Assessment of Sulphate Reduction Bacteria in lisan soil and Brackish water, case study from lower Jordan Valley

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COMMITTTEE DECISION

This thesis (Assessment of Sulphate Reduction Bacteria in lisan soil and Brackish water, case study from lower Jordan Valley.)

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Dedication

I dedicate this study to

- My beloved Family and Friends whom support me and give the power

- My father Mohammad who was the source of generosity which taught me how to rise up, who has given me infinite givens, May god have his compassion upon my father and send him to unlimited heaven.

- My mother Khawla the flower that never fades, who is my source of love , inspiration and strength, who continually provide her support , who taught me to love my language since my eyes opened to this world.

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- My daughter Joanna and my son Jalal, who are the flowers of my life.

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List of Abbreviations

Symbol	Description
SRB	Sulphate-reducing bacteria
PCR	Polymerase chain reaction
WHO	World Health Organization
RO	Reverse Osmosis
PWA	Palestinian Water Authority
TEAP	Terminal Electron Accepting Processes
WWT	Wastewater Treatment
MCM	Million Cubic Meter
EC	Electrical Conductivity
TDS	Total Dissolved Solids

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Abstract

Jericho area is suffering from limited water resources and high salinity of groundwater about 348 gL^{-1}. The lack of sufficient water presents a serious challenge to the people in Jericho. This is the most important problem facing the agricultural sector in Jericho; so the use of desalination techniques is essential and present one of promising and important step to compact and manage these problems.

Sulphide is a common constituent of many waste and saline water. The formation of sulphide upon reduction of sulphate and other sulphur containing compounds is one of the solutions by precipitating sulphur compounds. One method for that is using sulphate-reducing bacteria (SRB) to reduce sulphate.

This study focuses on isolation of SRB from north of Jericho area, and measuring their efficiency in sulphate reduction from prepared standard sulphate solute in distilled water (with SO_4^{-2} concentration of 250mg/l), in order to use SRB in the future as a new tool for reducing salinity rather than expensive existing techniques.

Water and soil samples were collected from the north of Dead Sea area and SRB were isolated from the water and soil samples and cultured on a specific selective media (Postgate C media) under anaerobic conditions.

Results showed isolation of four species of SRB, Two had PCR positive(With expected band size on gel electrophoresis) results , and the other two had PCR negative results (Two unknown bacterial isolates which not detected by universal primer have been used in this work without band on gel electrophoresis).

To confirm the isolated bacteria is SRB, Polymerase chain reaction (PCR) was used with specific primers to amplify 16S rDNA. For measuring the efficiency of isolated bacteria in sulphate reduction, bioreactor method was used.

Results of this experiment indicate that isolated bacteria belong to SRB as was confirmed by PCR with specific primers to amplify 16S rDNA; it was identified as *Desulfobacter latus* strain PTUKS (MK829591) as being determined with 98% homology with *Desulfobacter latus* (GenBank accession Sequence ID is: gi|343201416|NR_042142.1) using BLAST analysis , the other was identified as *Desulfovibrio vulgaris* strain PTUKS (MK829604) as being determined with 99% homology with *Desulfovibrio vulgaris* (GenBank accession Sequence ID is: gi|77539416|AB237496.1) using BLAST analysis.

Bioreactor results showed that the reduction percentages of sulphate concentrations were higher than achieved by previous studies and reached to 43% reduction percentage for *Desulfobacter latus* strain PTUKS (MK829591).

الملخص :

منطقة أريحا تعاني من محدودية مصادر المياه و ارتفاع نسبة ملوحة المياه الجوفية التي تصل الى 348 غرام /لتر . قلة المياه المتوفرة تشكل تحدي كبير لسكان منطقة أريحا، حيث تعتبر هذه من أهم المشاكل التي يواجهها القطاع الزراعي في أريحا ولا بد من اللجوء لاستخدام تقنيات لتحلية المياه. يعد السلفيت مكون شائع الوجود في المياه المالحة والمياه العادمة، اختزال السلفات و مركبات الكبريتات الموجودة تشكل احد الحلول لتخفيف ملوحة المياه و يمكن تحقيق ذلك عن طريق استخدام البكتيريا المختزلة للسلفات.

هذه الدراسة هدفت إلى عزل البكتيريا المختزلة للسلفات من منطقة شمال البحر الميت وتحديدها، ودراسة كفاءتها في اختزال السلفات الموجود في المياه المالحة من خلال جمع عينتين تربة وعينتين ماء من المشروع الانشائي العربي في المنطقة.

النتائج أظهرت عزل نوعين من البكتيريا المختزلة للسلفات من ضمن الأنواع الستة المعروفة عالميا و تم عزل نوعين بكتيريا جديدة (التي لم يتم تحديدها بالمحددات التي تم استخدامها)، بالإضافة إلى ذلك تم تسجيل نسب اختزال للسلفات أعلى من الدراسات السابقة، كما ان الانواع المعزولة كانت قادرة على تحمل درجات ملوحة أعلى في المياه والتقليل من نسب السلفات فيها، ما يفتح المجال أمام استخدام المياه في مجالات عديدة أهمها القطاع الزراعي.

تمت تنمية البكتيريا في بيئة خاصة لنموها في ظروف لا هوائية ،كما وتم استخدام تفاعل البوليميراز المتسلسل لمضاعفة الحمض النووي باستخدام محددات مخصصة للبكتيريا المختزلة للسلفات للتأكد من أن الأنواع المعزولة هي بكتيريا مختزلة للسلفات، ثم تم استخدام طريقة المفاعلات الحيوية لقياس كفاءة الأنواع المعزولة.

(Polymerase chain reaction (PCR) with specific primers to amplify rDNA)

أظهرت النتائج أن الأنواع المعزولة تنتمي للبكتيريا المختزلة للسلفات و تم تأكيد ذلك عن طريق PCR وتسلسل الحمض النووي ل 16S rDNA ، و تم تعريف تسلسل الحمض النووي الخاص بالنوعين المعروفين عالمياً على موقع بنك الجينات (http://blast.ncbi.nlm.nih.gov/Blast.cgi) تحت اسم:

Desulfobacter latus strain PTUKS (MK829591) ،

Desulfovibrio vulgaris strain PTUKS (MK829604)

من ناحية أخرى ، ظهرت كفاءة البكتيريا في اختزال السلفات في المفاعلات الحيوية حيث كانت اعلى من النسب المحققة في دراسات سابقة ووصلت نسبة اختزال السلفات الى 43% تم تحقيقها باستخدام النوع المعزول :

Desulfobacter latus strain PTUKS (MK829591) .

Chapter 1 – Introduction

1.1 Mediterranean region and Arab world water deficiency

There are many water-related challenges facing Arab regions, climate changes, population growth, mismanagement of water resource ,and salinization of ground water are among real challenges. To overcome these challenges, there is a need for more sustainable water use (Connor, 2015 ; Al-Zubari, 2017).

Population growth have reduced freshwater resources availability, that 16 of 22 Arab countries falling below the water scarcity level of 1,000 m^3 per capita per year and able have an average only 292 m^3 per capita per year in 2011 (Connor, 2015).

Almost 75% of the Arab population lives under the water scarcity level of 1,000 m^3 per capita per year, and nearly half lives under extreme water scarcity level of 500 m^3 per capita per year. During 2012 and 2013, intensive flash floods destroyed infrastructure in Gaza Strip, Oman, Tunisia and Saudi Arabia (Connor, 2015).

Nowadays, Arab countries are experiencing an alarming future of increasing water demands , water scarcity and supply costs, which threats preservation and sustainability of their past socio-economic achievements, their future development and socio-economic development efforts (Al-Zubari, 2017; Badran *et al*., 2017).

1.2 Water in Palestine

Palestine is among the countries faces problem of water availability. This problem was magnified by climate change and population growth. The gap deficit between need and the actual consumption is presented in Table1.1. It reached 66 MCM in the West Bank in 2012.While the needed quantities to provide a per capita supply rate of 150 L/c/d based on the World Health Organization (WHO) standard supply rate in the West Bank is almost with gap (deficit) of about 40 MCM (Palestinian Water Authority, 2012).

Table 1.1: Supply and Demand Quantities in Palestine (for 150 l/c/d).

Palestinian part	Population	Needed Quantities (MCM)	Supplied Quantities (MCM)	Deficit (MCM)	Actual Consumption (MCM)	Actual Deficit (MCM)
West Bank	2,338,361	128.2	88.3	39.9	62.3	65.9
Gaza Strip	1,580,167	86.4	97.7	-11.3	54.9	31.5

Palestinians live in the West Bank have an average of the actual water consumption per capita amounts nearly one third of internationally daily amount of water for consumption, hygiene, and cleaning needs , that is very low water consumption rate (Aliewi & Mimi, 2006).

Since the Oslo Accords (Application of the Peace Agreement signed in Oslo between the Palestinian Territories and Israel in 1993(Arnon & Bamya, 2015)), Israel had the asymmetric power that ensured its control of land and water over Palestine, and the Israeli government imposes

restrictions on water consumption and movement of Palestinians vehicles, affecting the development of the agriculture as well as the agricultural trade. (Beltrán & Kallis, 2018).

1.3 Study Problem

The Jordan Valley region, a 250 m below sea level, has low rainfall (100-300 mm annually) which runs along the Jordan River from northern Hebron, with higher altitude ranging from 400-1000 m above sea level (Isaac & Gasteyer, 1995). The major problem in the lower Jordan Valley is the increasing salinization (mainly chloride and sulphur content) of local ground water. The high levels of salinity limit the utilization of ground water for both domestic and agriculture applications (Marie &Vengosh, 2001).

The annually abstraction of the ground water wells in Jericho and Dead Sea area with electrical conductivity (EC) more than 2 mS/cm is about 8 MCM/a. The total volume of brackish water in the area in 2023 will equal 83,073,600 MCM (Amer, 2013).

The Dead Sea water has a currently salinity of about 348 gL^{-1}(Perl *et al*., 2017). The total dissolved solids (TDS) value of Arab Project wells is nearly 3,664 mg/L. Khayat *et al*. (2006) showed that about 10-15% of high dissolved solids in brackish groundwater taken from Arab Project in the

east of Jericho composed mainly of SO_4^{-2} concentrations, the results showed that Sulphate content values were between (200 - >300 mg/L). This high salinity, especially the high sulphate ratio, is an obstacle to agricultural practices in the Jericho area**,** management of the water resources under these conditions is required for decreasing salinity concentration.

Water quality classifications are given in Table 1.2. Few generally-used irrigation water exceed 2 dS/m in EC. With water that exceed about 10 dS/m in EC, only very tolerant crops can be successfully produced (Rhoades *et al*., 1992). In fact, suitability of saline water for irrigation depends on : Soil type, irrigation method, crops type, and climate and management practices.

Table 1.2: Classification of saline water (Rhoades *et al*., 1992).

Water class	Electrical conductivity dS/m	Salt concentration mg/l	Type of water
None- saline	<0.7	<500	Drinking and irrigation water
Slightly saline	0.7-2	500-1500	Irrigation water
Moderately saline	2-10	1500-7000	Primary drainage water and groundwater
Highly saline	10-25	7000-15000	Secondary drainage water and groundwater
Very highly saline	25-45	15000-35000	Very saline groundwater
Brine	>45	>35000	Seawater

Sulphate as one of the causative agent of salinity can discharge into water through atmospheric deposition and in industrial wastes, and from natural sources. However, the presence of high levels of sulphate in drinking-water may cause noticeable taste and may destroy distribution systems by the process of corrosion(World Health Organization (WHO), 2011).

There was an indication of the presence of sulphate-reducing activity in the sediments of the Dead Sea (Nissenbaum, 1975). Only few studies like Häusler *et al*. (2014) and Oren (1999) have investigated the presence of SRB in the Dead Sea without isolating or detecting the bacterial strain or characterization nor its efficiency in sulphate reduction. There are no further studies about identification and isolation of the SRB in the Dead Sea area in spite of these findings.

