

ENRICHMENT OF AUTOTROPHIC DENITRIFIERS AND INVESTIGATION
OF THEIR USE IN BIO-DESULFURIZATION OF GROUND TIRE RUBBER

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GROUND TIRE RUBBER**

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ABSTRACT

ENRICHMENT OF AUTOTROPHIC DENITRIFIERS AND INVESTIGATION OF THEIR USE IN BIO-DESULFURIZATION OF GROUND TIRE RUBBER

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Many new methods have been developed for the recycling of waste tires. Among these developed methods, bio-desulfurization takes attention with its low energy requirement, selectively breaking S bonds without damaging the rubber main chain and being an environmentally friendly application. This thesis aims to investigate the enrichment and use of sulfur-driven autotrophic denitrifiers for bio-desulphurization of ground tire rubber (GTR).

In the first part of the study, sulfur-based autotrophic denitrifiers were enriched from seed sludges, taken from three different treatment plants. Elemental sulfur (S^0) and thiosulfate ($S_2O_3^{2-}$) were selected as sulfur sources for the enrichment operation. Average nitrate removal efficiencies of the enrichment reactors (ERs), namely ER1, ER2, ER3 and ER4 were 96.0%, 96.9%, 97.6% and 87.2%, respectively. It was observed that ER4, the only one fed with S^0 , was more sensitive to shock loads and had relatively lower nitrate removal efficiency.

In the second part of the study, bio-desulphurization of GTR was investigated using enriched autotrophic denitrifiers, namely EC1, EC2, EC3 and EC4 obtained from ER1 to ER4, respectively. Thus the effects of seed sludge type in addition to electron donor were also investigated. An increase in the sulfate concentration was

used as a bio-desulphurization indicator. Results revealed that abiotic nitrate removal via GTR was a significant process and zinc oxide content of GTR could react with the sulfate being produced through bio-desulfurization, as also stated in the literature. Bio-desulphurization was only obtained with EC3, enriched from biological nutrient removal sludge of the Leather Organized Industrial Zone Wastewater Treatment Plant, as 3.9%, when the possible zinc sulfate formation was not taken into account, which could cause an under-estimation of the desulfurization ratio. Considering the possible zinc sulfate formation, bio-desulphurization by EC2 (enriched from anaerobic digester) and EC3 were calculated as 3.6% and 8.2%, respectively, which are comparable to the literature data. Not considering the possible zinc sulfate formation could cause an underestimation of the desulfurization ratio. Electron donor effect on bio-desulfurization could not be detected because bio-desulfurization was not obtained by EC1 ($\text{S}_2\text{O}_3^{2-}$ -fed) and EC4 (S^0 -fed) cultures. It can be concluded that it is possible to achieve bio-desulfurization of GTR with autotrophic denitrifiers, when excess nitrate was fed.

Keywords: Bio-desulfurization, Microbial Desulfurization, Autotrophic Denitrification, Ground Tire Rubber (GTR), Sulfur

ÖZ

OTOTROFİK NİTRAT GİDERİCİLERİN ZENGİNLEŞTİRİLMESİ VE ÖĞÜTÜLMÜŞ LASTİĞİN BİYO-DESÜLFÜRİZASYONUNDA KULLANIMLARININ ARAŞTIRILMASI

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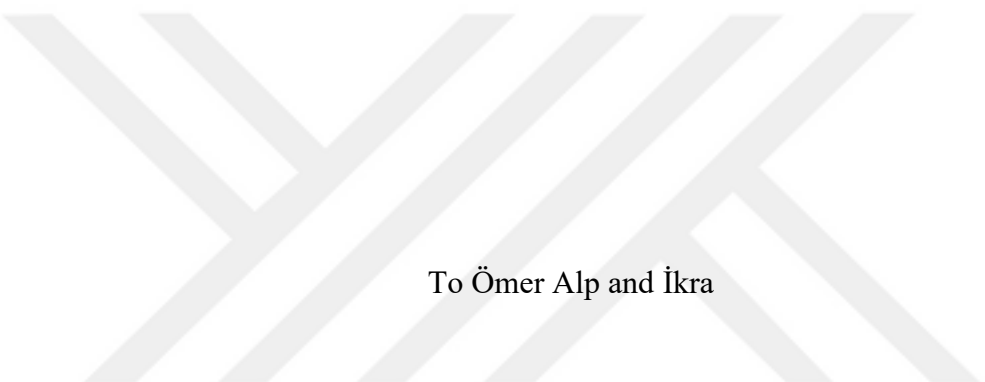
Atık lastiklerin geri dönüşümü konusunda birçok yeni yöntem geliştirilmiştir. Geliştirilen bu yöntemler arasında biyo-desülfürizasyon düşük enerji gereksinimi, kauçuk ana zincirine zarar vermeden seçici olarak S bağlarının koparılması ve çevreye duyarlı bir uygulama olması ile dikkat çekmektedir. Bu tez çalışmasının amacı öğütülmüş araç lastiğın (ÖAL) biyo-desülfürizasyonu için kükürt bazlı ototrofik nitrat gidericilerin kullanımının araştırılmasıdır.

Çalışmanın ilk bölümünde üç farklı arıtma tesisinden alınan aşı çamurları kullanılarak kükürt bazlı ototrofik nitrat gidericiler zenginleştirilmiştir. Bu işlem için kükürt kaynağı olarak elementel kükürt ve tiyosülfat seçilmiştir. Zenginleştirme reaktörleri (ZR), ZR1, ZR2, ZR3 ve ZR4 için ortalama nitrat giderim verimleri sırasıyla %96,0, % 96,9, %97,6 ve %87,2 olarak gerçekleşmiştir. S^0 ile beslenen tek zenginleştirme reaktörü olan ZR4'ün şok beslemelerine karşı daha hassas olduğu ve nitrat giderim veriminin düşük olduğu görülmüştür.

Çalışmanın ikinci bölümünde GTR'nin biyo-desülfürizasyonu, sırasıyla ZR1'den ZR4'e elde edilen ZK1, ZK2, ZK3 ve ZK4 isimli zenginleştirilmiş ototrofik nitrat gidericiler kullanılarak araştırılmıştır. Böylece elektron vericiye ek olarak aşı çamuru türünün etkileri de araştırılmıştır. Biyo-desülfürizasyon göstergesi olarak

sülfat konsantrasyonunda bir artış kullanılmıştır. Sonuçlar, GTR yoluyla abiyotik nitrat gideriminin önemli bir proses olduğunu ve GTR'nin çinko oksit içeriğinin, literatürde de belirtildiği gibi, biyo-desülfürizasyon yoluyla üretilen sülfat ile reaksiyona girebileceğini ortaya koymuştur. Biyo-desülfürizasyon, olası çinko sülfat oluşumu dikkate alınmadığında, yalnızca Deri Organiza Sanayi Bölgesi Atıksu Arıtma Tesisinin biyolojik nütriyent giderme ünitesinden alınan çamurla zenginleştirilen, ZK3 ile % 3,9 oranında elde edilmiştir. Olası çinko sülfat oluşumu göz önüne alındığında, ZK2 (anaerobik çürütücülerden zenginleştirilmiş) ve ZK3 ile biyo-desülfürizasyon, literatür verileriyle karşılaştırılabilir olarak, sırasıyla, % 3,6 ve % 8,2 olarak hesaplanmıştır. Muhtemel çinko sülfat oluşumunun dikkate alınmaması kükürt giderim oranının olduğundan daha düşük hesaplanmasına neden olabilir. Elektron vericinin biyo-desülfürizasyona etkisi, ZK1 ($S_2O_3^{2-}$ beslemeli) ve ZK4 (S^0 beslemeli) kültürleri ile biyo-desülfürizasyon sağlanamadığı için tespit edilememiştir. Aşırı nitrat beslendiğinde, ototrofik nitrat gidericiler ile ÖAL'nin biyo-desülfürizasyonunun mümkün olduğu sonucuna varılabilir.

Anahtar Kelimeler: Biyo-desülfürizasyon, Mikrobiyal desülfürizasyon, Ototrofik Denitrifikasyon, Öğütülmüş Araç Lastiği (ÖAL), Kükürt



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LIST OF ABBREVIATIONS

ABBREVIATIONS

Ar	Argon Gas
BNR	Biological Nutrient Removal
CO	Carbon Monoxide Gas
CO ₂	Carbon Dioxide Gas
DBT	Dibenzothiophene
EC	Enriched Culture
ELT	End of Life Tire
ER	Enrichment Reactor
EU	European Union
EU-28	The 28 European Union Countries
FeSO ₄ .7H ₂ O	Ferrous Sulphate Heptahydrate
FTIR	Fourier Transform Infrared Spectroscopy
GTR	Ground Tire Rubber
G-IR	Ground Isopren Rubber
G-NR	Ground Natural Rubber
G-SBR	Ground Styrene-Butadiene Rubber
HRT	Hydraulic Retention Time
H ⁺	Proton
HCO ₃ ⁻	Bicarbonate

H ₂	Hydrogen Gas
H ₂ O _(g)	Water Vapor Gas
IC	Ion Chromatograph
IR	Isoprene Rubber
KNO ₃	Potassium Nitrate
K ₂ HPO ₄	Dipotassium Hydrogen Phosphate
LOD	Limit of Detection
LOQ	Limit of Quantification
Mpa	Mega Pascal
NaHCO ₃	Sodium Bicarbonate
Na ₂ S ₂ O ₃ .5H ₂ O	Sodium Thiosulfate Pentahydrate
NH ₃ -N	Ammonia nitrogen
NH ₄	Ammonium
NH ₄ Cl	Ammonium Chloride
NO ₂ ⁻	Nitrite
NO ₃ ⁻	Nitrate
NR	Natural rubber
N ₂	Nitrogen Gas
S	Sulfur
SBR	Styrene Butadiene Rubber
sCOD	Soluble Chemical Oxygen Demand

SEM-EDS	Scanning Electron Microscopy with Energy Dispersive Spectroscopy
S-NR	Natural Rubber sheets
S/I	Substrate to inoculum ratio
S/N	Sulfur to Nitrogen Molar Ratio
S^0	Elemental Sulfur
S^{2-}	Sulfide
SO_3^{2-}	Sulfite
SO_4^{2-}	Sulfate
$S_2O_3^{2-}$	Thiosulfate
$S_4O_6^{2-}$	Tetrathionate
TOC	Total Organic Carbon
TS	Total Solids
USA	United States of America
VS	Volatile Solids
v/v	Volume by volume
WLR	Waste Latex Rubber
WWTP	Wastewater Treatment Plant
Z_{SO_4}	Zinc Sulfate

CHAPTER 1

INTRODUCTION

Natural rubber (NR) has been used in different applications for hundreds of years. Although NR was used in a wider geography after the discovery by the European explorers, usage areas remained limited (Morawetz, 2000; Myhre and MacKillop, 2002).

After the invention of vulcanization by Charles Goodyear in 1839, the rubber started to be used in different application areas and its consumption significantly increased. The total amount of rubber consumed increased from 25 tons in 1830, to 6000 tons in 1860 (Morawetz, 2000). The development of the rubber industry and the consumption of rubber increased further with the discovery of the pneumatic wheel. 28.4 million tons of rubber were consumed in 2017 (Malaysian Rubber Board, 2018). Approximately 70% of the rubber produced is consumed in tire production (Asaro et al., 2018). Therefore, waste tires constitute the vast majority of rubber waste problem, which was 25.6 million tons globally, in 2015 (WBCSD, 2018). With the increasing production, the need for raw materials and the number of end of life tires (ELTs) discarded also increase. Increasing ELT amounts have made it necessary to take new measures in the field of waste management.

According to the measures taken, landfilling of discarded tires was banned in the EU countries, with the European Commission Directive published in 1999 (European Council, 1999). This situation has accelerated the researches with respect to the management of waste tires. Total amount of ELT arised in Turkey increased from 225000 tons in 2012 to 257000 tons in 2018. On the other hand, the total amount of ELT recovered at the same time period also increased from 58 % to 70 %, respectively. The major progress among the waste management strategies

was observed in the material recovery option. Material recovery from ELT was increased from 43 % in 2012 to 52 % in 2018 (ETRMA, 2019).

Material recovery option for tires is crucial because tires may contain up to 60 % of fossil fuel originated raw materials (Rowhani and Rainey, 2016). In order to save our limited fossil feedstock in an environmentally sustainable way, recycling of rubber and its use in newly prepared rubber compounds will be the most efficient option (Adhikari et al., 2000).

Recently, the devulcanization process takes attention among other material recovery options. Devulcanization is an environmentally friendly process that enables selective breakage of sulfidic (mono-, di-, poly-) crosslinks in rubber. Due to its selective breakage of sulfidic crosslinks, it is possible to reuse (revulcanize) the devulcanized rubbers as obtained or to mix with other compounds. Reuse of devulcanized rubber will lower the cost, as well as the required fossil feedstock amounts (Asaro et al., 2018). There are several devulcanization processes developed so far, these are chemical devulcanization, microwave devulcanization, ultrasonic devulcanization, thermo-mechanical devulcanization, bio-devulcanization and devulcanization in supercritical carbon dioxide environment. Due to the very stable sulfur crosslinks, high amounts of energy is required for devulcanization. Most of the devulcanization methods developed are harsh and they can cause main chain scissions in addition to the sulfur crosslinks. This situation may lower the properties of newly prepared rubber compounds, which is undesired (Allan, 2018). Due to the higher operational costs and unselective operations, the bio-devulcanization method takes attention.

The bio-devulcanization method is the selective breakage of sulfidic (mono-, di-, poly-) crosslinks in vulcanized rubber with the help of microorganisms. In this thesis study, the term ‘desulfurization’ mainly ‘bio-desulfurization’ would be used to define the devulcanization through sulfur removal from GTR. This process takes place on the surface of the rubber by oxidation or reduction of sulfidic crosslinks (Romine and Romine, 1998; Bredberg et al., 2001). Although the first proof of bio-

desulfurization of rubber, that is removal of the sulfur from the rubber, was reported in 1945 (Thaysen et al., 1945), studies on literature had extensively started in the 1990s.

The species of *Thiobacillus* bacteria have been widely studied in the literature (Christiansson et al., 1998, Romine and Romine, 1998, Jiang et al., 2010; Li et al., 2012a). The mixed culture of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* was reported as the cultures with the highest bio-desulfurization efficiency than pure cultures (Romine and Romine, 1998). In addition, the mixed culture of *Gordonia* sp and *Sphingomonas* sp. showed a higher desulfurization amount than their pure cultures (Cui et al., 2016). However, except for a few, most of the conducted studies were performed with pure cultures. In addition to that, to our knowledge, the majority of the ground tire rubber (GTR) desulphurization studies were performed under aerobic conditions. Only one study (Bredberg et al., 2001) has been conducted under anaerobic conditions so far. Bredberg et al. (2001) studied the anaerobic desulfurization of ground rubber with the thermophilic archaeon *Pyrococcus furiosus*. Bio-desulfurization was reported with the increase in hydrogen sulfide content in the head space of the reactors.

The contamination of surface and groundwater problem associated with the nitrogen-containing compounds, mainly nitrate and nitrite, create serious environmental problems (Zhou et al., 2011). Therefore, nitrogen-containing compounds must be removed from the water. Recently the autotrophic denitrification process, used for the removal of nitrate and nitrite, takes attention due to its advantages compared to heterotrophic denitrification. For example, additional carbon source is not required in the autotrophic denitrification process, which lowers the operational cost and results in less sludge production. There are two main autotrophic denitrification processes; these are sulfur- and hydrogen-based autotrophic denitrification. Sulfur-driven autotrophic denitrification receives more attention due to its ease of handling and lower cost than hydrogen (Zhang and Lampe, 1999). The autotrophic denitrification process can be conducted by using the reduced inorganic sulfur compounds as a sulfur source. These sulfur sources

include thiosulfate ($\text{S}_2\text{O}_3^{2-}$), elemental sulfur (S^0), sulfide (S^{2-}) (Batchelor and Lawrence, 1978; Oh et al., 2000; Park and Yoo, 2009). Accordingly, the use of S-driven autotrophic denitrification for removal of S from GTR, that is, bio-desulfurization of GTR seems promising due to the advantages abovementioned and potential GTR recovery for rubber material production.

The bio-desulfurization method has been investigated to solve the waste rubber problem in an environmentally friendly manner. However, to our knowledge, there is not a large scale feasible application established yet. In order to make progress in the bio-desulfurization method, different applications needs to be investigated. Although the mixed cultures were seen as more efficient than pure cultures, most of the studies in the literature were conducted by using pure cultures. Besides, different conditions and process approaches such as autotrophic denitrification, need to be investigated in order to better understand the limits and the efficiency of bio-desulfurization.

Considering the above mentioned points, the objective of this thesis study was to enrich the autotrophic denitrifiers and investigate their use in bio-desulfurization of GTR under anoxic conditions. Specific objectives are listed below:

- To enrich the autotrophic denitrifiers using different seed sludge types under anoxic conditions.
- To compare the denitrification performances of different enrichment reactors
- To investigate the effect of particle size of GTR on bio-desulfurization with autotrophic denitrifiers, under anoxic conditions
- To investigate the effect of seed sludge and electron donor on bio-desulfurization of GTR with autotrophic denitrifiers, under anoxic conditions

CHAPTER 2

LITERATURE REVIEW

2.1 Rubber

2.1.1 Brief History

The rubber comes from the word Ca-hu-chu, which means crying tree, in the language used by the South American natives. When European explorers first set foot in South America, the natives of the continent had known and used this material for centuries (Ahmed et al., 1996; Holst et al., 1998). After the discovery by the European explorers, the use of rubber, which was seen in a wider geography for different purposes continued until the early 1800s. The fact that the rubber softens and becomes sticky in warm weather and turns to a rigid material in the winter, resulted in its limited usage in that period. These problems were solved in 1839 with the discovery of the vulcanization system by Charles Goodyear (Morawetz, 2000).

2.1.2 Rubber Types

Rubber can be divided into two groups, natural and synthetic rubber. Natural rubber is obtained from plants, and synthetic rubbers are obtained from petroleum derivatives.

Although natural rubber can be obtained from more than 200 plants, *Hevea Brasiliensis* is the only tree of commercial importance. The molecular weight of natural rubber is in the range of 200,000-500,000 g/mole, and its chemical formula

is cis-1,4-polyisoprene, given in Figure 2.1. It also contains traces of protein, sugar, and fatty acids (Simpson, 2002).

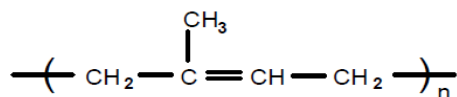


Figure 2.1 Chemical structure of natural rubber (Simpson, 2002)

The developments in the field of synthetic rubber gained momentum after Japan's control of the rubber plantations with the invasion of Southeast Asian countries during World War II. In this period, America increased its annual synthetic rubber production from 3760 tons in 1942 to 681056 tons in 1944. This period also led to the acceleration of research in the field of synthetic rubber (Morton, 1981).

2.1.3 Vulcanization

Vulcanization process creates mono-, di- and poly-sulfidic crosslinks between rubber chains and improves the mechanical properties of the rubber material (Imbernon and Norvez, 2016). As a result of the studies carried out in the following years, crosslinking was achieved by using peroxide. Peroxide is one of the most used crosslinking agents after sulfur (Akiba and Hashim, 1997). Illustration of the sulfur crosslinking mechanism is given in Figure 2.2.

With the discovery of vulcanization, rubber, which has limited use in practice has started to become an essential product of modern life. The importance of vulcanization can be understood by looking at the change in the consumption of rubber over the years. Global consumption of rubber increased from 25 tons in 1830 to 6000 tons in 1860. The development of the rubber industry and the consumption of rubber increased further with the discovery of the pneumatic wheel and its use in the automobile industry that Jonh Boyd Dunlop patented in 1888 (Morawetz, 2000).

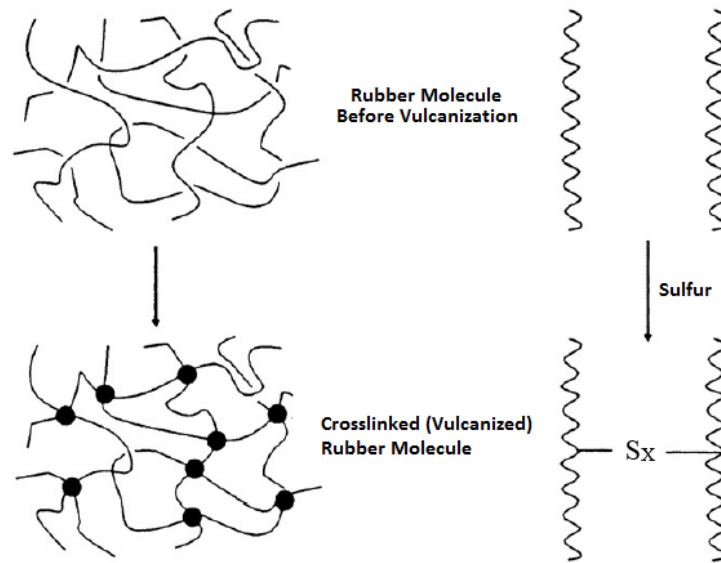


Figure 2.2 Sulfur crosslinking mechanism (Coran, 2005)

2.1.4 Rubber Industry Statistics and Trends

Recently, with the rapid industrialization and especially growth in the automotive industry, rubber consumption is increasing. The total amount of rubber consumed in 2017 was 28.4 million tons; natural rubber and synthetic rubber amount being 13.2 and 15.2 million tons, respectively (Malaysian Rubber Board, 2018). With the increase in consumption, the amount of waste rubber also increases. However, because of their bond structure, it is not possible to reuse vulcanized rubber wastes without pre-treatment or melting like thermoplastics. It also takes a very long time for them to decompose (Holst et al., 1998; Karaağaç et al., 2017). Therefore, the management of waste rubber is of great importance.

When the usage areas of rubber are analyzed, it can be seen that approximately 70% of the rubber produced is consumed in tire production (Asaro et al., 2018). Therefore, waste tires constitute the vast majority of rubber waste, and this occurred as 25.6 million tons globally in 2015 (WBCSD, 2018). Although there are

many methods for the management of waste tires, generally the easiest option, landfilling, is preferred. However, uncontrolled storage of waste tires in storage areas has adverse effects on the environment and human health, such as long-term fires and the growth of infectious diseases caused by animals (Asaro et al., 2018). Landfilling of used discarded tires was banned in the European Union (EU) countries, according to the European Commission Directive published in 1999 (European Council, 1999). This situation has accelerated the researches to investigate other feasible methods to manage waste tires.

2.2 End of Life Tire Management Strategies

According to the EU Waste Framework Directive, waste rubber is a general definition given to all end of life tires and general rubber goods (European Commission, 2018). End of life tires (ELT) are original or retreaded tires that were removed from the vehicle and have completed their useful life, or the discarded tires that appear during production and cannot be used as its original purpose again (Official Gazette No: 26357, date 25.11.2006).

The main methods used in waste tire management can be listed as landfilling, retreading, energy recovery and recycling. Activities on recycling mainly focus on raw material recovery.

2.2.1 Landfilling

Landfilling was used frequently in the management of discarded tires. However, it is not a feasible method because it causes serious problems for the environment and human health, and banned by regulations in many countries. Landfilling does not provide any financial gain, instead it causes several costs such as the transportation of the discarded tire to the landfill and the installation, arrangement and maintenance of the landfill (Adhikari et al., 2000). In addition, the areas where discarded tires are stored carry a risk of fire that cannot be easily controlled and

may last for weeks or even months. For example, the fire that started in Winchester, the USA in 1983 lasted nine months. Many toxic, carcinogenic and mutagenic pollutants spread to the atmosphere that seriously threatens human health as a result of burning tires (Downard et al., 2015). Besides, due to their hollow structure, waste tires create an environment suitable for the reproduction of virus and infectious disease carriers such as insects, mosquitoes and mice (Torretta et al., 2015).

2.2.2 Retread

Retreading is the most efficient method among all tire management methods in terms of sustainable use of raw materials; it provides raw material and energy saving (Amari et al., 1999). The amount of crude oil to be used to produce a new truck tire has been calculated as 83.3 L, while the amount needed to produce a truck tire by retreading is only 26.5 L. It has also been envisaged that the cost of tire retreading will be 30-50% cheaper than producing a new tire (more than 200 plants). In addition, the rubber parts formed during the retreading process can be used for construction, agriculture, etc. All waste can be used in industries or as a composite material without harming the environment and human health (Amari et al., 1999; Myhre and MacKillop, 2002; Isayev, 2013).

According to the current ELT management trend of Turkey, given in Figure 2.3, material recovery is the leading strategy, among the other methods such as unknown, energy recovery. Material recovery shows an increasing trend between 2016 and 2018, and its ratio was increased from 43% in 2017 to 52% in 2018. Besides, the energy recovery percentage is decreased from 22% in 2016 to %18 in 2018 (ERTMA, 2018). The total amount of ELT that was recycled and recovered in Turkey increased from 60% in 2014 to 70% in 2018 (Figure 2.4). Although the total recycled and recovered ELT percentage of the 28 EU countries (EU-28) decreased between these periods, from 97 to 94%, it is significantly higher than that of Turkey (ETRMA, 2019).

