Improving Evaporation Rate of Mine Wastewater

By

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Declarations

I, Londiwe Thandeka Precious Khumalo hereby declare that *Improving Evaporation Rate of Mine Wastewaters* is my own original work, that has not been submitted to any other university for obtaining an academic qualification. Furthermore, all the sources that I have used are accurately reported and acknowledged by complete reference.

Signature: LTP Khumalo Date:17 March 2018

Abstract

The treatment of mine water at the eMalahleni Water Reclamation Plant (EWRP) results in the production of large volumes of brine. Different brine management methods have been applied to dispose the brine but the evaporation pond method is regarded as the cheaper, most effective and less laborious method for brine disposal. Brine wastewater is pumped into the pond where it evaporates resulting in the mixture of salts. The rate at which evaporation occurs is influenced by many factors such as temperature, salinity, humidity and wind. Due to high salinities in brine the EWRP is currently experiencing a challenge with low evaporation rate. Here, a comparative study was done to determine the efficiency of using a chemical and a biological approach to enhance the evaporation rate of reject brine. The chemical approach involved the addition of various concentrations of methylene blue dye (100 to 300 ppm with 50 ppm increments) to 1L volumes of brine, and measuring the evaporation rate. On the other hand**,** the biological approach involved the isolation of pigmented halophilic bacteria from eMalahleni brine and Cerebos salt samples. Isolated bacterial strains were characterised based on their morphology, biochemical and salt tolerance characteristics. Furthermore, the strains were identified using 16S rRNA gene sequence analysis. Among the isolated halophilic bacterial strains, EP-3, an *Arthobacter agilis* isolated from the eMalahleni brine produced a darker pigment compared to the other strains. Therefore, EP-3 was evaluated for its effect on the evaporation of brine using a culture inoculum or the addition pigment extracted from an EP-3 culture. The addition of MB above 100 ppm overcame the effect of salt precipitation and resulted in higher evaporation (41%) rate. Addition of pigmented bacteria or bacterial extracted pigment to the brine respectively resulted in 18% and 24% increase in the evaporation rate.

Keywords: *eMalahleni Water Reclamation Plant, evaporation rate, methylene blue dye, halophilic microorganism, evaporation pond, biological pigment*

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Dedications

I would like to dedicate this thesis to my Aunty **Hlengiwe Nxumalo** *for inspiring me to pursue my master degree.*

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AMD	Acid mine drainage
Blast	Basic local alignment search tool
BOD	Biological Oxygen Demand
Bp	Base pairs
dH_2O	demineralised water
DNA	Deoxynucleotide Acid
DNTPS	Deoxyribonucleotides
DWAF	Department of water affairs
EDTA	Ethylenediamine tetra-acetic acid
et al.,	*et alia (*and Others)
EWRP	EMalahleni Water Reclamation Plant
HiPRO	Hi-Recovery precipitation reverse osmosis process technology
LB	Luria Bertani
MB	Methylene blue
NEB	New England Biolabs
OD	Optical density
ppm	Part per million
PCR	Polymerase Chain Reaction
rpm	Revolution per minute
RO	Reverse osmosis
SDS	sodium dodecyl sulphate
TAE	Tris-acetate EDTA
TDS	Total dissolved solids
TE	Tris-EDTA
Tris	Tris hydro methyl-aminomethane
µL	Microliter

UV	Ultraviolet
w/v	Weight per volume
WHO	World Health Organisation

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The mining industries in South Africa have created massive economic benefits and still play a significant role in ensuring the country's position in the global market (Dwa, 2010). However, despite such benefits, mining activities are still linked with the production of acid mine drainage (AMD), which poses a serious threat to the environment (Mapanda *et al.,* 2007; Sekar *et al.,* 2014). AMD is the highly acidic water that results from the oxidation of sulphur-rich mine wastes (Hobbs *et al.,* 2009).

In order to address and manage AMD generated from several mines within the Witbank area in Mpumalanga province, the eMalahleni Water Reclamation Plant (EWRP) was built and commissioned in 2007 to recover potable water from AMD (Hutton *et al.,* 2009). The EWRP produces 25000 m^3/d of potable water and 150 m^3/d of brine as a by-product using Precipitation Reverse Osmosis (HiPRO) process technology (Gunther and Naidu, 2009; Randall *et al.,* 2011; Sekar *et al.,* 2014). This process attains these high recoveries with membrane as the only recovering operation unity, resulting in reduced chemical and energy input (Hutton *et al.,* 2009).

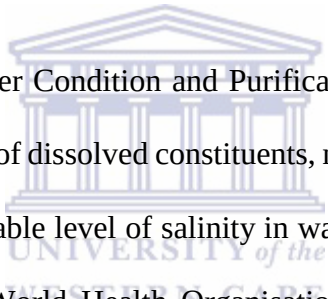
Numerous methods are used for brine disposal and these include deep well injection, evaporation ponds, disposal into surface water and the municipality sewer system (Ahmed *et al.,* 2000; El-Naas, 2011). However, evaporation ponds are considered the best method for brine management at the EWRP because they require low maintenance, little operator attention and they are relatively cheap and easy to build (Ahmed *et al.,* 2000; Morillo *et al.,* 2014). This method works by concentrating the reject brine, leading to the production of manageable solids as the brine evaporates completely. Evaporation ponds work

effectively in inland regions that receive high evaporation rate with relatively hot-dry climate (Ahmed *et al.*, 2000). The hotter the climate the faster the evaporation rate and large volumes of brine water lost through evaporation. Currently, the EWRP is facing a challenge with evaporation rates in their pond as the plant generates more brine than can be evaporated. The main objective of this study was to assess whether the brine evaporation rate of the eMalahleni pond can be improved. In this study, two different approaches, biological and chemical were compared.

The chemical approach entails the addition of methylene blue dye. Previous studies have shown that the addition of dyes in brine trap more solar radiation, increase temperature and vapour pressure thus increasing the rate of evaporation (Blochi *et al.*, 1951; Rajvanshi, 1981; Hoque *et al.*, 2010). However, this approach could have negative environmental impacts if the added organics are to be disposed. Therefore, an alternative biological approach was also considered, premised on the incorporation of pigment producing halophilic bacteria to enhance the evaporation rate of brine water. Halophilic bacteria require a high salt concentration for their growth and they can resist the osmotic pressure and denaturing effects of salt (Kivistö and Karp, 2011). These microorganisms have been noted to produce a bright red pigment in salt ponds (Zhiling and Guangyu, 2009). Carotenoids are not only produced by bacteria, algae species such as *Dunaliella* and *Spirulina* are also known to produce carotenoids and have been applied in the treatment of saline tannery wastewater (Dunn, 1998). These species grow and dominate saline water at an early stage, synthesizing and accumulating pigments. The pigment colours the brine, increases the extent of transparency and thus reducing the thickness of brine. This aids in the absorbance of solar radiation and increases the evaporation rate (Zhiling and Guangyu, 2009).

1.2. Brine

According to El-Nass, (2011) brine is any water body in a desalination process with high salinity compared to the feed water, whereas the reject brine is a highly saturated water discarded in the final stage of the desalination process as wastewater. Brine can be natural or anthropogenic in nature depending on the complexity of the chemical composition as well as the environmental factors (Moore and Runkles, 1968; Svensson, 2005). Studies have independently reported that natural brine contains dissolved molecules and ions (Nyamhingura, 2009). Moreover, the presence of Cl, Mg, Na and Ca ions as well as high levels of total dissolved solids is responsible for the salinity of brine waters making it unfit for human consumption and agricultural purposes.



According to the 2005 report of Water Condition and Purification, brine produced in industries using membrane treatment has 35000 mg/L of dissolved constituents, making it five times saltier than sea water (Peter and Cartwright, 2005). Acceptable level of salinity in waste water is 500 ppm but 1000 ppm can also be tolerated in special cases (World Health Organisation, 1988). The most significant part of desalination is the production of brine. About 185193 m^3 of brine is produced per month at Kromdraal mine and is estimated to increase to 523683 m^3 per month in future (Gunther and Naidu, 2009). Petrik *et al.,* (2015) estimated that the desalination of 1 m^3 of mine water produces an equal amount of reject brine. Other than reverse osmosis of AMD, brine is also produced from oil and gas production, evaporation cooling systems and process industries (Sekar *et al.,* 2014).

1.2.1 Chemical properties of brine

Due to the chemical composition of most brines in South Africa, the Department of Water Affairs (DWAF) rated brine as the second most harmful waste to be discarded into the environment (Gunther and Naidu, 2009). The chemical composition of brine waters is linked to the desalination method used,

the chemical properties of the feed water, the pre-treatment applied and the amount of water recovered (Ahmed *et al.,* 2000; Petrik *et al.,* 2015) and therefore varies. For example, the Secunda plant in South Africa treats brine water that contains Ca, Mg, Cl, Si, S, Na, Br, and Cr, V, Ti, P, Mn, Fe as trace elements (Petrik *et al.,* 2015). While the brine treated in Saldana region contains Fe, Al, As, Cd, Mn, Ni, Pb and Se (Mohamed *et al.,* 2005).

Various chemicals are employed during desalination processes for both the pre- and post -treatment stages. The pre-treatment of brine water may include chlorinification, acidification, de-chlorinification, flocculation and coagulation steps, depending on the quality of feed water. These processes involve the addition of chemicals such as sodium hypochlorite to inhibit bacterial growth, aluminium chloride to remove suspended solids from water, sodium hexamate phosphate to prevent scale formation from the membrane and pipes as well as hydrochloric acid to regulate the pH (El-Naas, 2011). The properties of the feed water are further affected if the water undergoes post-treatment (Swartz *et al.,* 2006; El-Naas, 2011).

1.3 Brine management techniques

The desalination of brackish and saline water has increased significantly in the past decade as the demand for potable water increases with the population growth. About 30 million cubic metres of desalinated water is produced per day and was expected to increase to 30 billion by 2015 (El-Naas, 2011). The largest desalination plant in South Africa, produces 15000 m^3 of desalinated water per day that is supplied to Mossel Bay municipality and PetroSA (Veolia Water Solutions & Technologies, 2011).

The disadvantage of the desalination process is the production of large volumes of reject brine. Due to its chemical composition brine water requires to be discarded properly. In coastal regions the ocean is considered the economical method for brine management (Svensson, 2005). However, for inland regions

the desalination plants are far away from the ocean therefore different methods are applied for brine disposal. These include release into deep wells, land application, evaporation ponds, the discharge of brine into surface waters and the release of brine into wastewater treatment plant (Ahmed *et al.*, 2000; De Vito *et al.*, 2011). Table 1 provides information about brine management methods used in the USA (Ahmed *et al.*, 2001; Mohamed *et al.*, 2005).

The selection of a method is dependent on the properties of the wastewater to be discarded, the area of operation and its economical aspect (Morillo *et al.*, 2014). Cost is an integral part to choosing the desalination method to use, as it can contribute up to 33% of the total cost for saline water disposal (De Vito *et al.*, 2011). The amount of brine to be discarded, the nature of environment, and the composition of the brine and the level of treatment prior to disposal are the main factors that determine the cost of brine disposal (Ahmed *et al.*, 2000; ESCWA, 1993; Ahmed *et al.*, 2001). It is considered cheaper to dispose of small volumes of water saturated with salts than to dispose of a large volume of slightly saline water (Hoque *et al.*, 2010). The following section focuses on different brine management techniques that are utilised in land regions.

Table 1: Brine disposal methods in the USA

Brine management methods	Percentage (%)
Surface water	48
Discard to municipality sewer	23
Land application	12
Deep well injection	10
Evaporation ponds	6

Adapted from: (Ahmed *et al.,* 2001; Mohamed *et al.,* 2005).

1.3.1 Sewer discharge

The blending of brine and a secondary influent from a wastewater treatment system is a technique used mostly by small reverse osmosis plants for reject wastewater management (Ahmed *et al*., 2000). This technique lowers both the biochemical oxygen demand (BOD) and total dissolved solids (TDS) and dilutes the brine concentration. This is also considered more economical than other methods because it uses the already available wastewater treatment facilities (Balasubramanian, 2013). The presence of high total dissolved solids in brine greatly affects the microorganisms in the system, impacts the infrastructure used and disallows the use of sewage wastewater for irrigation purposes (Ahmed *et al.,* 2000; Balasubramania, 2013).

In coastal regions reject brine is mostly discarded into the ocean or sea. Even though the effect of discarding brine waters into the oceans is largely unknown, the elevated temperature and salinity associated with the discharge may be detrimental to the marine environment (De Vito *et al.,* 2011). Furthermore, these brine waters contain high levels of elements such as Na, K, Ca and metals such as Fe, Li and Mn which have a potential to decrease the level of dissolved oxygen in the seawater resulting in negative impacts on the aquatic life (Svensson, 2005; Gunther and Naidu, 2009; El-Naas , 2011). As part of the environmental plan towards ensuring adequate protection and sustainability of water resources,

most desalination industries in the United Kingdom require a permit to manage the reject concentrate and such brines are not directly discarded without prior treatment (Svensson, 2005). In South Africa, the waste produced during the desalination process is assessed and classified according to Norms and Standard of waste disposal. Due to the nature of brine, it is mandatory that the brine residual must be discarded to a designated licenced landfill site that is managed by the site manager. This is done to prevent the pollution of water resources near the desalination plant (Chetty and Ladouce, 2013; Costley, 2013).

1.3.2 Land application

Reverse osmosis permeates are considered to be valuable for land applications such as irrigation, percolating pond and infiltration channels. Reject brine can be used to irrigate salt tolerant plants while for other plants the concentrate quality must meet the crop salt requirements (Balasubramanian, 2013). However, the increased levels of trace elements in reject brine make it largely unsuitable for irrigation purposes (Ahmed *et al.,* 2000). Site selection, pre-treatment, hydraulic loading rate, surface runoff, land application and the type of vegetation are factors that determine whether brine is suitable for irrigation purposes.

1.3.3 Deep-well injection

Deep well injection has been used for the disposal of saline, municipal and industrial toxic liquid waste globally (El-Naas, 2011; Balasubramanian, 2013). As a result, this method has been considered as an option for reject brine disposal in land regions (Glater and Cohen, 2003). In this approach brine is discarded into permeable subsurface rock formations as shown in Figure 1. The design criteria of this technique depend on two critical factors: site location and the depth of the well (Balasubramanian, 2013). Deep well injection is not suitable for areas that are vulnerable to volcanic activity or contain minerals, and the depth should be in the range of 330-2600 m below the surface to prevent contamination of the ground water (Ahmed *et al.*, 2000; Balasubramanian, 2013). However, this technique has challenges

including long transportation distances, conditioning of the brine, corrosion of the transporting pipes and seismic activity (Ahmed *et al.*, 2001; Glater and Cohen, 2003; El-Naas, 2011).

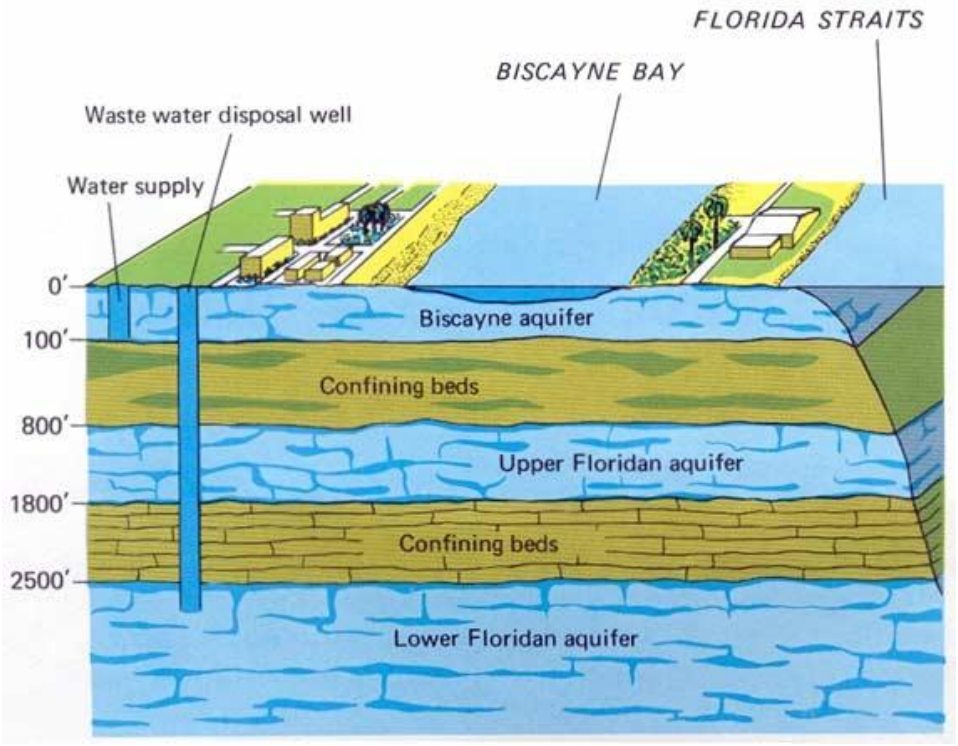


Figure 1: Deep well injection operation. The shaft containing multiple layers of protective casing and cement design to deposit the hazardous waste below the earth surface**. Adapted from:** Vernon, 1970

1.3.4 Zero liquid discharge

This method of discharge entails the pre-treatment and evaporation of the reverse osmosis effluent until the salts precipitate and crystallise. Crystallised salt is then removed from the pond, dewatered and disposed in a landfill site or sold to companies (Koppol *et al.*, 2003; Martinez and Pozuelo, 2011). Zero liquid discharge regulations require that the resulting solid waste should be regenerated with a minimal content of 10% moisture for proper landfilling disposal. What makes this technique unique is its ability to reduce disposal of brine to surface and ground water while minimising the use of freshwater. Therefore, this would help to overcome the shortage of freshwater availability (Balasubramanian 2013;

Koppol *et al.,* 2003). The cost of the zero liquid method is high due to energy consumption and the need for waste disposal, and is mostly applied in mechanical evaporation (Glater and Cohen, 2003).

