THE NATURE AND CONTROL OF ORGANIC COMPOUNDS IN SODA ASH EVAPORITE PRODUCTION

THESIS

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ABSTRACT

Solar evaporite systems are man-managed ecosystems which are highly vulnerable to biological, physical and chemical disturbances. The problems encountered in such systems are in many cases found to be associated with the microbial ecology and the design of the system. This project focussed on investigating the nature of organic compounds contaminating soda ash produced at a solar evaporite production system located at Sua Pan in Botswana. Several years after the plant was commissioned, problems, including accumulation of total organic carbon (TOC) and discolouration of the soda ash product were encountered. The salt produced also retained high moisture content and was coloured pink.

These phenomena impacted severely on the economic performance of the enterprise. This study was aimed at determining the origin and fate of these organic compounds within the system in order to elucidate the nature of the problem and also to conceptualise a remediation strategy suitable to reducing its impact. This was achieved by analysis of both dialysed and solvent extracts of the influent brine (well-brine), brine in the ponds (T-brine) and the bicarbonate filter cake.

Although complete identification of the organic compounds isolated was not undertaken in this study, spectroscopic analysis of compounds isolated, by UV, IR, NMR and MS, strongly indicated that fulvic acids, a component of the influent well-brine organics, contribute to the organic contamination of the final product. Part of this component, however, is degraded during the ponding process. It was shown that an extracellular polysaccharide (EPS) produced by *Dunaliella*

spp., which proliferates in the evaporation ponds, contributes in a major way to the accumulation of TOC in the system. This was demonstrated by relating the sugar profile of carbohydrates isolated from the pond brine and final product, being arabinose, xylose, 2-*o*-methyl hexose, mannose, glucose and galactose. Studies reported show that EPS production was enhanced when algal cultures were exposed to stress conditions of high illumination, increasing salinity and temperature, and nitrogen limitation.

Studies undertaken for the development of a remediation process for this system have shown that nutrient stripping and bacterial systems could be applied to deal with the dissolved TOC fraction, whereas adsorption systems could deal with the particulate fractions. Algal systems showed most potential for the removal of nutrients in the influent well-brine compared to chemical processes. Complete removal of ammonium and phosphorus removal efficiencies of approximately 50% were achieved in an unoptimised pilot-scale *Dunaliella*-based HRAP. While similar effects were demonstrated for chemical processes, some economic constraints were noted. The potential of halophilic bacterial systems for the degradation of organic compounds in brine was also demonstrated. The limitations on the performance of such systems, associated with the low metabolic diversity, and poor immobilisation of halobacteria, however, were noted. Although physico-chemical processes were found to have a very low impact on the dissolved TOC fraction of the brine, the removal of the particulate material was found to result in a 35% TOC reduction in the final soda ash product and the production of a white final product.

Apart from a description of the microbial ecology of the ponds and the identification of major contributions to the TOC of the final product, a number of remediation strategies were evaluated and are described. These include chemical and biological stripping of nutrients sustaining microbial

TOC production in the ponds, and also biological and physico-chemical processes for their removal once formed. Future studies to undertake the further development of these proposals has been described.

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ABBREVIATIONS

ANSAC = American Natural Soda Ash Corporation

ATP = Adenosine Triphosphate

BSA = Bovine Serum Albumin

C = Carbon

CS = Cooling Sump

E = Evaporation pond

EMS = Electrospray Mass Spectrometry

EPS = Extracellular Polysaccharide

GAC = Granular Activated Carbon

GC = Gas Chromatography

HRAP = High rate Algal Pond

HROP = High rate Oxidation Pond

IR = Infra Red

MEA = Monoethanolamine

MS = Mass Spectrometry

MWCO = Molecular Weight Cut Off

N = Nitrogen

NMR = Nuclear Magnetic Resonance

NS = N-brine storage pond

P = Phosphorus

PAC = Powdered Activated Carbon

SBBR = Sequencing Biofilm Batch Reactor

SEM = Scanning Electron Microscopy

ST = T-brine storage pond

TOC = Total Organic Carbon

UK = United Kingdom

US = United States

UV = Ultra Violet

X = Crystalliser pond

CHAPTER 1

INTRODUCTION

1.1 SOLAR EVAPORITES

Solar evaporites are minerals found in environments such as enclosed basins, saline lakes and sabkhas in semi-arid areas where annual evaporation exceeds total inflows of water (Gavish, 1980). These minerals are concentrated from brines by solar evaporation and crystallize into deposits that may be commercially exploited.

