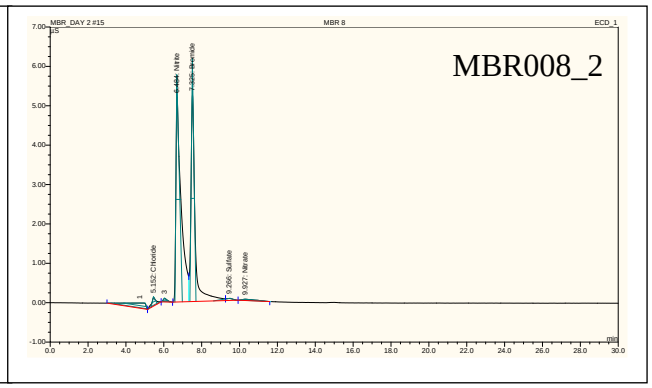


Sample 8



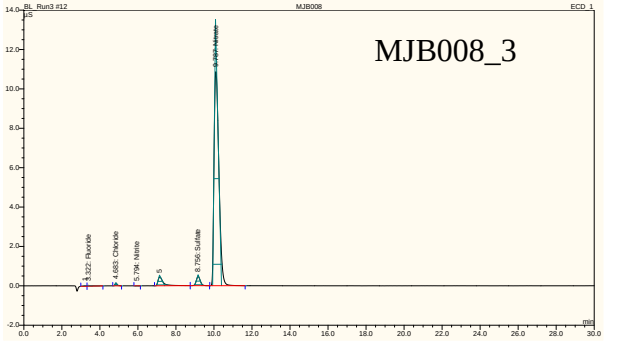


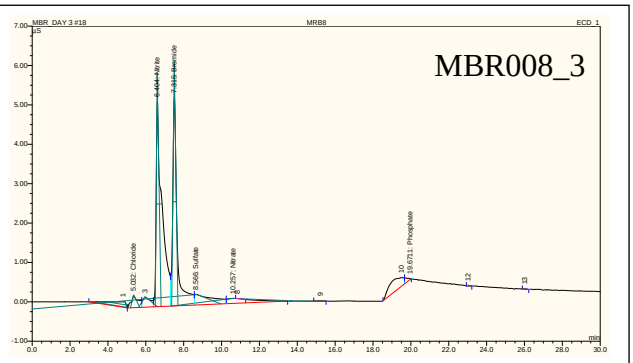




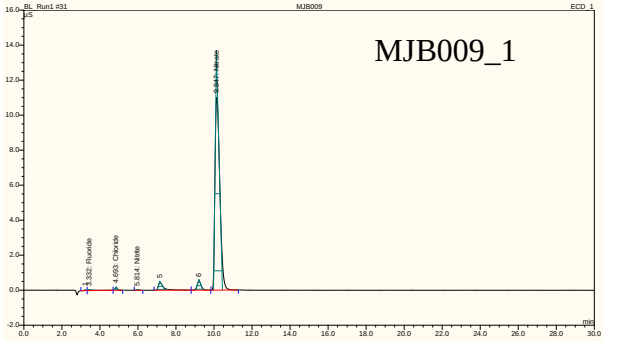
Control System	Bacterial System

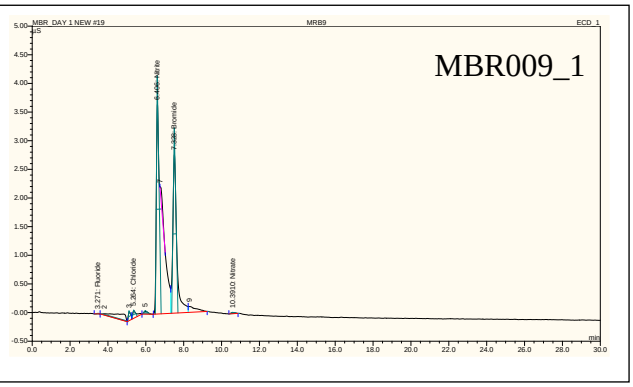
Sample 8

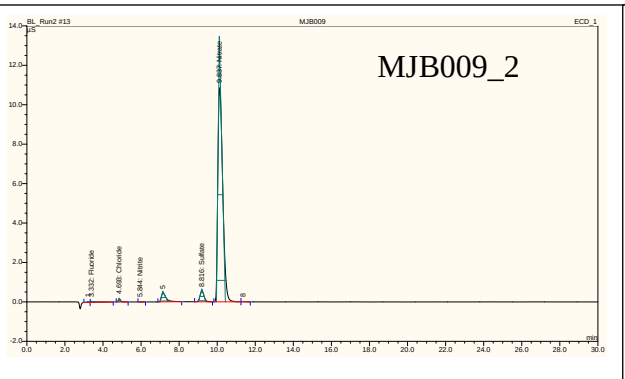


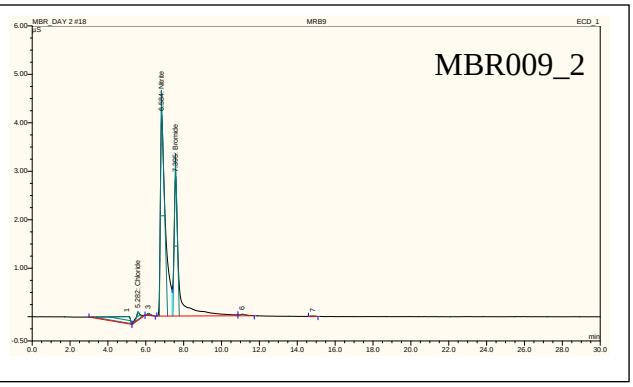


Sample 9



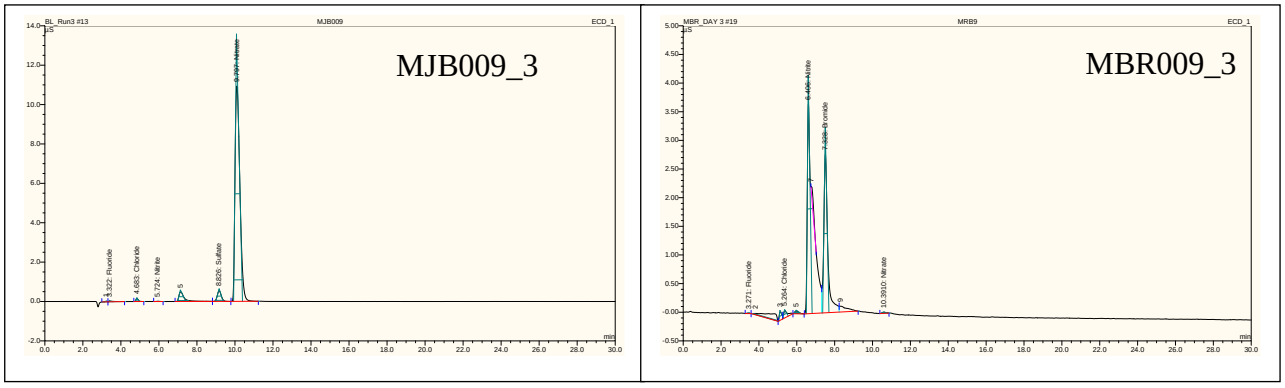




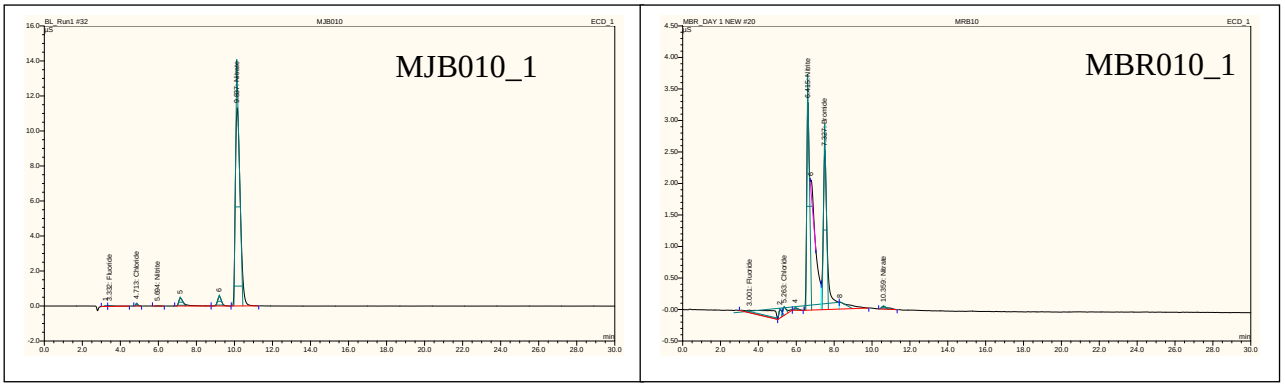


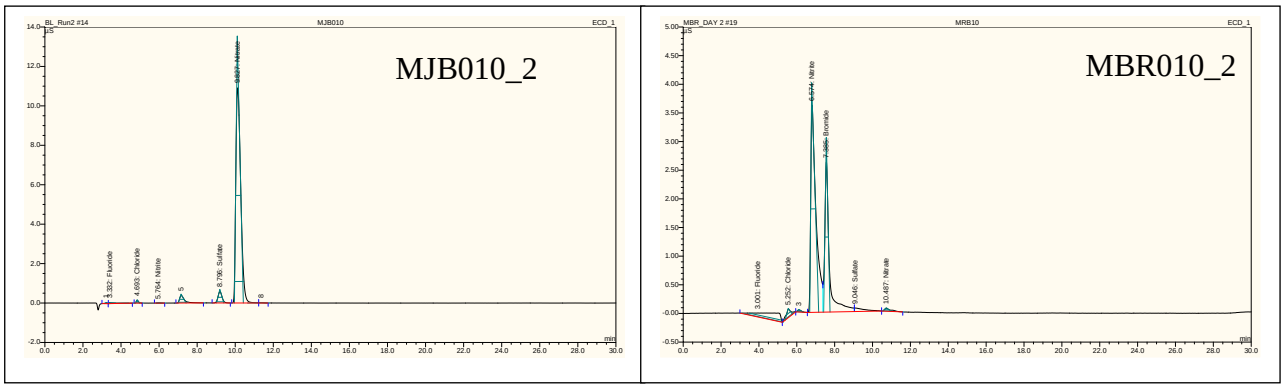
Control System	Bacterial System

Sample 9



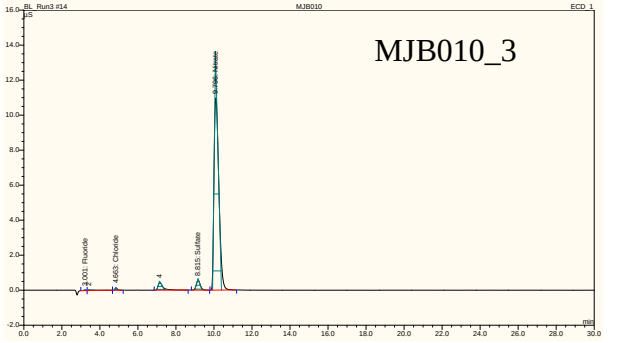
Sample 10

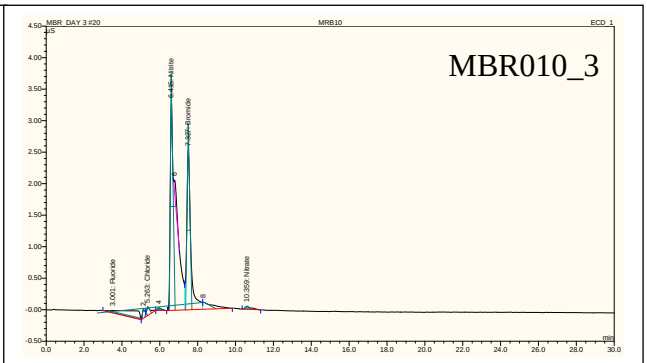




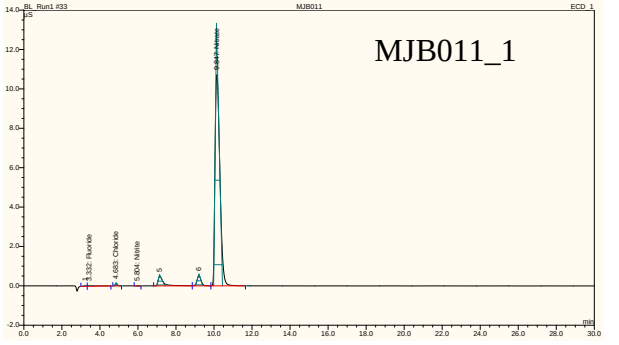
Control System	Bacterial System

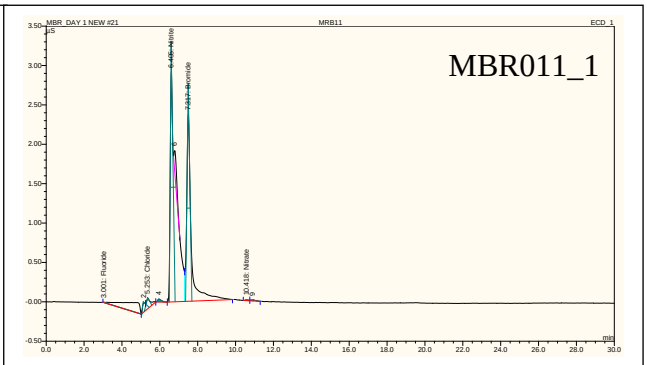
Sample 10