1.3.5 Evaporation pond

Inland region evaporation ponds are recognised as the most effective and economically viable method for brine disposal. Evaporation ponds are most effective in warm and dry climatic regions where a high evaporation rate may be achieved (Mickely, 1993; El-Naas, 2011; Balasubramania, 2013). Advantages include easy construction of the infrastructure, little operation-attention, low maintenance and most importantly the technique is not limited by the composition of the brine (Hoque *et al.*, 2010). Furthermore, no extensive mechanical equipment is required besides the pump to transport reject brine into the pond (Ahmed *et al.,* 2000; Ahmed *et al.*,2001; Glater and Cohen, 2003; Morillo *et al.,* 2014). The ideal size of the pond is dependent on accurate calculations of the evaporation rate per year (Ahmed *et al.*, 2000). Smaller ponds are favoured as they have higher evaporation rates and are easier to maintain than larger ponds. During the design of the evaporation pond the surface area and the depth of the pond are considered since the greater the surface area, the greater the rate of evaporation (Dama-fakir and Toerien, 2000; Dama-Fakir and Toerinaen, 2009). The depth of the pond is determined by the water and salt holding capacity, the surge capacity and the freeboard. The depth of the ponds range from 2.5-46 cm to maximize the evaporation rate (Petrik *et al.,* 2015). A study conducted by Bain *et al* (1969) showed that an increase in depth of the pond by 2.5-102 cm decreases the rate of evaporation by 40%.

The disadvantage of using evaporation ponds for the disposal of brine water centres around groundwater contamination via the seepage of the membrane permeates (Hoque *et al.*, 2010). This shortcoming is overcome by lining or sealing the pond with polymeric materials such as polyethylene (Ahmed *et al.*, 2001).

The main aim of an evaporation pond is to concentrate the saline effluent by reducing its volume through evaporation and thus leave salt crystals at the bottom (Ahmed *et al.,* 2000). The most significant aspect in the operation of an evaporation pond is the evaporation rate. Evaporation rate is defined as the amount of water evaporated from the water surface per unit of time (El-Naas, 2011). The rate of evaporation can be expressed either as mass or volume of water evaporated per unit of time (Al-Shammiri, 2002). This can be further equated to the depth liquid loss per unit of time in the whole area (Coleman, 2000). In the next section, various factors that impact the evaporation rate of brine waters will be highlighted.

1.4. Effect of climatic conditions

1.4.1 Humidity

Humidity refers to the amount of water vapour that is in the atmosphere. When air is saturated with water vapour it results in a decrease in the rate of brine evaporation (Patrick *et al.*, 2015). Wind, the salt composition and rainfall contribute to elevated levels of humidity. The chemical composition of the brine wastewater determines the level of humidity which in turn defines the level of evaporation. Evaporation of brine saturated with NaCl ceases when the level of humidity is greater than 70%, while with other salts such as MgCl, the evaporation stops at moderately low level of humidity (Dama-Fakir and Toerien, 2009).

1.4.2 Temperature

During the evaporation of brine the water molecules in the solution are continuously in motion, and the rate of movement of water molecules is dependent on the surrounding temperature. High temperatures weaken the bond between the water molecules which cause them to escape to the atmosphere as water vapour. These molecules remain in motion until they reach equilibrium vapour pressure that is dependent on the ambient temperature and chemical complexity of the liquid (Coleman, 2000). Other studies have

shown that an increase in the temperature of the brine wastewater is likely to enhance evaporation (Keyes and Gunaji, 1967; Hoque *et al*., 2010). However, the brine water is less effective in converting radiant energy into latent heat due to the exchange of sensible heat and long-wave radiation with the atmosphere. The overall result is that, with the same input of energy, the evaporation rate of freshwater is higher than that of brine water (Coleman, 2000). This then means high temperature is needed to increase the movement of water molecules to the atmosphere. The presence of salt in the brine decreases evaporation of water, and therefore the composition of waste brines is expected to impact on the evaporation of brines being treated.

Large facilities are concerned that this technology is one of the few treatment methods that offers decreasing returns to scale because of increasing boundary-layer resistance for larger ponds (Hoque *et al.*,2010). Considerable research has focused on finding ways to increase evaporation rates. Therefore, in the next section, the different methods which have been/are currently being devised to improve evaporation rates in wastewater brine ponds will be reviewed. But first the calculation involved in evaluating evaporation will be presented.

1.5 Calculation of evaporation rate

The rate of evaporation can be determined using different equations, and must consider the change of humidity in the air, heat and mass balance, weight change of water in the pan/pond, surface area etc. (Al-Shammiri, 2002). It is therefore also possible to measure or calculate evaporation rate from different bodies of water such as lakes, oceans and brine ponds (Calder and Neal, 1984; Kokya and Kokya, 2007; Abdelrady, 2013).

The standard pan equation, also termed the Brouwer and Heibloem equation, is mostly used to calculate daily pan evaporation rates, and is expressed as:

$$E_{pan}=P + (n_1 - n_2) \quad (1)$$

where E_{pan} is for daily pan evaporation measured in mm/day, P is the daily precipitation and n_1 and n_2 are water surface heights measured in the evaporation at the start and at the end pan before and after, respectively.

Other researchers have published methods for measuring the evaporation rate of freshwater bodies (Sartori, 2000; Tang and Etzion, 2004). One such equation used to measure evaporation of water from pure water is the Penman equation (1948) which is expressed as;

$$E_{mass} = \frac{mRn+PaCp(\delta e)ga}{\lambda v(m+\gamma)} \quad (2)$$

Where E_{mass} represents evaporation rate, m is the slope of saturation vapour and R_n is the net solar energy, Pa is the function of wind velocity and ga represent the momentum surface of aerodynamic conductance. δe represents vapour pressure deficit, λv is the latent heat vaporization and γ is the psychometric constant (Shuttleworth, 2007).

The reduced vapour pressure of brine waters renders this equation unsuitable to measure the evaporation rate of brine waters (Dama-Fakir and Toerien, 2010). This is due to the presence of solutes which increase the boiling point of saline water and lowers the vapour pressure (Mao, 1999; Ahmed *et al*., 2000). Akridge, (2008) modified the Penman equation to reflect the reduced vapour pressure of brine. The modified equation is expressed as;

$$\lambda E = \frac{\Delta}{\Delta+\gamma} R_n + \frac{y}{\Delta+\gamma} f(u)(es - e) \quad (3)$$

Where λ is the latent heat of vaporization, E is the evaporation rate measured in mm/day and Δ is the vapour pressure, y represents the psychometric constant and R_n net solar radiation. $f(u)$ is the function of wind and speed, es and e are the saturation vapour pressure of water and ambient water pressure,

respectively. Similarly, Calder and Neal, (1984) also modified the Penman equation to assess the activity of water in a saline solution at a given temperature. The revised equation is presented below;

$$\lambda E = \frac{\Delta H + \frac{Pc_p\left(e_{s\ (T)} - \frac{e}{aw}\right)}{r_a}}{\Delta + y/_{aw}} \quad (4)$$

Where E is the evaporation rate, λ is the latent heat of evaporation, Δ is the vapour pressure, P is the density of air and ^{c}P represents specific heat of air at constant pressure. $e_{s\ (T)}$ is the saturation vapour pressure of water at temperature T^{o}C, e vapour pressure of air, r_a aerodynamic resistance to the transport of water vapour from the surface into the atmosphere, H total available energy, aw presents activity of water in solution and y represents the psychometric constant.

Ladewing and Asquilth, 2012 has published an equation that does not require climate or system specification in order to calculate evaporation rate of saline water. The equation is presented below;

$$A = {}^{V}/_{E} \quad (5)$$

Where A is the surface area (cm^2),V being the volume of the brine lost over time (cm^3/hr) and

E is the evaporation rate (cm/hr).

1.6. Improving evaporation rates of saline water

The rate at which water escapes from its surface to the atmosphere is a most significant factor in evaporation pond operations (El-Naas, 2011). The rate of evaporation in saline solution can be improved by increasing temperature, the surface area, the vapour pressure and the wind speed, stirring the pond and by weakening the bond of water molecules and spraying the brine (Ahmed *et al.*, 2000; Coleman 2000; Mohamed *et al.*, 2005; Hoque *et al*., 2010).

1.6.1. Improving evaporation by increasing the surface area

The evaporation pond is the most effective method for dewatering of brine wastewater compared to other methods. However, there are shortcomings associated with this method. These include usage of expensive impervious liners to prevent leakage of brine to the underground water and the need for a large portion of land where high levels of evaporation are required (Hoque *et al*., 2010). To overcome these problems, researchers have used coloured solutions such Congo red and naphthol green to increase temperature and vapour pressure and consequently improve evaporation (Keyes and Gunaji, 1966; Rajvanshi 1981).

Other alternative methods of enhancing evaporation rates such as waive and wetted floating fin (Figure 2) were extensively reviewed by Hoque *et al.*, (2010). These methods were studied on saline solutions containing 16-18 g/L of total dissolved solids and highly concentrated with calcium. Waive requires water to be driven onto the fabric to increase surface area and was reported to increase the evaporation rate by 50% when applied in an open pan evaporation pond. The authors used the absorbent properties of the material to uplift water vertically. The effect of the absorbent on the evaporation rate of water has also been studied (Arnal *et al*., 2005). The results show that the presence of the absorbent in water enhances evaporation by 100%.

Spraying of brine into the atmosphere is another technique used to increase the evaporation rate by increasing surface area (Ahmed *et al*., 2000). Coleman, (2000) reported that the average evaporation rate of a saline water body at the surface is about 1400 mm per year. However, when the brine is sprayed over an empty and full pit the rate of transfer is increased from 61% (900 mm /year) to 128% (1800 mm/year) respectively. The author further mentioned that the results were averse to change as the spray could gather more energy from the heated rocks on the pond wall. Therefore, the effective evaporation rate from the nearly empty pond will be 45% (600 mm/year).

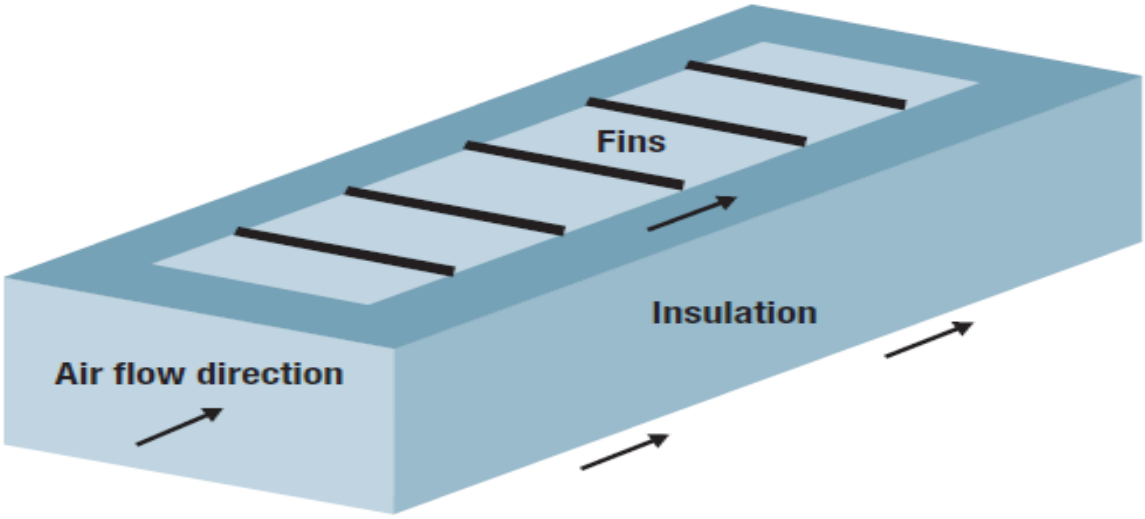


Figure 2: A schematic representation of the wetted fin technique. The evaporation is enhanced by using the floating fin, which increase the surface area, provide an additional area of exchange and also serve as a boundary layer breaker. In this method no pumping system is required as merely the absorbent properties of the material is used to push water up (Hoque *et al.*, 2010).

1.6.2 Increasing temperature /solar radiation

Although the above-mentioned methods are possible options, they suffer from certain drawbacks due to salt build-up in the pond, which requires constant flushing of the brine and leads to increased maintenance cost (Rajvanshi,1981). A second drawback is the need to maintain shallow depths, which requires the precise control of feed and which increases the capital cost which makes them technically and economically unattractive for development on a large scale, such as the evaporation pond system which forms the basis of this study. The major drawbacks are the reduced efficiency of distillation of the system. To try overcome these drawbacks, an alternative option, the incorporation of water soluble organic dyes has been proposed as a way of increasing the solar radiation and thereby the rate of evaporation (Rajvanshi, 1981; Coleman, 2000; Hoque *et al*., 2010).

The dye maximises the absorbance of solar energy in saline effluent in the thin upper layer (~2 cm), thus raising the surface temperature of the brine (Rajvanshi, 1981). Thus, the building up of salt at the bottom of the pond does not interfere with the solar radiation absorption near the surface. Since the introduction of large scale colouring of brines in this way, production figures for companies harvesting chlorides of potassium, sodium and magnesium increased by 40% (Blochi *et al*., 1951). This was largely due to a decrease in seepage, since because of the higher evaporation rates the brines remained in the pans for shorter periods. For the eMalahleni ponds, however, it was anticipated that the seepage would not be a major concern as the ponds are lined.

Studies to determine the effect of the addition of dyes (and related factors) on solar distillation have been conducted (Blochi *et al*., 1951; Rajvanshi, 1981). The related factors include the effect of different dye colours, the depth of the brine solution, ambient temperature, wind velocity and dye concentration. The ideal dye to be used depends on the nature of the brine. It must be water soluble, not contaminate the precipitated salt crystals, must be sufficiently opaque and not affected by the salinity of the brine. These

factors are very crucial in the salt recovery process, as they can increase the level of impurities and thus results in the addition of unnecessary cost for the removal of impurities. Reject brines sometimes contain natural contaminants such as chemical used for pre-treatment, by product formed during the treatment and heavy metals causing absorption of radiation. However, during evaporation the salt starts to crystallize, these contaminants often precipitate with it, leaving a clear brine. The dye is most efficient at these later stages of evaporation, due to the reflection of light from the salt layer at the bottom.

A scheme was developed to calculate the effect of a certain dye in increasing the evaporation (Rajvanshi, 1981). The test for three different coloured dyes (black, red and green) the solar energy absorbed for each was measured and found to be highest for black and lowest for red. As expected, since the black dye absorbed more radiation at the surface, increased evaporation rate was observed. Based on the result of the tested dyes, different regression lines were drawn and the following equations were published by Rajvanshi, (1981) for the calculation of the percentage increase in evaporation (*I*):

$$\mathrm{I} = \left(\frac{1}{\mathrm{S}}\left(1 - \frac{b}{M_c} - 1\right) * 100\% \right. \tag{6}$$

This equation varies with seasons since m_c (distillate output from control unit, kg/m^2-day) is season dependent. *b* (intercept of regression lines, kg/m^2-day) and *s* (slope of the regression lines) are derived from the following equations:

$$Regression\ line\ S = 1.444 - 1.028X \tag{7}$$

$$X = \frac{\int_{\lambda_1}^{\lambda_2} q_\lambda \ [1-\exp\ (-\alpha_\lambda x^*)]\ d_\lambda}{\int_{\lambda_1}^{\lambda_2} q_\lambda \ d\lambda} \tag{8}$$

Where X is the normalised absorption, λ_1 and λ_2 respectively presents the upper and lower limits wavelengths of solar spectrum, and λ is the wavelength (μM). X^* represent the path length of incident

solar radiation ray in a layer (M), σ is the Stefan Boltzman constant and q_λ represents spectral solar energy flux ($KW/m^2\mu M$).

$$Regression\ line;\ \ b = -2.857\ S + 2.571 \tag{9}$$

Thus, for any dye where *X* is known (which can be found from its absorption spectrum), the percentage increase in evaporation can be found.

The effect of increasing the amount of dye has also been determined (Rajvanshi, 1981). For all three dye colours, increasing the concentration resulted in improved evaporation rates. However, this was only true up until 500 ppm, after which there is no difference since around this concentration level about 99.9% of the incoming solar radiation is absorbed in the first 1.27 cm layer of the dye solution, thus making the evaporation independent of the nature of the dye. An interesting correlation between the depth of the dye-water system and concentration became apparent. By increasing the concentration of the dye, the effective depth of the pan is increased, while with decreasing concentration of the dye, the depth is decreased. It can therefore be concluded that for the same concentration of dye, increasing the basin depth will increase the evaporation productivity. Since greater concentration of dye increases the cost of evaporation, it is not advantageous to use greater concentrations of dye, and therefore needs to be carefully considered in relation to the depth. In the referenced study, the black dye (naphthylamine) at a concentration of 172.5 ppm was found to increase solar evaporation by 29% (Rajvanshi, 1981). Other studies have shown that the addition of less than 2 ppm naphthol green increases evaporation by 13% while methylene black naphthylamine at 175 ppm increased by 30% (USDI, 1970; Kalidasa Murugavel *et al*., 2008)**.** Methylene blue and Congo red dyes have also been applied for the solar evaporation of brine and methylene blue enhances evaporation more significantly compared to Congo red (Keyes and Gunaji, 1966; Rajvanshi, 1981).