Although a variety of products such as gypsum (calcium sulphate), thernadite (sodium sulphate), potash, borates, lithium, uranium, and silicates may be obtained from solar evaporites, the most significant products are halite (sodium chloride) and soda ash (sodium carbonate) (Glasby, 1986). Large operations for the production of salt and soda ash are distributed in many countries throughout the world.

1.1.1 Salt

Among the marine evaporites, salt is by far the most common and widely distributed mineral resource and is produced in many countries (Keyser, 1976). Salt has been an article of trade for centuries, for example, salt from North Africa sabkhas was mined and distributed by Arab caravans more than 2000 years ago (Williams, 1981). It is used by the chemical industry, in the production and preservation of foodstuffs and as animal feed (Smith *et al.*, 1973). Although salt

can be obtained from several sources such as solution mining, underground mining and evaporation of natural brines and seawater, solar salt-fields are the most important source of salt (Jones *et al*., 1981). Currently one third of worldwide production of salt comes from solar salt-works (Davis, 1990).

The annual production of salt amounts to 200 $Mt.year^{-1}$ (Tackaet and Sorgeloos, 1993) but only 10% is used for human consumption. The chemical industry is the largest consumer of salt which is used for the production of chlorine, soda ash, caustic soda, hydrochloric acid, sodium sulphate, sodium sulphide and metallic sodium (Smith *et al*., 1973; Keyser, 1976; Jones *et al*., 1981; Tackaet and Sorgeloos, 1993). The US is the largest producer of salt and in 1970, 54 companies operated 99 plants in 17 states producing more than 45 million tonnes of salt with a value of more than $285 million (Smith *et al*., 1973). Although deposits of rock salt are principal sources of salt in the US, salt lakes in the arid western states and solar evaporation of seawater also provide other sources. Other major producers of salt are Australia, where 3.6 million tonnes were produced in Western Australia in 1975 (Glasby, 1986) and China, Russia, Germany and the UK.

1.1.2 Soda Ash

Soda ash is used in the manufacture of glass and in the detergent, chemical, metallurgical and paper industries (Ehlers, 1998). The annual production of soda ash world wide is approximately 29 million tonnes (Taylor, 1991). Most countries except the US and few countries in Africa are dependent on synthetic soda ash manufactured from salt, ammonia and carbon dioxide by the Solvay process (Ehlers, 1998). The high cost of new plants however, is a limitation on production of soda ash by the Solvay process and therefore production from natural sources had become increasingly important (Smith *et al*., 1973).

Natural soda ash is in most cases deposited as trona ($NaCO_3.NaHCO_3.2H_2O$). These deposits have been found in the US in the Green River Formation of Eocene and Wyoming (Smith *et al*., 1973) and presently five large trona-refining plants operate in this area (Aitala and Aitala, 1997). Soda ash resources may also be found in the form of $NaHCO_3$ as in northwestern Colorado (Smith *et al*., 1973). In Searles and Owens lakes in the US, soda ash is extracted from brines. An operation owned by American Potash & Chemical Company produces 10% of American soda ash from deposits in Searles Lake (Aitala and Aitala, 1997). The US is currently producing 11.5 tonnes of soda ash per year, of which 4.5 million tonnes are exported (Aitala and Aitala, 1997) mainly by American Natural Soda Ash Corporation (ANSAC).

Apart from the US, the largest natural soda ash operation is at Lake Magadi in Kenya, operated by Magadi Soda Company (Eugster, 1980). This saline alkaline lake generates new trona at about the same rate as annual production and has been commercially exploited for many years. Salt is also obtained as a by-product from this operation. Carbonate-rich brine is pumped into evaporating ponds and thermonatrite ($Na_2CO_3.H_2O$) accumulates as a thick bottom deposit. Sodium chloride concentrations increase and halite forms a layer on top of the thermonatrite (Eugster, 1980). Soda ash is also extracted from brine deposits at Sua Pan in Botswana and Sua Pan is the most significant resource of natural soda ash discovered so far in southern Africa (Ehlers, 1998).