The brackish water treatment in Jordan Valley mainly applied two technologies: by reverse osmosis (RO) and /or Nano filtration. RO is actually was found to be more efficient, since highly reduction of the content of organic and inorganic matters present in raw was obtained. In addition that, water relatively in affordable price (0.26 €/m^3) (Afonso, Jaber, & Mohsen , 2004).

Nowadays, the Palestinian Water Authority (PWA) has established 3 desalination plants in the Jordan valley that use membrane technology and

RO to remove 70% of ground water salinity(Afonso, Jaber, & Mohsen , 2004).

1.4 Research Questions

As mentioned above, this study is trying to answer the following questions:

- Which species of SRB present in the study area?

- How efficient is each in the process of sulphate reduction?

- Is there any new SRB strain in this unique area?

1.5 Objectives

This study was carried out to fulfill the following objectives:

1- To isolation and molecular identification of sulphate reducing bacteria isolates.

2- To studying their efficiency in reducing the hazard effect of salinity by reducing SO_4^{2-} concentration, which can be used later on in irrigation.

Chapter 2 – Literature review

2.1 Sulphate reduction bacteria (SRB)

Sulphate reduction bacteria (SRB) are anaerobic microorganisms that perform anaerobic respiration using sulphate (SO_4^{2-}) as a terminal electron acceptor, reducing it to hydrogen sulphide gas (H_2S). Oxidation of organic compounds will provide energy for the growth of bacteria (Rzeczycka & Blaszczyk, 2005) as it clears in this interaction equation (1) :

SO_4^{-2} + organic matter = HS^- + H_2S + HCO_3^{-} ---------- (1)

The sulfate ion acts as an oxidizing agent for the dissimilation of organic matter. However, the sulfate reducers can only use sulfate in the absence of oxygen or nitrate; unlike the denitrifiers that are facultative organisms and prefer an aerobic environment (Hao *et al*., 1996).

Sulfate and organic matter are utilized by the SRB in a ratio of approximately 2:1, and may differ depending on the nature of the organics. The carbon source needed for heterotrophic SRB might originate from the soluble organics in the system. The preferred carbon sources for SRB are always low molecular weight compounds such as volatile acids (e.g., acetate), organic acids (e.g., lactate, formate, and malate), and alcohols (e.g., ethanol, propanol, and methanol) (Hao *et al*., 1996).

These bacteria can remove sulphate and heavy metals from waste streams(Cohen, 2006). However, SRB can cause problems, for example in industry,

by producing sulphide, which is highly reactive, corrosive and toxic (Muyzer & Stams, 2008). This bacterium has great potential to be used for solving problem of groundwater salinity caused by presence of sulphate in Jordan valley as had intended to explore in this work.

SRB can be used in the degradation of hydrocarbons; anaerobic microbial processes is found to be linked to one or more Terminal Electron Accepting Processes (TEAP), which involved in reduction of nitrate, iron, sulphate, manganese, and fermentation of acetate or reduction of bicarbonate to produce methane (methanogenesis) (Van Stempvoort *et al*., 2002; Stumm & Morgan ,1981).

Sulphate has greater natural abundance than other more energy-favored electron acceptors, and bacterial reduction may be a dominant TEAP in the natural attenuation of hydrocarbons in groundwater (Chapelle *et al*., 1996; Schmitt *et al*., 1996; Davis *et al*., 1999; Wiedemeier *et al*., 1999).

Muyzer and Stams (2008) found that some of the soil organisms can degrade sulphur-containing proteins into their constituent amino acids. The sulphur of the amino acids is converted to hydrogen sulphide (H_2S) by soil microbes. In the presence of oxygen, H_2S is converted to sulphur and then to sulphate by sulphur bacteria(Muyzer and Stams, 2008).

Hydrogen sulphide rapidly oxidizes to gases that dissolve in water to form sulphurous and sulphuric acids, forming what is called acid rain in aerobic condition that can kill sensitive aquatic organisms and damage stone buildings (Kellogg *et al*., 1972). H_2S itself under anaerobic conditions also causes the so-called depolarization of iron, and hence the corrosion of iron (Hao *et al*., 1996).

In order to control produced H_2S is the stripping of H_2S and subsequent removal of the odorous gas using a biofilter. The odor removal efficiency, however, the toxicity, corrosive properties, unpleasant odor, and high oxygen demand dictate a stringent control of H_2S release into the environment(Hao *et al*., 1996).

SRB activity is affected by salt, oxygen, sulphate concentration, temperature, pH, and organic matter composition (Visser *et al*., 1993; Bhattacharya *et al*., 1996; Vallero *et al*., 2003).

SRB prefer an environment with an optimum pH between 7.5 and 8.0, and are usually inhibited at pH values lower than 5.5 or higher than 9.27. The optimum temperature for sulfate reduction in sediments was around 30°C. Sulfide is known to be toxic to SRB, that the inhibiting level of H_2S resulting in an irreversible failure of the system(It was found that inhibitory levels of sulfate and sulfide for SRB is 1200 mg/1of sulfate and 120 to 140

mg/1 of sulfide). However, the sulfate reducers, can only use sulfate in the absence of oxygen or nitrate, nitrate inhibition of SRB is due to the requirement of the redox condition which depends on the redox potential in an aqueous environment(Hao *et al*., 1996).

There are 6 groups of SRB found worldwide based on Daly *et al*. (2000) as presented in Figure 2.1**.**

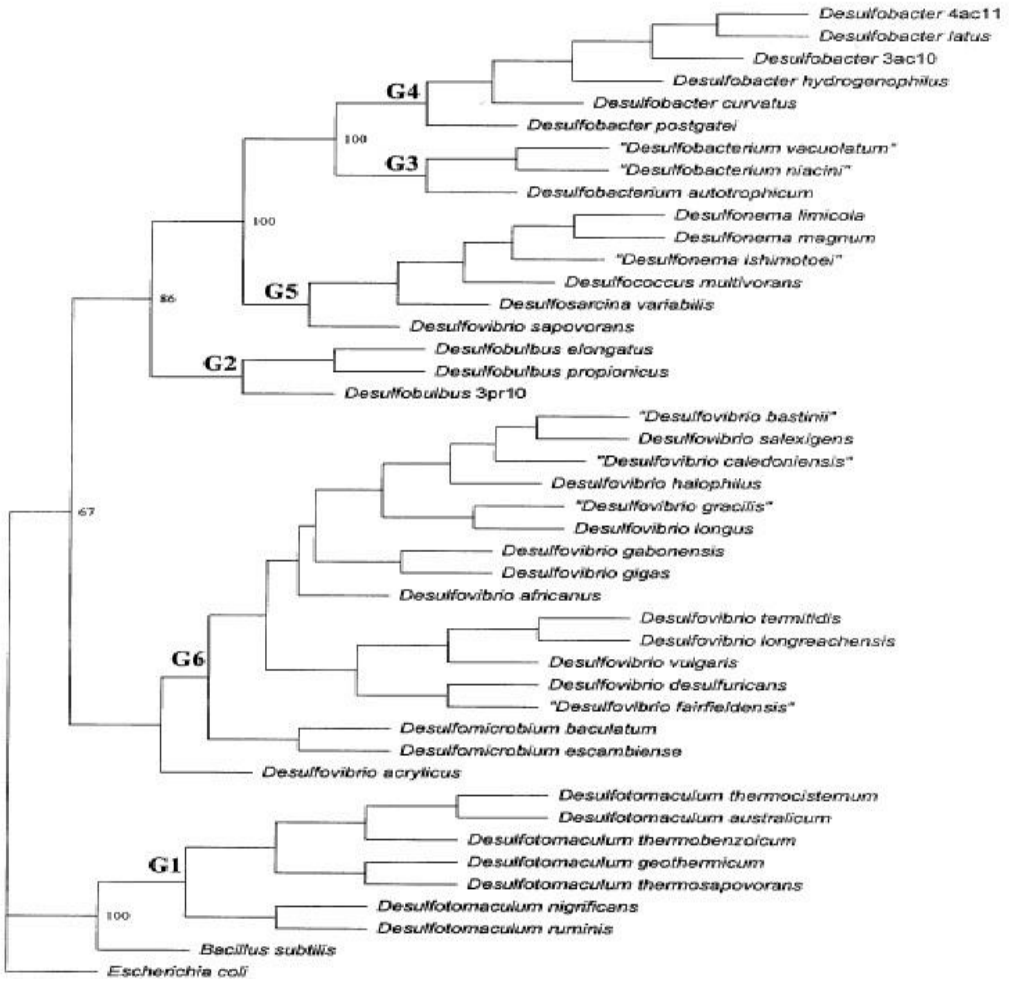

Figure 2.1 : The 6 Groups of SRB detected by Daly *et al*. (2000): *Desulfotomaculum sp ,Desulfobulbus sp , Desulfobacterium sp , Desulfobacter sp ,Desulfovibrio sp ,* and *(Desulfovibrio sp ,Desulfosarcina sp, Desulfococcus sp,* and *Desulfonema sp)* .

2.2 Application of SRB worldwide

Sulphate-reduction bacteria (SRB) have been observed existed in many different habitats (Postgate, 1984). It was detected in sewage water (Ishaq, 1965), wastewater treatment plants (De Beer *et al*., 1993; Lens *et al*., 1995; Manz *et al*., 1998), oil fields (Nilsen *et al*., 1996), arctic sediments (Knoblauch & Jørgensen, 1999), marine sediments (Mubmann *et al*., 2005), and it was noticed in soda lakes (Sorokin *et al*., 2011). The wide range present has allowed these bacteria to be applied extensively in biotechnology(Postgate, 1984).

Application of SRB to sulphate rich wastewater can be beneficial according to (Lens *et al*., 1998; Muyzer & Stams, 2008), there are many studies that discussed the use of SRB in wastewater treatment; sulphate removal from tannery wastewater (Zhao *et al*., 2011; Van Den Brand *et al*., 2015).

Many studies showed different efficiencies in sulphate removal from waste water using SRB. Jing *et al*. (2013) showed in their study 30% efficiency in sulphate removal from sulphate-rich wastewater using Desulfovibrio species of SRB, Mohan *et al*. (2007) observed 20% of sulphate removal efficiency from wastewater by SRB. Furthermore, Genschow *et al*. (1996) used biological sulphate removal from tannery wastewater and they

achieved approximately 30% in sulphate removal in the first stage of their study**.**

Key-parameter analyses including pH, organic substrates, sulphate, salt, temperature and oxygen revealed that the conditions are well suited for the application of SRB in domestic WWT. Since the application of SRB in WWT has environmental benefits, its application is worth considering for WWT when sulphate is present in the influent (Van Den Brand *et al*., 2015)

Drogaleva *et al*. (2015) have identified an isolate from Ust-Tegussky Oil Deposit and found that strains Y1 and Y2 are genetically close to *Desulfovibrio alaskensis* and *D. psychrotolerans*.

Roychoudhury *et al*.(2013) have obtained several sequences with <90% similarity to cultured strains show 96-98% identity to clone sequences derived from other hyper saline sites (Eder *et al*., 2002; Minz *et al*., 1999).

However, a similar phylogenetic distribution of sequences was obtained by Foti *et al*. (2007) in a study of Siberian soda lakes. Daffonchio *et al*. (2006) have demonstrated the presence of members of the *Desulfobulbaceae* family in deep hypersaline anoxic basins. The *Desulfohalobiaceae* are prominent in hypersaline environments (Roychoudhury *et al*., 2013).

2.2.1 Application in reducing sulphate in saline water

Few studies have discussed using of SRB in removing of sulphate in saline ground water. Wargin *et al*. (2007) investigated the occurrence of SRB in groundwater from Cretaceous and Quaternary formations in the Gdańsk region, Poland. Caumette, Cohen, & Matheron (1991) isolated a few strains of SRB from Solar Lake (Sinai), Egypt and maintained in pure culture. They found that Desulfovibrio genus strain showed the highest NaCl tolerance (about 18%). Foti *et al*. (2007) investigated the SRB presence in (hyper) saline soda lakes in Siberia, Russia. Good reduction rate of sulphate that was (between 12 and 423 mol/m^3 day^{-1}) was obtained for the most lakes and they isolated SRB strain was ASO3-1.