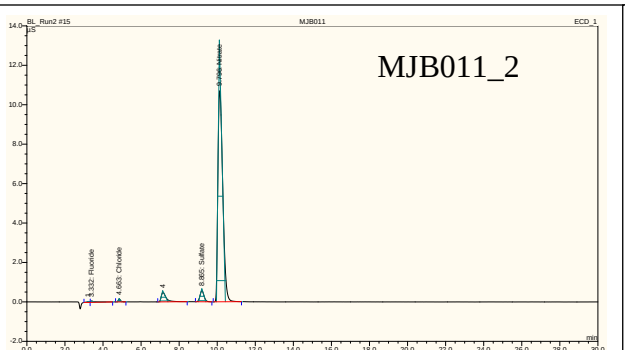


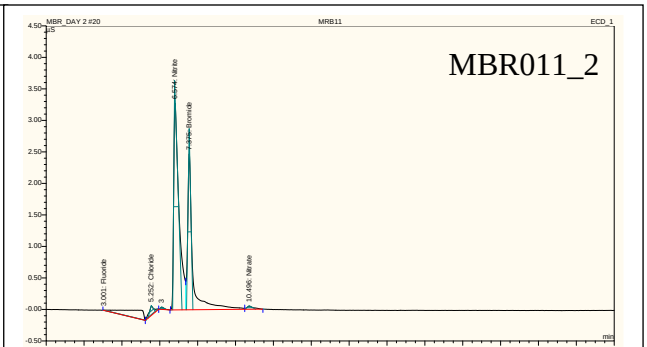


Sample 11



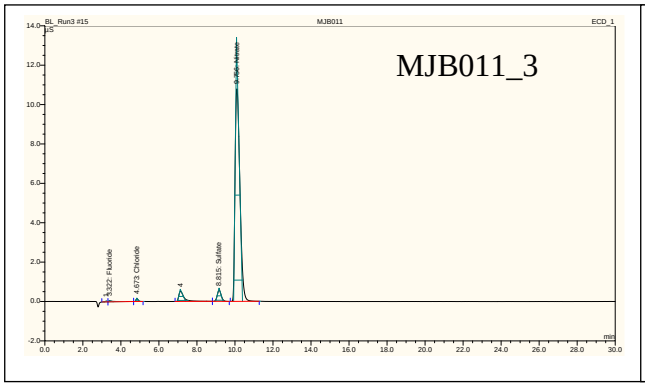


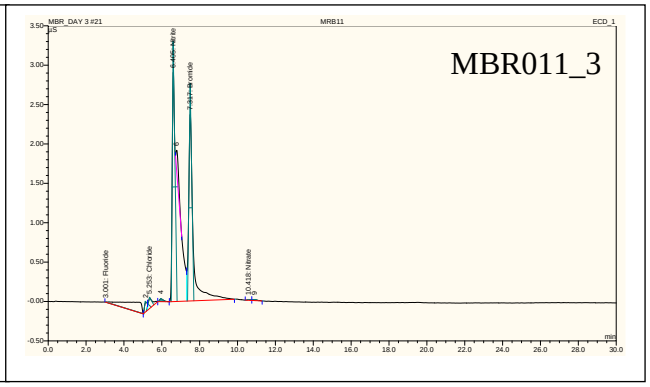




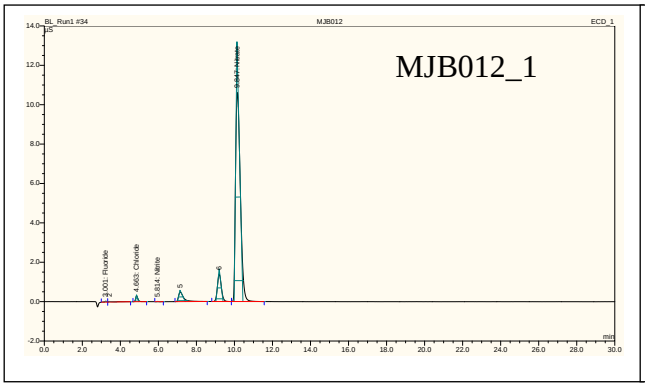
Control System	Bacterial System

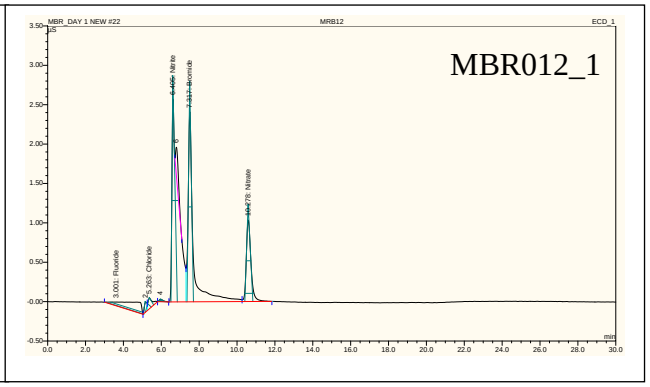
Sample 11

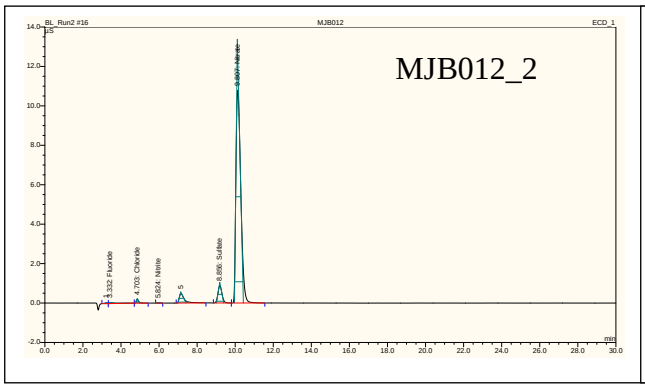


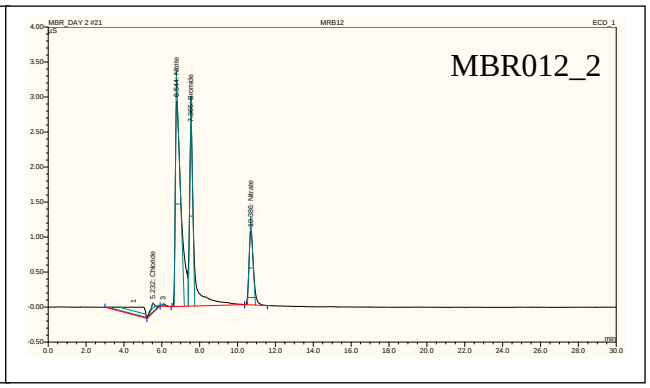


Sample 12



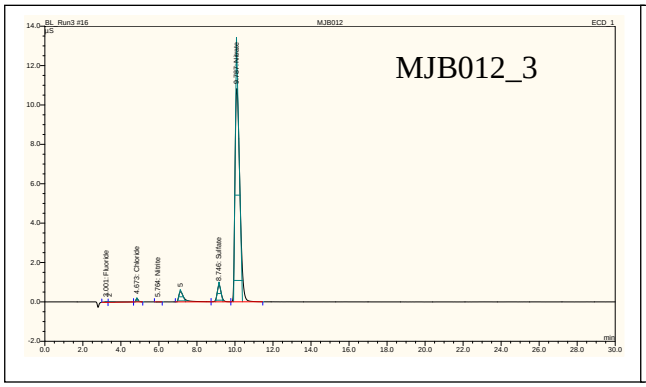


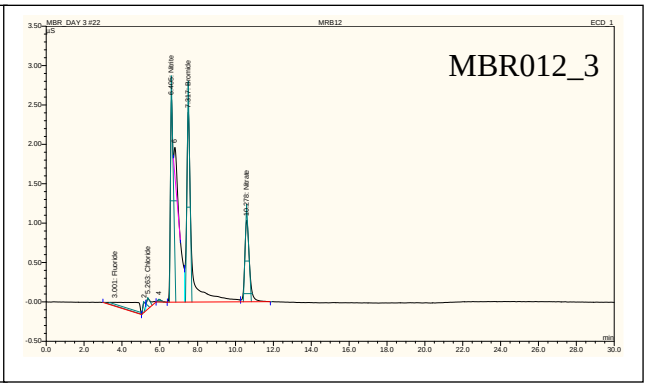




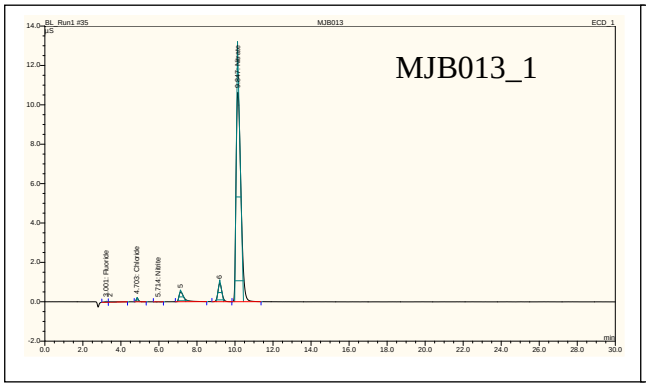
Control System	Bacterial System

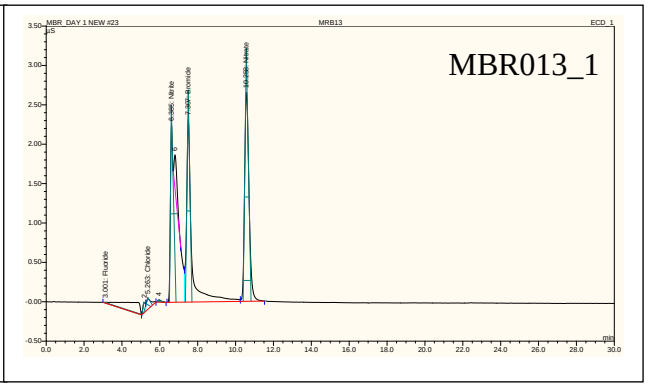
Sample 12

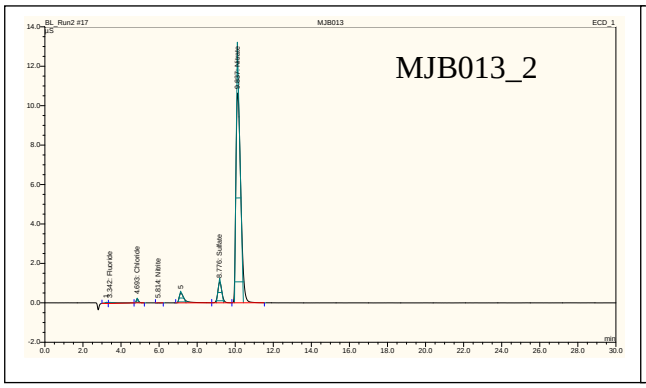


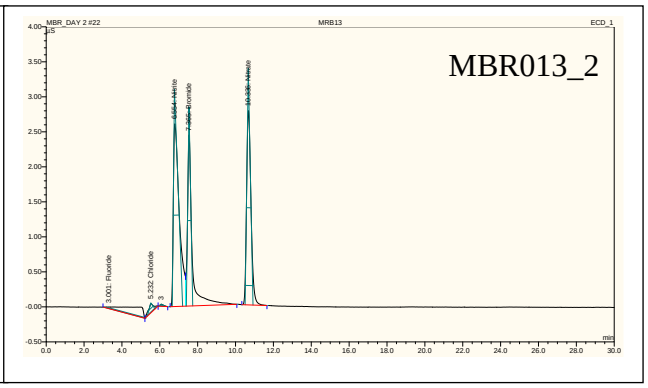


Sample 13



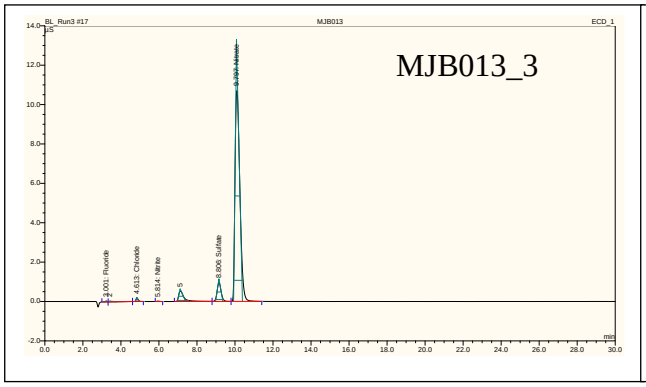


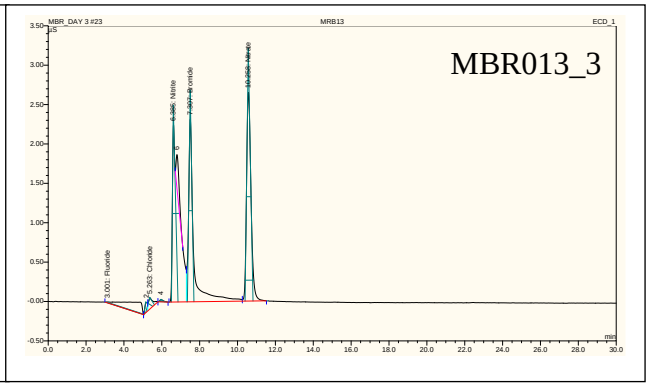