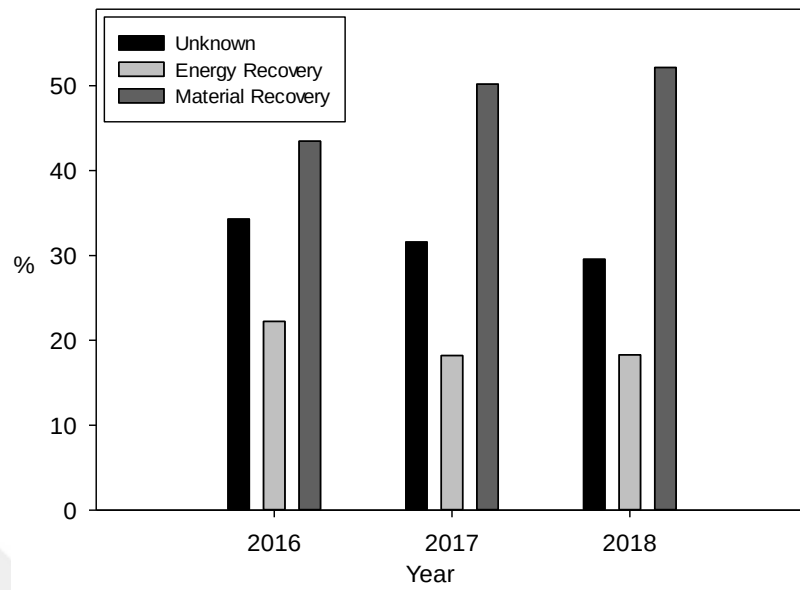


Figure 2.3 Current ELT management strategies in Turkey (ETRMA, 2019)

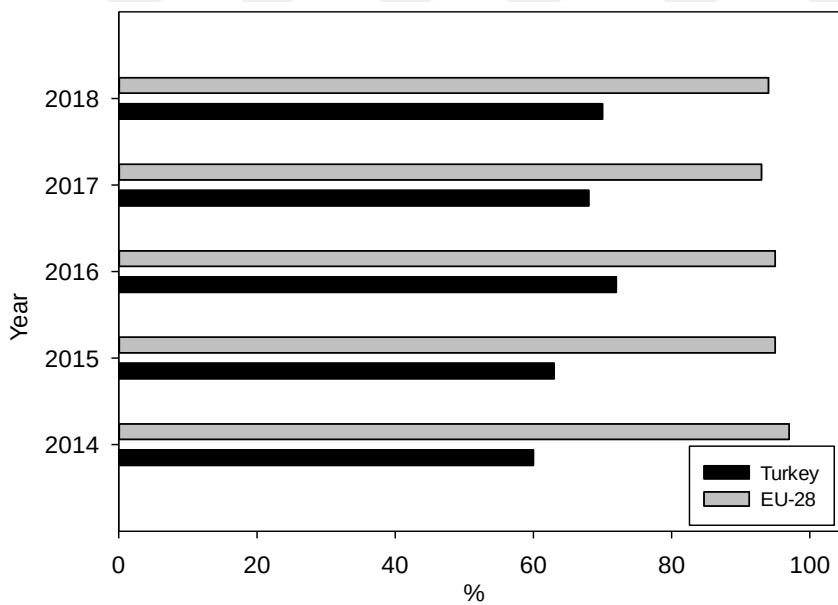


Figure 2.4 Total ELT recovered and recycled in Turkey and in EU-28 (ETRMA, 2019)

2.2.3 Reuse

It is possible to use waste tires directly without any process. They can be used as playground equipment in children's playgrounds, as marine fender, as a crash barrier on highways, as filler in erosion control applications (De et al., 2005). In addition, wave-breaking and artificial reef applications were carried out in the seas (Jang et al., 1998). However, in the 1970s, the artificial reef project, which was carried out using millions of waste tires and was predicted to create additional habitat for fish, caused an environmental disaster. These tires, which have been displaced by tropical storms and hurricanes, have damaged natural coral reefs located near the ocean and adversely affected the ocean wildlife. Today, many studies such as the "Osborne Reef Waste Tire Cleaning Project" are underway to clean these waste tire fields at the bottom of the ocean (Florida DEP, 2016).

2.2.4 Energy Recovery

Waste tires contain about 90% organic matter and their calorific value (32 MJ / kg) is quite high. For this reason, it can be used as an additional fuel, especially in the cement industry, power plants, iron and steel industry. However, as can be seen in Table 2.1, the energy to be obtained as a result of the incineration of the waste tire only meets 1/3 of the energy spent for the production of the tire. It should also be noted that emissions harmful to the environment are released at the end of this process. On the other hand, the energy required to grind the tires corresponds to approximately 2.5% of the energy required for production, and as a result of this process, no emissions polluting the environment occur (Sienkiewicz et al., 2012).

Table 2.1 Energy balances of selected tire applications (Sienkiewicz et al., 2012)

Energy balance for tire applications	Calorific Value (MJ/kg)
Energy required for tire production	87–115
Energy obtained from the combustion of tires	32
Energy required for grinding tires to particle size of < 1.5 mm	1.8–4.3

In Turkey, energy recovery from tires through the incineration process can be performed in licensed facilities in accordance with the regulations (Official Gazette No: 29036, date 20.06.2014). In parallel with the increase in licensed facilities in recent years, there was an increase in the amount of ELT used for energy recovery. 12750, 23250 and 46000 tons of ELT were incinerated in licensed facilities in 2014, 2015 and 2016, respectively (Figure 2.3) (ERTMA, 2018).

2.2.5 Recycle

In accordance with the Waste Management Regulation, 'recycling' is any process in which waste is processed and converted into products or materials for its main purpose or other purposes, while 'recovery' is defined as making the wastes ready for use for a beneficial purpose, in the market or a facility (Official Gazette No: 29314, date 04.02.2015). In this study, rubber recycling is defined as all processes where the discarded rubber is converted into a reusable condition in which it can be used to produce a new product or service (Sutanto et al., 2006). Recycling applications are listed under two main headings: grinding and raw material recovery.

Grinding

The vast majority of recycling processes start by grinding the waste rubber into granules or fine powder. Ground rubber can be used as inputs for other recycling processes or can be used directly. The cost of the mixture is reduced by decreasing the raw material usage. This could be done by adding the ground rubber into newly

prepared polymeric mixtures in certain proportions. Due to their damping characteristic, they can be mixed with concrete and asphalt as filling materials or can be used as acoustic barriers on highways. The use of ground rubber as safe ground material for playgrounds and running tracks is one of the widely used methods today (Imbernon and Norvez, 2016). Besides, it is stated in the literature that ground rubber can be used in green roof systems as a beneficial drainage layer (Pérez et al., 2012).

In order to granulate the waste tire; mainly ambient grinding, cryogenic grinding, and water jet grinding methods are used. Ambient grinding is the most preferred and the least costly method, but it leads to the production of large and irregular particles compared to the other methods. During the ambient grinding process, heat increases, which can cause the degradation of the rubber or fire. A cooling solution, such as water, is used to remove the heat generated (Dijkhuis, 2008; Isayev, 2013). In the cryogenic grinding process, the waste tire is cooled below the glass transition temperature with the help of liquid nitrogen. The fragile material obtained is then powdered by a hammer mill. With this method, properly ground milled rubber with smaller particle size can be produced and, the fiber contained in the tire can be separated. However, the high cost of liquid nitrogen used for cooling is a disadvantage for this method (Isayev, 2013). In the water jet grinding method, it is possible to convert the waste tire in to pulverized rubber by using ultra high-pressure water without any pre-treatment. The waste tire is pulverized by spraying the waste tire tread surface with high-speed water jets. Despite the high quality pulverized rubber, this method is costly and complicated (Zefeng et al., 2018).

Material Recovery

It is possible to obtain high-quality raw materials from rubber with the help of thermal, chemical, or biological processes. Methods used for raw material recovery can be listed as pyrolysis, gasification, liquefaction and devulcanization (Myhre et al., 2012). Thermal decomposition of rubber by heating (400–800°C) in an oxygen-free environment is called pyrolysis. Products obtained by pyrolysis are

hydrocarbons, carbon black, pyrolytic oil and steel (Reschner, 2008). Gasification that produces the gaseous product containing mainly H_2 and CO is a thermal transformation of organic matter by partial oxidation. Besides H_2 and CO, small amounts of CH_4 , CO_2 , N_2 , $H_2O_{(g)}$, and tar is produced (Lettieri and Al-Salem, 2011). In the tire liquefaction method, the tire is dissolved in a hot solvent, and a mixture containing carbon black and oil is obtained (Myongsook, 2010). The last raw material recovery method, which is devulcanization, is given in detail in Section 2.3.

2.3 Devulcanization Mechanism and Technologies

2.3.1 Devulcanization Mechanism

Devulcanization is an environmentally friendly process that enables selective breakage of sulfidic (mono-, di-, poly-) crosslinks in rubber. Desulfurization, another term used for the recovery of waste tires, is used for situations where sulfur is removed from rubber. Thus, it is frequently called devulcanization. In this thesis study, the term ‘desulfurization mainly bio-desulfurization’ would be used to define the sulfur removal from GTR. Main chain scission and degradation of the rubber are not desired in devulcanization applications. This situation may lower the mechanical properties of the mixture that would be prepared by devulcanized rubber (Asaro et al., 2018). Therefore, in the devulcanization process, it is necessary to pay attention to S-S, C-S, C-S_x, and C-C bond energies in the rubber structure. Different bonds in the rubber structure and their energy values are given in Figure 2.5 (Holst et al., 1998; Rajan, 2005).

Devulcanization process can be grouped as chemical devulcanization, microwave devulcanization, ultrasonic devulcanization, thermo-mechanical devulcanization, bio-devulcanization and devulcanization in a supercritical carbon dioxide environment. These processes are briefly mentioned below, while bio-devulcanization is given in Section 2.4.

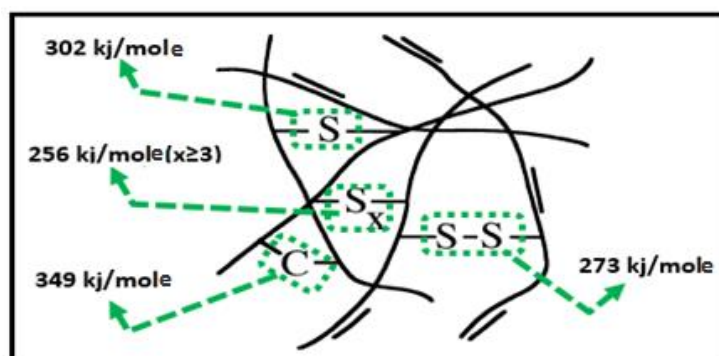


Figure 2.5 Network structure and bond energies of vulcanized rubber containing mono-, di- and poly- sulfidic crosslinks and carbon-carbon bonds (Holst et al., 1998; Rajan, 2005)

2.3.2 Chemical Devulcanization

This method consists of mixing granulated vulcanized rubber and devulcanization chemicals by heating in pressure and temperature-controlled system. After the time required for devulcanization is completed, the mixture is removed and rinsed, filtered, and dried to prevent any unwanted chemicals in it. The major disadvantage of this method is that chemicals used contain toxicity (Asaro et al., 2018; Lloyd 2004).

2.3.3 Microwave Devulcanization

The microwave devulcanization method is the process of breaking the sulfidic crosslinks by providing microwave energy to the vulcanized rubber in a controlled manner. The temperature of the rubber increases with the molecular motion created by microwave energy and this increase causes crosslink breakage. In order to absorb the required energy, the rubber needs to be polar enough. The amount of energy delivered is crucial in order not to break the C-C bonds in the rubber main chain (Figure 2.5). Therefore, post-treatment, that is cooling is required to prevent

rapid temperature rise (Myhre et al., 2012). The disadvantages of this method are its high cost and high sensitivity.

2.3.4 Ultrasound Devulcanization

Certain levels of ultrasonic waves can break the crosslinks in vulcanized rubber along with pressure and heat. For this purpose, rubber is feed to an extruder connected to an ultrasound source. Rubber crosslinks overstressed through extruder are exposed to ultrasonic waves at the extruder outlet. Strong ultrasonic waves generate acoustic cavitation in the rubber bonds and cause devulcanization by breaking the crosslink (Isayev et al., 1996; Edwards, 2016).

2.3.5 Thermo-mechanical Devulcanization

Thermo-mechanical devulcanization is based on the plasticization and heating of the waste rubber with the help of shear stresses. The applied shear stress ensures enough heat to break the bonds. Granulated waste rubber passes through the extruder, and the process continues until it becomes plasticized at a certain temperature, and devulcanization is then achieved. However, shear stresses in the system also cause the main chain scissions, results in a decrease in the mechanical properties of the recycled rubber, compared to the original, and some additives should be used to prevent this (Myhre et al., 2012).

2.3.6 Devulcanization in Supercritical CO₂ Environment

This method appears as an innovative, promising and clean technology. The most important reasons using supercritical carbon dioxide can be listed as being non-toxic and non-flammable and not reacting with chemicals. In addition, the critical temperature ($\sim 31.1^{\circ}\text{C}$) and low pressure ($\sim 7.38\text{ Mpa}$) are also crucial factors. In supercritical form, carbon dioxide swells the waste rubber and allows the

penetration of devulcanization agents, such as thiophenol-n-butylamine combination and diphenyl disulfide. It also allows the sulfidic crosslinks to be stretched and thus can be easily broken (Asaro et al., 2018).

2.4 Bio-devulcanization

The bio-devulcanization method is the selective breakage of sulfidic (mono-, di-, poly-) crosslinks in vulcanized rubber with the help of microorganisms. This process, which is carried out without damaging the main chain, takes place on the surface of the rubber by oxidation or reduction of sulfidic crosslinks (Romine and Romine, 1998; Bredberg et al., 2001).

Biological removal of sulfur, which is defined as bio-desulfurization, from rubber was first reported in 1945. According to the problems experienced by firefighters in rubber-coated hoses, it was observed that sulfur-oxidizing *Thiobacillus thiooxidans* bacteria oxidize the sulfur in the rubber and cause sulfuric acid formation (Thaysen et al., 1945). However, this finding has not been used for the bio-devulcanization of rubber for many years. When the studies summarized in Table 2.2 are analysed, it is seen that the first study after 1945 on this subject was performed in 1990 (Torma and Raghavan, 1990). The vast majority of the studies conducted in the last decade shows that the interest in this subject is increasing. Besides, to our knowledge, there is not an industrial scale application yet.

According to the literature, the bio-desulfurization ratio of the samples was measured as total or superficial. Total change in the sulfur content was measured by sulfate concentration or elemental analyses and varied between 0.035 and 21.9% (Torma and Raghavan 1990; Hu et al., 2014). On the other hand, superficial analyses were conducted with Scanning Electron Microscopy with Energy Dispersive Spectroscopy (SEM-EDS) and varied between 3% and 67% (Romine and Romine, 1998; Hu et al., 2014).

The reason for this wide range between 0.035-21.9% for total, 3-67% for superficial bio-desulfurization, may be primarily due to the type of microorganism used, as well as different environmental conditions such as rubber type, temperature, pH, time, detoxification method. To our knowledge, within the scope of the studies performed to date, the highest bio-desulfurization ratio (67%) on the surface of ground tire rubber (GTR) has been observed after 20 days, where the temperature is 30°C and the pH is 6.5, using the *Sphingomonas sp.* (Hu et al., 2014). When other studies are observed, *Alicyclobacillus sp.*, which has a 62.5% bio-desulfurization ratio, attracts attention together with *Sphingomonas sp.* (Yao et al., 2013; Yao et al., 2014). However, it should be noted that these results represent approximately 4µm outermost of the rubber particle. Therefore total bio-desulfurization ratio will change according to the particle size of the GTR.

Table 2.2 Summary of the bio-desulfurization performances and operational conditions of previous studies

Micoorganism	GTR Type ^a	Temperature (°C)	pH	Time (days)	Desulfurization Ratio (%)	Medium Control (%)	S-Bond (Crosslink) Scission	C-Bond (Mainchain) Scission	Crosslink Density	Reference
<i>Sulfolobus Acidocaldarius</i>	GTR	65-70	2.5-3.0	7	~13 ^c	-	-	-	-	Romine and Romine (1998)
<i>Thiobacillus ferrooxidans</i>	GTR	65-70	2.5-3.0	7	~6 ^c	-	-	-	-	Romine and Romine (1998)
Mixed <i>Thiobacillus</i> Culture	GTR	65-70	2.5-3.0	7	~11 ^c	-	-	-	-	Romine and Romine (1998)
<i>Thiobacillus thiooxidans</i>	GTR	65-70	2.5-3.0	7	~3 ^c	-	-	-	-	Romine and Romine (1998)
TH2 Lund (<i>thermophilic archaea</i>)	GTR	N.A.	2.0	20	1.6 ^c	0.6	-	-	-	Chritiansson et al. (1998)
<i>Thiobacillus ferrooxidans</i>	GTR	30	2.0	20	7.8 ^c	2.7	-	-	-	Chritiansson et al. (1998)
<i>Ceriporiopsis subVermispora</i>	S-NR	N.A.	N.A.	200	28.9 ^c	7	✓ ^f	-	✓	Sato et al. (2004)
<i>Thiobacillus ferrooxidans</i>	G-NR ^b	30	2.5	20	16 ^c	-	✓	-	-	Jiang et al. (2010)
<i>Thiomonas perometabolis</i>	GTR	30		30	33.8 ^c	-	-	-		Kim and Park (1999)

Table 2.2 Summary of the bio-desulfurization performances and operational conditions of previous studies (continued)

<i>Sphingomonas species strain</i>	WLR ^b	30	7.0	10	5.7 ^c	-	✓	-	✓	Yao et al. (2014)
Mixed <i>Thiobacillus</i> Culture	G-SBR	35	N.A.	6	0.035 ^c	0.0033	-	-	-	Torma and Raghavan(1990)
<i>Sphingomonas species strain</i>	WLR ^b	30	7.0	10	62.5 ^d	-	✓	-	✓	Yao et al. (2014)
<i>Acidithiobacillus ferrooxidans</i> YT-1	GTR ^b	30	2.5	20	19.9 ^d	-	-	-	-	Li et al. (2013)
<i>Thiobacillus ferrooxidans</i>	GTR ^b	30	2.5	30	-	-	✓	✓		Li et al. (2011)
<i>Alicyclobacillus sp.</i>	WLR ^b	50	2.5	10	62.5 ^d	-	✓	-	✓	Yao et al. (2013)
<i>Thiobacillus sp. Strain</i>	GTR ^b	30	7.0	20	-	-	✓	-	✓	Li et al. (2012a)
<i>Sphingomonas sp.</i>	GTR ^b	30	6.5	20	67 ^d	-	✓	-	✓	Hu et al. (2014)
Mixed Culture (<i>Gordonia sp</i> + <i>Sphingomonas sp.</i>)	GTR ^b	30	6.5-7.0	10	32.4 ^d	-	✓	-	✓	Cui et al. (2016)
<i>Gordonia sp.</i>	GTR ^b	30	7.0	10	19.4 ^d	-	✓	-	✓	Cui et al. (2016)
<i>Sphingomonas sp.</i>	GTR ^b	30	6.5	10	24.7 ^d	-	✓	-	✓	Cui et al. (2016)
<i>Gordonia amicalisa</i>	G-SBR ^b	30	N.A.	20	22.4 ^d	-	✓	-	✓	Hu et al. (2016)
<i>Gordonia amicalisa</i>	G-IR ^b	30	N.A.	20	0	-	-	✓	✓	Hu et al. (2016)
<i>Sphingomonas sp.</i>	G-SBR ^b	30	6.0	10	-	-	✓	✓	✓	Jiang et al. (2011)
<i>Pyrococcus furiosus</i> ^e	GTR ^b	N.A.	N.A.	10	-	-	-	-		Bredberg et al. (2001)

^a GTR: Ground tire rubber, G-SBR: Ground Styrene-Butadiene Rubber, WLR: Waste Latex Rubber, G-NR: Ground Natural Rubber, S-NR: Natural Rubber sheets, G-IR: Ground Isopren Rubber; ^b Ethanol leached, ^c Total desulfurization,

^d Superficial desulfurization, ^e Operated under anaerobic conditions (*Pyrococcus furiosus*), ^f: decrease

The highest total desulfurization ratio (21.9 %) was observed after 200 days, where the cultivation of Natural Rubber (NR) sheets was carried out statically at 70% humidity, using *Ceriporiopsis subVermispora* (Sato et al., 2004). Jiang et al. (2010) reported 16% decrease in the sulfur content of pulverized NR by 20 days of treatment at 30°C, using *T. ferrooxidans*. However, control reactors were not conducted and, thus, their effect on sulfate concentration i.e. potential abiotic reactions was not included in the results of this study.

The studies presented in Table 2.2 generally were carried out in batch reactors and under aerobic conditions, and effective results were obtained in 6-30 days (Yao et al., 2013; Hu et al., 2014; Yao et al., 2014). In the study of desulfurization of IR (Isoprene Rubber) and Styrene Butadiene Rubber (SBR) using *Gordonia amicalisa* microorganism, the surface desulfurization rate was 22.9% for SBR, but no desulfurization observed for IR. This situation is explained by the fact that *Gordonia amicalisa* microorganism has different degradation mechanisms on SBR and IR crosslinks. While *Gordonia amicalisa* breaks the carbon bonds on the IR surface, does not affect the carbon bonds on the surface of SBR, but caused the crosslinks to break down in the latter (Hu et al., 2016).

As mentioned above, the vast majority of the research in literature is carried out in an aerobic environment by sulfur-oxidizing microorganisms (Table 2.2). The first studies on bio-desulfurization primarily focused on fossil resources such as coal and crude oil, and in these studies, Dibenzothiophene (DBT) was used as a model. It was understood that sulfur connected to DBT was transformed into sulfate and removed from the environment by bio-desulfurization. The conversion of sulfur to sulfate was explained by Krawiec (1990) with sulfoxide/sulfone/sulfonate/sulfate transformation ('4S' mechanism). The DBT and '4S' mechanisms were also used as models in the bio-desulfurization studies of rubber (Romine and Snowden-Swan, 1993; Romine and Romine 1998). '4S' mechanism for DBT is given in Figure 2.6.

Bio-desulfurization of rubber can also perform in the context of reducing sulfur, under anaerobic conditions and, according to our knowledge, there is only one

study conducted yet (Bredberg et al., 2001). In this study, an anaerobic sulfur-reducing archaeon, *Pyrococcus furiosus*, was used, and an improvement in mechanical properties in the devulcanized rubber was observed after 10 days (Bredberg et al., 2001). The bio-devulcanization was defined by the increase in the hydrogen sulfide (H₂S) concentration in the medium (2.5%), and sulfur removal (total or on the surface) was not measured. Because of that, it is not possible to compare the efficiency of this study with the other conducted studies.

Bio-devulcanization studies show that microorganisms can penetrate 2-6 µm outermost of the GTR (Romine and Romine, 1998; Hu et al., 2014; Cui et al., 2016; Hu et al., 2016). The fact that bio-devulcanization takes place on the surface has also led to studies aiming to facilitate the attachment of microorganisms to the rubber surface. In these studies, the positive effect of surfactants on bio-devulcanization was achieved by increasing the attachment capacity of hydrophilic bacteria to the lipophilic GTR (Yao et al., 2013; Hu et al., 2014; Yao et al., 2014).

In order to increase the effect of microorganisms, the rubber must be ground and brought to smaller particle sizes before bio-devulcanization. In a study conducted for the optimization of the bio-devulcanization condition, using the Taguchi method, the size of the ground rubber was revealed as the most crucial factor that expresses the necessity of grinding as a pretreatment (Ghavipanjeh et al., 2018). However, the high cost of the grinding process prevents the grinding of waste rubber in very small sizes. It is seen that the size of the ground rubber used in the majority of bio-devulcanization studies ranged between 74 to 500 µm (Chritiansson et al., 1998; Romine and Romine, 1998; Kim and Park, 1999; Bredberg et al., 2001; Jiang et al., 2010; Jiang et al., 2011; Li et al., 2012a; Li et al., 2012b; Yao et al., 2013; Hu et al., 2014; Tatangelo et al., 2016; Allan, 2018).

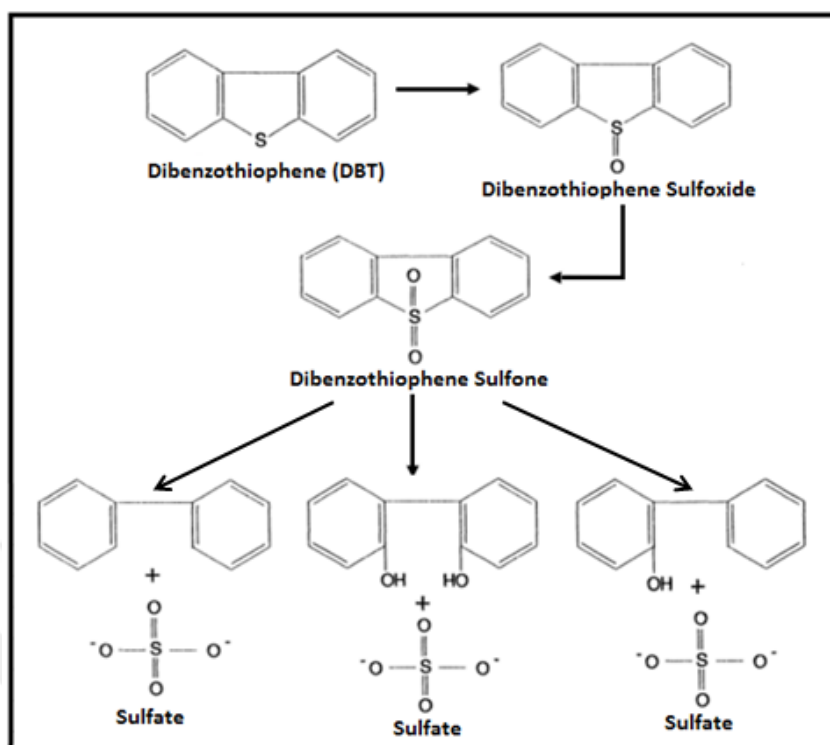


Figure 2.6 Oxidative pathway of DBT ('4S' Mechanism) (Romine and Romine, 1998)

Microorganisms used for bio-devulcanization are sensitive to additives used in rubber and different types of microorganisms attached to ground rubber. By removing them from the surface that is by the detoxification process, inhibition of microorganisms is prevented. In most of the studies, detoxification is performed by keeping the ground rubber in a solution containing 75% ethanol by volume for 24-72 hours (Jiang et al., 2010; Jiang et al., 2011; Li et al., 2011; Li et al., 2012a; Li et al., 2012b; Li et al., 2013; Hu et al., 2014; Yao et al., 2014; Cui et al., 2016; Hu et al., 2016). Besides ethanol and other non-environmentally friendly chemicals used for detoxification purposes, fungal detoxification of waste rubber was also researched. Although positive results have been obtained in this study (Bredberg et al., 2002), where 15 different types of wood-rotting fungi were used, fungal detoxification has not been widely applied in bio-devulcanization yet.