2.3 SRB in the Dead Sea area (Ground water and soil)

Few studies have investigated the presence of SRB in the Dead Sea water without detecting the bacterial strain or its characteristics or its efficiency in sulphate reduction (Häusler *et al*., 2014; Oren, 1999).

There was an indication of the presence of sulphate-reducing activity in the sediments of the Dead Sea (Nissenbaum, 1975). Isotopic analysis of sulphur in the Dead Sea (one of the most hyper saline lakes in the world) indicated an active sulphate reduction, extremely halophilic SRB community in the sediments of the Dead Sea. (Häusler *et al*., 2014)

In spite of these findings, there are no further studies about identification the SRB in the Dead Sea. Identification of SRB present in the Dead Sea and sequencing had not been done before in any of these studies.

Chapter 3 - Study Area

3.1 Geology

The geology of Jericho district was studied by many geologists such as Abed and Wishahi (1999), Khayat (2005), and Qannam (2002). The geology of Jericho district is characterized by Jordan rift valley deposits which are mainly composed of Pleistocene Alluvial and Marl formations; this type of formation is favorable for groundwater protection, and the formation is covered structurally by minor faults (Qannam, 2002 ; Khayat, 2005).

3.1.1 Lisan formation

The lisan formation is exposed in the eastern part of the Jericho area as well as in the whole Jordan valley rift. It consists mainly of laminated aragonite chalk, gypsum and clay, with some gravel beds and sandstone(Amer, 2013) .

In general, the Lisan formation is a major source of soil and water salinity in the Jordan Valley. The permeability of the Lisan formation is generally very low(Salameh, 2001). However, the Lisan and Samara formation horizontally interfinger along the Jericho aquifer system (Flexer et al., 1989).

3.2 Salinity in Jericho area and Lower Jordan Valley

The Dead Sea water has a currently salinity about 348 gL^{-1}(Perl *et al.*,2017). Saline water was detected in wells springs and seepages along the western shore of the Dead Sea that was contain high concentrations of salts, as example; 5000 mg L^{-1} Cl in the Amiaz wells located 20 km northward. Sulphate shows values between 200 and >300 mg/l (Khayat *et al.*, 2006).

The chemical composition of Dead sea area ground water with ionic content of Na > Ca > Mg and CI > SO_4^{-2} > HCO_3, concentration was as mentioned arrangement (Yechieli, 2000).

Based on EC classification, brackish water in Jericho area are nearly 100 % more than 2000 µS/cm as shown in Table 3.1. So accordingly, salinity hazards appear high in Jericho based on TDS that 100% of the Jericho wells have very high salinity water(Amer, 2013).

Table 3.1: Volume of brackish water in Jericho area based on EC classification (Amer, 2013)

	Categories in µS/cm	Volume MCM annually	Percent of total volume
Jericho area	< 2000	129600	0.02%
	2000-3000	1779840	0.20%
	3000-4000	3166560	0.37%
	4000-5000	1620000	0.19%
	5000-6000	1101600	0.13%
	> 6000	734400	0.09%
Sum		8,532,000	1.000

The highest salinity in the Jericho area present in the groundwater from east Arab Project wells, with high salt content. The chemical data of some wells in the Arab Association Project are shown in Table 3.2. For example, chloride content for the well (code:19-14/066) is more than 1,600 mg/L (Table 3.2). A total dissolved solids (TDS) value of Arab Project well (code:19-14/066) is nearly 3,531 mg/L. The study of Khayat *et al*. (2006) shows that about 10-15% of high dissolved solids in brackish groundwater taken from Arab Project in the east of Jericho composed mainly of SO_4^{-2} concentrations. Sulphate source is mainly from Lisan formation which composed of Gypsum that consist the large part of its constituents (Khayat *et al*., 2006).

Table 3.2: Hydrochemistry of the wells from the Arab Project in Jericho-Palestine area (mg/l) (Khayat *et al*., 2006)

Site	Arab Project	Arab Project	Arab Project	Arab Project
Well code	19-13/069	19-14/067	19-14/073	19-14/066
pH	7.02	6.99	6.99	7.04
Depth(m)	132	73	80	85
T(C)	26.3	26.4	26.4	26.6
Na	460.10	639.00	359.30	577.00
K	79.600	104.10	79.600	82.600
Mg	140.00	220.80	164.60	213.70
Ca	116.7	167.3	128.3	186.1
NH_4	0.017	0.000	0.000	0.010
B	0.8180	1.3200	0.9730	1.0780
Ba	0.1600	0.1534	0.2205	0.0990
Sr	1.960	2.8430	1.8330	3.5600
Cl	1,173	1,861.7	1,125.0	1,614.0
Br	10.90	14.00	7.600	15.70
SO_4^{-2}	103.8	183.4	95.9	300.9
NO_3	24.510	38.860	40.410	29.6900
PO_4	0.012	0.043	0.016	0.035
HCO_3	418.46	366.61	405.65	439.2
Calc. TDS	2,597.32	3,664.92	2,479.09	3,531.67
Tritium (TU)	0.9	1.0	0.9	<0.6

The SO_4^{-2} concentra0tion in the groundwater from this well(code: 19-14/066) is 300.9 mg/L, and this groundwater is highly saturated with dolomite and calcite (Khayat, *et al*., 2006). This means that 10% of TDS is from SO_4^{-2} content that is hazard in such high percentage.

3.3 Potential use of Saline water in Agriculture in Jericho

Almost 50,000 dunums in Jericho and Al-Aghwar are cultivated lands that form 2.9% of the total cultivated area of the West Bank. All the agricultural area is irrigated and forms 33.2% of total irrigated lands. Of the total cultivated area, 75 % is cultivated with vegetable crops, 14 % with fruit trees, and 11 % with field crops and forage. (Joint Council for Services, Planing & Development for solid waste management in Jericho (JCspd), 2012).

Date palm plantation has been expanded rapidly in the period between 2001to 2011 in Jericho district. According to the Ministry of Agriculture, the area in Jericho city planted with palm is shown in Figure 3.1.

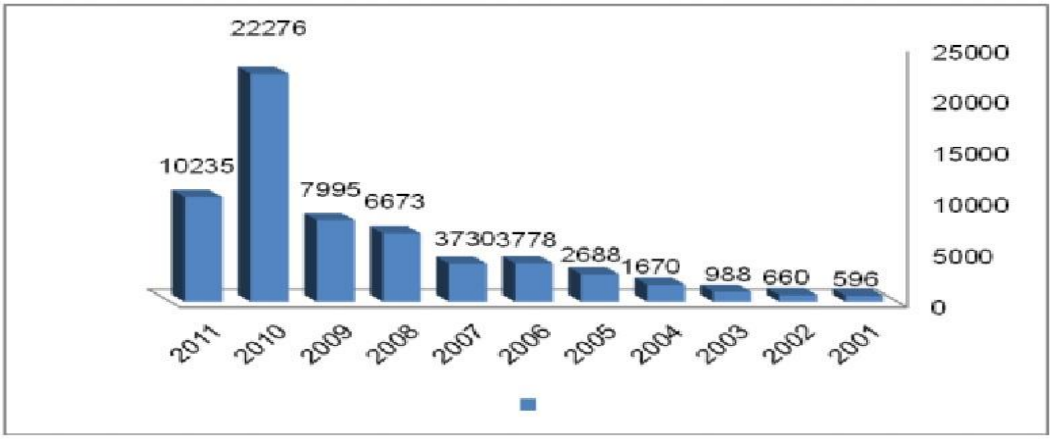

Figure 3.1: Cultivated area with date palm in dunums in Jericho from 2001-2011.

In Jericho, ground water wells are used basically for irrigation. The water has medium to high salinity hazard based on Rhoades *et al*. (1992) and Wilcox (1995) classification.

Bananas have the largest area of fruit trees about 72.4% of the fruit trees. Bananas require large quantity of water, about 17,000 CM/yr/hectare. Average annual total amount of water used by all crops in Jericho district is about 18.42 MCM (ARIJ, 1995).

The physical and chemical characteristics of soils degraded by irrigation with saline water that impede water penetration or otherwise cause the soil environment tube less favorable as a medium for root development (Eaton, 1942). Irrigation with saline water contains high percent of sulphate may affect plant growth adversely as (Machado, & Serralheiro, 2017; Machado *et al*.,2012) :

(1) Decreasing in moisture percentages in the green plants ;

(2) Increasing in the amount of tip burning;

(3) Increasing in plant roots to try uptake more water;

(4) The extent of salts accumulation in plant (high sulphate can cause health problems when person eats crops have accumulated sulphate in high quantity); and (5) Reduced yields as a result.

Chapter 4 - Materials and Methods

Materials and methods are presented in Table 4.1.

Table 4.1: Materials and methods of this study

<table>
<tr><td></td><td>Step</td><td colspan="2">Both water and soil samples</td><td>Water samples</td><td colspan="2">Soil samples</td></tr>
<tr><td rowspan="4">4.1</td><td rowspan="4">Samples collection</td><td colspan="2">Soil and water samples were collected from the Arab Construction Project Association in Jericho city.</td><td rowspan="4">Two water samples were collected from two wells in the Arab Construction Project Association (wells code of 19-14/066 and 19-14/067 with highest sulphate concentrations in the study area. (Khayat et al. ,2006)).</td><td colspan="2" rowspan="4">Two soil samples were taken; one from lisan white soil (0-30 cm) and the other from more depth layer (30-60 cm) of lisan soil.</td></tr>
<tr><td colspan="2">Bottles were washed before sampling.</td></tr>
<tr><td colspan="2">The date of sampling was 14th of February, 2018.</td></tr>
<tr><td colspan="2">Samples were analyzed in the Palestine Technical University. Sulphate was measured in the next day of sampling. Sulphate parameter measurements were done using spectrophotometer (HACH advanced spectrophotometer).</td></tr>
<tr><td>4.2</td><td>Preparation of Enrichment media</td><td>Nutrient medium for sulphate-reducing bacteria (Using Postgate C media)</td><td>The SRB isolation and subsequent cultivation for the production of active SRB cultures were carried out using Postgate's nutrient medium C (Postgate, 1984). The chemical composition of the complete medium is shown in Table 4.2.</td><td></td><td>Enrichment media was done as follows:</td><td>A- 100 to 200 g of sieved white Lisan soil (0-30 cm) were mixed with 3-5 g calcium sulphate in a bowl. Filter paper was torn into small pieces and mixed with the soil. Tap water was added to the soil mixture until it reached a cream-like consistency.</td></tr>
</table>

			The pH of the media was adjusted to 7.5 using 5M NaOH and 1N HCl. Solidified with 1.5 g/l of agar and autoclaved at 121°C for 15 min.			**B-** The mixed soil was applied to the bottom of a measuring cylinder in a thickness of about 2-5 cm; subsequently the column filled uniformly with soil paste at a height of about 15-25 cm (The column is appropriate if it has no air bubbles, and after standing for 24 hours about 0.5 cm layer of water covers the paste). To avoid dehydration and make environment suitable to SRB growth, the top of the measuring cylinder closed with plastic film.
						C- The column was placed for at least 4-6 weeks at room temperature. During the incubation period, the enrichment of sulphate reducing bacteria followed-up by colour changes and forming black to brown pots observed in the column due to bacterial growth and H_2S precipitation(Figure 4.1).
4.3	**Detection of Sulphate-reducing bacteria (SRB)**	For an aerobic conditions, special bags called An aero Gen™2.5L was used that make anaerobic condition for more than two days.		For first culturing step, 1.5 ml was taken from each ground water sample and centrifuged at 6000 rpm for 5 minutes in 2 ml eppendorf. Then, 150 µL was inoculated on Medium C and incubated at 30ºC in anaerobic condition.	Soil samples were sieved and eluted with distilled water (10g/50ml) at room temperature, supernatant were aliquoted in 1.5 ml eppendorf of that mixture, and 150 µL was inoculated on Medium C and incubated at 30ºC in anaerobic conditions.	

