5-2020

NITRATE AND HERBICIDES REMOVAL FROM GROUNDWATER USING IMMOBILIZED ALGAE

Sara Mollamohammada
University of Nebraska - Lincoln, smollamohammada@unomaha.edu

NITRATE AND HERBICIDES REMOVAL FROM GROUNDWATER USING IMMOBILIZED ALGAE

by

Sara Mollamohammada

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Civil Engineering

(Environmental Engineering)

Under the Supervision of Professor Mohamed Dahab

Lincoln, Nebraska

May 2020

NITRATE AND HERBICIDES REMOVAL FROM GROUNDWATER USING IMMOBILIZED ALGAE

Sara Mollamohammada

University of Nebraska, 2020

Advisor: Mohamed Dahab

To improve crop yield and control broadleaf weeds, many farmers apply nitrate-based fertilizers and chemical herbicides to their fields. Excessive use of these chemicals can cause contamination in waterbodies and impact on the public health. The physical and chemical methods have been suggested to remove these herbicides are usually costly. In this study, the capability of immobilized algal beads in removing nitrate and several herbicides of environmental concern from synthetic and actual groundwater and surface water was evaluated.

The experiments were performed in batch, sequencing batch and continuous flow modes and the effect of different operational conditions (light intensity, cultivation method, algal density, and temperature) on the performance of algal beads were assessed and the capability of the system in real working scenarios was evaluated.

The results from batch studies showed that *Scenedesmus species* cells, immobilized in sodium alginate are capable of removing 90% of nitrate from synthetic and actual groundwater with the beads/water ratio of 12.5%. The beads performance improved in heterotrophic conditions and the presence of light enhanced the nitrate removal. Immobilized algal beads were capable of uptaking nitrate for 100 consecutive days, and

the equal volumetric ratio of alga: alginate led to the highest nitrate uptake in sequencing batch mode.

Immobilized beads showed higher nitrate and atrazine uptake at room temperature (20 °C) in continuous flow reactor. When tested with the actual groundwater and surface water samples, atrazine, oxadiazon, triallate, cycloate, alachlor, simetryn and ametryn were partially removed through adsorption, bioaccumulation, and biodegradation. The presence of herbicides and toxins in the water lowered the efficiency of the immobilized beads.

Embedding immobilized algal beads with nanoclay was found to be an effective method to accelerate nitrate and atrazine uptake rate. Nearly 100% of 10 mg/L nitrate and 90% of 0.1 mg/L atrazine were removed in 3 days at the concentration of 0.3 mg Nanoclay per bead. The nitrate uptake capacity of the nanoclay-embedded algal beads was found to be through three mechanisms of adsorption, biological assimilation, and extracellular enzyme activity resulting from toxicity.

`

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to my advisor and co-advisor, Drs. Mohamed Dahab and Ashraf Aly Hassan, who supported me throughout my PhD. This work would not have been possible without their support, encouragement, and valuable recommendations.

I am also grateful of my committee members, Drs. Xu Li and Yulie Meneses for their time, and valuable feedback throughout the preparation and review of this document.

I'd like to acknowledge the financial support of University of Nebraska-Lincoln Research Council Seed Grant, and Entech Instruments who supported this work. A special thanks to Sage Dunham and Victoria Noad for their valuable technical support.

I'd like to also thank my colleagues, Naji Albakay, Chris Duerschner, Mitham Al-Faliti, Nicolette Holcomb, Carlos Felix, and Carlos Coria, who helped me with the experiments and analytical measurements.

I would like to give a heartfelt and special thanks to Sima Dabir, Nima Sabet, and Zahra Noee who truly encouraged me with their support and inspiring words throughout the completion of this work.

Last by not least, I'd like to thank my parents, Ali-Akbar and Masoumeh, and my sister, Parisa for their endless support and unconditional love during all these years, and my friends, Fahimeh, Fatemeh, Nargess, Soha, Elham, Atefeh, and Bella for their encouragement, and good wishes.

.

`

TABLE OF CONTENTS

`

`

`

`

`

List of Figures

`

`

`

List of Tables

`

`

CHAPTER 1

INTRODUCTION

1.1 Background

Agricultural contaminants can impair the quality of our water resources. Fertilizers and herbicides do not remain stationary on the land where they are applied and can enter streams, rivers, and groundwater through runoff and infiltration (USGS, 2010).

Nitrogen is an essential nutrient for plants and is used in various forms as fertilizer to enhance plants growth. Excess nitrogen in soil can infiltrate into the groundwater, commonly in the form of nitrate (Peña-Haro, Llopis-Albert, Pulido-Velazquez, & Pulido-Velazquez, 2010).

As it is shown in Figure 1.1, Midwest and parts of the western and northeastern United States are high-risk of groundwater contamination by nitrate.

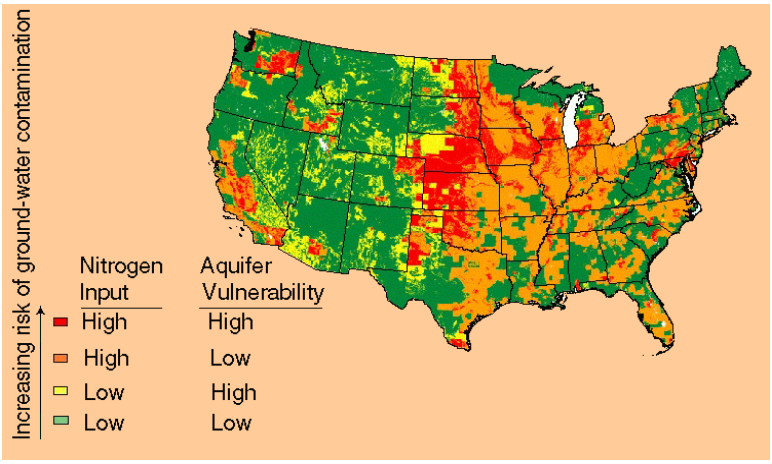


Figure 1.1 Areas in the United States with the highest risk of nitrate contamination (Dubrovsky, 2010).

`

Herbicides are synthetic organic compounds (i.e pesticides) used to control weeds, insects, and a variety of other agricultural purposes. Many herbicides are water-soluble and may dissolve in groundwater. Groundwater with elevated levels of nitrate or herbicides is not safe for human consumption and can cause adverse environmental impacts when discharged to the streams (Ator & Ferrari, 1997; Wick, Heumesser, & Schmid, 2012).

Several technologies have been applied for the treatment of groundwater: including reverse osmosis, ion exchange, and chemical and biological methods.

Currently, the best method to eliminate nitrate and herbicides is reverse osmosis. In a reverse osmosis system, a high-pressure pump is used to force the water across a semi-permeable membrane, leaving about 85-95% of nitrate behind in the reject stream. The amount of pressure required for the system depends on the quality of the feed water. The membranes of the system need to be replaced on a regular basis (Eisenberg & Middlebrooks, 2013)

Ion Exchange process in another method for nitrate remediation which works by passing nitrate contaminated water through a tank, filed with resin beads. The resin is saturated with chloride which chemically trades places with nitrate ions. Once exhausted, the resin needs to be recharged by backwashing, typically with a strength sodium chloride solution (Ghurye, Clifford, & Tripp, 1999).

The initial cost of constructing these removal systems can range from $350 to $1000 per resident. Annual operating costs depend on the concentration of the contaminants in raw water, the flow rate of water treated, maintenance costs, and chemicals costs (Eisenberg & Middlebrooks, 2013). In addition to the costs, reverse osmosis systems need special

`

expertise for operation and maintenance, that might be problematic in plenty of the rural areas (Pirsaheb, Khosravi, Sharafi, & Mouradi, 2016).

Nitrate decomposition can also be done through chemical processes. Metals such as zero-valent iron (ZVI), zinc, chromium, cadmium, aluminum and lead can chemically reduce nitrate in the groundwater (Huno, Rene, van Hullebusch, & Annachhatre, 2018). Nanoscale ZVI (nZVI) is found to be the most attractive metal for denitrification due to its high specific surface area and surface reactivity (Peng et al., 2015). Due to its electron donation properties nZVI acts as denitrification agent in this process and reduces nitrate to ammonium. Although it is a fast process, there are some limitations associated with nitrate reduction by ZVI. The formation of iron oxides on the surface of ZVI at high pH condition may inhibit the further reduction of nitrate. Moreover, the end reduction product of this process is mainly ammonium, which itself would be of concern (Y. Liu & Wang, 2019).

Biological processes have been applied to remove nitrate from contaminated water. Many bacteria are capable of reducing nitrate to nitrogen gas. Most denitrifying bacteria are heterotrophic and use organic substances (e.g. methanol, ethanol, methane) as electron donors to convert nitrate to nitrogen (Sharma & Sobti, 2012). In a natural heterotrophic reactive media, dissolved organic carbon in pore waters of the aquifer is used for denitrification process. In such environment, the organic carbon insufficiency may limit the denitrification rate (Huno et al., 2018).

Using microalgae can be a sustainable and effective approach for biological treatment of groundwater. Algae can uptake nitrate and herbicides and create biomass that concentrates the nitrogen and leaves the water with lower level of nitrate (Wick et al., 2012).

`

To overcome the complications of using suspended algae, microalgae can be used in immobilized form. Immobilization of algae in polymeric matrices makes them easier to handle and can be used repeatedly for product generation. It allows higher cell densities and easier harvesting of biomass from its liquid environment (De-Bashan & Bashan, 2010).

1.2 Research Objectives

The main goal of this study was to introduce an economical, efficient, and feasible alternative technology to remove nitrate, atrazine and potentially other herbicides of environmental concern from waterbodies using immobilized algae. This process will lower the health risks associated with high concentration of nitrate and herbicides and will produce valuable biomass which can be used as a source of green energy. The development of such a system starts from using the ideal conditions in the lab followed by being tested using actual water samples.

The specific objectives of this study are addressed below:

1. Optimizing an immobilized algae-based biological system for nitrate removal in batch reactors. In this phase, the optimum experimental conditions that maximizes the uptake rate of nitrate were investigated and used to design the reactors on the next phases. The ideal laboratory conditions were used in this activity. Objective 1 was completed by performing the following sub-tasks:

    1.1. Examine nitrate removal under autotrophic and heterotrophic conditions and under various light intensities: To simulate the autotrophic and heterotrophic growth of algae, batch studies were performed with and without addition of an organic carbon source. At autotrophic condition, the dissolved atmospheric CO_2

`

was the only source of carbon. The heterotrophic growth was simulated by adding glucose to the bulk solution. The effect of light on the heterotrophic growth of algal beads was evaluated by measuring the nitrate uptake rate and dry cell weight of the algae in complete darkness, medium and high intensity light.

1.2. Optimize the required algal beads in batch reactor for nitrate removal: Four different ratios of *algal* beads / water (50%, 25%, 12.5%, and 6.25% by volume) were evaluated for their nitrate removal efficiency from synthetic contaminated groundwater.

1.3. Test the performance efficiency of two algae species (*Chlorella sorokiniana* vs *Scenedesmus species)* for nitrate reduction

2. Evaluating different reactors configuration (sequencing batch and continuous flow reactor) for the removal of nitrate and atrazine from synthetic groundwater. The Kinetics and rates obtained from objective 1 were used to develop the different reactor configurations studied in objective 2.

Objective 2 was completed by performing the following sub-tasks:

2.1. Evaluate the performance of nitrate removal under sequential batch conditions and with different ratios of algae: alginate. In this sub-task, sets of experiments with four different ratios of algae to alginate (75:25, 50:50, 25:75, and 10:90 (v:v %)), were performed to assess the minimum number of initial algae needed without compromising the performance.

2.2. Evaluate the removal of nitrate and atrazine in continuous flow conditions and under temperature variation. The immobilization system that was optimized in

`

objective 1 was tested in continuous flow mode and in two different temperatures (20 and 35 °C) to evaluate the stability and steadiness of the system.

3. Evaluating the performance of the algal-based biological system in real working scenarios, by utilizing actual water samples collected from sites with high concentration of nitrate and agricultural residue herbicides. Actual water samples, obtained from Wahoo creek, the water treatment utilities in Hastings, NE, and Rockford lake were utilized to evaluate the system performance under real working scenarios. The experiments listed in each sub-task were performed in continuous flow mode:

3.1. Evaluate the performance of the immobilized system in nitrate removal using actual groundwater, collected from the Hastings water treatment utilities

3.2. Evaluate the performance of the system in removing nitrate and herbicides from actual surface water, collected from Wahoo Creek, NE.

3.3. Evaluate the performance of the system in removing nitrate and herbicides from water samples contaminated with cyanobacteria, and collected from Rockford lake, NE.

4. Utilization of nanoclay embedded algal beads as a combined physical-biological approach for maximizing the uptake of nitrate and atrazine from synthetic and actual groundwater. Nanoclay material was chosen for use was treated with 25-30% w/w trimethyl stearyl ammonium (TMSA). This modification was done in order to produce cations on the surface of the clay and add a positive surface charge to it. The overall positive charge resulting from this modification likely facilitated adsorption of the negatively-

`

charged nitrate ions on the surface of the nanoclay and, hence, increased the nitrate uptake rate.

The following sub-tasks were performed here:

4.1. Optimize the concentration of nanoclay inside of the beads to maximize nitrate uptake: To assess the impact of nanoclay dosage on the performance of the algal beads, the beads were embedded with nanoclay in different concentrations (0.0030 to 0.60 mg per bead) and tested for nitrate uptake.

4.2. Examine the effect of TMSA on algal cells and nitrate removal rate: To assess the toxicity of TMSA on the immobilized algal cells and investigate the mechanism of nitrate uptake on activity 4.1, the algal beads were embedded with TMSA and tested for nitrate uptake.

4.3. Examine the efficiency of the nanoclay based immobilized system on the removal rate of nitrate and atrazine using synthetic and actual groundwater.

1.3 Thesis Organization

The dissertation structure is detailed in Figure 1.2. This work is presented in seven chapters and covers the bioremediation of waterbodies in lab conditions and in real working scenarios. The remainder of this chapter provides an overview on the structure and organization of this dissertation.

Chapter 2 provides a comprehensive literature review about preparation and application of immobilized microalgae in water and wastewater treatment. Passive and active immobilization techniques are described and the capability of the immobilized algae in

`

removing nutrients, metals and organic compound from water and wastewater will be discussed.

Chapter 3 fulfills the first and second objectives of this research. It provides the results of development and optimization of batch and sequencing batch reactors in removing nitrate from synthetic groundwater. The effect of algae specie, volume of algal beads, and algae starvation on the nitrate removal rate are assessed and the lifetimes of algal beads are investigated using sequencing batch reactor.

Chapters 4 and 5 evaluate the bioremediation system in real working scenarios and fulfill Objectives 2 and 3. The results obtained from Chapter 3 are used to design the experimental conditions in Chapters 4 and 5. Chapter 4 evaluates the effect of temperature on the removal rate of nitrate and atrazine from synthetic and actual groundwater in a continuous flow reactor. Chapter 5 provides further evaluation of the removal of nitrate and herbicide residue from impaired surface water in a continuous flow reactor.

Chapter 6 evaluates a physical-biological approach to improve the capability of the immobilized system in nitrate removal. It fulfills objective 4. In this chapter, a hybrid biological adsorption approach is applied and evaluated for treatment of groundwater, contaminated with nitrate and atrazine. The performance of the immobilized algal beads embedded with nanoclay, as well as the mechanism of nitrate uptake and the adsorption kinetics are assessed in this chapter. Chapter 7 presents the conclusion of the research and provides recommendations for potential future work.

`

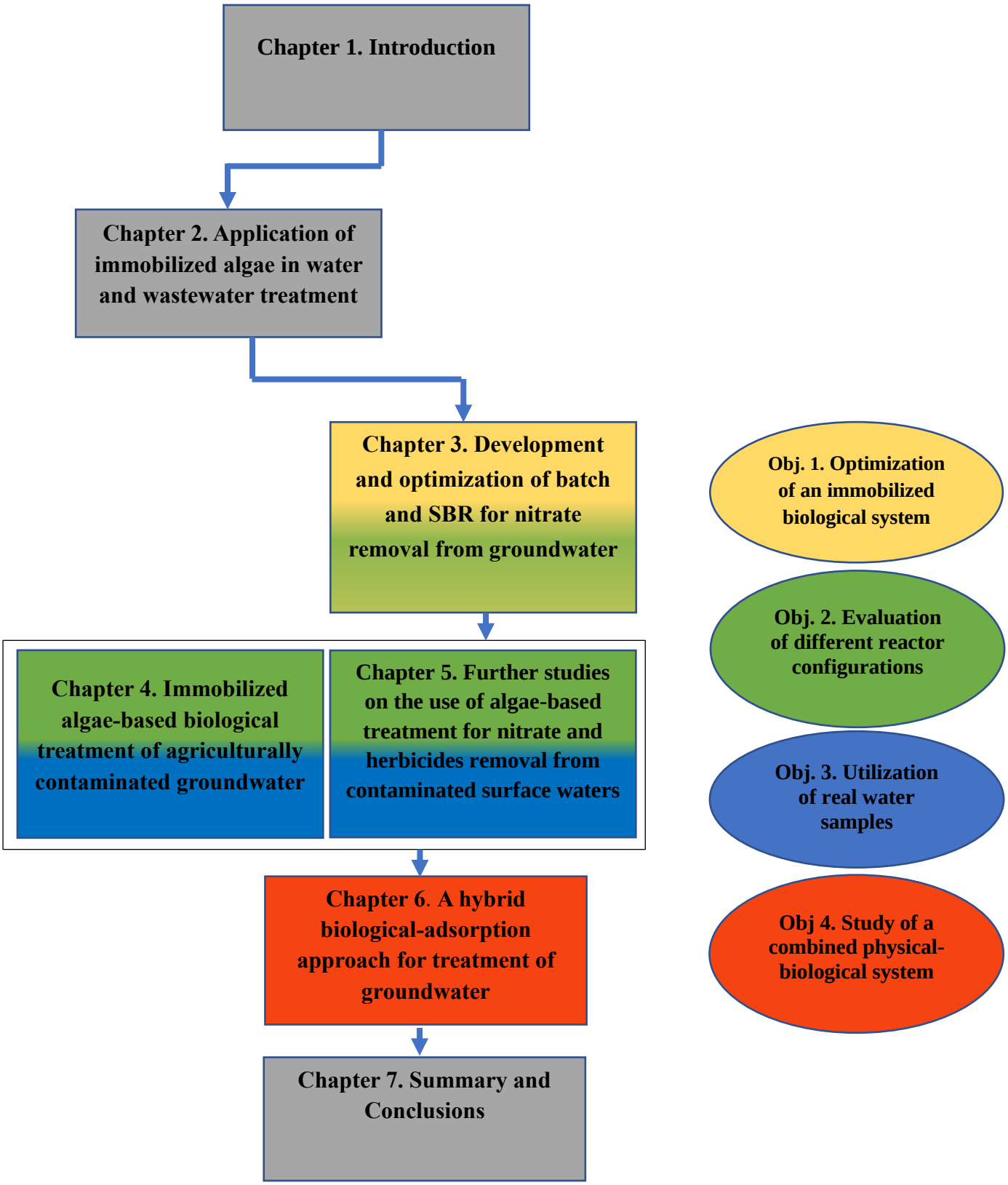


Figure 1.2 Schematic diagram of the dissertation structure.

`

References

Ator, S. W., & Ferrari, M. J. (1997). *Nitrate and selected pesticides in ground water of the Mid-Atlantic Region*: US Geological Survey.

Chen, R., Li, R., Deitz, L., Liu, Y., Stevenson, R. J., & Liao, W. (2012). Freshwater algal cultivation with animal waste for nutrient removal and biomass production. *Biomass and Bioenergy, 39*, 128-138.

De-Bashan, L. E., & Bashan, Y. (2010). Immobilized microalgae for removing pollutants: review of practical aspects. *Bioresource Technology, 101*(6), 1611-1627.

Dubrovsky, N. M. (2010). *The quality of our Nation's waters—Nutrients in the Nation's streams and groundwater, 1992–2004 Geological Survey Circular* Retrieved from

Eisenberg, T. N., & Middlebrooks, E. J. (2013). *Reverse osmosis treatment of drinking water*: Elsevier.

Ghurye, G. L., Clifford, D. A., & Tripp, A. R. (1999). Combined arsenic and nitrate removal by ion exchange. *Journal-American Water Works Association, 91*(10), 85-96.

Huno, S. K., Rene, E. R., van Hullebusch, E. D., & Annachhatre, A. P. (2018). Nitrate removal from groundwater: a review of natural and engineered processes. *Journal of Water Supply: Research and Technology-Aqua, 67*(8), 885-902.

Liu, Y., & Wang, J. (2019). Reduction of nitrate by zero valent iron (ZVI)-based materials: A review. *Science of the Total Environment*.

Pacheco, J., Marín, L., Cabrera, A., Steinich, B., & Escolero, O. (2001). Nitrate temporal and spatial patterns in 12 water-supply wells, Yucatan, Mexico. *Environmental Geology, 40*(6), 708-715.

Peña-Haro, S., Llopis-Albert, C., Pulido-Velazquez, M., & Pulido-Velazquez, D. (2010). Fertilizer standards for controlling groundwater nitrate pollution from agriculture: El Salobral-Los Llanos case study, Spain. *Journal of Hydrology, 392*(3-4), 174-187.

Peng, L., Liu, Y., Gao, S.-H., Chen, X., Xin, P., Dai, X., & Ni, B.-J. (2015). Evaluation on the nanoscale zero valent iron based microbial denitrification for nitrate removal from groundwater. *Scientific reports, 5*, 12331.

Pirsaheb, M., Khosravi, T., Sharafi, K., & Mouradi, M. (2016). Comparing operational cost and performance evaluation of electrodialysis and reverse osmosis systems in nitrate removal from drinking water in Golshahr, Mashhad. *Desalination and Water Treatment, 57*(12), 5391-5397.

Sharma, S. K., & Sobti, R. C. (2012). Nitrate removal from ground water: a review. *Journal of Chemistry, 9*(4), 1667-1675.

USGS. (2010). Agricultural Contaminants. Retrieved from https://www.usgs.gov/mission-areas/water-resources/science/agricultural-contaminants?qt-science_center_objects=0#qt-science_center_objects

Wick, K., Heumesser, C., & Schmid, E. (2012). Groundwater nitrate contamination: factors and indicators. *Journal of Environmental Management, 111*, 178-186.

`

CHAPTER 2

APPLICATION OF IMMOBILIZED ALGAE IN WATER AND WASTEWATER TREATMENT[1]

2.1 Introduction

Immobilization is defined as a technique that restricts the mobility of cells by entrapping them within a polymer matrix or attaching them to a solid support (Razavi-Shirazi, 2002). Immobilization of the cells have advantages over free suspended cells: 1) immobilization facilitates harvesting biomass at the end of the process, 2) immobilized cells require smaller space and are easier to handle than free cells, and 3) immobilization protects the cells against harsh environments, such as toxicity, salinity, and pH (E. Eroglu et al., 2012). A suitable immobilization matrix provides a structure with clefts or holes in which the microorganism can take hold and be protected from exposure to the contaminated and/or harsh environment. This way, the microorganism can tolerate higher concentrations of the contaminants since they are not in direct contact with them. The matrix does not need to provide any nutrients for the microorganism (Razavi-Shirazi, 2002).

The immobilized algal cells have various applications, including nutrients removal from water and wastewater (Fares A AlMomani & Örmeci, 2016), biosorption of heavy metals from wastewater (Blanco, Sanz, Llama, & Serra, 1999), removal of organic pollutants from wastewater (Zümriye Aksu, 2005), and biofuel production (Chevalier & de la Noüe, 1985). Various immobilization techniques and the applications of immobilized algal cells are discussed in the following sections of this chapter.

---

[1] This chapter is based on a publication: Mollamohammada S., Aly Hassan, A., Dahab, M. (2019) Application of immobilized algae in water and wastewater treatment. In: Bioenergy: Technologies and Future Prospects for Sustainability (Eds.) The Energy and Resources Institute (TERI), India. (In Press).

`

2.2 Immobilization Techniques

Immobilization techniques can be classified into two main groups: “passive” and “active”. Both natural and synthetic materials are used for passive and active immobilization. A good carrier must be able to hold the cells and prevent them from diffusing to the bulk solution. It must be porous enough to allow the diffusion of the molecules from bulk solution to the cells. For the autotrophic living cells, the carrier must allow sufficient light transmission.

2.2.1 Passive immobilization

Passive immobilization involves growing the microorganisms on surfaces due to their natural tendency to attach to them (Robinson, Mak, & Trevan, 1986). This technique is simple and cheap; but depends on the physical interaction between the microorganism and the surface of the immobilization agent (Martins, Martins, Fiúza, & Santaella, 2013). The immobilization of microorganisms on properly chosen adsorbents stimulates metabolism and preserves their physiological activity (Nikovskaya, 1989).

Both natural and synthetic carriers can be used for passive immobilization. Loofa sponge is a non-toxic, cheap fiber medium, which has been previously used for immobilization of microorganisms. It is derived from the genus luffa. A loofa sponge is a natural polymer with a very low density and very high porosity. This polymer is autoclavable and stable over the pH variation (Ogbonna, Liu, Liu, & Tanaka, 1994).

The general protocol for immobilization in loofa starts with soaking it in boiling water for 30 minutes, washing it with tap water, and leaving it in Deionized (DI) water for 24 hours. The sponges are then oven-dried at 70 °C, autoclaved, and soaked in a culture medium for 10 minutes. The loofa sponges are washed at the end in order to remove free cells from them (N Akhtar, Iqbal, & Iqbal, 2004).

`

The immobilization of blue-green microalgae on the loofa sponge for cadmium biosorption has been studied by Saeed and Iqbal (Saeed & Iqbal, 2006). This immobilized system was found effective in increasing the biosorption of cadmium at equilibrium compared to free biomass. It was reported that 96% of adsorption occurred within the first 5 minutes and the equilibrium was reached after 15 minutes.

Even though loofa sponge has the advantages of being cheap, physically strong, and highly porous (N Akhtar, Iqbal, & Iqbal, 2003), the results of experiments using this carrier are not easily repeatable. Because of the variation in the skeleton of the fruits in different plants, loofa sponge samples can have different physical characteristics, which makes it hard to get consistent results (Lau, Tam, & Wong, 1998). It was suggested that choosing the sponges of similar sizes and structures, such as same diameter and pore sizes of the fibrous network, could help in achieving consistent results (Y.-K. Liu, Seki, Tanaka, & Furusaki, 1998).

The synthetic materials used for passive immobilization include glass, wood, and plastic. Some strains of algae have the tendency to attach to the submerged surfaces. A study performed by Danilov and Ekelund (Danilov & Ekelund, 2001) compared the effectiveness of glass slides and tubes, pieces of wood, and pieces of PVC plastic on settlement patterns of periphyton in lakes of different trophic status. It was found that periphyton's favorable attachment surface is glass tubes; as wood did not adsorb as much algae as glass did, and plastics only adsorb bacteria and not algae. In another study, the colonization of periphytic algae on glass slides was evaluated within a 5-week period at four different current velocities. It was found that there is an inverse relationship between the accumulation of algae and current velocity, as there was more attached biomass in the low-current locations

`

(Ghosh & Gaur, 1998). In another study, (Urrutia, Serra, & Llama, 1995) used *Scenedesmus obliquus* immobilized in polyvinyl foams to remove nitrate from water. They found that the adsorbed cells have a higher growth rate than the cells immobilized by active immobilization, details of which are discussed in the subsequent section.

2.2.2 Active immobilization

Unlike passive immobilization, active immobilization techniques do not need the microorganisms to be able to naturally attach to a surface. Active immobilization focuses on entrapping the cells within a natural or synthetic polymer.

Entrapment of the cells consists of capturing the cells in an either natural (agar, alginate, and carrageenan) or synthetic (polyurethane, polystyrene, silica gel, polysilicate, and polyvinyl alcohol) polymers (De-Bashan & Bashan, 2010). Flocculation is another form of active immobilization that uses chemicals to force the algae to form lumps. Flocculation was originally developed to avoid expensive harvesting methods at the end of the treatment process. Alkaline flocculants neutralize the negative surface charges of the microalgae and allow them to coalesce into a floc. Some of the flocculants, such as anionic aluminum, can be toxic to the microalgae; therefore, natural flocculant agents such as chitosan have been used in most studies (Ignacio Moreno-Garrido, 2013).

Natural polymers are found to have a higher nutrient diffusion rate and synthetic polymers have shown more stability in the wastewater (De-Bashan & Bashan, 2010; Eroglu, Smith, & Raston, 2015). A successful entrapment must allow suitable diffusion of nutrients/ products toward and from the immobilized cells (Nirupama Mallick, 2002).

Natural polymers

`

Sodium alginate ($NaC_6H_7O_6$), the salt of alginic acid is one of the most widely used natural polymers for algal immobilization. Alginate is extracted from the cells of brown algae (K. Y. Lee & Mooney, 2012). Sodium alginate is very viscous, and it gels when it comes in contact with a divalent cation such as calcium ion. The divalent cation provides cationic bridges between the guluronic-rich regions of the alginate along the biopolymer backbone (Sirlei Jaiana Kleinübing, Frederico Gai, Caroline Bertagnolli, & Meuris Gurgel Carlos da Silva, 2013). The typical process of immobilizing cells in alginate matrix includes making a 1%–4% alginate solution, mixing it with a concentrated suspension of cells and dropping the mixture into a 2%–4% calcium chloride solution (Katırcıoğlu, Aslım, Türker, Atıcı, & Beyatlı, 2008; Alejandro Ruiz-Marin, Leopoldo G Mendoza-Espinosa, & Tom Stephenson, 2010). The main advantage of using alginate as the immobilization agent is that the immobilization process does not impose extreme physical or chemical changes to the cells. Further, the permeability and transparency of the gel provides a very gentle environment for the cells to grow (Moreno-Garrido, 2008a). A scanning electron microscope (SEM) image of *Scenedesmus sp.* microalgae, immobilized in sodium alginate matrix, is shown in Figure 2.1.

'Green bioprinting' is a new immobilization process, which has been used to scaffold the microalgae immobilized in sodium alginate, using 3D bioprinting (Krujatz et al., 2015). 3D bioprinting process was started by preparing 30 g/L sodium alginate solution, followed by adding 90 g/L of methylcellulose to it. The mixture was then incubated for 2 hours. The incubation process increased the volume of methylcellulose. The biomass suspension was pelleted before the plotting process and re-suspended in the plotting paste. The mixture of microalgae-sodium alginate was then poured into a cartridge, which was connected to the

`

plotting device. The mixture was dispensed using a 250 μm needle with the pressure of 1.4 bar. Compared to suspension cultures, 3D immobilized microalgae showed very stable growth rates over a wide temperature range (26°C, 30°C, or 37°C). Algal growth was reported to be independent of the illumination conditions.

Agarose ($C_{24}H_{38}O_{19}$) is an unbranched polysaccharide, which is extracted from the cell walls of some strains of red algae, mainly from the genera *Gelidium* and *Gracilaria* (Ignacio Moreno-Garrido, 2013). Agar has been used to immobilize algal cells but is best known as a thermos-reversible gel. As an immobilizing agent, it has a major drawback. Agar melts at 85 °C and solidifies at around 35 °C–40 °C. Therefore, the species should be selected carefully for agar immobilization, as a wide variety of algae strains cannot resist temperatures over 30 °C (Moreno-Garrido, 2008a). Generally, Cyanophyte can tolerate higher temperatures than eukaryotic algae, but temperatures close to 50 °C could damage any non-thermophilic species (Ignacio Moreno-Garrido, 2013).

Different methods have been used to immobilize algal cells into agar. Agar has been used to immobilize *Chlorella vulgaris* (*C. vulgaris)* cells to remove inorganic ions (NO_3, NO_2, PO_4, Cr_2O_7) from wastewater. The immobilization was performed by mixing strains of *C. vulgaris* with a 2% (w/v) solution of agar at 35 °C, letting it cool down, and then cutting the solidified mixture into 0.3×0.3×0.3 cm pieces. Even though the agar-immobilized cells used in this study showed high metal and nutrient-uptake rate, their growth rate was slower than the cells immobilized in alginate or carrageenan (N Mallick & Rai, 1994).

Agar also has been used to immobilize *C. vulgaris* for biosorption of Cu (II). The immobilized agar beads in this study were prepared by dropping 2.5% (w/w) agarose solution into edible oil at 40 °C. An ice bath was used to drop the temperature to 15 °C.

`

The immobilized *C. vulgaris* cubes were made by dropping the agarose solution into the biomass and gelling the mixture into 3.6± 0.2 mm diameter spheres. It was reported that the efficiency of the agarose- *C. vulgaris* beads was lower than calcium-alginate beads in metal adsorption. However, there was no assessment on the number of alive cells within the beads (Z Aksu, Eğretli, & Kutsal, 1998).

Carrageenan is a family of sulphated polysaccharides that are extracted from red algae (*Rhodophyceae*). Both bacteria and microalgae have been immobilized in carrageenan.

In one study, three algae strains (*C. vulgaris, Chlorella kessleri* and *Scenedesmus quadricauda*) were immobilized in carrageenan by mixing the gels with each strain and dropping the cells-gel mixtures into a 0.3 M KCl solution (Travieso et al., 1996). The stability of alginate beads was found to be higher than carrageenan; as the carrageenan beads partially lost their structure after a week of usage.

Chitosan is a natural polymer, obtained from chitin, an organic non-toxic compound that forms the exoskeleton of crustacean and other organisms. Sometimes, it is obtained from shrimp shells, which are waste byproducts in shrimp food industry. The chitosan beads were prepared by mixing chitosan flakes with 1% acetic acid at pH 4. The centrifuged microalgae solution was added to the chitosan and the mixture of algae-chitosan was dropped into a 0.1 M NaOH solution and left in agitation for 3 minutes. The immobilized algal beads were picked up from the solution using a strainer (Sashenka Fierro, Maria del Pilar Sánchez-Saavedra, & Carmen Copalcua, 2008). One problem of using chitosan as an immobilizing agent is its weak stability. In one study, the high viscosity of chitosan was used with konjac flour (glucomannan, a dietary fibre, was obtained from the konjac plant) in order to enhance the stability of immobilized *Scenedesmus bicelularis* cells. It was

`

reported that konjac flour did not significantly improve the rheological properties of mixed chitosan solutions (Kaya & Picard, 1996).

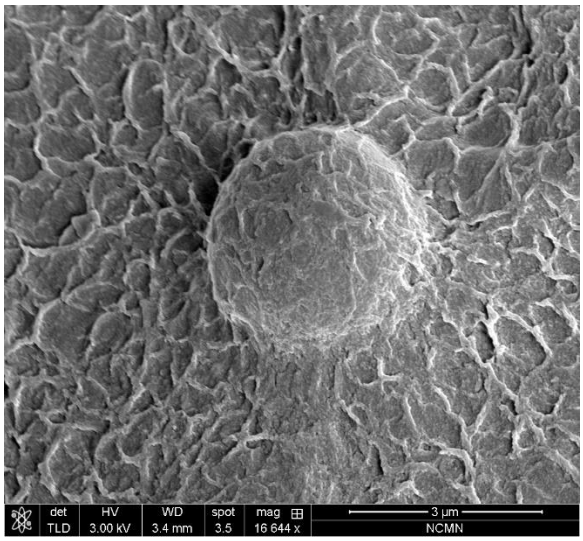


Figure 2.1 SEM image of *Scenedesmus sp.* cells immobilized in sodium alginate matrix.

Synthetic polymers

Most synthetic polymers contain compounds that are considered toxic for the living cells; therefore, using synthetic polymers in algae immobilization might increase the population of dead cells. Polyurethane and polystyrene foams, Polyvinyl alcohol (PVA), silica gel, and polysilicate are the main synthetic polymers used for algal immobilization.

Polyurethane and polystyrene have been used to immobilize *C. vulgaris, Chlorella kessleri,* and *Scenedesmus quadricauda* to remove nutrients from cattle manure (Travieso et al., 1996). To make immobilized cells using these synthetic foams, the cubes of polyurethane and polystyrene were submerged in microalgae culture and aerated for 10 days. Samples of cubes were taken daily and observed under the microscope to assess the colonization process and stability of the support media. The effect of using a natural support medium

`

(calcium alginate) was compared with polyurethane and polystyrene. It was found that polyurethane was a better medium for immobilization in the wastewater, as increasing the phosphate concentration in the wastewater caused the destruction of the alginate pellets (Travieso et al., 1996).

PVA is another synthetic polymer that is used in algal immobilization. PVA has been used to immobilize *Sargassum baccularia*. The immobilization was done by mixing the algae suspension with a 15% PVA solution (Hashim, Tan, & Chu, 2000). The mixture was first dropped into boric acid and then into the sodium phosphate solution to solidify. The lifetime of the immobilized beads was evaluated in multiple cycles of copper adsorption and desorption. Both desorbents (HCl and Ethylenediamine tetraacetic acid (EDTA)) used in this study were effective in stripping the adsorbed copper from the immobilized biomass over five cycles. However, the copper uptake in Cycles 2–5 was lower than that in Cycle 1, confirming that the desorbing agents decrease the lifetime of the immobilized biomass in multiple cycles of adsorption–desorption (Hashim et al., 2000).

Silica gel (SiO_2) is a porous synthetic polymer, which is produced from sodium silicate. Silica gel is a suitable matrix for algal immobilization because of its high porosity and adsorption capacity (Ignacio Moreno-Garrido, 2013).

(Carrilho, Nóbrega, & Gilbert, 2003) immobilized brown alga *Pilayella littoralis* on silica gel. 40 mg of powdered algae and 100 mg of silica gel were mixed and dried at 80 °C for 20 minutes. A few drops of DI water was added to make an algae-silica paste and the paste was dried again at 80 °C for 20 minutes. To achieve a better immobilization, the wetting/drying process was repeated 3–5 times. The final dried silica-algae was then sieved to discard the non-immobilized biomass.

`

A different approach was followed by (Rangsayatorn, Pokethitiyook, Upatham, & Lanza, 2004). The immobilization process started with mixing one gram of cyanobacterial cells (dry weight equivalent) with 25 ml of 6% sodium silicate solution, and 25 ml of DI water. The procedure was followed by adding the mixture to 20 ml of 18% HCl to form the gel. The gel was cut, and the immobilized particles were washed, and dried at 80 °C.

The procedure for immobilization of microalgae in polysilicate polymer has been described previously by (Stark & Rayson, 2000). The process was done by adding the biomass to a 6% $Na_2SiO_4 \cdot 5H_2O$ solution at a pH of 2.0. The incremental addition of the $Na_2SiO_4 \cdot 5H_2O$ was continued until the pH increased to 7.0. The mixture was then washed with DI water and baked overnight at 80 °C.

Due to the high temperature applied in these techniques, no living algal cells remain within the immobilization matrix (Ignacio Moreno-Garrido, 2013).