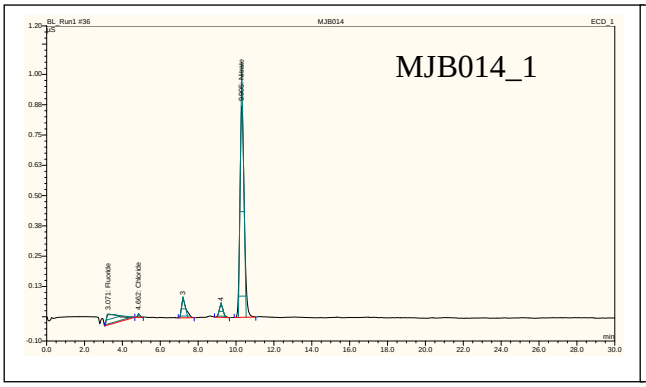
Control System	Bacterial System

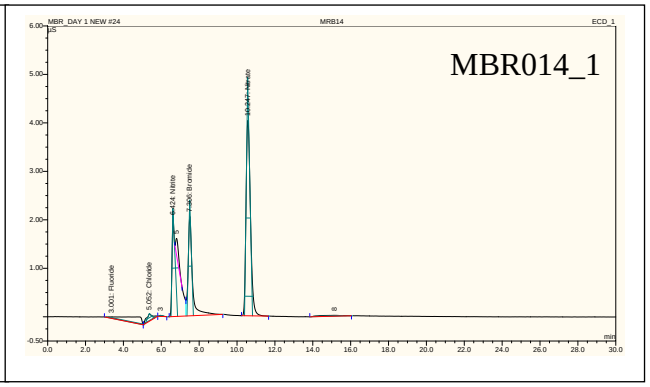
Sample 13

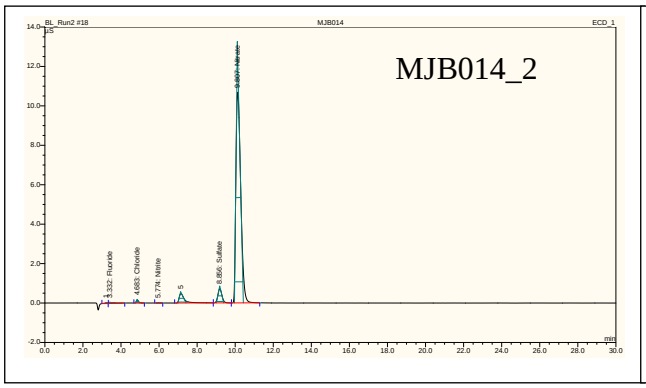


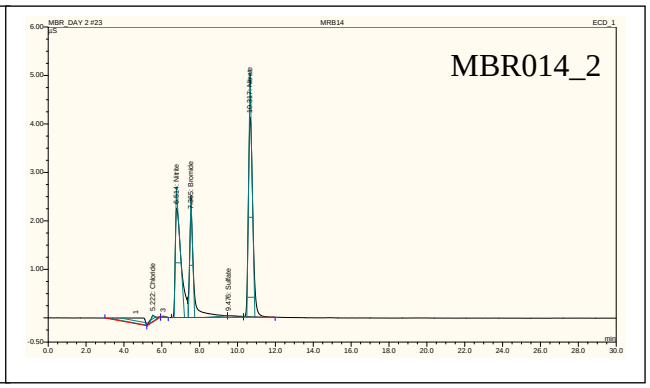


Sample 14



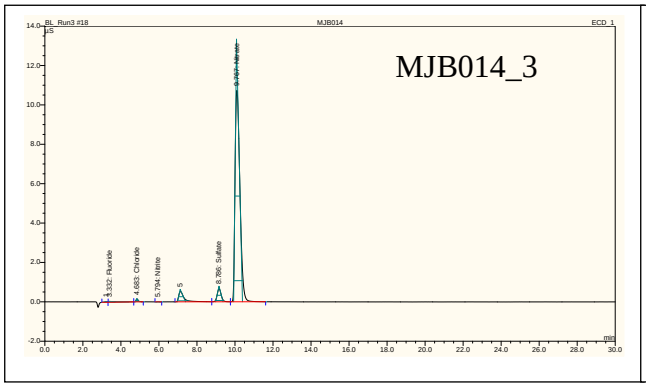


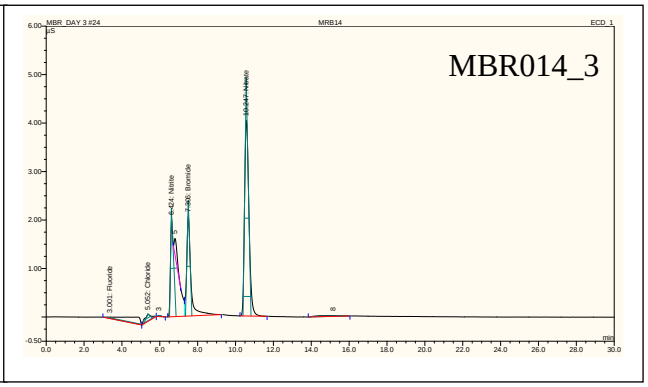




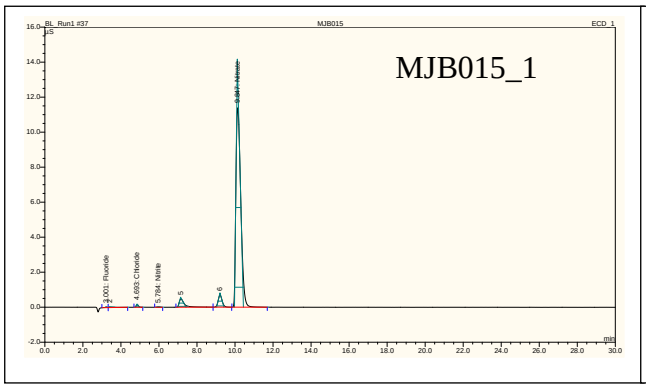
Control System	Bacterial System

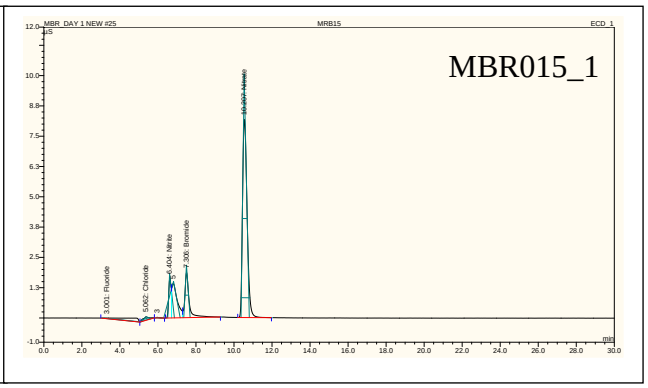
Sample 14

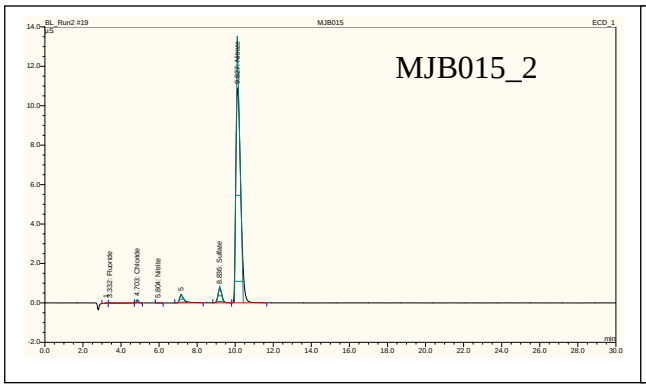


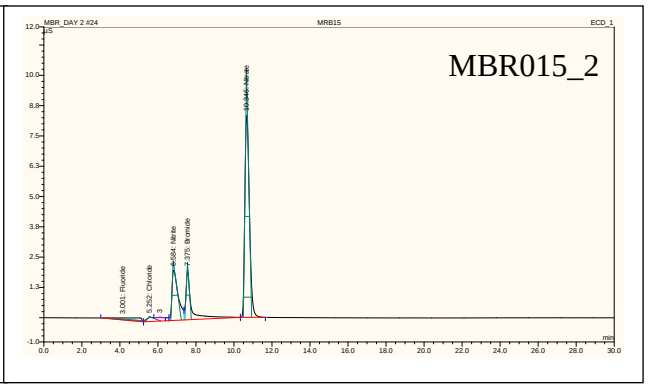


Sample 15



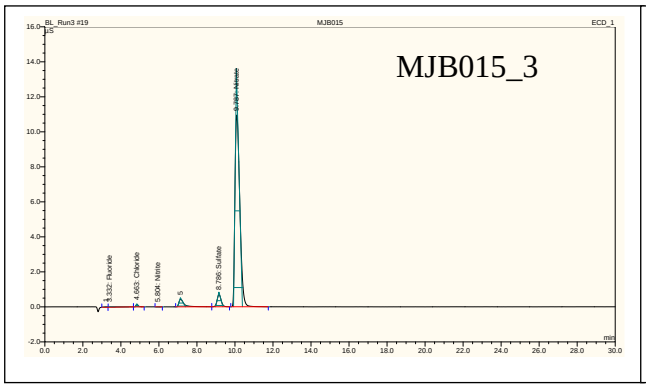


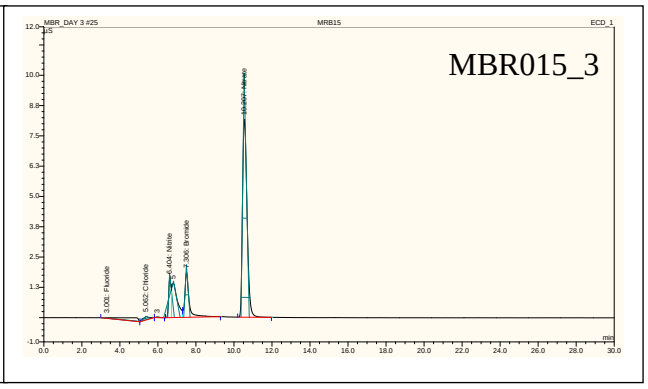




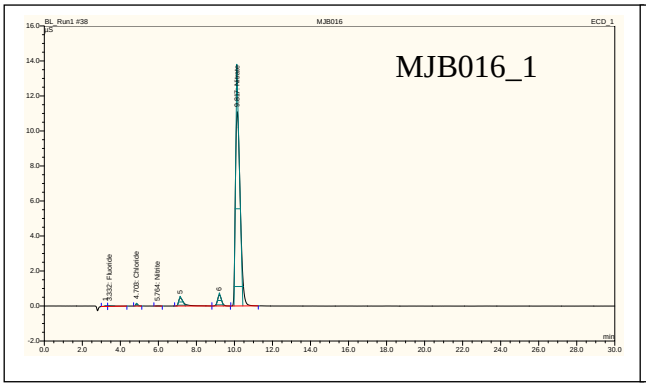
Control System	Bacterial System

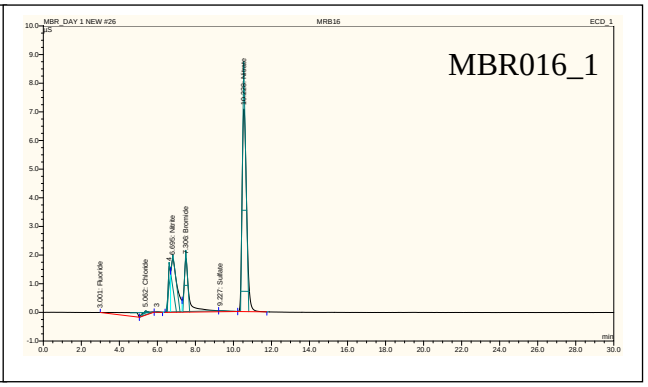
Sample 15