In bio-devulcanization studies, it is crucial to select microorganisms that will allow the sulfur crosslinks to be selectively broken, without damaging the main chain. When the conducted studies are examined, the main chain scission in C-C and C=C bonds was observed when *Thiobacillus ferrooxidans*, *Gordonia amicalisa*, and *Sphingomonas sp.* were used as microorganism (Jiang et al., 2011; Li et al., 2011; Li et al., 2012b; Hu et al., 2016). Despite the decrease in the sulfidic crosslinks, it is undesirable to damage the main chain or to degrade the rubber. Therefore, the results of these above mentioned studies were defined as undesirable for a recovery application.

The medium content used in bio-devulcanization studies is a crucial factor for microorganisms. According to the results of the study conducted to see the effect of Fe^{2+} on bio-devulcanization, with *Acidithiobacillus ferrooxidans* YT-1, when Fe^{2+} concentration increased from 2.78g/L to 11.1g/L the number of the microorganisms increased, and the sulfur content of rubber decreased (Li et al., 2013).

In a study investigating the effect of cultivation method, Yao et al. (2014) reported that half-submerged cultivation is much more beneficial than commonly used submerged cultivation method, due to its lower cost and improved desulfurization effect. In this study, an increase in the amount of waste latex rubber used and higher mechanical properties of the composite material was identified. Besides, lower S-C and S-S bonds on the surface of the desulfurized rubber were reported.

Bredberg et al. (2001) revealed that it is possible to analyze the desulfurization of rubber in 3 different ways: 1) increase in the amount of sulfur-containing product (sulfate, hydrogen sulfide) in the environment, 2) decrease in the amount of sulfur on the rubber surface, and 3) the change in the mechanical properties of the material containing bio-desulfurized rubber. Besides, Sato et al. (2004) proved that desulfurization was accomplished by microbial cleavage, which can be determined by changes in S-C, S-S, S-O bonds, and changes in cis-mono, trans-mono, cis-poly, and trans-polysulfide bonds.

2.5 Characterization of Rubber

Different characterization methods are available to evaluate the efficiency of the devulcanization process and to define the important characteristics of rubber. This section includes a review of the methods and analyses used in this thesis study. These are soluble (sol) fraction, crosslink density, elemental analysis, and FTIR analysis.

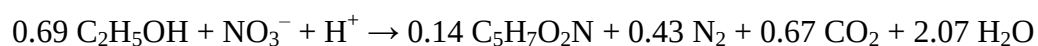
Sol fraction is defined as the soluble portion of rubber that can be removed with organic solvents such as acetone and chloroform, the remaining insoluble portion of rubber is called as gel fraction (ASTM D297-15, 2019). There is an inverse relation between crosslink density and sol fraction. With the breakage of sulfidic bonds during the devulcanization process, crosslink density of rubber decreases. On the contrary, sol fraction increases due to the formed free polymers (Charlesby, 1954).

Crosslink density, which is a measure of rubber and sulfur crosslinking, could be determined with different methods. Swelling method is reviewed in this section. Swelling method can be performed following the removal of the soluble portion of rubber (ASTM D297-15, 2019). After removal of the sol fraction, the rubber sample swells in a solvent such as toluene, benzene, and cyclohexane. Crosslink density, calculated with respect to the change in the weight of swollen rubber by Flory-Rehner equation (Flory, 1953; ASTM D6814-02, 2018).

Elemental analysis is beneficial for the determination of the efficiency of the devulcanization process, especially for rubber compounds with higher sulfur content. However, in bio-devulcanization studies, this method is not helpful due to the limited effect of bacteria on the rubber surface.

2.6 Sulfur-Driven Autotrophic Denitrification

Nitrogen-containing compounds, mainly nitrate and nitrite, create serious environmental problems. The main problem is the contamination of surface and groundwater sources, which can cause eutrophication. Besides, nitrate is dangerous for human health (Zhou et al., 2011). Therefore, nitrogen-containing compounds must be removed from the wastewater. There are several physicochemical methods such as ion exchange, reverse osmosis, distillation, and electrodialysis that can be used for the removal of nitrate from water. Although high purity water can be produced through these methods, generated brine and high operational cost are disadvantages of them. The biological removal of nitrate, that is denitrification, which is widely used and well-developed (Tchobanoglous et al., 2003). Heterotrophic denitrification can be used as an efficient method for the removal of nitrate from water. The main advantage of heterotrophic denitrification is to enable both removals of nitrate and organic material at the same time. The stoichiometric reaction for the removal of nitrate in the presence of ethanol is given in Equation 2.1 (McAdam and Judd, 2006).



Equation 2.1

The heterotrophic denitrification method is widely applied for the treatment of domestic wastewaters due to its biodegradable organic matter content. Biodegradable organics are used as carbon and electron donor source in the heterotrophic removal mechanism. Unlike the domestic wastewaters, in some cases, biodegradable organics can be limited, which prevents complete denitrification. Groundwater, leather-, and fertilizer-processing industry wastewater, landfill leachate can be given as examples of these water sources (Shao et al., 2010). In order to achieve complete denitrification in carbon limited waters, the additional carbon source is required. However, additional carbon requirement, which is necessary for bacterial activity, increases the cost of the process. In order to change the carbon source which increases the operational cost and causes

effluent contamination, inorganic electron donors were widely studied (Park and Yoo, 2009).

Recently, the autotrophic denitrification process takes attention due to its advantages compared to heterotrophic denitrification. These advantages are, no need for additional carbon source, which lowers the operational cost, and less sludge production, which minimize the handling cost. Sulfur and hydrogen-based autotrophic denitrification are the main processes that the research studies focus on. Sulfur-driven autotrophic denitrification receives more attention due to its ease of handling and lower cost than hydrogen (Zhang and Lampe 1999).

The autotrophic denitrification process can be conducted by using the reduced inorganic sulfur compounds as a sulfur source. These sulfur sources include thiosulfate ($\text{S}_2\text{O}_3^{2-}$), elemental sulfur (S^0), sulfide (S^{2-}), tetrathionate ($\text{S}_4\text{O}_6^{2-}$), and sulfite (SO_3^{2-}) (Batchelor and Lawrence, 1978; Oh et al., 2000; Park and Yoo, 2009). Inorganic carbon sources, like HCO_3^- and CO_2 , were used as a carbon source for autotrophic denitrifiers, which means organic carbon is not required (Zhang and Lampe 1999). Among the sulfur sources given above, elemental sulfur was mainly studied due to its lower cost, ease of handling, high sulfur content to mass ratio, and less sulfate production. However, the availability of elemental sulfur for microorganisms is limited due to its low solubility (Park and Yoo, 2009).

Thiosulfate and sulfide are other commonly used and practical examples of sulfur sources for autotrophic denitrification (Batchelor and Lawrence, 1978). Thiosulfate was the most readily available electron donor for microorganisms. The autotrophic denitrification with sulfide is also called as anaerobic chemolithotrophic desulfurization (Sun, 2012).

During the sulfur-driven autotrophic denitrification reaction, high amounts of proton are produced. In order to overcome this problem, alkaline addition is required. In addition, high concentrations of sulfate in the effluent stream can cause environmental problems. Sulfur-limestone autotrophic denitrification process was used to prevent these problems. However, the hardness of the effluent stream

increases with the addition of limestone, which prevents the widely usage of the method (Sun, 2012).

Denitrification rates and electron donor information of selected literature studies are given in Table 2.3 (Park and Yoo, 2009). Cardoso et al. (2006) compared the nitrate removal performances of the thiosulfate, sulfide and elemental sulfur in the same conditions. As seen in Table 2.3, the nitrate removal rate of thiosulfate was 4.6- and 9.5-fold higher than that of sulfide and elemental sulfur, respectively (Cardoso et al., 2006).

Table 2.3 Denitrification rate for various processes categorized by electron donor

Electron Donor	Denitrification Rate	Reactor Type	Reference
Thiosulfate	2258 ^a	Batch	Yamamoto-Ikemoto et al. (2000)
Thiosulfate	4500 ^a	Packed bed	Claus and Kutzner (1985)
Sulfur	560 ^a	Sulfur packed bed	Sierra-Alvarez et al. (2007)
Sulfur Limestone	80 ^a	Batch	Zhang and Lampe (1999)
Hydrogen	552 ^a	Fluidized bed	Kurt et al. (1987)
Thiosulfate	7.65 ^b	Batch	Cardoso et al. (2006)
Sulfide	1.56 ^b	Batch	
Sulfur	0.65 ^b	Batch	

^a (g-N m⁻³ d⁻¹), ^b (mM-N/day)

CHAPTER 3

MATERIALS AND METHODS

This chapter includes characteristics of GTR (Ground Tire Rubber), and seed sludges, experimental set-ups and analytical methods used in this thesis study. Experimental set-ups involve the enrichment of autotrophic denitrifiers from different seed sludges and bio-desulfurization sets (Set-1 and Set-2), where these enriched cultures were further used.

3.1 Ground Tire Rubber

Ground tire rubber (GTR) samples used in this thesis study, namely, GTR1 and GTR2, were supplied from two different companies, a local company from Ankara and Ün-Sal Rubber Company from Adana, respectively. These companies manufacture rubber powder and granules originated from car, truck and bus tires, which are free from steel wire and textile.

The sulfur content of GTR1 and GTR2 was determined by elemental analysis (Section 3.5.2) and found as 2.00 ± 0.31 and 2.24 ± 0.13 wt%, respectively. Theoretical accessible sulfur content GTRs used in each bio-desulfurization set was calculated according to the calculation given in Appendix A. GTR amounts used in the bio-desulfurization sets and theoretical accessible sulfur contents are also summarized in Table A.1. The penetration depth of the microorganisms was assumed as 6 μm as shown in the calculations (Appendix A).

The particle size distribution of GTR1 and GTR2 is given in Figure 3.1. GTR1, which has the particle size range of 75-1180 μm and average particle size of 712 μm was used in Set1. GTR2, with particle size range of 150-850 μm and an average particle size of 426 μm , was used in Set-2. GTR1, which has a wide

particle size distribution, was used to see the effect of particle size on bio-desulfurization (Set-1).

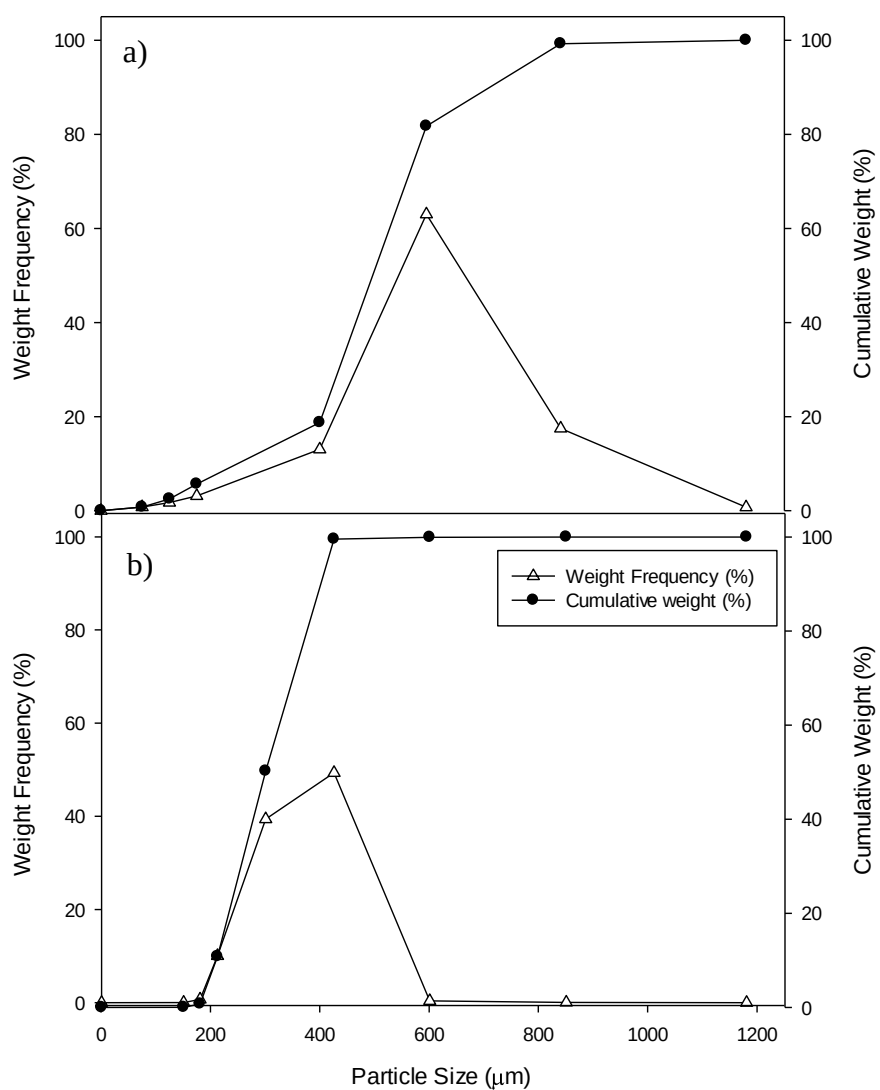


Figure 3.1 Particle size distributions of a) GTR1, and b) GTR2

3.2 Seed Sludge

Seed sludges used in experimental studies were obtained from 3 different sources. One of them was collected from the biological nutrient removal (BNR) system of the Pasakoy Advanced Biological Wastewater Treatment Plant (WWTP) in İstanbul and called S1. The other was taken from the anaerobic digester effluent lines of Ankara Central Domestic WWTP and was called S2. Finally, BNR sludge of the Leather Organized Industrial Zone WWTP was used in experimental studies and called as S3. Each sludge was used separately as inoculum source for the enrichment of sulfur-driven autotrophic denitrifiers. Characteristics of seed sludges are given in Table 3.1.

Table 3.1 Characteristics of seed sludges used in enrichment reactors

Parameter	Seed Sludge Type ^a		
	S1	S2	S3
TS (g/L)	21.82±0.4	10.35±0.2	13.41±0.9
VS (g/L)	10.53±0.2	4.25±0.0	3.55±0.0
pH	8.00	7.40	7.80
sCOD (mg/L)	310±22	1402±17	717±26

^aS1: BNR sludge of Pasakoy Advanced Biological WWTP, S2: Anaerobic digester effluent of Ankara Central Domestic WWTP, S3: BNR sludge of Leather Organized Industrial Zone WWTP

3.3 Synthetic Media

The synthetic medium used for the enrichment of sulfur-driven autotrophic denitrifiers was prepared using distilled water and Na₂S₂O₃.5H₂O (5000 mg/L), KNO₃ (2000 mg/L), K₂HPO₄ (2000 mg/L), NaHCO₃ (1000 mg/L), MgCl₂.6H₂O (500 mg/L), NH₄Cl (500 mg/L) and FeSO₄.7H₂O (10 mg/L) (Koenig and Liu, 2001). The effect of electron donor on bio-desulfurization was tested by replacing Na₂S₂O₃.5H₂O with elemental sulfur (8.74 g/L).

It is expected from the microorganisms to consume sulfur from GTR; for this reason, sulfur source in the synthetic media ($\text{S}_2\text{O}_3^{2-}$ or S^0), which was used as an electron donor for autotrophic denitrification in enrichment studies, was not used in bio-desulfurization sets (Set-1 and Set-2). Besides, in Set-1, the initial concentration of KNO_3 was determined based on the theoretical maximum accessible sulfur content of GTR, as explained in Appendix A. According to the results of the preliminary studies, given in Appendix B and C, it was decided to use excess KNO_3 in Set-2.

3.4 Experimental Set-ups

Before bio-desulfurization experiments, three enrichment reactors were conducted to enrich the sulfur-driven autotrophic denitrifiers. After sufficient enrichment of autotrophic denitrifiers, enriched cultures were used in bio-desulfurization sets, namely, Set-1 and Set-2, as given below.

- Set-1: The effect of particle size on bio-desulfurization efficiency
- Set-2: The effect of seed sludge and electron donor on bio-desulfurization efficiency

3.4.1 Enrichment of Sulfur-driven Autotrophic Denitrifiers

In order to enrich the autotrophic denitrifiers, obtained seed sludge sources were cultivated with nitrate-containing synthetic media. Thiosulfate or elemental sulfur was used as electron donors. Enrichment reactors (ERs), namely, ER1, ER2, ER3 and ER4, were used to supply seed sludge for bio-desulfurization experiments.

Enrichment reactors, constructed of borosilicate glass, were operated in anaerobic sequencing batch mode. Enrichment Reactor 1 (ER1) and Enrichment Reactor 4 (ER4) were operated with an effective volume of 4L. Enrichment Reactor 2 (ER2) and Enrichment Reactor 3 (ER3) were operated with an effective volume of 0.8L.

50% exchange ratio was applied for all reactors (Udert et al., 2003). Initially the operational conditions such as HRT, cycle time and exchange ratio were determined according to the nitrate removal efficiency. This means that, the cycle time and HRT were not fixed at the beginning. Only after the NO_3 removal efficiency increased to the values of 85-95%, the HRT, exchange ratio and cycle time were set to the values given in Table 3.2. Accordingly, ER1 and ER4 were operated with 48 h cycle time and 4 days of hydraulic retention time (HRT). ER2 and ER3 were operated with 72 h cycle time and 6 days of HRT. One cycle of each reactor consisted of 0.5 h of feeding, 1 h of settling and 0.5 h of withdrawal periods. 70 h of reaction time was applied for ER1 and ER4, and 46 h of reaction time was applied for ER2 and ER3. Reactors were operated under these operational conditions for 267 days (Table 3.2).

The initial VS concentrations of the enrichment reactors (ERs) are given in Table 3.3. In order to enrich the autotrophic denitrifiers, KNO_3 (139 mg/L of NO_3^- -N) was used as nitrate source for each ER (Koenig and Liu, 2001; Yang et al., 2018). Besides, $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (645 mg/L of $\text{S}_2\text{O}_3^{2-}$ -S) was used as a sulfur source for ER1, ER2 and ER3 (Oh et al., 2000; Koenig and Liu, 2001; Yang et al., 2018).

Operation of ER4 was performed in three phases (Table 3.3). Thiosulfate ($\text{S}_2\text{O}_3^{2-}$) is the most readily degradable form of sulfur for autotrophic denitrifiers, and nitrate degradation rate is 9.5-fold higher with thiosulfate than that of with elemental sulfur (Cardoso et al., 2006). Therefore thiosulfate was used as a sulfur source in Phase 1 to enrich the autotrophic denitrifiers. Elemental sulfur acclimation of ER4 was performed by gradually decreasing the thiosulfate concentration and one-time addition of elemental sulfur (S^0) in Phase 2 (Hashimoto et al., 1987). Finally elemental sulfur was used as the only sulfur source in Phase 3.

The initial pH of the ERs was set to the range of 7-8. Reactors were flushed with Ar gas (100%) and capped with rubber stoppers to achieve anaerobic conditions after each feeding period of each cycle. In order to define the effectiveness of autotrophic denitrification, the N_2 content of the produced biogas was crucial; for

this reason, Ar was selected for flushing instead of N₂ gas. ERs were operated in a temperature-controlled room at 30-32°C and mixed with magnetic stirrers at 175 rpm. Illustration of the enrichment reactors are given in Figure 3.2.

Table 3.2 Operational conditions of the enrichment reactors (ERs)

Reactor Name	Seed Source ^a	Effective Volume (L)	Exchange Ratio (%)	HRT (days)
ER1	S1	4	50	4
ER2	S2	0.8	50	6
ER3	S3	0.8	50	6
ER4	S1	4	50	4

^aS1: BNR sludge of Pasakoy Advanced Biological WWTP, S2: Anaerobic digester effluent sludge of Ankara Central Domestic WWTP, S3: BNR sludge of Leather Organized Industrial Zone WWTP

Table 3.3 Initial KNO₃⁻-N and S₂O₃²⁻-S concentrations of the ERs

Reactor Name	KNO ₃ ⁻ -N (mg/L)	S ₂ O ₃ ²⁻ -S (mg/L)	S ⁰ (g/L)	VS (g/L)
ER1	139	645	-	1.00
ER2	139	645	-	1.03
ER3	139	645	-	0.90
ER4 – Phase 1	139	645	-	1.38
ER4 – Phase 2	139	645→0 ^a	8.74 ^b	1.38
ER4 – Phase 3	139	-	8.74 ^b	1.38

^aER4 - Phase2: S₂O₃²⁻-S concentration was gradually decreased from 645 mg/L to 0, ^bone-time addition

The electron donor of ER4, that was S⁰ in Phase 2 and Phase3, was fed higher than the stoichiometric value, in order to increase the denitrification rate (Hashimoto et al., 1987; Sierra-Alvarez et al., 2007; Şahinkaya et al., 2015).

Nitrate and sulfate concentrations were measured periodically to investigate the enrichment and performance of autotrophic denitrifiers.

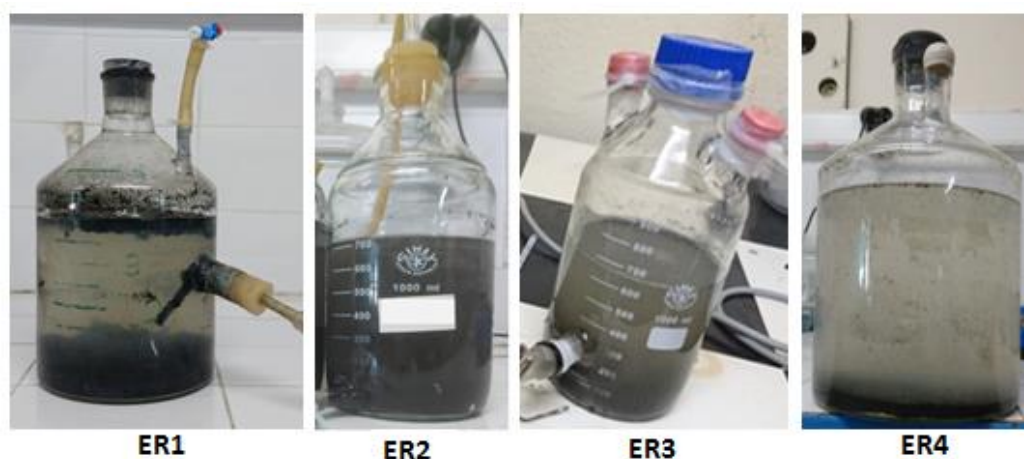


Figure 3.2 Illustration of the enrichment reactors during settling period

3.4.2 Bio-desulfurization Sets

After sufficient enrichment of autotrophic denitrifiers, enriched cultures (EC) were used in bio-desulfurization sets. Enriched cultures, namely, Enriched Culture 1 (EC1), Enriched Culture 2 (EC2), Enriched Culture 3 (EC3), and Enriched Culture 4 (EC4) were obtained from ER1, ER2, ER3 and ER4, respectively. Information on seed and sulfur sources of the ECs are summarized in Table 3.4.

Table 3.4 Seed and sulfur source information of ECs

	EC1	EC2	EC3	EC4
Seed Source	S1	S2	S3	S1
Enrichment Reactor	ER1	ER2	ER3	ER4
Sulfur Source	$\text{S}_2\text{O}_3^{2-}$	$\text{S}_2\text{O}_3^{2-}$	$\text{S}_2\text{O}_3^{2-}$	S^0

Each bio-desulfurization set contained control, test, and blank reactors. All reactors contained synthetic media. In addition to the synthetic media, control reactors contained only the EC, blank reactors contained only GTR, and test reactors contained GTR and EC. The initial pH of reactors was set to the range of 7-8. After

the required constituents were added, reactors were capped with rubber stoppers and flushed with Ar for 5 minutes to obtain anaerobic conditions. All reactors were incubated at 30-32°C on a magnetic stirrer at 175 rpm.

3.4.2.1 Set-1: The Effect of Particle Size on Bio-desulfurization Efficiency

The purpose of Set-1 was to investigate the effect of particle size on bio-desulfurization efficiency. Grinding GTR to smaller sizes is a costly process, so it may not be feasible to make an effort to grind GTR to small particle sizes. Although cost analysis is out of the scope of this study, it is still important to see if there is a statistically significant difference between GTR that is not grinded (factory-originated) and grinded small-sized GTR.

In this experimental study, four different particle sizes were selected to see their effect on bio-desulfurization. GTR1, which has a particle size range of 75-1180 µm were distributed into three different size ranges, 75-400, 400-595, and 595-841 µm, by sieve analyses. Besides particle size range of 75-1180 µm was also used as it was supplied from the company without any sieving. In order to remove the harmful additives and kill the bacteria attached to GTR, an ethanol leaching method was used before the experiment (Christiansson et al., 2000). For this purpose, GTR was immersed in 75% ethanol (v/v) for 24 hr, then filtered and dried (Jiang et al., 2010; Jiang et al., 2011; Li et al., 2011; Li et al., 2012a; Li et al., 2012b; Li et al., 2013; Hu et al., 2014; Yao et al., 2014; Cui et al., 2016; Hu et al., 2016).

Batch reactors with 70 ml effective and 110 ml total volume were used in the experiment. Test and blank reactors for each particle size were prepared. Test reactors were conducted in triplicate while control and blank reactors were conducted in duplicate. Each reactor contained synthetic medium, and their concentrations are given in Table 3.5. NO_3^- -N concentration in the synthetic medium was determined according to the theoretical maximum accessible sulfur content (Appendix A), and given in Table 3.5. Accordingly, the initial NO_3^- -N

concentration in the reactors was 208 mg/L in the test, blank and control reactors. The theoretical maximum accessible sulfur content was determined as 73.9, 35.8, 24.9 and 49.7 mgS/L for 75-400, 400-595, 595-841 and 75-1180 μm particle size ranges, respectively.