			On the other hand, 50 ml of water were filtered in sterile filter paper (4.5 mm), the whole filter paper were inoculated on medium C in Petri dishes, and kept as above conditions (Figure 4.2).	After 6 weeks from making the enriched media, 150 µL from the bacterial growth (shown as black pots) at the top of cylinder was inoculated on Medium C and kept as above conditions.
	Bacterial subculture	Subculturing was done several times during the experiment to be sure that SRB still alive at all steps of the study on the same medium composition.		
		About 50 µL from SRB growth in Postgate C broth were taken and cultured on Postage C media in petridish in order to make single colonies of SRB.		
	Identification of the selected SRB	**Four single colonies of SRB were selected (tow has PCR positive results with correct expected band size), identification was done at two levels;** **1 Morphology level** The grown and isolated SRB strains would primarily be identified based on Gram stain reaction by microscopic examination using the light microscope Nikon Eclypse 400.		
		2 Molecular identification of SRB To identify the isolated bacteria in this work belong to which group, PCR amplification method of 16S rDNA were used with SRB group-specific primers based on Daly *et al*. (2000) and Marangoni *et al*. (2013) (Table 4.3). The 16S rDNA-targeted PCR primer sequences used in this study based on this classification are shown in Table 4.4.		

	Colony Polymerase Chain Reaction (CPCR)	Colony PCR directly was applied to PCR reaction using specific primers shown in Table 4.4. Reactions were carried out in a thermal cycler as follows; 95ºC for 1 min (denaturation), annealing for 1 min (Table 4.3 for temperature) and 72ºC (extension) for 30 cycles. Each reaction tube contained: 12.5 µL of master mix, 1 µL of each primer (1 µL of each forward primer and reverse primer), 4.0 µL of supernatant (contains DNA), 6.5 µL of distilled H_2O with total volume 25 µL reaction mix. The master mix was (MyTaq^{TM} Red Mix). DNA did not extracted before PCR as Postgate C media is very specific for growth of SRB only. Subsequently, the PCR product was separated by 2% agar-gel electrophoresis and sequenced in Bethlehem University.
4.4	**Bioreactors design**	To determine that our isolates have the capacity to reduce sulphate; four bioreactors were constructed manually. Each bioreactor has Postgate's nutrient medium C (with sulphates), distilled water contain standard sulphate with concentration of 250 mg/L and inoculated with different colony. Each colony was obtained from PCR positive and PCR negative isolates(the positive has a clear strand when separate on gel electrophoresis and the negative has not any strand on it)(Table 4.5 and Figure 4.3).
		The pH was adjusted to 7.5 for all bioreactors, and the Bacterial initial Optical Density (OD_{600}) were measured for all bioreactors using spectrophotometer at wavelength of 600nm(At this wavelength the cells will not be killed as they would under too much UV light)(Table 4.5).
		Total liquid phase volume in each bioreactor was 250 ml. During the experiments, sulphate concentration, presence of hydrogen sulphide and SRB were monitored by precipitation of black precipitates of H_2S , and pink colour become colourless due to using resazurin indicator and sulphate concentration was recorded (Figure 4.3).
4.5	**SO_4^{-2} analysis**	The concentrations of sulphate ion were measured using HACH advanced spectrophotometer, with Sulphate kits. The presence of bacteria was confirmed again by the microscopic observation (after the Gram stained of the microscopical preparations, the magnification – 400x) using the light microscope Nikon Eclypse 400.

Figure4.1: The enrichment media in the column after preparation

Figure 4.2: The SRB in Petridishes for enrichment in anaerobic conditions.

Table 4.2: Composition of Modified Postgate C Medium (1.0L) (Postgate, 1984)

Component	**Quantity**
Potassium phosphate	0.5g
Ammonium chloride	1.0g
Sodium sulphate	4.5g
Calcium chloride	0.04g
Magnesium chloride	0.06g
Sodium lactate (50% m/v)	9.4ml
Yeast extract	1.0g
Ascorbic acid	0.1g
Ferrous sulphate	0.04g
Agar-Agar	1.9g
Resazurin (0.025% m/v)	4.0ml
Sodium chloride	35g
Sodium citrate	0.3g

Table 4.3: The 16S rDNA-targeted PCR primer sequences specific for SRB subgroups (Daly *et al*., 2000).

Primer pair	**Sequence**	**Product size**	**Annealing temperatu**	**Specificity**	**Genera**
DFM140 DFM842	TAG MCY GGG ATA ACR SYK G ATA CCC SCW WCW CCT AGC AC	700	58	Group 1	*Desulfotomaculum* sp.
DBB121 DBB1237	CGC GTA GAT AAC CTG TCY TCA TG GTA GKA CGT GTG TAG CCC TGG TC	1120	66	Group 2	*Desulfobulbus* sp.
DBM169 DBM1006	CTA ATR CCG GAT RAA GTC AG ATT CTC ARG ATG TCA AGT CTG	840	64	Group 3	*Desulfobacterium* sp.
DSB127. DSB1273	GAT AAT CTG CCT TCA AGC CTG G CYY YYY GCR RAG TCG STG CCC T	1150	60	Group 4	*Desulfobacter* sp.
DCC305 DCC1165	GAT CAG CCA CAC TGG RAC TGA CA GGG GCA GTA TCT TYA GAG TYC	860	65	Group 5	*Desulfovibrio* sp. *Desulfosarcina* sp. *Desulfococcus* sp. *Desulfonema* sp.
DSV230 DSV838	GRG YCY GCG TYY CAT TAG C SYC CGR CAY CTA GYR TYC ATC	610	61	Group 6	*Desulfovibrio* sp.

*Ambiguities: R (G or A); Y (C or T); K (G or T); M (A or C); S (G or C); W (A or T).

Table 4.4: The 16S rDNA-targeted PCR primer sequences used in this study based on Daly *et al*. (2000) study.

SRB group	Primer used for SRB group
Group 1	TAG ACT GGG ATA ACA CCT G ATA CCC GCA ACA CCT AGC AC
Group 2	CGC GTA GAT AAC CTG TCC TCA TG GTA GGA CGT GTG TAG CCC TGG TC
Group 3	CTA ATA CCG GAT GAA GTC AG ATT CTC AAG ATG TCA AGT CTG
Group 4	GAT AAT CTG CCT TCA AGC CTG G CTC TCT GCA GAG TCG CTG CCC T
Group 5	GAT CAG CCA CAC TGG GAC TGA CA GGG GCA GTA TCT TCA GAG TCC
Group 6	GAG TCT GCG TCT CAT TAG C GTC CGA CAT CTA GTA TTC ATC

Table 4.5: The experimental design and treatments

Bioreactor	Used isolate	OD_{600}
A	Group 6 of known SRB was isolated from the lisan white soil (0-30 cm) sample.	0.131
B	Unknown isolate from the well water sample (well code 19-14/067).	0.168
C	Unknown isolate from the lisan white soil (0-30 cm) sample.	0.152
M	Group 4 of known SRB was isolated from the well water sample (well code 19-14/066).	0.1485

Figure 4.3: Two out of the four bioreactors used in this study.

Chapter 5 - Results and discussion

5.1. Culture Enrichment.

In this work, isolation and detection of known and unknown isolates were reported for the first time, that both were able to reduce sulphate concentrations.

The SRB enriched in the column are shown in Figures 5.1 and 5.2. After 6 weeks, the SRB appeared at the top of water in the column as black to brown pots.

Figure 5.1: The enrichment media in the column after preparation.

Figure 5.2: The growth of SRB in the enrichment media in the column after 6 weeks.

5.1.1. Isolation of Bacteria

Bacterial growth was detected within 2 - 4 days after culturing on selective media (Postgate C medium). This was indicated using Resazurin indicator by color change from pink to colorless (Erb, & Ehlers, 1950) and formation of black precipitates (due to presence of S^{2-} in the grown colonies and Fe^{+2} in the medium) (Figure 5.3).

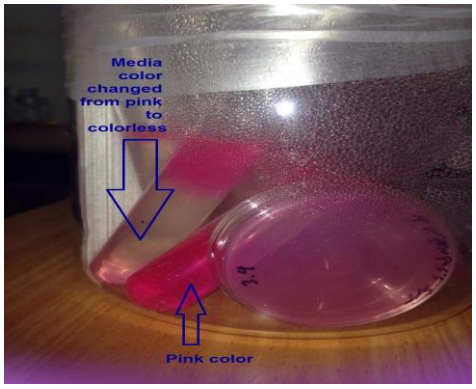

Figure 5.3: SRB growth on selective medium (Postgate C medium) bacteria.

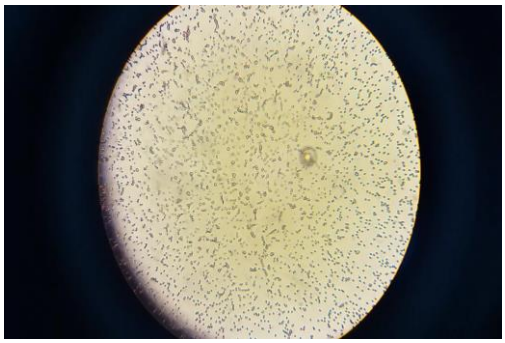

Figure 5.4: Gram stains of SRB with Pink colonies (400x).

The microscopic observation, as in Figure 5.4, showed gram negative bacteria and this agrees that *Desulfobacter* group and *Desulfovibrio* group.

5.2 Polymerase chain reaction (PCR)

The abundance and activity of SRB isolated from soil and water samples collected from the Arab Construction Project Association in the Dead Sea area have been described. For this purpose, a polyphasic approach has been used, which included both culture-dependent (i.e., enrichment) and independent (i.e., PCR, and sequencing) techniques.

Classical identification of SRB is time consuming; Molecular tools open the opportunity to detect these isolates. Polymerase chain reaction (PCR) was one of these tools.

Two isolates of SRB related to group 4 and group 6 of known SRB groups were detected and isolated(Table 4.3). Results of amplification of 16S

rDNA was amplified with specific primers showed the presence of 610 bp band which is corresponding to group 6 (*Desulfovibrio* sp.) of SRB (Table 4.3), it was found in the lisan white soil sample (0-30cm)(lane 4 in Figure 5.5). Group 4 (*Desulfobacter* sp.) was also found with expected band size of 1150 (lane 3 in Figure 5.5). However, the group 4 of SRB included the genus *Desulfobacter* sp., was found in the well water sample(well code 19-14/066) (Figure 5.5).

Other artifact bad was also detected (lane 2, 5-8 in Figure 5.5). This may be attribute din contamination with samples or other new bacteria are present. These bands will be further studied in another research to give clear cut if it is artifact or new type of bacteria that has potential in sulphate reduction.