In a very recent study, the efficiency and stability of the bacteria entrapped within the Silica High Internal Phase Emulsion [Si(HIPE)] shell were investigated (Roucher et al., 2018). The Si(HIPE) beads were synthesized by adding 5.02 g of Tetraethyl-ortho-silane (TEOS) to 16.02 g of an aqueous solution of tetradecyltrimethylammonium bromide (TTAB) (35%). 5.88 g of 37% HCl solution was then added to the mixture and left under stirring for 5 minutes. Later, 35 g of dodecane was added to the mixture and transferred drop by drop to polydimethylsiloxane oil to form droplets. The droplets were left at room temperature for 3 days to form the Si(HIPE) beads.

The Si(HIPE) beads were sterilized and transferred to M9 medium and left under static vacuum for 3 days to get prepared for microbial colonization. To colonize the beads with *E. coli* bacteria, one colony was inoculated into a flask containing M9 medium and

`

chloramphenicol. The flask was then placed in an incubator overnight until an optical density (OD_{600}) of 0.6 was achieved. The Si(HIPE) beads were then placed into the culture medium for bacterial colonization. The incubation was continued overnight at 25 °C. Finally, a sodium silicate shell was synthesized to confine bacteria inside the Si(HIPE) beads. The sodium silica shell was prepared using Phosphate-buffered saline (PBS) and 200 mM of sodium silicate solution which were acidified with HCl to the pH to 7. The bacteria beads were added to the solution and agitated until the gelation started. To check the stability of the bacteria entrapped in the silica shell, Si(HIPE) beads were observed for bacteria leakage over a period of 6 days. After 3 days, the leakage was only observed for one bead, and 75% of the beads kept their impermeability. After 6 days, the leakage was observed for 50% of the beads, which gave the silica coating the half-life of 6 days. On 6th day, the beads were all crushed on agar plate and large amounts of colonies were observed, which indicated the bacteria viability after 6 days of immobilization in the silica shell.

All the preparation methods are summarized in Table 2.1.

Table 2.1 Preparation methods for immobilizing microalgae in natural and synthetic polymers.

Immobilization matrix	**Preparation method**	**References**
Loofa sponge	Soak the sponge cuts in boiling water for 30 minutes Wash with tap water and leave them in DI water for 24 hours Dry the sponge cuts at 70°C Autoclave and soak in culture medium for 10 minutes	(N Akhtar et al., 2004)
Glass, wood, plastic	Clean the substrates with 99% ethanol Place them at well-illuminated locations in the lake Measure algal communities on the substrates after 9 weeks of exposure	(Danilov & Ekelund, 2001)
Sodium/ Calcium alginate	Make a 1%–4% sodium/calcium-alginate solution Mix it with a concentrated suspension of algae Drop the mixture into a 2%–4% calcium chloride solution	(Katırcıoğlu et al., 2008; Alejandro Ruiz-Marin et al., 2010)

`

3D bioprinting of alginate beads	Make a 30 g/L sodium alginate solution Add 90 g/L of methylcellulose to it. Incubate the mixture for 2 hours Pellet the biomass suspension before the plotting process and re-suspend in the plotting paste Pour the mixture of microalgae- sodium alginate into a cartridge, which is connected to the plotting device Dispense the mixture using a 250 μm needle with the pressure of 1.4 bar	(Krujatz et al., 2015)
Agarose (1)	Mix the algal suspension with a 2% (w/v) solution of agar at 35 °C Let it cool down and cut the solidified mixture	(N Mallick & Rai, 1994)
Agarose (2)	Drop 2.5% (w/w) agarose solution into edible oil at 40 °C. Use an ice bath simultaneously to drop the temperature to 15 °C Drop the agarose solution to algae suspension and gel the mixture into spheres	(Z Aksu et al., 1998)
Carrageenan	Mix the carrageenan gel with algal solution Drop the cells-gel mixtures into a 0.3 M KCl solution using a syringe Continue mixing at 100 rpm until pellets are formed Keep the formed pellets in the solution until their utilization	(Travieso et al., 1996)
Chitosan	Mix the chitosan flakes with 1% acetic acid in PH 4. Add the centrifuged microalgae solution to the chitosan Drop the gel mixture into a 0.1 M NaOH solution Leave the mixture in agitation for 3 minutes	(Sashenka Fierro et al., 2008)
Silica gel (1)	Dry 40 mg of powdered algae and 100 mg of silica gel at 80 °C for 20 minutes and mix them Add DI water to make algae-silica paste and dry it at 80 °C for 20 minutes Repeat the wetting/drying 3–5 times for better immobilization Sieve the final dried silica-algae	(Carrilho et al., 2003)
Silica gel (2)	Mix one gram of biomass (dry weight equivalent) with 25 ml of 6% sodium silicate solution and 25 ml of DI water Add the mixture to 20 ml of 18% HCl to form the gel Cut the gel and dry the pieces at 80 °C	(Rangsayatorn et al., 2004)
Silica High Internal Phase Emulsion [Si(HIPE)] shell	Add 5.02 g of TEOS to 16.02 g (35% aqueous solution) of TTAB Add 5.88 g of 37% HCl solution to the mixture and leave it for 5 minutes under stirring Add 35 g of dodecane to the mixture and transfer to polydimethylsiloxane oil to form droplets Leave the droplets for 3 days to form Si(HIPE) beads Transfer the beads to M9 medium and leave under static vacuum for 3 days Prepare the sodium silica shell using PBS and 200 mM of sodium silicate solution, which were acidified with HCl to lower the pH to 7	(Roucher et al., 2018)

`

	Add the beads to the solution and agitate until it forms gel	
Polyciliate	Add the biomass to a 6% solution of $Na_2SiO_4 \cdot 5H_2O$ at pH of 2. Continue adding $Na_2SiO_4 \cdot 5H_2O$ until the pH increases to 7 Wash the mixture with DI water and bake it overnight at 80 °C	(Stark & Rayson, 2000)
Polyurethane and polystyrene	Submerge PU cubes in microalgae culture and aerate for 10 days Take samples of cubes every day and observe under the microscope to check the colonization process, and stability of the support media	(Córdoba, Hernandez, & Weiland, 1995)
Polyvinyl alcohol (PVA)	Mix the algae solution with 15 % PVA solution. Drop the mixture into boric acid and then to the sodium phosphate solution Wait until the mixture solidifies	(Hashim et al., 2000)

2.3 Cultivation Methods and Application of Immobilized Algae

As a sustainable treatment system, immobilized algal beads have been widely used to remove nutrients (nitrate and phosphate), metals, and organic compounds from water and wastewater. Depending on the algae strain, there are three main cultivation modes which can be used for immobilized algal beads: photoautotrophic, heterotrophic, and mixotrophic growth (Jinghan Wang, Haizhen Yang, & Feng Wang, 2014). Generally, microalgae perform photosynthesis by fixing dissolved carbon dioxide and absorbing light. Therefore, they are considered photoautotrophs. Some strains of microalgae, however, use organic compounds as their carbon and energy sources without depending on light as their source of energy. They are called heterotrophic algae. During heterotrophic growth, assimilation of organic carbon generates energy by oxidative phosphorylation, followed by consumption of oxygen as the final electron acceptor. Hence, heterotrophic growth is an aerobic process (Perez-Garcia & Bashan, 2015).

Mixotrophic growth is another form of cultivation where the algae uses both inorganic and organic carbon sources in the presence of light (Kang et al., 2004). Mixotrophic algae

`

strains are more flexible because they gather both carbon and energy through organic or inorganic sources and light, simultaneously (Perez-Garcia & Bashan, 2015).

Depending on the algae strain, the three mentioned cultivation modes can be used with immobilized algae. However, heterotrophic growth is an easier choice, especially on a large scale, as it does not have the complications of light availibility. Heterotrophic cultivation of algae makes the process cheaper and simpler to construct (Octavio Perez-Garcia, Froylan ME Escalante, Luz E de-Bashan, & Yoav Bashan, 2011).

2.3.1 Nutrient Removal

One of the challenges facing microalgae-based biological treatment system is the loss of biomass in a continuous flow bioreactor. Another problem which makes the system economically unfavorable is the high costs of harvesting biomass at the end of the treatment process, which needs either centrifugation or filtration. Immobilization techniques solve both problems. Most immobilization techniques that are used to immobilize bacteria can also be adapted for algae (Ignacio Moreno-Garrido, 2013). Microalgae cultivation in wastewater has been an alternative system for biological wastewater treatment, as it has the advantages of removing nutrients and producing biomass. Microalgae can grow rapidly and remove nutrients from wastewater, and the remaining biomass can be used to produce algal metabolites or biogas (Delgadillo-Mirquez, Lopes, Taidi, & Pareau, 2016).

Strains of *S. obliquus* and *C. vulgaris* immobilized in sodium alginate have been used in batch and semi-continuous modes to remove nutrients from urban wastewater (Alejandro Ruiz-Marin et al., 2010). The immobilized *S. obliquus* were found more efficient in ammonia removal from urban wastewater and they grew faster than *C. vulgaris* treating urban and/or artificial wastewater; thus, suggesting that *S. obliquus* could be a suitable

`

choice to design semi-continuous bioreactors. Compared to *C. vulgaris, S. obliquus* was able to remove nitrogen and phosphorous for longer periods (181 hours), as explained in Table 2.2.

In another study, the growth rate and nutrient removal efficiency of *Scenedesmus intermedius* and *Nannochloris sp.* isolated from pig manure and immobilized in calcium alginate were evaluated (Jimenez-Perez, Sanchez-Castillo, Romera, Fernandez-Moreno, & Pérez-Martınez, 2004). Nitrate and phosphate removal efficiency of free cells were found to be higher than immobilized cells. Phosphate and nitrate removal rates, using species isolated from wastewater, were higher than the rates obtained with commercial species. Since the isolated species were adapted to high-nutrient concentrations they were better candidates for nutrient removal.

Immobilized algal cells can also be used as an alternative system for the biological treatment to remove nitrate from groundwater (Carlos Garbisu, Gil, Bazin, Hall, & Serra, 1991). The nitrate uptake of *Phormidium laminosum* cells, immobilized in polyurethane foam from water was studied in batch and continuous flow reactors (Carlos Garbisu et al., 1991). Entrapment in PU matrix was found to be toxic to the cells and caused the death of the immobilized cells. Therefore, the experiments were performed by adsorbing the cells onto PU foams. In this study, chlorophyll was used as an indicator of algal biomass. The immobilized *P. laminosum* cells removed 1.1 $\mu Mmg^{-1}chl\ h^{-1}$ of nitrate while the free cells were able to uptake nitrate at 2.6 $\mu Mmg^{-1}chl\ h^{-1}$. The lower rate of nitrate uptake in immobilized cells was reported to be due to the limitation in diffusion of nutrients into the cells.

`

Mats of electrospun chitosan nanofibers were also found to be effective in immobilizing *C. vulgaris*, and a durable system for removing nitrate from wastewater. The immobilized *C. vulgaris* mats were able to remove around 87% nitrate from the bulk solution in 15 days (initial nitrate concentration was 30 mg/L NO_3-N). It was discussed that the nanofiber mat in the liquid initiated the removal of nitrate, while the growth of algae subsequently consumed the remaining nitrate with a slower rate (E. Eroglu et al., 2012).

In another work, the effect of immobilizing *Scenedesmus sp.* (strains 1 and 2) and *S. obliquus* cells in chitosan matrix was evaluated on their viability, growth, and nitrate and phosphate uptake.

Table 2.2 Summary of studies done on nutrient removal using immobilized algae.

Immobilized algae	**Medium**	**Results**	**References**
S. obliquus and *C. Vulgaris*	Sodium alginate	Immobilized *S. obliquus* were more efficient in ammonia removal and grew faster than *C. vulgaris***.** *S. obliquus*. They able to remove nitrogen and phosphorous for longer periods (181 hours)	(Alejandro Ruiz-Marin et al., 2010)
S. intermedius and *Nannochloris sp.*	Calcium alginate	Nutrient removal efficiency of free cells was higher than immobilized cells. Using species isolated from wastewater will result in higher nitrate and phosphate removal	(Jimenez-Perez et al., 2004)
P. laminosum	Polyurethane foam	Free *P. laminosum* cells removed twice more nitrate than immobilized beads. The lower rate of nitrate uptake was reported to be due to the limitation in diffusion of nutrients into the cells	(Carlos Garbisu et al., 1991)
C. vulgaris	Chitosan	Immobilized mats removed around 87% nitrate from the bulk in 15 days	(E. Eroglu et al., 2012)
Scenedesmus sp. and *S.obliquus*	Chitosan	Immobilized *Scenedesmus sp*. had a higher growth rate than its free living. Immobilized cells removed 70% nitrate and 94% phosphate within 12 hours while free cells removed 20% nitrate and 30% phosphate within 36 hours of treatment	(Sashenka Fierro et al., 2008)

It was found that immobilized *Scenedesmus sp*. (strains 1) had a higher growth rate than its free living. Also, the immobilized cells were able to remove 70% nitrate and 94%

`

phosphate within 12 hours of incubation while free-living cells removed 20% nitrate and 30% phosphate within 36 hours of treatment (Sashenka Fierro et al., 2008).

2.3.2 Metal Removal

Industrial processes are known to release heavy metals in the natural water systems, which increases the concerns about the effects of toxic metal as environmental contaminants (Jalali, Ghafourian, Asef, Davarpanah, & Sepehr, 2002). Many algae species are known to have capability to sorb metals, and because of that, there is immense potential for using them in wastewater treatment. Algal cells are able to remove metals from multi-metal solutions; and dead cells are reported to be more efficient in metal sorption than live cells (Mehta & Gaur, 2005). Metal uptake by algal cells can either happen actively (bioaccumulation) and/or passively (biosorption) (Jalali et al., 2002).

Biosorption of four metals [Cu(II), Fe(II), Ni(II) and Zn(II)] using the strain of *P. laminosum* immobilized in polysulphone and epoxy resin was previously evaluated (Blanco et al., 1999). According to this study, the biosorption rate is dependent on the wetting of biomass beads, as the rate of metal biosorption decreased with the use of dry biomass beads. Also, using smaller immobilized beads increased the rate of metal biosorption. The cells immobilized in polysulphone showed the potential to be reused for at least 10 consecutive biosorption/desorption cycles without any loss of efficiency after reconditioning with 0.1 M solution of NaOH.

In another study, biosorption of nickel on free (live and dead) and immobilized dead *C. vulgaris* cells and blank alginate beads was evaluated (Al-Rub, El-Naas, Benyahia, & Ashour, 2004). The results indicated that immobilization increases the nickel removal because of the surface sorption contribution by the beads. The blank alginate beads resulted

`

in higher nickel removal than the free algal cells, which was another indicator that immobilization enhanced nickel sorption.

Another study was conducted to investigate the biosorption mechanism of radioisotopes of metals (^{60}Co, ^{54}Mn and ^{65}Zn) using *Chlorella salina* immobilized in calcium-alginate matrix. It was found that the accumulation of metal in algae includes a rapid biosorption, which does not depend on temperature, light, or metabolic inhibitor, followed by a slower accumulation, which is dependent on the cellular metabolism (Moreno-Garrido, 2008a).

One suitable tool to select the metal-tolerate algae strains is isolating them from the polluted water. The strain of *Anacystis nidulans*, isolated from polluted waters, was able to grow in a medium containing Cr with the maximum concentration of 100 μM (Khattar, Sarma, & Singh, 1999).

In another study, the efficiency of the *Chlorella sorokiniana* (LSIBCS), isolated from wastewater and immobilized in loofa sponge, was investigated in removing Cr (III) from aqueous solution. Comparing the biosorption capability of the free biomass of *C. sorokiniana* (FBCS) and LSIBCS showed 17.79% increase in the uptake when the algal cells were used in immobilized form. The highest biosorption rate for FBCS and LSIBCS were reported to be 58.80 and 69.26 mg Cr(III)/g biosorbent, respectively (Nasreen Akhtar, Iqbal, Zafar, & Iqbal, 2008). Table 2.3 summarizes the results on metal removal from the previous studies.

2.3.3 Organic Compounds Removal

In recent years, the amount of hazardous organic compounds being discharged into the environment has increased (Zümriye Aksu, 2005). Some strains of microalgae can biodegrade hazardous organic pollutants, as shown in Table 2.4.

`

Table 2.3 Summary of studies done on metal removal using immobilized algae.

Immobilized algae	**Medium**	**Metals removed**	**Results**	**References**
P. laminosum	Polysulphone and epoxy resin	Cu(II) Fe(II) Ni(II) Zn(II)	Cells showed the potential to be reused in at least 10 consecutive biosorption/desorption cycles Using smaller immobilized beads increased the biosorption rate	(Blanco et al., 1999)
C. vulgaris immobilized	Alginate	Ni	Immobilization increased the nickel removal. Blank alginate beads showed higher Ni removal than the free cells	(Al-Rub et al., 2004)
C. salina	Calcium alginate	^{60}Co ^{54}Mn ^{65}Zn	Accumulation of metal in algae included a rapid biosorption, which does not depend on temperature, light, or metabolic inhibitor, followed by a slower accumulation, which is dependent on the cellular metabolism	(Moreno-Garrido, 2008a)
Chlorella sorokiniana	Loofa sponge	Cr(III)	Metal uptake increased by 18% when using immobilized cells. Maximum biosorption capacity was 69 mg and 59 mg Cr(III)/g biosorbent for immobilized and free cells, respectively	(Nasreen Akhtar et al., 2008)

Dried *C. vulgaris* has been used for biosorption of three vinyl sulphone type reactive dyes: Remazol Black B (RB), Remazol Red RR (RR) and Remazol Golden Yellow RNL (RGY) in batch reactor. It was found that the dye sorption was highly dependent on the pH and temperature. The optimum pH was 2 and the temperature was found to impact on the process in an inversely proportional way. The maximum biosorption capacity occurred at 35 °C for RB and at 25 °C for RR and RGY (Zümriye Aksu & Tezer, 2005).

The capability of *Prototheca zopfii*, immobilized in polyurethane was evaluated in degradation of n-alkanes. Immobilization of *P. zopfii* in polyurethane cubes improved the volumetric biodegradation rate of hydrocarbons compared to the ones with free and alginate-immobilized cells. The improvement in removal rate was due to the affinity of

`

algal cells to the substrates in the foam cubes, hydrophobic interaction between the foam cubes and the substrate, and cell attachment to the foam cubes due to the hydrophobic interaction (Yamaguchi, Ishida, & Suzuki, 1999).

Table 2.4 Summary of studies done on organic pollutant removal using free and immobilized microorganisms.

Algae strain	**Medium**	**Organic compounds removed**	**Results**	**References**
Dried *C. vulgaris*	Vinyl sulphone	Remazol Black B Remazol Red RR Remazol Golden Yellow RNL	Dye sorption was highly dependent on pH and temperature. The optimum pH was 2 and the maximum biosorption capacity occurred at 35 °C for RB and at 25 °C for RR and RGY	(Zümriye Aksu & Tezer, 2005)
Prototheca zopfii	PU	n-alkanes	Immobilization improved the biodegradation rate for hydrocarbons	(Yamaguchi et al., 1999)
Oil-degrading yeast cells, *Yarrowia lipolytica* 180	PU foam	Crude oil	Immobilized cells absorbed 7–9 times their own weight of crude oil. Less than 5% of the absorbed oil was released when they were left on the water for more than 10 days	(Oh et al., 2000)
Activated sludge cells	PVA	2,4,6-trichlorophenol (TCP)	99.9 to 91% TCP removal efficiency was observed at loading of 300 to 600 mg/L-d with corresponding hydraulic retention times of 24.5 to 12.3 minutes, respectively. Immobilized cells stayed permeable during 166 days of experiments	(Razavi-Shirazi & Veenstra, 2000)

In another study, oil-degrading yeast cells, *Yarrowia lipolytica 180* immobilized in polyurethane foam (PUF) were used to absorb and degrade oil on water surface (Oh, Maeng, & Kim, 2000). PUF-immobilized cells were capable of absorbing crude oil up to

`

7–9 times their own weight. The immobilized cells showed high floatability in seawater. Less than 5% of the absorbed oil was released from the beads when they were left on the water for more than 10 days (Oh et al., 2000).

In another work, a biological permeable barrier medium was designed to remove 2,4,6-trichlorophenol (TCP) from groundwater (Razavi-Shirazi & Veenstra, 2000). PVA-immobilized activated sludge cells were found to be a successful permeable barrier. They removed 99.9% and 91% TCP, at loading rates of 300 and 600 mg/L-d with corresponding hydraulic retention times of 24.5 and 12.3 minutes, respectively. The immobilized cells stayed permeable during 166 days of continuous reactor experiments.

2.4 Conclusions

This chapter summarized the fundamentals and recent research advances of the microalgae immobilization techniques with examples of their application in water and wastewater treatment. The following are the conclusions drawn from this section:

1. Natural polymers are found to have a higher nutrient diffusion rate and synthetic polymers have shown more stability in the wastewater. Among the immobilization carriers, alginate is the most suitable one as it's permeable and transparent enough for light and nutrient diffusion, has high stability and does not impose extreme physical or chemical condition changes to the cells.

2. Heterotrophic growth is the most suitable choice for algae cultivation, as it doesn't have the complications of light availibility and makes the process cheaper and simpler to operate.

3. Immobilization of microalgae is a sustainable technique to remove nitrate, phosphate, organic pollutants (Dyes, n-alkanes, crude oil and TCP) and metals (Cu, Fe, Ni, Zn, Co,

`

Mn, Cr) from water and wastewater. When used in wastewater treatment, choosing species isolated from wastewater will enhance the pollutants removal rate.

`

References

Akhtar, N., J. Iqbal, and M. Iqbal. 2003. Microalgal-luffa sponge immobilized disc: a new efficient biosorbent for the removal of Ni (II) from aqueous solution. *Letters in Applied Microbiology* 37**(2)**:149–153.

Akhtar, N., J. Iqbal, and M. Iqbal. 2004. Removal and recovery of nickel (II) from aqueous solution by loofa sponge-immobilized biomass of Chlorella sorokiniana: characterization studies. *Journal of Hazardous Materials* 108**(1–2)**:85–94.

Akhtar, N., M. Iqbal, S. I. Zafar, and J. Iqbal. 2008. Biosorption characteristics of unicellular green alga Chlorella sorokiniana immobilized in loofa sponge for removal of Cr (III). *Journal of Environmental Sciences* 20**(2)**:231–239.

Aksu, Z. 2005. Application of biosorption for the removal of organic pollutants: a review. *Process Biochemistry* 40**(3–4)**:997–1026.

Aksu, Z., G. Eğretli, and T. Kutsal. 1998. A comparative study of copper (II) biosorption on Ca-alginate, agarose and immobilized C. *vulgaris* in a packed-bed column. *Process Biochemistry* 33**(4)**:393–400.

Aksu, Z. and S. Tezer. 2005. Biosorption of reactive dyes on the green alga *Chlorella vulgaris*. *Process Biochemistry* 40(3–4):1347–1361.

Al-Rub, F. A., M. El-Naas, F. Benyahia, and I. Ashour. 2004. Biosorption of nickel on blank alginate beads, free and immobilized algal cells. *Process Biochemistry* 39**(11)**:1767–1773.

AlMomani, F. A. and B. Örmeci. 2016. Performance of *Chlorella Vulgaris*, *Neochloris Oleoabundans*, and mixed indigenous microalgae for treatment of primary effluent, secondary effluent and centrate. *Ecological Engineering* 95:280–289.

Blanco, A., B. Sanz, , M. J. Llama, and J. L. Serra. 1999. Biosorption of heavy metals to immobilised Phormidium laminosum biomass. *Journal of Biotechnology* 69**(2–3)**:227–240.

Carrilho, E. N. V., J. A. Nóbrega, and T. R. Gilbert. 2003. The use of silica-immobilized brown alga (*Pilayella littoralis*) for metal preconcentration and determination by inductively coupled plasma optical emission spectrometry. *Talanta* 60**(6)**:1131–1140.

Chevalier, P. and J. de la Noüe. 1985. Wastewater nutrient removal with microalgae immobilized in carrageenan. *Enzyme and Microbial Technology* 7**(12)**:621–624.

Córdoba, L. T., E. S. Hernandez, and P. Weiland. 1995. Final treatment for cattle manure using immobilized microalgae. I. Study of the support media. *Resources, Conservation and Recycling* 13**(3–4)**:167–175.

Danilov, R. A. and N. Ekelund. 2001. Comparison of usefulness of three types of artificial substrata (glass, wood and plastic) when studying settlement patterns of periphyton in lakes of different trophic status. *Journal of Microbiological Methods* 45**(3)**:167–170.

de-Bashan, L. E. and Y. Bashan. 2010. Immobilized microalgae for removing pollutants: review of practical aspects. *Bioresource Technology* 101**(6)**:1611–1627.

de-Bashan, L. E., Y. Bashan, M. Moreno, V. K. Lebsky, and J. J. Bustillos. 2002. Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae Chlorella spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium Azospirillum brasilense. *Canadian Journal of Microbiology* 48**(6)**:514–521.

`

Delgadillo-Mirquez, L., F. Lopes, B. Taidi, and D. Pareau. 2016. Nitrogen and phosphate removal from wastewater with a mixed microalgae and bacteria culture. *Biotechnology Reports* 11:18–26.

Eroglu, E., V. Agarwal, M. Bradshaw, X. Chen, S. M. Smith, C. L. Raston, and K. S. Iyer. 2012. Nitrate removal from liquid effluents using microalgae immobilized on chitosan nanofiber mats. *Green Chemistry* 14**(10)**:2682–2685.

Eroglu, E., S. M. Smith, and C. L. Raston. 2015. 'Application of various immobilization techniques for algal bioprocesses'. Biomass and biofuels from microalgae. *Springer* 19–44.

Fierro, S., M. del Pilar Sánchez-Saavedra, and C. Copalcua. 2008. Nitrate and phosphate removal by chitosan immobilized Scenedesmus. *Bioresource Technology* 99**(5)**:1274–1279.

Garbisu, C., J. M. Gil, M. J. Bazin, D. O. Hall, and J. L. Serra. 1991. Removal of nitrate from water by foam-immobilized *Phormidium laminosum* in batch and continuous-flow bioreactors. *Journal of Applied Phycology* 3**(3)**:221–234.

Ghosh, M. and J. P. Gaur. 1998. Current velocity and the establishment of stream algal periphyton communities. *Aquatic Botany* 60**(1)**:1–10.

Hashim, M., H. Tan, and K. Chu. 2000. Immobilized marine algal biomass for multiple cycles of copper adsorption and desorption. *Separation and Purification Technology* 19**(1–2)**:39–42.

Jalali, R., H. Ghafourian, Y. Asef, S. Davarpanah, and S. Sepehr. 2002. Removal and recovery of lead using nonliving biomass of marine algae. *Journal of Hazardous Materials* 92**(3)**:253–262.

Jimenez-Perez, M., P. Sanchez-Castillo, O. Romera, D. Fernandez-Moreno, and C. Pérez-Martınez. 2004. Growth and nutrient removal in free and immobilized planktonic green algae isolated from pig manure. *Enzyme and Microbial Technology* 34**(5)**:392–398.

Kang, R., J. Wang, D. Shi, W. Cong, Z. Cai, and F. Ouyang. 2004. Interactions between organic and inorganic carbon sources during mixotrophic cultivation of Synechococcus sp. *Biotechnology Letters* 26**(18)**:1429–1432.

Katırcıoğlu, H., B. Aslım, A. R. Türker, T. Atıcı, and Y. Beyatlı. 2008. Removal of cadmium (II) ion from aqueous system by dry biomass, immobilized live and heat-inactivated Oscillatoria sp. H1 isolated from freshwater (Mogan Lake). *Bioresource Technology* 99**(10)**:4185–4191.

Kaya, V. M. and G. Picard. 1996. Stability of chitosan gel as entrapment matrix of viable Scenedesmus bicellularis cells immobilized on screens for tertiary treatment of wastewater. *Bioresource Technology* 56**(2–3)**:147–155.

Khattar, J., T. Sarma, and D. Singh. 1999. Removal of chromium ions by agar immobilized cells of the cyanobacterium Anacystis nidulans in a continuous flow bioreactor. *Enzyme and Microbial Technology* 25**(7)**:564–568.

Kleinübing, S. J., F. Gai, C. Bertagnolli, and M. G. C. d. Silva. 2013. Extraction of alginate biopolymer present in marine alga Sargassum filipendula and bioadsorption of metallic ions. *Materials Research* 16**(2)**:481–488.

Krujatz, F., A. Lode, S. Brüggemeier, K. Schütz, J. Kramer, T. Bley, M. Gelinsky, and J. Weber. 2015. Green bioprinting: Viability and growth analysis of microalgae immobilized in 3D-plotted hydrogels versus suspension cultures. *Engineering in Life Sciences* 15**(7)**:678–688.

Lau, P., N. Tam, and Y. Wong. 1998. Effect of carrageenan immobilization on the physiological activities of *Chlorella vulgaris*. *Bioresource Technology* 63**(2)**:115–121.

Lee, K. Y. and D. J. Mooney. 2012. Alginate: properties and biomedical applications. Progress in Polymer Science 37**(1)**:106–126.

`

Liu, Y.-K., M. Seki, H. Tanaka, and S. Furusaki. 1998. Characteristics of loofa (*Luffa cylindrica*) sponge as a carrier for plant cell immobilization. Journal of Fermentation and Bioengineering 85**(4)**:416–421.

Mallick, N. 2002. Biotechnological potential of immobilized algae for wastewater N, P and metal removal: a review. *Biometals* 15**(4)**:377–390.

Mallick, N. and L. Rai. 1994. Removal of inorganic ions from wastewaters by immobilized microalgae. *World Journal of Microbiology and Biotechnology* 10**(4)**:439–443.

Martins, S. C. S., C. M. Martins, L. M. C. G. Fiúza, and S. T. Santaella. 2013. Immobilization of microbial cells: A promising tool for treatment of toxic pollutants in industrial wastewater. *African Journal of Biotechnology* 12**(28)**.

Mehta, S. and J. Gaur. 2005. Use of algae for removing heavy metal ions from wastewater: progress and prospects. *Critical Reviews in Biotechnology* 25**(3)**:113–152.

Moreno-Garrido, I. 2008. Microalgae immobilization: current techniques and uses. *Bioresource Technology* 99**(10)**:3949–3964.

Moreno-Garrido, I. 2013. 'Microalgal immobilization methods'. Immobilization of Enzymes and Cells. *Springer* 327–347.

Nikovskaya, G. 1989. The adhesive immobilization of microorganisms in water purification. *Khim. Tekhnol. Vody* 11**(2)**:158–169.

Nunez, V. J., D. Voltolina, M. Nieves, P. Pina, A. Medina, and M. Guerrero. 2001. Nitrogen budget in *Scenedesmus obliquus* cultures with artificial wastewater. *Bioresource Technology* 78**(2)**:161–164.

Ogbonna, J. C., Y.-C. Liu, Y.-K. Liu, and H. Tanaka. 1994. Loofa (*Luffa cylindrica*) sponge as a carrier for microbial cell immobilization. *Journal of Fermentation and Bioengineering* 78**(6)**:437–442.

Oh, Y.-S., J. Maeng, and S.-J. Kim. 2000. Use of microorganism-immobilized polyurethane foams to absorb and degrade oil on water surface. *Applied Microbiology and Biotechnology* 54**(3)**:418–423.

Perez-Garcia, O. and Y. Bashan. 2015. 'Microalgal heterotrophic and mixotrophic culturing for bio-refining: From metabolic routes to techno-economics'. Algal biorefineries. *Springer* 61–131.

Perez-Garcia, O., F. M. Escalante, L. E. de-Bashan, and Y. Bashan. 2011. Heterotrophic cultures of microalgae: metabolism and potential products. *Water Research* 45**(1)**:11–36.

Rangsayatorn, N., P. Pokethitiyook, E. Upatham, and G. Lanza. 2004. Cadmium biosorption by cells of *Spirulina platensis* TISTR 8217 immobilized in alginate and silica gel. *Environment International* 30**(1)**:57–63.

Razavi-Shirazi, F. 2002. Biological permeable barrier to treat contaminated groundwater using immobilized cells. Google Patents.

Razavi-Shirazi, F. and J. N. Veenstra. 2000. Development of a biological permeable barrier to remove 2, 4, 6-trichlorophenol from groundwater using immobilized cells. *Water Environment Research* 72**(4)**:460–468.

Robinson, P., A. Mak, and M. D. Trevan. 1986. Immobilized algae; a review. *Process Biochemistry*. 21(4): 122-127

`

Roucher, A., M. Morvan, D. Pekin, M. Depardieu, J. L. Blin, V. Schmitt, M. Konrad, J. C. Baret, and R. Backov. 2018. From compartmentalization of bacteria within inorganic macrocellular beads to the assembly of microbial consortia. *Advanced Biosystems* 2**(3)**:1700233.

Ruiz-Marin, A., L. G. Mendoza-Espinosa, and T. Stephenson. 2010. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater. *Bioresource Technology* 101**(1)**:58–64.

Saeed, A. and M. Iqbal. 2006. Immobilization of blue green microalgae on loofa sponge to biosorb cadmium in repeated shake flask batch and continuous flow fixed bed column reactor system. *World Journal of Microbiology and Biotechnology* 22**(8)**:775–782.

Shirazi, F. 2018. A new economic reality for clean and safe drinking water. Retrived from https://www.microvi.com/drinkingwater

Stark, P. C. and G. D. Rayson. 2000. Comparisons of metal-ion binding to immobilized biogenic materials in a flowing system. *Advances in Environmental Research* 4**(2)**:113–122.

Travieso, L., F. Benitez, P. Weiland, E. Sanchez, R. Dupeyron, and A. Dominguez. 1996. Experiments on immobilization of microalgae for nutrient removal in wastewater treatments. *Bioresource Technology* 55**(3)**:181–186.

Urrutia, I., J. L. Serra, and M. J. Llama. 1995. Nitrate removal from water by Scenedesmus obliquus immobilized in polymeric foams. *Enzyme and Microbial Technology* 17**(3)**:200–205.

Wang, J., H. Yang, and F. Wang. 2014. Mixotrophic cultivation of microalgae for biodiesel production: Status and prospects. *Applied Biochemistry and Biotechnology* 172**(7)**:3307–3329.

Yamaguchi, T., M. Ishida, and T. Suzuki. 1999. An immobilized cell system in polyurethane foam for the lipophilic micro-alga *Prototheca zopfii*. *Process Biochemistry* 34(2):167–171.

`

CHAPTER 3

DEVELOPMENT AND OPTIMIZATION OF BATCH AND SEQUENCING BATCH REACTORS FOR NITRATE REMOVAL FROM GROUNDWATER[2]

3.1 Abstract

The treatment efficiency of *Chlorella sorokiniana* and *Scenedesmus species*, immobilized in sodium alginate was evaluated for removing nitrate from groundwater. The experiments were performed initially in batch mode and the best-performing conditions were replicated in sequencing batch reactor mode. *S. sp.* showed a higher nitrate uptake in short term than *C. sorokiniana*. Immobilized *S. sp.* and *C. sorokiniana* cells showed 90% nitrate removal in 9 and 12 days, respectively. The optimal ratio of algal beads/water was found to be 12.5% (v:v). Comparatively, suspended *S. sp.* cells were able to remove only up to 35% of nitrate in 8 days. Alginate immobilized *S. sp.* beads were capable of uptaking nitrate for 100 consecutive days in sequencing batch reactor mode. When tested in actual groundwater, 90% of nitrate was eliminated in 2 days without need for any additional carbon source. Immobilized algal beads can be a low-cost alternative technique to remove nitrate from groundwater as they are water-insoluble, non-toxic, easy to harvest and offer high removal efficiency.

3.2 Introduction

[2] This chapter is based on the publication: Mollamohammada, S., Aly Hassan, A., & Dahab, M. (2020). Nitrate Removal from Groundwater Using Immobilized Heterotrophic Algae. *Water, Air, & Soil Pollution, 231*(1), 1-13.

`

Nitrate is the most common contaminant in the groundwater's aquifers (Wheeler, Nolan, Flory, DellaValle, & Ward, 2015). About 21% of public water supply and 98% of self-supplied water in 2010 was extracted from groundwater (Survey, 2010). The extensive use of nitrate based fertilizers is the largest contributor to nitrate contamination, therefore, the highest concentration of nitrate in groundwater has been observed in agricultural areas (Fields, 2004; Lawniczak et al., 2016). US Environmental Protection Agency (US EPA) has set a maximum contaminant level (MCL) of 10 mg/l NO_3-N for nitrate. (Agency, 2018). Exposure to drinking water with a nitrate level above the MCL is a potential health risk for infants and sometimes, for adults. Methemoglobinemia, which is the result of nitrate reduction to nitrite in infants' digestive system is reported to be common between infants who have consumed water with elevated levels of nitrate (M. Liu, Liu, Bawa, & Chen, 2012). Moreover, exposure to drinking water contaminated with nitrate and atrazine, a type of herbicide used as weed killer, is reported as a potential health risk, because it can form nitrosamine. Nitrosamine can increase the risk of developing Non-Hodgkin Lymphoma (Rhoades et al., 2013b).

Reverse osmosis (RO) and ion exchange are generally considered to be the best available nitrate treatment technologies, however, they are associated with high capital and operating costs. Furthermore, RO membranes retain nitrate and other contaminants into a concentrated waste brine that poses a disposal problem (Y. Zhang et al., 2012) Most available ion exchange nitrate removal resins suffer from the inherent natural low selectivity for NO_3. Most anionic resins are more selective for SO_4 (than NO_3) which is also a common groundwater constituent; usually in higher concentrations than NO_3.

`

Therefore, there is a need to find an economical, sustainable water treatment technique which can diminish the nitrate concentration.

Microalgae treatment is a sustainable and effective approach for the removal of nitrate from groundwater. Algae is capable of uptaking nitrate and creating biomass that concentrates the nitrogen. Strains of *Chlorella vulgaris* and *Scenedesmus sp.* have already shown promising results in nitrate removal as immobilized and free cells in wastewater (F. A. AlMomani & & Örmeci, 2016). However, designing an effective algae photobioreactor is challenging because of the biomass loss in a continuous flow reactor and the difficulty in harvesting algal biomass at the end of treatment process. Algal biomass harvesting requires expensive centrifuging and filtration operations (I. Moreno-Garrido, 2013). Thus, the immobilization approach of microalgae into polymeric matrices will help solve both problems. Immobilized algal cells occupy smaller space and are easier to handle. The immobilization of algae facilitates the harvesting of biomass and protects the cells from the harsh environments (e.g. metal toxicity, pH and salinity) (E. Eroglu, Agarwal, V. et al., 2012).