EC1 was used as inoculum with the VS concentration of 0.67 g/L in the control and test reactors. 3.5 g of GTR1, corresponding to 5% (w/v) of the effective volume of the reactor, was used for each particle size range studied. Reactor configuration of Set-1 is given in Table 3.6.

Table 3.5 Synthetic medium constituents and their concentrations

Constituent	Concentrations (mg/L)	
	Set-1	Set-2
KNO ₃	1500	6000
MgCl ₂ .6H ₂ O	500	500
K ₂ HPO ₄	2000	2000
FeSO ₄ .7H ₂ O	10	10
NH ₄ Cl	500	500
NaHCO ₃	1000	1000

Table 3.6 Reactor configuration of Set-1

Reactor	Inoculum ^a	GTR1	Medium
C	EC1	-	X
B1 (75-400 μm GTR)	-	X	X
B2 (400-595 μm GTR)	-	X	X
B3 (595-841 μm GTR)	-	X	X
B4 (75-1180 μm GTR)	-	X	X
T1 (75-400 μm GTR)	EC1	X	X
T2 (400-595 μm GTR)	EC1	X	X
T3 (595-841 μm GTR)	EC1	X	X
T4 (75-1180 μm GTR)	EC1	X	X

^aEC1= Enriched culture 1

According to the results of Set-1, two preliminary experimental studies (Preliminary Study 1 and Preliminary Study 2) were conducted to define the reason and overcome the abiotic consumption of NO_3^- -N (Appendix B and C).

3.4.2.2 Set-2: The Effect of Seed Sludge Type and Electron Donor on Bio-desulfurization Efficiency

In this experimental study, cultures were enriched from four different ERs (Table 3.4). The purpose of Set-2 was to investigate the effect of seed sludge type and electron donor on bio-desulfurization efficiency. ECs obtained from EC1, EC2, and EC3 were used to see the effect of seed sludge. EC1 and EC4 were used to see the effect of the electron donor.

GTR2 that has a particle size range of 150-850 μm was used in Set-2. Before the experimental study, GTR2 was detoxified by immersing in 75% ethanol (v/v) for 72 hr and in every 24 hr, ethanol was replaced with fresh ethanol (Hu et al., 2016). The average particle size of the GTR used (GTR2) in the experiment was 426 μm .

Batch reactors with 60 ml total and 40 ml effective volume were used in the experiments. Test, blank, and control reactors for each variable were conducted triplicate. Each reactor contained synthetic medium, and their concentrations are given in Table 3.5 (Section 3.4.2.1). Accordingly the initial NO_3^- -N concentration was 832 mg/L in the test, blank and control reactors while the theoretical maximum accessible sulfur content was calculated as 41.6 mg-S/L.

EC1, EC2, EC3, and EC4 were used as inoculums with VS concentrations of 0.57 g/L in the reactors. 1 g of GTR2, corresponding to 2.5% (w/v) of the effective volume of the reactor, was used in test and blank reactors. Reactor configuration of Set-2 is given in Table 3.7.

Table 3.7 Reactor configuration of Set-2

Reactor ^a	Inoculum ^b	GTR2	Medium
B	-	X	X
C1	EC1	-	X
C2	EC2	-	X
C3	EC3	-	X
C4	EC4	-	X
T1	EC1	X	X
T2	EC2	X	X
T3	EC3	X	X
T4	EC4	X	X

^a B: Blank, C: Control, T: Test, ^b EC1= Enriched culture 1, EC2= Enriched culture 2, EC3= Enriched culture 3, EC4= Enriched culture 4

3.5 Analytical Methods

3.5.1 Reactor Performance

pH, nitrate (NO_3^-), sulfate (SO_4^{2-}), nitrite (NO_2^-), and soluble chemical oxygen demand (sCOD) were measured on cycle basis in order to analyze the performance of the ERs. To determine the required initial conditions and to investigate the performance of bio-desulfurization sets, total solids (TS), volatile solids (VS), ammonia nitrogen ($\text{NH}_3\text{-N}$), nitrate, sulfate, nitrite, and total organic carbon (TOC) were analyzed.

pH

pH was measured by using a pH meter (Oakton PC 450) and a pH probe (Hanna HI 1230B).

Nitrate (NO_3^-), Sulfate (SO_4^{2-}), and Nitrite (NO_2^-)

Nitrate (NO_3^-), sulfate (SO_4^{2-}), and nitrite (NO_2^-) were measured by using Shimadzu Prominence HIC-SP ion chromatograph (IC). The working conditions of the IC were set to have the highest pressure limit of 150 bar, oven

temperature of 45°C, and the flow rate of 0.8 ml/min. Before analysis, samples were filtered through 0.45 µm cellulose acetate filter.

The calibration curves for IC analysis are given in Figure D.1 (Appendix D). In addition, the limit of detection (LOD) and the limit of quantification (LOQ) values for low and high concentration calibrations are calculated and given in Table D.1 (Appendix D).

Total Organic Carbon (TOC)

Total Organic Carbon (TOC) content of the samples was determined using Shimadzu 5000A model TOC analyzer employing the 680 C combustion catalytic oxidation method. The calibration curve for TOC analysis is given in Figure E.1. LOD and LOQ values for calibration are given in Table E.1 (Appendix E).

Soluble Chemical Oxygen Demand (sCOD)

sCOD of the samples, which were initially filtered through 0.45 µm cellulose acetate filter, was measured with EPA approved digestion method (0-1500 mg/L). Aqualytic AL 38 heater and PC Multidirect Spectrophotometer were used for the analysis.

Total Solids (TS) and Volatile Solids (VS)

TS and VS contents were measured according to Standard Methods (2540 B, 2540 D) (APHA, 2005).

3.5.2 GTR Characterization

In order to evaluate the performance of the bio-desulfurization, GTR characterization was performed at the beginning and end of the experimental periods. Particle size distribution and elemental analysis were conducted at the beginning of the bio-desulfurization sets. Soluble (sol) fraction was conducted at the end of the Set-2.

Elemental Analysis

Sulfur content of GTR was analyzed in Central Laboratory of Middle East Technical University by using LECO CHNS-932 elemental analysis instrument.

Soluble (Sol) Fraction

The non-crosslinked free polymer content of rubber is defined as sol fraction (Section 2.5). After the bio-desulfurization experiments, the sol fraction of rubber was expected to increase due to the crosslink breakage. Sol fraction of GTR was measured with a Soxhlet extraction apparatus, according to ASTM D 297.

Particle Size Distribution

Particle size distribution analysis was conducted according to ASTM D 5644-01.



CHAPTER 4

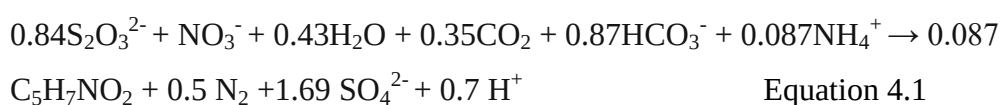
RESULTS AND DISCUSSION

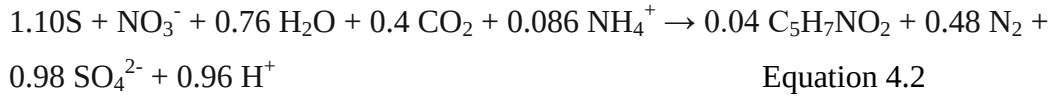
This chapter includes the results and discussion of the enrichment of the autotrophic denitrifiers and two bio-desulfurization sets conducted namely, Set-1 (the effect of particle size on bio-desulfurization) and Set-2 (the effect of seed sludge type and electron donor on bio-desulfurization). Characterization results of the GTRs used in the thesis study, after the bio-desulfurization sets were also given and discussed.

4.1 Enrichment of Autotrophic Denitrifiers

Four different reactors were operated to enrich the sulfur-driven autotrophic denitrifiers, namely, ER1, ER2, ER3 and ER4. pH, NO_3^- -N removal efficiency, $(\Delta\text{SO}_4^{2-}\text{-S})/(\Delta\text{NO}_3^-\text{-N})$ molar ratio, and NO_3^- -N and NO_2^- -N concentrations were observed to determine the performance of the ERs. Besides, kinetic studies were conducted to compare the effectiveness of autotrophic denitrifiers with the literature data.

Reduced inorganic sulfur compounds can be used as a sulfur source for the autotrophic denitrification process. These sulfur sources include thiosulfate ($\text{S}_2\text{O}_3^{2-}$), elemental sulfur (S^0), sulfide (S^{2-}), tetrathionate ($\text{S}_4\text{O}_6^{2-}$), and sulfite (SO_3^{2-}) (Batchelor and Lawrence, 1978; Oh et al., 2000; Park and Yoo, 2009). In this study, thiosulfate and elemental sulfur were used as sulfur sources (electron donor). Stoichiometric equations for $\text{S}_2\text{O}_3^{2-}$ and S^0 driven autotrophic denitrification is given in Equation 4.1 and Equation 4.2, respectively (Hashimoto et al., 1987).





Equation 4.2

The following sections cover the results of pH analyses of all ERs (Section 4.2.1) and removal performances of each ER (Section 4.1.2). It should be noted that, as mentioned before in Section 3.4.1, ERs were operated for almost one year. To indicate the final conditions (removal performances) of the enriched cultures before their use in bio-desulfurization sets, the data related to the last 3 months are given herein and the related performances were discussed. On the other hand, the pH of the ERs was represented (Figure 4.1) from the beginning of the enrichment operation, to be able to discuss the Phase differences of ER4 operation.

4.1.1 pH of the enrichment reactors (ERs)

Optimum pH for sulfur-driven autotrophic denitrifiers is in the range of 6 to 8 (Lawrence et al., 1978; Madigan et al., 2010). According to Equation 4.1 and Equation 4.2, proton (H^+) is generated during the reaction, which consumes alkalinity (HCO_3^-) and causes a decrease in pH. The denitrification process is inhibited when pH values are lower than 6 and higher than 9 (Trouve et al., 1998; Oh et al., 2000). In order to obtain optimum conditions for microorganisms, by considering the pH decrease, the initial pH for the ERs was set to the range of 7-8.

The cycle basis initial and effluent pH values of each ER are given in Figure 4.1. As seen in Figure 4.1, although fluctuations can be observed at the beginning of the enrichment operation, the pH values of the reactors are in accordance with the optimum pH values of the autotrophic denitrifiers. The average initial and effluent pH values of the ERs are given in Table 4.1. Compared to the initial pH values, the effluent pH values of the ERs decreased during the cycles as expected from Equation 4.1 and Equation 4.2.

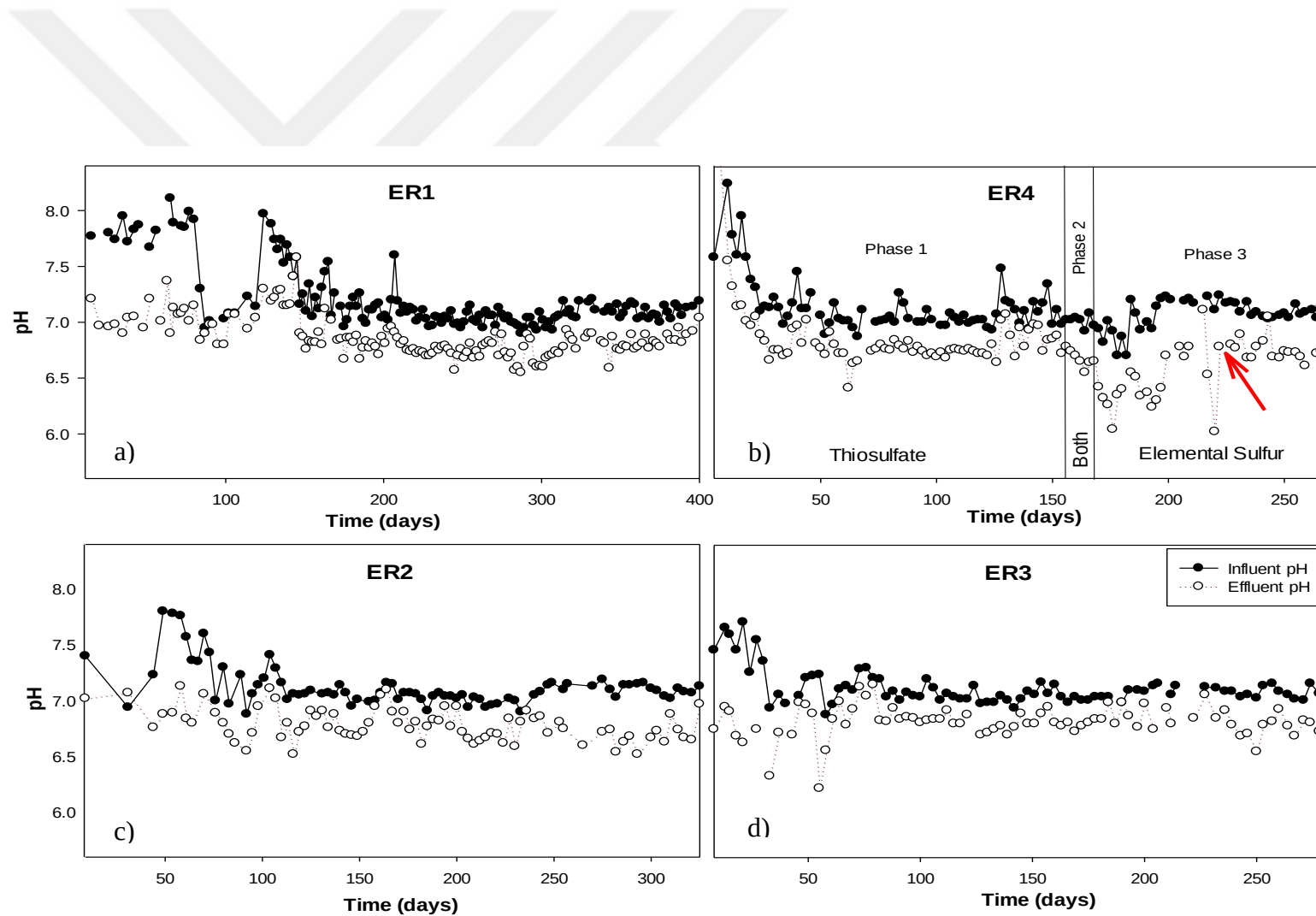


Figure 4.1 Cycle basis initial and effluent pH values of the a) ER1, b) ER4, c) ER2, and d) ER

Seed sludge for both ER1 and ER4 was taken from Paşakoy Advanced Biological WWTP. The difference between these ERs was the sulfur source (electron donor) used in the medium. $\text{S}_2\text{O}_3^{2-}$ was used for ER1 while S^0 was aimed/used for ER4.

ER4 operation was divided into three phases (Figure 4.1b), as mentioned in Section 3.4.1. Phase 1, Phase 2, and Phase 3 covered the cycles of 0-155, 156-168, and 169-266, respectively. In Phase 1, $\text{S}_2\text{O}_3^{2-}$, which has a higher degradation rate, was used as electron donor in order to enrich the autotrophic denitrifiers (Cardoso et al., 2006). S^0 became solely sulfur source in Phase 3. In between, in Phase 2, both sources were used (Table 3.3, Section 3.4.1). As can be seen from Figure 4.1b, steady-state conditions were achieved after cycle 222, which was stated with a red arrow.

Table 4.1 Average cyclic initial and effluent pH values of ERs

Reactor Name	Initial pH	Effluent pH
ER1	7.2±0.3	6.9±0.2
ER2	7.1±0.2	6.8±0.2
ER3	7.1±0.2	6.8±0.1
ER4	7.1±0.2	6.8±0.3

As seen in Figure 4.2, the pH decrease in Phase 3 (from 7.1±0.1 to 6.6±0.3) is slightly higher than Phase 2 (from 7.0±0.1 to 6.6±0.1) and much higher than Phase 1 (from 7.1±0.2 to 6.9±0.3). This situation can be explained by higher H^+ production in S^0 -driven autotrophic denitrification (Equation 4.2), compared to $\text{S}_2\text{O}_3^{2-}$ -driven counterpart (Equation 4.1).

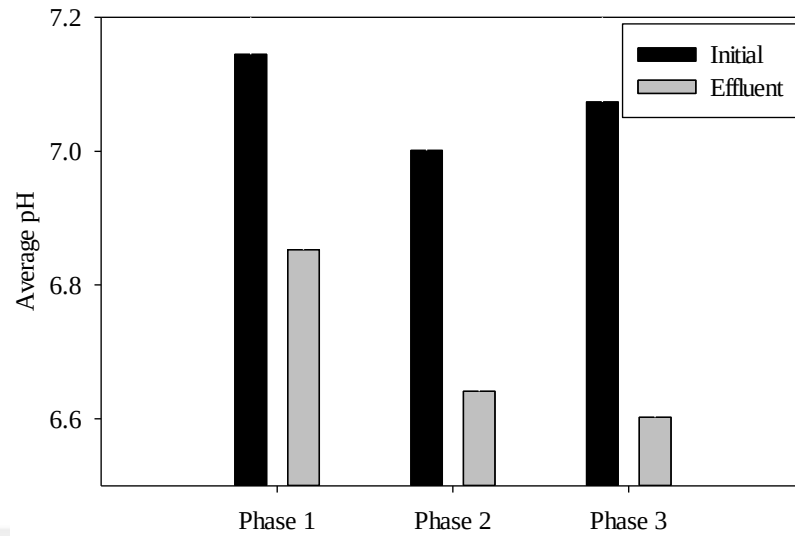


Figure 4.2 Average pH values of each phase in ER4 operation

4.1.2 Performance of the ERs

During the enrichment operation of all ERs, it was observed that nitrate decreased while the effluent sulfate concentration increased. This reveals that a sulfur source in the feed was used as an electron donor in the denitrification process. There was no carbon source added to the reactors. Therefore, denitrification was sulfur-driven autotrophic denitrification but not heterotrophic denitrification.

Three parameters were analyzed in order to investigate the performance of the ERs. These are NO_3^- -N removal efficiency, $\Delta\text{SO}_4^{2-}\text{-S}/\Delta\text{NO}_3^-$ -N molar ratio and NO_3^- -N initial and effluent concentrations.

NO_3^- -N removal efficiency and $\text{SO}_4^{2-}\text{-S}/\text{NO}_3^-$ -N molar ratios of the ERs were calculated using Equation G.1 and Equation G.2, respectively (Appendix G). $\text{SO}_4^{2-}\text{-S}/\text{NO}_3^-$ -N molar ratio values were then compared with the theoretical value, which is 1.69 for $\text{S}_2\text{O}_3^{2-}$ (Equation 4.1) and 0.98 for S^0 (Equation 4.2).

In order to determine the effect of the sulfur source (S^0) on the enrichment performance, ER4 operation was observed from the beginning of Phase 2 and compared with ER1. Besides, ER1, ER2, and ER3 operations were analyzed for the same periods (the last 3 months, 120 days) to compare the enrichment performances of the reactors.

4.1.2.1 Performance of the ER1

Analyses revealed that NO_3^- -N removal efficiency of ER1, which was seeded with S1 sludge, was relatively stable during the enrichment operation (Figure 4.3a). The average removal efficiency was $96.0 \pm 7.6\%$, and complete removal was achieved in almost half of the cycles represented in Figure 4.3a. There was a dramatic decrease in removal efficiency to 53% on Day 340. This decrease was due to the change in the initial NO_3^- -N concentration (Figure 4.3c). The initial NO_3^- -N concentration was increased from 139 to 294 mg-N/L between Days 340-344 due to an experimental error in the preparation of the feed solution. Despite a two-fold increase in N concentration on Day 340, the effect of shock loading was temporary, as also stated in literature (Yamamoto-Ikemoto et al., 2000). The autotrophic denitrifiers adapted to these new conditions, besides the removal efficiency recovered and increased back to 97% on Day 342.

Except for temporary changes, NO_3^- -N removal efficiency was relatively stable. However, fluctuations were observed at ΔSO_4^{2-} -S/ ΔNO_3^- -N molar ratio with the change in NO_3^- -N initial concentrations (Figure 4.3b). According to the reaction given in Equation 4.1, the theoretical sulfur to nitrogen (S/N) molar ratio was calculated as 1.69. Moreover, Oh et al. (2000) stated the experimental S/N ratio as 1.63, which is fairly similar to the theoretical value. Due to the increase in the initial NO_3^- -N concentration, the S/N molar ratio was decreased to 0.96 on Day 340, which was lower than the theoretical value. $S_2O_3^{2-}$ is the limiting substance when the S/N ratio was lower than the theoretical value (Campos et al., 2008).

Therefore, NO_3^- -N removal efficiency decreased, due to the deficiency of $\text{S}_2\text{O}_3^{2-}$ in the medium (feed). The difference between the theoretical and experimental ΔSO_4^{2-} -S/ ΔNO_3^- -N molar ratios could be attributed to the fluctuations in the initial NO_3^- -N concentrations. Yet, the stable initial NO_3^- -N concentrations resulted in stable ΔSO_4^{2-} -S/ ΔNO_3^- -N molar ratio values close to the theoretical value of 1.69. This indicated the well enrichment of $\text{S}_2\text{O}_3^{2-}$ -driven autotrophic denitrifiers in ER.

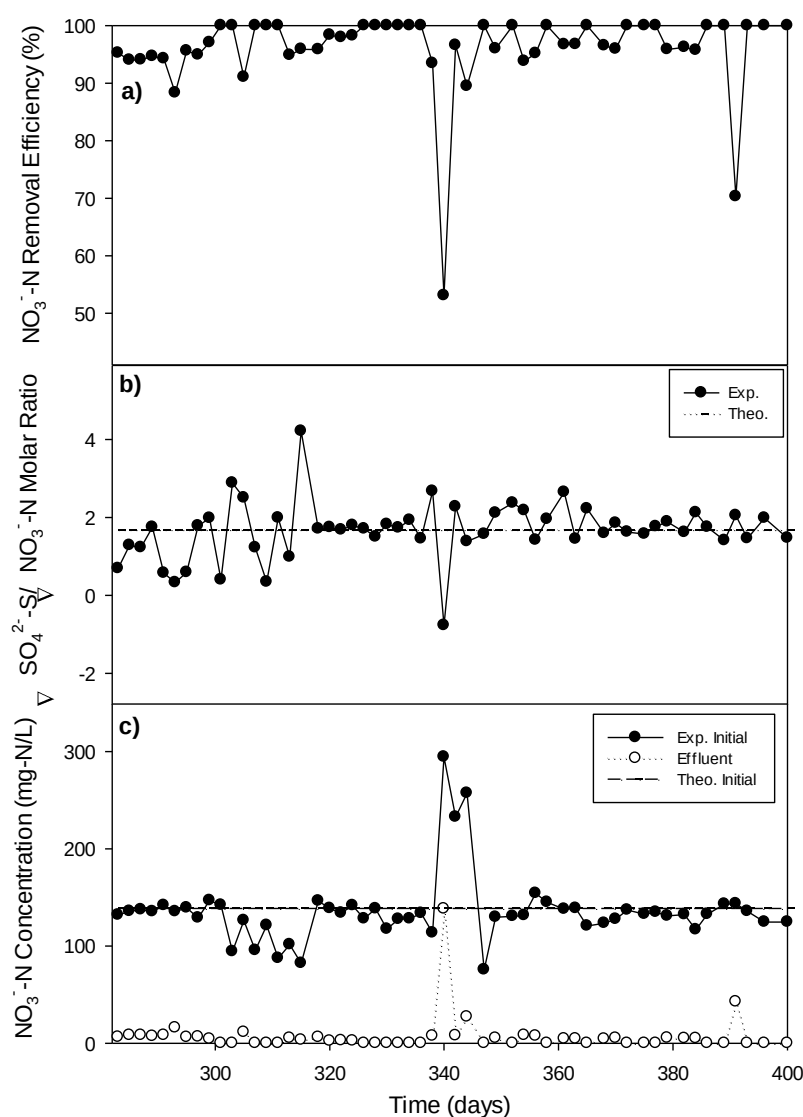


Figure 4.3 a) NO_3^- -N removal efficiency, b) ΔSO_4^{2-} -S/ ΔNO_3^- -N molar ratio and c) Initial and effluent NO_3^- -N concentrations of ER1

4.1.2.2 Performance of the ER2

The average removal efficiency of ER2, which was seeded with S2 sludge, was $96.9 \pm 3.3\%$ (Figure 4.4a). Except for Day 269 (84%), the minimum removal efficiency was 93% and complete (100%) removal was achieved as the maximum.

For a better representation of the conditions and the results, the operation period of ER2 was divided into three parts, namely, Part 1, Part 2, and Part 3. Part 1 corresponds to the Days 208-260, Part 2 corresponds to the Days 261-289, and Part 3 corresponds to the Days 290-325.

Due to the limitations experienced during the pandemic period, ER2 could not be set with the same frequency. Therefore, the cycle period was extended from 72 h to 96 h in Part 1, then decrease from 96 h to 84 h in Part 2. Volume exchange ratio was increased from 50% to 66.7% in Part 1, then was decreased to 58.3% in Part 2. Accordingly, despite the same synthetic medium usage, the initial concentration at the beginning of the cycles increased (Figure 4.4).

The initial concentration of the ER2 at the beginning of the operation (Part 1) was set to 139 mg-N/L. Concentration was increased from 139 to 185 mg-N/L at Part 2. With the increase in the initial NO_3^- -N concentration, the removal efficiency was decreased to 84% (Day 269). Yet, the removal efficiency recovered and increased to 98% on Day 274.

The initial NO_3^- -N concentration was then decreased from 185 to 162 mg-N/L at Part 3. The decrease in the initial NO_3^- -N concentration did not significantly affect the removal efficiency.

Similar fluctuations as observed for removal efficiencies were obtained for ΔSO_4^{2-} -S/ ΔNO_3^- -N molar ratios especially during the first two parts (Figure 4.4b). While significant fluctuations can be seen between Days 261-289, fairly similar results were obtained between Days 290-325 (Figure 4.4b), indicating the enrichment of the autotrophic denitrifiers.