1.2 SOLAR SALT-WORKS

The manufacture of salts by solar evaporation of seawater is an ancient art (Baas-Becking, 1931) and the principle of this production is the same today as it was in ancient times (Larsen, 1980).

Seawater or brine is pumped into a series of shallow ponds and is retained in each pond for a certain period while evaporation takes place. A salinity gradient is established in the pond series (Davis, 1990) and as the brine flows downstream, fractional precipitation of salts occurs. The least soluble salts, $CaCO_3$ and $CaSO_4$ precipitate and the NaCl rich brine is pumped into crystallizer ponds where NaCl is crystallized out, harvested, washed and dried. The mother liquor (bittern) has to be drained off before all the sodium chloride has crystallized out to reduce contamination with bromides and other salts which precipitate at these elevated salinities (Tackaet and Sorgeloos, 1993) or may be pumped to other ponds for precipitation of magnesium or potassium salts (Larsen, 1980).

Production of salt is determined by many factors including (Jones *et al*., 1981):

(a) climate - high sunshine, a prevailing wind and low rainfall are required.

(b) extent of brine loss by leakage.

(c) the degree of solar absorption and its consequent effect on evaporation.

(d) the microbial ecology.

New and well-established solar saltworks often experience problems which may cause production of low quality salt or production may be in quantities considerably less than design capacity (Davis, 1993). These have been related in part to the operation of biological systems not favourable to salt production, biological management systems or the design of the salt-field (Davis, 1993).

Biological problems found in most solar saltworks have been the subject of detailed review by Davis (1980; 1990; 1993), Jones *et al*. (1981) and Larsen (1980). In systems where there is

inadequate biological productivity and biomass formation, brine leakage may occur due to failure to establish a stable bottom mat, and inadequate solar energy absorption and evaporation may occur due to clear brines. Excessive production of organic matter, on the other hand, may lead to production of low quality salt and yields substantially less than design capacity.

A case study of biologically-related salt production problems has been reported for Dampier, Western Australia, where soft hopper crystals high in calcium were produced as a result of viscous brines. The high viscosity was caused by biological productivity, and in particular the dissolved extracellular polysaccharide produced by *Synechococcus* (*Coccochloris*) (Roux, 1996) and some species of purple photosynthetic bacteria occurring in microbial mats (Burnard and Tyler, 1992). Agitation of microbial mats caused viscosity increases. Two factors found to be a requirement for stable microbial mats were high light intensities and the correct type of substratum (Burnard and Tyler, 1992). Overproduction of mucilage by cyanobacteria has also been documented at Port Alma, another saltfield in Australia (Jones *et al*., 1981).

Because of problems of this type, the development of biological management systems for solar evaporite production has become increasingly important in recent years and extensive studies have been undertaken on the microbial ecology of solar evaporite systems (Borowitzka, 1981).

1.2.1 Microbial Ecology of solar saltworks

Hypersaline environments are normally characterized by low species diversity (Larsen, 1980; Oren, 1988; Ollivier *et al*., 1994). The high concentrations of solute ions enhance hydrophobic interactions causing aggregation or collapse of proteins. This also interferes with essential electrostatic interaction within or between macromolecules due to charge shielding, and salt

hydration limits the availability of free water required to sustain essential biological processes (Woolard and Irvine, 1994).

Organisms growing in ecosystems having a salt concentration above that found in seawater are referred to as halophiles and these include prokaryotic bacteria and eukaryotic algae (Lowe *et al*., 1993). Halophilic bacteria can either be moderate; requiring salt concentrations in the range 2-20% (0.3-3.4M) or extreme halophiles; requiring at least 15% (2.6M) (Lowe *et al*., 1993). Bacteria which can tolerate high salt concentrations but do not require them are referred to as halotolerant (Borowitzka, 1981). These organisms have adapted to live in environments with high salt concentrations.

1.2.1.1 Algae

A number of different primary producers develop in the dilute brines occurring in the initial stages of evaporation ponds. Cyanobacteria (*Spirulina*, *Oscillatoria*, *Coccochloris* and others) tend to form mats on the bottom of ponds while green algae such as *Dunaliella viridis* and *Dunaliella salina* grow as planktonic forms (Larsen, 1980). As the brine becomes more concentrated, the number of species as well as biological productivity is found to decrease, leaving *D. viridis* and *D. salina* as the dominating primary producers (Larsen, 1980). *Dunaliella salina* is the major and sometimes the only primary producer in highly saline lakes and salt crystallizing ponds of solar salt-works (Borowitzka and Borowitzka, 1988).