1.7. Biological processes for the Evaporation of brines

Extremophiles, including halophiles, produce different biomolecules which are of interest in biotechnology industries, because they function exceptionally well in harsh conditions common to industrial processes (Kerkar, 2004; DasSarma *et al.*, 2010). This includes enzymes such as DNase, protease, hydrolytic enzymes, lipases, amylases, gelatinase and pigments (Yoon *et al*., 2003; Joo and Kim, 2005; Udomsil *et al*., 2010; DasSarma *et al.*, 2010; Ventosa *et al*., 1998). More fitting to the focus of this study is the pigmented nature of halophilic microorganisms, and its application in the salt mining industry. The colouration of the pigments is noticeably intense in hyper-saline ponds when the brine approaches salt saturation (Jehlička *et al.*, 2013). An example of this effect is the intense red colouration in salt ponds due to high salinities caused by evaporation of water and the presence of dense microbial communities of halobacteria and haloarchaea. The pigments increase solar energy absorbance of the brine thus resulting in an increased evaporation rate (Zhiling and Guangyu, 2009). The concept of using of dyes in improving evaporation rates, together with the examples of pigment producing halo-tolerant or halophilic microorganisms led to the idea of whether microorganisms could be used as a source of dye to increase the evaporation rates of brine wastewaters. Such a biological approach, as opposed to the anthropogenic addition of dyes, could offer an advantage from a cost perspective if the pigmented culture can maintain its growth in the evaporation pan thus continuously contributing to the absorption of solar radiation and increased evaporation. In contrast to synthetic dyes, bacterial pigments are safer, non-carcinogenic, and biodegradable and thus exhibit high compatibility with the environment (Venil *et al.*, 2013).

The notion of using microorganisms to improve the evaporation of hypersaline water in solar ponds has been applied worldwide for the commercial production of table salt. Companies such as Northern solar works in China, Artisanal salt production in Aveiro/Portugal and Veolia Water Solutions and technologies in South Africa (Zhiling and Guangyu, 2009; Rodrigues *et al.*, 2011; Veolia Water

Solutions and Technologies, 2011) rely on this principle. The common method utilised by salt producing companies, includes pumping sea water into the evaporation pond, where the sea water undergoes physical processes such as condensation and crystallisation as a result of natural evaporation. Accompanying these processes are ecosystem changes that promotes the growth of pigmented microorganisms. Salinas biological systems are mainly composed of microorganisms that are either suspended in water (planktonic) or attached to the pond walls (benthic) and each plays a crucial role in the salt purification process (Davis, 2006). Understanding the ecology of these communities is therefore of ultimate significance to salt production from such ponds (Rodrigues *et al*., 2011). Each community includes producer organisms such as algae, cyanobacteria and other bacteria that produce organic constituents through photosynthesis and control the entire biological system (Davis, 2009).

When balanced, the biological system produces optimal quantities of organic constituents distributed amongst the plankton and benthic communities promoting the production of high quality salt. The planktonic community contributes to salt production by colouring the brine, increasing radiant energy absorption and water evaporation (Moosvi, 2006; Rodrigues *et al*., 2011). Furthermore, this biological system has an ability to protect the pond against leakage, inhibit growth of undesirable mucilage producers and fresh groundwater infiltration (Davis, 1980; Davis, 2000; Rodrigues *et al*., 2011). An unbalanced biological system lacks the necessary organic productivity, resulting in insufficient brine colouring, low evaporation rate and pond leakage (Davis, 1980; Rodrigues *et al*., 2011).

Algae blooms are common in salt works, not the least because of the increase of nutrients concentration with the evaporation (Cantrell *et al.,* 2006). The occurrence of algae bloom in low salinity pond is advantageous, as they colour the brine, increase solar radiation absorption, and thus resulting in faster evaporation. At high salinity, if present in large numbers, algae and their dissolved organic material and

decomposition products inhibits early precipitation of gypsum, because of the increased viscosity of the water. In this incident, gypsum that precipitates later in a crystalliser pond along with sodium chloride, will contaminate the salt and reduce its quality (Levens and Sorgeloos, 1996; DasSarma and DasSarma, 2002). Furthermore, the build-up of dying algae that turn black when oxidized could also contaminate the salt and be the cause of small salt crystals production. In extreme conditions, the brine viscosity can be too high and completely inhibit salt precipitation (Levens and Sorgeloos, 1996).

Artemia are not only important for the control of the algal blooms. However, their metabolites and decaying animals are an ideal substrate for the development of halophilic bacteria (*Halobacterium*) in a pond. A high concentration of halophilic bacteria turns the brine to bright red, increasing solar energy absorption while reducing the concentration of dissolved organic matter. This in turn results in reduced viscosity levels, stimulates the production of large salt crystals and improves salt quality (Levens and Sorgeloos, 1996).

The high salt content in the type of wastewaters generated by coal mines renders it unsuitable for conventional biological treatment unless it is diluted. However, recent studies have shown that using halophiles and halotolerant microorganisms can overcome such limitations (Amoozegar *et al*., 2007; Haddadi and Shavandi, 2013). Wastewater from manufacturing industries is highly concentrated with nutrients, heavy metals and sometimes with dye (Tahir and Rauf, 2004; Gandhi *et al*., 2008). These contaminants need to be reduced before the water is discarded, as this could potentially lead to serious environmental problems (Chen *et al*., 2011).

Zhuang *et al.,* (2010) reported that the addition of halo bacteria during the decontamination of 5% saline water enhanced the removal of ammonia, phosphate and COD up to 51%, 31% and 73%, respectively. The authors further explained that halophilic and halotolerant bacteria have the ability to reduce or

decolorize azo dyes. Therefore, there may be scope to develop the halophiles isolated from the brine evaporation ponds for usage in a wide range of biotechnological applications. The following sections will address saline environments with respect to microbial composition and activity, and a summary of known pigmented halophilic bacteria is presented.

1.8 Saline environments harbouring microorganisms

Saline environments, including brines, are considered as extreme environments. An extreme environment is described as an environment with reduced physicochemical (temperature and pH) and geochemical characteristics (high pressure and salinity) that are unfavourable to most forms of life on earth (Rampelotto, 2010; Morozova et al.,2011). However, microorganisms are not only able to withstand such conditions but they thrive on these conditions for survival and are referred to as extremophiles (Rothschild and Mancinelli, 2001; Cowan et al., 2013). Hypersaline environments are classified by having a salt concentration greater than that of seawater (typically between 3.3-3.5 % of TDS). Based on their origin they can be classified as either thalassophonean (derived from seawater) or athalassohaline (influenced by the geology of the area where they develop) (Kerkar, 2004; Grant, 2004; Gostinčar et al., 2011; Schneegurt, 2012). Great microbial diversity has been observed to thrive in saline conditions, even where sodium chloride concentrations reach up to 35 M (DasSarma and Arora, 2002; Kerkar, 2004). Such microorganisms adapted to grow and reproduce in salt saturated environments are termed halophiles (Antón et al., 2002; Mormile et al., 2003), and are classified into 3 groups based on their growth response to NaCl Table 2 (Kerkar, 2004).

Table 2: Different categories of halophilic microorganisms and optimum growth in NaCl

Classes	NaCl concentration (M)
Slight halophiles	0.2-0.85
Moderate halophiles	0.85-3.4
Extremely halophiles	3.4-5.1

Adapted from: (Kerkar, 2004)

Microbial communities that can thrive in high salt conditions have an ability to regulate osmotic pressure and thereby resist the denaturation effect of salt in the environment (Kivistö and Karp, 2011). Furthermore, a common phenomenon in such conditions is the occurrence of gradients in salinity due to evaporation, and halophiles can adjust their osmotic equilibrium as the outside salinity changes. These gradients result in the formation of very diverse microbial communities, where halophilic and halotolerant microorganisms from all 4 domains of life (eukaryote, bacteria, archaea and viruses) can be found (Cowan *et al.,* 2013). Hypersaline habitats are mostly dominated by bacteria and archaea with minor eukaryotes, mostly represented by the halotolerant green algae *Dunaliella*; photosynthetic, heterotrophic protists; and only a few fungal species (Ma *et al*., 2010; Cowan *et al*., 2013; Ventosa *et al*., 2015). Halophilic bacteria are distributed over a large number of phylogenetic groups and most of them are moderate rather than extreme halophiles (Ventosa *et al*., 1998). Even though extreme hypersaline environments are home to a number of phylogenetic groups, it is mostly dominated by haloarchaea that often display a bright red pigment (Mormile *et al*., 2003; López-López *et al*., 2010; López-López *et al*., 2013).

Saline environments harbour a dense microbial population that often display high activities of photosynthesis, dissimilatory sulphate reduction and other microbial processes, thereby imparting a profound effect on biogeochemical cycles (Foti *et al*., 2007). As evaporation occurs in such habitats it results in the formation of evaporate deposits such as halite and gypsum. These evaporates entrap and

preserve microorganisms, thus enabling them to retain their viability for a long time (Mormile *et al*., 2003).

Microbial survival within the salt crystals has become an interesting topic and relevant to a number of disciplines including geology, biogeography and in space studies (Imhoff, 1986; McGenity *et al*., 2001; Lowenstein *et al*., 2011). Not only that, most of the studies in biotechnology have focused on salt-adapted microorganisms because they produce a large variety of stable and unique biomolecules (Ventosa and Nieto, 1995; Joo and Kim, 2005; Ali Amoozegar *et al*., 2007; Jehlička *et a*l., 2013).

The microbiology of brines associated with the eMalahleni Reclamation pond was analysed using a metagenomics approach at different treatment stages. The results show an increase in microbial diversity among the 3 phases of brine treatment, with an exception of the RO stage, where there was a decrease in the microbial diversity correlated to reduced conductivity and pH levels. Seven phyla were detected with proteobacteria being the most dominant (Sekar *et al*., 2014). However, due to the evaporation of surface water and the generation of salinity gradients in the evaporation ponds, it is expected that the microbial communities will undergo diversity changes associated with the brine evolution. Furthermore, considering the evaporation ponds are non-sterile and open air environments, they are prone to constant inoculation, and therefore temporal changes in the community structures will also be expected. The presence (and accumulation) of other toxic elements in the brines will also have a significant impact on the microbial composition and activity. Considering the composition of brine varies from process to process, the identification and selection of the most appropriate organism to develop may have to be optimised on a case by case scenario.

1.9 Mode of survival in salt saturated environments

Microorganisms adapted to bloom in hypersaline environments, have developed a mechanism to survive in such extreme conditions. Halophiles use two adaptive mechanisms to withstand high saline conditions (Figure 3) (Kerkar, 2004). They maintain high internal ionic concentration using K^+ while pumping out Na^+. If microbes use this type of mechanism the entire metabolic machinery has to adapt to high ionic conditions of various salt concentration (Imhoff, 1986). This is mostly observed in aerobic halophiles, Archaea and halophilic bacteria (DasSarma and Arora, 2002; Kerkar, 2004). Some maintain the osmotic balance by using low concentration of salt while accumulating compatible solutes such as amino acids, sugars, polyols and ectoines (Joo and Kim, 2005; DasSarma and Arora, 2002). Proteomics studies have shown that the proteins from halophiles are highly negatively charged with a hydrated carboxyl group (Joo and Kim, 2005). These proteins remain active and soluble in salt concentrations higher than 2 M, whereas the non-halophilic proteins aggregate and become rigid (Joo and Kim 2005; Rainey and Oren, 2006; Rubiano-Labrador *et al*., 2014).

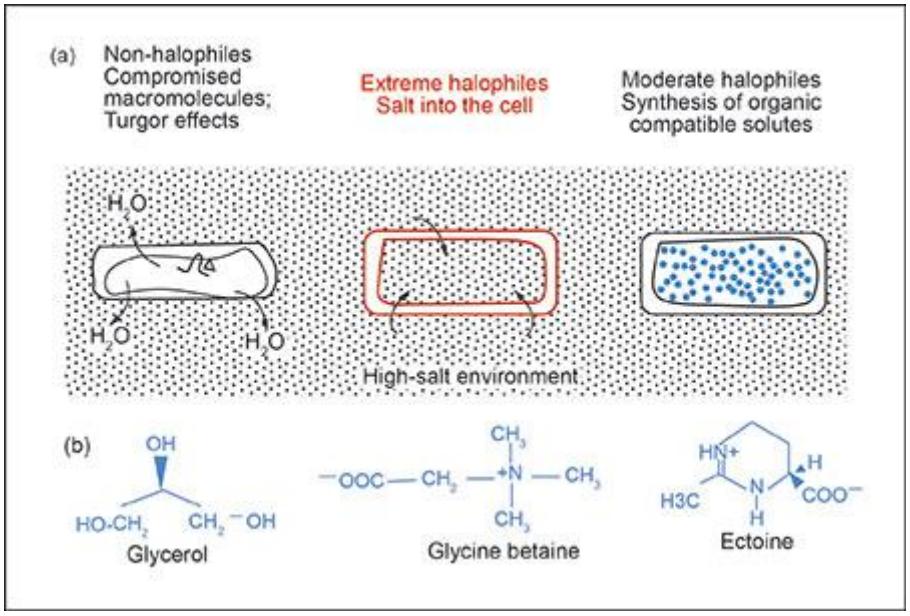


Figure 3: Survival mechanisms and compatible solutes used by microorganism to counteract osmotic stress in extremely hypersaline environments. Adapted from (Kagemann, 2015).

1.10 Pigmented Halophilic Bacteria

A wide range of microorganisms can produce a variety of different pigments, and these could be sourced for the objectives of this study. Different microbes (yeast, bacteria, algae and fungi) produce pigments such as riboflavin, beta-carotene, canthaxanthin, carotenoids, prodiogiosin, phycocyanin, melanin and chlorophyll (Joshi *et al*., 2003 Venil and Lakshmanaperumalsamy, 2009; Khanafari *et al*., 2010). The pigments are produced for different purposes. For instance, cyanobacteria produce phycobilin for photosynthesis (Ahmad *et al*., 2012), while *Pantoea stewart* produces carotenoid for protection against ultra violet radiation (Ahmad *et al*., 2012; Mohammadi *et al*., 2012). However, since the intention of this study was to apply the pigmented microorganisms to the brines, we focused more on those that are produced by halophilic/halotolerant microorganisms.

Microorganisms growing in salted crystalliser ponds are well known for the production of red-orange and red-purple pigments (Zhiling and Guangyu, 2009). These are the archaeal C_{50} carotenoids (α-bacterioruberin and derivatives); the C_{40} β-carotene of the green-algae *Dunaliella salina*; salinixthanin, the carotenoid pigments of a red extremely halophilic bacterium *Salinibacter* (Oren *et al*., 2004); and bacteriorhodopsin, the retinal proton pump produced by *Halobacterium* and other archaea, also referred to as a purple membrane (Stoeckenius *et al*., 1979). The first three pigments are lipophilic, whereas bacteriorhodopsin is a protein. Other popular and commercially valuable pigments produced by halophilic bacteria include astaxanthin (Soliev *et al.,* 2011), lycopene (Yatsunami *et al.,* 2014) and zeaxanthin (Kirti *et al.,* 2014). Despite the fact that the pigments from halophiles mainly consist of carotenoids, different molecular structures have been isolated from halophilic microorganisms as represented in Figure 4.

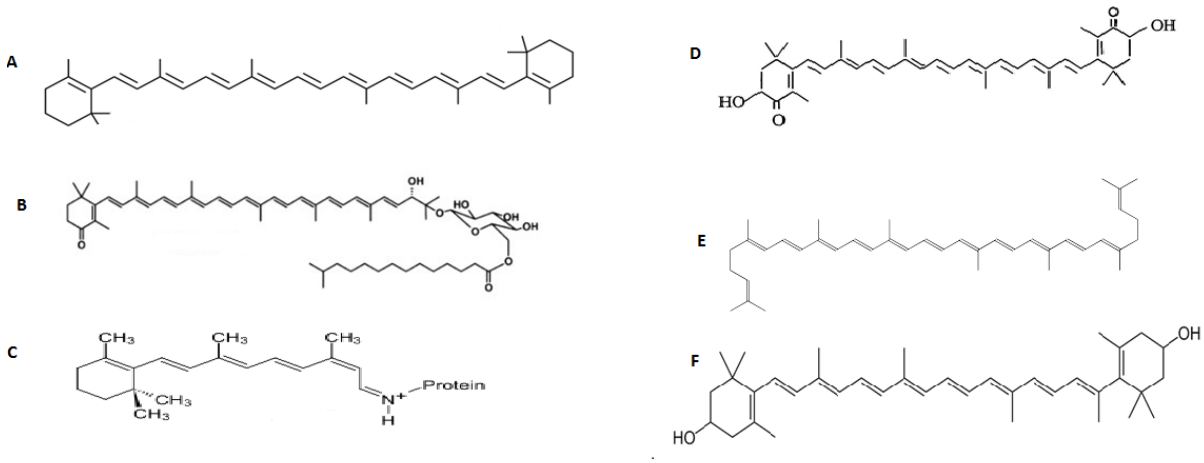


Figure 4: Molecular structures of some common pigments from halophiles. (a) β-carotene; (b) salinixthanin; (c) bacteriorhodopsin; (d) astaxanthin; (e) lycopene and (f) Zeaxanthin.

1.11 Aims and Objectives of the current study

The main aim of this study was to assess two different approaches for the improvement of the evaporation rate of brine; that being the use of a chemical and a biological approach.