The difference between the theoretical and experimental $\text{SO}_4^{2-}\text{-S}/\text{NO}_3^-\text{-N}$ molar ratios could be attributed to the fluctuations in the initial $\text{NO}_3^-\text{-N}$ concentrations.

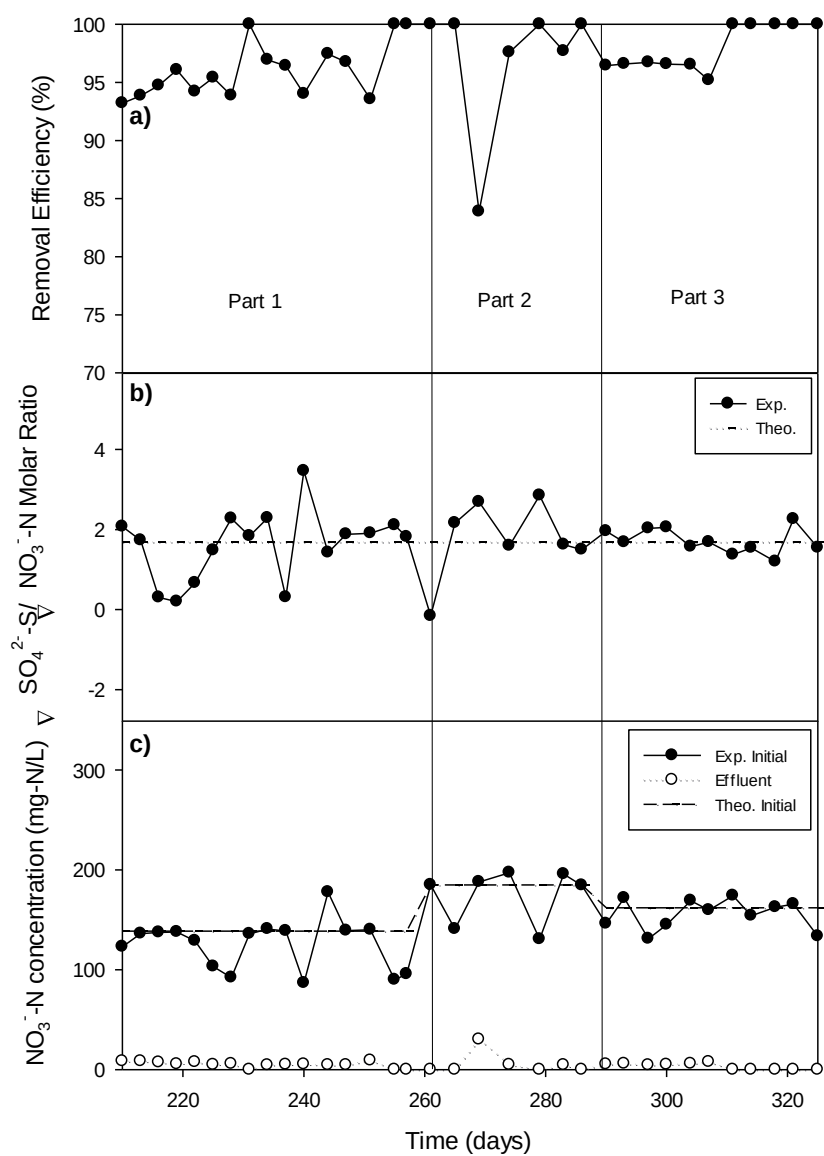


Figure 4.4 a) $\text{NO}_3^-\text{-N}$ removal efficiency, b) $\Delta \text{SO}_4^{2-}\text{-S} / \Delta \text{NO}_3^-\text{-N}$ molar ratio and c) Initial and effluent $\text{NO}_3^-\text{-N}$ concentrations of ER2

4.1.2.3 Performance of the ER3

NO_3^- -N removal efficiency of ER3, which was seeded with S3 sludge, was relatively stable during the enrichment operation, being usually greater than 95% (Figure 4.5a). The average removal efficiency of ER3 was $97.6 \pm 2.4\%$. The minimum and maximum removal efficiencies obtained were 91 and 100%, respectively.

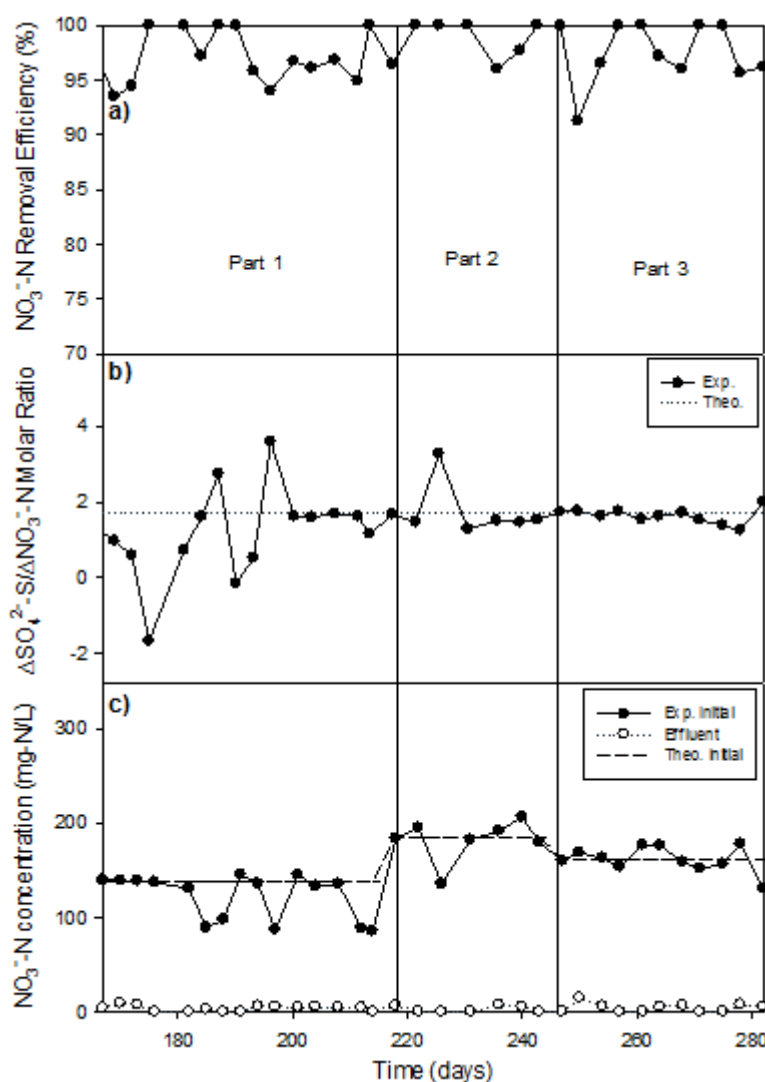


Figure 4.5 a) NO_3^- -N removal efficiency, b) $\Delta\text{SO}_4^{2-}\text{-S}/\Delta\text{NO}_3^-$ -N molar ratio and c) Initial and effluent NO_3^- -N concentrations of ER3

Similar to ER2, the operation period of ER3 was divided into three parts, due to the limitations experienced during the pandemic period. Part 1 corresponds to the Days 165-217, Part 2 corresponds to the Days 218-246, and Part 3 corresponds to the Days 247-282. By keeping the HRT (6 Days) constant, the initial NO_3^- -N concentration was set to 139, 185, and 162 mg-N/L at Part 1, Part 2, and Part 3, respectively (Figure 4.5c).

Change in the initial NO_3^- -N concentration did not significantly affect the removal efficiency. ΔSO_4^{2-} -S/ ΔNO_3^- -N molar ratios were close to the theoretical ΔSO_4^{2-} -S/ ΔNO_3^- -N molar ratio of 1.69 most of the time and almost constant despite the changes in initial NO_3^- -N concentrations during Part 2 and Part 3 (Figure 4.5b).

4.1.2.4 Performance of the ER4

S^0 was one of the most studied electron donors for autotrophic denitrification operation, due to its lower price, ease of usage, and lower sulfate production among other substrates (Kimura et al., 2002; Park and Yoo, 2009). ER4 operation was evaluated starting from Phase 2 where the electron donors were both $\text{S}_2\text{O}_3^{2-}$ and S^0 , as mentioned in Section 4.1.3. As can be seen in Figure 4.6b, after Phase 2, S^0 became solely sulfur source on Day 170 (Phase 3). After fluctuations in ΔSO_4^{2-} -S/ ΔNO_3^- -N molar ratio between Days 170-190, steady-state conditions were achieved by Day 191 (Phase 3). Autotrophic denitrifiers seemed to acclimate to the operational conditions and S^0 . During the following days, ΔSO_4^{2-} -S/ ΔNO_3^- -N molar ratios was always obtained around 0.75 ± 0.17 , which was 23 % lower than the theoretical value (0.98, Equation 4.2).

The average removal efficiency of ER4 was $87.2 \pm 17.9\%$ (Figure 4.6a). The minimum and maximum removal efficiencies were 29 and 100%, respectively.

Although there was not a significant change in the operational conditions on Day 213 (Phase 3), the removal efficiency decreased dramatically to 54%. Due to limitations experienced during the pandemic period, IC analyses could not be

performed between Days 213-221 (Phase 3). Therefore, with the addition of the feed on each cycle, the initial NO_3^- -N concentration gradually increased and accumulated in the reactor (Figure 4.6c). Accordingly, removal efficiency decreased to 29% on Day 217 (Phase 3). After IC analyses were conducted, on Day 223 (Phase 3), the initial concentration of NO_3^- -N was adjusted. The removal efficiency recovered and increased to 94% on Day 224 (Phase 3). Although the initial concentration of NO_3^- -N (145 mg/L) was quite similar to the theoretical value (139 mg/L) the removal efficiency decreased to 40% on Day 245 (Phase 3).

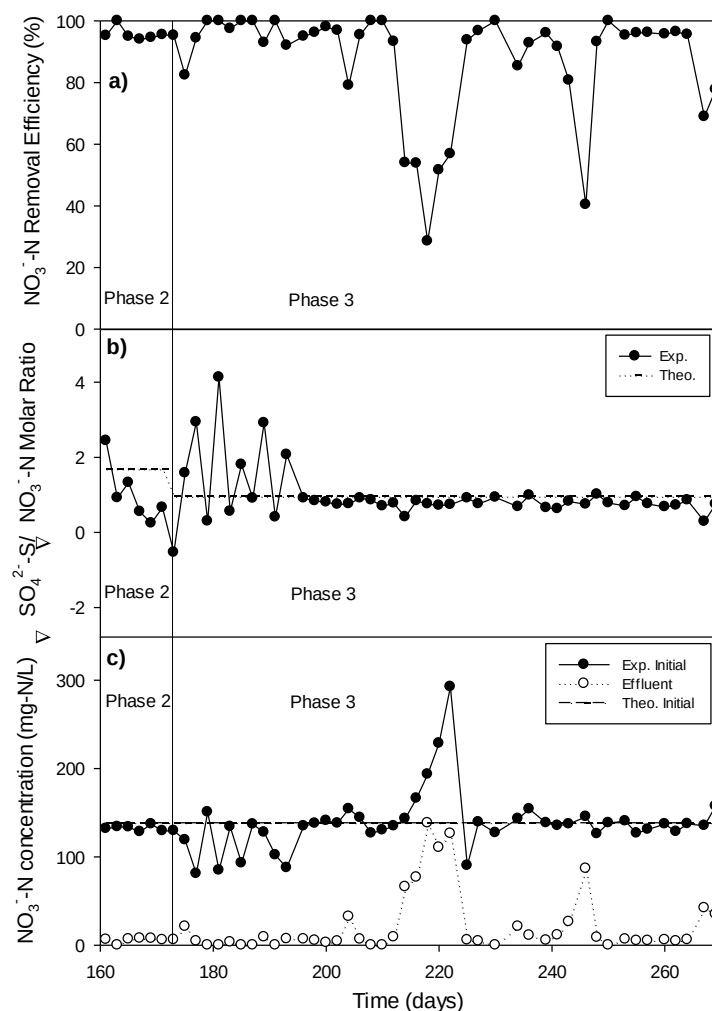


Figure 4.6 a) NO_3^- -N removal efficiency, b) ΔSO_4^{2-} -S/ ΔNO_3^- -N molar ratio and c) Initial and effluent NO_3^- -N concentrations of ER4

A decrease in the removal efficiency, on Day 245, could be attributed to the depletion of S^0 , which was added to the reactor at every 4 cycles. Moreover, the solubility of elemental sulfur could be the reason for the decrease in removal efficiency. Despite its many advantages, the solubility of S^0 is low, which adversely affects the denitrification process by limiting the availability for microorganisms (Park and Yoo, 2009). S/N molar ratio becomes lower than the theoretical value of 0.98 when the sulfur source is the limiting substance (Campos et al., 2008). Therefore, NO_3^- -N removal efficiency might have decreased due to the deficiency of soluble S^0 .

4.1.2.5 Comparison of the Performances of the ERs

The discrepancy term was used to evaluate the relative differences between theoretical and experimental ΔSO_4^{2-} -S/ ΔNO_3^- -N molar ratios (Equation G.3). In order to compare the discrepancies of ERs, the last 11 cycles were chosen, in which steady-state conditions were reached for each reactor. Moreover, the initial concentration of ER2 and ER3 was adjusted in this period.

Sun and Nemati (2012) used the discrepancy term in order to define the differences between experimental (produced) and theoretical (stoichiometric) sulfate concentrations, which is another version of ΔSO_4^{2-} -S/ ΔNO_3^- -N molar ratios.

Average NO_3^- -N removal efficiencies and discrepancies were calculated and represented in Table 4.2.

Table 4.2 Average removal efficiency and discrepancy results of ERs

Reactor	Average NO_3^- -N Removal Efficiency (%)	Discrepancy (%)
ER1	96.0±7.6	11.4±5.9
ER2	96.9±3.3	13.2±9.4
ER3	97.6±2.4	8.9±7.8
ER4	87.2±17.9	22.7±18.0

The initial NO_3^- -N concentration of ER4 was 139 mg/L, which corresponds to 9.9 mM. Sun (2012) investigated the S^0 -driven autotrophic denitrification with 9.9 mM of initial nitrate, and complete removal was achieved. In this study, 7.6% discrepancy between theoretical and experimental sulfate production was observed, which was considerably lower than ER4 (22.7%) operation. In addition to higher discrepancy value, ER4 was susceptible to shock loadings. An increase in the initial nitrate concentration directly affected and decreased the NO_3^- -N removal efficiency.

The discrepancy value of ER1, ER2, and ER3 was slightly different from each other, although $\text{S}_2\text{O}_3^{2-}$ was used as an electron donor for all ERs. Although initial conditions were the same, different results could be obtained due to the origin of the seed sludge (Campos et al., 2008). However, except for a slight difference in discrepancy value, removal efficiencies were very similar for these three ERs. Accordingly, kinetic studies were performed to better compare the performances of ER1, ER2 and ER3. For having relatively lower and fluctuating removal efficiency and almost an average of 23% discrepancy in experimental and theoretical molar ratios, ER4 was not included in the kinetic study.

4.1.3 Kinetic Study

Kinetic studies were conducted to compare the effectiveness of the enriched S-driven autotrophic denitrifiers with the literature data. As mentioned in Section 4.1.3.5, ER4 was not included in the kinetic studies.

This study was performed by measuring nitrate, nitrite, and sulfate concentrations and cumulative gas production volume. The reduction of nitrate in the denitrification process takes place through $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$ reaction sequence (Francis et al., 2007; Cui et al., 2019). The intermediate products NO and N_2O were taken as zero due to their negligible emissions, as stated in literature (Zhang et al., 2015; Yang et al., 2016). NO_2^- was not detected in the

effluent in the kinetic study experiments. According to these pieces of information, it was assumed that a complete reduction of NO_3^- to N_2 gas was achieved. Yang et al. (2018) calculated the denitrification rate through the slope of the cumulative gas production data.

The kinetic study was completed after the gas productions of ERs ceased. Gas production amounts of ERs were recorded during the experiment. The initial NO_3^- -N concentrations, theoretical and experimental produced gas amounts of ER1, ER2 and ER3 are given in Table 4.3. Gas volumes produced in the experiment were close to the theoretical values. The difference between theoretical and experimental results was calculated as 2-4%, which indicates a high performance for three ERs.

Table 4.3 Initial nitrate concentrations and produced biogas results of ERs

Reactor	NO_3^- -N initial (mg/l)	Theoretical N_2 production (ml)	Experimental N_2 Production (ml)
ER1	82.2	147	150
ER2	86.7	156	161
ER3	86.9	156	163

According to the recorded gas volumes during the experiment, cumulative gas production results of each ER were obtained and given in Figure 4.7.

The denitrification rate of ER1, ER2, and ER3 was calculated as 716, 770, and 397 mg N/(L.d), respectively. The denitrification rates of the ERs in this study were comparable with literature studies (Table 4.4). Specific denitrification rate of ER1, ER2 and ER3 was calculated considering Figure 4.7 and VS content used in the kinetic study as 314, 461 and 227 mg-N/(mg N/gVS.d), respectively. Specific denitrification rates obtained from related literature results and this experimental study are also shown in Table 4.4.

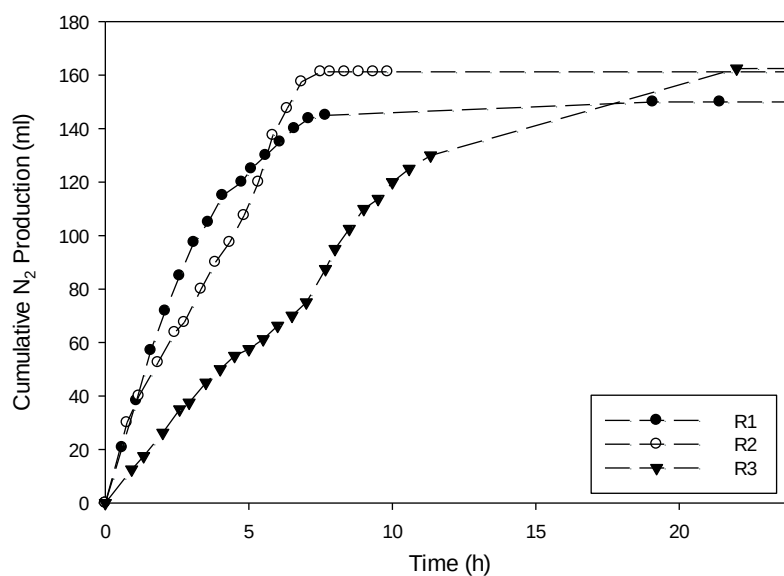


Figure 4.7 Cumulative N₂ production volumes of ER1, ER2, and ER3

Table 4.4 The results of denitrification and specific denitrification rate experiment with similar studies in literature

Denitrification Rate (mg N ₂ -N/l d)	Specific Denitrification Rate (mg N ₂ -N/gVS d)	Reference ^a
-	21	Yang et al. (2018)
53	66	Di Capua et al. (2016)
107	-	Cardoso et al. (2006)
-	4227 ^b	Claus and Kutzner (1985)
2258	-	Yamamoto-Ikemoto et al. (2000)
716	314	This study (ER1)
770	461	This study (ER2)
397	227	This study (ER3)

^aAll studies were performed in batch reactors with thiosulfate electron donor,

^b Stated as maximum specific denitrification rate

The denitrification rate of ER1 was 716 mg N/l d, which was slightly lower than ER2 and higher than ER3. The denitrification rate of ER1 is also in the range of literature studies, represented in Table 4.4. Specific denitrification rates followed the same order, being highest in ER2 and followed by ER1 and then ER3.

Campos et al. (2008) stated that the results of the enrichment studies with the same initial conditions could be different due to the origin of the seed sludge. Although electron donor and initial conditions of all ERs were similar, different denitrification trend was observed for ER3. A similar trend could be seen for ER1 and ER2.

Nitrogen removal from wastewaters is one of the aims of advanced biological treatment plants (Burdick et al., 1982). Both ER1 and ER3 sludges were originated from biological nutrient removal (BNR) operations. Yet, ER1 was collected from an urban WWTP's BNR while ER3 was taken from a leather industry treatment plant's BNR.

Sulfide is used as a treatment agent in beamhouse process of leather industry (Ozgunay et al., 2007), besides nitrate content is expected in leather industry wastewater (Bharagava et al., 2018). Therefore ER3 sludge was expected to be accustomed to sulfur and nitrate sources; however, the lowest denitrification rate (397 mgN/l.d) was obtained for ER3. On the other hand, the highest removal efficiency (97.6%) and the lowest discrepancy (8.9%) value was also obtained from ER3 (Table 4.3). This can be attributed to the microbial consortium, which is capable of high nitrate removal but with a lower denitrification rate. On the other hand, denitrification rate of ER3 was still in the range of illustrated literature data in Table 4.4.

The highest denitrification rate (770 mg N/l.d) was obtained in ER2. The seed source of ER2 was a domestic wastewater treatment plant. Typical domestic wastewater contains nitrogen-containing substances as well as organic substances (Tchobanoglous et al., 2003). Due to its organic content, heterotrophic denitrification was expected to be the mechanism responsible for denitrification. However it should be noted that the synthetic medium (Section 3.3), which is used for the enrichment operation, was not containing any organic substances. Therefore, heterotrophic denitrification was not expected for our study. This

mechanism is further discussed in Section 4.3.1. The reason of the higher denitrification ratio in ER2 could be attributed to its microbial consortium enriched in the system.

To conclude, enrichment reactor performances and kinetic studies revealed that all four enriched cultures (EC1 to EC4) have high removal efficiencies above 87%. EC1, EC2 and EC3 (from ER1, ER2 and ER3, respectively) have comparable denitrification and specific denitrification rates, with literature data. All four ECs can be used in the bio-desulfurization sets. In order to see the effect of these 4 seed sludge types on bio-desulfurization, this time from GTR, Set-2 was conducted. In Set-1, the EC1 with high removal efficiency, relatively low discrepancy, high rate and high amount of seed sludge was used.

4.2 Results of Set-1: The Effect of Particle Size on Bio-desulfurization Efficiency

The effect of particle size on bio-desulfurization efficiency was investigated to determine the optimum GTR particle size to use in the following sets of this thesis study. The effect of microorganisms could be enhanced by increasing the surface area where they are attached. Therefore, grinding the waste tire to smaller particle sizes is crucial. Ghavipankeh et al. (2018) stated that the particle size is the most important parameter among the properties they investigated. However, grinding rubber to smaller particle sizes is a costly process. In this context, optimum particle size could be defined as the maximum size, which has the bio-desulfurization efficiency that is insignificantly (statistically) different than those obtained using smaller sizes.

An increase in the amount of sulfur-containing product (SO_4^{2-}) in the medium could be used as an indication of bio-desulfurization (Torma and Raghavan, 1990; Christiansson et al., 1998; Romine and Romine, 1998; Jiang et al., 2010). According to the reactions given in Equation 4.1 and Equation 4.2 (Section 4.2),

SO_4^{2-} must be produced through autotrophic denitrification, where NO_3^- is consumed. Therefore, NO_3^- -N and SO_4^{2-} -S concentrations were measured in order to check the bio-desulfurization of GTR and the results are given in Table 4.5. Four different particle size ranges were treated with EC1 for 20 days.

As seen from Table 4.5, NO_3^- -N was depleted at the end of the experiment in both blank and test reactors, while SO_4^{2-} -S concentration did not increase. Although there was no seed sludge in the blank reactors, the nitrate was completely consumed, indicating the presence of an abiotic reaction.

In order to determine the possible mechanisms that caused the abiotic consumption of nitrate, the composition of the GTR was further analyzed. However, the information about the composition of GTR used in the experimental studies was not available; therefore, the composition of a typical tire was used instead. The composition of a typical truck tire is given in Table 4.6.

Table 4.5 NO_3^- -N and SO_4^{2-} -S concentrations at t=0 and t=20 of Set-1

Reactor Name	NO_3^- -N (mg/L)		SO_4^{2-} -S (mg/L)	
	t=0	t=20	t=0	t=20
Control	205±1.7	192±10.9	80±8.5	78±7.8
B1 (75-400µm)	197±0.1	0	11±0.3	12±1.1
T1 (75-400µm)	205±1.7	0	80±8.5	69±0.8
B2 (400-589µm)	197±0.1	0	11±0.3	11±0.1
T2 (400-589µm)	205±1.7	0	80±8.5	70±2.3
B3 (589-841µm)	197±0.1	0	11±0.3	12±0.0
T3 (589-841µm)	205±1.7	0	80±8.5	73±2.6
B4 (75-1180µm)	197±0.1	0	11±0.3	6±0.6
T4 (75-1180µm)	205±1.7	0	80±8.5	70±0.8

Paraffinic and microcrystalline waxes are used in order to protect the tire from the ozone attack. The protection mechanism could be explained with the protective layer that is generated through the migration of the paraffinic and microcrystalline waxes to the surface due to their poor solubility (Brendan, 2004). Microorganisms

are sensitive to these additives used in rubber (Sato et al., 2004). By removing the additives from the surface, which is obtained by the detoxification process, inhibition of microorganisms is prevented (Bredberg et al., 2002). In Set-1, in order to remove these additives from the surface, the detoxification process was previously performed by keeping the GTR in a solution containing 75% ethanol by volume for 24 hours, as stated in literature (Jiang et al., 2010; Jiang et al., 2011; Li et al., 2011; Li et al., 2012a; Li et al., 2012b; Li et al., 2013; Hu et al., 2014; Yao et al., 2014; Cui et al., 2016). Although detoxification had been performed, abiotic consumption was still observed. This was attributed to the additives, which could not be removed via the detoxification process due to the non-effectiveness of the procedure. Therefore, the detoxification procedure was modified by increasing the time from 24 hr to 72 hr (Hu et al., 2016). According to this new procedure, Preliminary Study 1 was conducted (Appendix B). Results of Preliminary Study 1 revealed that change in detoxification procedure did not prevent the abiotic consumption of nitrate. Therefore, Preliminary Study 2 was conducted to see the effect of excess nitrate (835 mg/L as NO_3^- -N) on abiotic consumption. The results of the second preliminary study revealed that excess nitrate in the feeding medium could overcome the problem associated with abiotic consumption of nitrate that is after abiotic nitrate consumption, there would be still nitrate in the medium available for autotrophic denitrification and bio-desulfurization. Therefore, as a result, Set-1 and Preliminary Studies 1 and 2 indicated that abiotic nitrate consumption is critical and autotrophic denitrification would be possible only if enough nitrate is supplied. Unfortunately, the effect of particle size would not be determined in Set-1.