Dunaliella is a unicellular, motile green alga belonging to the division Chlorophycophyta, order Volvocales. It grows in salt concentrations higher than any other eukaryotic alga and is able to

adapt very quickly to sudden changes in salt concentrations (Borowitzka, 1981). *Dunaliella* cells have no cell wall and therefore can change shape when salt-stressed. These cells accumulate high intracellular concentrations of glycerol (Borowitzka and Borowitzka, 1988; Ben-Amotz and Avron, 1989) to balance the concentration of salts in the external medium. This allows them to grow over a wide range of salt concentration (Borowitzka, 1981). The two major halophilic species occurring in the solar ponds are *D. salina*, whose cells accumulate carotenoids at high salinity and *D. viridis*, which remains green at all salinities. *D. salina* predominates in high salinity ponds and the â-carotene produced which act as a photoprotectant is responsible for the orange colour seen in some hypersaline water bodies (Post, 1977; Larsen, 1980; Borowitzka, 1981).

1.2.1.1.1 Production of Extracellular Polysaccharides by algae

Planktonic algae have been reported to respond to nutrient deficiency through a variety of physiological changes; changes in cellular content or release of extracellular carbohydrates (Myklestad, 1995). Extracellular release may also be a function of salinity change (Sudo *et al*., 1995), temperature and light (Claus, 1988). Huntsman (1972) and Guillard and Wangersky (1958) have reported that conditions favouring release are growth-phase dependent, with more release occurring in the stationary phase. Carbohydrates can constitute up to 80% of the extracellular carbon released by algae (Myklestad, 1995) with monosaccharides constituting up to 50% of this fraction (Claus, 1988). The released carbohydrates provide a substrate for bacterial growth, and organic material produced by *Dunaliella* was reported to be a major source of nourishment for halobacteria in the Dead Sea (Oren and Shilo, 1985).

Production of exopolysaccharides is common in cyanobacteria and is believed to play a major role in enabling the survival and growth of these organisms under a wide range of conditions in which they exist. These polysaccharides play essential roles such as protection against dessication, predation and antibacterial agents, chelation of cations essential to cell life and adhesion to solid surfaces (Vincenzini *et al*., 1990). Production of polysaccharide slime by cyanobacteria from saltpans has been reported to increase the viscosity of the medium (DePhilippis *et al*., 1993) which affects solar evaporation.

Release of organic matter by *Dunaliella salina* (Giordano *et al*., 1994) and other *Dunaliella* species (Guillard and Wangersky, 1958; Huntsman, 1972; Bell, 1983) has been reported. Release of organic matter has a major significance in the hypersaline ponds of solar salt-works since organic matter in salt crystallisers is detrimental to salt production (Giordano *et al*., 1994). Interference with crystal growth resulting in small crystals or hollow crystals retaining contaminants may occur (Davis, 1990).

1.2.1.2 Bacteria

Marine salt-works are habitats for a variety of halophilic or halotolerant bacteria which develop throughout the entire gradient of salt concentrations (Ollivier *et al*., 1994). The intermediate salinity ponds (10-20% NaCl) contain the greatest numbers of organisms most of which are moderately halophilic, whereas the high salinity ponds are inhabited by extreme halophiles which include the aerobic members of the *Archaea* (halobacteria) (Davis, 1980; Ollivier *et al*., 1994).

Halophilic or halotolerant bacteria are found both in the archaebacterial and in the eubacterial groups (Oren *et al*., 1992).