The specific objectives were:

- Isolate, identify and characterise pigmented halophilic bacteria
- Determine the effect of using pigmented halophilic bacteria (culture and pigment) on the evaporation of brine
- Determine the effect of methylene blue supplementation on evaporation of brine

CHAPTER 2: EFFECT OF METHYLENE BLUE ON THE EVAPORATION RATE OF BRINE

2.1 Introduction

Evaporation ponds are lined detection basins designed to hold and evaporate water, usually through simple exposure to sunlight (Hoque *et al*., 2010). This method of evaporation evolved years ago and is considered to be cost effective, less laborious and relatively easy to construct (Murugavel *et al*., 2008; Ahmed *et al*., 2000). Application of such artificial ponds ranges from the disposal of brine (from desalination plants) to pre–hazardous waste containment procedures (for easy transportation, storage and treating of water), as well as concentrating of brine (in salt evaporation ponds) from sea and or waste water (Akridge, 2008; Velmurugan *et al*., 2009; Balasubramanian, 2013; Ruskowitz *et al*., 2014).

Evaporation ponds are currently the method of choice at the eMalahleni Water Reclamation Plant (EWRP) for brine management. Two thousand five hundred cubic meters of potable water plus 150 m^3 of reject brine are produced per day at the EWRP using the Hi Recovery Precipitating Reverse Osmosis (HiPRO) process (Hutton *et al.*, 2009). Despite the advantages over other brine management techniques, there are certain drawbacks associated with the evaporation pond system, leading to the need for an economically viable, more sustainable and greener approach for brine disposal (Ahmed et al., 2000).

Some of the drawbacks include the high cost of impervious liners required to prevent leakage of brine to the groundwater and the requirement for a large piece of land when high evaporation rates are required. Moreover, the performance of the evaporation pond is dependent on the atmospheric conditions, since solar radiation is one of the major factors that influences the viability of this technique (Rajvanshi ,1981; Ahmed *et al*., 2000; Hoque *et al*., 2010).

As part of the measures aimed at addressing the disadvantages associated with the disposal of brine using evaporation ponds, a number of studies have looked at the possibility of using dyes to maximise the absorption of radiant energy (Keyes and Gunaji, 1966; Blochi *et al*., 1966; Rajvanshi, 1981). The premise behind the use of dyes in this context is that a coloured solution absorbs more radiant energy compared to the clear brine solution. This increases the temperature of the solution, lowers surface tension, increases vapour pressure and consequently enhances the evaporation rate of brine (Ahmed *et al*., 2000; Murugavel *et al*., 2008; Hoque *et al*., 2010). Patel *et al*., (2010) evaluated the effect of different coloured dyes (black, blue and red) on distillation using a single slope active solar still coupled with an evacuated glass tube solar collector. The results showed that the addition of black dye increases the distillate output by 30.4% with the conclusion that black and blue dyes are the appropriate dyes to enhance the efficiency of evaporation. Similarly, Rajvanshi (1981) evaluated the effect of black napthylamine, red carmoisine and dark green dyes at various concentrations. Among the tested dyes, black napthylamine was found to increase the distillate out put by 29% at the concentration of 172.5 ppm with no photochemical degradation. Other researchers observed an increased evaporation with the addition of absorbing material (ink and dye) when compared to the evaporation of water without any absorbing material (Priya and Mahadi, 2013). Considering the success and growing interest in the use of dyes for concentrating brine, this study seeks to evaluate the effect of various methylene blue (MB) concentrations on the evaporation rate of brine. Methylene blue was selected as per request by a commercial partner, specifically for the EWRP process.

2.2 Material and Methods

2.2.1 Sample collection

Reject brine samples were collected by collaborators on the 16th of May 2011 at the eMalahleni Water Reclamation pond (10 m deep with a capacity to hold 10000 mega litres) (Figure 5), situated in the Mpumalanga province, South Africa (S 25°56'41.4, E 29°11'67.0). All the samples were collected using 25L (6 × 25L) containers and the samples were stored in Kruger laboratory until transported to the University of the Western Cape after two days. Upon arrival at the University of the Western Cape, the samples were stored in the refrigerator at 4°C. After the samples were collected, the pH and electrical conductivity (EC) of the brine were measured on site as the pH of the solution can change within 24 hours as the new batch of brine is deposited. Samples were then filtered using a 0.45 µm pore membrane Whatman® filter paper. The filtered brine samples were then divided into two portions, one for anions analysis while the other portion was acidified with concentrated nitric acid before cation analysis. The sample were acidified in order to stabilize analyte concentrations for only a limited period of time and to prevent loss of targeted analyte. After acidifying, both samples were kept in a refrigerator at 4°C until they were analysed.

Figure 5: The eMalahleni Water Reclamation Plant.Adapted from (Petrik *et al*., 2015).

2.2.2 Chemical analysis of brine

Two litres of brine solution was analysed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to determine its chemical composition, at the University of Stellenbosch analytic unit. Physical qualities such as pH, electrical conductivity, organic matter (ammonia and total carbon), nitrate and nitrite were analysed using Bemlab (Pty) Ltd water analytical services 3. Total carbon was analysed using the LECO CR-421 carbon analyser, while nitrate, nitrite and ammonia were conducted following the South African National Standards 13395 and 5217, respectively, in a flow analyser model AA3.

2.2.3 Preparation of synthetic brine

Due to the instability as well as difficulties of attaining and storing natural brine, synthetic brine resembling the eMalahleni brine was prepared to be used for the chemical aspect of the project. The chemicals below (Table 3) were used to produce synthetic brine solutions containing the major and the minor elements that were detected in the analysis of the brine. The pH of the solution was adjusted with 0.2 M KOH solution. The chemicals used in this study were analytical grade and were supplied by Merck Chemicals and Laboratory, Sigma-Aldrich Chemical Company and Kimix Chemical and Laboratory supplies.

Table 3: Chemicals used to prepare synthetic brine solution

Major Elements	Purity	g/L
Sodium Sulphate	99.0%	10.356
Calcium Sulphate	99.5	4.148
Potassium Chloride	99.5	1.452
Magnesium Sulphate	99.9	5.58
Sodium Chloride	99-100	0.38
Sodium Carbonate	≥ 99.0	0.148
Minor Elements		**mg/L**
Sodium Phosphate	96	85
Barium Chloride	99.9	16.7
Iron Chloride	≥ 99.0	23.3
Aluminium Sulphate	99.9	62.5
Sodium Selenium	98	76.9
Sodium Molybdate	≥ 99	0.48

2.2.4 Preparation of solutions

Methylene blue (MB) was purchased from Merck Millipore, Cape Town. The chemical structure of the dye is illustrated in Figure 6. Stock solutions were prepared by dissolving 2 g of MB into 2000 mL of synthetic brine. The desired concentrations for the experiments were achieved by successive dilutions. MB solutions were prepared using synthetic brine to ensure that the concentration and chemical composition of synthetic brine was not altered.

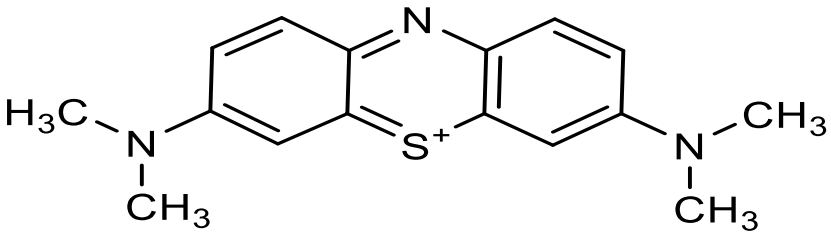


Figure 6: Chemical structure of methylene blue

2.2.5 Measuring Evaporation Rates

Varying MB concentrations in brine were evaluated for their effect on the evaporation rate of brine with time. Five MB concentrations in the brine were tested. The final concentrations in the brine ranged from 100 to 300 ppm, with 50 ppm increments. Figure 7 shows the evaporation set-up with solutions that were evaporated at room temperature and infra-red lights producing 240 Watts. The lights were placed 36 cm above the pans, pan measurements were 17.8 cm width X 27.9 cm length X 2.5 cm height. The infra-red lights were equally distributed to ensure that each pan received equal heat. The experiments were conducted in a closed laboratory that was accessed only once a day for taking measurements. The temperature was not monitored, but it was expected to fluctuate under the control of the internal air conditionings. However, while it may have fluctuated throughout a 24 hour period, this fluctuation was expected to be consistent. In addition, each experiment together with the controls were conducted simultaneously therefore the evaporation rates are comparative. For each concentration of MB, solutions were prepared to make a final volume of a litre that was added to the pan and allowed to evaporate. The experiments were performed in triplicate and brine with no MB added was used as a control. The changes in the volumes were measured at 6 hour intervals, by weighing the pan using a Bright Led Display Economy Weight Measurement Scale (KETE 10), until the pan was completely dry. As demonstrated by the various equations in the literature review, each is only valid for a specific system or a climate similar to those used in the respective studies. In this study, the environmental factors were not monitored. Therefore, the evaporation was calculated based on the time taken for each experiment to completely evaporate.

Figure 7: Set up of evaporation pans containing brine-methylene blue solution.

2.2.6 Reconstitution of methylene blue experiments

Methylene blue dye crystals trapped within the synthetic brine matrix at the end of the first evaporation rate experimental runs were reconstituted by adding 800 mL of freshly prepared synthetic brine in each pan. Methylene blue was reconstituted twice in this manner and all the experiments were done in triplicate. The reconstitution process was done in order to assess whether the remaining methylene blue would still be effective in increasing the rate of evaporation of the brine if the fresh brine is added. The evaporation rate of brine with the reconstituted MB was measured as explained in section 2.2.5 above. A standard curve correlating methylene blue concentration versus OD_{665} was determined using MB standards prepared with synthetic brine and the concentrations range from 0.05-0.2 mg/L. To determine the amount of reconstituted methylene blue in the solution during the evaporation experiment, 500 µL of

the MB-brine solution was transferred into 1.5 mL micro centrifuge tubes and centrifuged at 3000 rpm (1549 × g) for 3 minutes. After centrifugation, the sample was transferred into the cuvette and the absorbance at 665 nm was recorded. Where necessary, dilutions were conducted to maintain the absorbance readings in the linear range of the standard curve (Figure 8). The synthetic brine was used as a blank.

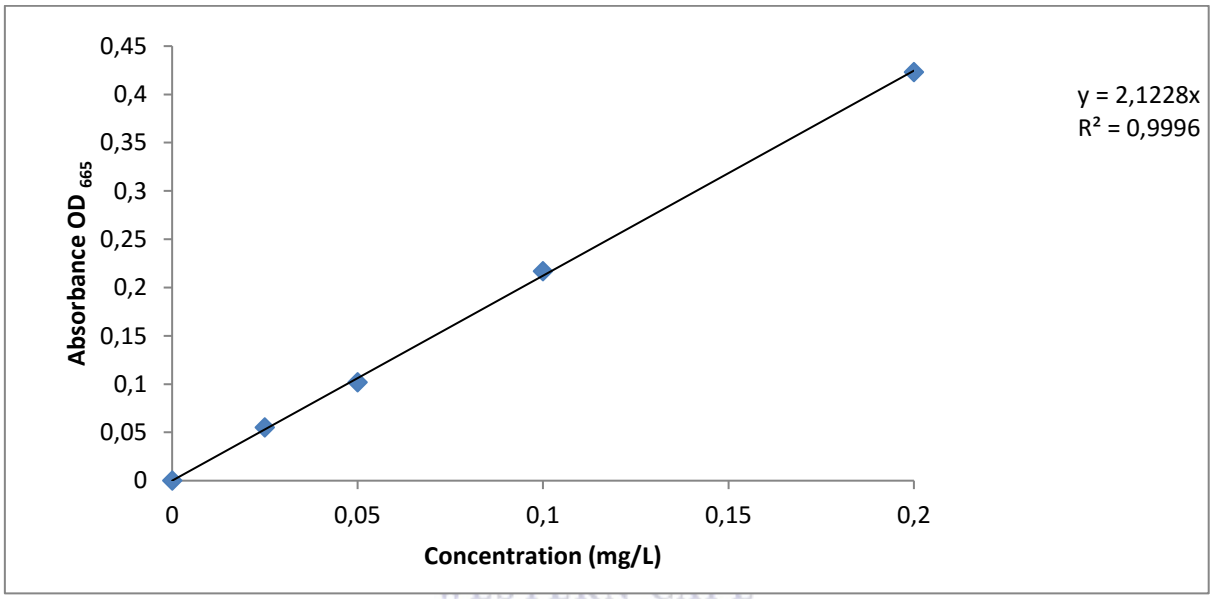


Figure 8: The standard curve of methylene blue dye made in synthetic brine.

2.3 Results and Discussion

2.3.1 Brine Analysis

The physical parameters and the chemical composition of the sampled eMalahleni brine was investigated and it was discovered that the brine pre-dominantly contain Na^{+}, SO_4^{2-}, K^{+}, Mg^{2+}, Cl^{-}, Si^{2+}, NH_4^{+} and Ca^{2+} while Fe^{2+}, Co^{2+} and Mo^{2+} were present in smaller quantities (Table 4). The high levels of Na (3973 mg/L) as the major cation and SO_4^{2-} (14520 mg/L) as the major anion, indicates that eMalahleni brine is a Na_2SO_4 type brine. Similar results were reported previously (Dama-Fakir and Toerien, 2000; Randall *et al*., 2011). It is well understood that the characteristics of the brine are influenced by the feed water, desalination process, percentage recovery and chemical additives used during the treatment (El-Naas, 2011), therefore it is not unexpected for brines to have different characteristics. The high concentration of SO_4 was expected since the brine is a by-product of AMD treatment, which is known to be laden with heavy metals, iron, and sulphate. Heavy metals such Mn, Cu, Cr, Pb, Ba, Ni and Zn were found as minor level concentrations, which are attributed to the brine undergoing the pre-treatment process to remove these metals prior to RO treatment. This is done to protect RO components from metals fouling (Hutton *et al*., 2009). The pH was found to be 7.76 while the EC was 21.9 mS/cm which shows high levels of free ions due to the presence of dissolved species. Nitrite and nitrate were found to be 0.01 and 122.12 mg/L respectively, and a very low total carbon level.

Table 4: Chemical analysis of eMalahleni concentrated reject brine

Major elements (mg/L)		Minor elements (mg/L)	
Ca^{2+}	1200	Al^{3+}	0.03
K^{+}	710	Cr^{2+}	0.01
Na^{+}	3973	Se	0.03
Si^{2+}	1.14	Fe^{2+}	0.07
Mg^{2+}	459	Mn^{2+}	0.01
SO_4^{2-}	14520	PO_4^{3-}	0.03
Cl^{-}	1072.78	Ba^{2+}	0.21
NH_4^{+}	45.38	Co^{2+}	0.05
NO_3^{-}	122.12	Ni^{2+}	0.07
NO_2^{-}	0.01	Cu^{2+}	0.004
Total C	0.01	Zn^{2+}	0.008
pH	7.76	Mo^{2+}	0.01
EC	21.9 mS/cm	Cd^{2+}	0.0003
		Pb^{2+}	0.0001

2.3.2 Evaporation results

The effect of increasing concentrations of MB on the evaporation rate of brine with time is shown in Figure 9 below. This was done to give insight to which concentration of MB can be applied without significantly increasing the cost of brine management and at what concentration the saturation point is reached. More information regarding the effect of dye on the evaporation rate of brine can be found in Chapter 1 (Section 1.6.2).

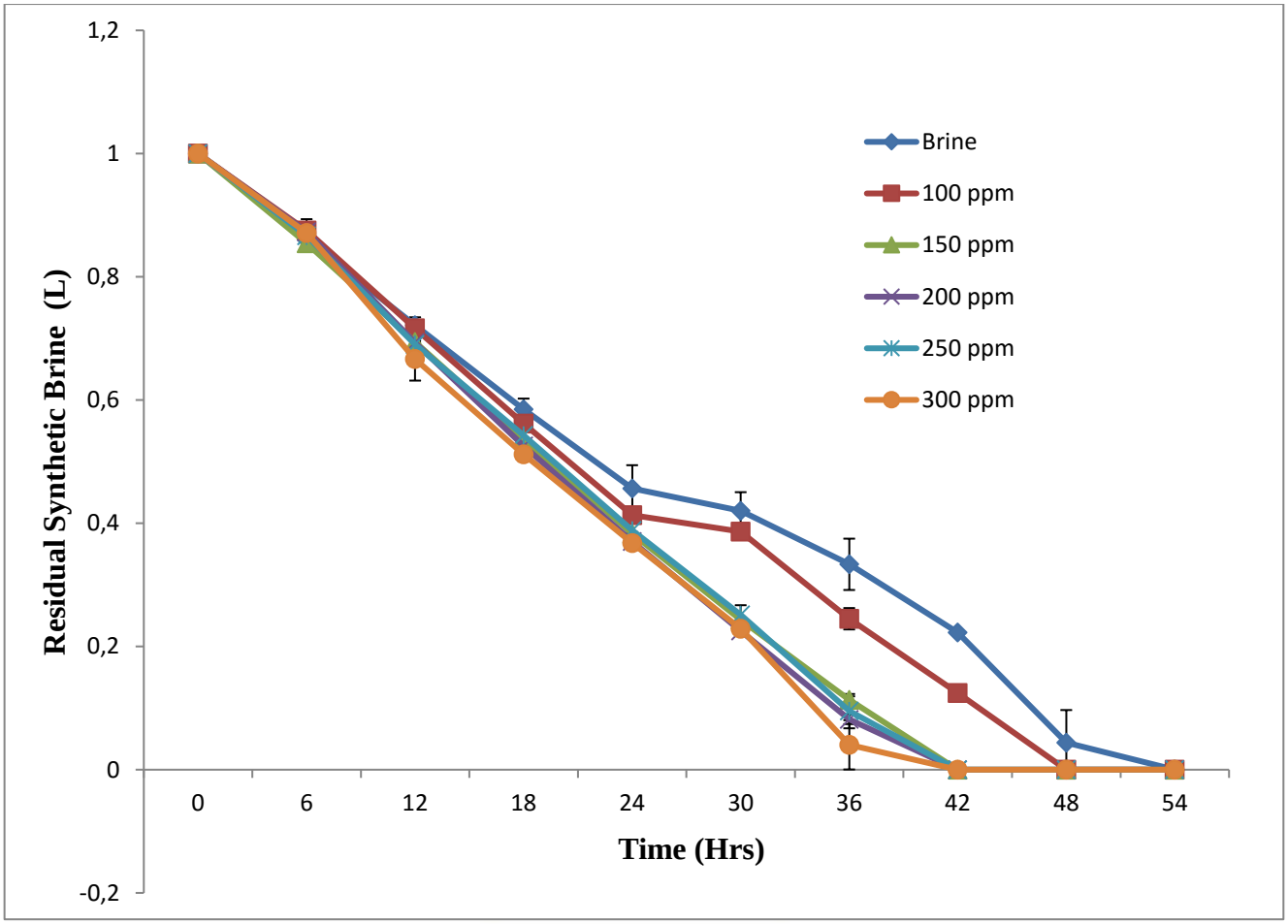


Figure 9: Effect of various methylene blue concentrations on the evaporation rate of synthetic brine. The error bars represent the standard deviation of triplicates run independently.