Table 4.6 Composition of a typical truck tire tread (Rodgers and Waddell, 2013)

Component	Weight (phr) ^a
Natural Rubber	100
Carbon black	50
Paraffin wax	1
Microcrystalline wax	1
Aromatic oil	3
Poly Dihydro trimethylquinoline	1
Stearic acid	2
Zinc oxide	5
Sulfur	1.2
Other	1.8

^a phr: Parts per hundred rubber

4.3 Results of Set-2: The Effect of Seed Sludge Type and Electron Donor on Bio- desulfurization Efficiency

This study (Set-2) was conducted to investigate the effect of seed sludge type and electron donor on bio-desulfurization. Accordingly, enriched cultures (ECs) from three different sources were compared with each other. In other words, EC1 (enriched from Paşakoy Advanced Biological WWTP sludge), EC2 (enriched from Ankara Central Domestic WWTP sludge) and EC3 (enriched from Leather Organized Industrial Zone WWTP sludge) fed with $S_2O_3^{2-}$ were used. In addition, EC4, which was enriched from Paşakoy Advanced Biological WWTP like EC1 but acclimated to S^0 , was also used to compare with EC1 and then investigate e-donor effect. Batch reactors were operated for 20 days.

GTR1, which has the particle size range of 75-1180 μm and average particle size of 712 μm was used in Set1. In Set-2, for not having enough GTR1, GTR2 which has a lower particle size range (between 150-850 μ) was used. Therefore, the effect of particle size could not be investigated in this set. However, in Preliminary Study 1, the particle size effect was studied for GTR2 with 2 size ranges as 150-416 μ and

150-850 μ (Appendix B). The results had shown that particle size range difference did not affect the nitrate removal performance. Therefore, in Set-2, the range of 150-850 μ (average of 426 μ) was used.

4.3.1 Bio-desulfurization Performance of Set-2

Initial and effluent concentrations of NO_3^- -N, SO_4^{2-} -S, and NO_2^- -N were measured for all reactors of Set-2. Change in the concentrations was calculated by subtracting the initial concentrations of the ions from the effluent concentration, and given in Figure 4.8. Unlike the previous studies, Set-1, Preliminary Studies 1 and Preliminary Study 2 (Appendix B and Appendix C), NO_2^- -N was detected in the effluent of Set-2 reactors. Therefore, NO_2^- -N concentrations were also discussed in the results.

In order to simplify the illustration, the change in the NO_3^- -N, SO_4^{2-} -S, and NO_2^- -N concentrations of the reactors conducted with the same seed sludge type were represented together (Figure 4.8). As an example, S2-1 label in the axis of Figure 4.8 corresponds to Blank (B), Control (C1) and Test (T1) batch reactors inoculated with EC1. Similarly, S2-2 label corresponds to B, C2 and T2 batch reactors inoculated with EC2. Blank (B) was used to measure the abiotic consumption of NO_3^- -N by GTR. Since the GTR type used in Set-2 was the same (GTR2), only one blank reactor (in triplicate) was conducted for all different reactor types.

Control reactors were conducted to indicate any potential background matrix due to seed sludge. Although S source was not added to the synthetic medium of Set-2, there might be still some S source ($\text{S}_2\text{O}_3^{2-}$ or S^0) left in the seed sludge resultant of enrichment studies. This phenomenon, was more likely to happen for S2-4, which was inoculated with EC4. S^0 was used as an electron donor in EC4 operation, and it was fed to the reactor with higher concentrations than the stoichiometric value, in order to increase the denitrification rate (Hashimoto et al., 1987; Sierra-Alvarez et

al., 2007; Şahinkaya et al., 2015). As a result, an increase in the SO_4^{2-} -S concentration of control reactors could be expected, especially in S2-4.

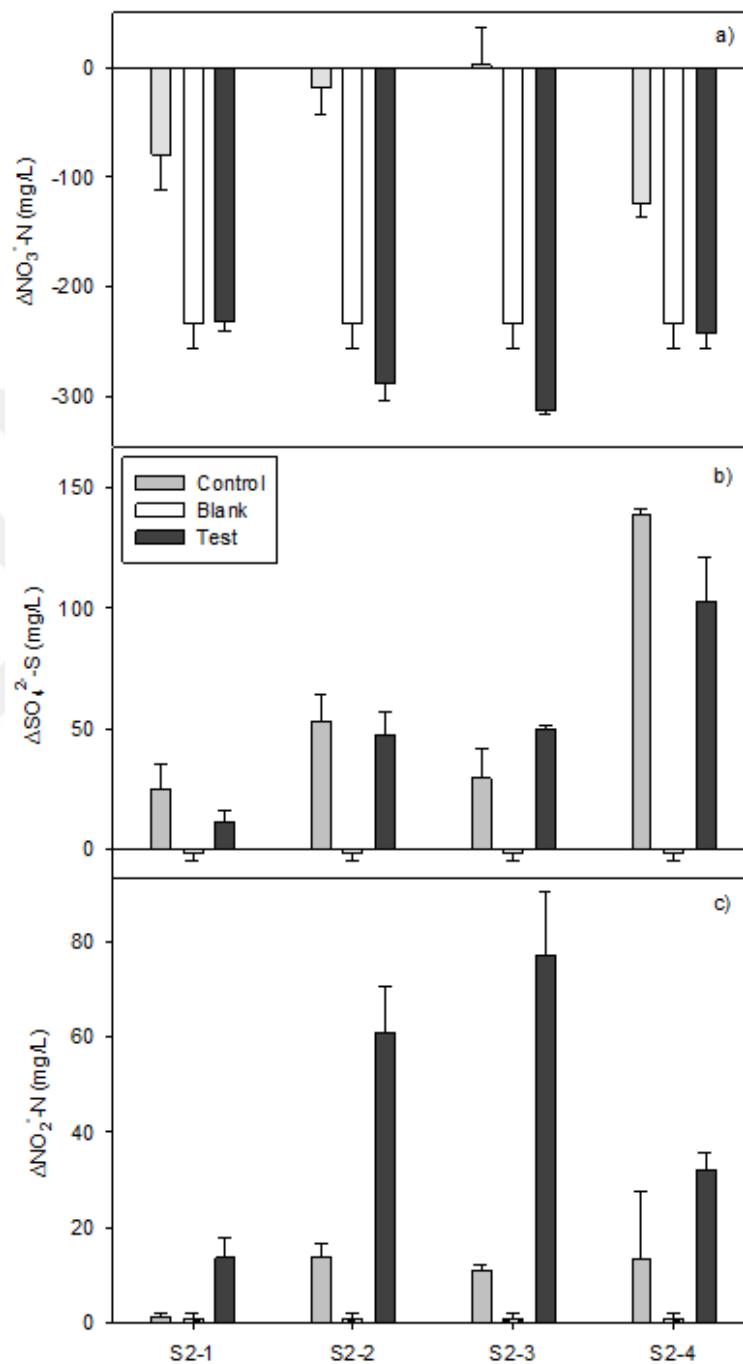


Figure 4.8 Changes in average a) $\text{NO}_3^- \text{-N}$, b) $\text{SO}_4^{2-} \text{-S}$ and c) $\text{NO}_2^- \text{-N}$ concentrations in Set-2

According to the results of S2-1, NO_3^- -N concentrations of both B and T1 reactors decreased by 233 ± 23 and 232 ± 10 mg/L, respectively (Figure 4.8a). Decrease in NO_3^- -N concentration of B was attributed to abiotic consumption. NO_3^- -N decrease in T1 was expected to be higher than that of B, if the removal of nitrate were due to a different mechanism in addition to abiotic consumption. However, NO_3^- -N decrease in B was slightly higher than that of T1. This indicated that there was not another reaction other than abiotic consumption. NO_2^- -N concentration of T1, on the other hand, slightly increased (by 14 ± 4 mg/L), while B and C1 remained unchanged (Figure 4.8c). According to the reduction mechanism of NO_3^- to N_2^- , as mentioned in Section 4.1.3, NO_2^- was produced as an intermediate product (Section 4.1.4) (Francis et al., 2007; Cui et al., 2019). Therefore, an increase in NO_2^- -N concentration could be attributed to an incomplete reduction of NO_3^- . As seen in Figure 4.8b, the effluent SO_4^{2-} -S concentration of reactor B was approximately the same with its initial concentration, while both effluent SO_4^{2-} -S concentrations of C1 and T1 increased as 25 ± 10 and 11 ± 5 mg/L, respectively. In order to determine the exact change in a parameter's concentration resultant of bio-desulfurization of GTR, that is, free of seed sludge effect (as in Control reactors) and abiotic reaction effect (as in B reactors), the total change in the concentrations of parameter of concern in B and C reactors were subtracted from that of T reactor; the final concentration was defined as 'net' concentration. For example 'net' ΔSO_4^{2-} -S concentration can be calculated by subtracting the total ΔSO_4^{2-} -S concentration in Control (C1) and Blank (B1) reactors from that of in Test (T1) reactor. In this experiment, an increase in SO_4^{2-} -S concentration of C1 was higher than T1, which gives a negative result (-12 mg/L) for net ΔSO_4^{2-} -S. Negative result conflicts with sulfur-driven (GTR based) autotrophic denitrification reaction mechanism, in which SO_4^{2-} must be produced as a product. Negative sulfate concentrations would be further discussed.

S2-2 results revealed that NO_3^- -N concentrations of both B and T2 reactors decreased as 233 ± 23 and 289 ± 15 mg/L, respectively. Higher decrease in NO_3^- -N concentration in test reactors is an indication of a different mechanism in addition

to abiotic consumption observed in B reactor, which could be attributed to sulfur-driven (GTR related) autotrophic denitrification reaction. ΔNO_2^- -N concentration of T2 was 61 ± 10 mg/L which was significantly higher than that of C2 (14 ± 3 mg/L). An increase in NO_2^- -N concentration could be attributed to an incomplete reduction of NO_3^- . However, SO_4^{2-} -S production of T2 (47 ± 10 mg/L) was slightly lower than C2 (53 ± 11 mg/L), which was expected to be higher. Therefore, net ΔSO_4^{2-} -S was -4 mg/L, which conflicts with the sulfur-driven (GTR related) autotrophic denitrification reaction.

As seen in Figure 4.8, the biggest change in NO_3^- -N and NO_2^- -N concentrations among the test reactors was seen in T3, as 313 ± 4 and 77 ± 13 mg/L, respectively. Both ΔNO_3^- -N and ΔNO_2^- -N of T3 were higher than those of C3 and B. Moreover, ΔSO_4^{2-} -S of T3 (50 ± 2 mg/L) was higher than that of C3 (29 ± 13 mg/L). Considering the experimental results, the change in SO_4^{2-} -S and NO_3^- -N concentrations might be attributed to sulfur-driven (GTR based) autotrophic denitrification.

The results of S2-4 revealed that the change in NO_3^- -N and SO_4^{2-} -S concentrations of C4 were the highest among the control reactors, as 124 ± 12 and 138 ± 3 mg/L, respectively. The reason for high concentration changes in C4 was attributed to the enriched culture (EC4) that was used as inoculum for S2-4. As mentioned earlier (Section 3.4.2), the electron donor of EC4, that was S^0 , was fed more than the stoichiometric value to the reactor in order to increase the denitrification rate (Hashimoto et al., 1987; Sierra-Alvarez et al., 2007; Şahinkaya et al., 2015). Therefore, high concentration changes in C4 was attributed to the excess S^0 which could have been left in the seed sludge (ER4) and thus in C4 of Set-2, and caused a decrease in NO_3^- -N concentration through S^0 -driven (EC related) autotrophic denitrification. Accordingly, SO_4^{2-} -S concentration increased in C4. The ΔNO_3^- -N of T4 (243 ± 12 mg/L) was slightly higher than that of B (233 ± 23 mg/L). Besides, ΔNO_2^- -N of T4 (32 ± 3 mg/L) was higher than that of C4 (13 ± 14 mg/L). These might indicate potential bio-desulfurization. However, a negative result for net

$\Delta\text{SO}_4^{2-}\text{-S}$ (-34 mg/L) conflicts with the sulfur-driven (GTR related) autotrophic denitrification, where net $\Delta\text{SO}_4^{2-}\text{-S}$ is expected to increase.

Du et al. (2016) explained the major reason for the aforementioned incomplete denitrification (accumulation of $\text{NO}_2^- \text{-N}$) with the reduction rates. Accumulation of $\text{NO}_2^- \text{-N}$ might have occurred due to its lower reduction rate than $\text{NO}_3^- \text{-N}$.

The average initial and effluent pH values of the test reactors (T1, T2, T3 and T4) were measured as given in Figure 4.9. As seen in Figure 4.9, the effluent pH values of all reactors slightly increased. It should be noted that $\text{S}_2\text{O}_3^{2-}$ and/or S^0 -driven autotrophic denitrification, as shown in Equation 4.1 and 4.2 (Section 4.1), is known to result in pH decrease.

Overall, the results of Set-2 could not be explained with the $\text{S}_2\text{O}_3^{2-}$ and/or S^0 -driven autotrophic denitrification, given in Equation 4.1 and/or Equation 4.2, which were well describing the conditions in the enrichment operations. Because Equation 4.1 and Equation 4.2 do not indicate any NO_2^- production and pH increase, both of which were the cases observed in Set-2. It should be noted that Sato et al. (2004) explained the microbial scission as a selectively breakage of **monosulfide** bonds from the main chain of the rubber. Therefore two other denitrification reactions, where HS^- (sulfide) is used as S source, were proposed instead of Equation 4.1 and 4.2. The proposed mechanism includes sulfide-driven complete and incomplete autotrophic denitrification and are given in Equation 4.3 (Mora et al., 2015, Cui et al., 2019) and Equation 4.4, respectively (Cardoso et al., 2006; An et al., 2010; Tang et al., 2010; Sun, 2012; Lee and Wong, 2014). These two reactions might have occurred in the reactors.

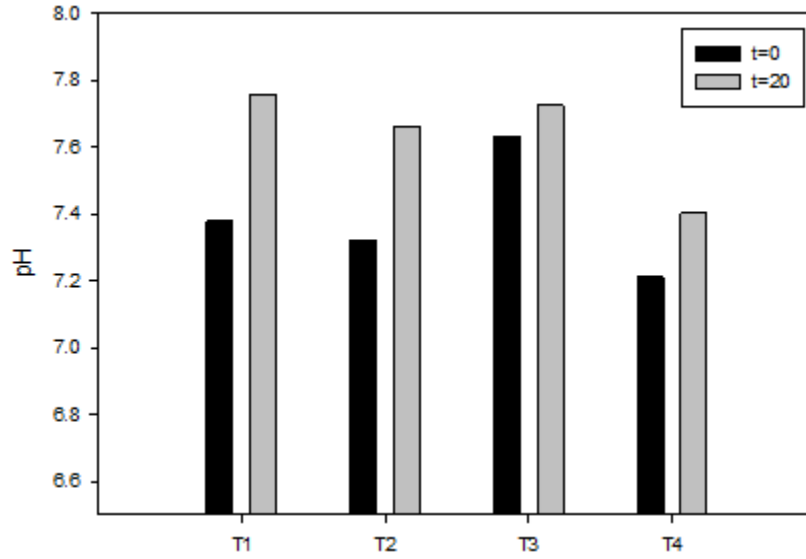
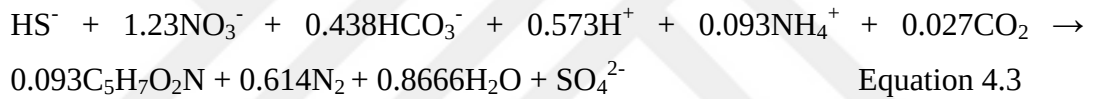


Figure 4.9 Average initial (t=0) and effluent (t=20) pH values of T1, T2, T3 and T4



According to Equation 4.3 and Equation 4.4, autotrophic denitrifiers oxidize sulfide crosslinks to sulfate, and nitrate is used as an electron acceptor. Nitrite is produced due to incomplete denitrification (Equation 4.4). In order to compare the results of Set-2 with the proposed reactions, net concentrations of the test reactors should be calculated by excluding the control and blank reactor effects. This calculation was made by subtracting the average ΔNO_3^- -N, ΔSO_4^{2-} -S and ΔNO_2^- -N concentrations of control (C_{av}) and blank (B_{av}) reactors from those of the corresponding test reactors (T_{av}), as seen in Equation 4.5. The net concentrations of the test reactors were calculated and given in Table 4.7.

$$\text{Net T} = T_{av} - B_{av} - C_{av} \quad \text{Equation 4.5}$$

Table 4.7 Net concentrations of the test reactors in Set-2

Reactor Name	Net Concentrations (mg/L)		
	$\Delta\text{NO}_3^- \text{-N}$	$\Delta\text{SO}_4^{2-} \text{-S}$	$\Delta\text{NO}_2^- \text{-N}$
T1	81	-12	11
T2	-37	-4	46
T3	-82	22	65
T4	115	-34	18

According to Equation 4.3 and 4.4, NO_3^- is used as an electron acceptor, which means a decrease in $\text{NO}_3^- \text{-N}$ concentration is expected. As seen in Table 4.7, a decrease in $\text{NO}_3^- \text{-N}$ concentrations was only seen in T2 (by -37 mg/L) and T3 (by -82 mg/L). In addition, $\text{NO}_2^- \text{-N}$ concentration of both T1 (by 46 mg/L) and T2 (by 65 mg/L) reactors increased, which is consistent with the proposed mechanism (Equation 4.4). The $\text{SO}_4^{2-} \text{-S}$ concentration of T3 (by 22 mg/L) increased. However, $\text{SO}_4^{2-} \text{-S}$ concentration of T2 did not increase, which conflicts with the proposed mechanism.

It is seen Table 4.7 that the net $\Delta\text{NO}_3^- \text{-N}$ concentrations of both T1 (81 mg/L) and T4 (115 mg/L) were positive, which means their concentrations increased. In addition, net $\Delta\text{SO}_4^{2-} \text{-S}$ concentrations of both T1 (-12 mg/L) and T4 (-34 mg/L) were negative, which was expected to increase according to Equation 4.3 and 4.4. Although $\text{NO}_2^- \text{-N}$ concentrations of both T1 (11 mg/L) and T4 (18 mg/L) were consistent with the proposed reactions(s), net $\text{NO}_3^- \text{-N}$ and net $\text{SO}_4^{2-} \text{-S}$ results conflicts with the reactions.

The conflict in the $\text{SO}_4^{2-} \text{-S}$ concentration of T2 reactor, was explained with zinc sulfate formation. As seen in Table 4.6 (Section 4.2), a typical truck tire contains 5 phr of zinc oxide (Rodgers and Waddell, 2013). Although different metal ions could be present in the tire formula, such as; aluminum, magnesium and barium, only zinc oxide was discussed due to its higher amount in the tire. Zinc oxide and zinc salts, which are present in a tire, can leach out through the desulfurization process, and they can react with the sulfate, where zinc sulfate precipitate is

produced accordingly. Bredberg et al. (2001) analyzed the precipitate in the flasks and found that the precipitate was composed of zinc and sulfur. As a result, the $\text{SO}_4^{2-}\text{-S}$ concentration could have decreased due to zinc sulfate formation. The conflict in the net $\Delta\text{SO}_4^{2-}\text{-S}$ concentration of T2 reactor could be attributed to zinc sulfate formation. Possible decrease in $\text{SO}_4^{2-}\text{-S}$ concentration through zinc sulfate formation was calculated as 24 mg/L (Appendix H). In order to simplify the discussion, the decrease in $\text{SO}_4^{2-}\text{-S}$ concentration (24 mg/L) through zinc sulfate formation was represented as Z_{SO_4} .

The net $\Delta\text{SO}_4^{2-}\text{-S}$ concentrations of the test reactors were calculated after the addition of Z_{SO_4} (24 mg/L) effect and given in Table 4.8. When Z_{SO_4} was taken into consideration, T2 reactor showed a similar increasing trend with the proposed reaction. T3 reactor was still consistent with the proposed reaction, after the addition of Z_{SO_4} effect. The proposed reactions possible to occur in T2 and T3 are further discussed below, considering the stoichiometric ratios (Section 4.3.1.1).

Although net $\Delta\text{SO}_4^{2-}\text{-S}$ concentration of T1 became positive after the addition of Z_{SO_4} effect, net $\Delta\text{NO}_3^-\text{-N}$ concentration still conflicts with the proposed reactions. In addition, the net $\Delta\text{NO}_3^-\text{-N}$ and $\Delta\text{SO}_4^{2-}\text{-S}$ concentrations of T4 reactor were not consistent with the proposed reactions still after the addition of Z_{SO_4} effect.

Table 4.8 Net concentrations of the test reactors after the assumption of ZnSO_4 formation

Reactor Name	Net Concentrations (mg/L)		
	$\Delta\text{NO}_3^-\text{-N}$	$\Delta\text{SO}_4^{2-}\text{-S}^a$	$\Delta\text{NO}_2^-\text{-N}$
T1	81	12	11
T2	-37	20	46
T3	-82	46	65
T4	115	-10	18

^a The effect of ZnSO_4 formation was added

In order to investigate the possibility of the proposed reactions, the stoichiometric ratios of the reactions were compared with the experimental ratios. The

calculations were made according to the net concentrations of the test reactors, with and without Z_{SO_4} effect (Table 4.7 and Table 4.8). The net $\Delta NO_3^- - N / \Delta NO_2^- - N$, $\Delta NO_3^- - N / \Delta SO_4^{2-} - S$ molar ratios were illustrated in Table 4.9. According to Equation 4.4, the $\Delta NO_3^- - N / \Delta NO_2^- - N$ molar ratio could be -1.00. However, $\Delta NO_3^- - N / \Delta NO_2^- - N$ molar ratio is not applicable for Equation 4.3. According to Equation 4.3 and 4.4, the $\Delta NO_3^- - N / \Delta SO_4^{2-} - S$ molar ratio could be -1.23 and -4.00, respectively. The discrepancy term was used to define the difference between the experimental and theoretical (proposed reactions) molar ratios. The calculation was made with the nearest theoretical ratio (NTR) and experimental ratio (ExR) using Equation 4.6, and results are given in Table 4.10.

As seen in Table 4.9, molar ratios of T1 and T4 reactors were not comparable to those of the proposed reactions either with or without the Z_{SO_4} effect. The reason of this situation is due to the negative net $\Delta NO_3^- - N$ (Table 4.7 and 4.8) value. Therefore discrepancy values of T1 and T4 were not calculated and were not included in Table 4.10.

The $\Delta NO_3^- - N / \Delta NO_2^- - N$ molar ratio of T2 and T3 reactors were close to the theoretical ratio of the proposed reaction, especially Equation 4.4. 20% discrepancy and 25% discrepancy were calculated for T2 and T3, respectively.

Table 4.9 The net $\Delta NO_3^- - N / \Delta NO_2^- - N$ and net $\Delta NO_3^- - N / \Delta SO_4^{2-} - S$ molar ratios of the test reactors and proposed reactions

Reactor Name	$\Delta NO_3^- - N / \Delta NO_2^- - N$ Molar Ratio	$\Delta NO_3^- - N / \Delta SO_4^{2-} - S$ Molar Ratio	
		Without Z_{SO_4} effect	With Z_{SO_4} effect
T1	7.08	-16.01	14.87
T2	-0.80	20.53	-4.24
T3	-1.25	-8.44	-4.05
T4	6.32	-7.79	-27.11
Equation 4.3	-	-1.23	-1.23
Equation 4.4	-1.00	-4.00	-4.00

The discrepancy value of $\Delta\text{NO}_3^- \text{-N} / \Delta\text{SO}_4^{2-} \text{-S}$ molar ratio for T2 and T3 reactors with the Z_{SO_4} effect was calculated as 6 and 1%, respectively (Equation 4.6). However, the discrepancy value for T3 reactor increases to 111% without the effect of Z_{SO_4} and $\Delta\text{NO}_3^- \text{-N} / \Delta\text{SO}_4^{2-} \text{-S}$ ratio of T2 is not comparable with the proposed mechanism, due to the negative net $\Delta\text{SO}_4^{2-} \text{-S}$ value.

$$\text{Discrepancy (\%)} = \frac{|\text{NTR} - \text{ExR}|}{|\text{NTR}|} * 100 \quad \text{Equation 4.6}$$

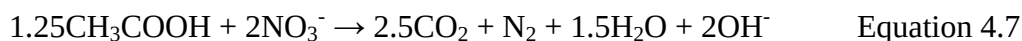
To conclude, the proposed reaction, Equation 4.4 and experimental results ($\Delta\text{NO}_3^- \text{-N} / \Delta\text{NO}_2^- \text{-N}$, $\Delta\text{NO}_3^- \text{-N} / \Delta\text{SO}_4^{2-} \text{-S}$ molar ratios) for T2 and T3 reactors were close when the effect of ZnSO_4 formation was taken into account. In other words, it is likely that Equation 4.4 might have taken place in addition to the ZnSO_4 formation in T2 and T3 reactors. Bio-desulfurization from GTR might have been achieved through sulfide-driven autotrophic denitrification, especially via Equation 4.4.