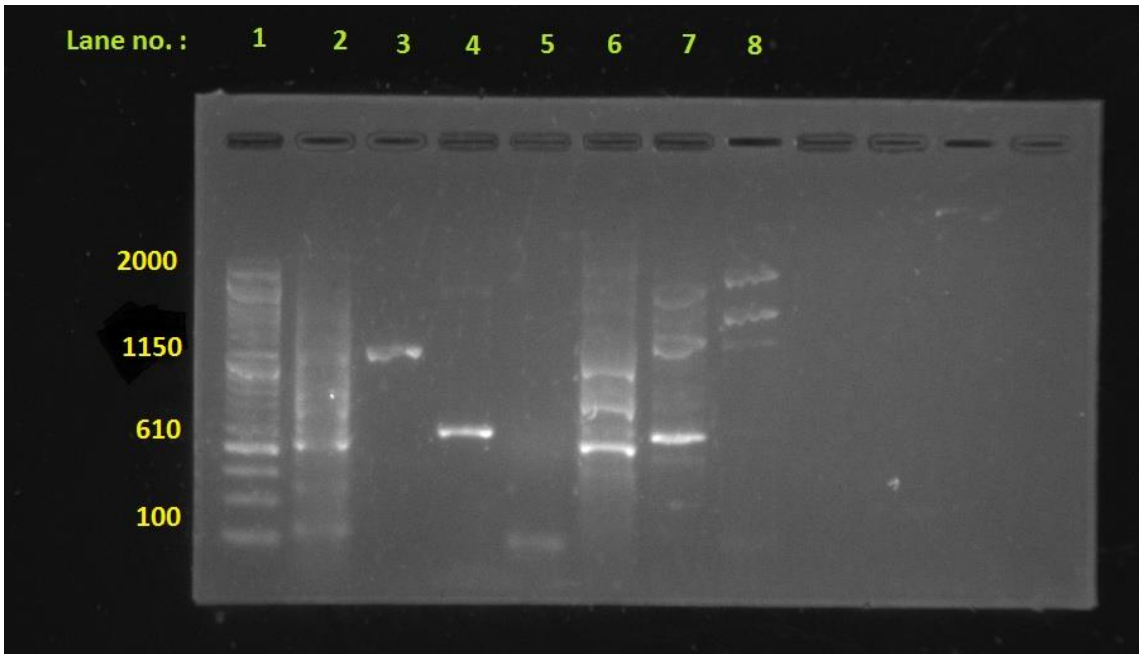

Figure 5.5: Ethidium bromide-stained gel of PCR products representing amplification of 16S rDNA. Lane 1 is 50 bp DNA marker, Lane 2-8 are DNA samples.

Colony PCR from enriched bacterial growth was effective for detection of SRB from the samples. This has advantages of time and cost reduction for the detection of SRB. Zhang & Fang (2001), and Hopkins *et al*. (2005) used PCR for detection of SRB from the samples in their study; they extracted DNA before doing the PCR.

In previous reports(like Drogaleva *et al*. , 2015; Roychoudhury *et al*., 2013), DNA extraction was done before then followed by PCR (Daly *et al*., 2000). They employed a similar methodology but they required DNA isolation from the bacteria as an additional step. Even at high salt concentrations of soil and water in the north of Dead sea area, substantial SRB were observed (Figure 5.5). This indicates again that a yet undiscovered group of SRB might be active in this area.

5.3. Sequencing of PCR products and BLAST search

DNA sequencing was performed only on bacteria showing 600 and 1150 bp only that belong to known group 4 and group 6 (Table 4.3). As stated above the other bands needed to be further studied. The obtained PCR product sequence was compared by Gene Bank data base (BLASTN analysis) with the nucleotide sequences of 16S rRNA gene of other strains in gene bank data base.

Sequence analysis of the 16S-rRNA gene and BLAST sequence comparison (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the isolated SRB belongs to group 4 was *Desulfobacter latus strain PTUKS* (submitted gene bank number MK829591), and showed similarity of 98% with *Desulfobacter latus* (GenBank accession Sequence ID is: gi|343201416|NR_042142.1)(See Appendix 2),while the other isolated SRB belongs to group 6 was identified as *Desulfovibrio vulgaris strain PTUKS* (submitted gene bank number MK829604), and showed similarity of 99% with *Desulfovibrio vulgaris* (GenBank accession Sequence ID is: gi|77539416|AB237496.1) (See Appendix 4).

Some isolates failed to be sequenced; the cause is not clear and could be many; the possible cause for that is the inhibitory contaminant (Contaminants can include residual primers, salts, RNA, ethanol, dNTP's, detergents, chromosomal DNA, proteins, buffer components, etc…).

Here, we report the using of 16S rRNA gene and sequencing for our identification, other researcher like Drogaleva *et al*. (2015) and Roychoudhury *et al*.(2013) have used the same technique for identification of SRB.

In this study 98-99 % similarity was obtained. Several sequences obtained by others reached to 96-98% identity to clone sequences derived from other hypersaline sites (Eder *et al*., 2002; Minz *et al*., 1999). Low sequence

identities also have been reported by other researchers (Mouné *et al*., 2003; Mubmann *et al*., 2005; Nakagawa *et al*., 2004).

5.4. Sulphate analysis:

The sulphate concentrations in the collected samples are reported in the Table 5.1. The result in sulphate concentration in the bioreactors (A, B, C, and M) is shown in the Table 5.2 and 5.3, results reported at room temperature and initial pH equals 7.5.

Table 5.1 : Sulphate concentration in the samples

Sample	lisan white soil (0-30 cm).	lisan soil (30-60 cm).	The well water sample (well code 19-14/067).	The well water sample (well code 19-14/066).
Sulphate concentration (mg/l)	249.18	171.79	188.30	304.19

Table 5.2: Sulphate concentration in the bioreactors A and B

Time(hours)	Date	Time	Conc. A (mg/L)	Conc. B (mg/L)
0	25/03/2018	14:00	305.16	314.52
18	26/03/2018	08:15	317.59	334.87
20	26/03/2018	10:15	332.89	337.58
22	26/03/2018	11:43	351.98	341.54
23	26/03/2018	01:00	352.34	341.72
24	26/03/2018	14:00	348.02	350.18
25	26/03/2018	15:00	319.57	350.54
42	27/03/2018	08:15	317.41	348.74
44	27/03/2018	10:00	317.04	346.76
46	27/03/2018	12:00	284.99	344.60
48	27/03/2018	14:00	281.57	336.50
49	27/03/2018	15:00	281.39	328.75
66	28/03/2018	08:00	274.18	321.73
68	28/03/2018	10:00	272.56	318.49
70	28/03/2018	12:00	272.38	306.42
72	28/03/2018	14:00	271.30	278.50
73	28/03/2018	15:00	263.56	278.14
90	29/03/2018	08:00	245.55	270.40
92	29/03/2018	10:00	245.37	268.78
94	29/03/2018	12:00	244.82	268.06
97	29/03/2018	15:00	243.56	248.25
162	01/04/2018	08:00	243.38	247.85
164	01/04/2018	10:00	243.38	247.85

Table 5.3: Sulphate concentration in the bioreactors C and M

Time(hours)	Date	Time	Conc. C (mg/L)	Conc. M (mg/L)
0	27/03/2018	12:00	393.63	406.19
2	27/03/2018	14:00	342.98	357.93
3	27/03/2018	15:00	342.80	349.28
20	28/03/2018	08:00	332.53	251.31
22	28/03/2018	10:00	330.19	247.89
24	28/03/2018	12:00	328.03	246.99
26	28/03/2018	14:00	263.37	246.63
27	28/03/2018	15:00	262.65	246.45
44	29/03/2018	08:00	259.77	242.12
46	29/03/2018	10:00	259.23	241.58
48	29/03/2018	12:00	258.87	240.86
51	29/03/2018	15:00	257.97	238.34
116	01/04/2018	08:00	257.07	236.18
118	01/04/2018	10:00	256.89	235.46
122	01/04/2018	14:00	256.89	235.28
140	02/04/2018	08:00	256.89	235.28
142	02/04/2018	12:00	256.89	235.28

Changes in sulphate concentration in the anaerobic bioreactors A, B, C, and M influenced by bacterial isolates types are shown in Figures 5.6 − 5.9.

The known SRB of group 6 which found in lisan white soil sample (0-30cm) was used in A bioreactor (Figure 5.6). The sulphate concentration initially started to increase slightly from 305.16 - 332.9 mg/L within the first 20 hours with 1.39 mg/L in average for each hour. Moreover, it increased significantly after 3 hours to be 352.3 mg/hour; the sulphate concentration increased while the oxygen consumption increased inside the bioreactor.

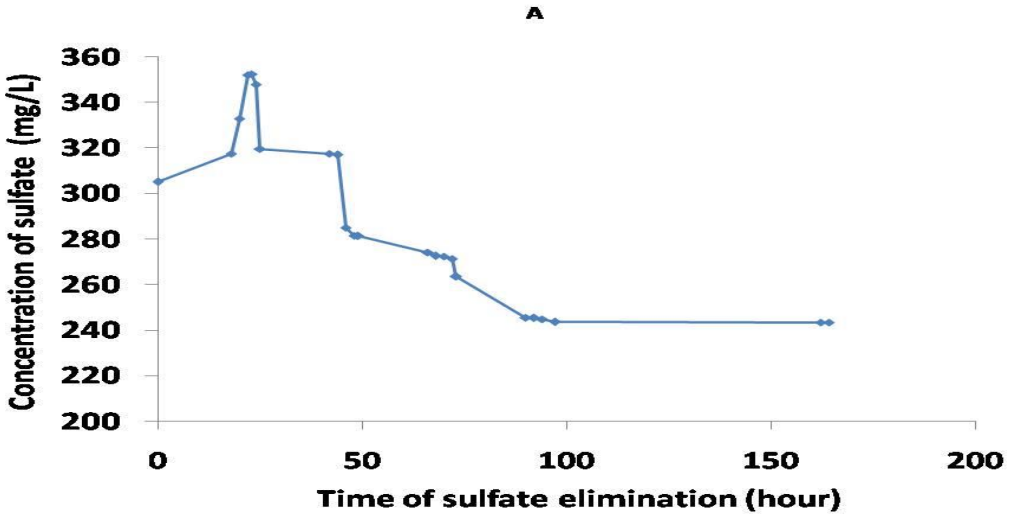

Figure 5.6: The biological sulphate reduction in the A bioreactor.

After the first 23 hours of the experiment, the concentration gradually decreased to 243.38 mg/L during time 23 to 100 hours while the process of bacterial reduction was started with time and sulphate removal efficiency increased rapidly during log and stationary phases of SRB growth with high Number of efficient SRB within this period. After 100 hours of the experiment, the sulphate concentration stabilized at 243.3839 mg/L and

remained constant till the end of the experiment while SRB died off rapidly because they lack nutrients and are poisoned by their own wastes. The efficiency of sulphate elimination during the experiment was **20%** (Figure 5.6).

The unknown isolate, which was isolated from the well water sample (well code 19-14/067) was used in B bioreactor. The sulphate concentration initially started to increase slightly as oxygen present in the bioreactor was consumed; it increased from 315 to 351 mg/L during the first 25 hours (Figure 5.7).

The concentration started gradually decreases to 247.8864 mg/L during time from 25 to 100 hours while the process of bacterial reduction was started with time and sulphate removal efficiency increased rapidly during log and stationary phases of SRB growth with high Number of efficient SRB within this period. After that, the sulphate concentration stabilized at 247.8864 mg/L and remained constant to the end of the experiment while SRB died off rapidly because they lack nutrients and are poisoned by their own wastes. Efficiency of sulphate elimination during the experiment was **22 %** (Figure 5.7).

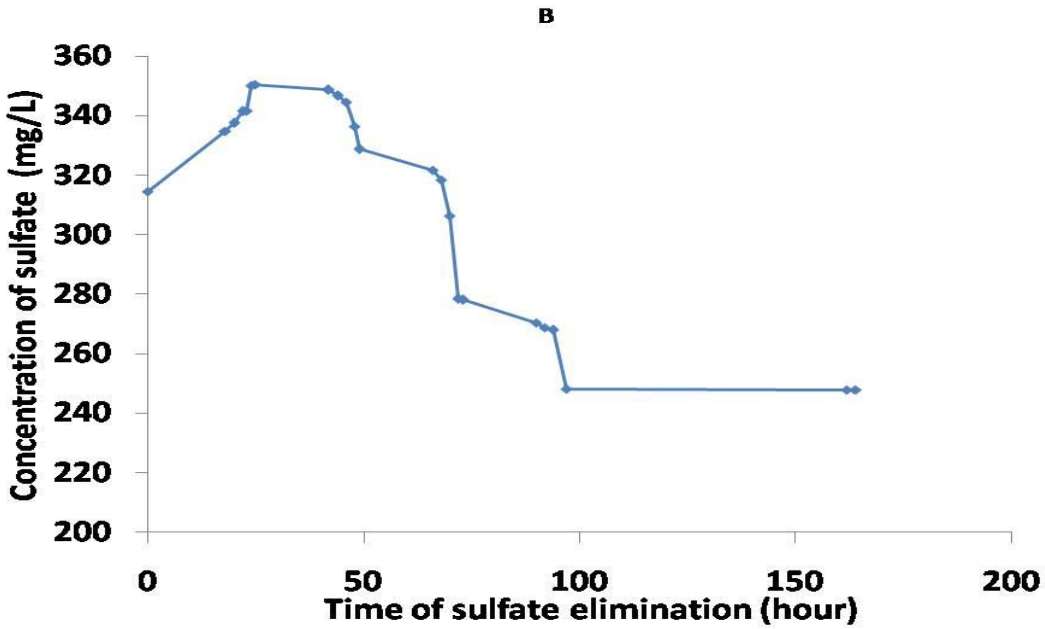

Figure 5.7: The biological sulphate reduction in the B bioreactor.