Figure 9, presents the effect of increasing MB concentrations on the evaporation rate of brine. After 6 hours of exposure there was no significant difference in the evaporation rate of coloured vs uncoloured brine. However, from 6 hours onwards, an increased rate of evaporation was observed from all the concentrations tested, compared to the synthetic brine control (Table 5).

The results indicate that the absorbing capacity of the synthetic brine increases with the addition of MB under laboratory conditions. Similar results were reported by Bloch *et al.*, (1951), the researcher observed a 19% increase in the evaporation rate through the addition of MB and they further recommended the addition of 0.19 g per 0.0283 cubic meter of brine. These findings support the hypothesis that dark coloured solutions absorb more radiant energy thereby causing an increase in the vibration of water molecules resulting in a large amount of water molecules escaping from the surface of the water (Ahmed

et al., 2000; Coleman, 2000; Rajvanshi, 1981). In this study the concentration of MB used was higher than the one used in the Bloch et al., (1951) study, therefore it was anticipated that the evaporation rate obtained would higher. The decrease in the evaporation rate of brine observed in the absence of MB dye could be due to the accumulation of dissolved salt and an increase in ion activity. Dissolved salts reduce the chemical partial molar free energy of brine by reducing the saturation vapour pressure and thus lowering vaporisation rate. On the other hand, ion activity results in a change of the chemical potential of water affecting the transformation of a liquid into the vapour state and consequently lowering the rate of evaporation (Dama-Fakir and Toerien, 2000).

From the concentration of MB used, it was also observed that 300 ppm MB had a higher evaporation rate compared to the 100 ppm MB solution, which would be expected on increasing the concentration of the dye. The rate of evaporation is linear for higher MB concentrations, but not at zero and 100 ppm. This could be linked to the precipitation of salt at the bottom of the pan that was observed between 24 and 48 hours in the zero and 100 ppm MB pans. Javed and Hafeez, (1998) discuss that the formation of crust prevents the escape of water molecules to the atmosphere and thus leads to a decline in the evaporation rate. It is therefore possible that the addition of MB at a concentration above 100 ppm delays the precipitation of salts and thus it increases the rate of evaporation.

Table 5: Evaporation rate of synthetic brine with various concentrations of methylene blue (mean± standard deviation)

Methylene blue concentration	Rate (L/hr)
0 ppm	0.0177±0.0006
100 ppm	0.023±0.0023
150 ppm	0.024±0.0001
200 ppm	0.025±0.0002
250 ppm	0.024±0.0004
300 ppm	0.026±0.0023

2.3.3 Evaporation of synthetic brine with the reconstituted methylene blue

The ability to reconstitute the methylene blue from precipitated salts in successive evaporation experiments, instead of replenishing the MB, could have important implications. Firstly, it would aid in keeping the MB concentration at manageable levels to prevent negative environmental impacts. Secondly, this would constitute an economic saving. The effect of reconstituted MB on the precipitated salt crystals mixed with fresh brine, and then measuring the evaporation rate of brine is presented in Figure 10 below. The evaporation rate of brine was observed to decrease with each successive reconstitution experiment. An evaporation rate of 0.025±0.0002 L/hr was noted when the initial 200 ppm concentration of MB was used. The evaporation rate decreased to 0.024±0.0028 L/hr following the first reconstitution (AV2) and then to 0.023±0.0021 L/hr (AV3) on the second reconstitution.

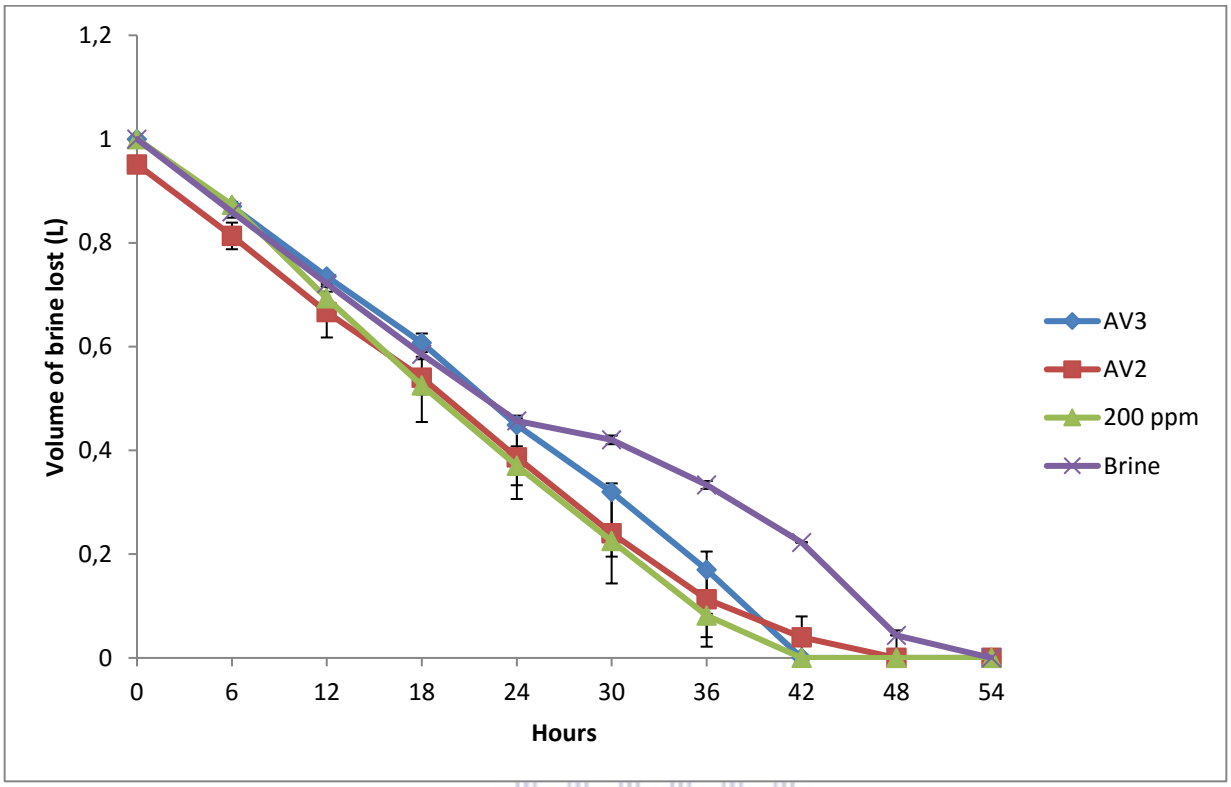


Figure 10: The effect of reconstituted MB on the evaporation rate of brine at a starting concentration of 200 ppm MB and reconstituted MB, AV2 (first reconstitute) and AV3 (second reconstitute). The error bars represent the standard deviation from the mean of triplicate experimental run independently.

2.4 Conclusion

The main objective of this study was to evaluate the effect of various MB concentrations (100 -300 ppm) on the evaporation rate of brine. The results showed that the evaporation rate linearly increases with an increase in the concentration of the dye, resulting in a 41% increase in evaporation when 200 ppm is used. Moreover, the addition of MB dye above 100 ppm delayed (Figure 9) the precipitation of salts out of the solution thus increasing the rate of evaporation. The increase in the evaporation is hypothesised to be due to MB absorbing more radiant energy, increasing the temperature of the brine which consequently results in a higher evaporation rate.

The reconstitution results from this study show that the MB can be fully reconstituted from the precipitated salts, as it gives higher evaporation rate compared to the control. Therefore, methylene blue dye may be considered as an alternative method for brine evaporation treatment as it offer high evaporation rate and a more cost saving option. Furthermore, the reconstitution treatment would keep the concentration of MB at manageable levels and thus preventing negative effect of the environments if the remaining salts would be discarded.

Even though a 41% increase in the evaporation rate of brine is significant, the experimental setup used in this study is expected to yield an over estimation compared to what should be expected in the natural setting. As stated in Chapter 1, the addition of dyes maximises the absorbance of solar energy in saline effluent in the thin upper layer (~2 cm), by raising the surface temperature of the brine (Rajvanshi, 1981). The depth of the evaporation pan used in this study ranged from 2.3- 2.5 cm, making this a suitable setup to determine the effect of MB on evaporation. However, the infrared producing lamps employed as the source of radiant energy resulted in a constant supply of heat, which will not be the case in an industrial application. In addition, due to the relatively shallow operating volumes of the evaporation pans used, the brine was more exposed to the heat, likely resulting in higher evaporation yields.

The results generated from this study further confirm the efficacy of MB to increase the evaporation rate of the synthetic brine. Results from this study further recommend that the addition of MB to brine can potentially be a practical method for significantly increasing the evaporation of brine. However, such a solution still poses a potential environmental risk. Hence, the work on Chapter 3 investigated alternative biological strategies to enhance brine evaporation, using pigmented halophilic bacteria.

CHAPTER 3: BIOLOGICAL MEDIATED EVAPORATION

3.1 Introduction

Brine water is characterised as being anoxic, metalliferous and highly saline, where the salinity has been shown to be as much as 500 g/L (Arnal *et al.*, 2005). These characteristics make brines rare and inhospitable environments on earth (Ollivier *et al.*, 1994; Dama-Fakir and Toerien, 2009; Bougouffa *et al.*, 2013; Kasedde *et al.*, 2014; Sekar *et al.*, 2014). Regardless of such harsh conditions, brine water harbours significant microbial communities (Ventosa *et al.*, 1998; Antón *et al.*, 2002; Foti *et al.*, 2007; Ma *et al.*, 2010; Bougouffa *et al.*, 2013; Sekar *et al.*, 2014). Culture based approaches have been acknowledged for only describing a small portion of the actual diversity in a natural environment, and the 16S rRNA analyses of environmental DNA samples has shown to be a powerful tool for microbial identification and diversity evaluation (Kambourova *et al.*, 2016).

Owing to the use of both culture dependent as well as the culture independent methods, a large diversity of halophilic organisms has been shown to exist in hypersaline habitats. The most well-known halophilic microorganisms include the archaea *Halogeometricum borinquense*, eukaryotic green algae *Dunaliella salina* and the halophilic bacteria *Sporasarcina aquimarina* and *Salinibacter ruber* (Schleper *et al.*, 1995 ; Yoon *et al.*, 2001; Oren *et al.*, 2004;).

A variety of pigments produced by these organisms play a crucial role in their protection against ultraviolet radiation, as an antioxidants (Mohammadi *et al.*, 2012) and as a membrane stabilizer (Yatsunami *et al.*, 2014). The pigments that are produced by halophilic organisms include bacteriorhodopsin (Stoeckenius *et al.*, 1979), β-carotenoids, phytoene, lycopene, salinxanthinin, by-products of bacterioruberin (de Lourdes Moreno *et al.*, 2012), melanin, prodiogiosin (Kumar *et al.*, 2015), chloronatronochrome and natrochrome (Takaichi *et al.*, 2004).

These organic compounds produced by halophilic microorganisms are not essential for growth and reproduction; therefore, they are referred to as secondary metabolites. Expression of the pigment by halophilic organisms is influenced by many environmental factors such as oxygen availability, pH, moisture content, aeration rate, salt concentration, temperature and strain variation (Cardona-Cardona *et al.*, 2010; Kumar *et al.*, 2015). For example, *Halobacterium sp.* exhibits maximum pigment production when grown in 25% of NaCl, however, no pigment is observed when the concentration of NaCl is less than 10% (Asker and Ohta, 1999). Whereas, other strains of *Halobacterium sp*. do not produce any pigmentation when subjected to NaCl concentrations above 15% (Khanafari *et al*., 2010).

Precursors such as amino acids (di-aspartic acid, l-glutamic acid, l-proline and l-alanine) (Williams *et al.*,1971) and quorum sensing systems are also known to induce pigment expression. The most known auto- inducer molecules include butanolides for actinomycetes and N-aclyhomoserine lactones and oligopeptides for Gram-negative and Gram- positive bacteria, respectively. These precursors can either stimulate the production of pigment by inducing the biosynthetic enzyme or increase the concentration of the inhibiting precursors (Demain, 1998).

Given that pigmented organisms improve the evaporation of sodium chloride based brines in the salt production process as explained in section 1.9 of Chapter 1, this study proposes whether a similar approach could be adopted to a completely different brine system, such as the sodium sulphate based brine generated from mining activities. Specifically, could the introduction of pigmented organisms to the evaporation pond in miming industries be used to improve evaporation of the reject brines?

Maintaining a balance between the physicochemical factors and the biological system is crucial in solar ponds for rapid salt production (Rodrigues *et al*., 2011). Thus, one of the parameters that needs to be optimised in the brine wastewater application is the selection of halophilic bacteria that are able to grow and produce pigment under the brine and evaporation pond conditions. This chapter focuses on the

isolation and identification of pigmented bacteria from two brine samples, i) mine wastewater brine generated as a by- product of a reverse osmosis process from AMD, and ii) a thassalomic brine sample from the Cerebros salt works on the West Coast, Saldana region. This chapter also for the first time assesses the effect that the pigmented halophilic bacteria have on the evaporation rate of synthetic brine.

3.2 Material and methods

3.3 General chemicals, reagents and enzymes

The chemicals, culture media and reagents utilised for experimental purposes of this study were supplied by Merck Chemicals and Laboratory Supplies (Darmstadt, Germany), Sigma Aldrich Chemical Company (Deissenhofen, Germany) and Kimix Chemical Laboratory Supplies, unless otherwise indicated. The oligonucleotides for Polymerase Chain Reaction (PCR), enzymes, and DNA size markers were purchased from Integrated DNA Technologies (Coralville, lowa, USA), New England bio lab (Ipswich, MA, USA) and White Scientific (Pty) Ltd (Cape Town, South Africa).

3.4 Sample Collection

Salt sediments and reject brine samples were collected from EWRP at eMalahleni municipality, (S 25°56’’41.4’’ E 29°11’67.0) (Mpumalanga province, South Africa) and Cerebos Salt Company (S32°47'3.84" E18°9'51.48") (Western Province, South Africa). Details regarding eMalahleni brine samples collection are found in Chapter 2 section 2.2.1. Salt sediments and sea water were collected from all Cerebos ponds (pond1-pond 5). From each pond, two samples were collected using 50 mL falcon tube. To avoid cross contamination a new pair of gloves was used when sampling a different pond. All the samples were labelled according to their pond number, placed in a cooler box and transported into the laboratory where they were stored at 4 ^{o}C. After 1 week, a portion of each samples were used for analysis and the remaining portion were stored at -80 ^{o}C for long term storage.

3.5 Media

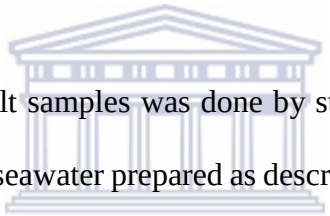
Media used in this study were prepared by mixing the components as indicated in Table 6, where necessary the pH was adjusted to 7.0 and the media autoclaved at 121 ºC for 15 minutes. For R2A brine agar, the eMalahleni brine was used to prepare the media instead of distilled water (dH_2O). The composition of the brine is presented in Table 4 of chapter 2. Unless stated otherwise, 15 g/L of agar was added to all agar based media and the recipes for media preparation were obtained from Sambrook and Russell, (2001) and Antón *et al*., (2002).