Table 4.10 Discrepancies between the theoretical and experimental net $\Delta\text{NO}_3^- \text{-N} / \Delta\text{NO}_2^- \text{-N}$, $\Delta\text{NO}_3^- \text{-N} / \Delta\text{SO}_4^{2-} \text{-S}$ molar ratios of the proposed reactions

Reactor Name	$\Delta\text{NO}_3^- \text{-N} / \Delta\text{NO}_2^- \text{-N}$ Discrepancy (%)	$\Delta\text{NO}_3^- \text{-N} / \Delta\text{SO}_4^{2-} \text{-S}$ Discrepancy (%)	
		Without Z_{SO_4} effect	With Z_{SO_4} effect
T2	20	-	6
T3	25	111	1

Other Possible Mechanisms

In addition to the autotrophic process, denitrification can also occur via heterotrophic denitrification. Organic carbon source is used as an electron donor in heterotrophic denitrification, and nitrate is converted to nitrogen gas (Chung et al., 2014). A possible stoichiometric reaction for heterotrophic denitrification is given in Equation 4.7 (Reyes-Avila et al., 2004).



In this study, total organic carbon (TOC) was measured in order to observe the effect of heterotrophic denitrification and results are given in Figure 4.10. As seen in Figure 4.10, the TOC value of the test reactors did not decrease at the end of the experiment, which means heterotrophic denitrification did not occur. On the other hand, an increase in the TOC value was observed in all test reactors. This situation could be explained with the possible organic chemicals leaching from GTR through bio-desulfurization and/or abiotic reaction. An increase in the TOC value should be investigated in detail further.

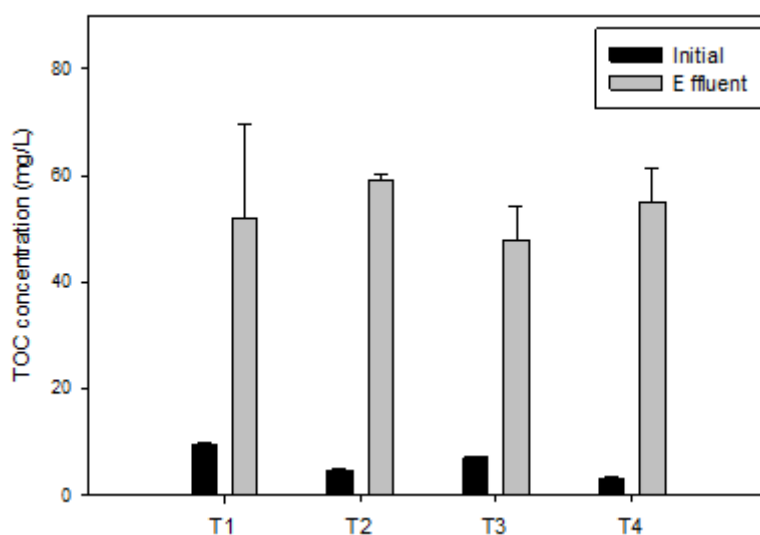


Figure 4.10 Initial and effluent TOC values of the test reactors in Set-2

Dissimilatory nitrate reduction to ammonium, which is an anaerobic process, was also investigated (Silver et al., 2001). NH_4^+ concentrations of the reactors were measured and results are given in Figure 4.11.

As seen in Figure 4.11, NH_4^+ concentrations of the test reactors did not increase at the end of the experiment. Instead, NH_4^+ concentration decreased at the end of the experiment, which could be attributed to the microbial growth-related ammonium

consumption, as shown in Equation 4.3. According to the sulfide-driven autotrophic denitrification mechanism (Equation 4.3), NH_4^+ was used, which caused a decrease in its concentration. Therefore, it could be concluded that dissimilatory nitrate reduction did not occur in this study.

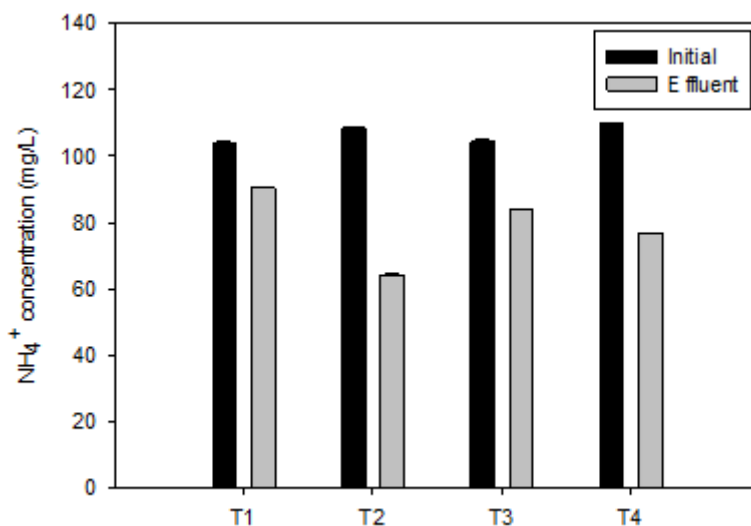


Figure 4.11 Change in the NH_4^+ concentrations of the test reactors is Set-2

To conclude, nitrate removal under anoxic conditions of Set-2 was not related to the heterotrophic denitrification and dissimilatory nitrate reduction. Thus, as proposed, the decrease in nitrate removal and the corresponding increase in sulfate concentration, following the stoichiometric ratios, were attributed to bio-desulfurization through sulfide-driven autotrophic denitrification.

The bio-desulfurization of rubber increases with the increasing sulfate concentration. Therefore, high $\Delta\text{SO}_4^{2-}\text{-S}$, which corresponds to higher sulfur removal from GTR was used as selection criteria. Accordingly, although similar results ($\Delta\text{NO}_3^- \text{-N} / \Delta\text{SO}_4^{2-}\text{-S}$ and $\Delta\text{NO}_3^- \text{-N} / \Delta\text{NO}_2^- \text{-N}$ molar ratio) were obtained for both T2 and T3 reactors, T3 was chosen as the most efficient reactor, due to higher $\Delta\text{SO}_4^{2-}\text{-S}$ concentration. Besides, the results obtained with the addition of ZnSO_4 effect were more consistent with the proposed reaction in T3. Apparently,

autotrophic denitrifier enriched from BNR treating leather industry wastewater was the most efficient in the bio-desulfurization of GTR. T2 reactor was selected as the second effective reactor.

4.3.1.1 Sulfur Conversion from GTR Regarding the $\Delta\text{SO}_4^{2-}\text{-S}$ Concentrations

Bredberg et al. (2001) stated that an increase in the amount of sulfur-containing product (sulfate) could be used to measure the desulfurization (bio-desulfurization). The sulfur conversion was calculated by using the net $\Delta\text{SO}_4^{2-}\text{-S}$ concentration data, with respect to the law of conversion of matter. Therefore, any $\text{SO}_4^{2-}\text{-S}$ produced was related to the sulfur desulfurized from GTR and react with nitrate via microbial means.

Based on the results of Preliminary Study 2, 1 g of GTR2, corresponding to 2.5% (w/v) of the effective volume of the reactor (40 ml), was used in Set-2 (Section 3.4.2.2). Besides sulfur content of GTR2 was determined by elemental analysis and found as 2.24 ± 0.13 wt%. According to these informations sulfur conversion (ΔS) was calculated with Equation 4.8.

$$\text{Sulfur Conv. } (\Delta\text{S})(\%) = \frac{(\Delta\text{SO}_4^{2-}\text{-S}) \frac{\text{mg}}{\text{L}} * \left(\frac{1000\text{mL}}{1\text{L}} \right) * \left(\frac{1\text{g}}{1000\text{mg}} \right)}{\left(\frac{2.5\text{ gGTR}}{100\text{ mL}} \right) * \left(\frac{2.24\text{ gS}}{100\text{ gGTR}} \right)} * 100 \quad \text{Equation 4.8}$$

The sulfur conversion from GTR, which corresponds to the increase in the $\Delta\text{SO}_4^{2-}\text{-S}$ concentration with and without the effect of Z_{SO_4} per g of S in GTR were calculated with Equation 4.8 for each test reactor of Set-2 and shown in Table 4.11. Besides, the results of the literature studies in which sulfur conversion was calculated through sulfate production are given in Table 4.11.

Table 4.11 Sulfur conversion percentages and its comparison with related literature

Sulfur conversion (%)		Reference
Without ZnSO ₄ formation	With ZnSO ₄ formation	
N.D.	2.2	This study - T1
N.D.	3.6	This study - T2
3.9	8.2	This study - T3
N.D.	N.D.	This study - T4
0.035	-	Torma&Raghavan (1991)
N.D.	-	Chritiansson et al. (1998)
1	-	
5.1	-	
16	-	Jiang et al. (2010)
21.9	-	Sato et al. (2004)

^a N.D.: Not detected

As seen in Table 4.11, sulfur conversion percentages range from 0.035 to 21.9%. This wide range in the conversion values is related to the conditions of the conducted studies. The material (GTR, NR, WLR) used in the bio-desulfurization study, particle size, reaction time and type of the microorganisms are the primary parameters that affect the conversion ratios. In addition, although control samples/reactors are crucial to calculate the correct results, some of the research studies did not include the control experiments which could result in an overestimation of the sulfur conversion value (Kim and Park, 1999; Jiang et al., 2010). Sato et al. (2004) investigated the fungal bio-desulfurization of Natural Rubber (NR) sheets for 200 days and observed 21.9% conversion of sulfur. Jiang et al. (2010) reported a 16% decrease in the sulfur content of pulverized NR by 20 days of treatment. However, control reactors were not conducted and the effect of seed sludge on sulfate concentration was not included in the results of that study.

The sulfur conversion results of the Set-2 also revealed that T3 with the highest conversion percentages was the most effective reactor among the test reactors. Besides, T3 was the only reactor that sulfur conversion was achieved without Z_{SO_4} effect, that is without $ZnSO_4$ formation and conversion percentage (3.9%) was in the range of the literature results (0-21.9%). If the $ZnSO_4$ formation is taken into consideration, T1 and T2 reactors have 2.2 and 3.6% of sulfur conversion, respectively.

As mentioned previously, the possible decrease in SO_4^{2-} -S concentration (Z_{SO_4}) through zinc sulfate formation was calculated by assuming the penetration depth for microorganisms as 6 μm (Appendix H). The penetration depth of the microorganisms can be calculated by using the sulfur conversion value (Romine and Romine, 1998). According to the sulfur conversion percentages (Table 4.11), penetration depth of T1, T2 and T3 was calculated by using Equation A.1 (Appendix A). The accessible volume fraction (V_{ac}) in Equation A.1 was taken as the sulfur conversion fraction. The penetration depth results are given in Table 4.12. As seen in Table 4.12, penetration depth for T3 with Z_{SO_4} was calculated as 5.9 μm which is close to the assumption made (6 μm). The penetration depth values for the reactors were comparable with literature studies, which were recorded between 2 to 6 μm (Romine and Romine, 1998; Hu et al., 2014; Cui et al., 2016; Hu et al., 2016).

Table 4.12 Sulfur conversion and corresponding penetration depth values of T1, T2 and T3 reactors

Reactor Name	Without Z_{SO_4}		With Z_{SO_4}	
	Sulfur Conversion (%)	Penetration Depth (μm)	Sulfur Conversion (%)	Penetration Depth (μm)
T1	-	-	2.2	1.5
T2	-	-	3.6	2.5
T3	3.9	2.7	8.2	5.9

4.3.2 Characterization of the GTR2 (Set-2)

The soluble (sol) fraction of the rubber sample, which is extracted by using the solvent, was investigated after the bio-desulfurization experiment. Although the sol fraction of GTR was expected to increase after bio-desulfurization, it could be the lowest increase among other desulfurization methods (Mangili et al., 2015), due to its superficial effect (Yao et al., 2013). Sol fraction that consists of both acetone and toluene extractable portion of the GTR, was calculated as total extractable material and results are given in Figure 4.12.

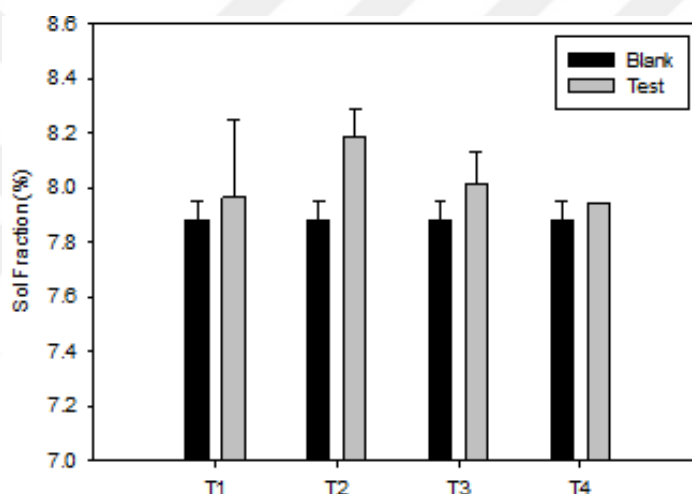


Figure 4.12 Sol fraction results of the blank and test reactors in Set-2 (T4 reactor was conducted in duplicate)

As seen in Figure 4.12, the sol fraction of test reactor samples (GTRs) were measured around 8%. However, only a small portion of this increase was due to bio-desulfurization. The effect of the blank reactor (containing only GTR and synthetic medium), as mentioned before, can be seen clearly with the sol fraction results of blank samples (7.86-8.00).

The sol fraction results of the test reactors were slightly higher than the blank reactors. This difference could be attributed to crosslink scission through bio-

desulfurization. The sol fraction differences between test and blank reactors are called as ΔSol and given in Table 4.13 with the related literature studies. As seen in Table 4.13, although sulfur removal from rubber was reported in literature studies, ΔSol values were low due to the superficial effect of microorganisms (Li et al., 2012b; Yao et al., 2013). ΔSol values of this thesis study were found to be low when compared with the ΔSol of the literature studies (Table 4.13). T2 reactor has the highest ΔSol value, then T3 has the second highest ΔSol result. Although T3 reactor was chosen as the best effective seed source according to sulfate results, the results of T2 were also higher when ZnSO_4 effect was included in results. This means that the effect of ZnSO_4 needs to be included in the calculations.

Table 4.13 Average ΔSol results of the test reactors and related literature

Sol Fraction (%)			Microorganism	Reference
Blank	Test	ΔSol^a		
2.30	3.10	0.80	<i>G. desulfuricans</i> 213E	Mangili et al. (2015)
8.67	9.13	0.46	<i>Acidithiobacillus Ferrooxidans</i>	Allan (2018)
1.95	2.64	0.69	<i>Alicyclobacillus</i> sp	Yao et al. (2013)
4.30	7.30	3.00	<i>Sphingomonas</i> sp.	Jiang et al. (2011)
4.69	7.43	2.74	<i>Thiobacillus ferrooxidans</i>	Li et al. (2011)
4.69	7.63	2.94	<i>Thiobacillus</i> sp. strain	Li et al. (2012a)
4.69	8.68	3.99	<i>Sphingomonas</i> sp.	Li et al. (2012b)
7.88	7.97	0.09	<i>Autotrophic Denitrifiers</i>	This Study-T1
7.88	8.19	0.31	<i>Autotrophic Denitrifiers</i>	This Study-T2
7.88	8.01	0.13	<i>Autotrophic Denitrifiers</i>	This Study-T3
7.88	7.94	0.06	<i>Autotrophic Denitrifiers</i>	This Study-T4

^a Difference in sol fraction between Test and Blank reactors

4.3.3 Electron Donor Effect

The electron donor effect was investigated by comparing the results of S2-1 and S2-4 reactors, which were inoculated with EC1 ($\text{S}_2\text{O}_3^{2-}$ electron donor) and EC4 (S^0 electron donor), respectively (Section 3.4.1). As seen in Table 4.7, net ΔNO_3^- -N of

T1 and T4 were 81 and 115 mg/L respectively. Besides, net $\Delta\text{SO}_4^{2-}\text{-S}$ of T1 and T4 were calculated as -12 and -34 mg/L without Z_{SO_4} effect and 12 and -10 mg/L with the effect of Z_{SO_4} , respectively. According to the proposed sulfide-driven complete and incomplete denitrification reactions (Equation 4.3 and 4.4), $\Delta\text{NO}_3^-\text{-N}$ is expected to be a negative value due to consumption. On the other hand, $\Delta\text{SO}_4^{2-}\text{-S}$ is expected to increase due to the conversion of sulfide to sulfate during bio-desulfurization. The proposed reactions could not explain the conditions occurring in T1 and T4 reactors. Therefore, it was not possible to compare the electron donor effect on the bio-desulfurization efficiencies.



CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

This thesis study aimed to enrich the autotrophic denitrifiers and investigate their use in the bio-desulfurization of GTR under anoxic conditions. The results and outcomes were summarized as follows;

Enrichment of sulfur-driven autotrophic denitrifiers from three different seed sludges, namely, S1 (BNR sludge of the Pasakoy Advanced Biological WWTP), S2 (Anaerobic digester effluent of Ankara Central Domestic WWTP) and S3 (BNR sludge of Leather Organized Industrial Zone WWTP) was successfully achieved.

The lowest NO_3^- -N removal efficiency among the ERs was obtained from ER4 (87.2%), which was acclimated with elemental sulfur (S^0) and S1 was used as a seed source. Removal efficiencies of ER1, ER2 and ER3, which were acclimated with $\text{S}_2\text{O}_3^{2-}$, and S1, S2 and S3 were used as seed source, were obtained as 96.0%, 96.9% and 97.6%, respectively.

The highest discrepancy ratio was calculated for ER4, by 22.7%. ER3, has the lowest discrepancy ratio (8.9%), which indicates the highest consistency with the $\text{S}_2\text{O}_3^{2-}$ -driven autotrophic denitrification mechanism. The discrepancy values of ER1 and ER2 were 11.4% and 13.2%, respectively.

Due to lower NO_3^- -N removal efficiencies and higher discrepancy ratios and susceptibility to shock loadings, ER4 was not included in the kinetic study.

Kinetic studies revealed that ER1 and ER2 had higher denitrification rates as 716 and 770 $\text{mgN}_2\text{-N/Ld}$ than ER3 (397 $\text{mgN}_2\text{-N/Ld}$). Although the highest removal efficiency (97.6%) and the lowest discrepancy (8.9%) value was

obtained for ER3, the denitrification rate was observed lower than others. This can be attributed to the microbial consortium, which is capable of high nitrate removal with a lower denitrification rate.

The abiotic consumption of nitrate could not be prevented by increasing the detoxification time from 24 h to 72 h.

Abiotic removal of NO_3^- -N was higher for GTRs with smaller particle sizes range with an average of 283 μm (Preliminary study 1). Besides abiotic removal of NO_3^- -N was proportional to the GTR amount. Abiotic NO_3^- -N removal of 2.5% and 5% GTR were 278 and 520 mg/L, respectively (Preliminary Study 2).

2.5 % w/v GTR amount was more efficient than 5 % w/v of GTR, due to lower abiotic NO_3^- -N removal. Besides, potential inhibitory chemicals leaching from the GTR would be lower for 2.5 % w/v of GTR.

Electron donor effect on bio-desulfurization could not be detected for EC1 ($\text{S}_2\text{O}_3^{2-}$ -fed) and EC4 (S^0 -fed) cultures.

The most effective seed source was determined as BNR sludge of leather industry wastewater with an 8.2% bio-desulfurization ratio.

Enrichment of the autotrophic denitrifiers was successfully achieved, with high removal rates and removal efficiencies. The increase in the net ΔSO_4^{2-} -S concentration proved that some part of the sulfur crosslinks on the surface of GTR was oxidized to SO_4^{2-} and bio-desulfurization was achieved. Nitrate removal under anoxic conditions was not related to the heterotrophic denitrification or dissimilatory nitrate reduction. As proposed, the decrease in nitrate removal and the corresponding increase in sulfate concentration, following the stoichiometric ratios, were attributed to bio-desulfurization through sulfide-driven autotrophic denitrification. It is likely that bio-desulfurization from GTR might have been

achieved through sulphide-driven autotrophic incomplete denitrification. In addition, ZnSO₄ might have been produced.

RECOMMENDATIONS

- ❖ A laboratory-made rubber compound with a known formula could be used, instead of a non-homogenous GTR, before the bio-desulfurization analyses. This will help the optimization of the process parameters before the GTR application and improve the understanding of the extent of the precipitation mechanism and potential ions taking place in the reaction.
- ❖ The abiotic nitrate reduction mechanism that occurs, during the bio-desulfurization process could be further investigated with a known formula of laboratory-made rubber compound.
- ❖ The effect of the substrate to inoculum (S/I) ratio on bio- desulfurization efficiency was not studied yet. Thus, S/I ratio should be further studied.
- ❖ According to the results of this study, sulfur removal from rubber is likely to occur via sulfide-driven autotrophic denitrification mechanism. Therefore, enrichment operation for S²⁻-driven autotrophic denitrification will be further investigated in order to see the effect of bio-desulfurization.
- ❖ Despite being comparable to the literature data, bio-desulfurization ratios obtained (3-8%) were low. Yet, this technique still can be used for some application areas because the optimum desulfurization ratio required changes according to the application area of the rubber. Therefore, product-specific usage areas can be defined for the bio-desulfurization products.
- ❖ After optimization with the abovementioned points and improve the bio-desulfurization efficiency, the experiments can be performed with nitrate-rich wastewater. With this approach, both valorization of nitrate and material recovery from GTR might be achieved.



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APPENDICES

A. Theoretical Maximum Accessible Sulfur Content and Average Particle Size Calculations

Although the sulfur content of GTR is constant for different particle sizes, accessible sulfur amount changes with respect to the total surface area and due to the limited effect of bacteria on the surface of GTR (Romine and Romine, 1998; Hu et al., 2014; Cui et al., 2016; Hu et al., 2016). There is an inverse relationship between the accessible sulfur amount and particle size of GTR. With decreasing particle size, available sites for microorganisms and accessible sulfur content of GTR increases. However, grinding GTR to lower particle sizes is a costly process; therefore, it is crucial to find the optimum particle size to achieve effective bio-desulfurization at a lower cost.

In this thesis study, GTR was sieved before its use in the bio-desulfurization sets. This process was essential for analyzing the effect of particle size and calculating the theoretical maximum accessible sulfur content of GTR. Besides, in order to determine the input NO_3^- -N concentration in bio-desulfurization sets, theoretical maximum accessible sulfur content of rubber must be calculated. This calculation was made by assuming the GTR particles as spherical and the penetration depth of microorganisms as 6 μm (Cui et al., 2016).

Illustration of the assumptions and equations used to calculate the theoretical maximum accessible sulfur content of GTR is given in Figure A.1 and Equation A.1, respectively. The sulfur contents of GTRs were analyzed with the LECO CHNS-932 analyzer in the Central Laboratory of Middle East Technical University. The maximum accessible sulfur content of GTR was calculated by multiplying the sulfur content with accessible volume fraction (Equation A.2).

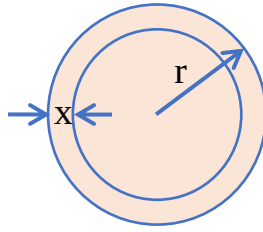


Figure A 1 Illustration of the GTR and penetration depth

r (μm)=Average particle radius of GTR

x (μm)= Penetration depth

V_{ac} = Accessible volume fraction

$$V_{ac} = 1 - \frac{(r-x)^3}{r^3} \quad \text{Equation A.1}$$

Ms = Sulfur content of GTR

Ms_{ac} = Maximum accessible sulfur content of GTR

$$Ms_{ac} = Ms * V_{ac} \quad \text{Equation A.2}$$

Average particle size (diameter) calculation was demonstrated (Equation A.3) by using the sieve analyses result of GTR1, given in Table A.1. Average particle sizes of GTR1 and GTR2 were calculated as 712 and 426 μm , respectively.

Table A 1.1 Sieve analyses result of GTR1

Size Range (μm)	Mean size (μm)	Mass Fraction (%)
0-75	37.5	0.00
75-125	100	0.79
125-175	150	1.74
175-400	287.5	3.19
400-595	497.5	13.15
595-841	718	63.50
841-1180	1010.5	17.63

$$\text{Average particle size (diameter) of GTR1 } (\mu\text{m}) = ((37.5*0) + (100*0.79) + \dots + (1010.5*17.5) + (1295*0.74)) / 100 = 712 \mu\text{m} \quad \text{Equation A.3}$$

The penetration depth was used as 6 μm as mentioned earlier. According to the Equation A.1 and assumption, the accessible volume fraction (V_{ac}) of GTR1 was calculated as 0.0494 (Equation 4.4), which means microorganism could be effective in 4.94 % of the outermost layer of GTR1. In other words, 4.94 % of the sulfur in GTR could be removed from GTR by microorganisms, through bio-desulfurization.

$$V_{ac} = 1 - \frac{(r-x)^3}{r^3} = 1 - \frac{(358-6)^3}{358^3} = 1 - 0.9506 = 0.0494 \quad \text{Equation A.4}$$

Sulphur content (M_s) of GTR1 was determined by elemental analysis (Section 3.5.2) and found as 2.00 ± 0.31 wt % (Section 3.1). The maximum accessible sulfur content of GTR1 (M_{sac}) was calculated by multiplying the sulfur content with accessible volume fraction and found as 0.988 mgS/gGTR (Equation 4.5).

$$M_{sac} = M_s * V_{ac} = 0.02 \frac{\text{gS}}{\text{gGTR}} * 0.0494 * \frac{1000\text{mg}}{1\text{g}} = 0.988 \frac{\text{mgS}}{\text{gGTR}} \quad \text{Equation A.5}$$

The accessible volume fraction and maximum accessible sulfur content of GTR2 was calculated with the same approach and found as 0.0821 and 1.840 mgS/gGTR, respectively.