The unknown isolate, which was isolated from the lisan white soil sample was used in C bioreactor. The sulphate concentration dropped rapidly from 393.6 to 343 mg/L during the first 2 hours with decreasing average of 25.3 mg/L in each hour due to short lag phase of this bacterium. Then it decreased to 328 mg/L within time from 2 to 24 hours (Figure 5.8).

After that, a rapidly decreasing from 328 to 263 during the next 2 hours was observed with decreasing average of 32.5 mg/L in each hour due to the rapid increase in the bacterial efficiency and growth that SRB double in numbers with time during log phase of SRB growth. It continue its decreasing during time from 26 to118 hours till it stabilized at 256.8914 mg/L until the end of experiment as a result of high Number of efficient SRB within this period, it stabilized as SRB died off rapidly because they

lack nutrients and are poisoned by their own wastes. Efficiency of sulphate elimination during the experiment was **34 %** (Figure5.8).

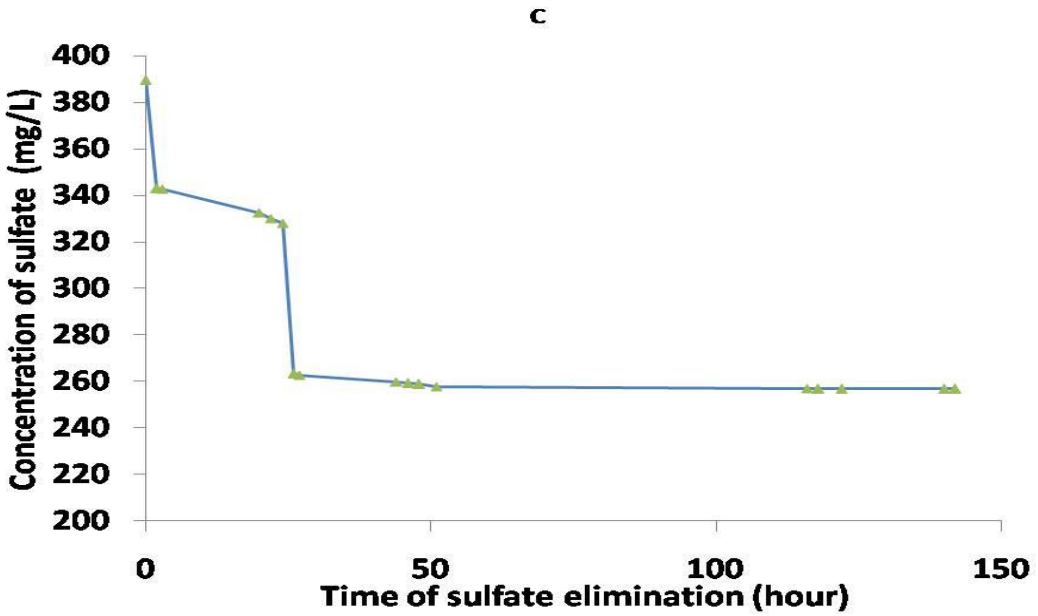

Figure 5.8 : The biological sulphate reduction in the C bioreactor.

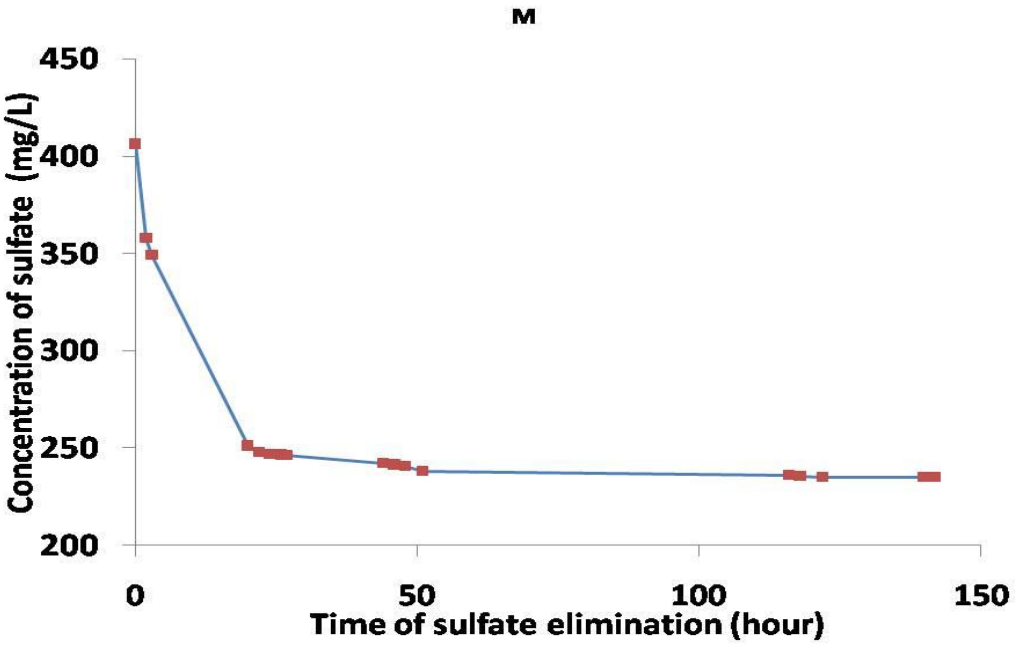

Figure 5.9 : The biological sulphate reduction in the M bioreactor.

The known SRB of the Group 4 which represented by the genus *Desulfobacter sp.* was isolated from the well water sample (well code 19-14/066) was used in M bioreactor. As shown in Figure 5.9, the sulphate concentration dropped rapidly from 406 to 251 mg/L during the first 20 hours with the decreasing average around 7.75 mg/L in each hour due to short lag phase of these bacteria.

The concentration continued its decrease to 235.4595 mg/L within time from 20 to 118 hour as a result of the increasing in bacterial growth and efficiency during log and stationary phases of SRB growth with high Number of efficient SRB within this period. After that the sulphate concentration stabilized at 235.4595 mg/L and remained constant to the end of the experiment while SRB died off rapidly because they lack nutrients and are poisoned by their own wastes. The efficiency of sulphate elimination during the experiment was **43%** (Figure 5.9).

The results of the experiments shown in Figures (5.6 - 5.7) showed that the sulphate concentration initially started to increase slightly as oxygen present in the bioreactor was consumed, but then the process of bacterial reduction was rapidly started with time, and lead to rapid decrease in the sulphate concentration.

Meanwhile, Figures 5.8 and 5.9 show direct decrease in sulphate concentration as bacterial reduction was rapidly started with time, and lead

to rapid decrease in the sulphate concentration due to the short lag phase of SRB used in C and M bioreactors.

Comparison between the biological sulphate reduction in A, B, C, and M bioreactors, and with estimated bacterial growth curve is shown in Figure 5.10.

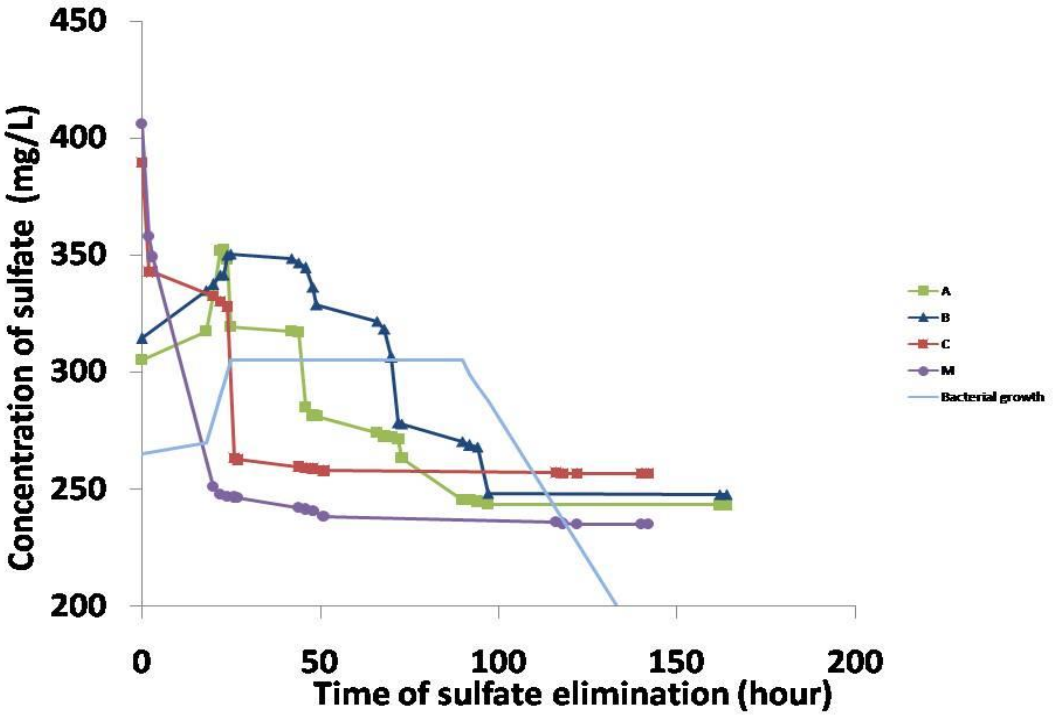

Figure 5.10: Comparison between the biological sulphate reduction in A, B, C, and M bioreactors, and with estimated bacterial growth curve.

Bacterial growth follows a regular pattern that consists of four phases as Figure 5.10 shows:

- First; Lag phase which bacteria exhibits little or no growth. Bacteria used in C and M bioreactors started directly rapid decreasing in sulphate concentration as the lag phase was short, while bacteria used in A and B

bioreactors have longer lag phase that continued to around 20 hours, sulphate concentration initially started to increase slightly as oxygen present in the A and B bioreactors was consumed.

- Second; Log phase which bacteria double in numbers with time. Bacteria used in bioreactors reduced sulphate rapidly during log phase from around 20 to 28 hours due to doubling of SRB.

- Third; Stationary phase which Number of bacteria is steady as the new organisms being produced is equal to number of organisms that are die. Gradually decrease in sulphate concentration in the bioreactors occurred during the stationary phase from 28 to 100 hours.

- Fourth; Death phase which Bacteria die off rapidly because they lack nutrients and are poisoned by their own wastes. Death phase started after time reached 100 hour and the sulphate concentration stabilized to the end of the experiment.

The initial concentration of sulphate used in the bioreactors was 305-406 mg/L (250 mg/L prepared with using standard sulphate and the gap (nearly 55-156 mg/L) comes from sulphate concentration in the medium used).

Figure 5.11 present the percent of sulphate reduction in bioreactors used in this study. In general, the results indicate the presence of active SRB communities with a high diversity in the study area. This reflect that there

are still a lot of undiscovered group of SRB that might be active in this area, but needs new different primers to isolate and identify these types. Moreover, the efficiency of sulphate elimination (or reduction) by SRB using complete Postgate's nutrient medium C was higher than previous studies.