1.2.1.2.1 Aerobes

Although dissolved oxygen may be low in the hypersaline environment of saltworks (Borowitzka, 1981; Norton, 1992), aerobic conditions still prevail in the brine (Larsen, 1980). The aerobic red coloured bacteria of the genera *Halobacterium* and *Halococcus* (halobacteria) have been reported to be the dominating bacteria in crystalliser ponds (Davis, 1980; Larsen, 1980). Brine in these ponds is usually red due to the presence of these bacteria which produce carotenoids, mainly bacterioruberin (approximately 10^8 cells/ml) (Post, 1981; Larsen, 1980; Grant and Larsen, 1989). The colour imparted in the brine aids solar absorption and hence increases the rate of brine evaporation (Davis, 1980). Absorption of light by pigments produced by halobacteria may also be beneficial by increasing the temperature of the brine therefore stimulating the metabolism of these bacteria having optimum temperature as high as 40-50^{0}C (Larsen, 1980).

1.2.1.2.1.1 Halobacteria

Halobacteria may be distinguished from other halophilic prokaryotes (the eubacteria) by their archaebacterial characteristics which are: possession of ether-linked phosphoglycerides, insensitivity to eubacterial inhibitors such as penicillin and chloramphenicol and lack of muramic acid-containing peptidoglycan in the cell envelope (Rodriguez-Valera, 1991). Archaebacteria also

possess eukaryote-like traits not found in eubacteria and these have been discussed by Gray and Doolittle (1982).

According to Grant and Larsen (1989), isolates of the Halobacteriaceae comprise essentially two groups:

1. Isolates that grow at, or close to, neutrality (pH 5-8) with Mg^+ requirement of at least 5mM. These include the genus *Halobacterium, Haloarcula, Haloferax* and *Halococcus*.
2. Haloalkaliphilic isolates that grow at high pH (8.5-11) with a very low Mg^+ requirement (<1mM). These include the genus *Natronobacterium* and *Natrococcus*.

In hypersaline natural waters, sodium, potassium, magnesium and calcium are the principal cations whereas chloride, sulphate and carbonates are the predominant anions. In most cases sodium and chloride predominates (Edgerton and Brimblecombe, 1981). Each halobacterial type has developed a mechanism to adapt or tolerate the ions concentrated in its own habitat (Cohen *et. al.*, 1983; Norton, 1992).

Halobacteria require at least 1.5M NaCl for growth, and for most strains the optimum is 3.5-4.5M NaCl (Norton, 1992). Sodium chloride protects the cells of halophilic bacteria from changes in osmotic pressure and prevents swelling, deformation, bursting and lysis as a result of osmotic shock (Mudryk and Donderski, 1991). In the absence of adequate salt, all except the coccal forms lyse immediately (Norton, 1992). In adapting to function in high intracellular salt concentrations, their enzymes and cell envelope proteins had lost the ability to function in low salt concentrations (Borowitzka, 1981).

Halobacteria show a typically aerobic chemoorganotrophic metabolism although some are also able to grow anaerobically fermenting amino acids or reducing nitrate (Hartmann *et al*., 1980; Javor, 1989; Rodriguez-Valera, 1991). Halobacteria have been reported to grow primarily on amino acids with only a limited ability to utilize carbohydrates, however, strains able to utilize a wide range of substrates including carbohydrates have been described (Gonzalez *et al*., 1978; Rodriguez-Valera *et al*., 1980; Rodriguez-Valera *et al*., 1983). Pure autotrophic growth has never been demonstrated but carbon dioxide fixation by anaplerotic reactions has been reported for bacteriorhodopsin-containing species (Javor, 1989).

Hypersaline environments are very stressful and the halobacteria have to cope with several stress factors including osmotic stress, low oxygen and periodic habitat disappearance (Norton, 1992). In environments up to 1.5M salt concentration, these microorganisms either use an ion-pumping mechanism to maintain low intracellular solute concentration or they accumulate organic osmotic solutes like glycine betaine or ectine to buffer the osmotic imbalance as occurs in the eubacteria. However, above this concentration these mechanisms are ineffective and energetically unfavourable (Dennis and Shimmin, 1997).

Extreme halophiles have adapted by balancing intracellular cation concentrations with the external environment by concentrating predominantly K^+ and extruding Na^+ ions. K^+ hydrates less water than Na^+ and intracellular K^+ may accumulate to concentrations of up to 5M (Norton, 1992).