Both synthetic and natural polymers can be used as immobilization agents (Moreno-Garrido, 2008a). Alginate is the most widely used polymer for immobilization of microalgae; as it is a non-toxic, transparent polymer. Transparency of the alginate matrix allows light transmission which is a critical factor for photosynthesis in autotrophic cells (Hatanaka et al., 1999; Selimoglu & Elibol, 2010). Several studies have used immobilized algal cells to remove nutrients from wastewater (M. Liu et al., 2012; Alejandro Ruiz-Marin et al., 2010) . The strains of *Scenedesmus obliquus* and *Chlorella vulgaris*, immobilized in sodium alginate were used to remove nutrients from wastewater in batch and semi-

`

continuous modes (Alejandro Ruiz-Marin et al., 2010). *S. obliquus* was reported as a better candidate as it showed higher N and P uptake than *C. vulgaris*. Additionally, *S. obliquus* was more effective in removing N and P for longer periods in semi-continuous mode than in batch cultures.

Chlorella vulgaris and *Chlamydomonas sp.*, immobilized in calcium alginate were used during the tertiary treatment of municipal wastewater. *C. vulgaris* was reported as the best specie with 72% and 99% of reduction in nitrate and orthophosphate, respectively (Shaker et al., 2015). *Chlorella sp.* immobilized in calcium alginate sheets removed 100% of NH_4-N and P from real domestic secondary effluents (E. Zhang et al., 2012).

As discussed in chapter 2, there are three main cultivation modes which can be applied for immobilized algal beads: Photo-autotrophic, heterotrophic, and mixotrophic growth (J. Wang, H. Yang, & F. Wang, 2014). Photo-autotrophic microalgae use dissolved carbon dioxide and absorb light to perform photosynthesis. In contrast, heterotrophic growth occurs when microalgae perform photosynthesis and use organic carbon as carbon and energy sources without the need of light (Perez-Garcia & Bashan, 2015). Mixotrophic cultivation uses both inorganic and organic carbon sources in the presence of light (Abreu, Fernandes, Vicente, Teixeira, & Dragone, 2012). In autotrophic growth, light penetration is inversely proportional to the cell concentration. Therefore, it is more difficult to reach a high density of microalgae biomass using autotrophic growth. As the cells achieve higher density, it gets harder for the light to penetrate the cells. The low biomass concentration in autotrophic growth also increases the harvesting cost at the end of the treatment process (Liang, Sarkany, & Cui, 2009). In large scale applications, heterotrophic cultivation is cheaper and easier to handle than autotrophic cultivation as it does not have the limitation

`

of light dependency for growth (O. Perez-Garcia, F. M. Escalante, L. E. de-Bashan, & Y. Bashan, 2011). The microalgae species that undergo heterotrophic growth can grow in light limited conditions and this can lead to the higher biomass production. Strains of *C. sorokiniana* and *S. sp.* can produce biomass under autotrophic, mixotrophic, and heterotrophic conditions (Di Caprio, Altimari, & Pagnanelli, 2018; Kim, Park, Cho, & Hwang, 2013; Rai & Gupta, 2016). Monosaccharides or organic acids can be used as carbon and energy sources in heterotrophic cultivation(Figueroa-Martinez, Nedelcu, Smith, & Reyes-Prieto, 2015) . While wastewater treatment using immobilized algae was previously reported, there are no reported studies on heterotrophic growth of immobilized microalgae to treat nitrate-contaminated groundwater. In this chapter, both batch and sequencing batch growth studies were conducted to develop and optimize an immobilized-based biological treatment system and evaluate the treatment efficiency of *C. sorokiniana* and *S. sp.* immobilized in sodium alginate for removing nitrate from groundwater.

3.3 Materials and Methods

3.3.1 Cultivation of algae and preparation of immobilized algal beads

Stock cultures of *C. sorokiniana* and *S. sp.* were obtained from the Biochemistry Department at the University of Nebraska-Lincoln. The composition of the nutrient solution is provided elsewhere (Sorial et al., 1997). The nutrient solution consisted of essential inorganic salts and vitamins necessary to grow micro-organisms: B^{+3}, Ca^{+2}, Cl^{-}, Co^{+2}, CU^{+2}, Fe^{+3}, K^{+}, Mg^{+2}, Mn^{+2}, Mo^{+6}, NH_4^{-}, Na^{+}, SO_4^{-2}, Zn^{+2}, p-aminobenzoic acid, biotin, cyanocobalamin, folic acid, nicotinic acid, panothenic acid, pyriodoxine hydrochloride, riboflavin, thiamin hydrochloride and thioctic acid concentration. In addition, a nutrient spike solution (0.29 mM $NaNO_3$ and 0.03 mM $NaH_2PO_4 \cdot H_2O$) was

`

added to the feed solution to achieve the target of 50 mg/L nitrate. The algal solution was kept at room temperature. Both artificial plant light and sunlight were used for algal growth and the intensity of light was ranged between 500-700 Lux.

Microalgae was cultivated in a 3-gallon container using bulk media solution. After approximately two weeks of cultivation, the algae were ready to be used for bead preparation, when the turbidity reached 30 NTU. To prepare alginate-algae beads, algae solution was centrifuged at 3500 rpm for 10 minutes. The supernatant was discarded, and the algal cells were resuspended in DI water to obtain a concentrated solution. All the growth medium nutrients used to cultivate microalgae, except nitrate, were added to the algae concentrated suspension. Next, the concentrated algae solution was mixed with 1.5% sodium alginate solution. The mixture was dropped into a 2% calcium chloride solution using a syringe pump (Harvard Apparatus, model 55-3333) and left overnight to form algal beads. The algal beads were uniform and had the diameter of 2.6 mm. The beads were rinsed and kept in DI water before being used for the experiments.

The water matrix used in all batch and sequencing batch experiments- except the one with actual groundwater- was DI water, containing 10 mg/L NO_3-N. Potassium Nitrate (99.9%, Sigma Aldrich) was used and the concentration of NO_3-N in all reactors was measured using UV-vis spectrophotometer (Spectronic, model Genesys 5). The actual groundwater sample used at the final stage of the experiments was collected from a pump station at city of Hasting, NE. The concentration of dissolved oxygen (DO) in each reactor was measured on daily basis, using DO meter (YSI, model 5100). A pH meter (Thermo Scientific, model Orion 3-star) and a conductivity meter (Hach, model HQ14D) were used to record pH and conductivity daily. The COD of the bulk solution was measured using TNT-plus vial tests

`

(Hach, TNT820). GE plant light kits were used as light sources. The intensity of light was measured using a digital light meter (Leaton, model 935976) and maintained between 780-1000 lux.

3.3.2 Viability and growth assessment

An assessment of the viability of algal cells within the beads was performed by observation of algal colonies under Ti-S Inverted Fluorescence microscope (Nikon, Melville, NY). The chloroplast in algae contains chlorophylls, which are light harvesting molecules, embedded in the thylakoid membrane. The emission spectrum of chloroplasts show strong autofluorescence in red, with a peak at 680 nm (Kodama, 2016). The microscopic images were used to isolate the live algal cells from the background using the color threshold function in ImageJ v 1.51 j8. The total area covered in red was recorded as population of live cells.

Detailed images of the beads were taken using Scanning Electron Microscopy (SEM). SEM images were acquired using a Nova Nano SEM 450 (FEI, Hillsboro, OR), with the voltage ranging between 2 to 5 kV. The air-dried algal beads were coated with a thin layer of gold before imaging.

The dry cell weight (DCW) measurements were performed in sequencing batch reactors to determine the density and growth rate of algal cells inside of the beads. Five beads from each reactor were dissolved in 5 ml of 4% sodium bicarbonate solution. The suspension was then filtered through a 3-μm (pore size) filter paper, leaving a layer of microalgae on the paper. The dry weight of the microalgae was measured after drying the filter paper at 100°C for 24 hrs.

`

All batch and sequencing batch studies were performed in 500 mL flasks, containing 400 ml DI water and 10 mg/L NO_3- N. All required macro- and micro- nutrients were supplied imbedded in the beads. Glucose, when needed, was added at a concentration of 125 mg/L. For the samples with actual groundwater, no additional nutrients or glucose was provided in the bulk solution.

3.4 Results and Discussion

3.4.1 Impact of autotrophic *vs.* heterotrophic conditions on nitrate removal

To simulate the autotrophic and heterotrophic growth of algae, batch studies were performed with and without addition of an organic carbon source. At autotrophic condition, 75 mL of *C. sorokiniana* beads were run for 34 days without adding any organic carbon source. The dissolved atmospheric CO_2 (~ 1.5 g/L) was the only source of carbon. Two types of controls were operated simultaneously: one reactor with alginate beads (no microalgae) and another reactor containing only deionized water. At day 34, glucose (125 mg/L) was added to the bulk solution to facilitate heterotrophic growth. In the first 5 days, immobilized *C. sorokiniana* beads showed 6.5% Nitrate removal which reached 33% after 34 days of autotrophic growth. As shown on Figure 3.1, the removal efficiency increased to 36%, only one day after adding glucose to the bulk (day 35) and reached to 82%, at day 40. Since glucose is the only source of organic carbon in the bulk, the COD concentration was used to measure the glucose consumption by algal cells. The change in concentration of COD in the solution indicates its complete consumption by algal cells.

The effect of light on the heterotrophic growth of algal beads was also evaluated by measuring the nitrate uptake rate and DCW in complete darkness, medium (300 Lux) and

`

high (700 Lux) intensity light. The highest DCW and nitrate uptake was observed in presence of high intensity light source.

Studies on microalgae suggest that only two enzymes, nitrate reductase (NR; EC 1.6.6.1-3) and nitrite reductase (NiR; EC 1.7.7.1), are responsible to catalyze nitrate to ammonium (Fernandez & Galvan, 2007). Different environmental variables affect nitrate assimilation. One of the key factors in nitrate assimilation is the presence of light, as most algae assimilate nitrate faster in the light than in the dark. Light also has an important effect in glucose uptake by microalgae cells. For *Chlorella* cells growing at the presence of glucose, the blue end of the visible spectrum controls many of the metabolic reactions (Karlander & Krauss, 1966) The blue light inhibits uptake of glycine, proline, and arginine, but activates the nitrate reductant enzymes and therefore, enhances uptake of oxygen and nitrate by microalgae (Octavio Perez-Garcia, Froylan ME Escalante, Luz E. de-Bashan, & Yoav Bashan, 2011).

`

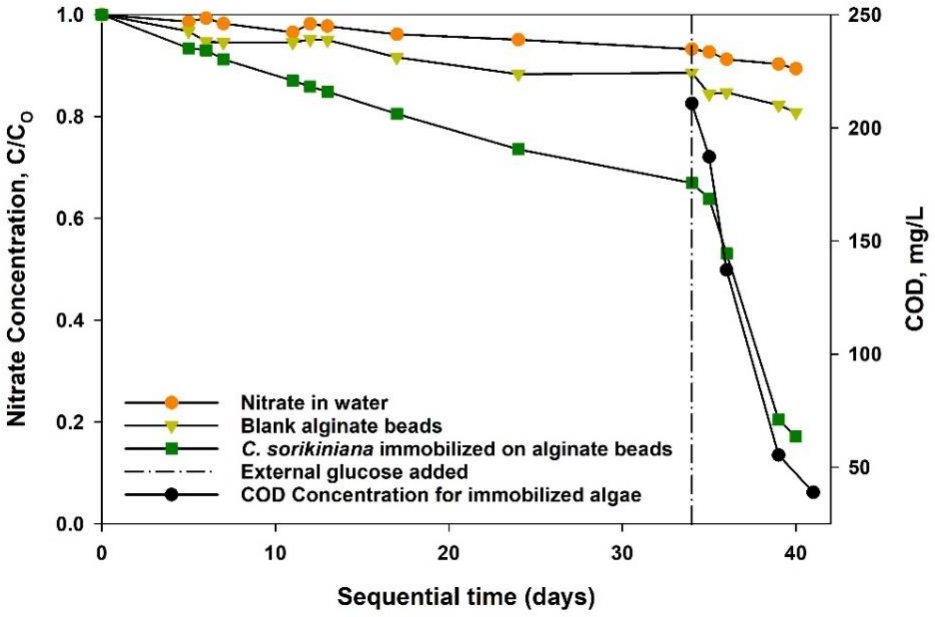


Figure 3.1 Percentage of nitrate present (left vertical axis) and change in concentration of COD (right vertical axis) of the solution over time. At the end of day 34, 50 mg glucose was added to each reactor.

3.4.2 Optimum volume of algal beads needed

To determine the optimum algal beads/water ratio that maximizes the nitrate removal rate, four different ratios of *C. sorokiniana* beads to water (50%, 25%, 12.5%, and 6.25%) by volume were evaluated. A sample containing 50% blank alginate beads was prepared for control treatments. As shown in Figure 3.2a, the removal of NO_3-N after 16 days in both control treatments, with no beads and with blank alginate beads were 28% and 31%, respectively. All other bioreactors with microalgae achieved higher removal than the control. The average of 50% NO_3-N was removed by immobilized *C. sorokiniana* beads in the first 5 days which reached the maximum of 99.9% within 16 days. Comparing the nitrate removal rate of samples containing different ratio of algae to water indicates that using higher volumetric ratio than 12.5% did not result in improved performance.

`

Therefore, the ratio of 12.5% was used in the batch reactors on the remaining experiments. Macro- and micro-nutrients required for growth were introduced to the solution only through the added bead volume. Since nitrate removal pattern is equal beyond 12.5%, this was an indication that this ratio provided sufficient nutrients. The average removal rate for the reactor containing 12.5% algal beads was 0.190 mg N/d.

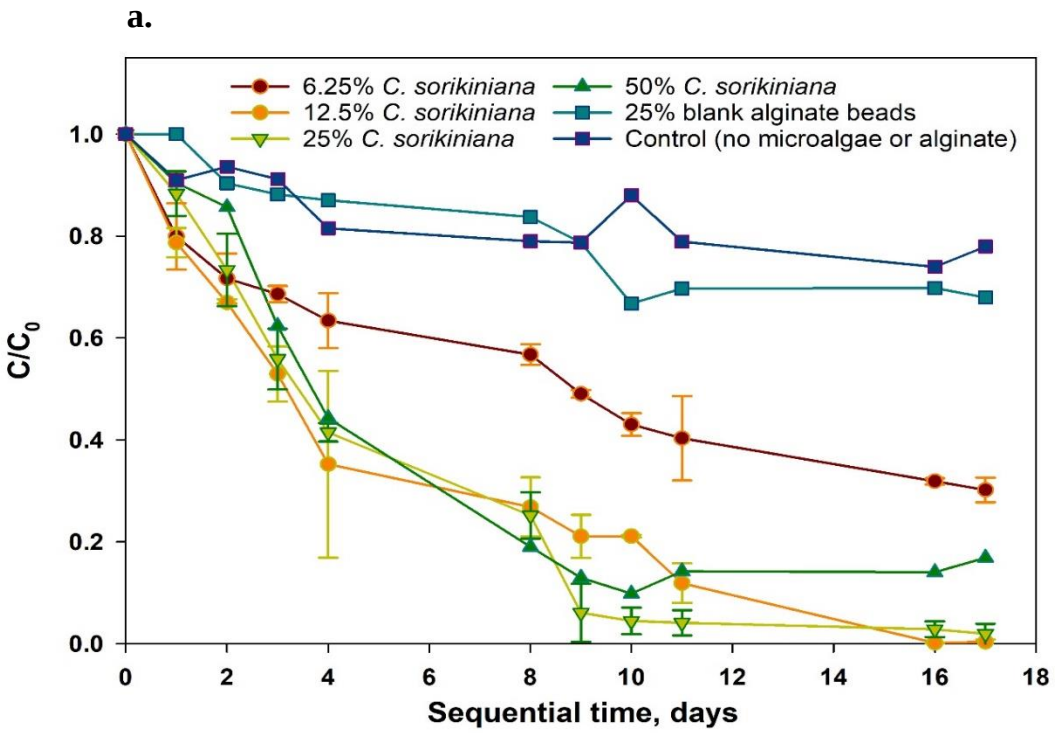
Figure 3.2b shows the average values of pH, DO and conductivity in the bulk solution for each reactor during 16 days of growth. There was a direct relationship between the volume of algal beads in the reactors and the pH of the bulk solution. This is explained by photosynthesis occurring in presence of increased algal cells numbers. The same trend occurred for DO. Since the algal cells had access to both organic carbon source (glucose) and inorganic carbon source (carbon dioxide from the air) and they were kept in presence of light, we surmise that they switched between autotrophic and heterotrophic growth, depending on the energy source (light or glucose) availability. The concentration of glucose used in the growth study (125 mg/L) was substantially lower than what was suggested by other literatures (5- 80 g/L) (Samejima & Myers, 1958; Shi, Liu, Zhang, & Chen, 1999); therefore, it is possible that the glucose initiated the heterotrophic growth but was the limiting factor for the long-term heterotrophic growth.

Moreover, there is a direct correlation between the volume of algal beads and conductivity of the bulk solution. The algal beads were kept in $CaCl_2$ solution overnight to solidify before application. By comparing the conductivity of the control treatment (with no microalgae or alginate-88 µs/cm) and the other reactors (362-1074 µs/cm), it is evident that the high value of conductivity is mostly because of the presence of calcium and chloride

`

ions. The average concentration of chloride in the batch reactor with 12.5% of algal beads was estimated to be 89±2.2 mg/L.

Figure 3.2c compares the COD consumption *vs*. time for three of the reactors. The reactors containing 6.25% and 12.5% algal beads follow a very similar trend in glucose consumption. In the first 48 hours, about 35% and 45% of the glucose was consumed by algal cells in the reactors with 6.25% and 12.5% algal beads, respectively. The reactor with 25% algal beads, however, had a sharp reduction on day 2. About 65% of the glucose was consumed at the first 48 hours by algal cells in this reactor. At the last four days (days 5-9), the glucose concentration stayed almost constant indicating algae switching from heterotrophic to autotrophic mode.



`

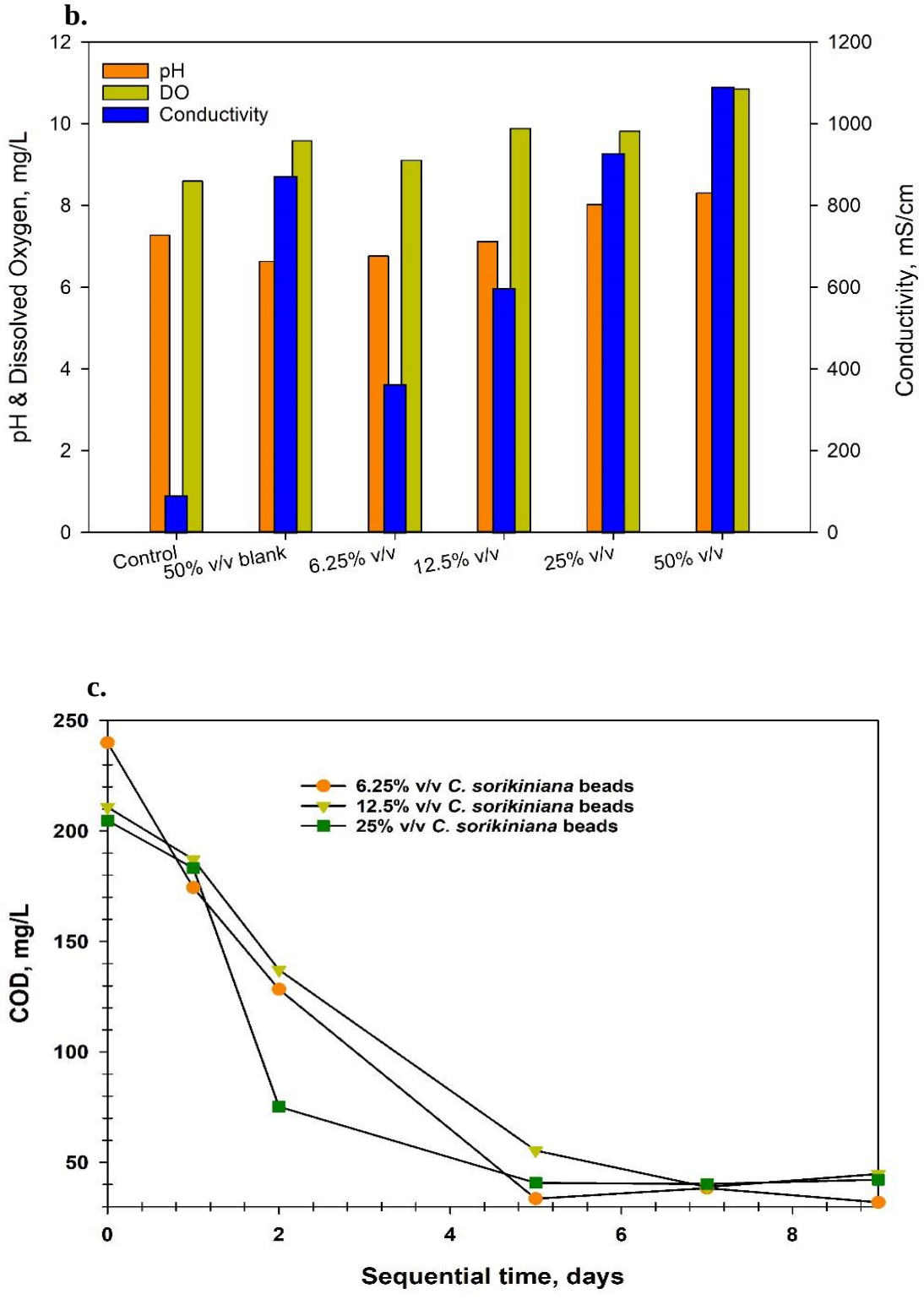


Figure 3.2 Effect of four different ratio of immobilized *C. sorokiniana* cells to water ratio: a. Percentage of nitrate present in the solution over time, b. The average pH, DO and conductivity, and c. Change in COD concentration of the bulk solution.

`

3.4.3 *Chlorella sorokiniana* vs. *Scenedesmus species*, effect of starvation and immobilization

Comparing the strains of *C. sorokiniana* and *S. sp.* indicates that immobilized *S. sp.* were slightly better than *C. sorokiniana* in nitrate removal (Figure 3.3). Consequently, *S. sp.* were further used in the sequencing batch reactor evaluation. *S. sp.* beads removed 60% of NO_3 after five days and 90% of NO_3 was eliminated in nine days. For *C. sorokiniana*, the removal rate was 58% in five days and 78% in nine days.

Moreover, the effect of starvation on nutrient removal was evaluated. *S. sp.* beads were stored without nutrients for 6 days until their utilization in bioreactors. While there was an increase in DO concentration and pH levels for both starved and non-starved *S. sp.* cells, the rate of increase (in both DO and pH) was higher in non-staved cells; which was an indicator of increased levels of photosynthesis occurred in cells without any starvation period. Between the biological compounds, RNA, DNA, and protein are indicators of physiological status due to their direct relationship to cell division and growth. Positive correlations between the ratio of RNA/DNA and growth rate have been obtained in a variety of organisms; such as microbial communities, natural and synthesized phytoplankton populations (Berdalet, Latasa, & Estrada, 1994). *S. sp.* is one of the microalgae species with the ability to produce and accumulate triglyceride (TAG) under nitrogen starvation conditions (Breuer, Lamers, Martens, Draaisma, & Wijffels, 2012). Under limited nitrogen conditions, the rate of DNA, RNA and chlorophyll production is impaired. As a result, less electrons are generated by photosynthesis which will mostly be used to produce TAG (Breuer, Lamers, Martens, Draaisma, & Wijffels, 2013). As shown in Figure 3.4 the starvation did not improve the nitrate removal rate. N- starvation was

`

studied in *Heterocapsa sp.*, grown in batch cultures, and it was reported that RNA was markedly impacted by N-starvation cells in the intermediate and stationary phases. The N-starved treatments also resulted in a remarkable decrease in the net photosynthetic rate and chlorophyll content per cell which could be the main reason of deterring performance of the starved immobilized cells (Berdalet et al., 1994).

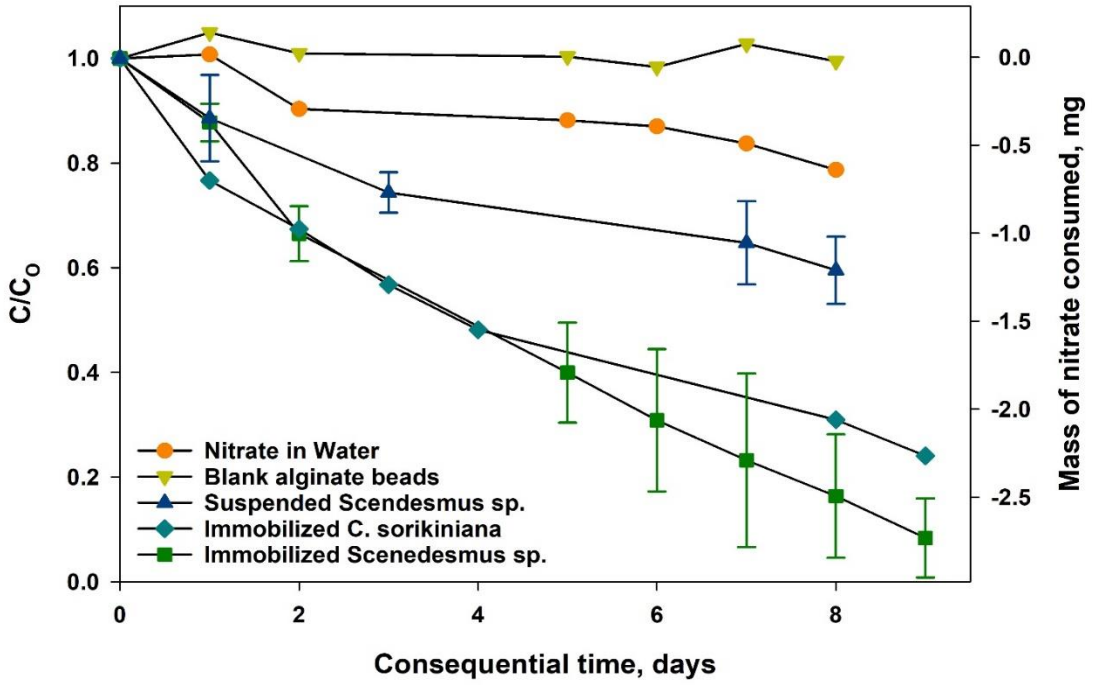


Figure 3.3 Nitrate concentrations present in the solution over time in presence of immobilized *C. sorokiniana, S. sp.* beads, and suspended *S. sp.*.

Nitrate removal by suspended algal culture was compared to immobilized cultures. The same mass of algae was used in both conditions. The nitrate removal rate for suspended and immobilized culture was the same at day 1 as seen in Figure 3.3. In the next 7 days, however, the nitrate uptake by immobilized cells was significantly higher than it was by the suspended culture. Suspended *S. sp.* cells were able to remove 58% of nitrate in 8 days whereas the immobilized beads reached 80% removal rate during the same time. Higher

`

rate of nitrate removal by immobilized cells could be explained by higher light intensity reaching the algal cell. For suspended cultures, as biomass density increases, the light penetration depth will decrease. This will result in smaller percentage of algal cells being effectively illuminated (J. F. Wang, Liu, & Liu, 2015). An overall light penetration throughout the reactor was estimated for both immobilized and suspended cultures. For the immobilized cells at day 0, 44% of the light penetrated the entire dimeter of the photobioreactor *vs.* 42% measured for the suspended cells. On day 8, these values changed to 35% and 20% for immobilized cells and suspended cultures, respectively. Light intensity and distribution inside the immobilized cells were not quantified

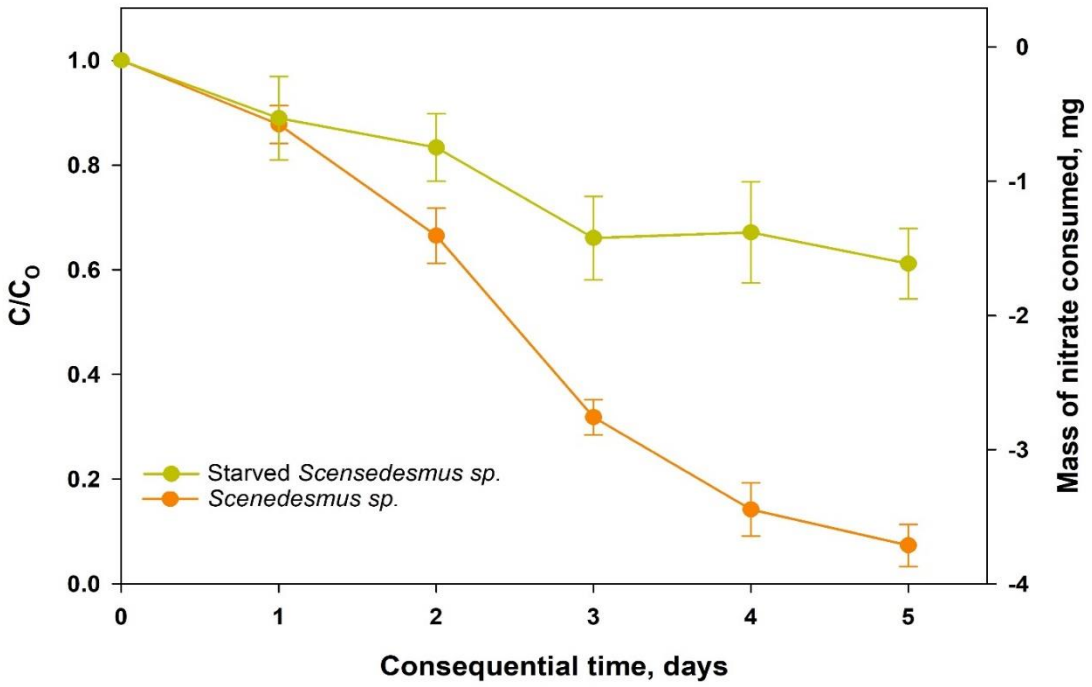


Figure 3.4 The impact of starvation on nitrate uptake rate by immobilized *S.sp* beads.

`

3.4.4 Nitrate removal in a sequencing batch reactor

While nitrate was successfully removed in batch reactors, it was essential to know how many cycles, the beads could be reused for water treatment. Therefore, the bead lifetime was assessed using sequencing batch reactors. Every 9 days, the treated water in each reactor was discarded and the reactors were filled back with 400 ml nitrate-contaminated water matrix and 125 mg/L glucose. Besides the nutrients embedded within the beads during manufacturing, no additional nutrients were added throughout these experiments. Additionally, four sets of experiments with different ratios of algae to alginate were used: 75:25, 50:50, 25:75, and 10:90 (v:v %), respectively. Different ratios were used to assess the minimum number of initial algae needed without compromising the performance. Maintaining high nitrate removal performance with smaller algae concentration results in reduced cost for the required initial algae cultivation. Figure 3.5 shows the removal efficiency and the performance of the immobilized *S. sp.* beads over their lifetime until they failed to remove additional nitrate. The ratio of 75:25 (algae: alginate) was discontinued as alginate was not sufficient to support the bead structurally. The higher initial algae concentration within the beads (50:50) showed the maximum removal efficiency at the first 75 days of operation (periods 1-7). The performance obtained by the 25:75 ratio kept improving over time. The performance is summarized in Figure 3.5 by dividing the data in three distinct periods of operation. By period 4, the performance of 25:75 ratio was almost equal to the 50:50 ratio. Similar performance between both ratios was maintained throughout period 6 but 25:75 ratio deteriorated without a known reason thereafter. The 10:90 ratio, which has initially the lowest algae concentration took longer time to catch up to the same performance as 50:50 ratio. By period 6, the performance of

`

10:90 ratio was comparable to 50:50 ratio and surpassed it by period 8. At period 10, all the beads showed a deteriorating performance.

The initial algal density has a major impact on nutrient removal from water. The higher algal density within the beads causes more nutrients as well as glucose assimilation. The initial average algal density for the beads with 50:50, 25:75, and 10:90 ratios were 8.14, 7.04 and 6.42 mg/L, respectively. Toward the end of the treatment, nitrate removal efficiency of the 50:50 ratio beads was lower than that of the 10:90 ratio (Figure 3.5). It was observed that the dense algal mass in the concentrated culture were metabolically less active towards the end of the treatment process. The high algal density causes self-shading, an accumulation of auto-inhibitors, which reduces photosynthetic efficiency (Xu, Shen, & Chen, 2015). These results suggest that having a concentrated culture can shorten the retention time for nitrate removal and algal cells can be replaced before self-shading substances accumulated to a toxic level.

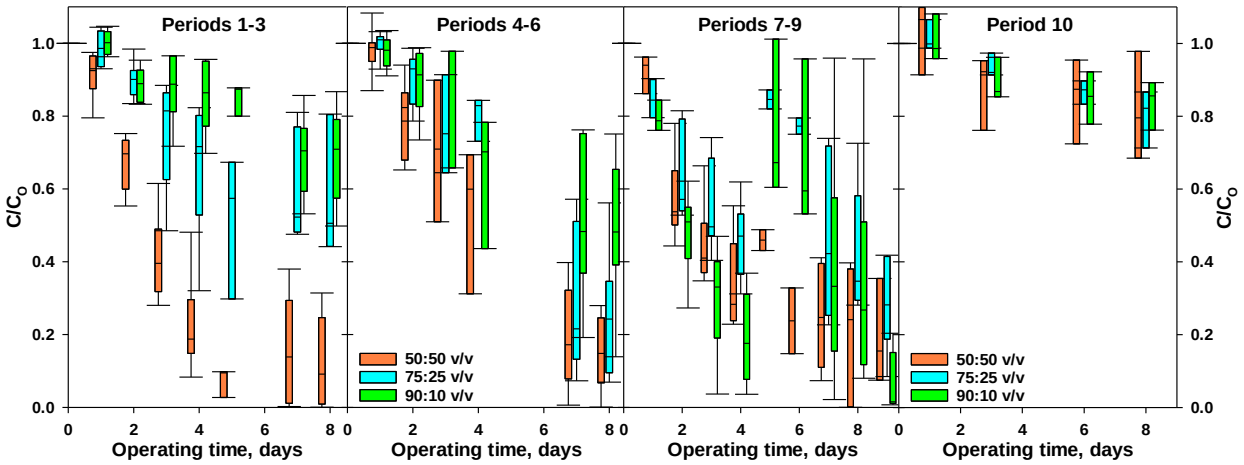


Figure 3.5 Nitrate removal in a sequence batch reactor operated with different volumetric ratio of alginate to algae. Box plots summarize several periods for data clarity.

`

shows the concentration of different elements in the water matrix upon the preparation of the beads (period 1) and at the end of the treatment process (period 10). As it's shown, Na, Mg, P, K, Ca, Zn and Cu were the dominant elements in the water. The nutrients that were added to the algae solution prior to making the beads include all these elements. The more algae content in the beads means more nutrients and that's why the highest metal concentration is observed in water containing 50:50 ratio beads. Most of these elements were partially removed by algal beads at period 10. The only exception was Fe, which was not detectable at period 1, but was diffused from the beads to the bulk solution at period 10. There are also trace amounts of Co, As, Se and Cd in period 1 which were all removed at the end of the treatment process (period 10).

`

Table 3.1 The concentration of elements in water matrix used in sequencing batch reactors at periods 1 and 10.

	Samples	Na	Mg	P	K	Ca	Fe	Cu	Zn	CO	As	Se	Cd
		C (ppm)					C (ppb)			C(ppt)			
Period 1	50:50	62.64±6.90	3.52± 0.57	0.27±0.07	78.93±0.05	43.49±0.20	ND	6.87±0.06	25.90±13.32	494.10±28.64	108.60±52.93	1629.73±87.39	191.83±5.94
	75:25	57.39±0.85	0.12± 0.06	0.12±0.01	40.95±0.03	32.64±6.40	ND	5.30±0.61	10.53±1.17	419.40±26.60	104.23±26.36	1770.70±34.66	229.90±37.22
	90:10	52.50±0.88	0.06± 0.01	0.05±0.02	39.36±0.07	21.90±1.12	ND	3.67±0.15	ND	ND	115.27±23.64	3178.33±23.46	250.13±11.82
Period 10	50:50	4.34± 0.32	ND	0.09±0.01	52.29±4.65	6.02± 1.22	17.59±1.44	ND	8.96± 0.14	ND	ND	ND	ND
	75:25	4.83± 0.12	0.03± 0.01	0.06±0.02	39.66±9.87	14.17±0.12	40.09±2.34	ND	10.57±0.76	ND	ND	ND	ND
	90:10	4.56± 0.65	0.17± 0.04	0.06±0.02	50.32±7.23	16.89±6.72	45.60±10.55	ND	8.05± 1.33	ND	ND	ND	ND

3.4.5 Algal growth, pH, DO, and reaction rates within beads

Algal density of immobilized *S. sp.* in sequencing batch mode was measured periodically using dry cell weight (DCW). Figure 3.6 shows the change within the first two periods. The density of the algal cells is gradually increasing over time at the first period (days 0-9). The increase of cells density within the beads indicates that algae can still perform photosynthesis after immobilization, which agrees with previous reports of *C. vulgaris* immobilized in alginate (A. Ruiz-Marin, L. G. Mendoza-Espinosa, & T. Stephenson, 2010). During the second period (days 9-17), however, the algal density increased slightly until day 14 after which it remained mostly constant. This indicates that the stationary phase of growth was reached. The slight algal density decrease could be explained by insufficient nutrients in the bulk solution. At the end of every period of sequencing batch operation, treated water was discarded and the beads were washed out. During this process, some nutrients that diffused from the beads to the bulk solution were washed out and never recovered.

Figure 3.7 shows the changes in pH and DO of the bulk solution within selected periods of growth studies in sequencing batch mode. As shown, there was a large drop in the concentration of DO every week. The color of the beads turned from green at period 1 to white at period 10. When algal cells photosynthesize, they use carbon source and produce oxygen and increase the concentration of DO in the solution. Photosynthesis also increases the pH of water as it removes carbon dioxide.

`

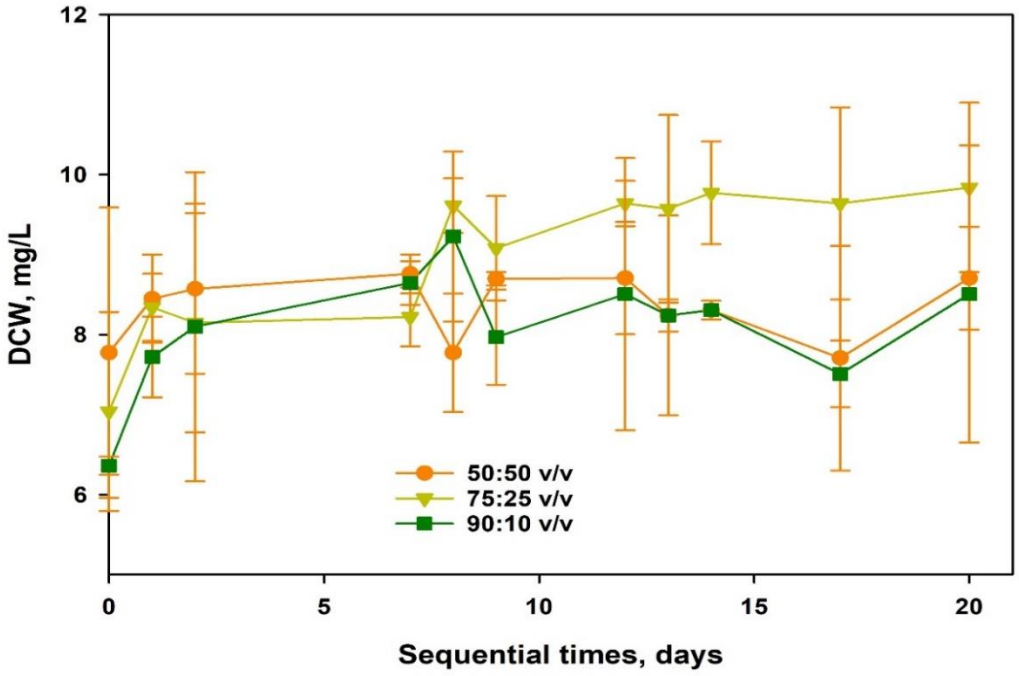


Figure 3.6 DCW of the algae in the beads with different volumetric ratio of alginate: algae, used in a sequence batch reactor.