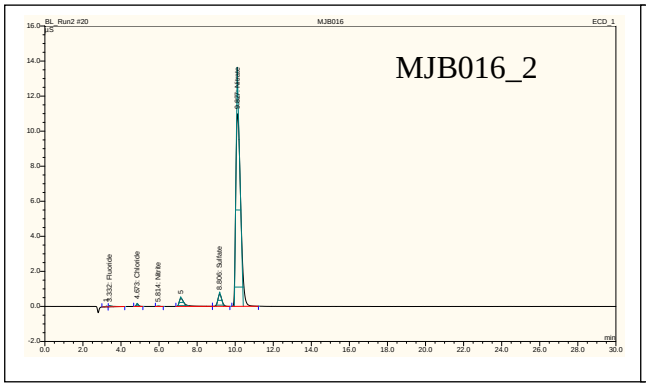


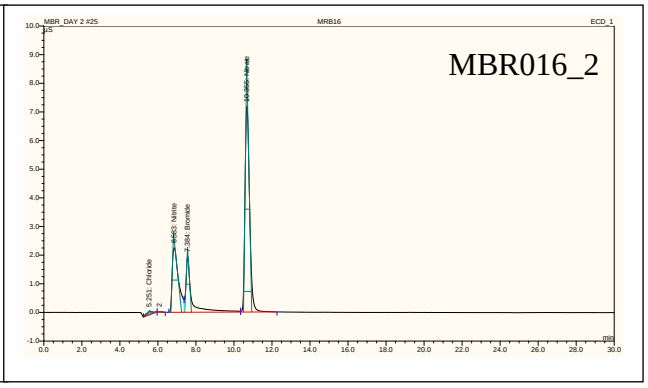


Sample 16



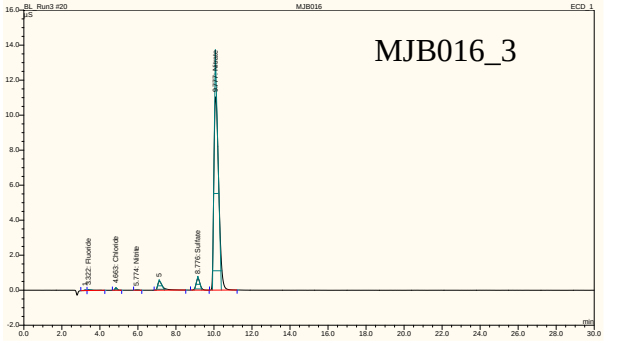


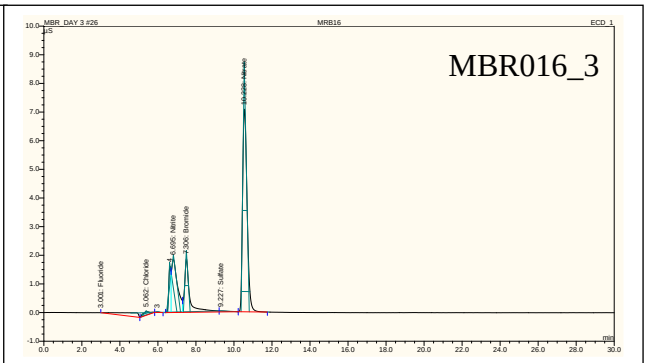




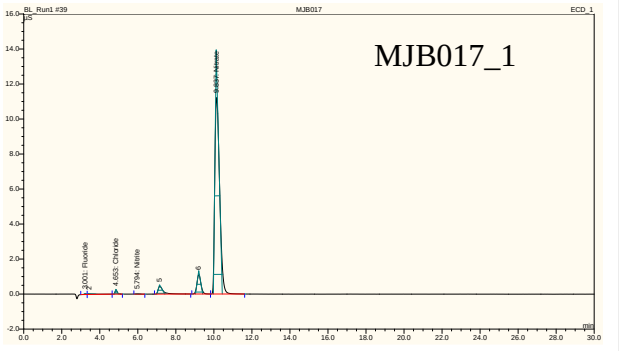
Control System	Bacterial System

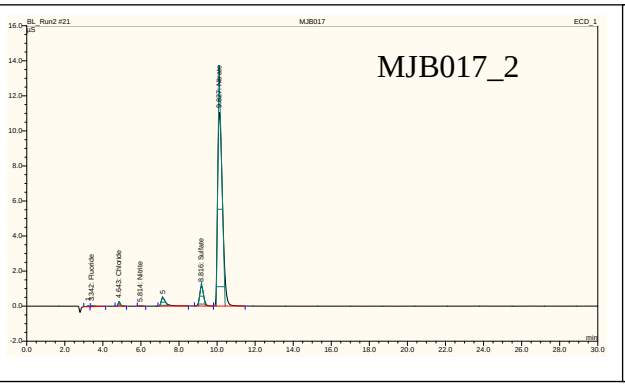
Sample 16





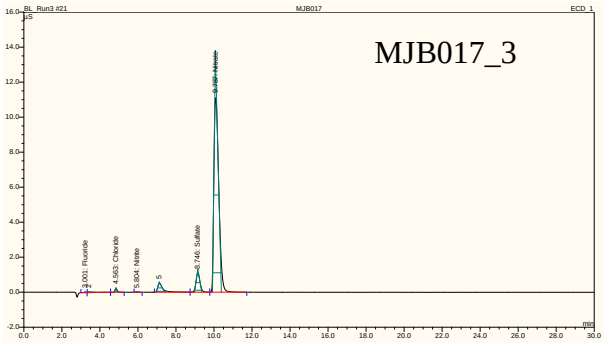
Sample 17



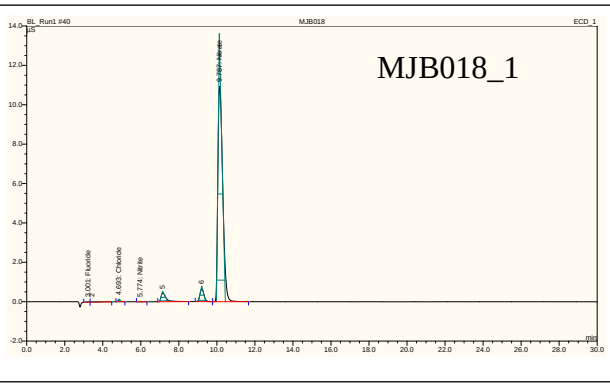


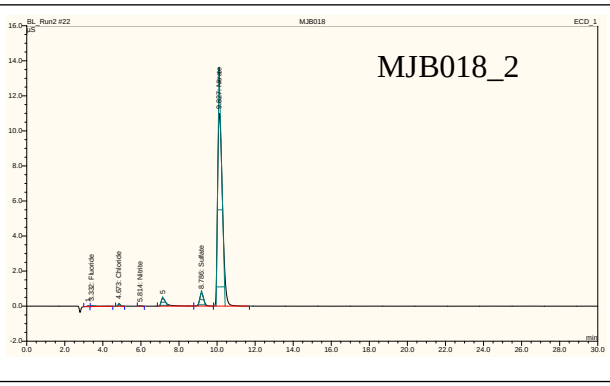
Control System	Bacterial System

Sample 17



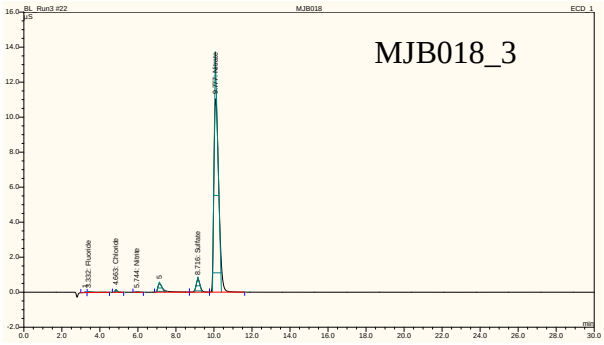
Sample 18





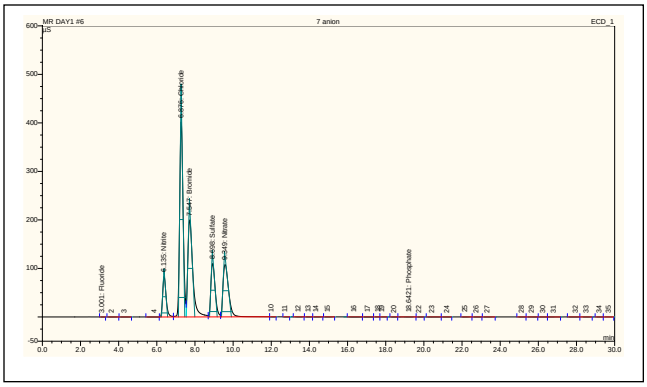
Control System	Bacterial System

Sample 18

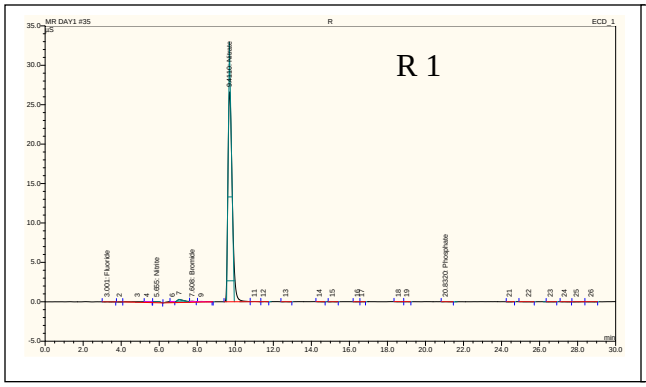


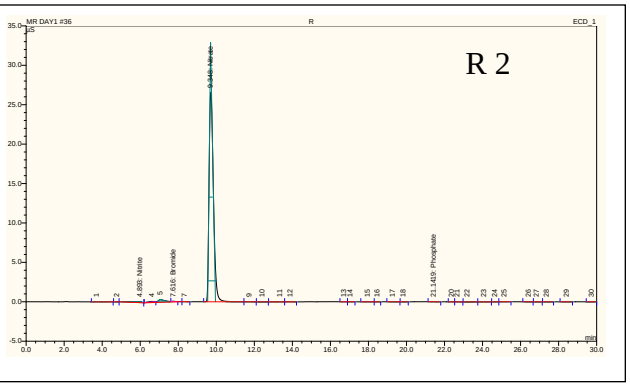
Chromatographs of Repeated Experiment using Bacteria