Calculation of Initial NO_3^- Concentration

The initial KNO_3 concentration of Set-1 and Preliminary Study 1 was calculated with respect to the maximum accessible sulfur content of GTRs. Due to the usage of different particle sizes in Set-1 and Set-2, the lowest particle size was used for

calculations. The stoichiometric ratio of KNO_3/S , was found as 1.1, in Equation 4.2 (Section 4.1). The initial weight of both GTR1 and GTR2 in the reactors were 3.5 g. Considering the calculated maximum accessible sulfur contents of GTRs and amounts of GTRs in the reactors, the sulfur amount potential to be used through bio-desulfurization was found as 3.46 mg. The effective volume of the reactors was 70 ml; thus S concentration to be used during bio-desulfurization becomes 49.4 mg/L. Accordingly, the initial KNO_3 concentrations for Set-1 and Preliminary Study 1 were calculated as 1200 and 1800 mg/L, respectively.



B. Preliminary Study 1: The Effect of Detoxification on Abiotic Consumption of Nitrate

The results of Set-1 revealed that there might have been an abiotic consumption of nitrate by unreacted additives on the surface of GTR. Therefore, a preliminary study was performed to overcome the adverse effect of abiotic consumption of nitrate by modifying the detoxification method.

Detoxification procedure, performed by immersing GTR in 75% ethanol (v/v), was modified by increasing the time from 24 hr to 72 hr (Hu et al., 2016), and replacing the ethanol with fresh ethanol in every 24 hr. By this modification, it was aimed to remove any additives and chemicals found on the surface of GTR that cause the abiotic removal of nitrate added as an electron acceptor of bio-desulfurization reaction.

Although GTR1 was expected to be free from textiles, a small quantity was observed during the sieve analyses (Figure B.1). Therefore, GTR1 was replaced with GTR2, due to its textile content. The particle size distribution of GTR2, ranging between 150-850 μm was given in Figure 3.1b. The average particle size of GTR2 was calculated as 426 μm . Preliminary Study 1 was performed with two particle size ranges, namely, 150-416 μm and 150-850 μm , with average sizes of 283 μm and 426 μm , respectively. With this approach, it was possible to research the particle size effect on bio-desulfurization.

Batch reactors with 110 ml total volume and 70 ml effective volume were used. Test (T), blank (B) and control (C) reactors for each variable (average particle sizes of 283 μm and 426 μm) were prepared in duplicate. Each reactor contained medium, and their concentrations are given in Table B.1. EC1 was used as inoculum with the VS concentration of 0.64 g/L in the reactors. 3.5 g of GTR2 was used for each particle size, corresponding to 5% (w/v) of the effective volume of the reactor. Reactors were operated in a constant temperature room at 30-32°C, on a shaker at 175 rpm, and the initial pH for reactors were set between 7-8. After

optimum conditions were set and required constituents (Section 3.3) were added, reactors were capped with rubber stoppers and flushed with Ar for 5 minutes to obtain anaerobic conditions. Reactor configuration of Preliminary study 1 is given in Table B.2.



Figure B 1 Textile parts observed in sieve analyses

Table B 1 Medium constituents and their concentrations

Constituent	Concentrations (mg/L)
KNO ₃	1800
MgCl ₂ .6H ₂ O	500
K ₂ HPO ₄	2000
FeSO ₄ .7H ₂ O	10
NH ₄ Cl	500
NaHCO ₃	1000

Table B 2 Reactor configuration of Preliminary Study 1

Reactor	Inoculum ^a	GTR	Medium
Control	EC1	-	X
B1 (283 µm GTR)	-	X	X
B2 (426 µm GTR)	-	X	X
T1 (283 µm GTR)	EC1	X	X
T2 (426 µm GTR)	EC1	X	X

^aEC1= Enriched culture 1

Reactors were incubated for 32 days. On Day 20, all control, test and blank reactor types conducted were taken out of the incubator and analyzed for their NO_3^- -N content. According to the results given in Table B.3, it was observed that although the detoxification method was changed, all of the initial NO_3^- -N concentration was still consumed in the blank (1116 mg/L) and test (1049 mg/L) reactors. Consumption of NO_3^- -N in blank reactors was an indication of an abiotic reaction. For this reason, additional NO_3^- -N (450 mg/L) was added to the remaining replicates, and the incubation lasted 12 days more until Day 32.

Table B 3 Consumption of NO_3^- -N at Day 20 and 32

Reactor Type ^a	ΔNO_3^- -N Concentration			
	Day 0-20		Day 21-32	
	283 μm	426 μm	283 μm	426 μm
Control	41	41	7	7
Blank	252	252	105	73
Test	237	237	162	134

^aControl: Seed+Medium, ^bBlank: GTR+Medium, ^cControl: Seed+GTR+Medium

As seen in Table B.3, after the addition of 450 mg/L of NO_3^- -N on Day 20, nitrate consumption was still observed between Days 21-32. However, this time, consumption was limited in both blank reactors and test reactors. NO_3^- -N consumption, between Days 21-32, for the average particle size of 283 μm was measured as 105 and 162 mg/L for blank and test reactors, respectively. In the same period, NO_3^- -N consumption for blank and test reactors for the average particle size of 426 μm were measured as 73 and 134 mg/L, respectively, for the average particle size of 426 μm . This means that after a concentration (357 mg/L for 283 μm and 325 mg/L for 426 μm) abiotic reaction ceased. This might be due to the consumption of the oxidizing chemicals on the surface of the GTR. Besides, higher NO_3^- -N consumption observed in the test reactors than those of blank reactors could be an indication of denitrification/bio-desulfurization, which was observed for both particle sizes.

NO_3^- -N removal of both blank and test reactors were higher in 283 μm particle size of GTR than those for 426 μm particle size. However, when the effects of blank reactors were excluded by subtracting the blank reactor concentration from that of test, results became similar for both particle sizes. There was not a significant difference (4 mg/L NO_3^- -N) between the two particle sizes (283 μm and 426 μm particle sizes) when the effects of blank reactors were excluded. As a result of this study, GTR2 with a particle size range of 150-850 μm (average size of 426 μm) was decided to be used in Set2 and Set3.

Microorganisms use nitrate as their electron acceptor during bio-desulfurization process. Lack of nitrate may lead to less amount of sulfur consumption from GTR, which would result in an under-estimation of the bio-desulfurization efficiency. For this reason, it was concluded at the end of the Preliminary Study 1 that excess NO_3^- -N is required for bio-desulfurization sets (Set1-Set2) to achieve effective bio-desulfurization. Apparently, modification in detoxification process did not prevent the abiotic removal of nitrate.

C. Preliminary Study 2: The Effect of Excess Nitrate on Bio-desulfurization

Although the detoxification procedure was changed in Preliminary Study 1, the consumption of nitrate remained unchanged and the need for excess nitrate was realized. For this reason, Preliminary Study 2 was conducted to see the effect of excess nitrate on bio-desulfurization. Besides, two different GTR amount was used to see the effect of the particle amount.

Batch reactors with 60 ml total volume and 40 ml effective volume were used. Test (T), blank (B) and control (C) reactors for each variable (GTR content of 2.5% and 5% w/v) were prepared. Each reactor contained a medium, and the concentrations of the constituents used in the medium are given in Table C.1. The initial KNO_3 concentration was 6000 mg/L. EC1 was used as inoculum with the VS concentration of 1.14 g/L in the reactors. GTR2 with the particle size range of 150-416 μm that has 283 μm of average, was used. 2 g and 1 g of GTR were used, which were corresponding to 5 and 2.5 % (w/v) of the effective volume of the reactors, respectively.

Table C 1 Medium constituents and their concentrations

Constituent	Concentrations (mg/L)
KNO_3	6000
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	500
K_2HPO_4	2000
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	10
NH_4Cl	500
NaHCO_3	1000

Reactors were operated at 30-32°C, and the initial pH of reactors were set to a pH range of 7-8. Reactors were capped with rubber stoppers and flushed with Ar for 5 minutes to obtain anaerobic conditions. Reactor configuration of Preliminary Study 2 is given in Table C.2.

Table C 2 Reactor configuration of Preliminary Study 2

Reactor	Inoculum ^a	GTR	Medium
Control	EC1	-	X
B1 (2 g of GTR)	-	X	X
B2 (1 g of GTR)	-	X	X
T1 (2 g of GTR)	EC1	X	X
T2 (1 g of GTR)	EC1	X	X

^aEC1= Enriched culture 1

IC analyses were conducted at every 4-day intervals and results are given in Figure C.1. In this way, it was aimed to see whether the sulfate concentration would change, i.e. whether there was any SO_4^{2-} -S removal potential during the incubation period or not. At the end of 20 days, NO_3^- -N was not consumed totally, and higher sulfate production was observed in test reactors compared to their corresponding blank reactors. When the SO_4^{2-} -S concentration at the beginning and end of the incubation period were considered, the increase in SO_4^{2-} -S was calculated in T1, T2 and C reactors as 62, 46 and 35 mg/L, respectively (Figure C.1b). As seen in Figure C.1b, the biggest change in SO_4^{2-} -S concentrations for both T1 and T2 reactors was seen on Day 4, as 97 and 82 mg/L, respectively. After Day 4, fluctuations were observed and SO_4^{2-} -S concentrations followed a decreasing trend. These fluctuations could be attributed to the reaction of SO_4^{2-} -S with the leached chemicals which were formed due to the bio-sulfurization. This situation was explained in detail in Section 4.3.1.

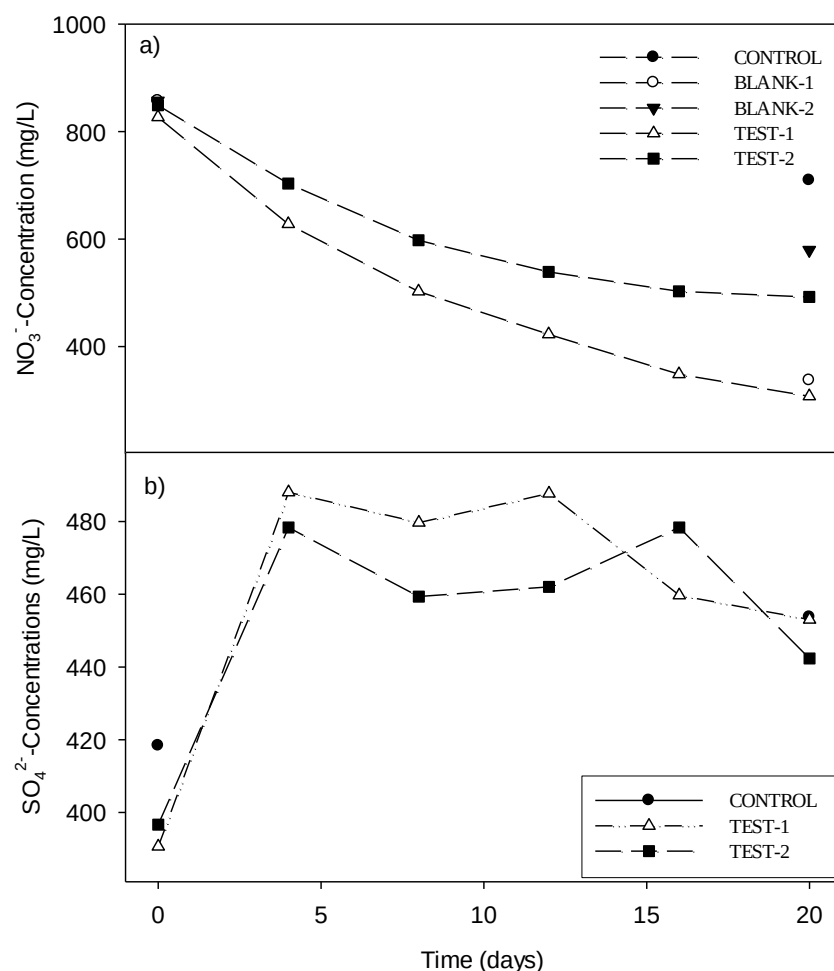


Figure C 1 Change in a) NO₃⁻-N and b) SO₄²⁻-S concentrations in Preliminary Study 2

Change in NO₃⁻-N concentration of T2 (357 mg/L) was higher than that of B2 (278 mg/L), which is an indication of a different mechanism than abiotic consumption (Figure C.1a). However, change in NO₃⁻-N concentration of T1 (519 mg/L) was approximately same with that of B1 (520 mg/L). According to these results, there is no other mechanism than abiotic consumption for T1 reactor.

The accessible sulfur amount is expected to increase proportional with the GTR amount. Therefore, higher GTR amount might have resulted in an increase in NO₃⁻-N consumption and SO₄²⁻-S production. Moreover, the abiotic consumption of

NO_3^- -N, which is directly related with the GTR amount, was also expected to increase with the increase in GTR amount. Meanwhile, it should be noted that this situation also causes an increase in the concentrations of the leached chemicals inside the reactor, which may inhibit the microorganisms (Holst et al., 1998; Bredberg et al., 2002).

In order to compare the effect of GTR amount on bio-desulfurization, both SO_4^{2-} -S and NO_3^- -N concentration changes were observed. As aforementioned, change in SO_4^{2-} -S concentration of T1 (62 mg/L) was higher than T2 (46 mg/L). Higher SO_4^{2-} -S production could be attributed to higher initial accessible sulfur content. Change in NO_3^- -N concentration was also resulted as higher in T1 (519 mg/L) than that of T2 (357 mg/L). However, it must be noted that two different blank reactors (B1 and B2) were used in this study, which were used to see and exclude the effect of abiotic consumption. When the effect of abiotic consumption was excluded by subtracting the blank results from test reactors, higher NO_3^- -N consumption was calculated in T2 reactor (79 mg/L) than that of T1 reactor (-1 mg/L). This might be attributed to the inhibition of microorganisms due to higher amount of leached chemical concentration in the reactor with higher GTR amount (T1).

These results indicated that, together with the abiotic consumption of nitrate, a portion of nitrate was also consumed by microorganisms and sulfate produced. The increase in sulfate was observed mainly in the first 4 days, then a decrease in sulfate concentration was observed. Because the only S source was GTR, the increase in sulfate concentration was attributed to the bio-desulfurization. Lower amount of mass such as 1g (2.5% w/v) might be better to use to minimize abiotic nitrate removal and potential inhibitory chemicals leaching from the GTR.

D. Ion Chromatography (IC) Calibration Results

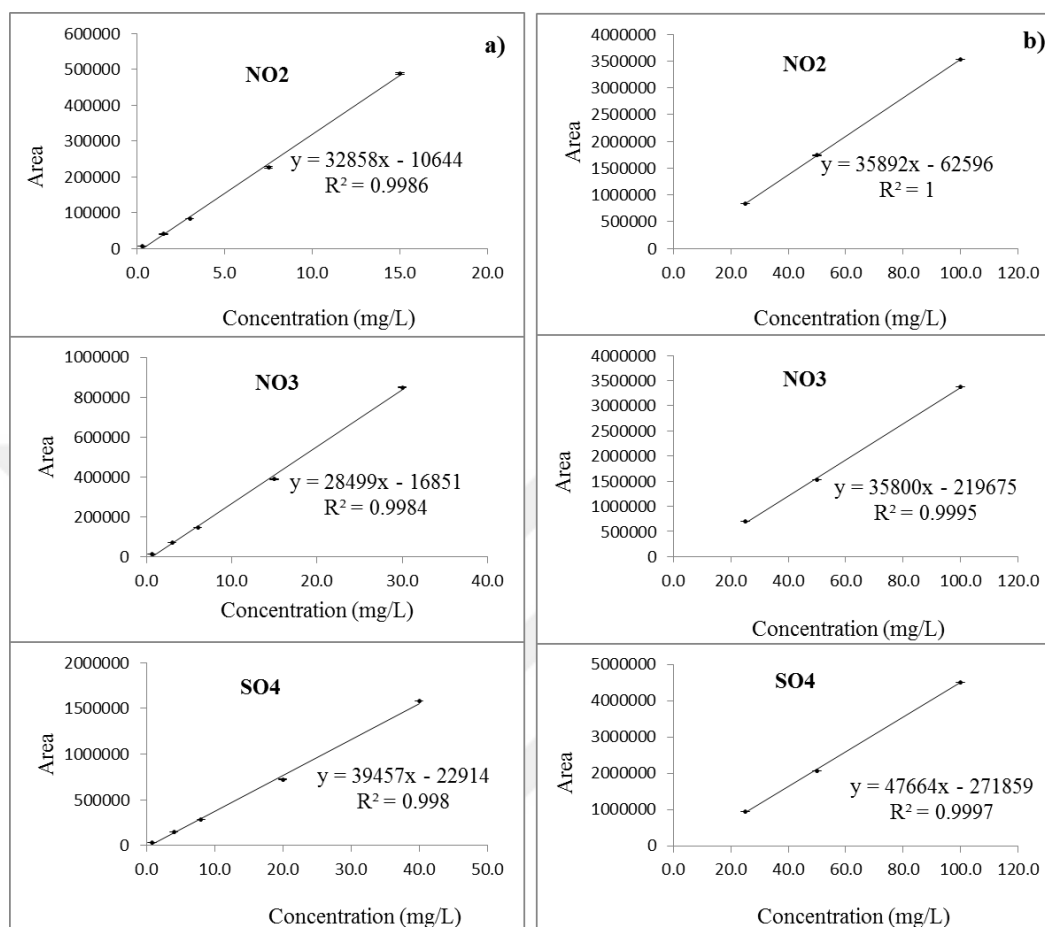


Figure D 1 Calibration curves used for IC analysis

Table D 1 Low and high calibration LOD and LOQ values of IC

Ion	Low Calibration		High Calibration	
	LOD (mg/L)	LOQ (mg/L)	LOD (mg/L)	LOQ (mg/L)
NO ₂ ⁻	0.01	0.05	0.61	1.84
NO ₃ ⁻	0.01	0.03	0.20	0.61
SO ₄ ²⁻	0.00	0.01	0.13	0.40

E. Calibration curve for TOC analysis

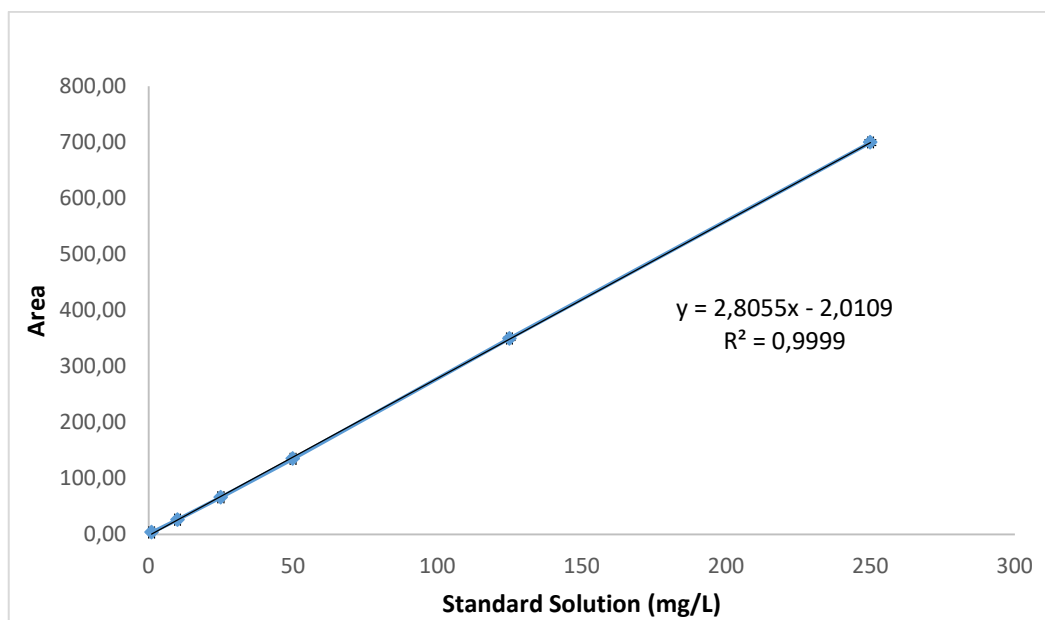


Figure E 1 Calibration curve used for TOC analysis

Table E 1 LOD and LOQ values of TOC

LOD (mg/L)	LOQ (mg/L)
0.023525253	0.071288644

F. Calibration curve for Nessler method

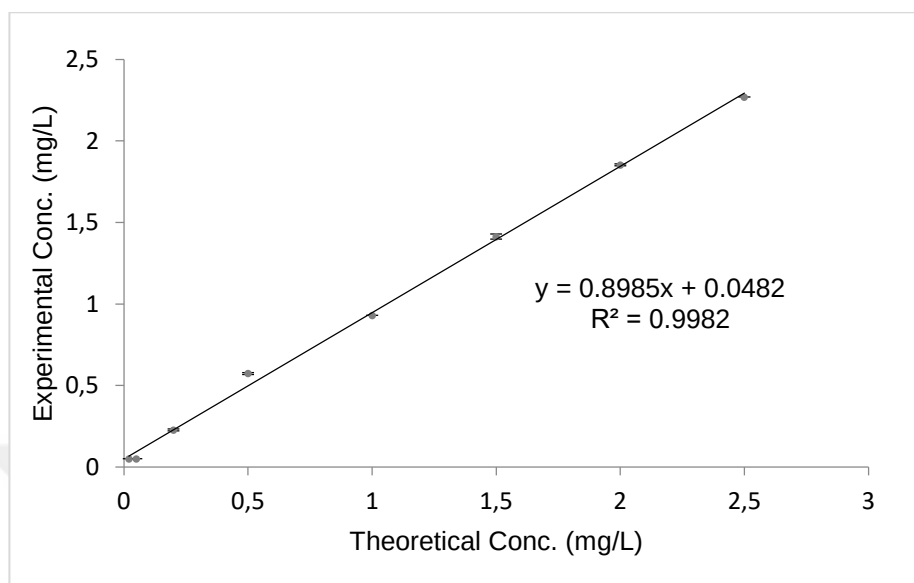


Figure F.1 Calibration curve used for Nessler NH_4^+ analysis

G. Calculation of the Performance Parameters

$\text{NO}_3^- - \text{N}_{\text{initial}}$ = Samples taken at the feeding period of a cycle

$\text{NO}_3^- - \text{N}_{\text{effluent}}$ = Samples taken at the withdrawal period of a cycle

$$\text{NO}_3^- - \text{N RE (\%)} = \frac{(\text{NO}_3^- - \text{N}_{\text{initial}}) - (\text{NO}_3^- - \text{N}_{\text{effluent}})}{\text{NO}_3^- - \text{N}_{\text{initial}}} * 100 \quad \text{Equation G.1}$$

Experimental $\text{SO}_4^{2-} - \text{S} / \text{NO}_3^- - \text{N}$ molar ratio = $E_{\text{S/N}}$

Theoretical $\text{SO}_4^{2-} - \text{S} / \text{NO}_3^- - \text{N}$ molar ratio = $T_{\text{S/N}}$

For $\text{S}_2\text{O}_3^{2-} \rightarrow T_{\text{S/N}} = 1.69$

For $\text{S}^0 \rightarrow T_{\text{S/N}} = 0.98$

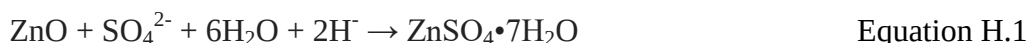
$$E_{\text{S/N}} = \frac{\text{SO}_4^{2-} - \text{S}}{\text{NO}_3^- - \text{N}} = \frac{(\text{SO}_4^{2-} - \text{S}_{\text{effluent}}) - (\text{SO}_4^{2-} - \text{S}_{\text{initial}})}{(\text{NO}_3^- - \text{N}_{\text{initial}}) - (\text{NO}_3^- - \text{N}_{\text{effluent}})} \quad \text{Equation G.2}$$

Discrepancy = The term used for the evaluation of the relative difference between $T_{\text{S/N}}$ and $E_{\text{S/N}}$ values

$$\text{Discrepancy (\%)} = \frac{|(T_{\text{S/N}}) - (E_{\text{S/N}})|}{\max((T_{\text{S/N}}), (E_{\text{S/N}}))} * 100 \quad \text{Equation G.3}$$

H. Possible Zinc Sulfate (ZnSO₄) Formation

Typical truck tire contains 5 phr of ZnO, which corresponds to 3% in a total 166 phr of formula (Table 4.6). Possible zinc sulfate formation reaction of ZnO and SO₄²⁻ was given in Equation H.1. Due to its high solubility ZnO forms hydrates (Rohe et al., 2000).



In order to calculate the possible SO₄²⁻ consumption through ZnSO₄ formation, leached Zn²⁺ must be calculated. The calculation was made based on the penetration depth of the microorganisms in bio-desulfurization studies. It is assumed that Zn²⁺ can only leach from the volume where bio-desulfurization takes place. According to the assumptions made in Appendix A, the penetration depth was taken as 6 µm. Besides, the accessible volume fraction (Vac) calculation was made by using the Equation A.1 (Appendix A). Possible decrease in SO₄²⁻ concentration was calculated with Equation H.2 and given in Table H.1.

Table H 1 Required information for the calculation of possible decrease in SO₄²⁻ concentration

Weight of GTR Used in Set-2 (g)	Reactor Volume (ml)	Typical ZnO Content in Tire (%)	Zn ²⁺ content of ZnO (%)	Accessible Volume Fraction (%)	SO ₄ ²⁻ / Zn ²⁺ ratio of ZnSO ₄ (mg/mg)
1	40	3	80.34	8.21	1.4679

Possible decrease in SO₄²⁻ concentration through ZnSO₄ formation (mg/L) = D_{SO4}

$$D_{\text{SO4}} = 1\text{g} * \frac{1000\text{mg}}{1\text{g}} * \frac{3}{100} * \frac{80.34}{100} * \frac{8.21}{100} * 1.4679 \frac{\text{SO}_4^{2-}}{\text{Zn}^{2+}} * \frac{1}{0.04\text{L}} = 72.6 \frac{\text{mg}}{\text{L}} \text{SO}_4 \quad \text{Equation H.2}$$

$$D_{\text{SO4}} - S \left(\frac{\text{mg}}{\text{L}} \right) = 72.6 \frac{\text{mg}}{\text{L}} \text{SO}_4 * \frac{32 \text{ mg} - S}{96 \text{ mg} - \text{SO}_4} = 24 \frac{\text{mg}}{\text{L}} \text{SO}_4^2 - S$$