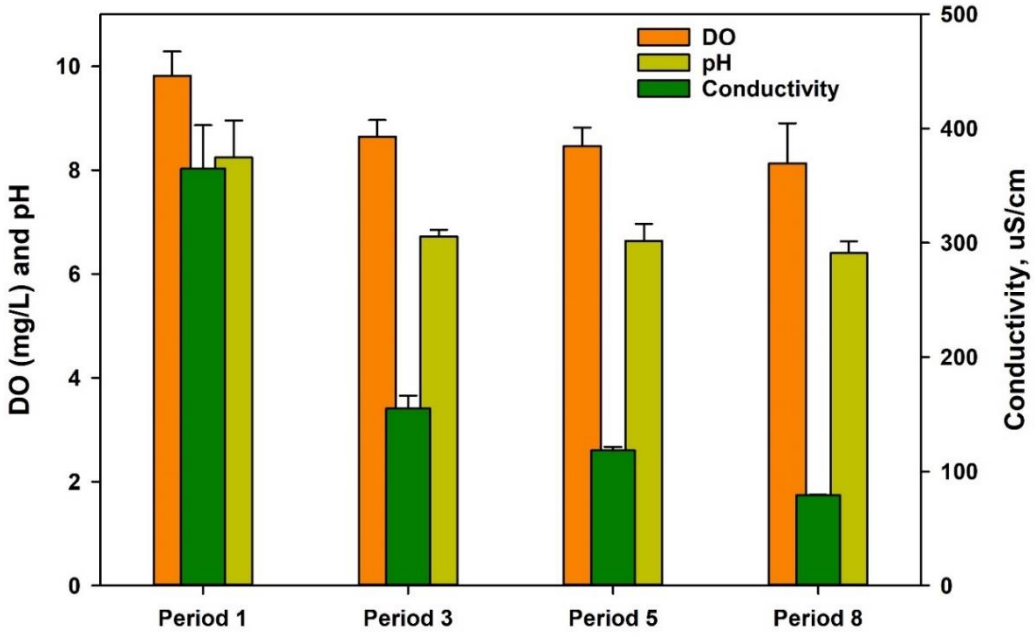


Figure 3.7 Average pH, DO and conductivity of the bulk solution over the 8 periods of the experiments in sequencing batch mode.

`

When photosynthesis slows down or stops, both DO, and pH tend to decrease. Therefore, the drop in the DO and pH, and change of color are all indicators that photosynthetic rate is decreasing in the beads which could be due to nutrient depletion, as it was explained in section 3.4. The nitrate uptake in each sequencing batch reactor was simulated using the first order reaction kinetics ($\ln(\frac{C}{Co}) = -kt$). The k value was maximum for the samples with 50:50 ratio beads (0.18 1/d) which was an indicator of faster nitrate removal rate by them. For the samples with 25:75 and 10:90 ratio beads, the k value was 0.10 and 0.07 respectively (Figure 3.8).

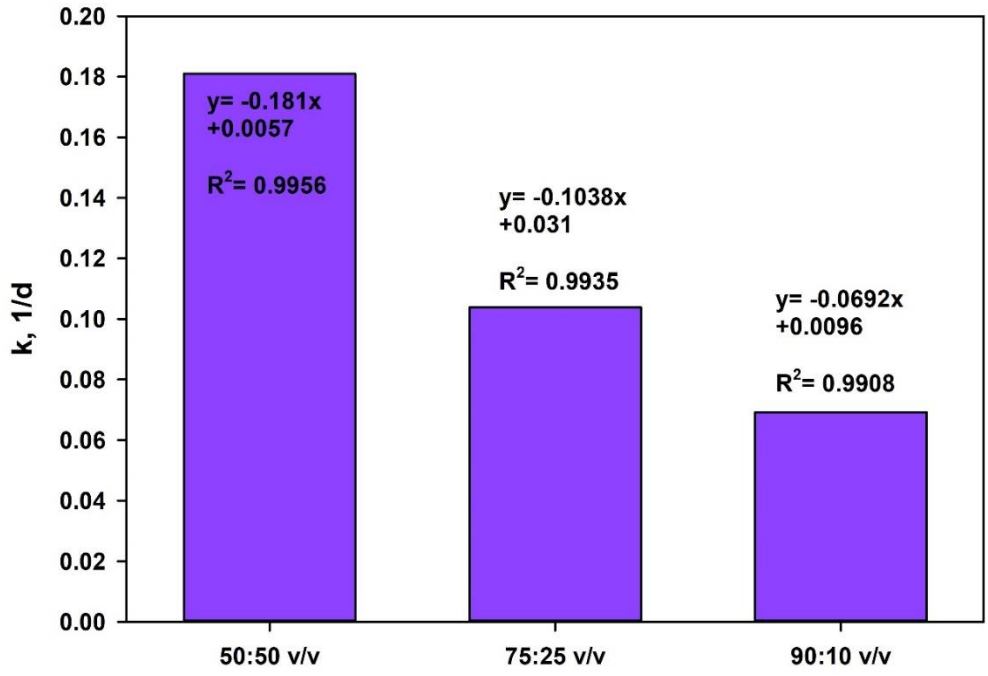


Figure 3.8 Average k value calculated for each reactor in a sequence batch reactor.

`

3.4.6 Viability of algal cells within the beads

Figure 3.9 shows optical images of *S. sp.* cells immobilized in sodium alginate (a) initially, (b) after 10 days, (c) after 20 days, and (d) after 112 days from the start of experiments in sequencing batch reactor. The red color is an indicator of healthy algal cells. The area covered with live cells was 104,385 pixels initially, 104,411 pixels after 10 days of growth, 75702 pixels after 20 days of growth and 0 pixels after 112 days. The results from microscopic images match the algal density results measured by means of DCW (section 3.3.5).

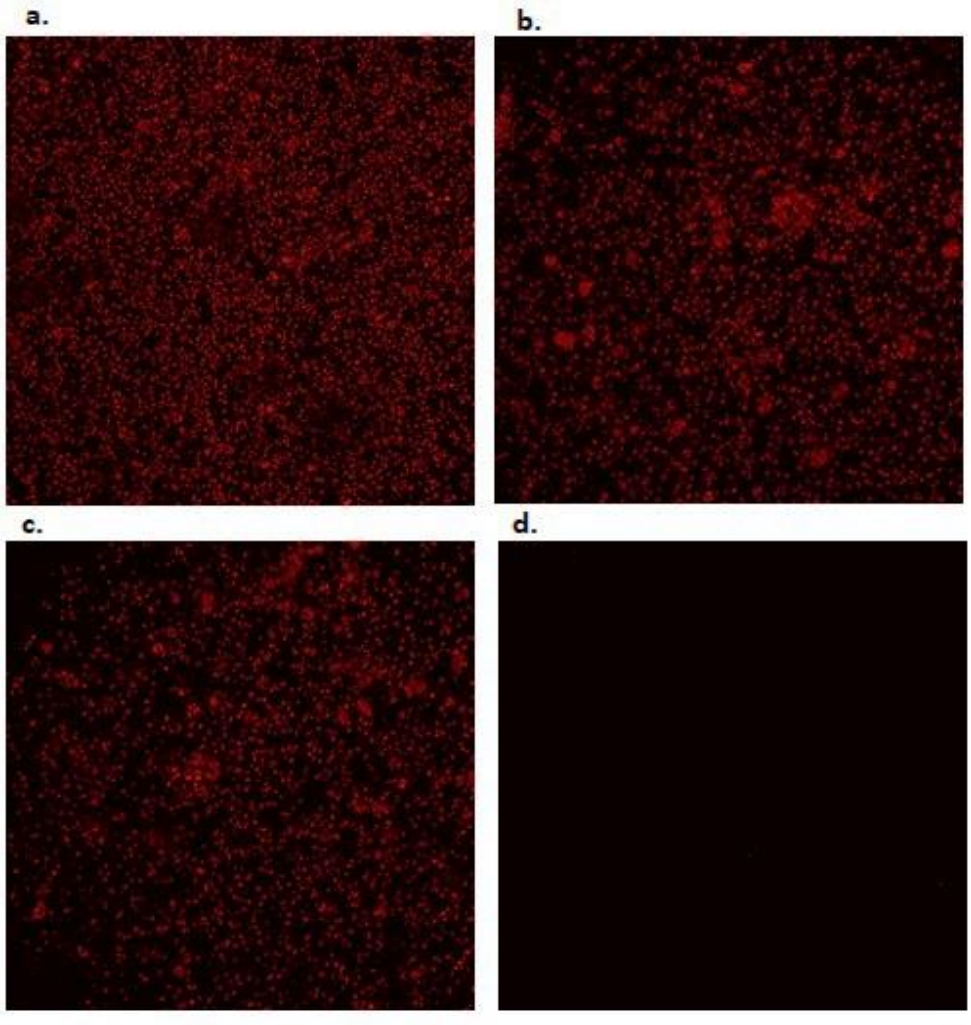


Figure 3.9 Optical images of immobilized *Scenedesmus* cells (a) initially, (b) after 10 days, and (c)after 20 days and (d) after 112 from the start of experiments in sequencing batch mode.

`

Detailed images of the algae within the bead are shown through a Scanning Electron Microscope (SEM) in Figure 3.10. As shown in Figure 3.10a, sodium alginate matrix is an effective support matrix for immobilizing *S. sp.* cells, which have the diameter of 3–4 μm. This porous structure of sodium alginate also allows the diffusion of nutrients between the bulk liquid and the algae, which allows photosynthesis of algal cells.

After 112 days of operation, however, sodium alginate matrix lost its firm structure due to the loss of the calcium ions in the solution in sequencing batch mode. Calcium ions provide cationic bridges between the guluronic-rich regions along the biopolymer backbone (S. J. Kleinübing, F. Gai, C. Bertagnolli, & M. G. C. D. Silva, 2013) and its depletion damages the structure of the beads. At day 112, no algal cells were observed in sodium alginate matrix and the beads were surrounded with strains of fungi. As shown in Figure 3.10b, the alginate mat became very porous with the diameter of 13 – 20 nm which allowed algal cells to escape from the matrix. Fungus are ubiquitous in the environment and the media is a great environment for them to quickly colonize and flourish. In our case, open bottles were used as reactors for 112 days which could cause fungus contamination in long term.

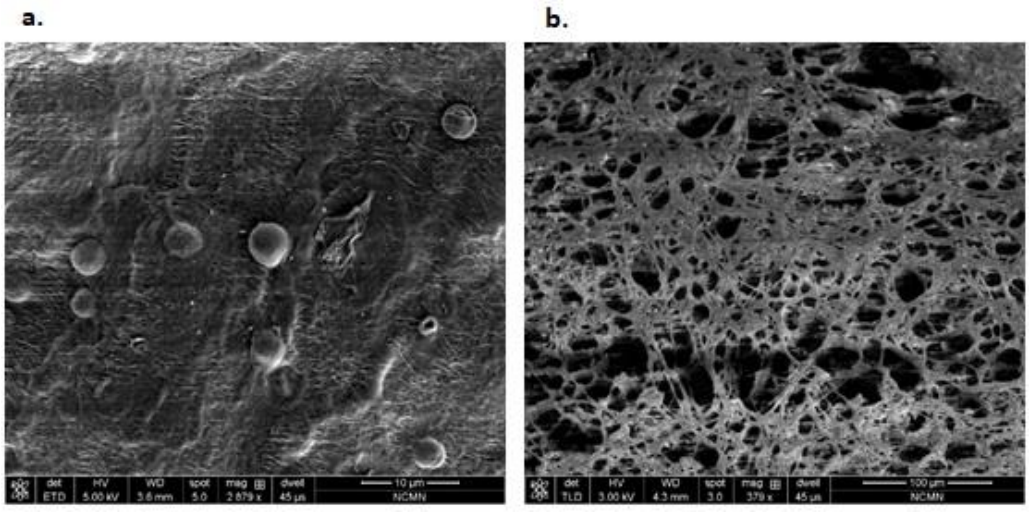


Figure 3.10 SEM images of immobilized *S. sp.* cells on the surface of sodium alginate at day 0 (a) and day 112 (b) of operation.

`

3.4.7 The application of actual groundwater

Immobilized *S. sp.* were tested using actual groundwater that was collected from wells in Hastings, NE. Hasting was chosen because it is experiencing nitrate contamination in its water supply aquifer. About 12% of the municipal wells in Hastings were reported to have nitrate concentration above MCL in 2016. High levels of nitrates migrating through the aquifer and into the city wells is a potential health risk for people in this town. The estimated cost of installing a traditional water treatment facility to eliminate nitrate contamination is $75 million. Currently, the city is working closely with water quality experts to find a sustainable and efficient solution to reduce the high level of nitrates (City of Hastings, 2016)

Upon collection, the concentration of nitrogen, phosphorous and COD in the groundwater sample was measured and found to be 8.9, 2.8 and 100 mg/L, respectively. As shown in Figure 3.11, 90% of nitrate was eliminated within two days without adding any external carbon source. The groundwater was rich in nutrients described above, which provided a more suitable environment for algal cells to grow without needing any additional nutrients/ carbon source.

`

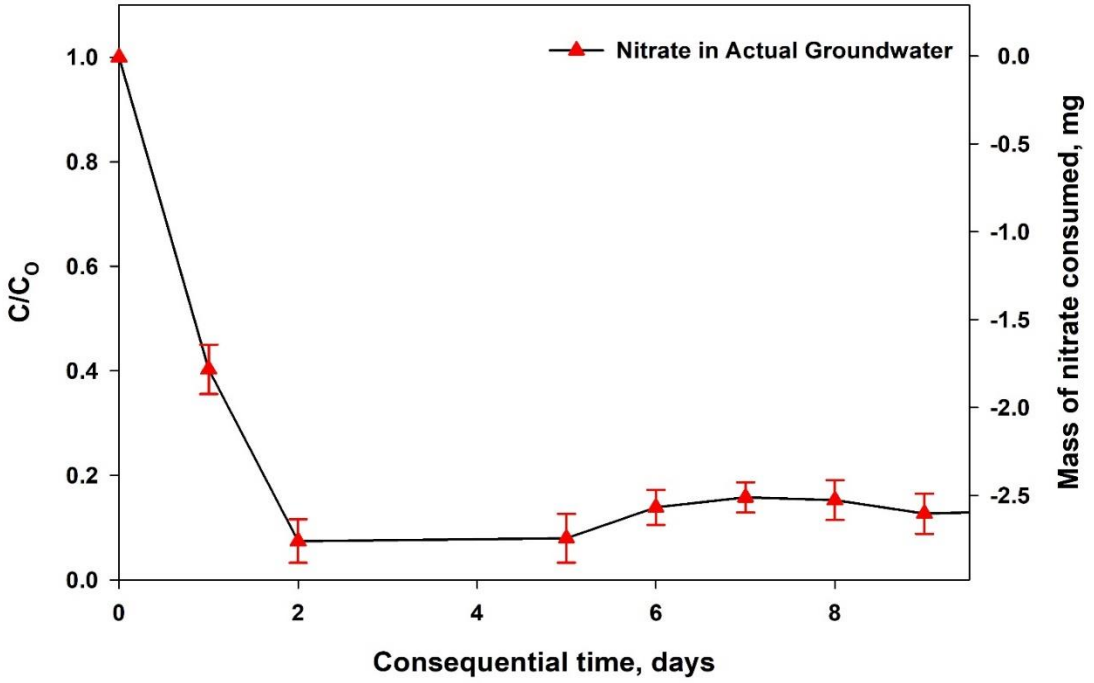


Figure 3.11 Percentage of nitrate present and mass of nitrate consumed over time in actual groundwater.

3.5 Conclusions

In this chapter, batch experiments were performed to find the optimum conditions for nitrate removal from synthetic groundwater. The optimum experimental conditions were used to design the sequencing batch reactors and perform the experiments with the actual groundwater. The following conclusions could be drawn from this study:

1. The results showed that immobilized *S. sp.* beads were more efficient in removing nitrate from water than the suspended cell system and can be used up to 100 days in sequencing batch mode.

2. Heterotrophic cultivation of immobilized *S. sp.* is an efficient biological method for nitrate removal as it exceeds the nitrate uptake rate. The presence of light increases the DCW concentration and enhances the nitrate uptake rate in heterotrophic condition.

`

3. The Initial algae concentration in the beads is an important factor in nitrate uptake rate. Higher algal density in the beads causes more nutrients and glucose assimilation. However, the algal beads must be replaced before the self-shading effect occurs.

4. Actual groundwater is more suitable for nitrate removal than simulated lab water as it is rich in nutrients that facilitate algae growth inside of the immobilization matrix. In natural water, no additional carbon source is needed for a successful removal.

`

References

Abreu, A. P., Fernandes, B., Vicente, A. A., Teixeira, J., & Dragone, G. (2012). Mixotrophic cultivation of Chlorella vulgaris using industrial dairy waste as organic carbon source. *Bioresource Technology, 118*, 61–66.

Agency, U. E. P. (2018). 2018 edition of the drinking water standards and health advisories tables. US EPA.

AlMomani, F. A., & & Örmeci, B. (2016). Performance of Chlorella vulgaris, Neochloris Oleoabundans, and mixed indigenous microalgae for treatment of primary effluent, secondary effluent and centrate. Ecological Engineering, 280–289.

Berdalet, E., Latasa, M., & Estrada, M. (1994). Effects of nitrogen and phosphorus starvation on nucleic acid and protein content of Heterocapsa sp. *Journal of Plankton Research, 16*(4), 303–316.

Breuer, G., Lamers, P. P., Martens, D. E., Draaisma, R. B., & Wijffels, R. H. (2012). The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains. *Bioresource Technology, 217-226*.

Breuer, G., Lamers, P. P., Martens, D. E., Draaisma, R. B., & Wijffels, R. H. (2013). Effect of light intensity, pH, and temperature on triacylglycerol (TAG) accumulation induced by nitrogen starvation in Scenedesmus obliquus. *Bioresource technology*, 1-9.

City of Hastings, NE, (2016). Aquifer storage and restoration project. https://www.cityofhastings.org/departments/utilities/water/asr-project.html .

Di Caprio, F., Altimari, P., & Pagnanelli, F. (2018). Effect of Ca^{2+} concentration on Scenedesmus sp. growth in heterotrophic and photoautotrophic cultivation. *New biotechnology*, *228*-235

Eroglu, E., Agarwal, V., Bradshaw, M., Chen, X., Smith, S. M., Raston, C. L., & Iyer, K. S. (2012). Nitrate removal from liquid effluents using microalgae immobilized on chitosan nanofiber mats. *Green Chemistry, 14*(10), 2682–2685.

Fernandez, E., & Galvan, A. (2007). Inorganic nitrogen assimilation in Chlamydomonas. *Journal of Experimental Botany, 58*(9), 2279–2287.

Figueroa-Martinez, F., Nedelcu, A. M., Smith, D. R., & Reyes-Prieto, A. (2015). When the lights go out: the evolutionary fate of free-living colorless green algae. *New Phytologist, 206*(3), 972–982.

Karlander, E. P., & Krauss, R. W. (1966). Responses of heterotrophic cultures of Chlorella vulgaris Beyerinck to darkness and light. I. Pigment and pH changes. *Plant physiology,* 41(1), 1–6.

Kim, S., Park, J. E., Cho, Y. B., & Hwang, S. J. (2013). Growth rate, organic carbon and nutrient removal rates of Chlorella sorokiniana in autotrophic, heterotrophic and mixotrophic conditions. *Bioresource Technology*, 8–13.

Kleinübing, S. J., Gai, F., Bertagnolli, C., & Silva, M. G. C. D. (2013). Extraction of alginate biopolymer present in marine alga Sargassum filipendula and bioadsorption of metallic ions. *Materials Research,* 16(2), 481–488.

Kodama, Y. (2016). Time gating of chloroplast autofluorescence allows clearer fluorescence imaging in Planta. *Plos One, 11*(3), ARTN e0152484.

`

Lawniczak, A. E., Zbierska, J., Nowak, B., Achtenberg, K., Grzeskowiak, A., & Kanas, K. (2016). Impact of agriculture and land use on nitrate contamination in groundwater and running waters in central-west Poland. *Environmental Monitoring and Assessment, 188*, (3), ARTN 172, 10.1007/s10661-016-5167-9.

Liang, Y., Sarkany, N., & Cui, Y. (2009). Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnology Letters, 7*, 1043–1049.

Liu, M., Liu, F., Bawa, M. L., & Chen, H. (2012). Nitrate in drinking water: a major polluting component of groundwater in gulf region aquifers, south of Togo. *International Journal of Physical Sciences, 7*(1), 144–152.

Moreno-Garrido, I. (2008). Microalgae immobilization: current techniques and uses. *Bioresource Technology, 99*(10), 3949–3964.

Moreno-Garrido, I. (2013). Microalgal immobilization methods. In *Immobilization of Enzymes and Cells* (pp. 327, Totowa–347). Humana Press.

Perez-Garcia, O., & Bashan, Y. (2015). Microalgal heterotrophic and mixotrophic culturing for bio-refining: from metabolic routes to techno-economics. In *Algal biorefineries* (pp. 61-131): Springer.

Perez-Garcia, O., Escalante, F. M., de-Bashan, L. E., & Bashan, Y. (2011). Heterotrophic cultures of microalgae: metabolism and potential products. *Water research, 45*(1), 11-36

Rai, M. P., & Gupta, S. (2016). Growth and lipid production from Scenedesmus sp. under mixotrophic condition for bioenergy application. In *Proceedings of the First International Conference on Recent Advances in Bioenergy Research, New Delhi* (pp. 159–167): Springer.

Rhoades, M. G., Meza, J. L., Beseler, C. L., Shea, P. J., Kahle, A., Vose, J. M., et al. (2013). Atrazine and nitrate in public drinking water supplies and non-Hodgkin lymphoma in Nebraska, USA. *USA. Environmental health insights*.

Ruiz-Marin, A., Mendoza-Espinosa, L. G., & Stephenson, T. (2010). Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater. *Bioresource Technology, 101*(1), 58–64.

Samejima, H., & Myers, J. (1958). On the heterotrophic growth of Chlorella pyrenoidosa. *Microbiology, 18*(1), 107–117.

Selimoglu, S. M., & Elibol, M. (2010). Alginate as an immobilization material for MAb production via encapsulated hybridoma cells. *Critical Reviews in Biotechnology, 30*(2), 145–159.

Shaker, S., Nemati, A., Montazeri-Najafabady, N., Mobasher, M. A., Morowvat, M. H., & Ghasemi, Y. (2015). Treating urban wastewater: Nutrient removal by using immobilized green algae in batch cultures. *International Journal of Phytoremediation, 17*(12), 1177–1182.

Shi, X.-M., Liu, H.-J., Zhang, X.-W., & Chen, F. (1999). Production of biomass and lutein by Chlorella protothecoides at various glucose concentrations in heterotrophic cultures. *Process Biochemistry, 34*(4), 341–347.

Sorial, G. A., Smith, F. L., Suidan, M. T., Pandit, A., Biswas, P., & Brenner, R. C. (1997). Evaluation of trickle bed air biofilter performance for BTEX removal. *Journal of Environmental Engineering-Asce, 123*(6), 530–537.

Survey, U. G. (2010). Groundwater use in the United States. USGS.

`

Wang, J., Yang, H., & Wang, F. (2014). Mixotrophic cultivation of microalgae for biodiesel production: status and prospects. *Applied Biochemistry and Biotechnology, 7*, 3307–3329.

Wang, J. F., Liu, J. L., & Liu, T. Z. (2015). The difference in effective light penetration may explain the superiority in photosynthetic efficiency of attached cultivation over the conventional open pond for microalgae. *Biotechnology for Biofuels, 8*, ARTN 49.

Wheeler, D. C., Nolan, B. T., Flory, A. R., DellaValle, C. T., & Ward, M. H. (2015). Modeling groundwater nitrate concentrations in private wells in Iowa. *Science of the Total Environment, 536*, 481–488.

Xu, X., Shen, Y., & Chen, J. (2015). Cultivation of Scenedesmus dimorphus for C/N/P removal and lipid production. *Electronic Journal of Biotechnology, 18*(1), 46–50.

Zhang, E., Wang, B., Ning, S., Sun, H., Yang, B., Jin, M., et al. (2012a). Ammonia-nitrogen and orthophosphate removal by immobilized Chlorella sp. isolated from municipal wastewater for potential use in tertiary treatment. *African Journal of Biotechnology, 11*(24), 6529–6534.

Zhang, Y., Ghyselbrecht, K., Vanherpe, R., Meesschaert, B., Pinoy, L., & Van der Bruggen, B. (2012b). RO concentrate minimization by electrodialysis: techno-economic analysis and environmental concerns. *Journal of Environmental Management, 107*, 28–36.

`

CHAPTER 4

IMMOBILIZED ALGAE-BASED TREATMENT OF AGRICULTURALLY CONTAMINATED GROUNDWATER[3]

4.1 Abstract

Scenedesmus species algal cells immobilized on alginate gel, were found effective in removing nitrate, atrazine, oxadiazon, and triallate from groundwater in a continuous flow reactor. A hydraulic retention time (HRT) of 7 days was provided in the reactors. The experiments with synthetic groundwater were performed at two temperatures (20 and 35 °C). The uptake rate of nitrate and atrazine were higher at room temperature due to the higher algal growth rate. When tested in actual groundwater, 92% of nitrate, 100% of magnesium, 99.9% of phosphorus, and 92% of zinc were successfully eliminated at the end of 29 days treatment operations. The beads removed 100% of oxadiazon and triallate in the first 10 days, but some of the herbicides diffused back into the solution toward the end of the treatment process.

4.2 Introduction

Application of agrochemicals such as fertilizers and herbicides in agriculture has increased yield of agricultural crops and minimized the economic losses but at the same time, caused environmental contamination (Subashchandrabose, Ramakrishnan, Megharaj, Venkateswarlu, & Naidu, 2013). Excessive application of nitrogen-based fertilizers in

[3] Mollamohammada S., Aly Hassan, A., Dahab, M. (2020) Immobilized algae-based treatment of agriculturally contaminated groundwater, *Water Environment Research* - journal, (In preparation).

`

agriculture has been the primary reason for high levels of nitrate in the groundwater (Majumdar & Gupta, 2000). Because of its high solubility in water and low retention by soil, nitrate can easily leach to the subsoil layers and contaminate groundwater.

Atrazine [6-chloro-N2-ethyl-N4-isopropyl-1,3,5-triazine-2,4-diamine] is the most common type of herbicide (Hayes et al., 2002), used to inhibit the photosynthetic electron transport in the weeds (Sene, Converti, Secchi, & Simão, 2010). Due to the presence of the C-Cl linkage, atrazine is a stable compound with a half-life time of 3-5 years (Bonora et al., 2013). Triallate [N,N-bis (propan-2-yl)[(2,3,3-trichloroprop-2-en-1-yl) sulfanyl] formamide] and oxadiazon [2-tert-butyl-4-(2,4-dichloro-5-isopropoxyphenyl)-1,3,4-oxadiazolin-5-one] are two other commonly- used herbicides with the same application as atrazine. Triallate belongs to thiocarbamate family (Wilson, 1984) and oxadiazon is an oxadiazole herbicide (Meister, 1981).

Water contaminated with nitrate and/or herbicides is of concern for human health if the water is consumed. One of the major health effects of drinking nitrate-contaminated water is methemoglobinemia or blue baby syndrome which is the result of nitrate conversion to nitrite in the infants' body. The nitrites combine with hemoglobin in the infant's blood to form methemoglobin, which is incapable of transporting oxygen from the infant's lungs to its body tissues – hence, "the blue baby syndrome." (Knobeloch, Salna, Hogan, Postle, & Anderson, 2000). A study performed in 2009 on a group of pregnant women showed that there is a correlation between high levels of atrazine in the drinking water and poor birth outcomes such as low body weight and incomplete bone formation (Ochoa-Acuña, Frankenberger, Hahn, & Carbajo, 2009). There are also studies that show correlation

`

between exposure to atrazine and Non-Hodgkin's lymphoma, prostrate and ovarian cancer (Dorsey, 2003).

The Environmental Protection Agency (EPA) set the maximum contaminant level (MCL) of 10 ppm for NO_3-N and 3 ppb for atrazine (EPA, 2018). The EPA has not established any MCLs for triallate and oxadiazon but they are both classified as highly toxic to fish and aquatic organisms (Company, 1989). The 96-hr lethal dose (LD)-50 in rainbow trout and bluegill exposed to triallate are reported to be 1.2 and 1.3 mg/L, respectively (Company, 1989; Meister, 1981). Moreover, an increase in tumors was seen in rats and mice fed oxadiazon in long term. Based on these results, U.S. EPA classified oxadiazon as likely to be carcinogenic to humans (Richert, Price, Chesne, Maita, & Carmichael, 1996).

Groundwater contaminated with nitrate and herbicides needs to be treated before being used as a source of drinking water. Pump-and-treat is a type of *ex-situ* remediation technique, used for contaminated groundwater. In this method, the contaminated water is extracted from the subsurface and sent to a treatment operation. The treated water would then be discharged back into the aquifer (Brusseau, 2019).

Once extraction wells transfer the contaminated water to the surface, the treatment methods are selected, based on the contaminants and the capacity of the treatment facility (Eastern Research Group & Information, 1996).

Reverse osmosis (RO) and ion exchange are currently the two most used methods to remove nitrate from groundwater. However, they both have disadvantages such as high energy requirements, the generation of waste products that need careful disposal, and high operation and maintenance costs (Bohdziewicz, Bodzek, & Wąsik, 1999).

`

Several methods have been tested to remove atrazine from water. Nanofiltration is one of the successful methods for atrazine removal. One study has reported the removals rate of 66%- 98% for atrazine contaminated water at an initial concentration of 3–5 µg/l (Organization, 2011a). UV radiation is another effective method that has been tested in reducing atrazine concentrations. In lab studies, 5-minutes irradiation time using 254 nm UV light removed 90% of atrazine. When tested at pilot plant scale, 70% of atrazine in a chalk borehole water was eliminated with UV dose of up to 700 W/m^3 (Organization, 2011b).

Biological treatment can be an economical and sustainable option for groundwater remediation. Strains of bacteria have been previously studied and applied for biological treatment of atrazine. In one study, a bacterial consortium consisted of *Rhodococcus sp. FJ1117YT, Bradyrhizobium japonicam CSB1, Arthrobacter 7 sp. CD7w* and *β-Proteobacteria CDB21* was found capable of degrading chloro-s-triazines (simazine and atrazine) and methylthio-s-triazines (simetryn and dimethametryn) over 4 and 9-days cultivation period, respectively. The mineralization occurred via the herbicide's 2-hydroxy 13 analogues (Yamazaki et al., 2008).

Even though strains of bacteria have shown promising results as bioremediation agents, they need high carbon and nutrient sources compared to algae (Subashchandrabose et al., 2013). Using microalgae can be an interesting alternative technique for bioremediation of waterbodies. Utilization of nutrients and organic compounds by microalgae is an eco-friendly treatment technique, as it produces valuable biomass that can be used for biofuel production (Ummalyma, Pandey, Sukumaran, & Sahoo, 2018).

`

Studies on the interactions between pesticides and algae have shown that microalgae is capable of bioaccumulation, biosorption or biotransformation of some environmental pollutants (Rath, 2012). Microalgal biomass has also shown to be effective in the removal of nitrate and heavy metals from aqueous solutions (Puranik & Paknikar, 1997; Volesky & Holan, 1995; B. Wang & Lan, 2011). Immobilization of microalgae is an attractive technique to retain biomass in a natural or synthetic polymer for a range of biological operations. The main advantages of using microalgae in immobilized form are easy separation of biomass from water, protecting cells from the harsh environment such as toxicity, and relatively high cell density (Al-Rub et al., 2004). These advantages make immobilized algal beads a great candidate for biological treatment of groundwater.

If immobilized microalgae is used as the remediation technique in groundwater pump and treat, nitrate, metals, and herbicides can also be removed simultaneously through assimilation, biosorption, bioaccumulation or biodegradation processes.

In this study, the possibility of using immobilized *scenedesmus sp.* for bioremediation of groundwater in continuous flow reactor (CFR) is evaluated.

4.3 Materials and Methods

4.3.1 Cultivation of algae

Scenedesmus. sp. was obtained from previous experiments at the Civil and Environmental Engineering Department at the University of Nebraska-Lincoln and cultivated using Bold's Basal Medium (BBM) at room temperature. The algae solution was illuminated using artificial plant light with the intensity of 600-700 Lux. The solution was used to prepare algal beads about two weeks after cultivation.

`

4.3.2 Preparation of algae- alginate beads

Immobilized algae-alginate beads were prepared by entrapping *Scenedesmus sp* in an alginate matrix according to the following steps: algae solution was centrifuged and a mixture of concentrated algae and a 2% sodium alginate solution was dropped into a 2% calcium chloride solution using a syringe pump (Harvard Apparatus, model 55-3333). The drops of algae-alginate gelled into 2.3 ± 0.1 mm diameter beads upon contact with the calcium chloride solution. The formed beads were kept in calcium chloride solution overnight to harden. The beads preparation procedure was described in our previous work (Mollamohammada, Hassan, & Dahab, 2020).

4.3.3 Experimental conditions

The experiments were performed using synthesized and actual groundwater. Synthesized groundwater was made by adding potassium nitrate (99.9%, Sigma Aldrich) and an analytical standard of atrazine (99.9%, Sigma Aldrich) to DI water at concentrations of 10 mg/L NO_3-N and 0.1 mg/L atrazine, respectively. The effect of the temperature on nitrate and atrazine removal efficiency was evaluated as one of the critical factors here. The reactors were operated at two different temperatures to represent different seasons; 20 °C to represent the fall and the spring, and 35 °C to represent summer. A summary of the experimental conditions is shown on Table 4.1.

The actual groundwater was collected from the City of Hastings Water Utilities, Hastings, NE. Upon collection, the water sample was stored in a refrigerator at 4°C. A complete analysis was done to quantify the concentration of nitrate, metal and non-metal ions and herbicides (Table 4.2). Prior to being used in the experiments, the suspended solids were

`

removed by filtering water through a Whatman syringe filter membrane. All the experiments with actual groundwater were performed at 20 $°C$.

Table 4.1 Summary of the experimental conditions.

Conditions		**Water source**	**Temperature**
I.	Synthetic Groundwater	DI water with 10 mg N/L nitrate and 0.1 mg/L atrazine (prepared in the lab)	20 °C
II.			35 °C
III.	Actual groundwater	City of Hastings, NE	20 °C

The treatment process was performed in continuous flow reactor (CFR), using four 500 mL flasks. A schematic of a CFR is shown on Figure 4.1.

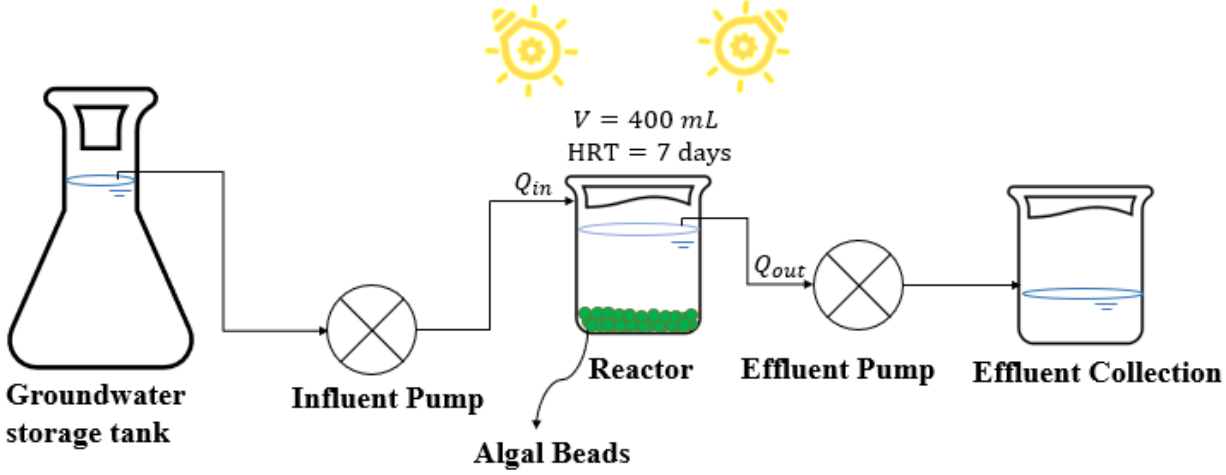


Figure 4.1 A schematic of a CFR.

The feed water was continuously pumped and accumulated in each reactor for the average of 7 days. Immobilized algal beads were added to the reactors at the beginning of the experiments. Light sources illuminated algae from above on an 18h: 6h light: dark cycle to simulate sunlight.

To prevent contamination, the flasks used in the experiments were autoclaved for 20 min at 121 $°C$. Each flask contained 400 mL of the aqueous solution and the ratio of the beads/

`

solution was 12.5% in all the reactors, based on the results from Chapter 3. Experiments were performed under heterotrophic conditions and glucose was used as an organic carbon source at a concentration of 125 mg/L.

No additional nutrients or glucose were provided in the beads or in the bulk solution of the reactors containing actual groundwater.

All experiments were performed in triplicates and the results presented in this work is the mean of three replicated studies.

Table 4.2 Analysis of the actual groundwater, collected from Hastings Utilities, NE.