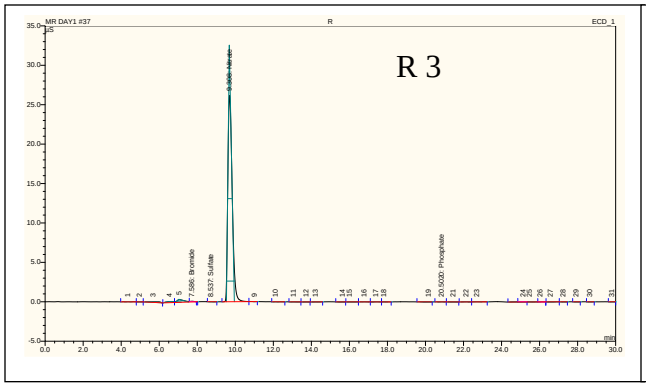
Seven anion standards



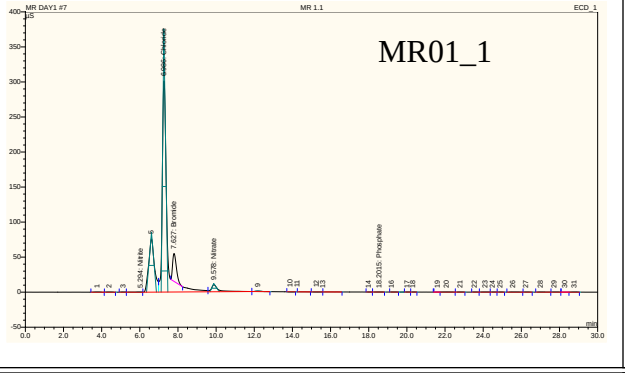
Reservoir solution

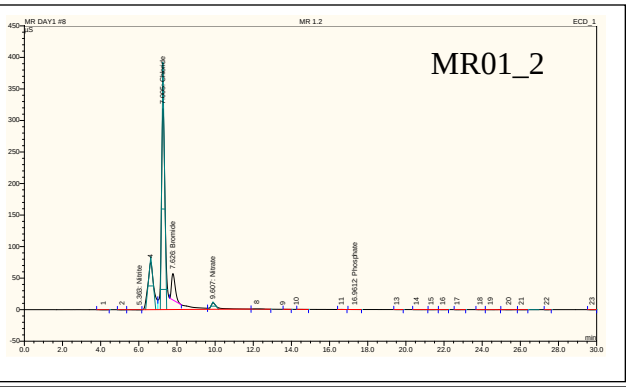


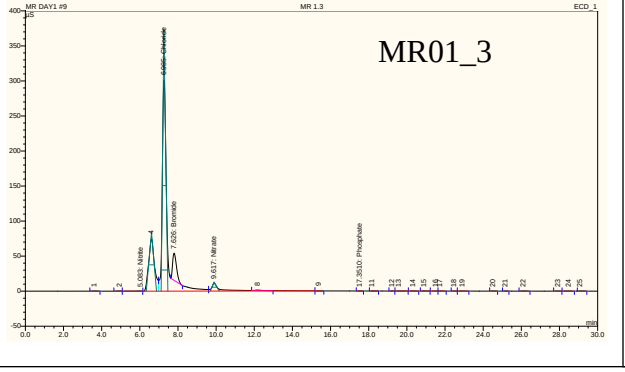




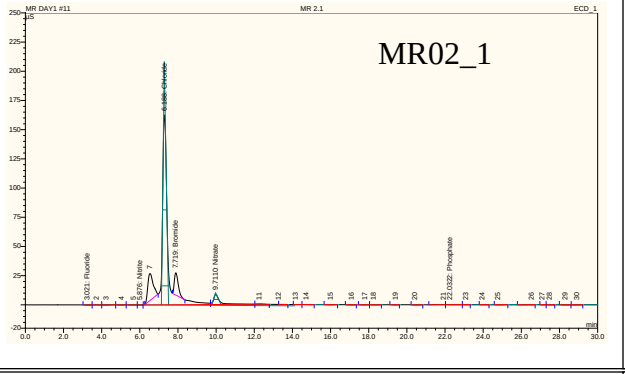
Sample 1

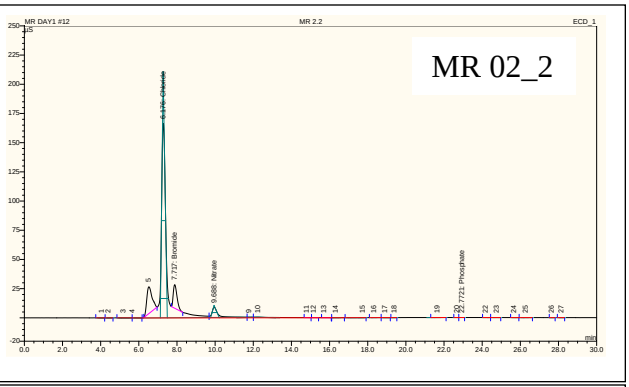


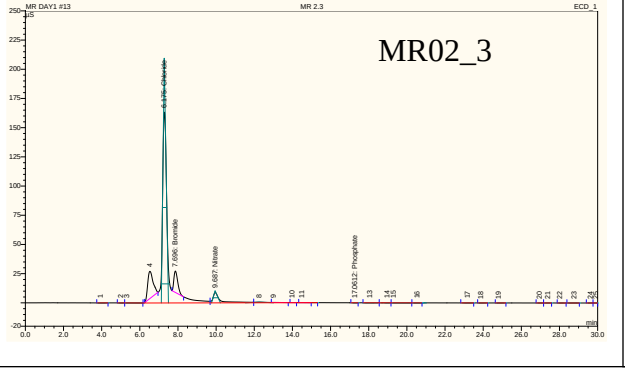




Sample 2







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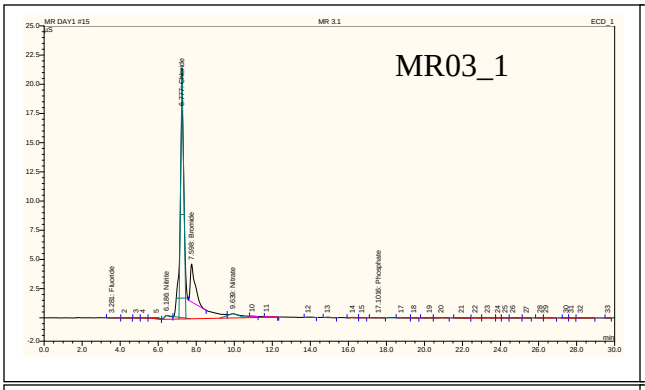
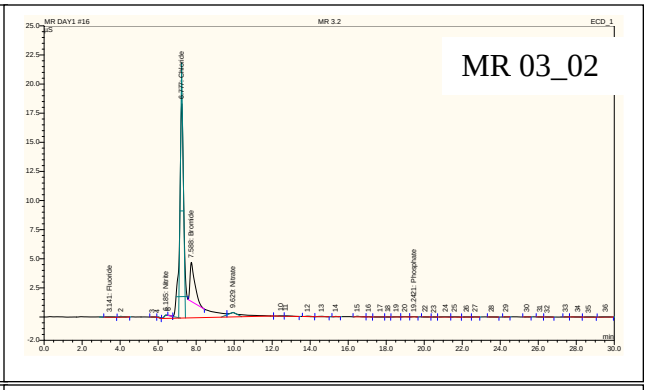
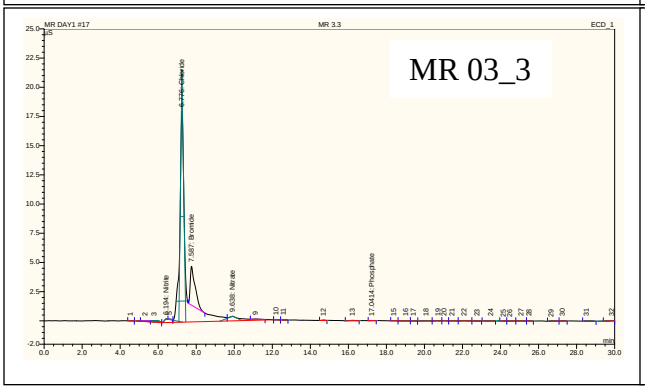
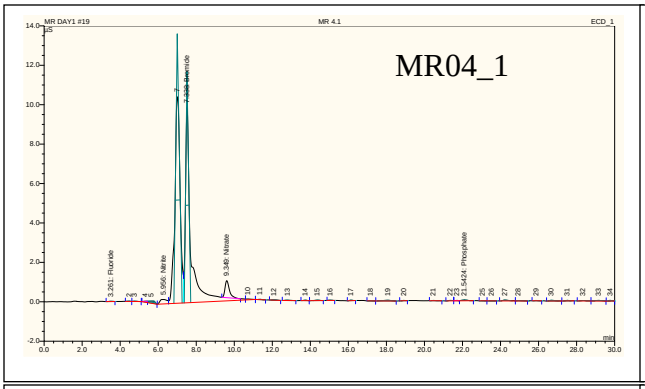
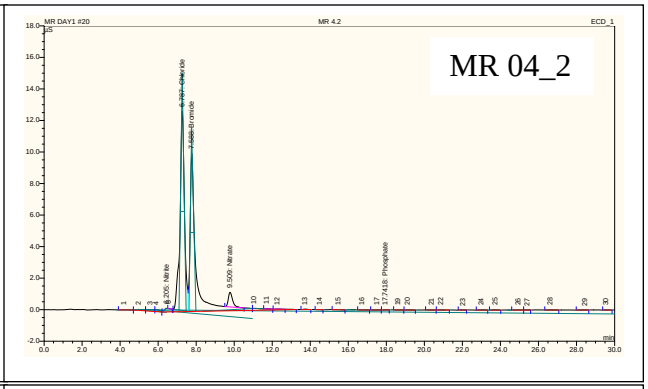


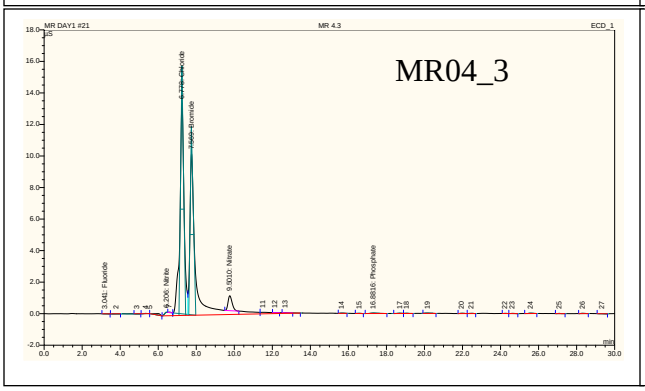




Sample 4







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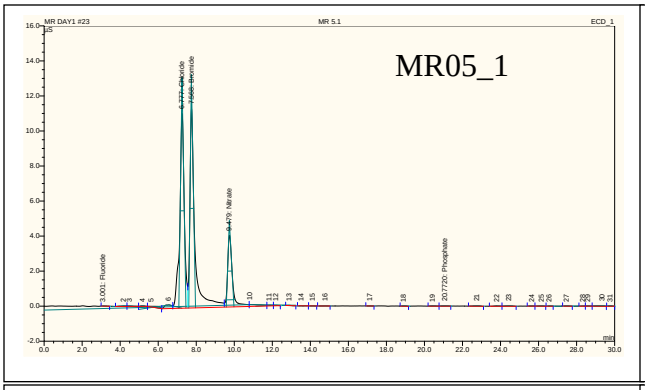
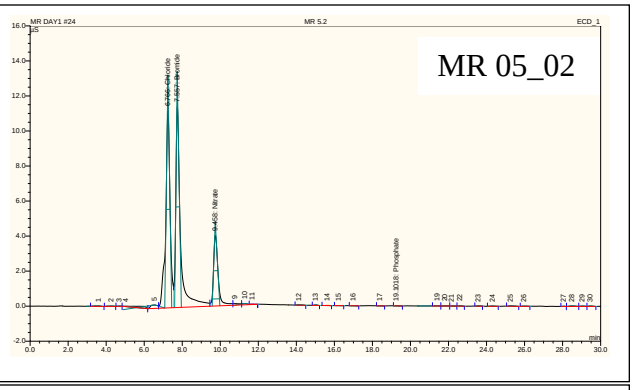
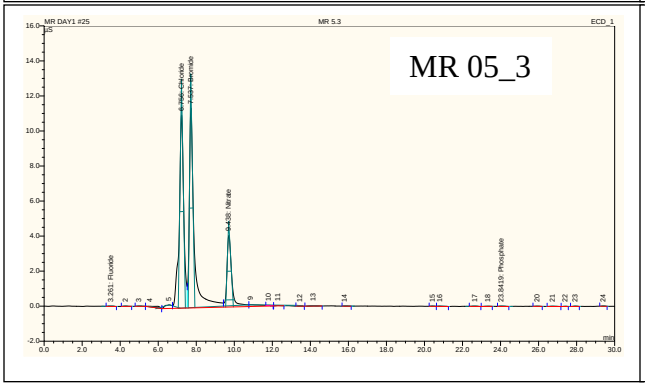
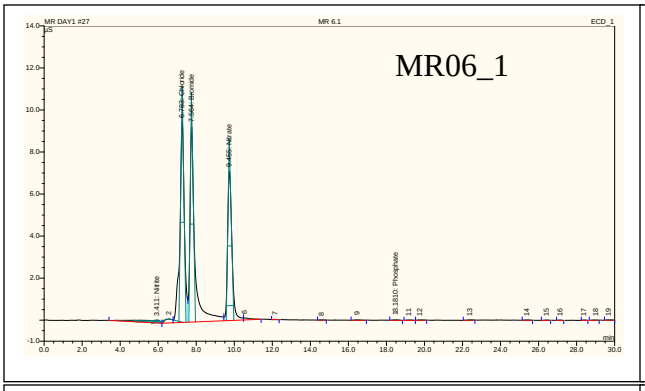
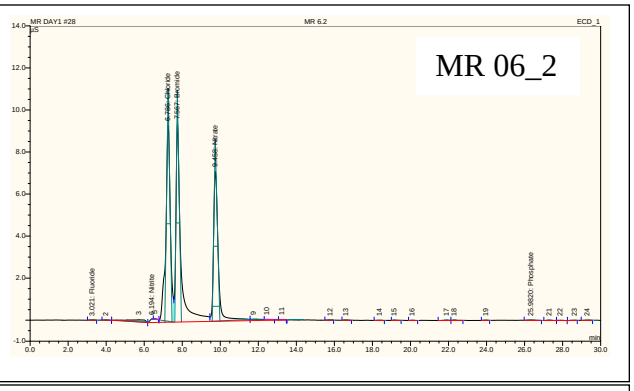