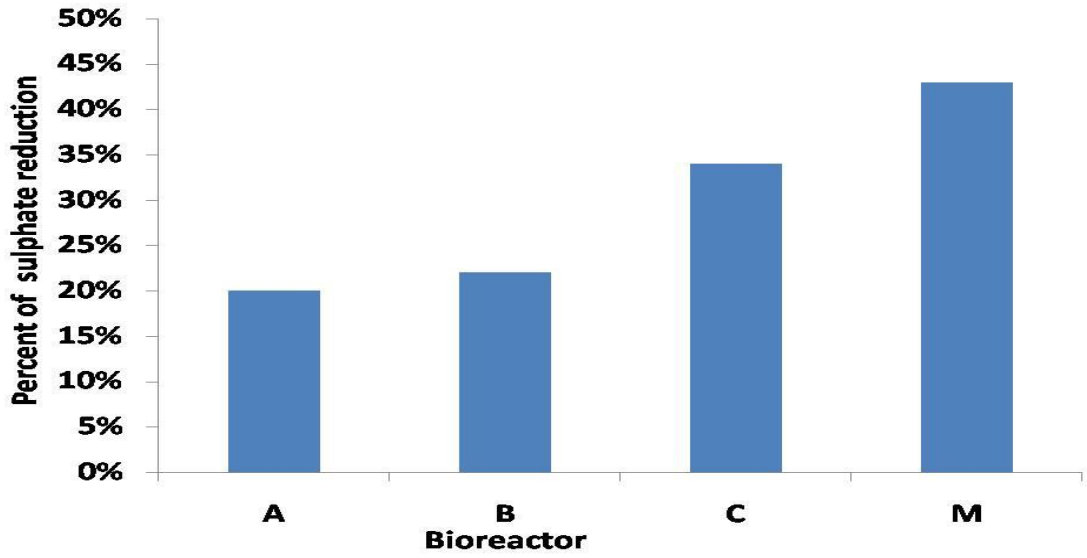

Figure 5.11 : Efficiencies of isolates used in (A, B, C, and M) bioreactors in sulphate reduction.

The general microbiology of hypersaline environments has been extensively studied. For instance, many studies on the biogeochemistry and community composition of hypersaline microbial mats (Baumgartner *et al*., 2006; Decker *et al*., 2005; Fourçans *et al*., 2004; Sørensen *et al*., 2004) and stratified communities within salt crusts (Oren *et al*., 1995; Sørensen *et al*., 2004) have been reported. In addition, a number of novel species of halophilic SRB have been isolated (Caumette *et al*., 1991; Krekeler *et al*., 1997; Ollivier *et al*., 1994). However, only few studies (Foti *et al*., 2007;

Kjeldsen *et al*., 2007) have investigated the nature and activity of the sulphate-reducing microbial community.

A study of Porter *et al*. (2007) has clearly demonstrated that sulphate reduction rates are affected by changes in salinity and sulphate concentration, and organic substrate addition. Based on that, it appears that these parameters are also major factors affecting the SRB community structure. This was reflected by presence of unknown isolates.

Mouné *et al*. (2003) found that specific rates of sulphate reduction influenced under stress and SRB have been shown to up-regulate components of the sulphate reduction pathway as part of a salt stress response (Although it has been shown that sulphate reduction occurs in situ at extremely high salinities (Foti *et al*., 2007; Porter *et al*., 2007) , thus in situ communities of SRB in hypersaline environments may be living under constant salt stress (Brandt *et al*., 2001) resulting in increased specific sulphate reduction rate.). In this study, fluctuation was seen in Sulphate reduction in the bioreactors; this might attributed to stress found in bioreactors.

Meanwhile, it must be noted that for effective sulfate transformation in the bioreactors is affected by prevailing anoxic environment and the induced anoxic conditions during the operation (Mohan *et al*. , 2007).

Based on the reduction of sulphate concentration with time, theoritical interaction was made for each bioreactor (depending on $\log_{10}$ of sulphate concentration in the bioreactor vs time) that to calculate how time would it take until sulphate concentration could reach zero (theoriticaly with refreshment of bacteria) (Figure 5.12).

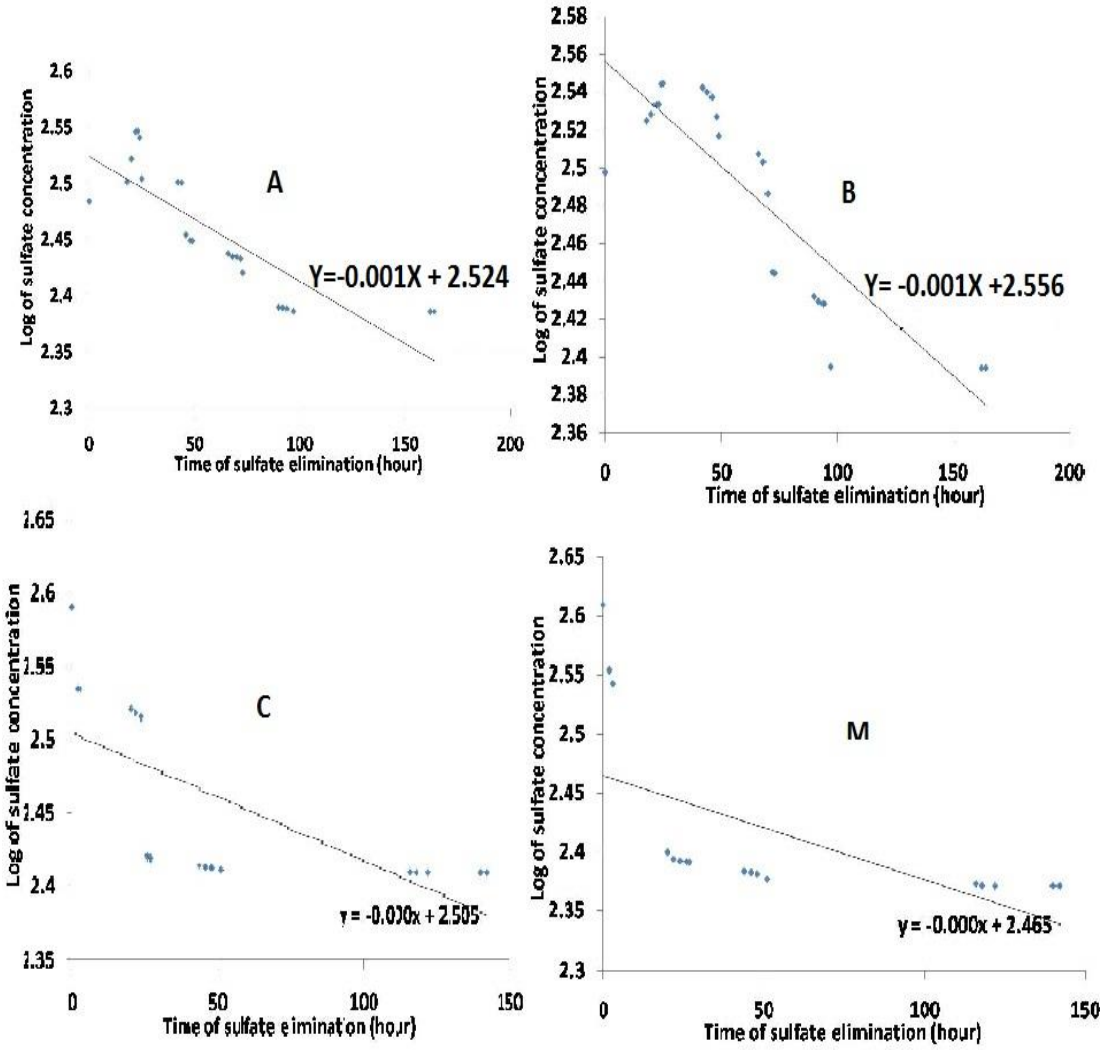

Figure 5.12 **:** Log_{10} of sulphate concentration in the A, B, C, and M bioreactors vs time**.**

Bioreactor results showed that the reduction percentages of sulphate concentrations for *Desulfovirbio sp.* in A bioreactor was 20%, and for the unknown isolate (The one which is not detected by universal primer we have used) was 22% was achieved in B bioreactor (Figure 5.11), which both were near the efficiency reduction percentage of 20% reported by Mohan *et al*. (2007) but much lower than that 30 % achieved by Jing *et al*. (2013) and Genschow *et al*. (1996).

The reduction percentages of sulphate concentrations for *Desulfobacter latus* in M bioreactor was 43%, while 34% reduction percentage of sulphate concentrations for the other unknown isolate was achieved in C bioreactor (Figure 5.11),which both were much higher than that 20% reported by Mohan *et al*.(2007) and 30 % achieved by Jing *et al*. (2013) and Genschow *et al*.(1996).

When these bioreactors (used in this study) reached time after 100 hours, sulphate removal was maintained and stabilized to the end of the experiment. There was no evidence of sulphate removal increasing with time extension. SRB usually competes effectively at low substrate levels (Isa *et al*., 1986). Also with time, the free sulphide concentration is increased and dissolved sulphides usually cause physical, chemical and biological constraints in anaerobic digestion, which may lead to process failure as reported (Chen *et al*., 2008).

Chapter 6 - Conclusions and Recommendations

6.1 Conclusions

Sulphate reducing bacteria (SRB) of known species and unknown species were isolated for the first time from unique high salinity environment of the Dead Sea, whereas other studies isolated such bacterial from relatively less salinity environment.

This work was done to identify and isolate Palestinian bacterial isolates from this unique environment. In this context the SRB belongs to groups 4 and 6 have been isolated. Both have capacity to reduce sulphate. In addition to that, two unidentified isolates were also isolated with a high potential capacity to reduce sulphate.

It was found that the efficiency in sulphate reduction as percentage for *Desulfobacter latus* in M bioreactor was 43%, while 20 % reduction percentage for *Desulfovirbio sp.* was achieved in A bioreactor. Most importantly, for the unknown isolates (The one which is not detected by universal primer have been used in this study) was 22% and 34% in B and C bioreactors, respectively.

6.2 Recommendations

1- Further studies of different SRB application in sulphate removal with high efficiency for the isolates from the Dead Sea area are needed. However, the effect of high chloride content on the SRB efficiency must be taken into account in any new application studies.

2- Further studded to be conducted to sequence and identified the new band presented in PCR products

3- Involvement of Ministry of Agriculture, Ministry of environment, and other related stakeholder to adopt the application of Palestinians SRB isolates in future work.

4- Exploring other areas in Palestine for the presence of SRB and test its efficiency.

5- Exploring the efficacy of application of two SRB isolates together in for their efficiency in sulphate reduction.