Detection Method	**Parameters**	**Groundwater**
TNT vials	COD	100±2
UV-VIS	Nitrate (ppm)	9.07±1.04
ICP-MS	Sodium (ppm)	49.30±0.21
	Magnesium (ppm)	21.34±0.18
	Potassium (ppm)	10.09±0.04
	Calcium (ppm)	120.45±0.29
	Sulfur (ppm)	26.18±0.19
	Phosphorous (ppm)	2.76±0.006
	Zink (ppb)	19.50±0.18
	Chromium (ppb)	1.06±0.07
	Vanadium (ppb)	2.83±0.07
	Arsenic (ppt)	1,706±36
	Molybdenum (ppt)	1,251±106
	Lithium (ppt)	24,882±257
	Boron (ppt)	61,544±1248
	Manganese (ppt)	11,873±114
	Nickel (ppt)	1,393±103
	Selenium (ppt)	2,299±42
GC-MS	Oxadiazon (ppb)	111.5±10.1
	Triallate (ppb)	190±12.4

4.3.4 Analytical Measurements

The concentration of NO_3-N in the reactors was measured using a UV-vis spectrophotometer (Thermo Scientific, Nano-Drop, model 2000). Vacuum-assisted sorbent

`

extraction (VASE) (Entech Instruments, Model 5800) was applied in combination with gas chromatography-mass spectrometry (GC-MS) (Agilent Technologies, Model 5977B) to measure the concentration of herbicides in the aqueous solutions. The concentration of dissolved oxygen (DO), Chemical Oxygen demand (COD) and pH were measured using a DO meter (YSI, model 5100), TNTplus vial tests (Hach, TNT820) and pH meter (Thermo Scientific, model Orion 3-star). Scanning Electron Microscopy and Energy dispersive X-ray spectroscopy (EDS) analysis were performed using Nova Nano SEM (FEI, model 450) in order to examine the morphological structure and elemental analysis on the beads. The concentrations of metals and non-metal elements in the groundwater were quantified using Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) (Agilent, 7500 CX).

4.4 Results and Discussion

4.4.1 Algae growth and Nitrogen Removal

The density of the algal cells inside of the immobilized matrix was measured using Dry Cell Weight (DCW). As it's shown in Figure 4.2a, the highest biomass concentration was observed in the reactor operating at 20 °C. Previous studies have also reported 20-25 °C as the optimal temperature for algal biomass production (Delgadillo-Mirquez et al., 2016; Martınez, Sánchez, Jimenez, El Yousfi, & Munoz, 2000). Rupture of algal cells occurred in the reactor operating at 35 °C toward the end of the treatment process. It could be contributed to the excessive temperature of the water which inhibited the growth of the cells (Martınez et al., 2000)

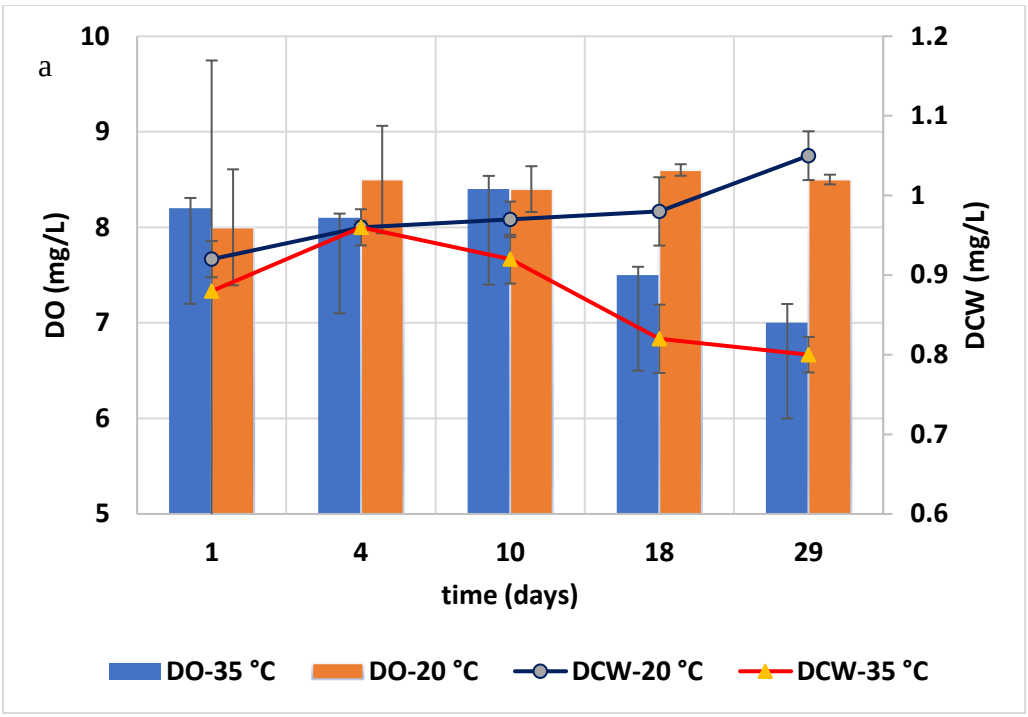
Exposure of algal cells to high temperature over a long period impacts structure and activity of the cell membrane, which disturbs metabolic processes and result in the retardation of growth (Béchet, Laviale, Arsapin, Bonnefond, & Bernard, 2017). Low

`

concentration of dissolved oxygen (DO) was also an indicator of retardation in growth of algal cells in temperature of 35 °C. When algal cells photosynthesize, they consume dissolved carbon dioxide and produce oxygen (Mollamohammada et al., 2020). A drop in pH value toward the end of the treatment operations is another indicator that photosynthetic is decreasing in algal cells.

The conductivity of the water was also measured in different days of the treatment. As shown in Figure 4.2b, the reactor operating at 35 °C has higher conductivity compared to the one operating at room temperature. The conductivity increased over time in both reactors. As it was explained in materials and methods, the immobilized beads were kept in calcium chloride solution to solidify. Increasing the conductivity over time is due to the presence of calcium and chloride ions in the bulk solution, which have partially diffused from the beads to the water.

`



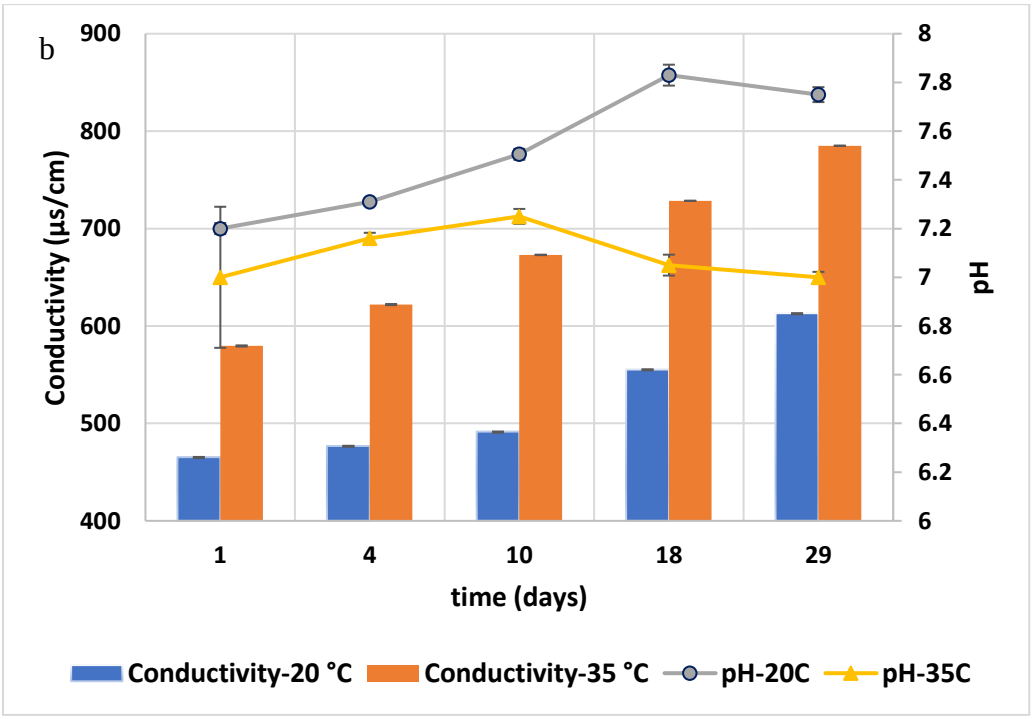


Figure 4.2 a) Growth of *Scenedesmus sp*, represented as DCW and changes in DO concentration and b) Changes in pH & conductivity of the CFRs operating at 20 and 35° C. Error bars represent standard deviations. Day 1 represents 7 days after the reactor's start up.

`

Figure 4.3 shows the average nitrate uptake in CFR during a period of 29 days as a function of time. As it is shown, the nitrate concentration in the bulk solution decreased over time in the reactor operating at room temperature; and reached steady state status at day 21. For the reactor operating at $35°C$, the nitrate concentration in the solution starts to rise after 16 days.

Nitrate uptake by algal beads requires energy and reducing power, which is provided by glucose, carbon dioxide and the light. Physical, chemical and biological factors impact the rate of nitrate uptake by algal beads and temperature is considered as one of the most important physical factors. (Pedersen, Kraemer, & Yarish, 2004). Generally, the optimum temperature for the algal growth is the same as the optimum temperature for the uptake of the nutrients (Pedersen, Kraemer, & Yarish, 2004). For *Scenedesmus sp*., $20°C$ was reported as the optimum temperature to achieve the maximum specific growth rate (Xin, Hong-Ying, & Yu-Ping, 2011).

A rapidly growing, healthy culture is needed to provide an efficient long-term nitrate uptake treatment system. Since the nitrate uptake rate is a function of algal growth, a high nitrate uptake rate may not be achieved under too low or too high temperature. According to these results, two different trends are recognizable based on the temperature of the water. At $20°C$ (spring or fall), it is useful to keep the culture until the end of 30 days projected. In $35°C$ (summer), however, it is best to replace the algal beads after 10 days of the treatment to keep the nitrate uptake rate at the maximum range.

`

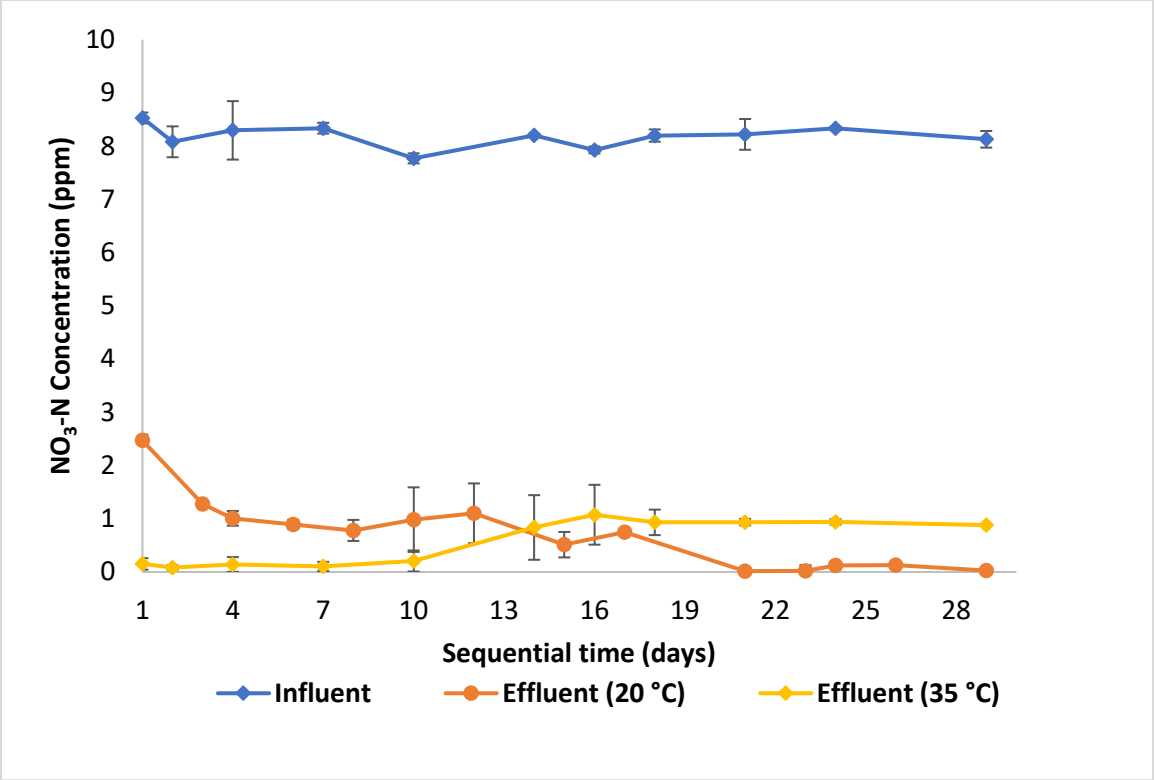


Figure 4.3 Changes in the concentration of NO_3-N in two CFRs, operating at 20 and 35° C.

4.4.2 Atrazine removal

The performance of the algal beads operating at 20° C and 35° C in removal of atrazine is summarized in Figure 4.4. A higher removal rate was observed in the reactor operating at 35° C at the beginning of the experiments (day 5). By day 15, the performance of the reactor operating at room temperature surpassed the 35 °C one. The solution was tested for the concentrations of two atrazine metabolites; Deethyl-atrazine (DEA), and Deisopropyl-atrazine (DIA) and none of them was detected in the solution. The algal beads were also tested for the presence of atrazine and nothing was detectable in the beads. Since the removal occurred during the period of 29 days, the microalgae had enough time to bioaccumulate, metabolize or facilitated the degradation of atrazine. Since the removal rate

`

by the control (alginate beads) is about half of what observed in algal beads, it is still possible that the removal of atrazine by algal beads happened through combination of adsorption on the surface of the alginate, followed by bioaccumulation and biodegradation by microalgae. Generally, sorption of organic compounds (e.g. atrazine) is a passive process that is performed by chemical partitioning into the hydrophobic biomass. The uptake of atrazine by the algal cells includes an initial sorption, which is due to the affinity of the atrazine to the surface of the algae, followed by membrane transport of atrazine into the algal cells. The environmental conditions may not have an impact on the first phase; however, transport of atrazine through the cell membrane into algal cells may be impacted by several factors, such as composition of the cell membranes and specific binding to photosystem II components of the chloroplast (Tang, Hoagland, & Siegfried, 1998).

High density of microalgae in the beads accelerated the atrazine bioaccumulation rate at the start of the experiments in the reactor performing at 35°C. The continuous excessive temperature however, lowered the density of the algal cells in the beads and decreased the removal rate.

`

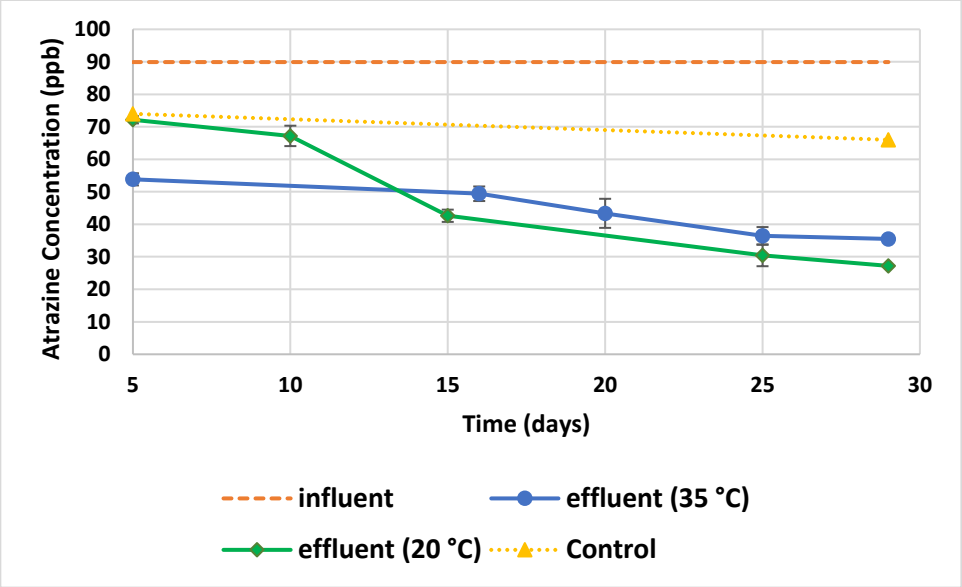


Figure 4.4 Change in concentration of atrazine in CFRs, operating at 20 and 35 °C.

4.4.3 Remediation of the Actual Groundwater

Table 4.3 shows the removal efficiency of nitrate and other ions from the actual groundwater after 29 days of operation in the CFR. Nutrient removal by algal beads includes the adsorption of nitrate on the surface of the bead, penetration into the alginate and then, sorption into the cells (MS Abdel Hameed, 2007).

The Nitrate removal rate depends on culture conditions such as the medium composition. In this case, groundwater was rich in nutrients and trace minerals and provided a suitable environment for microalgae to grow and assimilate nitrate.

Among the elements present in the water, the removal rate of Mg, P, and Zn was the highest (100%, 99.9% and 92%, respectively). SEM and EDS analyses were performed to study the surface morphologies of the beads after exposure to the groundwater (Figure 4.5).

`

Table 4.3 Removal of ions from the actual groundwater after 29 days of the operation in CFR.

Component	**Removal efficiency (%)**
Nitrate (NO_3)	92±2.1
Sodium (Na)	45±3.4
Magnesium (Mg)	99.9±1.02
Potassium (K)	8.03±0.88
Phosphorous (P)	97.2±2.5
Iron (Fe)	71.1±1.4
Zink (Zn)	96.6±1.2
Sulfur (S)	28±2.3

SEM provides more insight into the mechanism of metal sorption by algal beads and help in the detection of active sorptive areas, and EDS analysis can confirm the identity of the elements on the surface of the beads (W. W. Maznah, A. Al-Fawwaz, & M. Surif, 2012). The SEM images of algal beads exposed to the groundwater show that the surfaces of these beads were rough, and had more fractures compared to what we saw in the unexposed beads (Figure 4.5b). These changes could have happened due to the presence of the metal ions in the solution. Carboxyl, hydroxyl, sulphate, phosphate, amino, amido and phenolic groups are reported as functional groups on the surface of algal beads which can attract heavy metals (Petrovič & Simonič, 2016). Therefore, one can assume that the metals in the solution were attached to the functional groups and made changes on the surface of the beads (WO Wan Maznah et al., 2012)

`

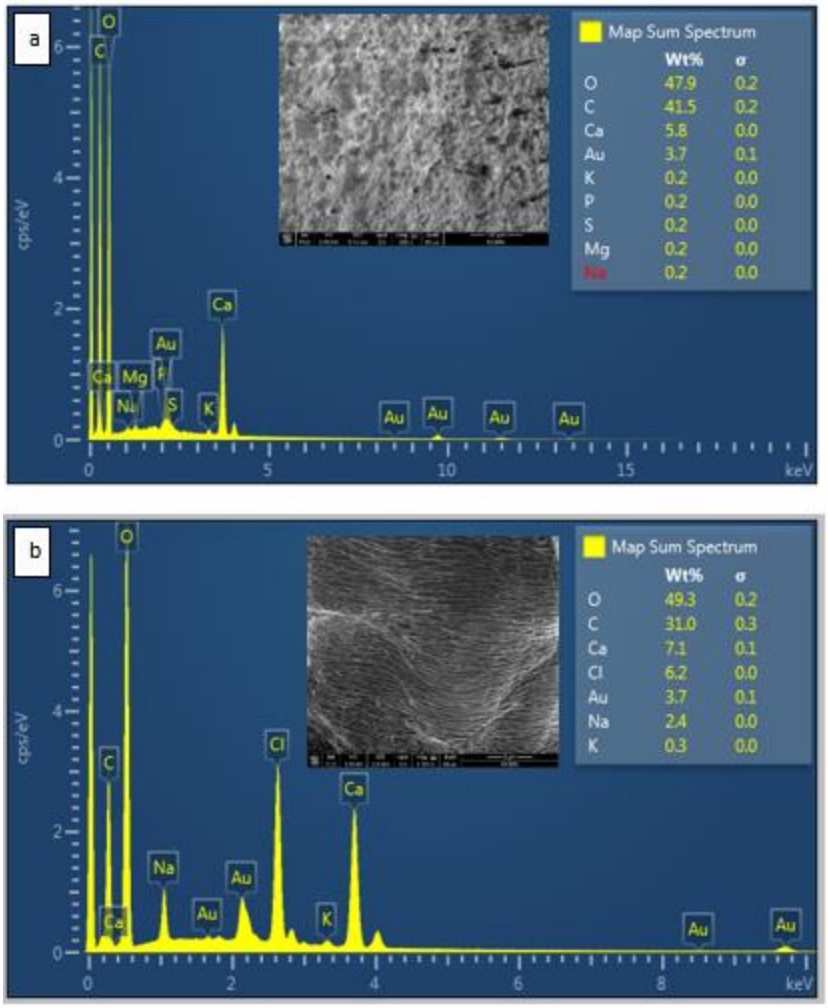


Figure 4.5 SEM and EDS analysis of immobilized *S. Sp* beads exposed to a) groundwater and b) an unexposed bead.

The EDS spectra of the immobilized *Scenedesmus sp*. beads confirmed the presence of some metal and non-metal elements on the surface of biosorbent. The surface of the beads was covered with gold prior to SEM/ EDS analyses and that's why we see the peak of gold within the EDS spectrum. The spectrum also showed peaks for C, O, and Ca, which could be explained by the composition of the algal beads. Alginate is one of the main components in algal beads which consists of β-D-mannuronic acid and α-L-guluronic acid units; which are rich in C and O. $CaCl_2$ was used in preparation of algal beads as the hardening material

`

and it explains the presence of Ca ions on the surface of the beads.. The other main elements on the surface of the beads exposed to the groundwater were K, P, S, Mg and Na, all of which existed in theses samples according to the ICP-MS analysis (Table 4.2) and removed by algal beads (Table 4.3). Biosorption can be the main mechanism controlling metal uptake by algal beads. In biosorption process, metals ions transfer from the solution to the surface of the beads, which contains active binding sites. The metal ions then diffuse into the pores of absorbent and finally on the internal surface of the beads (Petrovič & Simonič, 2016).

The algal beads were also tested for their capability in removing herbicides. No residual herbicides were found in the solution at days 1-10 of the operation. Therefore, 100% of oxadiazon and triallate were removed in the first 10 days of treatment in CFR. Partial uptake of herbicides was observed in the control, which was, likely, due to the adsorption on the surface of alginate. At days 1 and 10, the medium with algal beads contained less amounts of herbicides than the controls (w/o alga), indicating that the herbicides were accumulated by the algae.

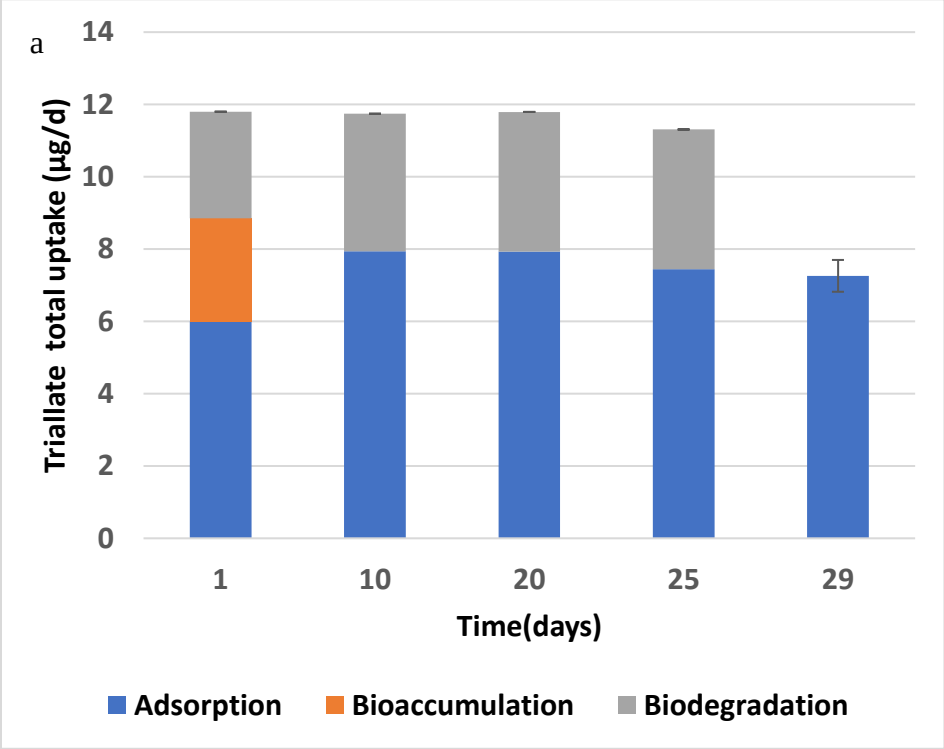
The rate of biodegradation $\frac{dB}{dt}(\mu g.d^{-1})$ was calculated using Equation 4.1:

$$\frac{dB}{dt} = \dot{m}_{in} - \dot{m}_{out} - \dot{m}_d - \dot{m}_c \qquad \text{Equation 4.1}$$

Where $\dot{m}_{in}$ is the mass of the herbicide entering the reactor, $\dot{m}_{out}$ is the mass of the herbicide in the effluent, $\dot{m}_d$ is the mass of herbicide adsorbed by the alginate beads, and $\dot{m}_c$ is the mass of the herbicide accumulated in microalgae. All mass rates are measured in $\mu g.d^{-1}$.

`

The accumulation rate of triallate reduced from day 1 to 10 and biodegradation rate started to increase. An unsteady accumulation rate by algal beads was an indicator of biodegradation occurring in the cells. For oxadiazon, however, no accumulation was detected in the beads and the majority of uptake, likely occurred through adsorption on the alginate beads (Figure 4.6b).



`

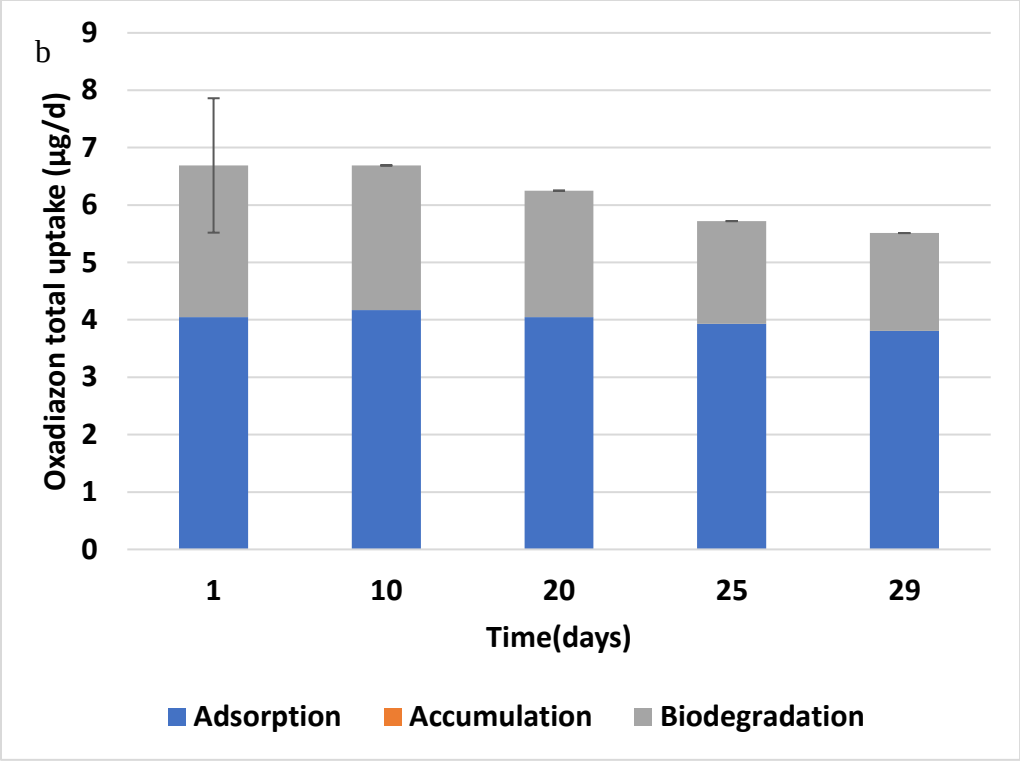


Figure 4.6 The uptake of a) Triallate and b) Oxadiazon by immobilized algal beads through adsorption, accumulation and biodegradation.

According to the previous studies, accumulation of xenobiotics may result in stress in algae cells, caused by toxicity of the herbicides. This stress can activate the defense mechanism of the cells and result in degradation of herbicides (Szewczyk, Kuśmierska, & Bernat, 2018). Therefore, although the herbicides may inhibit the growth of *Scenedesmus sp*, they can promote the accumulation and simultaneous degradation, and enhance the overall remediation rate.

4.5 Conclusions

In this chapter, the experiments in CFR were performed to assess the capability of immobilized algal beads removing nitrate and herbicides in continuous flow mode. The following conclusions could be drawn from this study:

`

1. The results show that the immobilized algal beads were more effective in nitrate and atrazine removal in room temperature (20 °C) than the high temperature (35 °C). The results suggest that during spring or fall the culture can be kept until the end of 30 days projected. During summer, it is best to replace the algal beads after 10 days of the treatment operation.

2. Immobilized *Scenedesmus sp.* beads were capable of simultaneous removal of nitrate, metals and herbicides from actual groundwater in the CFR. The removal of oxadiazon and triallate occurred through the combination of adsorption, bioaccumulation and biodegradation. Due to the possible toxicity of the herbicides to the algae, the removal efficiency decreased as the treatment process progressed.

`

References

Al-Rub, F. A., El-Naas, M., Benyahia, F., & Ashour, I. (2004). Biosorption of nickel on blank alginate beads, free and immobilized algal cells. *Process Biochemistry, 39*(11), 1767-1773.

Béchet, Q., Laviale, M., Arsapin, N., Bonnefond, H., & Bernard, O. (2017). Modeling the impact of high temperatures on microalgal viability and photosynthetic activity. *Biotechnology for biofuels, 10*(1), 136.

Bohdziewicz, J., Bodzek, M., & Wąsik, E. (1999). The application of reverse osmosis and nanofiltration to the removal of nitrates from groundwater. *Desalination, 121*(2), 139-147.

Bonora, S., Benassi, E., Maris, A., Tugnoli, V., Ottani, S., & Di Foggia, M. (2013). Raman and SERS study on atrazine, prometryn and simetryn triazine herbicides. *Journal of Molecular Structure, 1040*, 139-148.

Brusseau, M. L. (2019). Soil and groundwater remediation. In *Environmental and pollution science* (pp. 329-354): Elsevier.

Cermak, O., & Cermakova, M. (1992). Treatment of atrazine and other pesticides-laboratory studies. *Water Supply (United Kingdom)*.

Company, M. A. (1989). *Toxicology Information Summary for Triallate.* Retrieved from

Delgadillo-Mirquez, L., Lopes, F., Taidi, B., & Pareau, D. (2016). Nitrogen and phosphate removal from wastewater with a mixed microalgae and bacteria culture. *Biotechnology Reports, 11*, 18-26.

Dorsey, A. (2003). *Toxicological profile for atrazine*: Agency for Toxic Substances and Disease Registry.

Eastern Research Group, I., & Information, C. f. E. R. (1996). *Pump-and-treat Ground-water Remediation: A Guide for Decision Makers and Practitioners*: US Environmental Protection Agency, Office of Research and Development ….

EPA. (2018). *2018 Edition of the Drinking Water Standards and Health Advisories Tables*. Retrieved from

Hameed, M. A. (2007). Effect of algal density in bead, bead size and bead concentrations on wastewater nutrient removal. *African Journal of biotechnology, 6*(10).

Hayes, T. B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A. A., & Vonk, A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proceedings of the National Academy of Sciences, 99*(8), 5476-5480.

Knobeloch, L., Salna, B., Hogan, A., Postle, J., & Anderson, H. (2000). Blue babies and nitrate-contaminated well water. *Environmental health perspectives, 108*(7), 675-678.

Majumdar, D., & Gupta, N. (2000). Nitrate pollution of groundwater and associated human health disorders. *Indian journal of environmental health, 42*(1), 28-39.

Martınez, M., Sánchez, S., Jimenez, J., El Yousfi, F., & Munoz, L. (2000). Nitrogen and phosphorus removal from urban wastewater by the microalga Scenedesmus obliquus. *Bioresource Technology, 73*(3), 263-272.

Maznah, W. W., Al-Fawwaz, A., & Surif, M. (2012). Biosorption of copper and zinc by immobilised and free algal biomass, and the effects of metal biosorption on the growth and

`

cellular structure of Chlorella sp. and Chlamydomonas sp. isolated from rivers in Penang, Malaysia. *Journal of Environmental Sciences, 24*(8), 1386-1393.

Meister, R. T. (1981). *Farm chemicals handbook*: Meister Publishing Company.

Mollamohammada, S., Hassan, A. A., & Dahab, M. (2020). Nitrate Removal from Groundwater Using Immobilized Heterotrophic Algae. *Water, Air, & Soil Pollution, 231*(1), 1-13.

Ochoa-Acuña, H., Frankenberger, J., Hahn, L., & Carbajo, C. (2009). Drinking-water herbicide exposure in Indiana and prevalence of small-for-gestational-age and preterm delivery. *Environmental health perspectives, 117*(10), 1619-1624.

Organization, W. H. (2011). *Atrazine and its metabolites in drinking-water*. Retrieved from

Pedersen, A., Kraemer, G., & Yarish, C. (2004). The effects of temperature and nutrient concentrations on nitrate and phosphate uptake in different species of Porphyra from Long Island Sound (USA). *Journal of Experimental Marine Biology and Ecology, 312*(2), 235-252.

Petrovič, A., & Simonič, M. (2016). Removal of heavy metal ions from drinking water by alginate-immobilised Chlorella sorokiniana. *International journal of environmental science and technology, 13*(7), 1761-1780.

Puranik, P., & Paknikar, K. (1997). Biosorption of lead and zinc from solutions using Streptoverticillium cinnamoneum waste biomass. *Journal of biotechnology, 55*(2), 113-124.

Rath, B. (2012). Microalgal bioremediation: current practices and perspectives. *Journal of Biochemical Technology, 3*(3), 299-304.

Richert, L., Price, S., Chesne, C., Maita, K., & Carmichael, N. (1996). Comparison of the induction of hepatic peroxisome proliferation by the herbicide oxadiazon in vivo in rats, mice, and dogs and in vitro in rat and human hepatocytes. *Toxicology and Applied Pharmacology, 141*(1), 35-43.

Sene, L., Converti, A., Secchi, G. A. R., & Simão, R. d. C. G. (2010). New aspects on atrazine biodegradation. *Brazilian Archives of Biology and Technology, 53*(2), 487-496.

Subashchandrabose, S. R., Ramakrishnan, B., Megharaj, M., Venkateswarlu, K., & Naidu, R. (2013). Mixotrophic cyanobacteria and microalgae as distinctive biological agents for organic pollutant degradation. *Environment international, 51*, 59-72.

Tang, J., Hoagland, K. D., & Siegfried, B. D. (1998). Uptake and bioconcentration of atrazine by selected freshwater algae. *Environmental Toxicology and Chemistry: An International Journal, 17*(6), 1085-1090.

Ummalyma, S. B., Pandey, A., Sukumaran, R. K., & Sahoo, D. (2018). Bioremediation by microalgae: current and emerging trends for effluents treatments for value addition of waste streams. In *Biosynthetic Technology and Environmental Challenges* (pp. 355-375): Springer.

University, O. S. (2017). *Oxadiazon Roadside Vegetation Management Fact Sheet*. Retrieved from

Volesky, B., & Holan, Z. (1995). Biosorption of heavy metals. *Biotechnology progress, 11*(3), 235-250.

Wang, B., & Lan, C. Q. (2011). Biomass production and nitrogen and phosphorus removal by the green alga Neochloris oleoabundans in simulated wastewater and secondary municipal wastewater effluent. *Bioresource Technology, 102*(10), 5639-5644.

Wilson, R. G. (1984). Accelerated degradation of thiocarbamate herbicides in soil with prior thiocarbamate herbicide exposure. *Weed Science, 32*(2), 264-268.

`

Wu, M., Quirindongo, M., Sass, J., & Wetzler, A. Still poisoning the well: atrazine continues to contaminate surface water and drinking water in the United States, 2010. *The National Resources Defense Council*.

Xin, L., Hong-Ying, H., & Yu-Ping, Z. (2011). Growth and lipid accumulation properties of a freshwater microalga Scenedesmus sp. under different cultivation temperature. *Bioresource Technology, 102*(3), 3098-3102.

Yamazaki, K.-i., Takagi, K., Fujii, K., Iwasaki, A., Harada, N., & Uchimura, T. (2008). Simultaneous biodegradation of chloro-and methylthio-s-triazines using charcoal enriched with a newly developed bacterial consortium. *Journal of Pesticide Science*, 0806060043-0806060043.

`

CHAPTER 5

FURTHER STUDIES ON THE USE OF ALGAE-BASED TREATMENT FOR NITRATE AND HERBICIDES REMOVAL FROM CONTAMINATED SURFACE WATERS [4]

5.1 Abstract

The treatment efficiency of *Scenedesmus species*, immobilized in sodium alginate was evaluated for removing nitrate and herbicides from surface water. The experiments were performed in a continuous flow reactor and a hydraulic retention time (HRT) of 7 days was provided. The surface water was collected from two different locations: Rockford Lake, Beatrice, Nebraska with cyanotoxins, triallate and cycloate contamination and Wahoo Creek, Ashland, NE with ametryn, simetryn and alachlor. The average removal efficiencies of nitrate from Rockford Lake and Wahoo Creek were 85% and 80%, respectively, during 29 days of the operation. Nearly 100% of Mercury and Sulfur were eliminated at the end of the treatment process. The beads were able to remove 90-100% of ametryn, simetryn, alachlor, triallate and cycloate during the first 10 days, but the herbicides started to diffuse back into the solution toward the end of the treatment process. These results suggest that immobilized algal beads can be used as a natural, low-cost alternative for bioremediation of the contaminated surface water.

[4] Mollamohammada S., Aly Hassan, A., Dahab, M. (2020) Immobilized algae-based treatment of agriculturally contaminated groundwater, *Science of the Total Environment* - journal, (In preparation).

`

5.2 Introduction

The presence of excessive nutrient and xenobiotics residue in surface water is likely the result of excessive application of agrochemicals in the past decades (Szewczyk, Kuśmierska, & Bernat, 2018). Transport of fertilizers, herbicides and insecticides from agricultural fields to the waterbodies has decreased the water quality and caused environmental contamination(Gentry, David, Smith-Starks, & Kovacic, 2000). These contaminants have been previously observed in streams that receives large amount of agricultural runoff.

Most fertilizers that are used in agriculture contain nitrogen and are known as the primary source of high nitrate levels in waterbodies (Majumdar & Gupta, 2000). Among herbicides, triazines are the most heavily used in agricultural fields (Klementova & Keltnerova, 2015). Triazines are characterized by a structure of nitrogen-containing heterocycle (Szewczyk et al., 2018) and known for inhibiting photosynthesis in broadleaf weeds (Brooks et al., 2005).