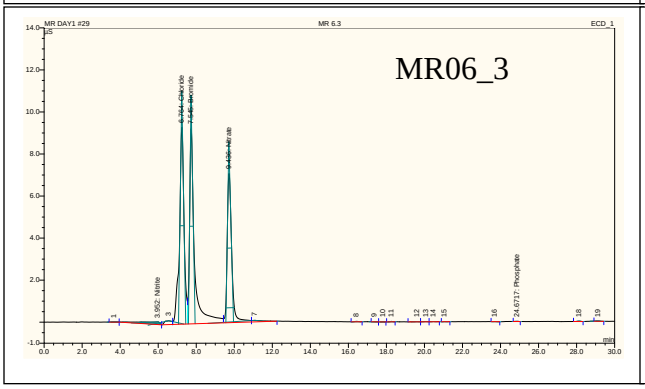




Sample 6







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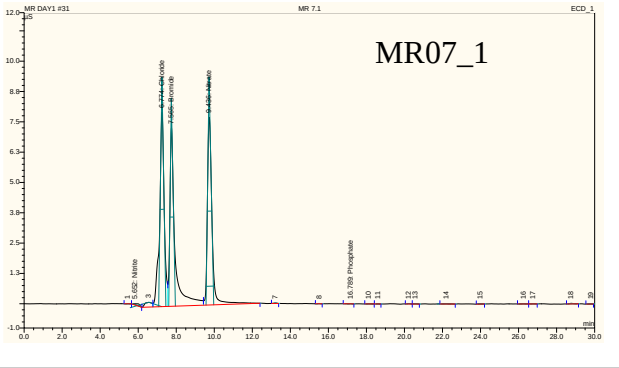

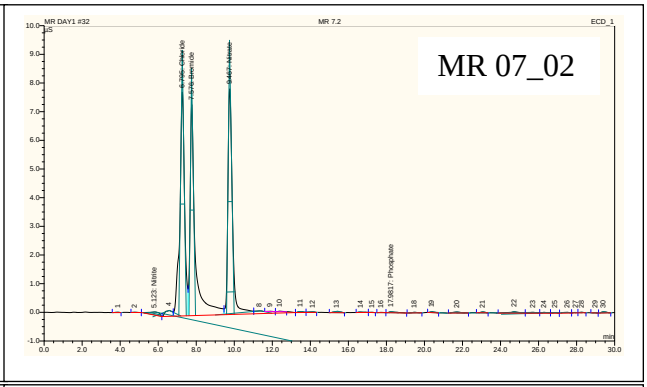

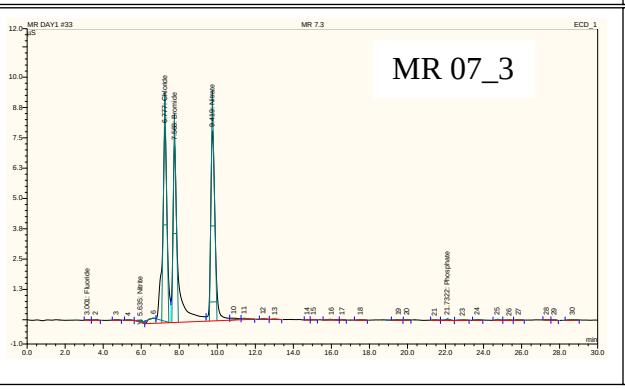

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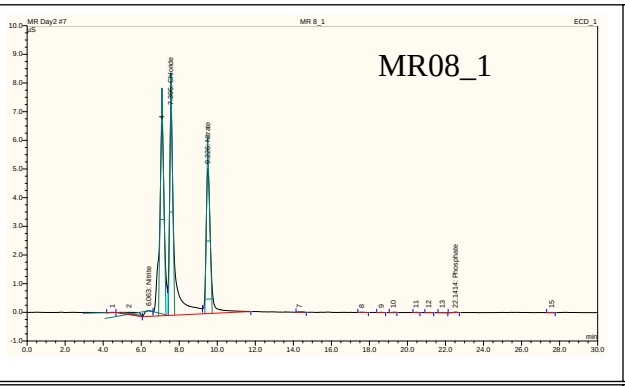

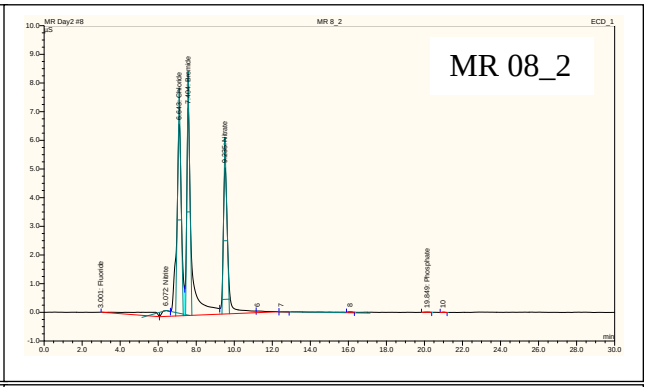

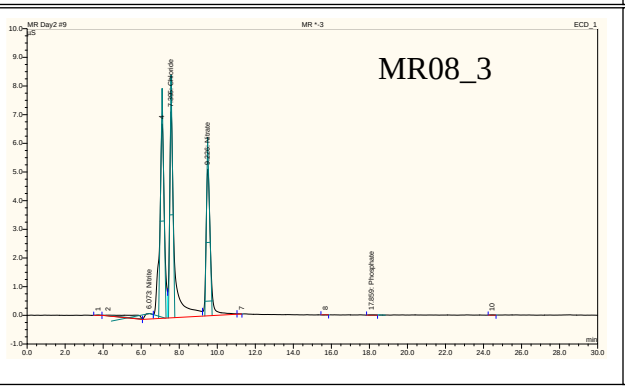

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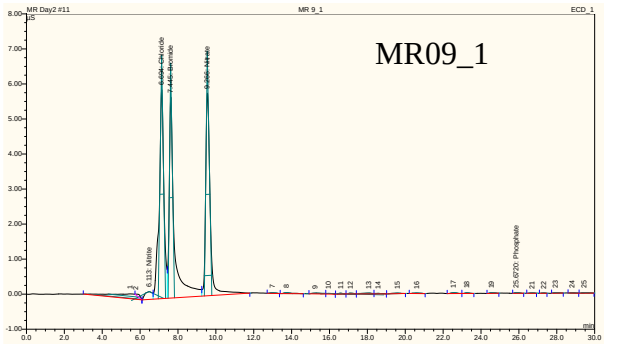
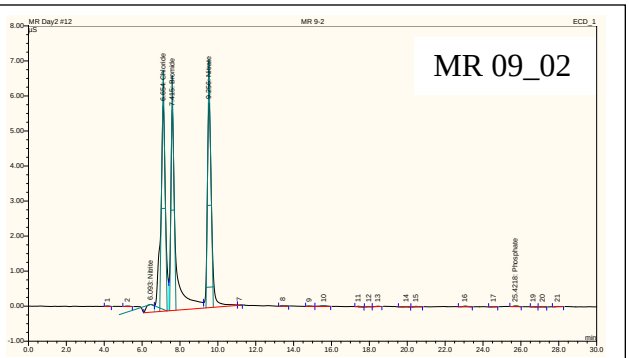
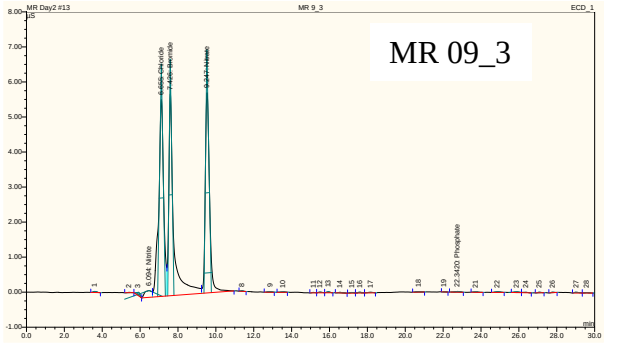
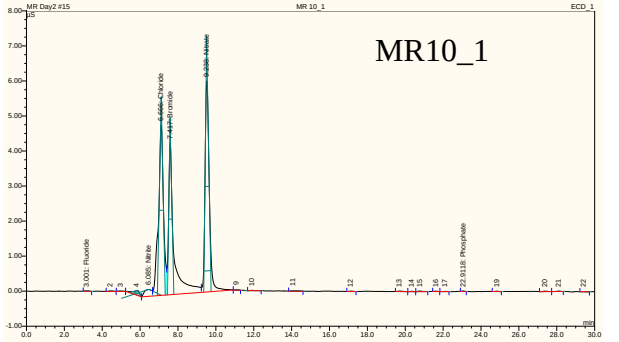
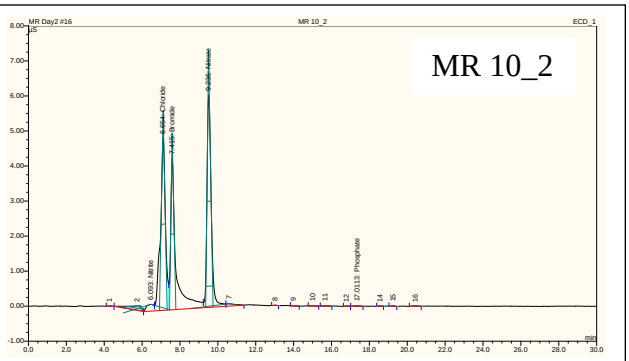