Table 6: The media used for culturing bacterial isolates

Constituent	Amount (g/L)
Tryptone soya Agar	
TSB	3
NaCl	18
MgCl	2
KCl	0.525
$CaCl_2$	0.075
Zobel $\frac{1}{4}$ Strength	
Yeast extract	0.525
Peptone	3.75
NaCl	18
MgCl	2
KCl	0.525
$CaCl_2$	0.075
R2A Broth	
Casein acid hydrolysate	0.5
Dextrose	0.5
K_2HPO_4	0.3
$MgSO_4$	0.024
Protease peptone	0.5
$C_3H_3NaO_3$	0.3
Starch, soluble	0.5
Yeast Extract	0.5
LB (Luria Bertani) Medium	
Tryptone	10
NaCl	5
Yeast extract	10

3.6 Bacterial isolation and growth

One litre of brine from the eMalahleni pond was vacuum filtered through 0.22 µm MF-Millipore™ membrane filters. The membranes were suspended in 1 mL of distilled water (dH_2O) and vortexed for 2 minutes to dislodge the embedded bacterial cells from the membranes. A serial dilution series ranging from 10^{-1} to 10^{-6} was then prepared from the bacterial suspension and inoculated by spreading onto R2A brine agar media. The R2A agar plates were later incubated at room temperature for 5 days. After incubation, the pigmented colonies were picked from the resulting mixed cultures and streaked onto fresh R2A brine agar media. This process was repeated until pure cultures were obtained.



Isolation of bacteria from Cerebos salt samples was done by suspending 1 g of Cerebos sediment salt sample into 10 mL of artificial sterile seawater prepared as described in Dyall-Smith, (2008). The sample was then mixed by vortexing to make serial dilutions. One hundred microliters of the 1 in 10 dilution was transferred to 900 µL of sterile sea water and a serial dilution series up to 10^{-6} set up. One hundred microliters of each dilution were spread plated onto 3 different growth media, namely R2A, Zobel and Tryptone Soy Agar prepared with dH_2O. As defined in Table 2 (Chapter 1), halophiles are classified based on their optimum growth in the presence of NaCl (> 0.2 M). All the three media used in this study have been used previously to isolate halophiles and are therefore, able to support the growth of bacteria with a wide range of tolerance to salt (Antón *et al.*, 2002; Yoon *et al.*, 2001). The plates were incubated at room temperature for a period of 5 days. Pigmented isolates growing on the plates were picked and streaked on R2A brine agar (Section 3.5). In this study the use of the term, halophiles refer to bacteria that are able to tolerate > 0.2 M NaCl.

3.7 Biochemical test and morphological characterisation

Pure pigmented isolates growing on R2A brine agar were selected for further characterisation. The isolates were Gram stained and visualised microscopically (Azhar et al., 2014) after observing the colony morphology. Cell shape and pigment coloration of the isolates were also recorded.

3.8 Salt tolerance

Tolerance to salt by the isolates was evaluated in R2A broth supplemented with various concentrations of Na_2SO_4 and NaCl. Each of the strains was cultured in 10 mL of R2A broth with Na_2SO_4 and NaCl concentrations ranging from 5 to 30 % (w/v). The cultures were incubated at 28 ^oC on a rotatory shaker set at 120 rpm for 48 hours. The growth of the cultures was monitored by measuring turbidity at 660 nm after 48 hours of growth.

3.9 Pigment extraction and analysis

The bacterial isolates were grown in 100 mL Tryptic Soy Broth (TSB) in an Erlenmeyer flask and incubated at room temperature with shaking set at 120 rpm for 3 days. At the end of the incubation period pigmented cells were harvested by centrifuging 50 mL of the culture for 10 minutes at 6500 rpm (7274 × g). The supernatant was removed and the cell pellet washed twice with sterile distilled water. The pigment was extracted from the pelleted cells using methanol, as follows. The pellet was re-suspended in 2 mL of methanol and the mixture incubated at 65 ^oC for 10 minutes. At the end of the incubation, bleached cells were pelleted by centrifugation at 6500 rpm for five minutes and the coloured supernatant filtered through Whatman number 1 filter paper. The filtered supernatant was transferred to a new pre-weighed tube and a scanning spectrophotometer was used to determine the wavelength at which the pigment absorbed maximally. The absorption range scanned was from 300 to 800 nm. After determining the maximum wavelength, the remaining samples were incubated in oven at 60 ^oC until they were completely dry.

Total carotenoid content was calculated using the following equation (Jalal *et al*., 2014):

C = D·V·F (10/2500) / dry weight of the sample [g] (10)

Where;

C = Total Carotenoid (mg/g)

D = Absorption at 495 nm

V = Volume of Sample Used

F = Dilution Factor of the Sample

2500 = Average extinction co-efficient for Carotenoid.

3.10 Bacteria identification using 16S rRNA gene analysis

3.10.1 Genomic DNA Extraction

Genomic DNA was extracted from bacterial isolates grown in 15 mL LB broth. Two day old cultures were pelleted by centrifugation at 2600 rpm (1163 × g) for 10 minutes. The pellet was resuspended in Tris-EDTA (TE) (25 mM Tris-HCL and 10 mM EDTA) buffer (pH 8). Two microliters of a 10 mg/mL RNase A stock and 30 µL of 10% sodium dodecyl sulphate (SDS) was added to the TE-cell suspension and the mixture incubated at 70 ^{o}C for 30 minutes for cell lysis. At the end of the incubation period, 3 µL of proteinase K at a concentration of 20 mg/mL was added to the tubes, and incubated at 37 ^{o}C for one hour. An equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) solution was added to the DNA lysate and the contents mixed by inverting the tubes three times. The tubes were centrifuged at 6500 rpm for 10 minutes, at the end of which the aqueous phase was transferred into a new tube. The phenol: chloroform: isoamyl alcohol step was repeated twice before DNA was precipitated from the aqueous phase. DNA was precipitated by adding 0.6 volumes of ice-cold isopropanol and 0.1 volume of 5 M sodium acetate (pH 5.2). The mixture was incubated at -20 ^{o}C overnight to remove excess salts and

the DNA pelleted by centrifugation at 8500 rpm (12438 × g) for 10 minutes. Seventy percent ice-cold ethanol was used to wash the DNA pellet followed by centrifugation at 8500 rpm for ten minutes. The 70% wash was done twice to remove salts from the DNA pellets. The DNA pellets were air-dried, re-suspended in 50 µL of TE buffer and stored at -20 ºC until further use.

3.11.1 Nucleic Acid Analysis

Qualitative and quantitative analysis of the DNA was done using a Nano Drop spectrometer (ND-100: Nano Drop Technologies, Inc., USA). The purity of the DNA was determined based on 260/280 ratio.

3.11.2 DNA agarose gel electrophoresis

DNA was resolved by agarose gel electrophoresis as described by Sambrook and Russell, (2001). Agarose gel was prepared in 1X Tris-Acetate-EDTA (TAE) (40 mM Tris-acetate and 1mM EDTA) buffer at the concentration of 0.8-1 % (w/v) of agarose. The agarose-TAE mixture was heated in a microwave until the agarose completely dissolved. The molten agarose was cooled down and ethidium bromide added to a final concentration of 0.5 µg/ml to ensure DNA visualisation. DNA samples were prepared for loading into the gel by adding 6X DNA loading dye (ThermoFisher Scientific™). Electrophoresis was performed in 1X TAE buffer at 90 V for 1 hour and the DNA visualised using the Alpha Imager UV transilluminator (Alpha Imager 2000, Alpha Inno tech, USA). The size of the DNA was estimated using lambda DNA digested with *Pst*I endonuclease as molecular weight marker.

3.11.3 Polymerase chain reaction

The extracted genomic DNA was used as a template for amplifying the 16S rRNA gene. The universal primer set U9F and U150R were used for amplification (Hansen *et al*., 1998; Reysenbach *et al*., 2009).

Fifty microliter reactions containing 1,25 units of NEB Taq polymerase, 10 X NEB PCR buffer, 0.2 mM of deoxynucleotide triphosphates (dNTPS), and 0.1 μM of each primer were set up. The following thermocycling pattern was used: initial denaturation at 95 °C for 4 min (1 cycle); 30 cycles of denaturation at 95 °C for 30 secs, annealing at 55 °C for 30 seconds, extension at 72 °C for 90 seconds, and final extension at 72 °C for 7 minutes. One thousand five hundred base pair amplicons from the PCR were confirmed by agarose gel electrophoresis on a 1% gel. The amplicons were excised from the gel and purified using the Nucleospin™ DNA clean-up kit. The purified 16S rRNA gene fragments were sequenced at the Central Analytical Facility, Stellenbosch University, and the sequences were queried on the NCBI BLASTn database to identify the isolates.

3.11 Biological mediated evaporation

The effect of pigments produced by the halophilic bacteria on the evaporation rate of brine was evaluated in 2 ways: i) inoculating synthetic brine with the pigmented EP-3 culture (seeding); and ii) adding extracted pigment from the culture to synthetic brine (pigment). In both approaches, the solutions were allowed to evaporate in the evaporation pans in the set up shown in Figure 11**.** The brine without anything added on it, brine with sterile TSB and the culture were used as controls. Infra-red lamps generating 240 Watts were used as a source of energy. The lamps were placed at a height of approximately 36 cm above the pans. The rate of evaporation was followed by recording the weight change in the pans because of water loss measured at 6-hour intervals until the solution was completely evaporated.

3.11.4 The effect of bacterial pigment on the evaporation rate of brine

The pigment was extracted from a three- day old culture of EP-3 as detailed in section 3.9 in Chapter 3. One gram of the extracted pigment was added to 1000 mL of synthetic brine, which was then allowed to

evaporate. The rate of evaporation was followed by weighing the evaporation pans at 6 hour intervals until the pans were completely dry.

3.11.5 The effect of the bacterial culture on the evaporation rate of brine

A starter culture was prepared by inoculating EP-3 into two 250 mL Erlenmeyer flasks containing 120 ml of TSB prepared with synthetic brine, and incubated with shaking at 120 rpm at 37 ^{o}C for 3 days**.** Two hundred millilitres of this culture was added to into 800 mL of synthetic brine (Figure 11). The inoculated brine was contained in sterilized evaporation pans which were put under infrared lights to aid in evaporation, under non-sterile conditions. All the experiments were done in triplicate. The rate of evaporation was followed by recording the weight change in the pans as a result of water loss. The weights of the pans were recorded at 6-hour intervals until the brine had completely evaporated.

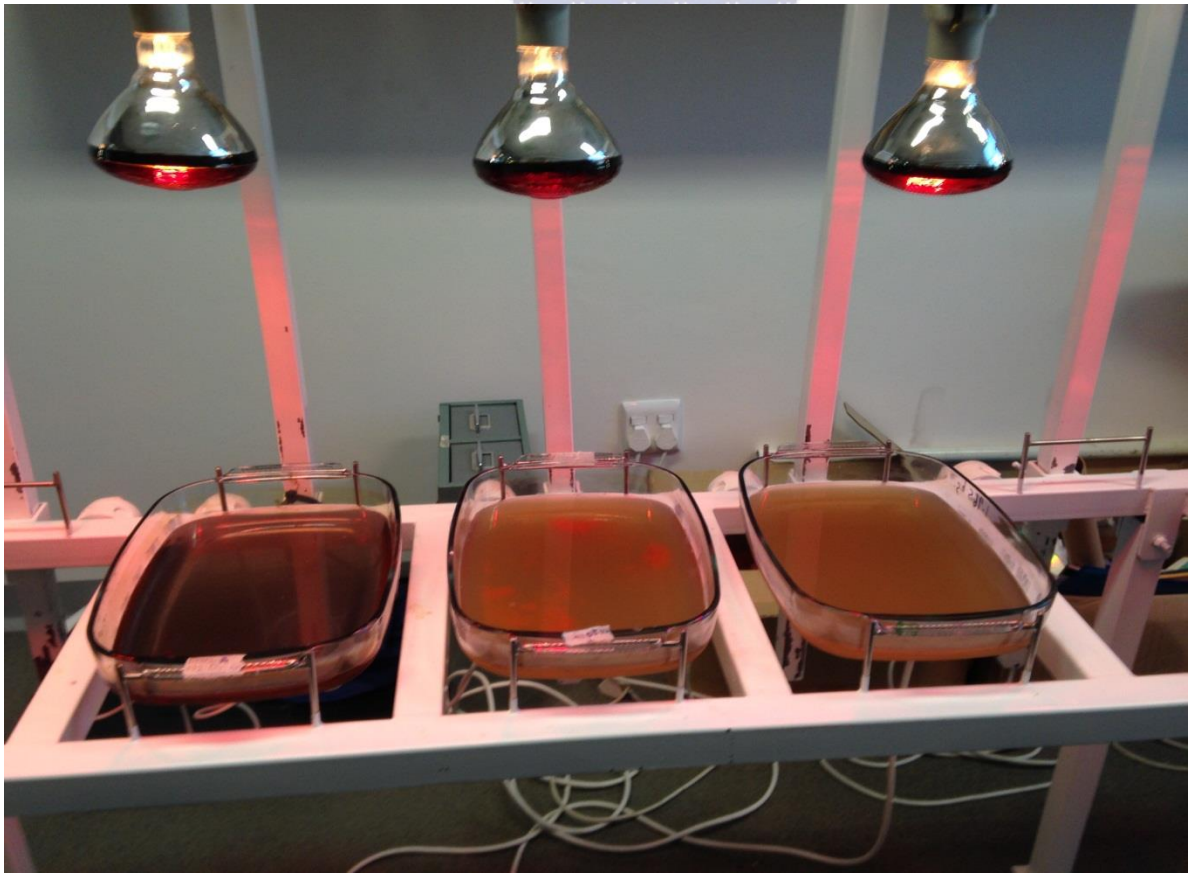


Figure 11: Experimental set up for biological mediated evaporation studies. Pan 1 contains 1 gram pigment that was dissolved in brine, pan 2 and 3 contained 200 mL of EP-3 culture in 800 mL of brine.

3.12 Results and Discussion

3.12.1 Isolation and characterisation of pigmented halophilic bacteria

Fourteen pigmented bacterial strains were isolated from both the eMalahleni brine (3 isolates) and Cerebos salt samples (11 isolates). Microscopic and phenotypic characteristics of the isolates are presented in Table 7. Pigmentation was yellow, orange, pink, cream and red. Nine out of the 14 isolates were cocci while the remainder were rod shaped. Of the 14 isolates, 10 were Gram -positive, 2 Gram-negative and two Gram-variable.

Table 7: Microscopic and phenotypic characteristics of the isolated bacterial strains

Source	Isolate	Gram Reaction	Morphology	Colony Colour
eMalahleni	Ep-1	Positive	Cocci	Yellow
	Ep-2	Gram Variable	Rods	Orange
	EP-3	Positive	Cocci	Red
Cerebos	Cp2-1	Positive	Cocci	Pink
	Cp2-2	Positive	Cocci	Pink
	Cp2-3	Positive	Cocci	Orange
	Cp4-1	Positive	Rods	Red
	Cp5-1	Negative	Rods	Orange
	Cp5-2	Positive	Cocci	Yellow
	Cp5-3	Gram variable	Cocci	Red
	Cp5-4	Positive	Cocci	Red
	Cp5-5	positive	Cocci	Yellow
	Cp5-6	Positive	Rods	Cream
	Cp5-7	Negative	Rods	Cream

In order to further characterise the isolates, the isolated strains were cultured in R2A agar supplemented with brine as represented in Figure 12. The EP-3 strain showed a dark red pigment while the others were cream and yellow.

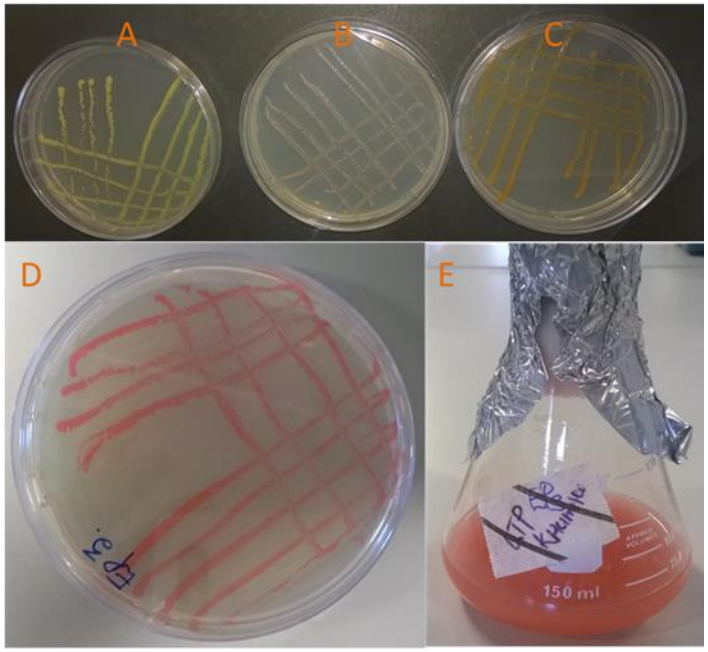


Figure 12: The pigment produced by some of the isolated bacteria on R2A media. Plate A–D represent EP-1, Cp-5-4, EP-2 and EP-3. Picture E is the two days old EP-3 culture in R2A broth.

To identify the isolated strain based on their 16S rRNA gene sequence, genomic DNA was extracted and the quality and the integrity of the DNA was validated using 1% agarose gel (Figure 13). Samples 2 and 5 had higher concentrations of RNA, therefore, 2µL of RNase was added and all the samples were used as template DNA for the PCR.