S-triazines or 1,3,5-triazines with the formula of $(HCN)_3$ are a group of herbicides that are highly soluble in water and have long half-life time (Navarro, Vela, Giménez, & Navarro, 2004; Szewczyk et al., 2018). S-Triazines can easily leach from the soil to the water bodies and are considered major risks to the environment (Prosen, 2012).

Ametryn [4-N-ethyl-6-methylsulfanyl-2-N-propan-2-yl-1,3,5-triazine-2,4-diamine], and simetryn [2-N,4-N-diethyl-6-methylsulfanyl-1,3,5-triazine-2,4-diamine] are two common types of S-triazine herbicide (Hayes et al., 2002). They bind to the quinone-binding protein in photosystem II, and inhibit the photosynthetic electron transport in weeds (Bonora et al., 2013; Yalkowsky, He, & Jain, 2016).

`

Ametryn is classified as a class III herbicides; meaning that it has moderate toxic effects on fish, humans and large mammals, but it is considered highly toxic to crustaceans and molluscs (Navaratna et al., 2012). Simetryn is toxic to the aquatic system and the studies have proven that the exposure of tadpoles to simetryn in concentrations founds in water used for rice cultivation can be highly toxic (Saka, 2010).

Alachlor (2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide), is another type of herbicide, which belongs to the chloroacetamide class (Dale, Renner, & Kravchenko, 2006; Wilson, 1984). Triallate [N,N-bis(propan-2-yl)[(2,3,3-trichloroprop-2-en-1-yl) sulfanyl]formamide] and cycloate [N-cyclohexyl-N-ethyl(ethylsulfanyl)formamide] are two other types of herbicides, that belong to thiocarbamate family. All three have the same application as S-triazine herbicides (Dale et al., 2006; Wilson, 1984).

The residues of alachlor in drinking water has been regulated under the Safe Drinking Water Act (SDWA). EPA has set the Maximum Contaminant Level (MCL) of 2 ppb for alachlor (EPA, 2018). Alachlor is known to increase the risk of developing cancer and other chronic diseases (W. J. Lee et al., 2004).

Triallate is considered highly toxic to aquatic organisms (Company, 1989). Cycloate is classified as “Not Likely Carcinogenic to Humans”, however, a study performed on rats showed that cycloate is a neurotoxicant and a large oral dose of this herbicide induced a cell-specific and highly localized forebrain lesion (Simpson et al., 2005).

Microorganisms have been studied and applied to remove herbicides from water. *Rhodococcus sp.* strain FJ1117YT was found to be effective in degrading simetryn. Methylsulfinyl and methylsulfonyl analogues were identified as the metabolites (Fujii,

`

Takagi, Hiradate, Iwasaki, & Harada, 2007). In another study, *Paecilomyces marquandii*, a microscopic fungus, was found capable of alachlor removal by N-acetyl oxidation (Szewczyk, Soboń, Słaba, & Długoński, 2015)

Eventhough bacteria have shown promising results in removing organic pollutants, they need high carbon and nutrient sources compared to algae (Subashchandrabose et al., 2013). Algae can be a sustainable alternative for contaminted water bioremediation. Algae can take up nutrients and organic compounds and produce valuable biomass which can be used to produce biofuel (Ummalyma et al., 2018).

Microalgae are shown to be able to assimilate, accumulate, sorb or transforme some environmental pollutants (Rath, 2012). In one study, the bioremediation of a mixture of herbicides including Atrazine, Molinate, Simazine, Isoproturon, Propanil, Carbofuran, Dimethoate, Pendimethalin, Metoalcholar, Pyriproxin was evaluated using the microalgae *Chlorella vulgaris*. The microalgae were able to remove 87% to 96.5% of the herbicides (Hussein, Abdullah, Badr El-Din, & Mishaqa, 2017).

Immobilization of microalgae is an attractive technique to retain biomass in a natural or synthetic polymer for a range of biological operations. The main advantages of using microalgae in immobilized form are easy separation of biomass from water, protecting cells from the harsh environment such as toxicity, and the production of relatively high cell density(Al-Rub et al., 2004). These advantages make immobilized algal beads a great candidate for biological treatment of waterbodies. In this treatment process, herbicides, heavy metals and nutrients can also be removed simultaneously.

In this study, immobilized microalgae were evaluated as a natural treatment system for the uptake of nitrate and herbicides from surface water. To assess the capability of the beads

`

in real working scenarios the experiments are performed during two stressed conditions: lake with algae bloom and creek at harvesting season with large concentration of herbicides.

5.3 Materials and Methods

5.3.1 Cultivation of algae

The strain of *Scenedesmus sp.* was cultivated in Bold's Basal Medium (BBM). A grow light was used to illuminate the microalgae solution at the intensity of 600-700 Lux. The solution was used to prepare the algal beads about two weeks after cultivation.

5.3.2 Preparation of algae- alginate beads

To prepare the immobilized algae- alginate beads, the algae solution was centrifuged at 3500 rpm, and the concentrated cells were mixed with a 2% sodium alginate solution. The mixture was then dropped into a 2% calcium chloride solution. Algal beads which are 2.3 ± 0.1 mm in diameter were formed upon contact with calcium chloride solution.

5.3.3 Experimental conditions

The experiments were performed using actual samples of surface water. The water samples were collected from two different locations: Rockford Lake, Beatrice, NE, and Wahoo Creek, Ashland, NE. Wahoo creek was chosen because it receives large amount of agricultural runoff. The sampling was performed in June because of the expected high concentration of nitrate and herbicides in the creek resulting from agricultural activities.

Rockford Lake was selected based on the weekly results from the Nebraska Department of Environmental Quality (now the Nebraska Department of Energy and Environment)

`

reports. The weekly reports identified cyanotoxins in the lake around late July of 2019 and it was when the samples were collected.

The samples were stored in a refrigerator at 4°C. A complete analysis was completed to quantify the concentration of nitrate, herbicides and other constituents (Table 5.1). Before starting the experiments, the suspended solids in the water were removed through filtration. All the experiments were performed at $20\ °C$.

The results from batch studies in Chapter 3 were used to select the operational parameters in continuous flow reactors (CFRs). The nitrate and herbicide impaired water was passed through a CFR. Four 500 mL flasks were used to test this process. A schematic of the CFR used in these experiments is shown on Figure 5.1.

The feed water was continuously pumped and allowed to accumulate in each reactor. Accumulated water stayed at an average HRT of 7 days. Algae beads were added at the start of the experiments. Light sources were introduced from above on an 18h: 6h light: dark cycle to simulate sunlight.

All flasks were autoclaved for 20 mins at $121\ °C$ prior to being used in the experiments. No additional nutrients or glucose was provided in the beads or in the bulk solution during the experiments.

The experiments were performed in triplicates and the results presented here is the mean of three replicated studies.

`

Table 5.1 Analysis of the water samples (based on three replicas).

Measurement Methodology	**Parameters**	**Wahoo Creek**	**Rockford Lake**
UV-VIS	Nitrate (ppm)	7.20±0.75	8.44±0.39
TNT vials	COD (ppm)	203.11±8.05	188.67±9.45
ICP-MS	Sodium (ppm)	29.12±0.17	38.21±0.54
	Magnesium (ppm)	10.86±0.11	11.61±0.21
	Potassium (ppm)	8.89±0.081	10.95±0.19
	Calcium (ppm)	30.36±0.82	30.09±0.45
	Phosphorous (ppb)	595.0±1.04	68.0±0.44
	Iron (ppb)	66.42±0.82	27.16±0.12
	Copper (ppb)	5.9±0.09	7.72±0.14
	Zink (ppb)	23.4±0.11	5.38±0.26
	Mercury (ppb)	32.74±0.89	11.71±0.07
	Molybdenum (ppb)	0.78±0.12	1,439±3.2
GC-MS	Simetryn (ppb)	1,448±12	ND
	Ametryn (ppb)	480±14	ND
	Cycloate (ppb)	ND	150±13
	Triallate (ppb)	ND	120±11
	Alachlor (ppb)	23±2	ND

`

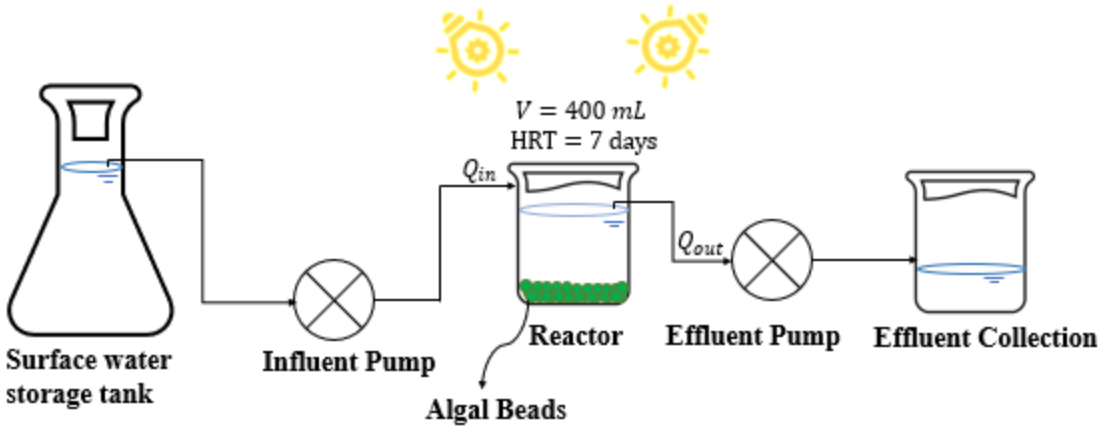


Figure 5.1 A schematic of a CFR designed and operated in this study.

5.3.4 Analytical Measurements

A UV-vis spectrophotometer (Thermo Scientific, Nano-Drop, model 2000) was used to quantify the concentration of NO_3-N in the reactors. The concentration of herbicides was measured using Vacuum-assisted sorbent extraction (Entech Instruments, Model 5800) in combination with gas chromatography-mass spectrometry (Agilent Technologies, Model 5977B). The dry cell weight (DCW) was measured to determine the growth of algal cells inside of the beads. Energy dispersive X-ray spectroscopy (EDS) and scanning electron microscopy (W. J. Lee et al.) analysis was performed using Nova Nano SEM (FEI, model 450) for the elemental analysis and examination of the bead's morphology. Inductively coupled plasma-mass spectrometry (Agilent, model 7500 CX) was used to measure the concentration of the heavy metals in the water.

5.4 Results and Discussion

`

5.4.1 Algal growth

Figure 5.2a shows the changes in DCW and dissolved oxygen (DO) for the two sets of CFRs. As shown, the algal cells had lower growth in the reactor containing water sample from Rockford Lake. In both reactors, DCW and DO concentration started to go down after 10 days of the operations and continued toward the end of the treatment process.

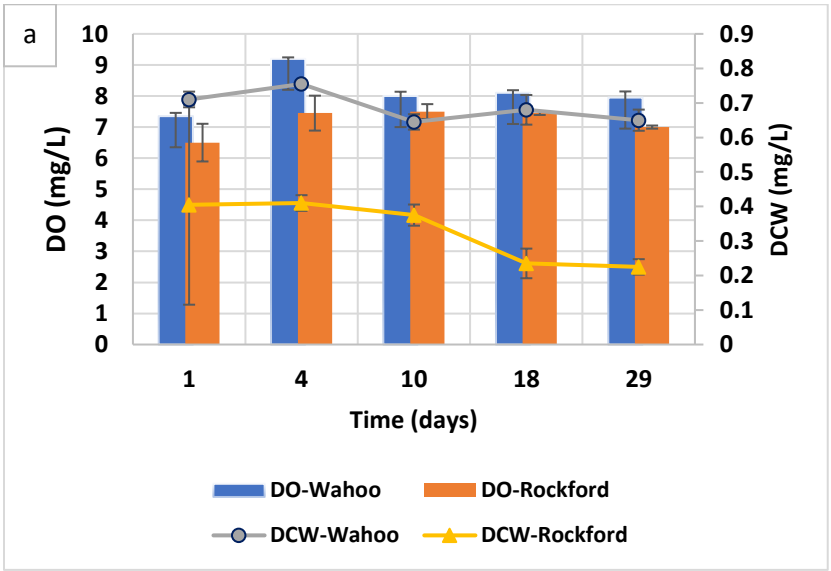
As shown in Table 5.1, the sample from Wahoo Creek contained simetryn, ametryn and alachlor. Studies have shown that these herbicides are toxic to green microalgae, which could have been the main reason for retardation of algal growth toward the end of the treatment process. The herbicides can inhibit photosynthesis by interfering in the Hill Reaction which is an important step in photosynthesis and involves transfer of electrons from water molecules to Hill reagents (non-physiological oxidants) as part of photosynthesis process. (Gaggi et al., 1995).

As was indicated in Materials and Methods, sampling from Rockford Lake was performed in summer, when the algal bloom-forming cyanobacteria was present. The bloom-forming cyanobacteria are known for producing toxins such as hepatotoxic peptides, dermatotoxic phenolic compounds and neurotoxic alkaloids which can colonize the bead and compete with the algae and inhibit the growth (Sivonen et al., 1990).

Algal cells use carbon source and produce oxygen when photosynthesizing. Photosynthesis also increases the pH of bulk solution as it removes carbon dioxide (Mollamohammada et al., 2020). Therefore, the drop in concentration of DO and pH are indicators that photosynthetic is decreasing in algal cells which occurred in both CFRs.

`

Conductivity of the waterbodies is affected by the discharges to the streams as well as bedrock they are flowing through. For example, streams that flow through granite bed rock have lower conductivity compared to the ones that flow through clay soil; because granite is made of materials that do not ionize into the water. (Bhateria & Jain, 2016). As shown in Figure 5.2b, Wahoo Creek had higher conductivity compared to Rockford Lake. In both samples, the conductivity increased over time. As was described in Materials and Methods section, the algal beads were kept in calcium chloride solution to solidify before being used in the experiments. It was possible that the increase in conductivity over time was because of the presence of calcium and chloride ions which may have partially diffused from the beads to the bulk solution.



`

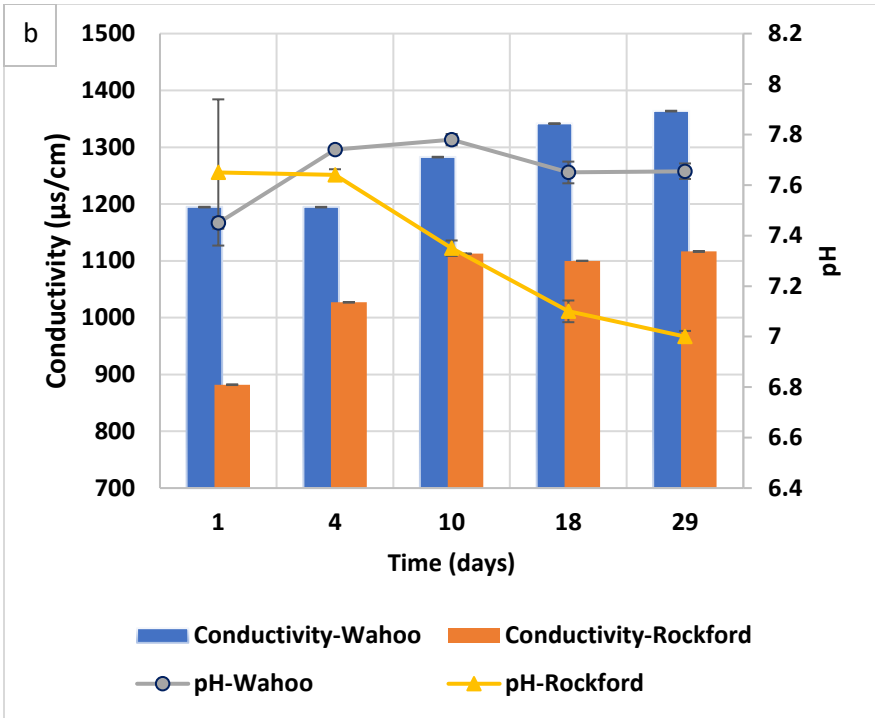


Figure 5.2 Changes in the concentrations of: a) DO & DCW and b) pH & conductivity of the CFRs over time.

5.4.2 Nitrate Removal

Figure 5.3 shows the removal efficiency of nitrate from Wahoo Creek and Rockford Lake during a period of 29 days as a function of time. As shown, the nitrate concentration decreased in the reactor containing the Wahoo Creek sample during the first 10 days; and then concentration increased after that. For the reactor containing sample from Rockford Lake, the lowest nitrate concentration was observed on day 8 and the reactor reached a steady state condition after 20 days.

Nitrate uptake by algal beads starts with adsorption on the surface of the bead, continued with penetration into the alginate and then, sorption into the cells (MS Abdel Hameed, 2007). Nitrate removal rate is very dependent on culture conditions such as medium composition. The herbicides and toxins presented in water samples inhibited the algal

`

growth and thus, lowered nitrate assimilation rate. This would explain the increase in concentration of nitrate in the bulk solution toward the end of the treatment period. Comparing the results with the control (alginate beads) shows that rather than assimilation by microalgae, nitrate removal may have taken place through adsorption on the surface of the alginate beads. As shown in Figure 5.3, the removal rate by the control treatment in the CFRs containing Rockford Lake sample is higher than the one with Wahoo Creek. It is concluded that cyanobacteria could also be partially responsible for the nitrate uptake.

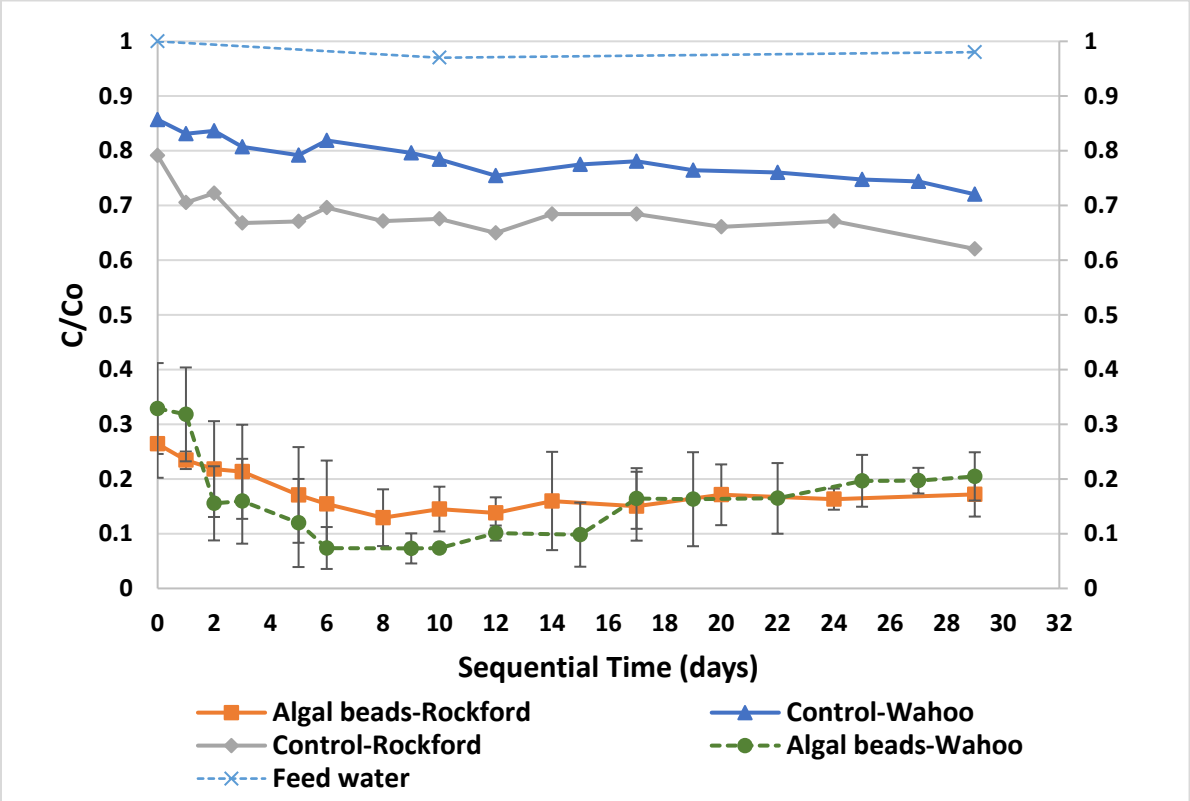


Figure 5.3 Changes in the concentration of NO_3-N in CFRs – Day 0 represents 7 days after the reactor's start up.

`

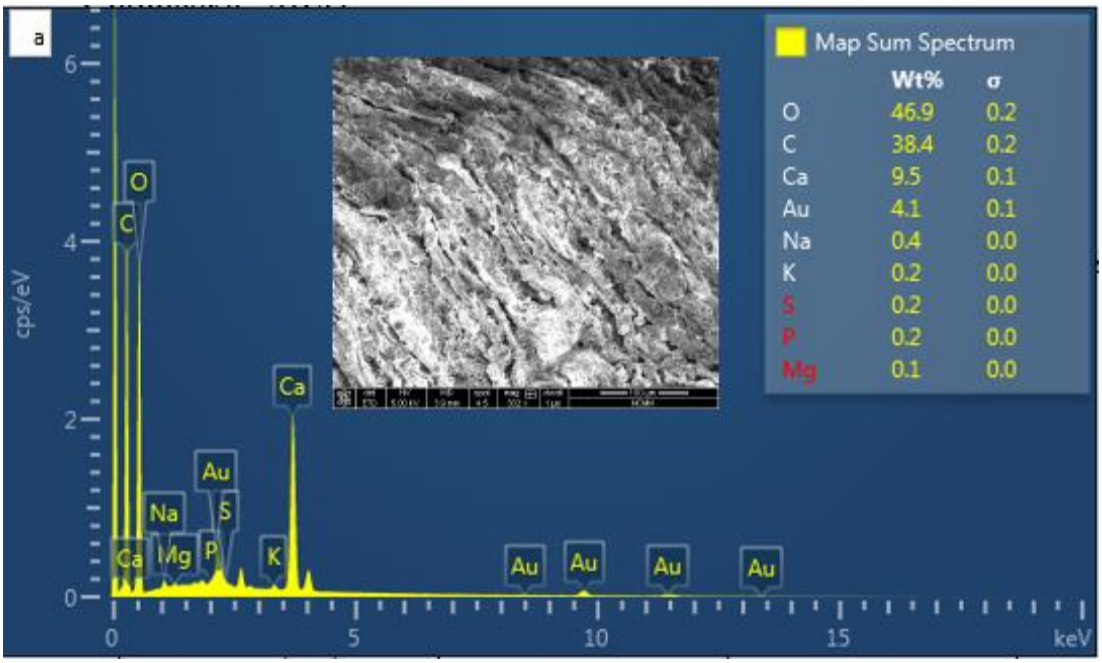
The removal efficiencies of the metals and non-metal ions present in the water are shown in Table 5.2. Hg and S were the only elements that were removed completely in both reactors.

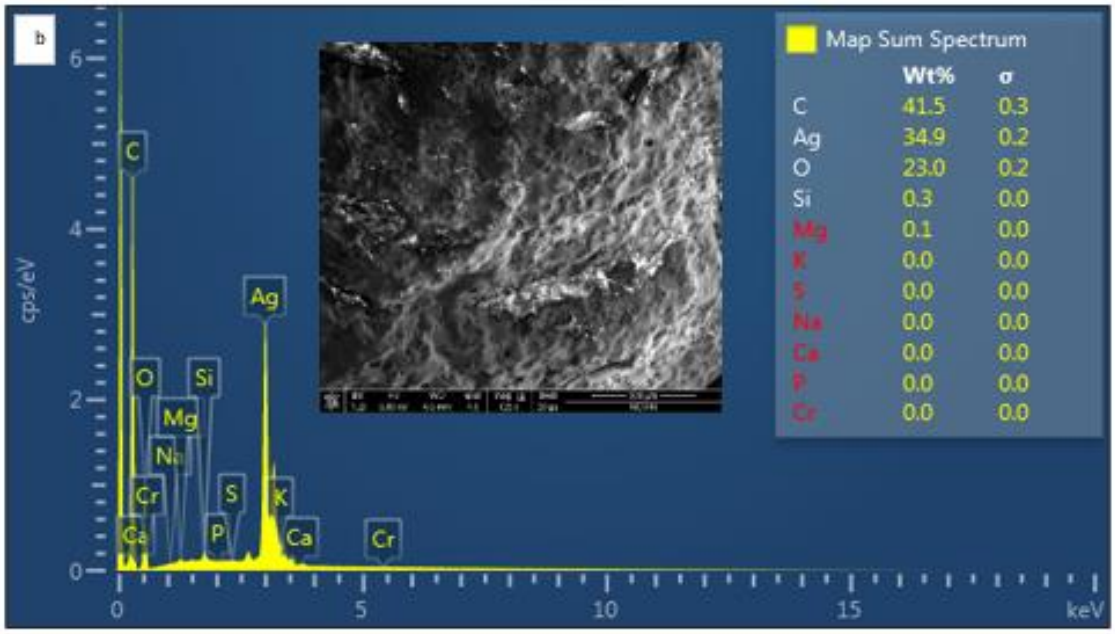
EDS analysis coupled with SEM was performed to evaluate the morphological changes on the surface of the beads after exposure to the water samples (Figure 5.4). SEM examines the mechanism of metal sorption by the beads and helps in detecting active sorptive areas and EDS analysis examines the distribution of elements on the surface of the beads (WO Wan Maznah et al., 2012; Michalak, Marycz, Basińska, & Chojnacka, 2014).

Table 5.2 Ions uptake by algal beads, exposed to water collected at Rockford Lake and Wahoo Creek based on influent and effluent concentrations measured at day 29.

Element	**Removal efficiency (%)**	
	Wahoo Creek	**Rockford lake**
Sodium (Na)	71.7	9.1
Magnesium (Mg)	8.8	59.0
Potassium (K)	69.2	0.0
Phosphorous (P)	100.0	12.1
Zink (Zn)	9.4	100.0
Mercury (Hg)	100.0	100.0
Sulfur (S)	100.0	100.0

`


a
Map Sum Spectrum
Wt% σ
O 46.9 0.2
C 38.4 0.2
Ca 9.5 0.1
Au 4.1 0.1
Na 0.4 0.0
K 0.2 0.0
S 0.2 0.0
P 0.2 0.0
Mg 0.1 0.0
cps/eV
keV
O
C
Ca
Au
Na
S
Mg
P
K


b
Map Sum Spectrum
Wt% σ
C 41.5 0.3
Ag 34.9 0.2
O 23.0 0.2
Si 0.3 0.0
Mg 0.1 0.0
K 0.0 0.0
S 0.0 0.0
Na 0.0 0.0
Ca 0.0 0.0
P 0.0 0.0
Cr 0.0 0.0
cps/eV
keV
C
Ag
O
Si
Mg
Na
Cr
S
K
Ca
P
Cr

`

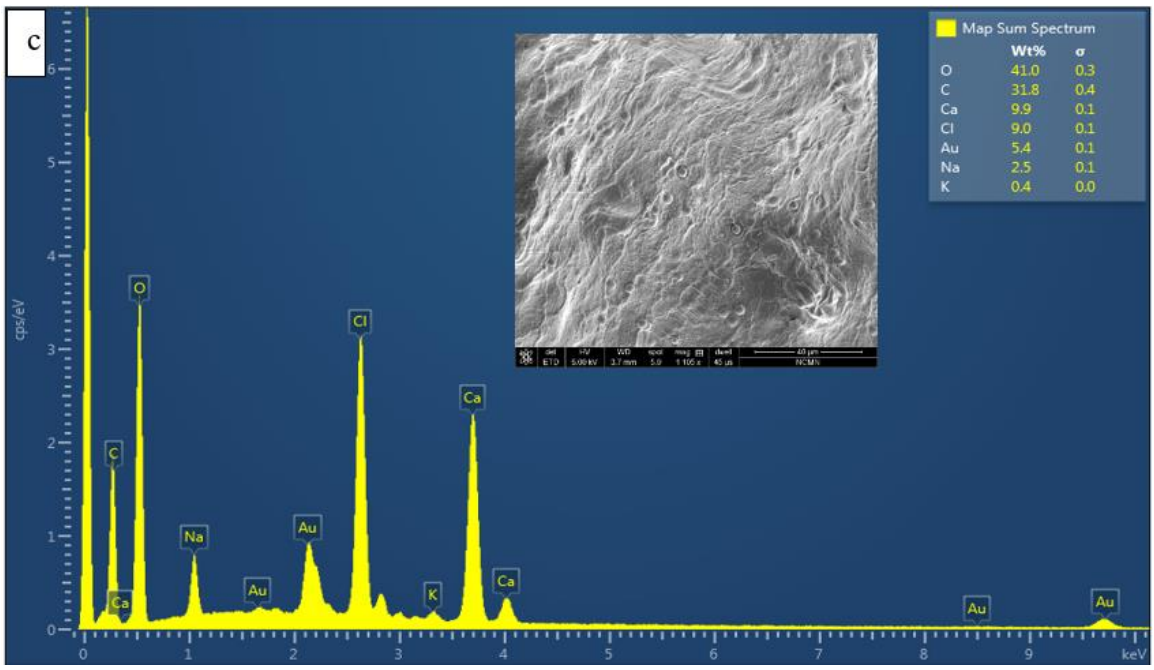


Figure 5.4 SEM and EDS analysis of immobilized *S. Sp* beads exposed to a) Rockford Lake and b) Wahoo Creek; and c) shows an SEM image of the unexposed bead.

The SEM images revealed significant changes in the morphology of the beads exposed to the water samples from Wahoo Creek and Rockford Lake. Figure 5.4a and 5.4b show that the surfaces of the beads were heterogeneous, with more fractures after being exposed to the surface water samples compared to an unexposed bead (Figure 5.4c)

It is possible that presence of the metal ions in the solution could have been the reason behind these changes on the surface of the beads.

The main mechanism for metal sorption by algal beads is the formation of the complexes between the ions and functional groups (carboxyl, carbonyl, amido, amino etc.) on the surface or inside the porous structure of the algae (Jin-Fen, Rong-Gen, & Li, 2000). Therefore, the attraction of the metals in the solution to the functional groups could've caused the changes on the surface of the beads (WO Wan Maznah et al., 2012).

`

The EDS analyses of the immobilized *Scenedesmus sp*. beads confirmed that some ions were present on the surface of the algal beads. The beads were coated with gold before observation and that would explain the presence of the Au peak within the EDS spectrum. The EDS spectrum also showed peaks for C and O which are due to the composition of organic matter and alginate in the beads. High intensity Ca peaks were observed on the surface of the beads exposed to the Wahoo Creek sample. $CaCl_2$ was used in to form the algal beads and the presence of Ca is because of the crosslinks between Calcium ions and the carboxylic groups present in the alginate (Pasparakis & Bouropoulos, 2006). No Ca was observed on the beads exposed to the Rockford Lake water which could be due to the possible diffusion of the Ca ions into the bulk solution during the treatment process.

Ag and Si were two dominant ions on the surface of the beads exposed to the Rockford Lake water. The presence of these two elements was possibly because of using a silver tape to attach the beads to the steel plate before performing EDS analysis. K, P, S, Mg and Na were the other five dominant ions on the surface of the beads exposed to the Wahoo Creek water which were existed in the samples according to the ICP-MS analysis (Table 5.1) and removed by algal beads (Table 5.2).

5.4.3 Herbicides removal

Herbicide uptake by algal beads may involve adsorption, bioaccumulation, biodegradation or the combination of all three (Hussein, Abdullah, Badr El-Din, & Mishaqa, 2017). The total uptake of the herbicides by *Scenedesmus sp.* beads through biodegradation, bioaccumulation and adsorption are shown in Figure 5.5. The rate of biodegradation $\frac{dB}{dt}(\mu g.d^{-1})$ was calculated using Equation 5.1:

`

$$\frac{dB}{dt} = \dot{m}_{in} - \dot{m}_{out} - \dot{m}_d - \dot{m}_c \quad \text{Equation 5.1}$$

Where $\dot{m}_{in}$ is the mass of the herbicide entering with the influent into reactor, $\dot{m}_{out}$ is the mass of the herbicide in the effluent, $\dot{m}_d$ is the mass of herbicide adsorbed by the control treatment (alginate beads), and $\dot{m}_c$ is the mass of the herbicide accumulated in microalgae biomass. All mass flow rates are measured in $\mu g.d^{-1}$.

As shown on Figure 5.5a-c, all triallate was removed after one day of starting the effluent reactor (7 days after the start-up of the treatment process) and the uptake started to decrease afterwards. For cycloate, the uptake was almost steady throughout the treatment process. For alachlor, the maximum uptake occurred at days 1 and 10.

For all three herbicides, partial removal was done in the control treatment and through adsorption on alginate.

The bioaccumulation rate of triallate and cycloate was reduced from day 1 to 10 and biodegradation started to increase. The unsteady accumulation can be an indicator of biodegradation occurring by the algal cells.

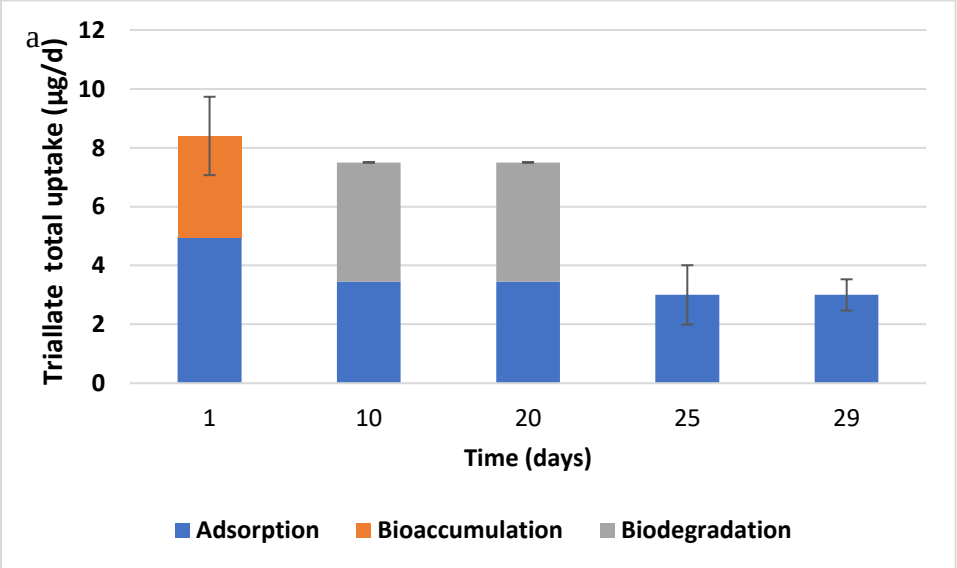
The highest uptake of ametryn and simetryn was observed at days 1 and it started to slowly decrease afterwards. As shown on Figure 5.5d to e, the accumulation rate was very low, and the majority of uptake was occurred possibly largely through adsorption on the surface of alginate or biodegradation.

Bioaccumulation is defined as transfer and storage of pollutants into the interior of living cells. In this process, nutrients such as nitrate, phosphate, and organic or inorganic carbon can also be removed from treated effluents (Barron, 1995). The toxicity of the herbicides accumulated in the cells may cause stress in the algal cells, resulting in activation of the

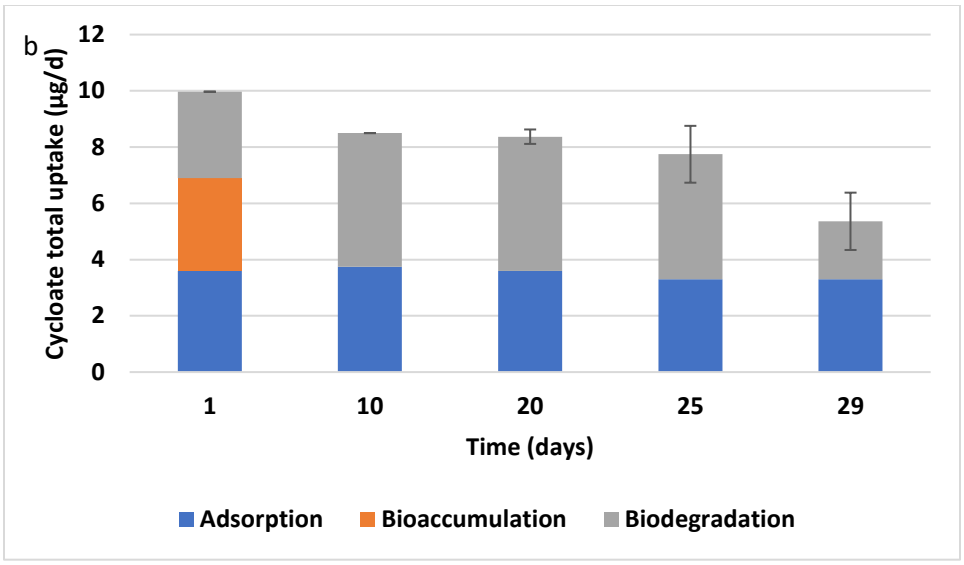
`

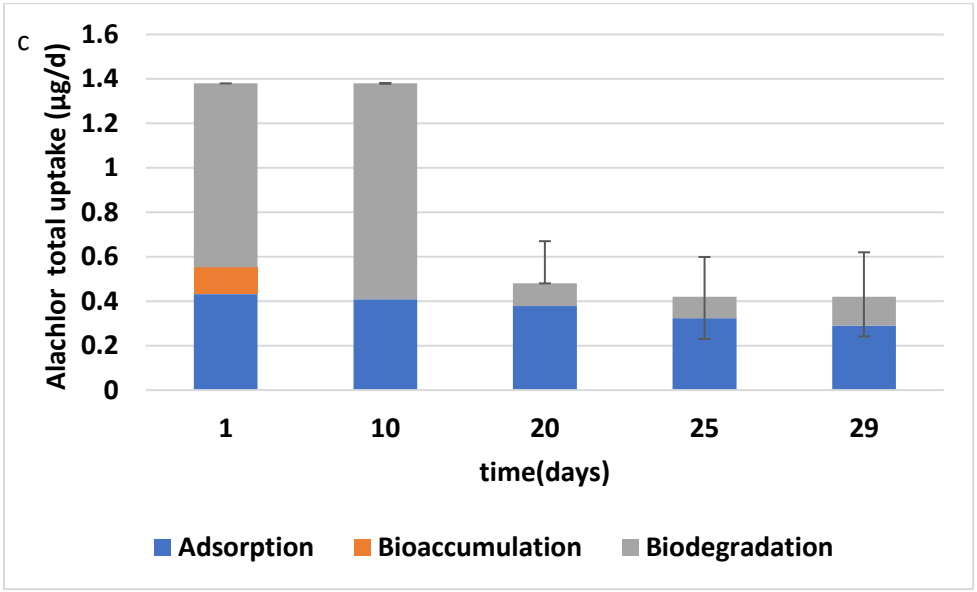
cell's defense mechanism. The defense mechanism can cause the degradation of the herbicides. (Szewczyk, Kuśmierska, & Bernat, 2018). Therefore, if given enough time, the accumulation of herbicides can result in degradation and increase the overall removal rate.