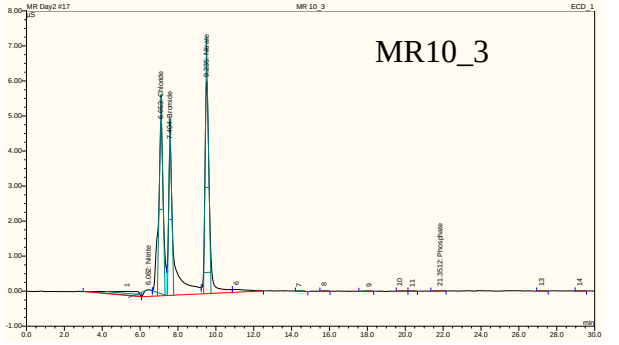




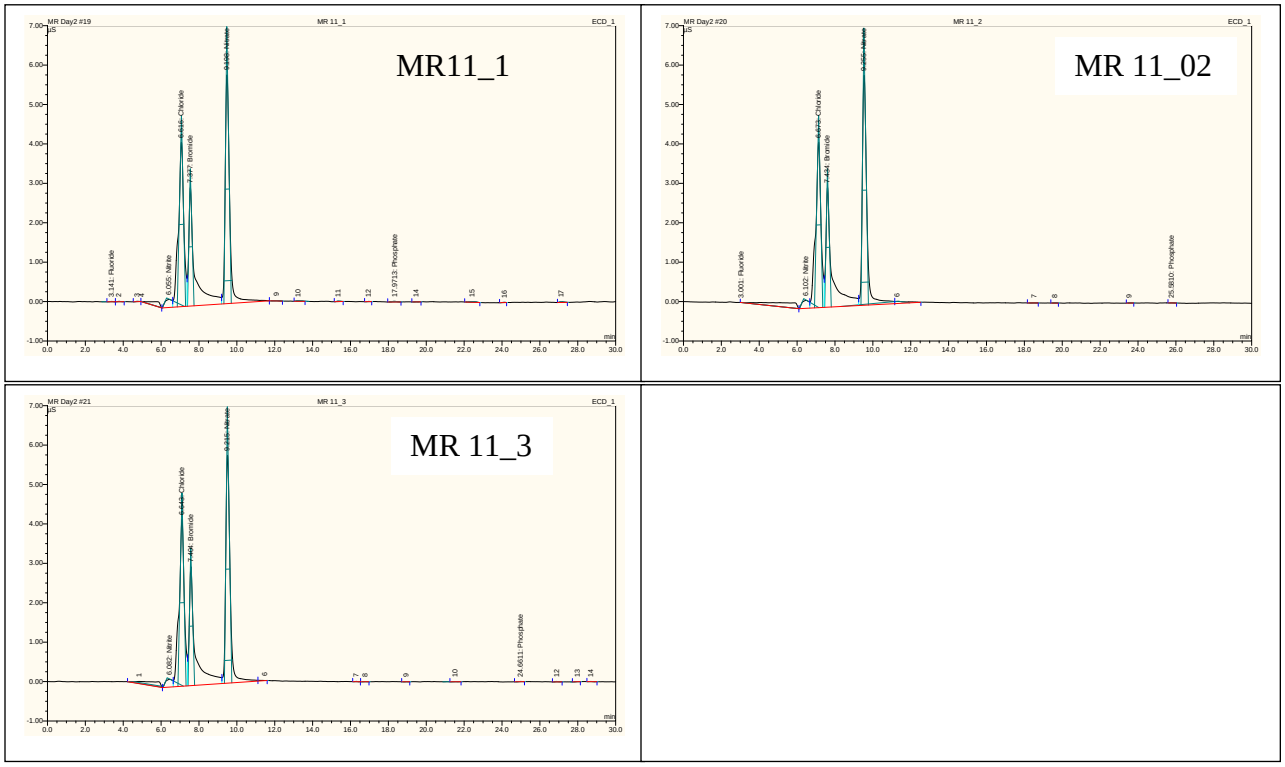
Sample 10



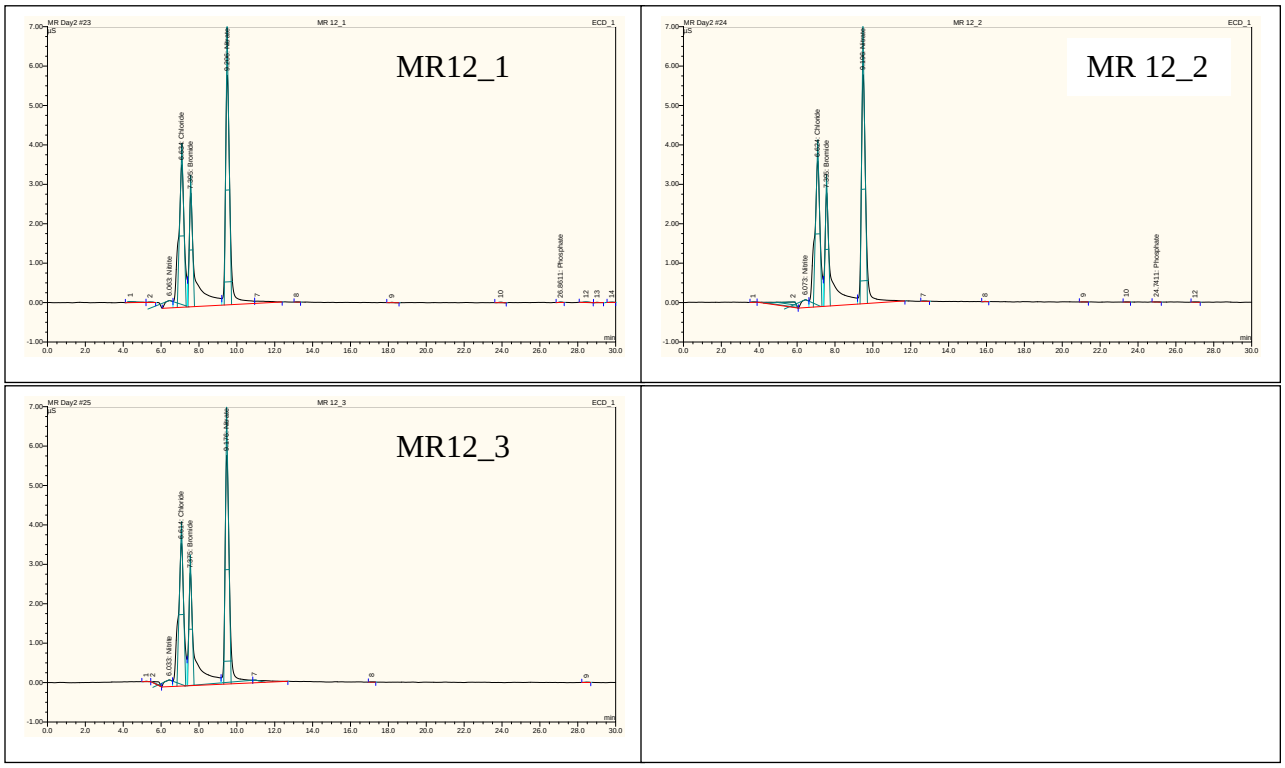




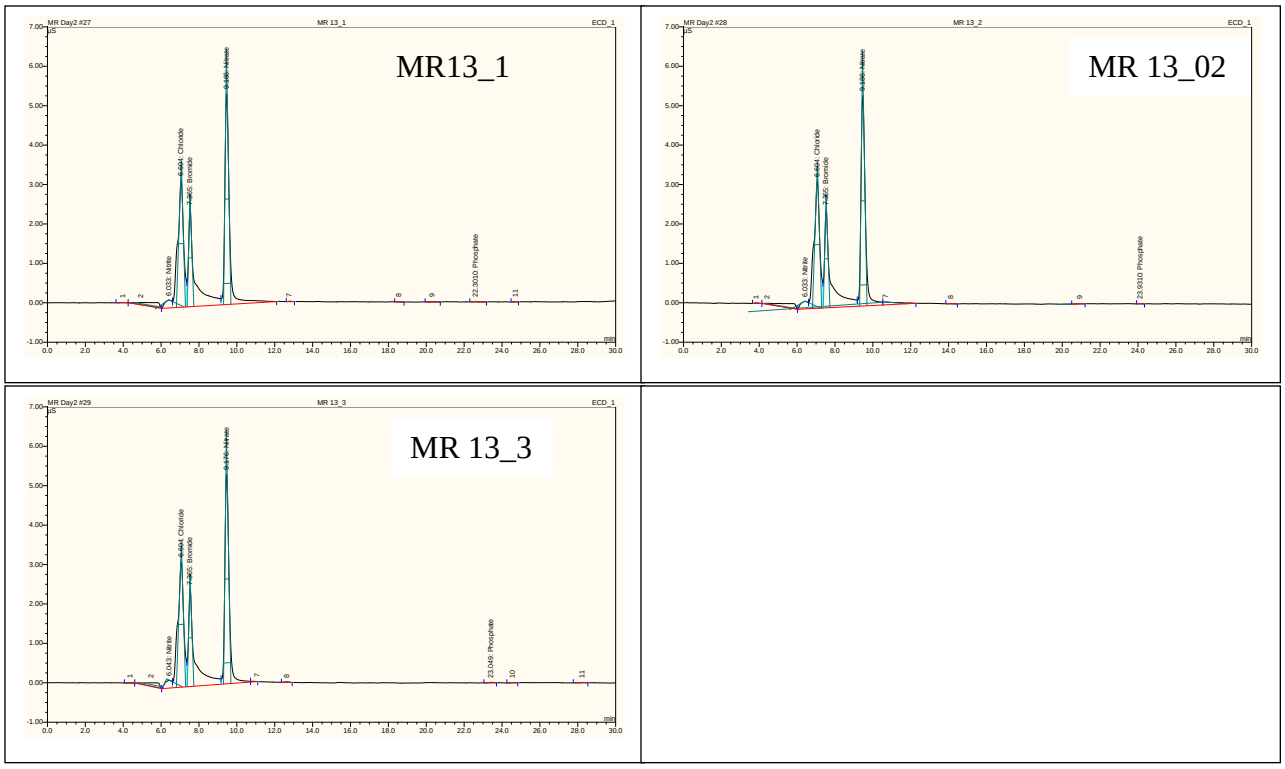
Sample 11



Sample 12



Sample 13



APPENDIX D. CALCULATION TABLES

Appendix Table 1: Calculation for Bacterial System

A	B	C	D	E	F	G	H	I
Sample	Date	Time	Temp	Pressure (Pump)	Pressure (Meter)	Mass B+L	Mass B+L+S	Amount of Nitrate (ppm)
MRB1	11/24/201	6:42 PM	23.5	500	0	99.160	160.000	16.77056334
MRB2	11/26/201	10:5 AM	22.9	500	0	98.960	154.100	3.399991928
MRB3	11/27/201	11:44 AM	24.5	500	0	99.130	190.000	2.860726408
MRB4	11/28/201	5:1 PM	24.4	500	0	99.505	149.885	2.436078854
MRB5	11/29/201	4:8 PM	20.4	500	0	99.720	174.000	2.26935032
MRB6	11/30/201	5:43 PM	18.1	500	0	99.885	164.000	2.107052699
MRB7	12/2/2011	8:41 AM	20.7	500	0	99.005	180.000	1.560470799
MRB8	12/4/2011	10:28 AM	19.2	500	0	99.625	182.000	0.320780845
MRB9	12/5/2011	5:14 PM	21.0	500	0	99.285	158.000	0.0155
MRB10	12/7/2011	9:42 AM	20.6	500	0	99.400	170.000	0.054726552
MRB11	12/8/2011	3:59 PM	21.1	500	0	99.260	164.000	0.03316288
MRB12	12/10/201	10:40 AM	21.6	500	0	100.005	194.000	0.834226021
MRB13	12/11/201	10:10 AM	19.8	500	0	99.875	176.000	2.108809371
MRB14	12/15/201	10:12 AM	18.2	500	0	99.485	224.000	3.199795496
MRB15	12/16/201	9:12 AM	21.0	500	0	99.535	164.000	6.61283432
MRB16	12/17/201	11:12 AM	20.7	500	0	99.640	134.340	5.700136437
Cell 1								13.61767207
Cell 2								13.63978066
Reservior								**19.25321128**

Time (Minutes)	Solute Flux (cm^3/s)	Solution Flux (cm/s) Jv	Lp (cm^2s/g)	Lp (m^3/N-s)	Jv (m/s)	Amount of Nitrate (mmoles)
0						
1377	0.000736383	1.59768E-05	4.63446E-13	4.63446E-14	1.59768E-07	0.000270476
3140	0.000292675	6.34995E-06	1.84197E-13	1.84197E-14	6.34995E-08	5.4835E-05
4678	0.000323749	7.02415E-06	2.03753E-13	2.03753E-14	7.02415E-08	4.61378E-05
5954	0.000141026	3.05973E-06	8.87552E-14	8.87552E-15	3.05973E-08	3.92891E-05
7341	0.000168642	3.6589E-06	1.06136E-13	1.06136E-14	3.6589E-08	3.66001E-05
8875	0.000120404	2.61231E-06	7.57767E-14	7.57767E-15	2.61231E-08	3.39825E-05
11213	0.000120389	2.61198E-06	7.57671E-14	7.57671E-15	2.61198E-08	2.51673E-05
14200	9.66843E-05	2.09769E-06	6.08487E-14	6.08487E-15	2.09769E-08	5.17355E-06
16045	6.09899E-05	1.32325E-06	3.83843E-14	3.83843E-15	1.32325E-08	2.49984E-07
18473	6.36966E-05	1.38198E-06	4.00877E-14	4.00877E-15	1.38198E-08	8.82629E-07
20290	5.31789E-05	1.15378E-06	3.34684E-14	3.34684E-15	1.15378E-08	5.34851E-07
22850	6.85594E-05	1.48748E-06	4.31482E-14	4.31482E-15	1.48748E-08	1.34544E-05
24259	5.23002E-05	1.13472E-06	3.29154E-14	3.29154E-15	1.13472E-08	3.40109E-05
28821	7.20048E-05	1.56223E-06	4.53166E-14	4.53166E-15	1.56223E-08	5.16063E-05
30200	3.55767E-05	7.71881E-07	2.23904E-14	2.23904E-15	7.71881E-09	0.000106652
31760	1.82095E-05	3.95077E-07	1.14602E-14	1.14602E-15	3.95077E-09	9.19318E-05
						0.000219626
						0.000219982
						0.000310516