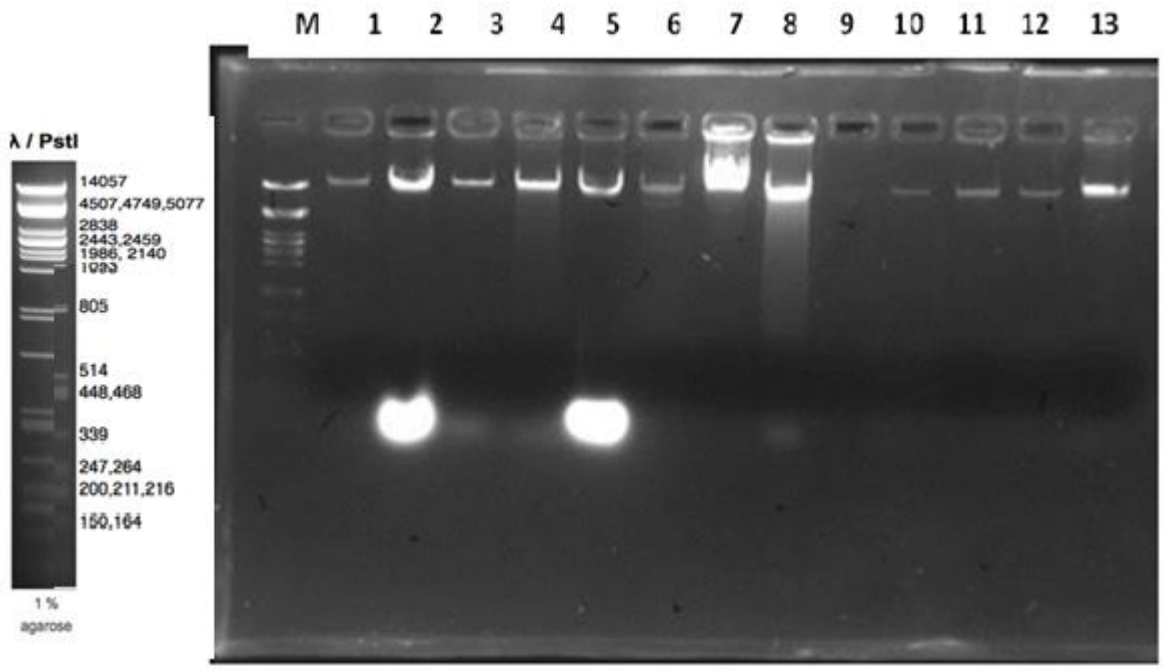


Figure 13: Agarose gel electrophoresis of gnomic DNA extracted from both eMalahleni and Cerebos isolates. Lane M represents the phage λ DNA digested with *Pst*I, lane 1-13 indicate the genomic DNA of Ep-1, Ep-2, EP-3, Cp-2-2, Cp-5-5, Cp-2-1, Cp-2-3, Cp-5-2, Cp-5-7, Cp-5-1, Cp-5-3, Cp-4-1 and Cp-5-6.

The 16S rRNA gene amplicons (1500 bp) amplified from the genomic DNA were confirmed on agarose gel electrophoresis as seen on Figure 14. The failed experiments were repeated until the 16S rRNA amplification for all the isolates was obtained.

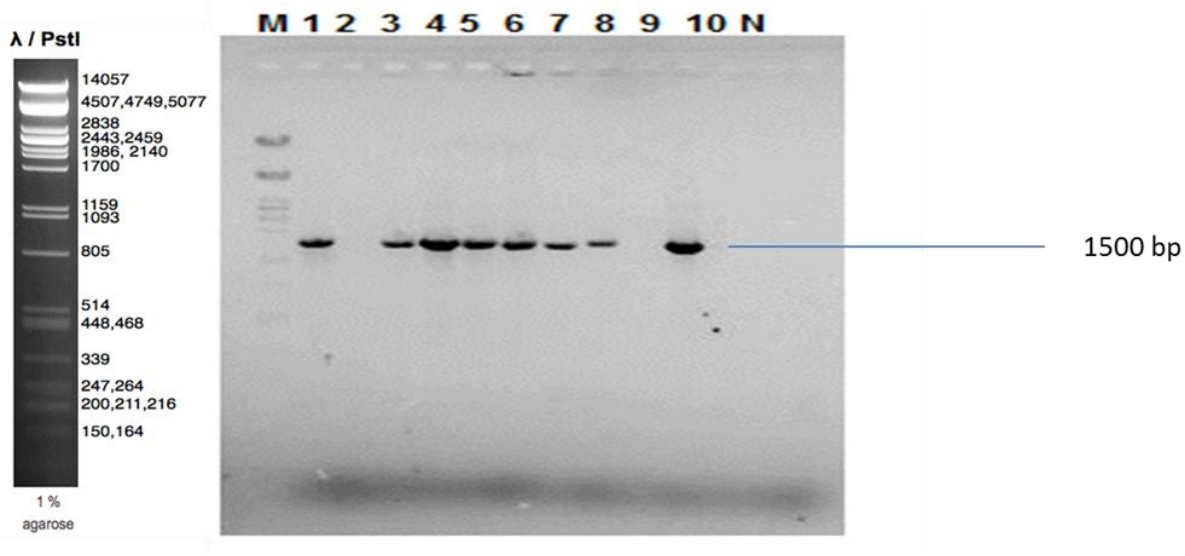


Figure 14: The agarose gel representation of 16S rRNA gene amplicons. Lane M is the phage λ DNA digested with *Pst*l, lane 1-10 shows the 1500 bp 16S rRNA gene of EP-1, Ep-2, EP-3, Cp-5-1, Cp-5-2, Cp-5-3, Cp-5-4, Cp-5-5, Cp-5-6, and CP-5-7 and lane N is the negative control

To identify the isolated bacterial strains the 16S rRNA gene amplicons were sequenced and the sequences were compared against the NCBI BLASTn database. Based on the 16S rRNA sequences analyses (Table 8) the isolates were identified to belong to the *Planoccocus*, *Sporosarcina*, and *Marinococcus*, *Staphylococcus*, *Microbacterium, Chromohalobacter* and *Arthobacter* genera. Other studies have shown that these strains may produce yellow, orange and red carotenoid pigments (Ventosa *et al*., 1998; Yoon *et al*., 2001; Yoon *et al*., 2003; Joghee and Gurunathan, 2014). Regarding intensity of the pigment produced by the isolates, EP-3 was the only one that produced a dark red pigment in R2A.

The 16S rRNA results of the Gram-variable strains (Cp-5-3 and EP-2; Table 8) revealed that the isolates were closely related to *Sporasarcina aquimarina (*query coverage of 99%). These results agree with the Gram stain of *S. aquimarina* recorded in Bergey's manual, 2009 (systematic bacteriology: volume 4) using a 3-day old culture. Correspondingly, Yoon *et al*., (2001) and Janarthine and Eganathan, (2012) independently reported similar results about this strain.

Table 8: Identification of pigmented halophilic bacteria based on 16S rRNA gene analysis

Source	Strain	Closest 16S rRNA BLAST hit (Sequence length/ identity%)	Accession Number
eMalahleni	EP-1	*Microbacterium oxydans* strain DSM 20578 (1399/99)	NR04493.1
	EP-2	*Sporosarcina aquimarina strain* SW28 (1425/99)	NR025049.1
	EP-3	*Arthrobacter agilis* strain DSM 20550(1391/99)	NR026198.1
Cerebos	CP2-1	*Staphylococcus capitis* strain JCM 2420 (1437/99)	NR113348.1
	CP2-2	*Microbacterium paraoxydans* CF36 (1528/97)	NR025548.1
	CP2-3	*Planococcus maritimus strain* N23 (1442/95)	KF318399.1
	CP4-1	*Planococcus maritimus strain* KMM 3738 (1550/97)	NR025049.1
	CP-5-1	*Planococcus maritimus strain TF-9* (1379/98)	NR114298.1
	CP-5-2	*Staphylococcus hominis* subp. novobiosepticum strain GTC 12228 (700/99)	NR041323.1
	CP-5-3	*Sporosarcina aquimarina* SW28 strain (1437/99)	NR025049.1
	CP5-4	*Planococcus maritimus* TF-9 D27 (1421/99)	NR0252471
	CP-5-5	*Micrococcus yunnanensis* strain YIM (1379/99)	NR116578.1
	CP5-6	*Planococcus maritimus strain* HNI107 (1217/97)	KF933638.1
	CP-5-7	*Chromohalobactercanadensis* strain DSM 6769 (1320/99)	NR114545.1

16S rRNA sequences from the strains isolated from eMalahleni brine showed that they were closely related to *Microbacterium oxydans*, *Sporasarcina aquamarina* and *Arthrobacter agilis.* The microbial ecology of eMalahleni reject brine has been studied before using metagenomics approaches. *Arthrobacter spp.* was identified among the halotolerant and halophilic species that were present in brine (Sekar *et al.*, 2014). The difference observed between eMalahleni and Cerebos isolates could be due to the type of brine (Na_2SO_4 vs NaCl respectively) variance in the chemical composition of the samples. Also, the media that was used during the isolation stage could have played a role in selecting the type of bacteria that were isolated.

From the results in Table 8, it may be concluded that the Cerebos samples were mostly dominated by strains related to *Planococcus maritimus*. This was not surprising at all since other researchers have isolated this strain from a number of hypersaline habitats, such as sea water from the tidal flat in Korea and in the intertidal sediments from the Clyde estuary in the United Kingdom (Shindo *et al*., 2008; Joghee and Jayaraman, 2014). Among the isolated strains, CP-5-6, CP-4-1, CP-2-2 and CP-2-3 BLASTn hit was found to be closely related to *Planococcus maritimus* with 97% (CP-5-6, CP-4-1 and CP-2-2) and 95% (CP-2-3) similarity. Similar to *P. maritimus*, these isolates were found to produce orange pigment and can grow in the presence of up to 17% NaCl (w/v). However, based on their nucleotide percentage it is very much possible that they represent novel species. Further characterisation would be required to confirm their novelty.

3.12.3 Salt tolerance

In order to ascertain the salt tolerance levels of the isolated bacteria, the isolated strains were grown on various NaCl and Na_2SO_4 concentrations (Tables 9 and 10). This was done to aid in the selection of the strain to be used for evaporation studies at the laboratory scale. The tested isolates were able to grow on medium containing up to 30% (w/v) of NaCl (Table 9). Five isolates, namely Cp-5-1, Cp-5-5, EP-2, EP-3 and CP-4-1 grew optimally at 5% NaCl (w/v), while CP-5-3 and Ep-1 grew optimally at 10% (w/v) NaCl. Isolate CP-5-2 was an exception as it grew optimally at 15% (w/v) NaCl. This was significantly higher than the salt concentrations in which most the isolates grew optimally. Based on their optimal growth in NaCl the isolates were classified as moderate halophilic bacteria according to Kerkar, (2004). This is not surprising since many moderate halophilic bacteria are able to grow over a wide range of salt concentrations. They grow up to 20% of NaCl while others can flourish even at 30% (Ventosa *et al*., 1998).

Table 9: Growth of the isolates at various NaCl (w/v) concentrations

Isolate	Sodium chloride range (%)	Optimum growth (%)	Category
CP-5-1	5-30	5	Moderate
CP-5-3	5-30	10	Moderate
CP-5-5	5-30	5	Moderate
EP-1	5-30	10	Moderate
EP-2	5-30	5	Moderate
EP-3	5-30	5	Moderate
CP-4-1	5-30	5	Moderate
CP-5-2	5-30	15	Moderate

Table 10: Growth of the isolates at various Na_2SO_4 (w/v) concentrations

Isolates	Sodium sulphate range (%)	Optimum Growth (%)
CP-5-1	5-20	20
CP-5-3	5-30	20
CP-5-5	5-30	5
EP-1	5-30	5
Ep-2	15-30	20
EP-3	5-30	20
CP-4-1	5-30	5
CP-5-2	5-30	10

Six of the isolates were able to grow in media supplemented with up to 30% (w/v) of Na_2SO_4. Isolate CP-5-1 grew from 5-20% while EP-2 did not grow when the concentration was below 15%. Comparing

the growth of isolates on NaCl and Na_2SO_4, the isolates seem to tolerate higher Na_2SO_4 concentrations. This was not surprising from the bacterial strains that were isolated from eMalahleni brine since Na_2SO_4 are the major ions present in Table. As for the Cerebos isolates, it was very unusual since they were isolated from NaCl-based saline water. However, studies have shown that SO_4 is the major inorganic compound in seawater and it can be found up to saturation. It is therefore, likely that these strains were already adapted to high SO_4 environments. All the isolates in this study are heterotrophs. Heterotrophic bacteria can assimilate sulphate for the biosynthesis of organic sulphur containing compounds, such as S-containing amino acids (Fry and Peel, 1954). *Micrococcus yunnanensis*, for example can oxidise sulphide to sulphur (Mullick, 2012; Alesia, 2014) and can tolerate about 200 g/L of sulphate (Alesia, 2014). The tolerance to Na_2SO_4 by the isolated bacteria suggests that they could withstand sulphate concentrated brine from eMalahleni as the growth medium, and thus could be the target for the evaporation experiments. However, EP-3 was chosen for the evaporation studies, based on its growth on various concentrations of Na_2SO_4, the most dominant type of salt in the eMalahleni brine. Moreover, among the isolated strains, EP-3 produced a dark red pigment that could potentially absorb more solar energy compared to the yellow, pink and orange pigment produced by the other isolates.

3.12.5 Pigment Identification

The red pigment produced by EP-3 was analysed spectrophotometrically, and absorbance peaks at 320 nm, 390 nm and a major peak at 495 nm were observed. A similar spectrum was observed by Fong *et al.*, 2001, when analysing a methanol extract prepared from an *Arthrobacter agilis* isolated from Antarctic sea ice. The authors observed two *cis* maxima between 368-372 nm and 384-390 nm and the longest wavelength maxima between 523-530 nm (Figure 15b), which the authors attributed to bacterioruberin as the main component. The occurrence of the first two peaks is characteristic of monocyclic or cyclic chromophore. Since a similar spectrum was observed for EP-3, which is also identified to be

Arthrobacter agilis, it is tempting to speculate that the red pigment it produces is also bacterioruberin. The total carotenoid content of *Arthrobacter agilis* was found to be 1.2± 0.41 mg/g.

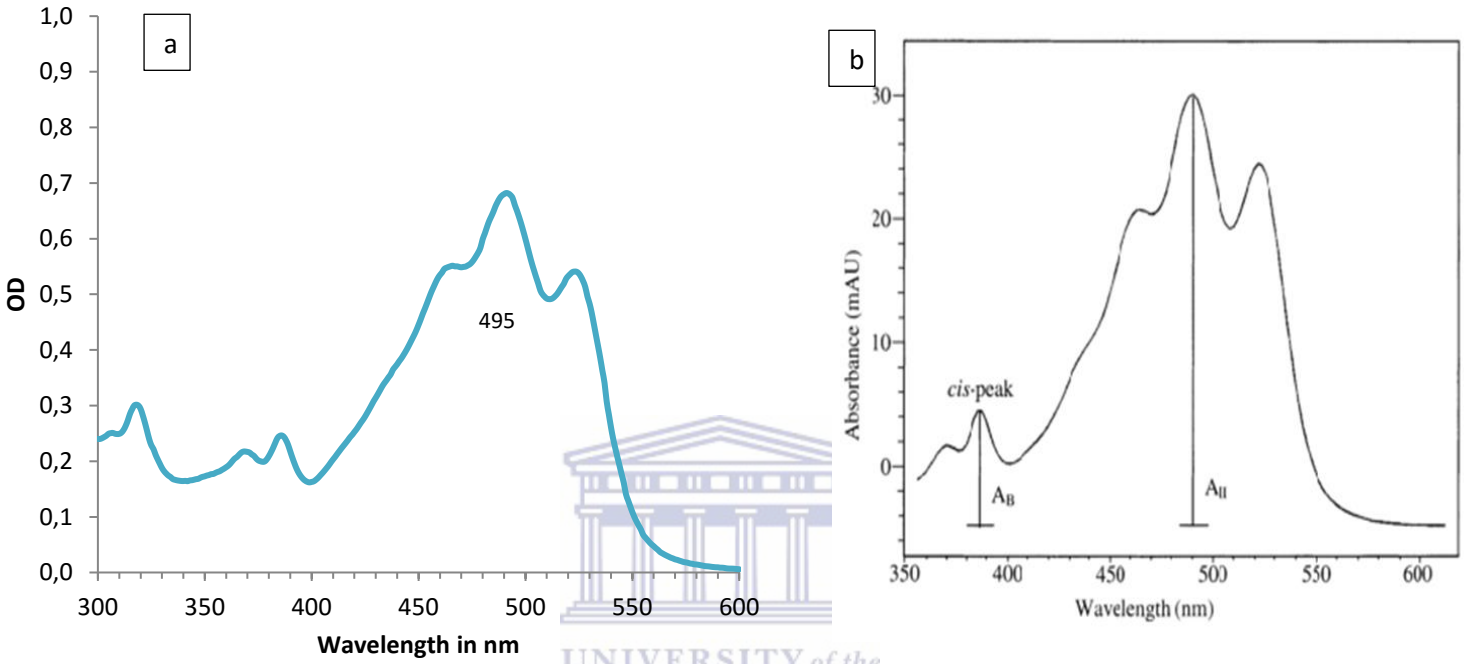


Figure 15: A visible absorption spectrum of a red pigment produced by *Arthrobacter agilis*. Figure a represent the absorption spectrum of a red pigment produced by *Arthrobacter agilis* (EP3) with its maximum absorption peak at 495. Figure b is the representation of absorption spectrum of a cis carotenoid. Tetra-anhydrobacterioruberin and bacterioruberin diglycoside displayed the characteristic 'three fingered' maxima of red carotenoids, with maxima at approximately 500 nm. (Fong et al., 2001)

3.12.6 Effect of bacterial pigment on the evaporation rate of brine

In this experiment, we were interested in determining whether the red pigment could be extracted from an EP-3 culture and evaluated for its ability to improve the evaporation rate of brine. From Figure 16 it was observed that the addition of pigment to the brine resulted in the complete evaporation of the brine within 48 hours, compared to the brine without pigment that took 54 hours. Based on this finding, the evaporation rate was calculated and the results are presented in Table 11. The results showed that the addition of the pigment extracted from EP-3 increased the evaporation rate of brine by 30%, suggesting

that the pigment resulted in the absorption of more solar energy, increasing the temperature of the brine and thus increasing evaporation.