Biodegradation of herbicides by algae is affected by two important factors: first, type of microalgae and the optimum conditions for its activity (such as optimum temperature, light intensity, etc.) and second, the composition of the herbicides. The molecular weight and functional groups of herbicides, their concentration, and toxicity to the algal cells can impact on the ability of algal beads to uptake herbicides (Rath, 2012). Algae have shown to be more capable of metabolizing compounds with low molecular weight (Hussein et al., 2017) which can explain why the degradation rate is the highest for simetryn. Surface area to biovolume ratio of the algae is an important factor that determines the algal capacity to uptake the herbicides. The higher the ratio, the greater potential for sorption of herbicides (Subashchandrabose et al., 2013).

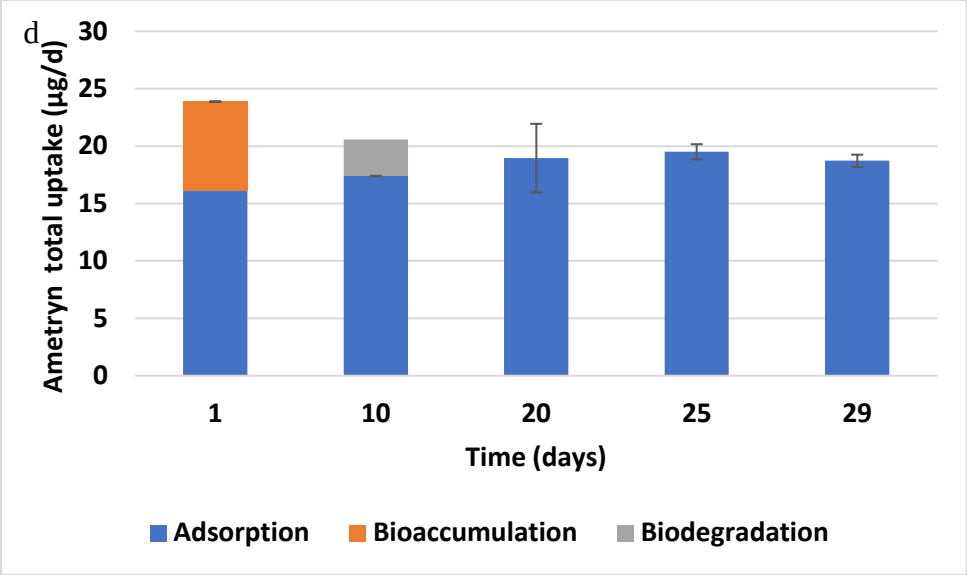


`


b
Cycloate total uptake (µg/d)
12
10
8
6
4
2
0
1
10
20
25
29
Time (days)
Adsorption
Bioaccumulation
Biodegradation


c
Alachlor total uptake (µg/d)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
10
20
25
29
time(days)
Adsorption
Bioaccumulation
Biodegradation

`



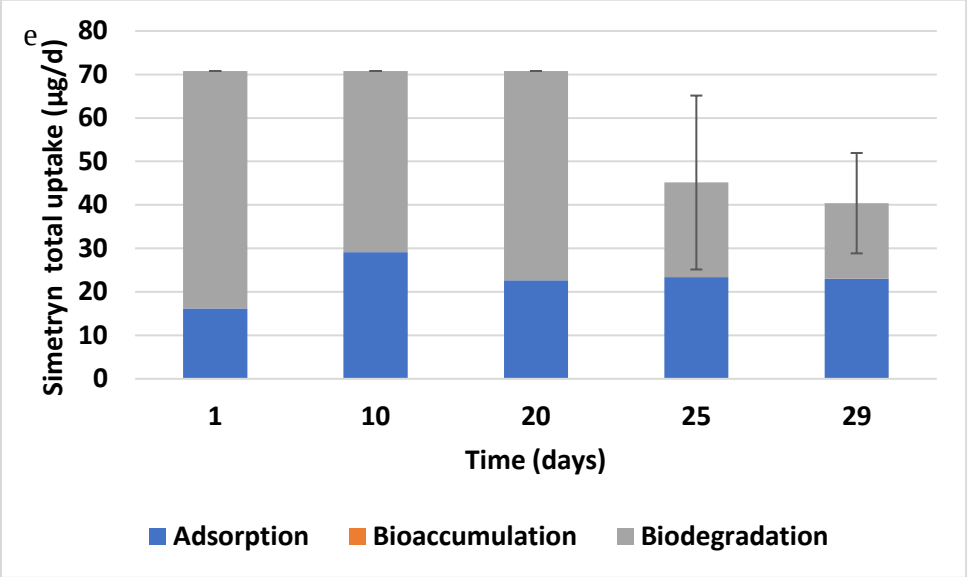


Figure 5.5 The uptake of: a) Triallate and b) Cycloate from Rockford lake and c) Alachlor, d)Ametryn, and e) Simetryn from Wahoo creek by immobilized algal beads through adsorption, bioaccumulation and biodegradation.

There are not many reported studies regarding the mechanisms and pathways of herbicide degradation by microalgae. Previous studies have shown that exposing microorganisms to xenobiotics will result in a significant increase in reactive oxygen species generation (Boyd, Jaynes, & Ross) which may induce an oxidative stress conditions (Szewczyk et al.,

`

2018). Microorganisms produce different types of oxidative enzymes such as peroxidase, polyphenoloxidase, laccase, and tyrosinase that can affect the transforming xenobiotics (Van Eerd, Hoagland, Zablotowicz, & Hall, 2003). It is reported that the biotransformation of herbicides by algae could be catalyzed by dealkylating enzymes such as cytochrome P450 (Subashchandrabose et al., 2013). In most cases, the polymerization products have less toxicity compared to the substrate which lowers the over all environmental contamination associated with them (Van Eerd et al., 2003).

5.5 Conclusions

In this chapter, the experiments in CFR were performed to assess the capability of immobilized algal beads removing nitrate and herbicides in stressed conditions and their effectiveness as an alternative treatment of surface water.

The following conclusions could be drawn from this study:

1. The results showed that the nitrate uptake rate by immobilized algae is very dependent on the culture conditions. Even though the algal cells are used in immobilized form, the herbicides and toxins present in the water can lower their growth and impair their capability in removing nitrate.

2. Despite the toxicity effect of herbicides and toxins, algae beads were still able to remove 90-100% of herbicides, and 93% of nitrate in the first 10 days of the operation.

3. Adsorption, bioaccumulation and biodegradation were the main three processes involved in the removal of herbicides. Further research is required to identify the mechanisms and pathways of herbicides' degradation by microalgae to assess the environmental impacts associated with them.

`

3. The immobilized algal based treatment is a natural alternative to treat the contaminated surface water. One of the important applications of this process can be the treatment of surface water that is used in groundwater recharge. Recharging groundwater with treated water can serve to dilute the concentration of nitrate and herbicides in the groundwater, resulting in lower potential health risks. Additional in-aquifer (*in-situ*) treatment can also be realized as a result of in-aquifer storage.

`

References

Al-Rub, F. A., El-Naas, M., Benyahia, F., & Ashour, I. (2004). Biosorption of nickel on blank alginate beads, free and immobilized algal cells. *Process Biochemistry, 39*(11), 1767-1773.

Barron, M. (1995). Bioaccumulation and bioconcentration in aquatic organisms. A: Hoffman DJ, Rattner BA, Burton GA i Cairns J. Hadbook of Ecotoxicology. In: Lewis Publishers, USA.

Bhateria, R., & Jain, D. (2016). Water quality assessment of lake water: a review. *Sustainable Water Resources Management, 2*(2), 161-173.

Bonora, S., Benassi, E., Maris, A., Tugnoli, V., Ottani, S., & Di Foggia, M. (2013). Raman and SERS study on atrazine, prometryn and simetryn triazine herbicides. *Journal of Molecular Structure, 1040*, 139-148.

Brooks, D. R., Clark, S. J., Perry, J. N., Bohan, D. A., Champion, G. T., Firbank, L. G., . . . Woiwod, I. P. (2005). Invertebrate biodiversity in maize following withdrawal of triazine herbicides. *Proceedings of the Royal Society B: Biological Sciences, 272*(1571), 1497-1502.

Committee, H. M. A. (1974). *Herbicide Report: Chemistry and Analysis, Environmental Effects, Agricultural and Other Applied Uses.* Retrieved from

Company, M. A. (1989). *Toxicology Information Summary for Triallate.* Retrieved from

Dale, T. M., Renner, K. A., & Kravchenko, A. N. (2006). Effect of herbicides on weed control and sugarbeet (Beta vulgaris) yield and quality. *Weed technology, 20*(1), 150-156.

Fujii, K., Takagi, K., Hiradate, S., Iwasaki, A., & Harada, N. (2007). Biodegradation of methylthio-s-triazines by Rhodococcus sp. strain FJ1117YT, and production of the corresponding methylsulfinyl, methylsulfonyl and hydroxy analogues. *Pest Management Science: formerly Pesticide Science, 63*(3), 254-260.

Gaggi, C., Duccini, M., Bacci, E., Sbrilli, G., Bucci, M., & Naby, A. H. E. (1995). Toxicity and hazard ranking of s-triazine herbicides using microtox® two green algal species and a marine crustacean. *Environmental Toxicology and Chemistry: An International Journal, 14*(6), 1065-1069.

Gentry, L. E., David, M. B., Smith-Starks, K. M., & Kovacic, D. A. (2000). Nitrogen fertilizer and herbicide transport from tile drained fields. *Journal of Environmental Quality, 29*(1), 232-240.

Hameed, M. A. (2007). Effect of algal density in bead, bead size and bead concentrations on wastewater nutrient removal. *African Journal of biotechnology, 6*(10).

Hayes, T. B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A. A., & Vonk, A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proceedings of the National Academy of Sciences, 99*(8), 5476-5480.

Holt, M. (2000). Sources of chemical contaminants and routes into the freshwater environment. *Food and chemical toxicology, 38*, S21-S27.

Hussein, M., Abdullah, A., Badr El-Din, N., & Mishaqa, E. (2017). Biosorption potential of the microchlorophyte chlorella vulgaris for some pesticides. *J Fertil Pestic, 8*(01).

Jin-Fen, P., Rong-Gen, L., & Li, M. (2000). A review of heavy metal adsorption by marine algae. *Chinese Journal of Oceanology and Limnology, 18*(3), 260-264.

`

Klementova, S., & Keltnerova, L. (2015). Triazine herbicides in the environment. *Herbicides, Physiology of Action, and Safety*.

Lee, W. J., Hoppin, J. A., Blair, A., Lubin, J. H., Dosemeci, M., Sandler, D. P., & Alavanja, M. C. (2004). Cancer incidence among pesticide applicators exposed to alachlor in the Agricultural Health Study. *American journal of epidemiology, 159*(4), 373-380.

Majumdar, D., & Gupta, N. (2000). Nitrate pollution of groundwater and associated human health disorders. *Indian journal of environmental health, 42*(1), 28-39.

Maznah, W. W., Al-Fawwaz, A., & Surif, M. (2012). Biosorption of copper and zinc by immobilised and free algal biomass, and the effects of metal biosorption on the growth and cellular structure of Chlorella sp. and Chlamydomonas sp. isolated from rivers in Penang, Malaysia. *Journal of Environmental Sciences, 24*(8), 1386-1393.

Maznah, W. W., Al-Fawwaz, A., & Surif, M. (2012). Biosorption of copper and zinc by immobilised and free algal biomass, and the effects of metal biosorption on the growth and cellular structure of Chlorella sp. and Chlamydomonas sp. isolated from rivers in Penang, Malaysia. *Journal of Environmental Sciences, 24*(8), 1386-1393.

Michalak, I., Marycz, K., Basińska, K., & Chojnacka, K. (2014). Using SEM-EDX and ICP-OES to investigate the elemental composition of green macroalga Vaucheria sessilis. *The Scientific World Journal, 2014*.

Mollamohammada, S., Aly Hassan, A., & Dahab, M. (2019). Nitrate removal from groundwater using immobilized heterotrophic algae. *water, air and soil pollution (in press)*.

Navaratna, D., Elliman, J., Cooper, A., Shu, L., Baskaran, K., & Jegatheesan, V. (2012). Impact of herbicide Ametryn on microbial communities in mixed liquor of a membrane bioreactor (MBR). *Bioresource Technology, 113*, 181-190.

Navarro, S., Vela, N., Giménez, M. J., & Navarro, G. (2004). Persistence of four s-triazine herbicides in river, sea and groundwater samples exposed to sunlight and darkness under laboratory conditions. *Science of the Total Environment, 329*(1-3), 87-97.

Plakas, K. V., & Karabelas, A. J. (2012). Removal of pesticides from water by NF and RO membranes—A review. *Desalination, 287*, 255-265.

Prosen, H. (2012). Fate and determination of triazine herbicides in soil. In *Herbicides-Properties, Synthesis and Control of Weeds*: IntechOpen.

Rath, B. (2012). Microalgal bioremediation: current practices and perspectives. *Journal of Biochemical Technology, 3*(3), 299-304.

Saka, M. (2010). Acute toxicity of rice paddy herbicides simetryn, mefenacet, and thiobencarb to Silurana tropicalis tadpoles. *Ecotoxicology and environmental safety, 73*(6), 1165-1169.

Simpson, M., Horner, S., Mistry, P., Duffel, S., Widdowson, P., Scanlon, Y., & Lock, E. (2005). Neuropathological studies on cycloate-induced neuronal cell death in the rat brain. *Neurotoxicology, 26*(1), 125-132.

Sivonen, K., Niemelä, S., Niemi, R., Lepistö, L., Luoma, T., & Räsänen, L. (1990). Toxic cyanobacteria (blue-green algae) in Finnish fresh and coastal waters. *Hydrobiologia, 190*(3), 267-275.

Subashchandrabose, S. R., Ramakrishnan, B., Megharaj, M., Venkateswarlu, K., & Naidu, R. (2013). Mixotrophic cyanobacteria and microalgae as distinctive biological agents for organic pollutant degradation. *Environment international, 51*, 59-72.

`

Szewczyk, R., Kuśmierska, A., & Bernat, P. (2018). Ametryn removal by Metarhizium brunneum: Biodegradation pathway proposal and metabolic background revealed. *Chemosphere, 190*, 174-183.

Szewczyk, R., Soboń, A., Słaba, M., & Długoński, J. (2015). Mechanism study of alachlor biodegradation by Paecilomyces marquandii with proteomic and metabolomic methods. *Journal of hazardous materials, 291*, 52-64.

Ummalyma, S. B., Pandey, A., Sukumaran, R. K., & Sahoo, D. (2018). Bioremediation by microalgae: current and emerging trends for effluents treatments for value addition of waste streams. In *Biosynthetic Technology and Environmental Challenges* (pp. 355-375): Springer.

Wilson, R. G. (1984). Accelerated degradation of thiocarbamate herbicides in soil with prior thiocarbamate herbicide exposure. *Weed Science, 32*(2), 264-268.

Yalkowsky, S. H., He, Y., & Jain, P. (2016). *Handbook of aqueous solubility data*: CRC press.

`

CHAPTER 6

A HYBRID BIGOLOGICAL-ADSORPTION APPROACH FOR THE TREATMENT OF CONTAMINATED GROUNDWATER USING IMMOBLIZED NANOCLAY-ALGAE MIXTURES [5]

6.1 Abstract

Scenedesmus species cells mixed with nanoclay and immobilized on alginate gel, were found effective in removing nitrate, atrazine and metals from aqueous solutions. Gel beads containing the hybrid mixture removed 100% of 10 mg/L N nitrate and 98% of 100 µg/L atrazine from synthetic groundwater in 3 days. The optimal amount of nanoclay was found to be 0.30 mg per bead. The experimental data fit well into Freundlich adsorption isotherm and followed pseudo first-order kinetics. When tested in actual groundwater, 91% of nitrate and 100% of Cr, Se and V were eliminated in 3 days without need for any nutrients or carbon source. Immobilized algal beads embedded with nanoclay are a natural low-cost alternative for groundwater treatment. The gel beads can be reused for at least two cycles without a compromise in performance. They are water-insoluble, easy to harvest, and offer high removal efficiency.

6.2 Introduction

Groundwater in rural areas is suffering from the overuse of nitrogen-based fertilizers and herbicides. Nitrogen is an essential nutrient for the plants and is used in various forms to enhance plants' growth. However, excess nitrogen in soil infiltrates into the groundwater

[5] Mollamohammada S., Aly Hassan, A., Dahab, M. (2020) Immobilized algae-based treatment of agriculturally contaminated groundwater, *Chemosphere* - journal, (under review).

`

commonly in the form of nitrate (Sebilo, Mayer, Nicolardot, Pinay, & Mariotti, 2013). Herbicides and pesticides are synthetic organic compounds used to control weeds, insects, and a variety of other agricultural purposes. Many herbicides are water-soluble and may dissolve in groundwater. Atrazine is one of the most commonly used triazine herbicide in the US which is utilized to control broadleaf weeds. It is a concern to the water supply because of its toxicity, relatively long half-life (60 to 100 days) and poor absorption by soil (K_{oc} = ~100). In most cases, the source of atrazine in groundwater is caused by leaching near wellheads due to loading the sprayers with atrazine, cleanup activities or back siphoning accidents during spraying operations (Isensee et al., 1988).

The US Environmental Protection Agency (US EPA) has set a maximum contaminant level (MCL) of 10 mg/L for NO_3-N and 3 ppb for atrazine (U. EPA, 2018).

High levels of atrazine were detected in public water systems serving people in Texas, Kansas, Kentucky, Missouri and Ohio (Naidenko, 2017).

Exposure to drinking water with a nitrate level above the MCL is a potential health risk. Methemoglobinemia, which is the result of nitrate reduction to nitrite in infants' digestive system is reported to be common in infants fed formula using well water with elevated nitrate contamination (Greer & Shannon, 2005). Additionally, strong evidence relating drinking nitrate- contaminated water and colorectal cancer, thyroid disease, and neural tube defects was documented (Ward et al., 2018). Nitrosamine is formed in water contaminated with nitrate and atrazine. Nitrosamines have been reported to increase the risk of developing Non-Hodgkin Lymphoma (Rhoades et al., 2013a).

`

Microalgal-based water treatment system is a sustainable solution to remove nitrates; however, these systems also have disadvantages, such as expensive biomass harvesting (E. Eroglu et al., 2012). Immobilization of microalgae in polymeric matrices is an attractive alternative that facilitates the biomass harvesting and makes the operation more flexible (S. Fierro, M. del Pilar Sánchez-Saavedra, & C. Copalcua, 2008). Algal immobilized gels can be manufactured using both synthetic (e.g. polyurethane, poly vinyl alcohol) and natural (alginate, carrageenan, agar, chitosan) polymers. A proper carrier must be porous enough to allow the diffusion of the molecules from bulk solution to the cells. For the autotrophic living algal cells, the carrier must be transparent and allow light transmission (Moreno-Garrido, 2008b).

Several studies have been performed to evaluate the capability of immobilized algal beads in removing nutrients from aqueous solutions. Immobilized *Chlorella vulgaris* and *Scenedesmus rubescens* removed more than 90% of phosphate, nitrate and ammonium from secondary, synthetic wastewater within 9 days (C. Garbisu, Gil, Bazin, Hall, & & Serra, 1991). In Chapter 3, the removal efficiency of nitrate was evaluated in groundwater using immobilized *Scenedesmus sp (S. sp)* beads. The immobilized beads were able to remove 90% of nitrate from groundwater in 2 days.

The mechanism of nitrate uptake by algal beads involves the adsorption of the nitrate onto the surface of the bead, penetration into the polymer and then, sorption into the cells (MS Abdel Hameed, 2007). Hence, a faster adsorption process could lead to a faster nitrate assimilation by microalgae which will increase the nitrate removal rate considerably.

Adding an adsorbent to the algal beads will accelerate this process especially if it is positively charged to attract the negatively charged nitrate ions. As an abundant low-cost

`

natural adsorbent, clay was used to aid microalgae in enhancing nitrate removal from groundwater. Clays have been successfully used in removing nitrate, heavy metals, and endocrine-disrupting chemicals from water (Rezvani & Taghizadeh, 2018; Srinivasan, 2011). Clays are primary made of particles with at least one dimension in the nanometer scale; therefore, they can be regarded as nanoclay (Yuan & Wu, 2007). The specific surface area of the nanoclay can reach to a few hundred m^2/g, due to their small size. Their high surface area, combined with nano-size dimension and peculiar charge are three most important factors behind their large tendency for taking up ions and organic compounds from water (Srinivasan, 2011; Yuan & Wu, 2007). Several studies explored the application of nanoclay as adsorbents for nitrate and atrazine removal. Clay mineral originated from a dam site in Morocco was optimized for the adsorption of nitrate. Highest nitrate removal of 72% was achieved at the pH = 5.1, using 1 g/L of adsorbent (El Ouardi, Qourzal, Alahiane, Assabbane, & Douch, 2015). Clay minerals modified by a cationic surfactant were used in batch mode for the adsorption of pesticides. The results showed an increase in the adsorption rate, compared to untreated clays, however, the adsorption coefficient of atrazine was found to be very low (Sanchez-Martin, Rodriguez-Cruz, Andrades, & Sanchez-Camazano, 2006) . In another study, high sorption values of atrazine (98% after 18 h) on dye-clay complexes were achieved; however, the concentration of adsorbent used was very high (50 gL^{-1}) (Srinivasan, 2011).

In this study, a mixture of algae (*S. sp)* and positively charged nanoclay particles, immobilized on alginate gel, (hybrid biological-adsorption system) are evaluated for the removal of nitrate and atrazine from synthetic and actual groundwaters. Because of its high surface area, nanoclay can accelerate the adsorption of nitrate and atrazine from the bulk

`

solution and result in a faster removal rate. Batch and sequential batch growth studies are conducted to evaluate the treatment efficiency. The nitrate uptake capacity of the hybrid system is quantified based on different uptake mechanisms of adsorption, biological assimilation, and extracellular enzyme activity resulting from toxicity.

6.3 Materials and Methods

6.3.1 Cultivation of algae

A sample of the microalgae strain *S. sp.* were cultivated in a sterilized Bold's Basal Medium (BBM) which contained nitrogen, phosphorus and the other macro and micronutrients; and kept at room temperature (20 °C). The light necessary for algal growth was provided by sunlight and artificial plant light. The intensity of light varied between 600-700 Lux. After about two weeks of cultivation, the microalgae were used for bead preparation.

6.3.2 Preparation of nanoclay embedded algal beads and experimental conditions

To prepare nanoclay embedded alginate-algae beads, the cells of *S. sp* were first harvested by centrifugation at 3500 rpm for 10 minutes. The supernatant was discarded, and the cells were resuspended in DI water and mixed with a 2% sodium alginate solution and sufficient amount of nanoclay. The nanoclay mineral used in the experiments was purchased form Sigma Aldrich (St. Louis, MO) and used as is after dissolution and sonication. The nanoparticles were treated with 25-30% w/w trimethyl stearyl ammonium (TMSA) - a cationic surfactant. All required growth medium nutrients, except nitrate, were added to the suspension.

`

A syringe pump (Harvard Apparatus, model 55-3333) was used to drop the mixture of algae, alginate, and nanoclay into a 2% calcium chloride solution. The uniform beads (diameter: 2.35 mm) were formed upon dropping the mixture into the calcium chloride solution and were left overnight to harden. As a control sample and blank beads were prepared without adding any algal cells. The experiments were performed using synthetic and actual groundwater samples. Synthetic groundwater was prepared using DI water mixed with target concentration of nitrate and/or atrazine. Potassium Nitrate (99.9%, Sigma Aldrich) and analytical standard atrazine (99.9%, Sigma Aldrich) were used to achieve concentration of 10 mg/L NO_3-N and 0.1 ppm atrazine, respectively. The actual groundwater sample was collected from a pump station at the City of Hastings, NE, and stored at 4°C in a refrigerator. Before being used in the experiments, the suspended solids in groundwater were removed by filtering through a Whatman syringe filter membrane of 0.45 mm pore size.

All experiments were carried out in 500 mL flasks at $20\ °C$. The flasks were autoclaved for 20 mins at $121\ °C$ in order to prevent any contamination. Each flask contained 400 mL of the aqueous solution. The ratio of the beads/solution was 12.5% in all reactors. The experiments with the synthetic groundwater were performed under heterotrophic conditions by adding glucose (125 mg/L) to each reactor as an organic carbon source. The concentration of Chemical Oxygen demand (COD) was used as a surrogate for determining glucose availability. When actual groundwater was used, no additional nutrients or glucose were provided in the beads or to the bulk solution. All experiments were performed in triplicates and each result presented here is the mean of three replicated studies

`

6.3.3 Analytical Measurements

UV-vis spectrophotometer (Thermo Scientific, Nano-Drop, model 2000) was used to measure the concentration of NO_3-N in all reactors. A new method based on vacuum-assisted sorbent extraction (VASE) (EnTech Instruments, 5800) used with gas chromatography-mass spectrometry (GC-MS, Agilent Technologies, 5977B) to measure the concentration of atrazine in the solutions. A Dissolved Oxygen (DO) meter (YSI, 5100) was used to measure the concentration of DO in each reactor. pH and conductivity of the bulk solution were measured using a pH meter (Thermo Scientific, Orion 3-star) and a conductivity meter (Hach, HQ14D). TNTplus vial tests (Hach, TNT820) were used to record the COD of the bulk solution. Zeta potential of the beads was determined using a Zeta Potential Analyzer (Brookhaven Instruments, ZetaPals).

Scanning Electron Microscopy (SEM) was used to examine the morphological structure of the beads. SEM images were taken using a Nova Nano SEM (FEI, 450), with the voltage ranging between 2 to 5 kV. The beads were air dried first and coated with a thin layer of gold before imaging. The analysis of metals in the groundwater was performed using Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) (Agilent, 7500 CX).

6.3.4 Viability and growth assessment

The microscopic images of the algal colonies were taken using a Ti-S Inverted Fluorescence microscope (Nikon, Melville, NY). An assessment of the viability of the algal cells within the immobilization matrix was performed by isolating the live cells from the background using the color threshold function in ImageJ v 1.51 j8.

6.4 Results and Discussion

`

6.4.1 Adsorption capacity of nanoclay on algae-alginate matrixes

To evaluate the impact of nanoclay on nitrate uptake rate by the algal beads, batch studies were performed using four different groups of alginate gel beads embedded with: nanoclay + algae (Hybrid), nanoclay (Adsorption), algae (Biological) and none (Control).

As shown on Table 6.1, the hybrid beads produced the highest nitrate removal rate. They were able to remove 100% of nitrate in three days. The biological beads took up 43% of nitrate in this time period. At day 3, the removal of NO_3-N in the adsorption and control groups were 20% and 10%, respectively. An increase in concentration of DO and pH, and reduction in COD were indicators of photosynthesis and glucose uptake by algal cells was highest in the biological beads. No photosynthesis was occurring in the adsorption and control beads because they did not have any algal cells. This could explain why the concentration of DO and COD was almost stable in these two groups of the beads.

It was evident that the nanoclay addition accelerated the nitrate uptake rate by the algal beads. The uptake occurred through the hybrid mechanism of consecutive nitrate adsorption on the nanoclay surface followed by assimilation by algal cells, as a physicochemical process. The availability of nitrate in a high density around algal cells increased assimilation rate and freed the surface for further adsorption. As described in Section 6.3.2, the nanoclay used in these experiments was treated with a cationic surfactant. The positively charged nanoclay enhances the adsorption properties of the beads and increases its tendency to adsorb the negatively charged entities which is nitrate in this case. Hence, making the dual mechanism of adsorption and assimilation of nitrate more efficient than each mechanism individually.

`

Two kinetics models, pseudo-first order and pseudo-second order were applied in order to understand the dynamics of nitrate adsorption on adsorption beads. The pseudo-first-order equation is shown as:

$$\frac{dq_t}{dt} = K_1(q_e - q_t) \quad \text{Equation 6.1}$$

where q_e and q_t are nitrate adsorbed per unit mass of nanoclay (mg /g) at equilibrium and at time t (day), respectively, and K_1 (l/day) is the rate constant of first-order adsorption.

The pseudo-second order process is:

$$\frac{dq_t}{dt} = K_2(q_e - q_t)^2 \quad \text{Equation 6.2}$$

Where q_e and q_t are defined in the same way as in the first order reaction and K_2 $(g.\frac{mg}{d})$ is the constant of the second-order adsorption. Graphically, these two models showed that the adsorption followed the pseudo-first order model. The pseudo-first order model had a higher correlation coefficient (0.910) compared to the second order-model (0.770). Also, the calculated $q_{e,cal}$ from the first order model agreed with the experimental value (Table 6.2).

`

Table 6.1 Percentage of nitrate present and change in concentration of COD (mg/L), DO (mg/L) and pH of the solution over time.

	Hybrid Group: Algae + nanoclay				Adsorption Group: Nanoclay				Control Group: None				Biological Group: Algae			
Day	**C/Co**	**COD**	**pH**	**DO**	**C/Co**	**COD**	**pH**	**DO**	**C/Co**	**COD**	**pH**	**DO**	**C/Co**	**COD**	**pH**	**DO**
0	1.00±0.00	156.8	7.71	8.6	1	160	7.02	7.8	1	160	8.36	8.2	1.00±0.00	172.8	6.34	8.8
1	0.95±0.19	-	7.76	9.33	0.96	-	6.51	8.2	1.01	-	6.56	8.8	0.88±0.03	-	6.98	9.2
2	0.15±0.01	139.5	7.54	9.34	0.87	161	6.3	8.1	0.9	155	6.44	8.3	0.67±0.04	115.2	7.15	9.4
3	0.00±0.00	-	7.09	9.4	0.8	-	6.17	8.1	0.9	-	6.17	8.3	0.57±0.08	-	7.22	9.5
5	0.00±0.00	152	6.99	9.43	0.75	155	5.94	8.1	0.88	152	5.64	8	0.40±0.11	115.8	7.6	9.9

Table 6.2 Kinetic parameters for the adsorption of nitrate on nanoclay-embedded alginate beads.

$q_{e,exp}\ (\frac{mg}{g})$	**First order adsorption**		**Second order adsorption**	
1.025	$K_1\ (\frac{1}{d})$	$q_{e,cal}\ (\frac{mg}{g})$	$K_2\ (g.\frac{mg}{d})$	$q_{e,cal}(\frac{mg}{g})$
	1.580	1.644	0.023	2.267

6.4.2 Impact of nanoclay dosage on nitrate uptake by the beads

Varying amounts of nanoclay were used in order to determine the effect of the nanoclay mass on the nitrate removal rate. The range changed from 0.0030 to 0.60 mg per bead and the removal efficiency of nitrate increased from 9.2% to 98.6%. The beads containing 0.30 and 0.60 mg of nanoclay showed the highest nitrate uptake (98.8% and 98.2%, respectively).

The process of nitrate uptake in immobilized algal beads appears to start with adsorption of nitrate on the surface of the bead, followed by penetration into alginate and sorption into cells (M. A Hameed, 2007). Therefore, adsorption on alginate gel and nanoclay, and nitrate assimilation by algae cells would be the three major processes involved in nitrate uptake. Figure 6.1 separates these three processes. The adsorption rate by alginate beads was calculated from the nitrate uptake by control beads. The adsorption rate by nanoclay and assimilation rate by microalgae are calculated using Equations 6.3:

$$A_{s,t} = A_{h,t} - A_{n,t} \qquad \text{Equation 6.3}$$

Where $A_{s,t}$ is the amount of nitrate assimilated by microalgae and $A_{h,t}$ and $A_{n,t}$ are quantity of nitrate uptaken by hybrid and adsorption beads, respectively, after t days of operation. Adsorption by nanoclay is calculated using Equation 6.4:

`

$$A_{d,t} = A_{n,t} - A_{c,t}$$ Equation 6.4

Where $A_{d,t}$ is the amount of nitrate adsorbed by nanoclay and $A_{c,t}$ is quantity of nitrate uptaken by control beads after t days of the operation.

Since the quantity of alginate is the same in all six groups of beads, the adsorption rate by alginate will be identical for all of them. According to Figure 6.1 the adsorption capacity of the nanoclay and assimilation rate by microalgae increases by increasing the dose up to 0.30 mg/bead and reaches an equilibrium at this point. There is no significant change in nitrate uptake rate at dosage higher than 0.30 mg/bead.

To determine the changes in surface charge of the hybrid beads, the zeta potential was measured at different days of the operation. As shown on Table 6.3, increasing the nanoclay concentration increased the zeta potential of the beads at the beginning of the treatment process (day 0). The nanoclay used in these experiments was treated with TMSA which contains a positive charge. Adding more nanoclay to the beads provides more positive charge and increases the zeta potential. As the treatment process progresses, the zeta potential becomes more negative which would confirm the presence of nitrate ions in the beads. The highest "negative" zeta potential and greatest nitrate uptake was observed in the beads with the concentration of 0.3 mg nanoclay/bead.

Increasing the nanoclay dose will provide more surface area and active sites for the adsorption of nitrate. At amounts higher than 0.30 mg/bead. However, the adsorbent particles may aggregate which would result in blockage of the active sites and no further nitrate uptake. Therefore, the concentration of 0.30 mg/bead was considered as an optimum dose and was used in the remaining experiments.

According to Figure 6.1, adding more nanoclay to the beads also increases the assimilation rate by microalgae. Because of that, we expected an increase in DO concentration which

`

did not occur. Therefore, it is possible that there was a fourth mechanism influencing the treatmnet process in the hybrid beads which could have been caused by the inherent toxicity of TMSA to the algal cells. Previous studies have shown that the toxicity of some metals (e.g. copper) to the algal cells can increase the activity of the nitrate reductant (NR) enzymes and the photosynthetis rate (Han, Kang, Park, Lee, & Brown, 2008). In this case, no increase in the photosynthesis rate was observed. Therefore, the toxicity may cause some algal cells to break down and secrete some of the NR enzymes to the outside of the cells. These NR enzymes function faster outside of the cells and help the nitrate uptake rate to be faster than the assimilation process. The accuracy of this theory will be evaluated in section 6.4.3.

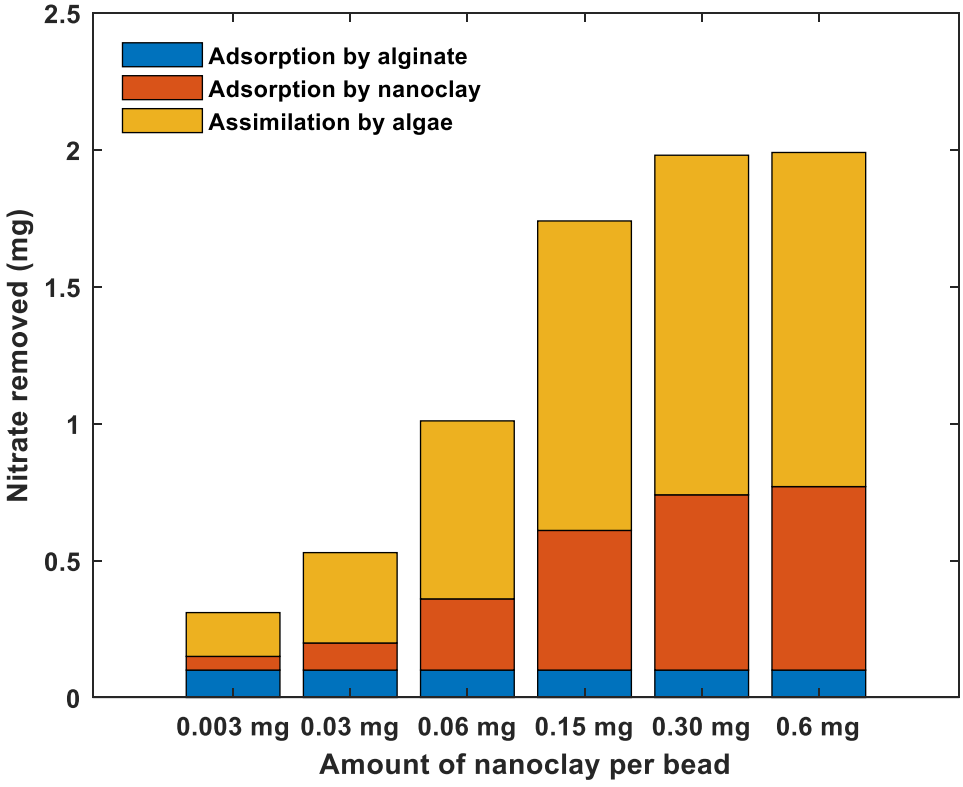


Figure 6.1 The mass of nitrate removed by hybrid beads embedded with different amount of nanoclay after 3 days of operation.

`

Table 6.3 Change in zeta potential of the hybrid beads, embedded with different amount of nanoclay, during 5 days of the treatment process.

	Zeta Potential (mV)					
Day	0.003 mg	0.03 mg	0.15 mg	0.30 mg	0.60 mg	Control-no nanoclay
0	-0.14±0.02	-1.14±-0.1	3.2±-1.2	3.4±1.3	4.9±0.1	-1.4±-0.3
1	-0.42±-0.02	-1.35±-0.2	-2.3±-1.1	-5.2±-1.1	-6.8±-2.4	-2.8±1.1
5	-1.1±-0.2	-5.2±-2.8	-7.9±-2.5	-11.7±-2.2	-10.6±-1.4	-4.5±-1.3

The equilibrium of the adsorption on the adsorption beads was evaluated using the Langmuir and Freundlich isotherm models. The Langmuir isotherm is based on the assumptions that adsorption only occurs at specific homogeneous sites within the nanoclay-alginate matrix and the adsorbent is saturated after one layer of nitrate molecules forms on the surface of it. The Freundlich isotherm describes the adsorption of multilayer surfaces (Wu et al., 2012). The linearized form of the Langmuir and Freundlich isotherms can be seen in Equations 6.5 and 6.6, respectively:

$$\frac{C_e}{q_e} = \frac{1}{q_0 K_L} + \frac{C_e}{q_0} \qquad \text{Equation 6.5}$$

$$\log\ q_e = log\ K_f + \frac{1}{n} log\ C_e \qquad \text{Equation 6.6}$$

Where q_e is the amount of nitrate adsorbed per gram of nanoclay (mg/g), C_e is the equilibrium solution concentration of nitrate (mg/L), q_0 (mg/g) is the maximum capacity of nitrate required to form a complete monolayer on the surface, and K_L represents the Langmuir constant.

`

K_f is the Freundlich adsorption constant, related to the adsorption capacity of the adsorbent and n is the Freundlich factor, related to the surface heterogeneity. q_e was calculated by Equation 6.7:

$$q_e = \frac{(C_0 - C_e)}{m} \cdot V \quad \text{Equation 6.7}$$

Where: C_0 and C_e (mg/L) are the initial and equilibrium nitrate concentrations, respectively, V (L) is the volume of the solution and m (g) is the mass of the nanoclay.