C(0, t) Nitrate	C(e, t) Nitrate	erfc {(x-Jvt)/2√(Dt)} x=0.4	erfc {(x-Jvt)/2√(Dt)} x=0.8	erfc {(x-Jvt)/2√(Dt)} x=1.2	C(x=0.4, t) Nitrate	C(x=0.8, t) Nitrate
0.000466035	0.000149637	4.9538E-11	2.64998E-10	6.51231E-10	0.000312603	0.000321679
0.000466369	0.000197845	4.05878E-11	4.1309E-10	1.17501E-09	0.000312229	0.000327954
0.00046666	0.000216673	1.05151E-11	4.11048E-10	1.39173E-09	0.000310961	0.0003279
0.000466902	0.00022675	3.42171E-13	3.47064E-10	1.43218E-09	0.00031053	0.000325217
0.000467165	0.000234747	3.33038E-11	2.42257E-10	1.36162E-09	0.000311929	0.000320795
0.000467456	0.000241424	1.58089E-10	1.18499E-10	1.17956E-09	0.000317236	0.000315553
0.000467899	0.000248951	6.15154E-10	2.45326E-12	7.80357E-10	0.000336739	0.000310621
0.000468465	0.000255821	1.87063E-09	1.8434E-10	2.59094E-10	0.000390544	0.000318402
0.000468814	0.000259104	3.14122E-09	6.00451E-10	4.95389E-11	0.000445199	0.000336261
0.000469274	0.000262676	5.52937E-09	1.64144E-09	4.44853E-11	0.000548282	0.000381099
0.000469618	0.000264932	7.93616E-09	2.87453E-09	3.29212E-10	0.000652516	0.000434391
0.000470103	0.000267653	1.23689E-08	5.41309E-09	1.29111E-09	0.000845162	0.000544498
0.00047037	0.000268968	1.53886E-08	7.27028E-09	2.16049E-09	0.000976803	0.0006253
0.000471234	0.000272558	2.84226E-08	1.59556E-08	7.06282E-09	0.001547791	0.001005084
0.000471495	0.000273483	3.34538E-08	1.94965E-08	9.28437E-09	0.001769175	0.001160603
0.00047179	0.000274458	3.98201E-08	2.40783E-08	1.22753E-08	0.002049943	0.001362308

C(x=1.2, t) Nitrate	σ Nitrate	Time (Seconds)	Jv * 10^7(m/s) Bacteria	Change in Concentration	Amount of Nitrate (mmoles)	% Nitrate Reduction
					0.000310516	
0.000337948	0.513906851	82620	1.597675447	4.00405E-05	0.000270476	12.89482439
0.000360117	0.404274314	188400	0.634995152	0.000255681	5.4835E-05	82.3406711
0.000369376	0.365835154	280680	0.702414724	0.000264378	4.61378E-05	85.14157986
0.000371181	0.346214271	357240	0.305972665	0.000271227	3.92891E-05	87.34717063
0.000368289	0.331121609	440460	0.365889517	0.000273916	3.66001E-05	88.2131474
0.000360657	0.318858606	532500	0.261230921	0.000276533	3.39825E-05	89.0561103
0.000343781	0.305431272	672780	0.261197902	0.000285349	2.51673E-05	91.89501985
0.0003216	0.293591109	852000	0.209768551	0.000305342	5.17355E-06	98.33388591
0.00031264	0.288095144	962700	0.132325225	0.000310266	2.49984E-07	99.91949404
0.000312429	0.28225658	1108380	0.138197621	0.000309633	8.82629E-07	99.71575398
0.000324703	0.278655933	1217400	0.115378249	0.000309981	5.34851E-07	99.82775424
0.000366325	0.274413293	1371000	0.148748243	0.000297062	1.34544E-05	95.66708628
0.00040406	0.272408038	1455540	0.113471738	0.000276505	3.40109E-05	89.04698627
0.00061797	0.267111589	1729260	0.156223342	0.00025891	5.16063E-05	83.38047787
0.000715334	0.265795258	1812000	0.077188098	0.000203864	0.000106652	65.65338427
0.000846728	0.264433351	1905600	0.039507748	0.000218584	9.19318E-05	70.39387555

Appendix Table 2: Calculation for Control System

Sample	Date	Time	Temp	Pressure (Pump)	Pressure (Meter)	Mass B+L	Mass B+L+S	Time (Minutes)
								0
MJB001	6/4/2011	11:10 AM	20.7	500	0	22.700	179.620	1494
MJB002	6/5/2011	10:52 AM	20.6	500	0	22.585	157.559	2915
MJB003	6/6/2011	10:42 AM	21.2	500	0	22.340	159.048	4344
MJB004	6/7/2011	9:35 AM	21.3	500	0	22.615	151.365	5717
MJB005	6/8/2011	9:34 AM	20.7	500	0	22.975	158.579	7155
MJB006	6/8/2011	5:32 PM	20.7	500	0	22.125	66.340	7633
MJB007	6/9/2011	3:46 PM	20.6	500	0	22.765	144.750	8967
MJB008	6/10/2011	12:46 PM	20.2	500	0	22.550	137.765	10227
MJB009	6/11/2011	12:19 PM	20.4	500	0	22.445	152.397	11639
MJB010	6/12/2011	10:31 AM	19.7	500	0	22.125	141.840	12971
MJB011	6/13/2011	3:36 PM	21.0	500	0	22.480	132.110	14715
MJB012	6/14/2011	4:49 PM	21.3	500	0	22.180	149.020	16227
MJB013	6/15/2011	6:15 PM	20.9	500	0	22.690	120.605	17752
MJB014	6/16/2011	6:23 PM	21.0	500	0	22.135	112.365	19200
MJB015	6/17/2011	6:16 PM	20.7	500	0	22.185	139.990	20632
MJB016	6/18/2011	4:0 PM	20.7	500	0	22.520	126.865	21935
MJB017	6/20/2011	8:57 AM	20.7	500	0	22.465	222.860	24392
MJB018	6/21/2011	8:49 AM	20.4	500	0	22.760	108.850	25823
Reservior								
Cell 1								
Cell 2								
Cell 3								
Cell 4								

Solute Flux (cm^3/s)	Solution Flux (cm/s) Jv	Lp (cm^2s/g)	Lp (m^3/N-s)	Jv (m/s)	Time (Seconds)	Amount of Nitrate (mmoles)
0.001750558	0.000145476	4.2199E-12	4.2199E-13	1.45476E-06	89640	0.000284927
0.000771721	6.41321E-05	1.86031E-12	1.86031E-13	6.41321E-07	174900	0.000291731
0.000524509	4.35881E-05	1.26438E-12	1.26438E-13	4.35881E-07	260640	0.0002906
0.000375343	3.1192E-05	9.04803E-13	9.04803E-14	3.1192E-07	343020	0.000288463
0.000315872	2.62498E-05	7.61444E-13	7.61444E-14	2.62498E-07	429300	0.000283412
9.65435E-05	8.02303E-06	2.32728E-13	2.32728E-14	8.02303E-08	457980	0.000290026
0.000226729	1.88418E-05	5.46555E-13	5.46555E-14	1.88418E-07	538020	0.000286878
0.000187763	1.56036E-05	4.52622E-13	4.52622E-14	1.56036E-07	613620	0.000291799
0.000186087	1.54643E-05	4.48582E-13	4.48582E-14	1.54643E-07	698340	0.00029072
0.000153824	1.27832E-05	3.70809E-13	3.70809E-14	1.27832E-07	778260	0.000294921
0.00012417	1.03189E-05	2.99326E-13	2.99326E-14	1.03189E-07	882900	0.000285116
0.000130277	1.08263E-05	3.14046E-13	3.14046E-14	1.08263E-07	973620	0.00028518
9.19286E-05	7.63951E-06	2.21604E-13	2.21604E-14	7.63951E-08	1065120	0.000282585
7.83247E-05	6.50899E-06	1.8881E-13	1.8881E-14	6.50899E-08	1152000	0.000284439
9.51637E-05	7.90836E-06	2.29402E-13	2.29402E-14	7.90836E-08	1237920	0.000295777
7.92835E-05	6.58867E-06	1.91121E-13	1.91121E-14	6.58867E-08	1316100	0.000294042
0.000136927	1.1379E-05	3.30076E-13	3.30076E-14	1.1379E-07	1463520	0.000297083
5.55642E-05	4.61753E-06	1.33943E-13	1.33943E-14	4.61753E-08	1549380	0.000293226
						0.000310516
						0.000322964
						0.000323054
						0.000320456
						0.000381962

Reservoir Concentration (mmoles)	C(0, t) Nitrate	C(e, t) Nitrate	erfc {(x-Jvt)/2√(Dt)} x=0.4	erfc {(x-Jvt)/2√(Dt)} x=0.8	erfc {(x-Jvt)/2√(Dt)} x=1.2	C(x=0.4, t) Nitrate
0.000310516	0.000474777	0.000168	1.94892E-11	2.71478E-11	2.20088E-10	0.00031138
0.000310516	0.000483298	0.000208	6.15999E-10	1.29386E-10	4.28368E-12	0.00033934
0.000310516	0.000491788	0.000227	2.82989E-09	1.35309E-09	4.15017E-10	0.00044946
0.000310516	0.000499833	0.000238	7.442E-09	4.54799E-09	2.36298E-09	0.00069212
0.000310516	0.000508106	0.000246	1.58965E-08	1.10288E-08	7.04834E-09	0.00116127
0.000310516	0.000510816	0.000248	1.9706E-08	1.40713E-08	9.38316E-09	0.00137961
0.000310516	0.000518255	0.000254	3.3439E-08	2.53711E-08	1.84152E-08	0.00219204
0.000310516	0.000525098	0.000258	5.11974E-08	4.04355E-08	3.09419E-08	0.00328615
0.000310516	0.000532531	0.000261	7.75174E-08	6.32797E-08	5.04855E-08	0.00497195
0.000310516	0.000539291	0.000264	1.0943E-07	9.1471E-08	7.51206E-08	0.00709135
0.000310516	0.000547742	0.000267	1.63034E-07	1.39553E-07	1.17897E-07	0.01078612
0.000310516	0.000554678	0.00027	2.2169E-07	1.92825E-07	1.65973E-07	0.01497146
0.000310516	0.000561285	0.000272	2.9362E-07	2.58765E-07	2.26111E-07	0.02025388
0.000310516	0.000567187	0.000274	3.74929E-07	3.33864E-07	2.95181E-07	0.02637589
0.000310516	0.000572659	0.000275	4.68878E-07	4.21173E-07	3.76027E-07	0.03360223
0.000310516	0.00057732	0.000276	5.66944E-07	5.12765E-07	4.61307E-07	0.04128092
0.000310516	0.000585286	0.000279	7.87307E-07	7.19803E-07	6.55324E-07	0.05890427
0.000310516	0.000589436	0.00028	9.38705E-07	8.62767E-07	7.90031E-07	0.07122706

C(x=0.4, t) Nitrate	C(x=0.8, t) Nitrate	C(x=1.2, t) Nitrate	σ Nitrate	Jv *107 (m/s)
0.00031138	0.00031172	0.00032031	0.47664486	1.454760568
0.00033934	0.00031657	0.00031072	0.39907468	0.641320878
0.00044946	0.00037695	0.00033089	0.36906816	0.435880993
0.00069212	0.00054373	0.00043168	0.35493114	0.311919743
0.00116127	0.00090076	0.00068773	0.34719906	0.262498412
0.00137961	0.00107392	0.00081957	0.34559502	0.080230257
0.00219204	0.00173808	0.00134669	0.34278794	0.188418299
0.00328615	0.00266066	0.00210889	0.34171916	0.15603592
0.00497195	0.00411578	0.00334641	0.3416877	0.154643303
0.00709135	0.00597852	0.00496536	0.3423878	0.127831807
0.01078612	0.00927737	0.00788587	0.3439532	0.103188896
0.01497146	0.01306256	0.01128675	0.34564566	0.108263438
0.02025388	0.01788642	0.0156685	0.34749684	0.076395145
0.02637589	0.02352103	0.02083172	0.34928722	0.065089891
0.03360223	0.03021504	0.02700953	0.35102258	0.07908356
0.04128092	0.03736569	0.03364705	0.35253273	0.06588671
0.05890427	0.05388043	0.04908172	0.35512099	0.113789788
0.07122706	0.06549012	0.05999512	0.35644228	0.046175311