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Appendices:

Appendix 1: The sequence of SRB group 4 *Desulfobacter latus PTUK S*

resulted in this work :

The sequence is:

CGGTGATAATATCCATTCAAGCCTGGCTGGAGAGTTTGATCCTGG
CTCAGAATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAA
CGAGAAAGGGATTGCTTGCAATCCTGAGTAGAGTGGCGCACGGG
TGAGTAACACGTAGATAATCTGCCTTCAAGCCTGGGATAACTATT
CGAAAGGGTAGCTAATACCGGATAAAGTCGATTCACATAAGTAAA
TTGATGAAAGATTGCCTCTTCTTGAAAGCAATTGTTTGGGGATGA
GTTTGCGTACCATTAGCTAGTTGGTGGGGTGAAGGCCTACCAAG
GCTGCGATGGTTAGCTGGTCTGAGAGGATGATCAGCCACACTGG
AACTGGAACACGGTCCGGGCTCCTACGGGAGGCAGCAGTGAGGA
ATTTTGCGCCATGGGGGCAACCCAGACGCAGCAATGCCGCGTGA
GTGAAGAAGGCCTTTGGGTCGTAAAGCTCTGTCAACAAGGAAGA
AATTAGGAATTATTAATAGTTGTTTCTATTGACGGTACTTGTTGAG
GAAGCGCCGGCTTACTCCGTGCCAGCAGCCGCGGTAACACGGGG
GGCGCAAGCGTTATTCGGAATTATTGGGCGTAAAGGGCGCGCAG
GCGGTCTTGTCCGTCAGGTGTGAAAGCCCGGGGCTCAACCCCGG
AAGAGCACTTGAAACAGCAAGACTTGAATACGGGAGAGGAGAGA
GGAATTCCTGGTGTAGAGGTGAAATTCGTAGATATCAGGAGGAA
CACCGATGGCGAAGGCATCTCTCTGGACCGATATTGACGCTGAG
GCGCGAAGGCGTGGGGAGCGAACGGGATTAGATACCCCGGTAGT
CCACGCAGTAAACGTTGTACGCTCGGTGTAGCGGATATTAAAATC
TGCTGTGCCAAAGCTAACGCATTAAGTGTACCGCCTGGGAAGTAC
GGTCGCAAGACTAAAACTCAAAGGAATTGGCGGGGGCCCGCACA
AGCGGTGGAGCATGTGGTTTAATTCGATAAAACGCGAAGAACCTT
ACCTGGGTTTGACATCCTGTGAATATCCCGTAATTGGGATAGTGC
CTTCGGGAGCACAGAGACAGGTGCTGCATGGCTGTCGTCAGCTC
GCGTCGTGAGATGTTTGGTTAAGTCCAGCAACGAGCGCAACCCTT
ATCGTCAGTTGCCAGCACTTCGGGTCTCTCGGCATAGTCGATGCC
CTACGCAAAAAAAAGAAAAGAAAA

Appendix 2: NCBI Blast, description of sequencing producing alignments:

Desulfobacter latus strain DSM 3381 16S ribosomal RNA, partial sequence

Sequence ID: gi|343201416|NR_042142.1 Length: 1533 Number of Matches: 1

▶ See 1 more title(s)

Range 1: 1 to 1150 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1994 bits(2210)	0.0	1132/1150(98%)	0/1150(0%)	Plus/Plus

```
Query  35    AGAGTTTGATCCTGGCTCAGAATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAAC  94
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     AGAGTTTGATCCTGGCTCAGAATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAAC  60

Query  95    GAGAAAGGGATTGCTTGCAATCCTGAGTAGAGTGGCGCACGGGTGAGTAACACGTAGATA  154
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    GAGAAAGGGATTGCTTGCAATCCTGAGTAGAGTGGCGCACGGGTGAGTAACACGTAGATA  120

Query  155   ATCTGCCTTCAAGCCTGGGATAACTATTCGAAAGGGTAGCTAATACCGGATAAAGTCGAT  214
             ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  121   ATCTGCCTTCAAGCCTGGGATAACTATCCGAAAGGGTAGCTAATACCGGATAAAGTCGAT  180

Query  215   TCACATAAGTAAATTGATGAAAGATTGCCTCTTCTTGAAAGCAATTGTTTGGGGATGAGT  274
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   TCACATAAGTAAATTGATGAAAGATTGCCTCTTCTTGAAAGCAATTGTTTGGGGATGAGT  240

Query  275   TTGCGTACCATTAGCTAGTTGGTGGGGTGAAGGCCTACCAAGGCTGCGATGGTTAGCTGG  334
             ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  241   TTGCGTACCATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCTGCGATGGTTAGCTGG  300

Query  335   TCTGAGAGGATGATCAGCCACACTGGAACTGGAACACGGTCCGGGCTCCTACGGGAGGCA  394
             |||||||||||||||||||||||||||||||||||||||||| | ||||||||||||||||
Sbjct  301   TCTGAGAGGATGATCAGCCACACTGGAACTGGAACACGGTCCAGACTCCTACGGGAGGCA  360

Query  395   GCAGTGAGGAATTTTGCGCCATGGGGGCAACCCAGACGCAGCAATGCCGCGTGAGTGAAG  454
             |||||||||||||||||||| |||||||||||||| |||||||||| ||||||||||||||
Sbjct  361   GCAGTGAGGAATTTTGCGCAATGGGGGCAACCCTGACGCAGCAACGCCGCGTGAGTGAAG  420

Query  455   AAGGCCTTTGGGTCGTAAAGCTCTGTCAACAAGGAAGAAATTAGGAATTATTAATAGTTG  514
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  421   AAGGCCTTTGGGTCGTAAAGCTCTGTCAACAAGGAAGAAATTAGGAATTATTAATAGTGG  480

Query  515   TTTCTATTGACGGTACTTGTTGAGGAAGCGCCGGCTTACTCCGTGCCAGCAGCCGCGGTA  574
             |||||||||||||||||||| ||||||||||||| ||||||||||||||||||||||||||
Sbjct  481   TTTCTATTGACGGTACTTGTGGAGGAAGCGCCGGCTAACTCCGTGCCAGCAGCCGCGGTA  540

Query  575   ACACGGGGGGCGCAAGCGTTATTCGGAATTATTGGGCGTAAAGGGCGCGCAGGCGGTCTT  634
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   ACACGGGGGGCGCAAGCGTTATTCGGAATTATTGGGCGTAAAGGGCGCGCAGGCGGTCTT  600

Query  635   GTCCGTCAGGTGTGAAAGCCCGGGGCTCAACCCCGGAAGAGCACTTGAAACAGCAAGACT  694
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   GTCCGTCAGGTGTGAAAGCCCGGGGCTCAACCCCGGAAGAGCACTTGAAACAGCAAGACT  660

Query  695   TGAATACGGGAGAGGAGAGAGGAATTCCTGGTGTAGAGGTGAAATTCGTAGATATCAGGA  754
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   TGAATACGGGAGAGGAGAGAGGAATTCCTGGTGTAGAGGTGAAATTCGTAGATATCAGGA  720

Query  755   GGAACACCGATGGCGAAGGCATCTCTCTGGACCGATATTGACGCTGAGGCGCGAAGGCGT  814
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   GGAACACCGATGGCGAAGGCATCTCTCTGGACCGATATTGACGCTGAGGCGCGAAGGCGT  780

Query  815   GGGGAGCGAACGGGATTAGATACCCCGGTAGTCCACGCAGTAAACGTTGTACGCTCGGTG  874
             ||| |||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  781   GGGTAGCGAACGGGATTAGATACCCCGGTAGTCCACGCAGTAAACGTTGTACACTCGGTG  840

Query  875   TAGCGGATATTAAAATCTGCTGTGCCAAAGCTAACGCATTAAGTGTACCGCCTGGGAAGT  934
             | ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  841   TGGCGGATATTAAAATCTGCTGTGCCCAAGCTAACGCATTAAGTGTACCGCCTGGGAAGT  900

Query  935   ACGGTCGCAAGACTAAAACTCAAAGGAATTGGCGGGGGCCCGCACAAGCGGTGGAGCATG  994
             |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  901   ACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG  960

Query  995   TGGTTTAATTCGATAAAACGCGAAGAACCTTACCTGGGTTTGACATCCTGTGAATATCCC  1054
             |||||||||||||   |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   TGGTTTAATTCGAGCCAACGCGAAGAACCTTACCTGGGTTTGACATCCTGTGAATATCCC  1020

Query  1055  GTAATTGGGATAGTGCCTTCGGGAGCACAGAGACAGGTGCTGCATGGCTGTCGTCAGCTC  1114
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  GTAATTGGGATAGTGCCTTCGGGAGCACAGAGACAGGTGCTGCATGGCTGTCGTCAGCTC  1080

Query  1115  GCGTCGTGAGATGTTTGGTTAAGTCCAGCAACGAGCGCAACCCTTATCGTCAGTTGCCAG  1174
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  GCGTCGTGAGATGTTTGGTTAAGTCCAGCAACGAGCGCAACCCTTATCGTCAGTTGCCAG  1140

Query  1175  CACTTCGGGT  1184
             ||||||||||
Sbjct  1141  CACTTCGGGT  1150
```

Appendix 3: The sequence of SRB group 6 *Desulfovibrio vulgaris PTUK S* resulted in this work :

The sequence is:

CGTGTCGGAGCCCGCGTTCCATTAGCTAGTTGGTGAGGTAACGGCCCA
CCAAGGCGACGATGGGTAGCCGGTCTGAGAGGATGACCGGCCACACTA
GGACTGGAACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTGAGGGATGA
AGGTCCTCGGATCGTAAACCTCTGTCAGGAGGGAAGAACGGCCACGGT
GCTAATCAGCCGTGGTCTGACGGTACCTCCAAAGGAAGCACCGGCTAA
CACCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTGTATCGG
AATCACTGGGCGTAAAGCGCACGTAGGCTGCTTGGTAAGTCAGGGGTG
AAAGCCCGCGGCTCAACCGCGGAATTGCCTTTGATACTGCCGAGCTAG
AGTCCGGGAGAGCGTAGTGGAATTCCAGGTGTAGGAGTGAAATCCGTA
GAGATCTGGAGGAACATCAGTGGCGAAGGCGACTACCTGGACCGGTAC
TGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCA
GGTAGTCCACGCCGTAAACGATGGACACTAGGTGCC GACCGTCATCTA
GGATGCATCAAAAAAGAAAA

Appendix 4: NCBI Blast, description of sequencing producing alignments:

Desulfovibrio vulgaris gene for 16S rRNA, partial sequence

Sequence ID: gi|77539416|AB237496.1 Length: 605 Number of Matches: 1

Range 1: 1 to 605 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1065 bits(1180)	0.0	599/605(99%)	0/605(0%)	Plus/Plus

```
Query  8    GAGCCCGCGTTCCATTAGCTAGTTGGTGAGGTAACGGCCCACCAAGGCGACGATGGGTAG  67
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GAGCCCGCGTTCCATTAGCTAGTTGGTGAGGTAACGGCCCACCAAGGCGACGATGGGTAG  60

Query  68   CCGGTCTGAGAGGATGACCGGCCACACTAGGACTGGAACACGGCCCAGACTCCTACGGGA  127
            |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  61   CCGGTCTGAGAGGATGACCGGCCACACTGGGACTGGAACACGGCCCAGACTCCTACGGGA  120

Query  128  GGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTGAGG  187
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTGAGG  180

Query  188  GATGAAGGTCCTCGGATCGTAAACCTCTGTCAGGAGGGAAGAACGGCCACGGTGCTAATC  247
            |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  181  GATGAAGGTCCTCGGATCGTAAACCTCTGTCAGGAGGGAAGAACCGCCACGGTGCTAATC  240

Query  248  AGCCGTGGTCTGACGGTACCTCCAAAGGAAGCACCGGCTAACACCGTGCCAGCAGCCGCG  307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AGCCGTGGTCTGACGGTACCTCCAAAGGAAGCACCGGCTAACACCGTGCCAGCAGCCGCG  300

Query  308  GTAATACGGAGGGTGCGAGCGTGTATCGGAATCACTGGGCGTAAAGCGCACGTAGGCTGC  367
            |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  301  GTAATACGGAGGGTGCGAGCGTTAATCGGAATCACTGGGCGTAAAGCGCACGTAGGCTGC  360

Query  368  TTGGTAAGTCAGGGGTGAAAGCCCGCGGCTCAACCGCGGAATTGCCTTTGATACTGCCGA  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TTGGTAAGTCAGGGGTGAAAGCCCGCGGCTCAACCGCGGAATTGCCTTTGATACTGCCGA  420

Query  428  GCTAGAGTCCGGGAGAGCGTAGTGGAATTCCAGGTGTAGGAGTGAAATCCGTAGAGATCT  487
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GCTAGAGTCCGGGAGAGGGTAGTGGAATTCCAGGTGTAGGAGTGAAATCCGTAGAGATCT  480

Query  488  GGAGGAACATCAGTGGCGAAGGCGACTACCTGGACCGGTACTGACGCTGAGGTGCGAAAG  547
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GGAGGAACATCAGTGGCGAAGGCGACTACCTGGACCGGTACTGACGCTGAGGTGCGAAAG  540

Query  548  CGTGGGGAGCAAACAGGATTAGATACCCAGGTAGTCCACGCCGTAAACGATGGACACTAG  607
            |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  541  CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGACACTAG  600

Query  608  GTGCC  612
            |||||
Sbjct  601  GTGCC  605
```