3.12.7 Effect of bacterial culture on the evaporation rate of brine

The possibility of using the EP-3 bacterial culture (seeding) to enhance evaporation rate of brine was also investigated using the method detailed in section 3.11.2. The concept being that the addition of an endogenous bacteria able to proliferate in the brine pond conditions would potentially continue to produce the pigment. From a sustainability point of view, such a scenario would be preferred to the supplementation of pigment. Figure 16 illustrates the amount of water loss during the course of the experiment when the EP-3 culture is inoculated. Inoculation of the culture resulted in the complete evaporation of brine within 48 hours, whereas the un-inoculated control brine took 54 hours. It was observed that from 0-24 hours the brine evaporated at the same rate as the two experiments (brine+EP-3 and brine+ pigment). However, after 24 hours of exposure it begins to slow down as the salts precipitate out and thus delaying the evaporation. Comparing the two controls (brine and brine +TSB), it was observed that brine without the media evaporated faster. This could be due to the nutrients and turbidy of the culture that requires more energy for converting liquid molecules to vapour. A comparison of the evaporation rate of the brine inoculated with the culture is presented in Table 11.

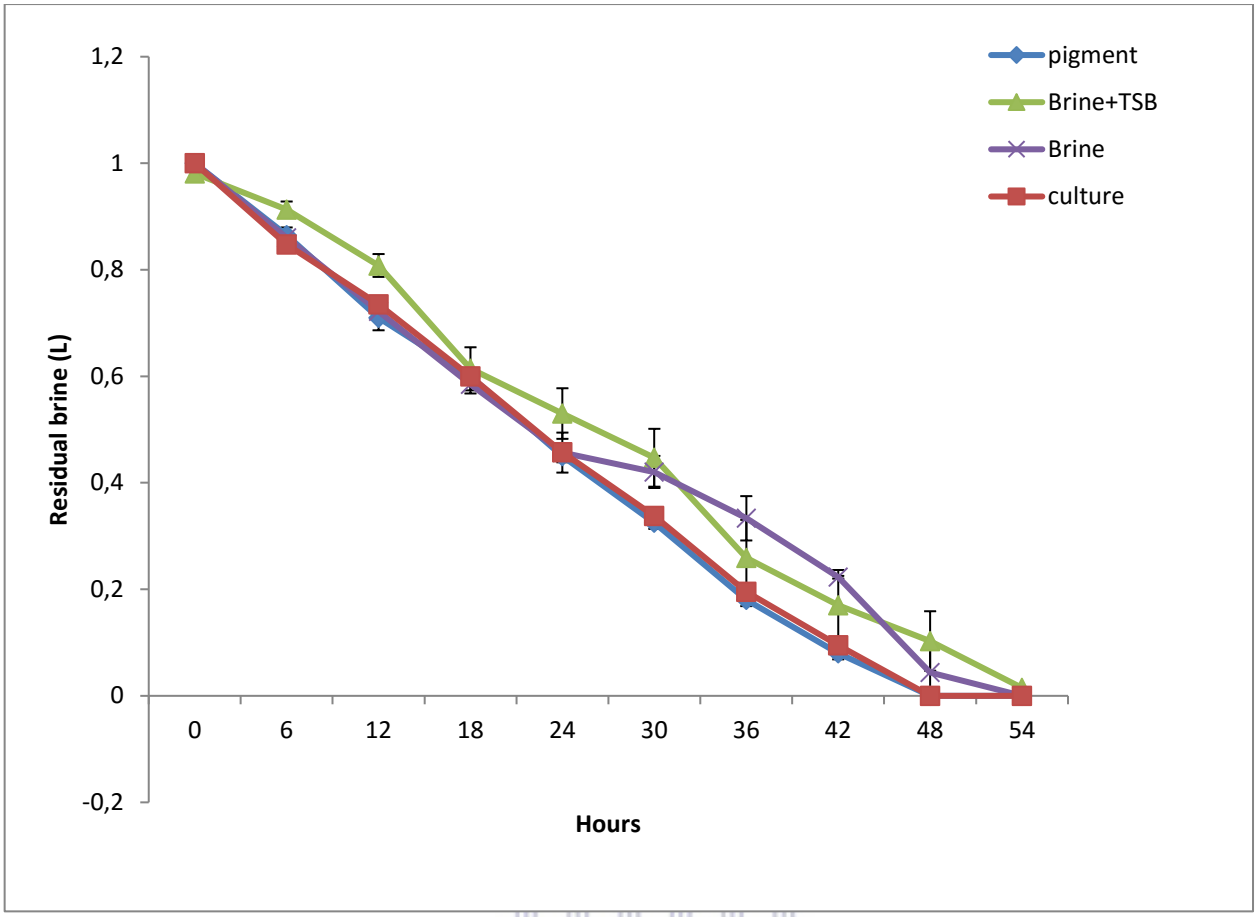


Figure 15: Effect of biological pigment and bacterial culture on the evaporation of synthetic brine. The error bars represent the standard deviation of triplicate experiments run independently.

Table 11: Comparison of the evaporation rate of synthetic brine using pigment and the culture of EP3

Sample	Evaporation rate (L/hr)
Brine	0.0177±0.0006
Brine+ TSB	0.018±0.003
Culture	0.021±5.77350E-05
Pigment	0.022±0.001

The evaporation rate increase between 18% -24% could be observed when the pigment and culture is added, with the pigment being marginally faster than culture. The reduced evaporation rate observed in the brine+EP-3 experiment could be due to the cells affecting the solar absorption and scattering the solar

energy. In order to assess the actual contribution of the pigment to the evaporation, a non-pigmented mutant would offer the most useful comparison. .

3.13 Conclusion

In this study, fourteen moderately halophilic bacteria were isolated from two different environments; Cerebos salt samples and eMalahleni brine. The isolates were identified using 16S rRNA gene analysis. The Cerebos samples were mostly dominated by *P. maritimus* while very few species were isolated from the eMalahleni brine. This could be because the eMalahleni brine sample had been in storage at 4 ^{o}C for approximately two years. Various pigments were noted to be produced by the isolates. However, only *A. agilis* (EP-3) isolated from eMalahleni brine produced a dark red pigment that was identified as bacterioruberin and thus it was evaluated for its ability to improve the evaporation rate of the synthetic brine. This was assessed independently using both bacterial pigment and the seeding approaches. The evaporation rate increase between 18-24% could be observed when the pigment and culture is added, with the pigment being marginally better than culture. Irrespective, and increased evaporation was observed compared to the 2 controls. Thus, it may be feasible to seed an evaporation pond with a pigmented culture and/or promote its proliferation in such a system. Given the number of environmental factors that contribute to a pond system, it was not feasible to assess this in this study. However, one can consider applying the cultures as inoculum into the industrial pond, the nutrients present in the inoculum might also promote the growth of other halophilic microorganisms such as *Dunaliella spp,* and halobacteria that may further contribute to the intensity of the pigment and subsequently improve evaporation. In contrary, the inoculum could promote the growth of non-pigmented halophiles, resulting in no pigment production and subsequently no evaporation rate enhancement. However, the cost of cultivating the EP-3 strain in the media prior to the addition into the pond could also contribute to high cost of brine management if this were to be applied in the eMalahleni evaporation pond.

In contrast to the seeding approach, the increase in the evaporation rate of brine that was observed when using a bacterial pigment was anticipated, based on previous studies that have assessed a range of coloured dyes (Keyes and Gunaji, 1967; Rajvanshi, 1981; Patel et al., 2013). Therefore, as the part of future work the growth and pigment production by EP-3 inoculum should be assessed and monitored in order to accurately determine the impact of inoculum on the evaporation rate of brine. Furthermore, this could help in determining the minimum inoculum to be used so as to cut the cost of cultivating the strain.

It should be noted that, when choosing the best approach to be applied in enhancing evaporation of brine, cost and feasibility of each method should be taken into consideration. A previous study showed that brine management accounts for approximately 33% of the total cost in desalination (De Vito *et al*., 2011). Comparing the cost of the two biological approaches, seeding involves the cost media while pigment includes the extra cost of the solvents that are used during extraction. Therefore, in future assessments of the applicability of any of these approaches, a cost analysis is crucial.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSION

Despite the significant contribution of the mining industry to the economy of South Africa, the disposal of the industrial waste, Acid Mine Drainage, has become a serious environmental threat. For example, at the eMalahleni Plant Reclamation, about 150 m^3 of concentrated brine per day is generated; the plant is facing challenges with low evaporation rate as the pond is unable to treat the large volumes of brine being produced.

Indeed, various methods of improving evaporation rate of brine, such as increasing the Waive, wetted floating fin, and sparing of the brine have been studied (Hoque *et al*., 2010). As much as these methods reportedly improved evaporation rate of brine, they suffer from certain drawbacks that include the use of a large fabric and pumping systems that utilise more electricity (Tang and Etzion, 2004; Hoque *et al*., 2010). Because of these mentioned shortcomings these techniques are technically and economical unattractive for application at a large scale, unlike an evaporation pond.

From the study findings, it is important to note that the application of both approaches resulted in a substantial increase in the evaporation rate of brine with the use of MB being 17% more effective compared to the two biological approach used. Each of the approaches applied in this study have advantages and limitations. The chemical approach is a simple method to apply since it only requires MB powder to be dissolved in brine to the desired concentration (200 ppm), which can be added directly to the pond. It is a darker pigment that would aid in the absorption of radiant energy to enhance the evaporation rate of brine. Furthermore, MB can be reconstituted thus making it an economical option. However, its toxicity to human health and the environment represents the major limitation to its application on a large scale.

Using the biological approach, the addition of the EP-3 culture (seeding) to the actual pond would allow the strain to proliferate and produce the pigment and improve evaporation rate. Consequently, the salts

that precipitate out can be safely discarded without harming the environment. However, the adaptability of the EP-3 strain to the evaporation pond conditions could constitute a great challenge. The ability of the EP-3 strain to proliferate and maintain pigment production under changing conditions, as would be experienced in the evaporation pond (pH and temperature), could significantly impact the performance of this approach. Therefore, the results obtained in this study might not be reproducible in the actual pond. To mitigate limitations associated with the expression of the pigment synthesis genes (in relation to changing brine conditions and thus regulatory signals within the ponds), one could consider manipulating the EP-3 carotenoid biosynthetic pathway, or EP-3 could be engineered. For example, the biosynthetic pathway responsible for the production of a darker pigment (such violacein or melanin from another organism) could be expressed in EP-3. Thus, the constitutive expression of pigment would overcome any regulation issues.

In a scenario where the ponds are supplemented with a culture inoculum, thus introducing nutrients, it is conceivable that this could promote the growth of non-pigmented microorganisms, which might out-compete the EP-3 organism in the pond. This would then compromise the level of pigment produced and the evaporation could not be improved. Therefore, the implication of adding nutrients to the actual pond should be investigated prior to the implementation of this method. This was not investigated in this study because all the evaporation studies were done on the synthetic brine other than the actual brine. On-going studies are assessing this using the actual brine wastewater.

Unlike the chemical method, the pigment approach is laborious and time consuming as it requires the large-scale cultivation of the EP-3 strain in liquid media followed by the extraction of the pigment. In this study, one gram of pigment was added in 1000 ml of brine to give it a slightly red colour. Scaling this up to a real brine pond scenario would constitute an enormous challenge in terms of the volume (media and solvents for extracting the pigment) required to make this a viable process. Another challenge

associated with the application of both pigment and culture is the stability of the pigment, due to the change of pH as the evaporation progresses (Joshi *et al.*, 2003). The EP-3 growth and pigment stability should be monitored at various pH and temperatures, as this could negatively affect the efficiency of the evaporation rate in the natural setting. The study by Rajvanshi, (1981) showed high evaporation with no photochemical degradation when the black dye was used. However, similar assessments would need to be conducted with the EP-3 pigment.

The major constrain associated with the application of these methods, whether it be the chemical or biological approach at a large scale, is the cost which accounts for about 30% of the total cost in desalination (Ahmed *et al.* 2000; De Vito *et al.*, 2011). Therefore, the cost and feasibility of the method should be well thought out when selecting the best approach to improve evaporation rate of brine in the pond. For arguments sake, an over simplified cost analysis was conducted to estimate the cost of applying chemical and biological approaches in a 500 m^2 pond with a 20 cm depth. A range of dyes (congo red, naphylamine, napthol green and methylene blue), based on the concentrations proposed by Svensson, (2005) were assessed (using the Sigma Aldrich prices) Table 12. The cost of applying a chemical approach is dye dependent; however, for all the dye is substantially lower compared to the biological approach.

The cost of the two biological approaches (pigment and seeding) should be considered when choosing the approach to apply. Apart from the cost of the media and solvents, both of these approaches inherit additional costs that are not accountant for in the simplified calculation presented in Table 12. For example, there is the need for microbiological capacity (Infrastructure, such as a laboratory, bioreactor and relevant training/expertise). In addition, large quantities of water will be used to prepare the cultures. Based on the calculations in Table 12, about 20 000L of the culture would need to be added into the pond for seeding approach, and this would results in a 20% volume increase in the pond. This is obviously counter-

productive, but could be over comed by applying lyophilized culture preparation. The viability of the EP-3 after lyophilisation would have to be assessed, but then again imposes the need for specialised equipment or capacity.

The cost of the biological approach could; however, be reduced substantially by using a variety of waste product for example; corn steep juice, as a carbon and nitrogen source instead of R2A media used and on which calculations are based. This would form part of follow-up study. Without further experimentation, the most cost effective and convenient method to apply is the chemical approach (MB dye). Although Naphthol green is the cheapest, it only offers slight improvement of 13%. The second economical and effective dye is naphthylamine that gives an evaporation rate that is equivalent to the biological method but with 50% less cost. Methylene blue is the most effective method and the 17% extra improvement may be worth the higher cost. However, this cost-effectiveness and convenience of the chemical approach would still have to be weighed against the potential environmental and health hazard, and the downstream cost to discard or treat the dyes.

Table 12: Estimated annual cost of applying the indicated dyes at the optimum concentration assuming a 20 cm depth in a 500m^2 evaporation pond.

Dye	Quantity	prices	Required concentration	Evaporation rate (%)	cost
Naphthylamine	25G	R 734	172.5 ppm	29	R513800
Naphthol green	25G	R 308	2 ppm	13	R2464
Methylene blue	25G	R 1 196	200 ppm	41	R956800
Congo red	25G	R 2 590	200 ppm	Less effective	R2072000[†]
R2A (Biological medium)	500G	R2035	200ml/L	30	R1237280[*]

[†]Less effective = evaporated at the same rate as the brine. [*] This indicates only the cost of the reagents used to prepare the media. However; there are additional costs that will have to be considered for inoculum preparation .This includes the cost of water and infrastructure required to cultivate large volumes of the inoculum.

Pond depth has a significant effect on the evaporation (Murugavel *et al.*, 2008). Previous studies have observed high evaporation rate of coloured brine in deep ponds compared to the shallow ones (Rajvanshi, 1981; Murugavel *et al.*, 2008). Other researchers have suggested that for maximum evaporation, the pond depth ranging from 25-45 cm are ideal (Mickely *et al.*, 1993). In this study, the MB related rate is dependent on the depth of the litre pan that was used and cannot necessarily be extrapolated for the eMalahleni pond or any other pond. Furthermore, factors that greatly influence evaporation rate of saline waters, such as humidity, wind speed, ambient temperature, and the density of the brine were not considered during this study. Therefore, although the proof of concept was established through this study, further investigation is still required to determine the effectiveness under more natural conditions. In conclusion, this study for the first time revealed the potential application of endogenous pigment producing halophilic bacteria (A. agilis) to improve evaporation of mine wastewater brine in the evaporation pond. In as much as the study was conducted specifically for eMalahleni pond, the principle can still be applied by other compaies generating and treating reject brine using evaporation ponds.

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Appendix A

The following calculations are used to estimate the cost of applying dyes in based on (Svesson, 2005).

Given: Size of the pond 20cm top of 500 m^2

Concentration of the napthol green 2 ppm

Volume of water =area * height

=(500 m^2) *(0.2 m)

=100 m^3

=100 000 dm^3

=100 000 L

$v = \frac{m}{c}$

$m = cv$

Napthol green

(2 mg/mL)*100 000 = 200 000 mg/1000

200g Since 100 g of napthol green cost $44.15, therefore the cost of 200g will be

100g $44.15

200g X

=$88.3≈ R1320 However , when using Sigma aldrich prices it costs R2464 to use the exact concentration of napthol green in the same size of the evaporation pond.

Methylene blue

200 mg/mL *100 000 L= 200 00000 mg /1000
=20 000g

25 R1196
20 000 X
R956800

Naphylamine

172.5 mg/mL*100 000 L =17250000 mg/1000
=17250g

25	R734
17250	X

= R513800

Congo red

200 mg/mL *100 000 L= 200 00000/1000

=20 000g

25 g	R2590
20000 g	X

=R2072000

Biological Media (R2A)

200 mL of culture was added in 1 litre of brine

Therefore 20000 L of culture will require R304 000 g of R2A media

500 g	R
304000	X

=R1.2 million