Nitrate adsorption data on nanoclay alginate matrix fitted well to the Freundlich model and higher R^2 was derived in Freundlich isotherm ($R^2 = 0.998$) as compared to Langmuir model ($R^2 = 0.912$). Using Freundlich model, n and K_f were found to be 0.335 and 0.165, respectively.

In the Freundlich isotherm, the 1/n term describes the linearity of the adsorption process, where 1/n=1 indicates that the reaction between solute and adsorbent is linear, while 1/n<1 indicates that the adsorption is unfavorable at lower equilibrium concentration and the shape of the nonlinear adsorption isotherm is convex. The term 1/n>1 indicates that the concave shape of nonlinear adsorption isotherm and it means that the adsorption is more favorable at lower equilibrium concentrations (Mohsenipour, Shahid, & Ebrahimi, 2015). The obtained value of 1/n was computed to be 3.0 suggesting that adsorption of nitrate on the nanoclay-alginate matrix was more favorable at lower concentrations of nitrate.

6.4.3 The role of TMSA on nitrate removal

TMSA is a widely used cationic surfactant. Due to its chemical stability and heat and pressure resistance, it is used as a raw material for producing hair conditioner, fabric softener, silicone oil and rubber (Henton & E., 1989; Murray, 2001). TMSA is reported to be toxic to the *Chlorella* cells. Once inside the cell, TMSA may affect thylakoid

`

organization and chlorophyll synthesis which will result in impairment of photosynthetic capacity and lower the number of live cells. Although the mechanisms of TMSA toxicity may vary in different algae species, it is generally accepted that such toxicity impacts the structure of algal lipid membranes, binding with or denaturing membrane proteins. This will cause increased membrane permeability and leakage of compounds like ions and amino acids that are significant for cell viability (Qv & Jiang, 2013).

The toxicity of the TMSA to algal cells might be the main reason behind the enhanced nitrate removal rate observed in hybrid beads (Section 6.4.2). This toxicity may break down the cells, releases the NR enzymes and make them more readily available for nitrate uptake. To check the accuracy of this theory, the algal cells were mixed with different quantity of TMSA and immobilized in alginate and tested for nitrate uptake. The nanoclay that was used in this study contained ~30% (by wt.) TMSA. This meant that the hybrid beads with the concentration of 0.06, 0.15 and 0.30 mg nanoclay/bead contained 0.02, 0.04 and 0.09 mg TMSA, respectively. These ratios were used to make TMSA- embedded algal beads.

Live cell assessment was then performed to check the toxicity of TMSA to the algal cells. Figure 6.2 compares the number of live cells in algal beads, embedded with 0.02, 0.04 and 0.09 mg TMSA over time. As it's shown, at day 0, the number of live cells is slightly higher in the cells embedded with lower mass of TMSA. In all three groups, the number of live cells decreases over time.

`

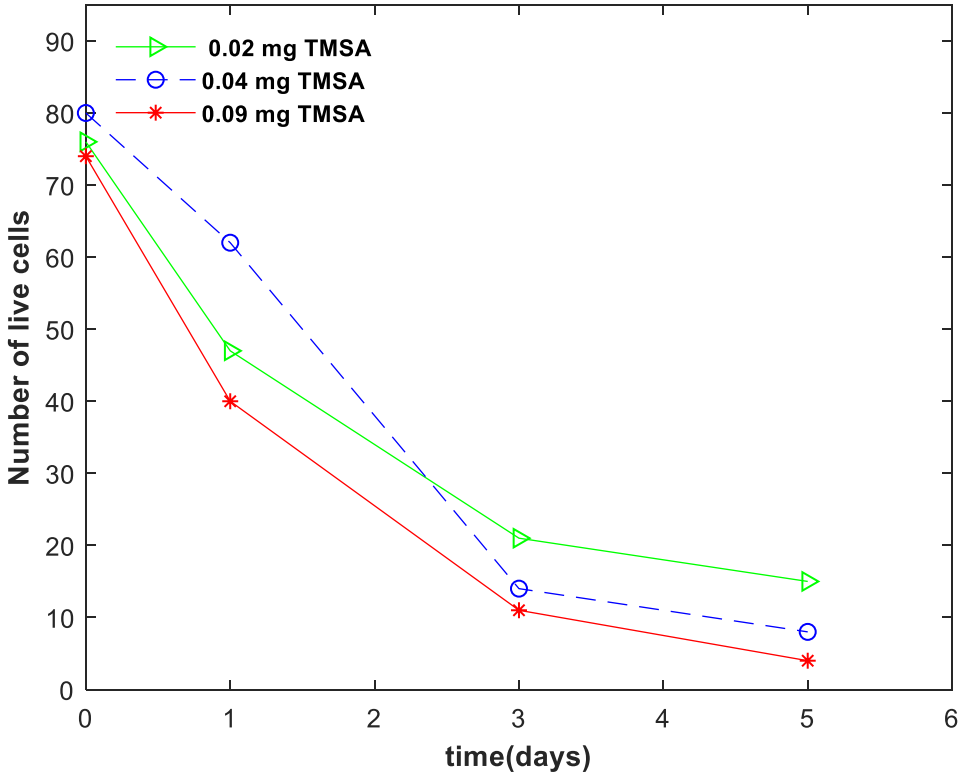


Figure 6.2 Change in the number of live cells in algal beads with 0.02 g, 0.04 and 0.09 mg TMSA per bead over time.

The amount of nitrate uptake by TMSA embedded beads was measured one day after the start of the experiments. As it's shown on Figure 6.3a, the beads containing 0.02, 0.04 and 0.09 mg TMSA, removed 0.78±0.44, 0.69±0.31, and 0.65±0.24 mg of nitrate respectively. This is about twice higher than the removal observed by the hybrid beads, embedded with nanoclay, and containing the respective mass of TMSA. After 3 days of the experiments, the removal rate reached its maximum which was 3.27±0.05 mg nitrate. (Figure 6.3b).

In the TMSA embedded beads, there was a direct contact between the algal cells and TMSA. Due to the apparent inherent toxicity of TMSA to the algal cells, the main mechanism controlling the treatments here can be the death of the cells and the subsequent

`

release of the NR enzymes to the bulk solution which accelerates the nitrate removal rate. It is possible that the extracellular enzymes activity is the fourth mechanism controlling the treatment in hybrid beads; and the main reason behind the high nitrate uptake observed in Section 6.4.2, above.

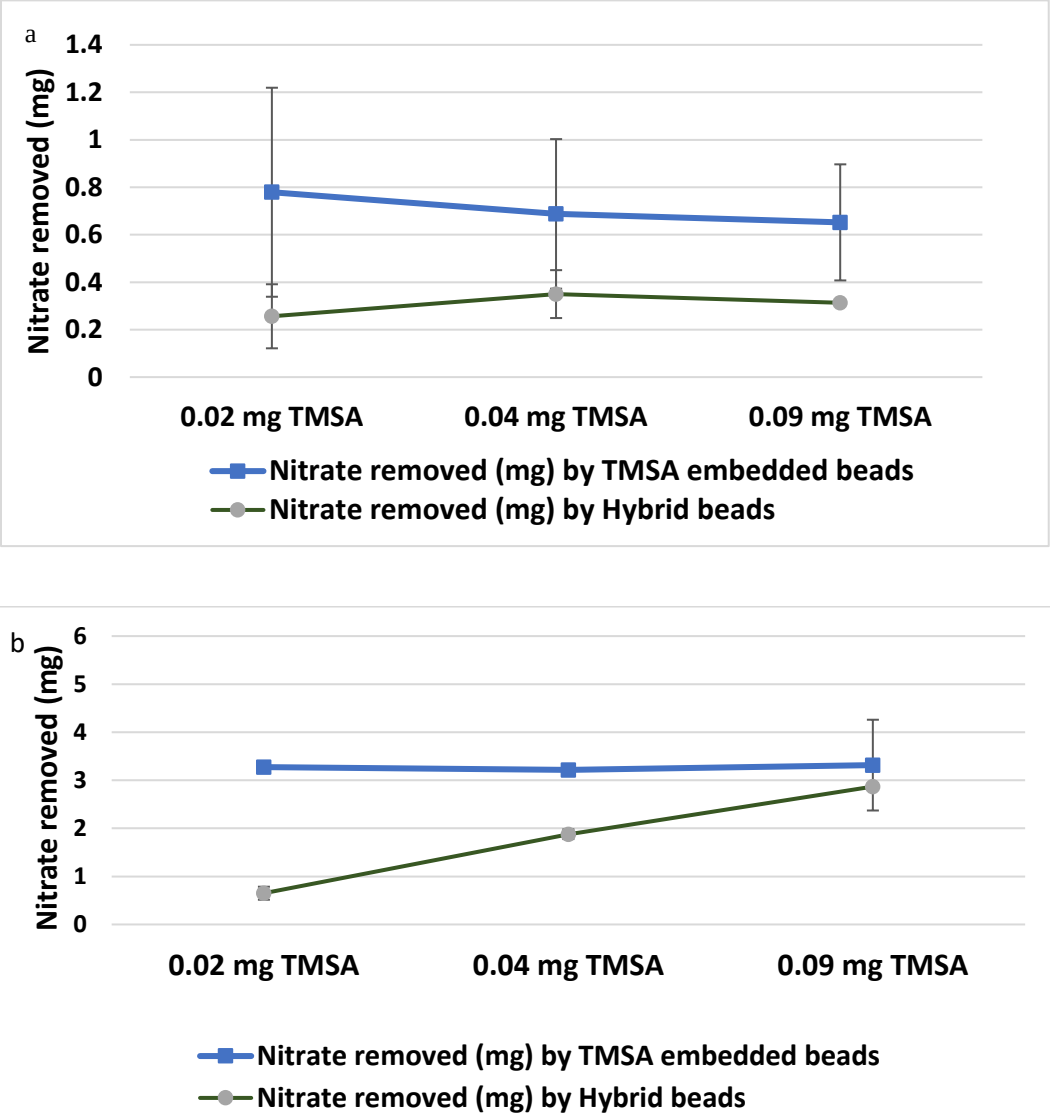


Figure 6.3 Mass of nitrate removed after: a) 1 day and b) 3 days of operation as affected by the mass of TMSA and nanoclay embedded in the beads.

`

6.4.4 Evaluation of the performance of nitrate removal under sequential batch conditions

While nitrate was successfully removed in batch reactors, the durability of the treatment system was tested in sequential batch mode. The experiments were conducted in triplicate reactors for three consecutive treatment periods. At the end of each period, the treated water in each reactor was discarded and the reactors were filled back with synthetic groundwater and glucose. In addition to the nutrients embedded within the beads during manufacturing, no additional nutrients were provided. As shown in Figure 6.4 the nitrate removal rate decreased from 96.2% to 47.5% over periods 1 to 3. Over time, the turbidity of the solution increased from 1.2 NTU (day 1) to 10.6 NTU (day 25) which could be a sign of nanoclay leaching out of the beads and entering the groundwater matrix. In sequential batch mode, nutrients and calcium may get washed out from the reactors by the discarding of the treated water at the end of each period. Calcium is an essential ion for the stability of the beads. When the beads form, calcium ions bind to the blocks of alginate chains and the strength of the alginate gel is largely dependent on the number of cross-links forms (Li, Fang, Vreeker, Appelqvist, & Mendes, 2007). Since they were never replaced, the algal cells may have faced calcium and nutrient deficiency, which lead to the loss of beads strength, lower nitrate assimilation and reduced performance. The beads may have longer lifetime when they are used with actual groundwater that is rich in nutrient and calcium. However, it is also possible that the fragile beads ultimately break up due to repeated physical handling and possibly, shear forces within the reactor contents.

`

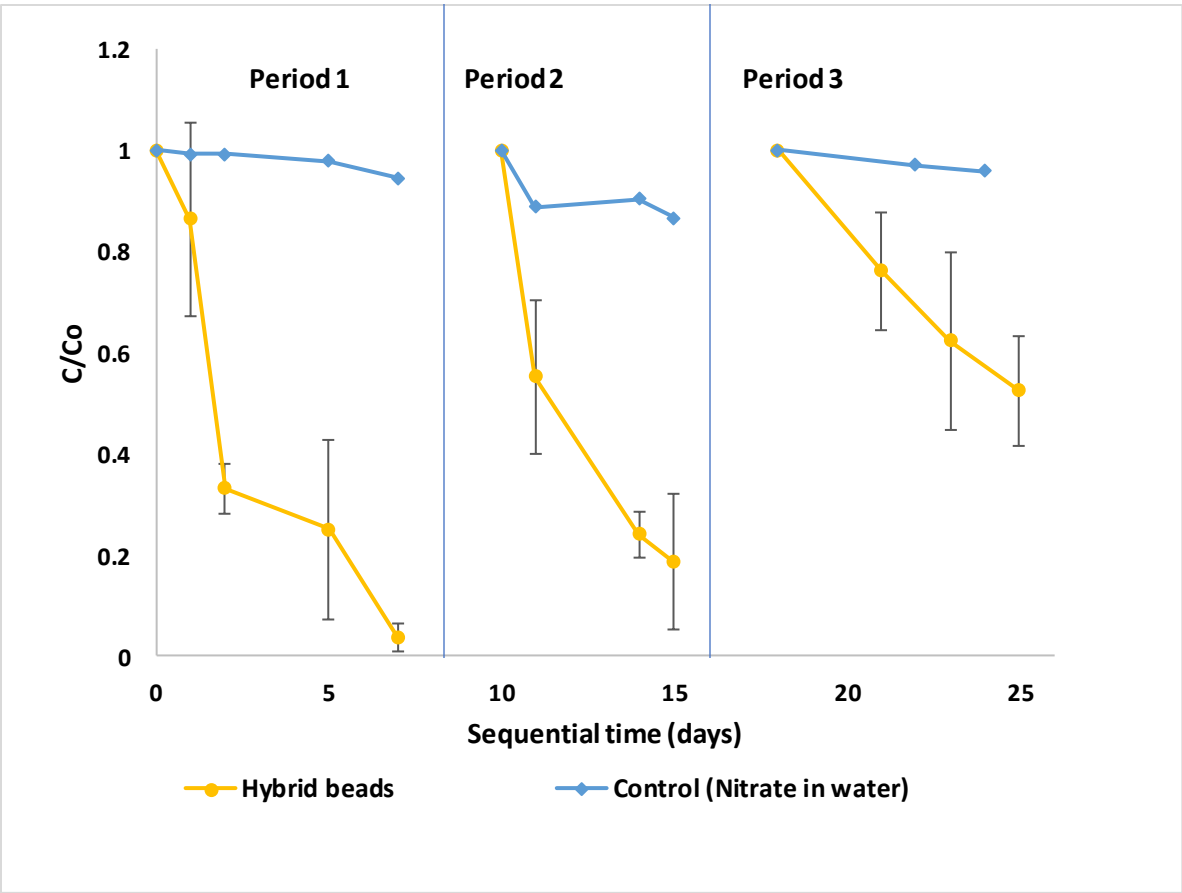


Figure 6.4 Nitrate present in the solution in sequencing batch mode.

6.4.5 Examination of the efficiency of the beads on the removal rate of atrazine

Figure 6.5 provides a graphical representation of the capability of the beads in removing atrazine from groundwater. Atrazine was reduced during first 24 hours of treatment by 86% when using the hybrid beads. The removal by adsorption beads is estimated at 64%. Therefore, the net percentage of atrazine removal by immobilized algal cells would be 22%. On day 3, the removal efficiency reached the maximum of 98% for the hybrid beads and 96% for the adsorption beads. The removal rate for the adsorption beads stayed constant until day 5, but it decreased to 85% for the hybrid beads which was a sign of

`

atrazine migration from the beads back to groundwater. When tested with biological beads, 50% of atrazine was removed after 2 days of operation. The removal rate decreased to 40% at day 5.

According to (Boyd et al., 1989), clays modified using cations with large alkyl groups, (e.g., TMSA) contain paraffin-like layer of organic phase in the interlayer of the clay lattice. This layer acts as the partition medium for the sorption of the organic compounds. Sorption of atrazine on TMSA modified nanoclay was a function of the organic phase in the interlayer of nanoclay, following the adsorption of cationic surfactants (TMSA) in the lattice (Dutta & Singh, 2015). The mechanism of atrazine removal by algae was not previously reported, and it may involve biosorption, bioaccumulation, or biodegradation, or a combination of these mechanisms. Atrazine is considered toxic to many strains of green algae. However, the toxicity of the atrazine depends on the strain of algae and the exposure time. For *Scenedesmus*, the EC_{50} ranges between 21-49 ppb (Larsen, Stay, Shiroyama, & DeNoyelles Jr, 1986). The initial concentration of atrazine used in these experiments was 100 ppb, which is higher than the reported inherent toxicity of atrazine to the algal cells. Therefore, it is postulated that, after 3 days of exposure, the atrazine molecules slowly started to leave the cells and transfer to the bulk solution which caused 11% reduction in uptake rate for hybrid and biological beads.

`

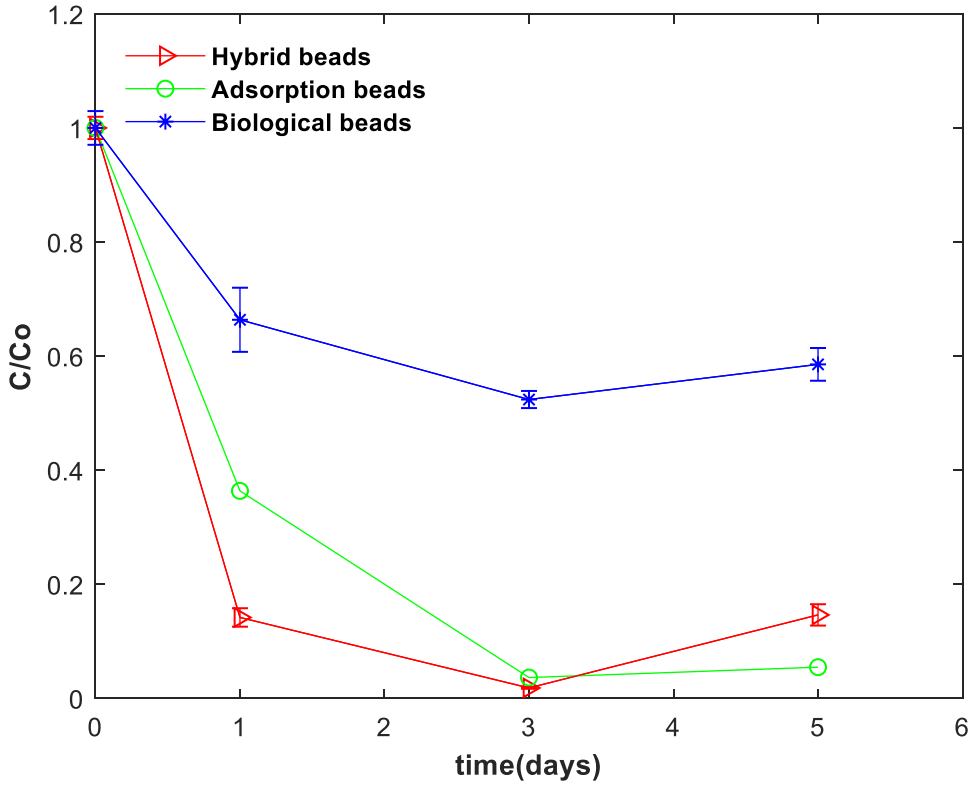


Figure 6.5 Percentage of atrazine present in the solution over time.

The solid/water distribution coefficient K_d was calculated to evaluate the effectiveness of the adsorption on alginate beads in uptaking nitrate and atrazine. K_d was calculated using the following equation:

$$K_d = = \frac{C_i - C_e}{C_e} \times \frac{V}{m} \qquad \text{Equation 6.8}$$

Where, C_i and C_e are the initial and final (equilibrium) concentrations of the contaminant in solution (mg/L), V is the volume of the solution (L), and m is the mass of the adsorbent (g) (Hamoudi & Belkacemi, 2013). The distribution coefficients are shown in Table 6.4.

`

Table 6.4 Distribution coefficient (K_d) for nitrate and atrazine adsorption from groundwater

Matrix	K_d $\left(\frac{L}{g}\right)$- Nitrate adsorption	K_d $\left(\frac{L}{g}\right)$ - Atrazine adsorption
Adsorption beads (nano clay + alginate)	0.148	3.85
Control beads (Alginate)	0.109	0.231

The higher K_d values represent more effectiveness of the adsorbent material in removing target contaminants from the aqueous solution. As shown in Table 6.4, adding nanoclay to the alginate beads increased the distribution coefficient from 0.231 to 3.85 L/g and improves the adsorption of atrazine significantly. There is also a slight increase in nitrate removal when alginate beads are mixed with nanoclay. However, the greatest performance in nitrate uptake occurred when nanoclay was added to the mixture of algae and alginate and the uptake occurs through the hybrid mechanisms of adsorption, biological assimilation, and extracellular enzyme activity; as discussed in Section 6.4.2, above.

6.4.6 Application of actual groundwater

The capability of the hybrid beads was tested in removing nitrate and metals from actual groundwater. The same procedure that was outlined in the Materials and Methods section was used to test the raw groundwater for the concentration of nitrate and metals prior to the start of the experiments.

In these experiments. The hybrid and biological beads removed 91% and 93% of nitrate from groundwater within 3 days without requiring any external organic carbon source. The removal rate remained constant for biological beads and it reached 99% after 6 days in hybrid beads. The uptake of nitrate by adsorption and the control beads during this period was 35% and 12%, respectively (Table 6.5).

`

Table 6.5 Percentage of nitrate present in the solution, pH, and COD of the bulk solution over time in actual groundwater.

	Adsorption Beads			Hybrid Beads		
Day	**C/Co**	**COD (mg/L)**	**pH**	**C/Co**	**COD (mg/L)**	**pH**
0	1.00	180.00	7.32	1.00±0.00	166.33	7.31
1	0.90	90.00	7.64	0.89±0.09	86.00	7.62
2	0.76	115.60	8.16	0.58±0.07	138.00	8.42
3	0.72	121.00	8.37	0.09±0.07	120.00	8.06
5	0.69	112.50	8.27	0.02±0.03	194.80	7.74
6	0.65	123.20	8.10	0.01±0.01	196.80	8.03

	Biological beads			Control beads		
Day	**C/Co**	**COD (mg/L)**	**pH**	**C/Co**	**COD (mg/L)**	**pH**
0	1.00±0.00	172.00	7.30	1.00	164.50	7.42
1	0.40±0.04	146.00	8.59	0.94	162.00	7.25
2	0.08±0.03	133.00	8.18	0.92	158.20	7.12
3	0.07±0.03	132.00	9.50	0.93	160.00	7.68
5	0.07±0.02	124.60	10.03	0.88	162.40	7.77
6	0.07±0.02	110.00	10.02	0.88	160.00	7.93

Some of the metals and none-metal ions that were present in the groundwater were fully or partially removed by hybrid beads (Table 6.6).

SEM analysis was performed to study the surface morphologies of the hybrid beads before and after exposure to the groundwater. SEM images show that the fresh beads had porous, heterogeneous surface. The pores and cavities on the beads facilitate the sorption of nitrate and metals (Figure 6.6a). After exposure to the groundwater the size of the pores decreased, and beads became smoother and compact (Figure 6.6b).

`

The main process involved in the removal of metals by immobilized beads is thought to be biosorption, which is described as follows: the metal ions are first transferred from the solution to the biosorbent surface, which contained different functional groups that act as active binding sites (Petrovič & Simonič, 2016).

Table 6.6 Ion removal efficiency of Hybrid beads in groundwater.

Ions	**Removal Efficiency (%)**
Mg	14.0
P	40.6
S	4.2
V	100.0
Cr	100.0
Mn	54.4
Zn	100.0
As	27.8
Se	100.0
Fe	72.0

a. b.

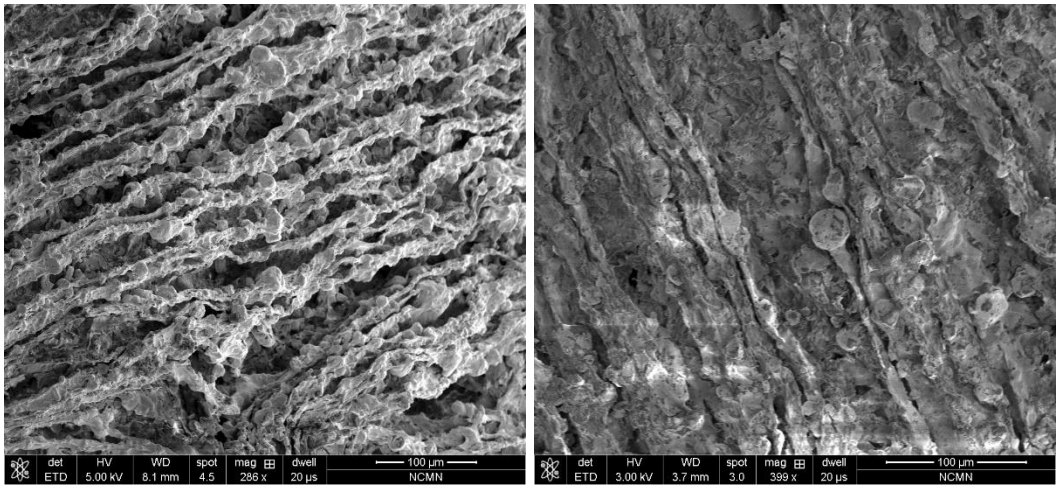


Figure 6.6 SEM images of immobilized nanoclay-embedded *S. sp*. cells on the surface of sodium alginate at day 0 (a) and day 3 (b) of operation.

`

The metal ions then diffuse into the pores of adsorbent and eventually on the surface of the material (W. W. Maznah, A. T. Al-Fawwaz, & M. Surif, 2012). In the hybrid beads, the surface properties of clay and the abundant functional groups of the gel provided adequate adsorption for the uptake of metals and facilitate the treatment process.

6.5 Conclusions

Based on the studies reported in this chapter, the following conclusions could be made:

1. This study investigated the capability of nanoclay embedded *Scenedesmus. sp.* beads to remove nitrate, atrazine along with some metal constituents from aqueous solutions. The experimental data show that embedding the algal beads with nanoclay accelerates the nitrate removal rate.

2. From the studies reported here, it was established that pseudo-first-order kinetic model described the kinetic rate, and the equilibrium data fitted well the Freundlich adsorption isotherm.

3. The initial concentration of atrazine used in these experiments was 100 ppb, which is higher than the inherent toxicity of atrazine to the algal cells. Therefore, after 3 days of exposure, the atrazine molecules slowly started to leave the cells and transfer to the bulk solution.

4. Nanoclay embedded beads can be a potential technique for remediation of water contaminated with nitrate or atrazine owing to its ability to improve the uptake capacity as well as apparent high selectivity for nitrate and herbicides.

`

References

Boyd, S. A., Jaynes, W. F., & Ross, B. S., 1989. Immobilization of organic contaminants by organo-clays; application to soil restoration and hazardous waste containment. *In Baker, R.A. (Ed.), Organic substances and sediments in water, Lewis Publishers, New York* (pp. 181-199).

Dutta, A., & Singh, N., 2015. Surfactant-modified bentonite clays: preparation, characterization, and atrazine removal. *Environmental science and pollution research, 22*(5), 3876-3885.

El Ouardi, M., Qourzal, S., Alahiane, S., Assabbane, A., & Douch, J., 2015. Effective removal of nitrates ions from aqueous solution using new clay as potential low-cost adsorbent. *Journal of Encapsulation and Adsorption Sciences, 5*(04), 178-190.

EPA., 1995. *National Primary drinking water regulations*

EPA., 2018. *2018 Edition of the Drinking Water Standards and Health Advisories Tables*.

Eroglu, E., Agarwal, V., Bradshaw, M., Chen, X., Smith, S. M., Raston, C. L., & Iyer, K. S., 2012. Nitrate removal from liquid effluents using microalgae immobilized on chitosan nanofiber mats. *Green Chemistry, 14*(10), 2682-2685.

Fierro, S., del Pilar Sánchez-Saavedra, M., & Copalcua, C., 2008. Nitrate and phosphate removal by chitosan immobilized Scenedesmus. *Bioresource Technology, 99*(5), 1274-1279.

Garbisu, C., Gil, J. M., Bazin, M. J., Hall, D. O., & Serra, J. L., 1991. Removal of nitrate from water by foam-immobilizedPhormidium laminosum in batch and continuous-flow bioreactors. *Journal of Applied Phycology, 3*(3), 221-234.

Greer, F. R., & Shannon, M., 2005. Infant methemoglobinemia: the role of dietary nitrate in food and water. *Pediatrics, 116*(3), 784-786.

Hameed, M. A., 2007. Effect of algal density in bead, bead size and bead concentrations on wastewater nutrient removal. *African Journal of biotechnology, 6*(10), 1185– 1191

Hamoudi, S., & Belkacemi, K., 2013. Adsorption of nitrate and phosphate ions from aqueous solutions using organically-functionalized silica materials: Kinetic modeling. *Fuel, 110*, 107-113.

Henton, D. E., 1989. Process for coagulating a grafted rubber compound. In: Google Patents.

Isensee, A. R., Helling, C. S., Gish, T. J., Kearney, P. C., Coffman, C. B., & Zhuang, W., 1988. Groundwater residues of atrazine, alachlor, and cyanazine under no-tillage practices. *Chemosphere, 17*(1), 165-174.

Larsen, D. P., Stay, F., Shiroyama, T., & DeNoyelles Jr, F., 1986. Comparisons of single-species, microcosm and experimental pond responses to atrazine exposure. *Environmental Toxicology and Chemistry: An International Journal, 5*(2), 179-190.

Li, L., Fang, Y., Vreeker, R., Appelqvist, I., & Mendes, E., 2007. Reexamining the egg-box model in calcium− alginate gels with X-ray diffraction. *Biomacromolecules, 8*(2), 464-468.

Maznah, W. W., Al-Fawwaz, A., & Surif, M., 2012. Biosorption of copper and zinc by immobilized and free algal biomass, and the effects of metal biosorption on the growth and cellular structure of Chlorella sp. and Chlamydomonas sp. isolated from rivers in Penang, Malaysia. *Journal of Environmental Sciences, 24*(8), 1386-1393.

Mohsenipour, M., Shahid, S., & Ebrahimi, K., 2015. Nitrate adsorption on clay kaolin: batch tests. *Journal of Chemistry, 2015*, 1-7

Mollamohammada, S., Aly Hassan, A., & Dahab, M., 2019. Nitrate removal from groundwater

`

using immobilized heterotrophic algae. *water, air and soil pollution (In press)*.

Moreno-Garrido, I., 2008. Microalgae immobilization: current techniques and uses. *Bioresource Technology, 99*(10), 3949-3964.

Murray, A. M., 2001. Hair conditioning composition. In: Google Patents.

Petrovič, A., & Simonič, M., 2016. Removal of heavy metal ions from drinking water by alginate-immobilised Chlorella sorokiniana. *International journal of environmental science and technology, 13*(7), 1761-1780.

Qv, X. Y., & Jiang, J. G., 2013. Toxicity evaluation of two typical surfactants to Dunaliella bardawil, an environmentally tolerant alga. *Environmental toxicology and chemistry, 32*(2), 426-433.

Rezvani, P., & Taghizadeh, M. M., 2018. On using clay and nanoclay ceramic granules in reducing lead, arsenic, nitrate, and turbidity from water. *Applied Water Science, 8*(5), 131.

Rhoades, M. G., Meza, J. L., Beseler, C. L., Shea, P. J., Kahle, A., Vose, J. M., Spalding, R. F., 2013. Atrazine and nitrate in public drinking water supplies and non-Hodgkin lymphoma in Nebraska, USA. *Environmental health insights, 7*, 15–27

Sanchez-Martin, M., Rodriguez-Cruz, M., Andrades, M., & Sanchez-Camazano, M., 2006. Efficiency of different clay minerals modified with a cationic surfactant in the adsorption of pesticides: influence of clay type and pesticide hydrophobicity. *Applied Clay Science, 31*(3-4), 216-228.

Sebilo, M., Mayer, B., Nicolardot, B., Pinay, G., & Mariotti, A., 2013. Long-term fate of nitrate fertilizer in agricultural soils. *Proc Natl Acad Sci U S A, 110*(45), 18185-18189.

Srinivasan, R., 2011. Advances in application of natural clay and its composites in removal of biological, organic, and inorganic contaminants from drinking water. *Advances in Materials Science and Engineering, 2011*, 1-17.

Ward, M. H., Jones, R. R., Brender, J. D., De Kok, T. M., Weyer, P. J., Nolan, B. T.,Van Breda, S. G., 2018. Drinking water nitrate and human health: an updated review. *International journal of environmental research and public health, 15*(7), 1-31.

Wu, X., Hui, K., Hui, K. S., Lee, S., Zhou, W., Chen, R., Son, Y., 2012. Adsorption of basic yellow 87 from aqueous solution onto two different mesoporous adsorbents. *Chemical Engineering Journal, 180*, 91-98.

Yuan, G., & Wu, L., 2007. Allophane nanoclay for the removal of phosphorus in water and wastewater. *Science and Technology of Advanced Materials, 8*(1-2), 60-62

`

CHAPTER 7 SUMMARY AND CONCLUSIONS

7.1 Research Motivation and Summary of Conclusions

The presence of nitrate and herbicides in surface and groundwater impairs their quality as ~~a~~ drinking water sources, and causes several health effects, such as methemoglobinemia and Non-Hodgkin Lymphoma. The contamination issue is exacerbated when considering that available water treatment methods such as reverse osmosis are typically very expensive and energy extensive and require special expertise which might be problematic in rural areas.

The main goal of this study was to determine the capability of the immobilized algal-based biological system, as a natural alternative technique to remove nitrate and herbicides from waterbodies. This process can lower the health risks associated with high concentrations of nitrate and herbicides and produces biomass which can be used as a source of green energy. The development of such a system started from using the ideal conditions in the lab, followed by testing using actual contaminated water samples.

The main objectives of this study were to optimize an immobilized algal-based system to remove nitrate from groundwater; evaluate the capability of the designed system in real working scenarios by using actual water samples, and finally, exploring a combined physical- biological approach for maximizing the uptake of nitrate from contaminated groundwater.

The two microalgae species (*Chlorella vulgaris* and *Scenedesmus species*) used in this work were acquired from Biochemistry Department at University of Nebraska-Lincoln.

`

Sodium alginate was used an immobilizing agent in all of the experiments. To prepare the immobilized algal beads, algae solution was centrifuged, supernatant was discarded, and the algal cells were resuspended in DI water to obtain a concentrated solution. Next, the concentrated algae solution was mixed with sodium alginate solution. The mixture was dropped into a calcium chloride solution using a syringe pump and left overnight to form algal beads.

The immobilized algal-based system was first tested in batch mode, using synthetic groundwater. The optimum operational parameters that maximized the uptake rate of nitrate in batch mode were then used to design the sequencing batch and continuous flow reactors. The capability of the system in real working scenarios was evaluated later, using actual groundwater and surface water samples, contaminated with nitrate, herbicides and cyanotoxins.

Finally, to improve the capability of the algal beads in removing nitrate and herbicides, a combined physical-biological system was designed by embedding the algae cells with nanoclay, a natural material with high absorbance capacity. The capability of nanoclay-embedded algal beads was tested in batch reactor, using synthetic and actual groundwater.

The following are the principal conclusions drawn from this study, as related to the research objectives:

Objective 1: Optimizing an immobilized algae-based biological system for nitrate removal

1. Immobilized *Scenedesmus sp.* beads were more effective in nitrate uptake from synthetic groundwater than the suspended cell system.

`

2. Among different cultivation methods, the heterotrophic cultivation of immobilized *Scenedesmus. sp.* was more effective in nitrate removal.

3. The Initial algae concentration in the beads is an important factor in nitrate uptake rate, and the highest nitrate uptake rate was achieved in the beads made with the equal volumetric ratio of algae: alginate.

4. Higher algal density in the beads caused more nutrients and glucose assimilation. However, the self-shading effect may have reduced photosynthetic activity of the cells toward the end of the treatment. Therefore, it is suggested that the algal beads should be replaced before the self-shading effect occurs.

5. Actual contaminated groundwater was found to be more suitable for nitrate removal than synthetic groundwater as it may have contained more nutrients and trace minerals and facilitated algae growth inside of the immobilization matrix.

Objectives 2 : Evaluating different reactors configuration

1. Immobilized algal beads kept functioning for up to 100 days in sequencing batch mode.

2. The immobilized algal beads were more effective in nitrate and atrazine removal at room temperature (20 °C) than at the high temperature of 35 °C in a continuous flow reactor.

3. According to these results, the culture can be kept until the end of 30 days projected during spring or fall. However, during summer, it may be necessary to replace the algal beads more frequently (after 10 days of the treatment operation).

Objectives 3: Evaluating the performance of the algal- based biological system in real working scenarios

`

1. The results using actual contaminated water samples showed that the immobilized *Scenedesmus. Sp.* beads were capable of simultaneous removal of nitrate, metals, and herbicides in continuous flow mode.

2. Even though the algae cells used in this study were in an immobilized form, herbicides present in the water tended to degrade their growth rate and impair their capability in removing nitrate and herbicides. It was observed that the removal of herbicides was the highest during the first 10 days of the treatment operation and it slightly went down afterwards.

3. Adsorption, bioaccumulation and biodegradation were probably the main three processes involved in the removal of herbicides.

4. The algal based system can be a great option to treat the surface water for reuse purposes including artificial recharge of groundwater aquifers. Recharging the aquifers with the treated water serves to dilute the concentration of nitrate and herbicides in the groundwater, thus lowering the potential health risks.

Objective 4: Utilization of nanoclay-embedded algal beads as a combined physical-biological approach.

1.The experimental data showed that embedding the algal beads within nanoclay accelerates the nitrate removal. The removal rate in this case appeared to follow a pseudo-first-order kinetic model. The performance rate and equilibrium data appeared to best fit the Freundlich adsorption isotherm.

`

2. The initial concentration of atrazine used in these experiments was higher than the inherent toxicity limit of atrazine to the algal cells. Therefore, after 3 days of exposure, the atrazine molecules slowly left the cells and transferred back into the bulk solution.

3. Nanoclay embedded beads can be a potential technique for treatment of water contaminated with nitrate or atrazine because of their exceptional uptake capacity as well as high selectivity for this anionic contaminant and herbicides.

7.2 Recommendations for Further Study

Further studies will be needed to help improve the stability and the sorption properties of the immobilized algae. This can be done by evaluating and comparing the effect of other immobilization matrices on performance of microalgae. This work can lead to more effective use of immobilized algae, and as a result, accelerate the development of more effective biosorbents.

To gain a better understanding of herbicide removal by algae, the pathway of herbicide degradation and their metabolites as well as the enzymes participating would need to be studied.

Economic studies are needed in order to compare the feasibility of immobilized algal system in comparison with other techniques, such as ion exchange and reverse osmosis. . The bioremediation system can also be tested in full scale in the aquifers with nitrate concentrations higher than the regulatory level.

`