Another mechanism of adaptation involves modification of soluble intracellular proteins and macromolecules. Proteins which contain more acidic amino acid residues are more hydrated than other amino acids, reduce protein hydrophobicity thereby preventing aggregation or structural

collapse and form salt bridges positioned such that they are protected from shielding by solute ions and therefore provide structural rigidity and stabilized three dimensional structure. Bacteriorhodopsin can absorb light and create a proton gradient which can be used to extrude excess Na^+ ions from the cell through a sodium-proton anti-port system (Norton, 1992) and this promotes passive accumulation of K^+.

Halobacterium have two alternative ways of producing adenosine triphosphate (ATP). Aerobically they respire using a respiratory chain as an electron acceptor. Under conditions of very low oxygen supply and illumination a purple membrane containing bacteriorhodopsin is synthesized (Norton, 1992). The retinal associated protein can use light energy to create a proton gradient thereby enabling the cell to produce ATP.

Other rhodopsin-like pigments, halorhodopsin and sensory rhodopsin occur (Norton, 1992). Halorhodopsin acts as a light-driven inwardly directed chloride pump to accumulate a high intracellular KCl concentration. Photophosphorylation may therefore be of ecological significance in the natural habitat of these organisms since oxygen solubility is low in saline waters and anaerobic conditions may often develop (Brock and Peterson, 1976). However, Oren (1990) has shown that light plays a minor role in energy production in solar salterns.

1.2.1.2.2 Anaerobes

Microbiological research has focussed on the aerobic microflora in hypersaline environments and relatively little is known about the anaerobic flora (Ollivier *et al.*, 1994). The bottom floor of shallow ponds often contains black mud beneath the mat of cyanobacteria. This indicates anaerobic

activity of sulphate reducing bacteria (Larsen, 1980). Photosynthetic sulphur bacteria also develop in sediments rich in hydrogen sulphide. Isolates belonging to the genera *Ectothiorhodospira* and the purple bacteria identified as *Chromatium spp*. have been reported (Borowitzka, 1981). Mass occurrence of these bacteria occur in strong brines of high alkalinity such as Wadi Natrun in Egypt (Larsen, 1980).

Sulphate reduction occurs in marine sediments where sulphur is not limiting (Ollivier *et al*., 1994). Although methanogens are inhibited by sulphate reduction products, particularly hydrogen sulphide, methanogenesis has also been reported in marine and brackish sediments (Mathrani and Boone, 1985; Oremland and Polcin, 1982; Sowers and Ferry, 1983; Winfrey and Ward, 1983; Giani *et al*., 1984; Zhilina, 1986; King, 1988; Zhilina and Zarvazin, 1990). Another group of anaerobic bacteria belonging to the family *Haloanaerobiaceae* which ferment carbohydrates, have been reported in hypersaline environments (Ollivier *et al*., 1994) and one isolate, *Haloanaerobacter chitinovorans,* has been isolated from a solar saltern (Liaw and Mah, 1992).

1.3 BIOLOGICAL MANAGEMENT OF SOLAR SALT-WORKS

For the effective operation of salt-works, a competent biological management of the saline evaporation ponding process is now known to be crucial (Davis, 1990). In the brine of any solar salt-works, living organisms constitute an ecosystem essential to salt production which may either aid or decrease the production and quality of the product (Davis,1990). Biological management for most solar salt-works involves solving problems caused by organisms living in the ponds (Davis, 1993).

The biota of the ponds is organized into the planktonic and benthic communities. In low salinity ponds, photosynthetic algae and bacteria produce organic fuel for the biota in these and the downstream ponds. As salinity increases, the brine ecosystem changes from a nutritionally independent biota to a nutritionally dependent system. In a balanced system, enough organic material is produced to maintain the biota and allow bottom communities to develop. Non-mucilage producing bacteria dominate and impart important dark colours to the brine, thereby aiding solar absorption. The bottom community maintains a desired thickness and helps to prevent brine leakage. The depth of the ponds also influences the formation of the bottom mats. If the ponds are too deep then light transmission to the pond bottom is limited resulting in poor development of the bottom mat. On the other hand, if the ponds are too shallow then the mat formed may increase sufficiently to decrease pond surface area and volume (Davis, 1990).

In an unbalanced system excess biological activity, caused by excess nutrients entering the system, may cause the dominance of the planktonic forms and dark brine colours which may limit the

growth of algal mat due to shading (Jones *et al.*, 1981; Davis, 1990; 1993). Also mucilage producing algae such as *Cladospora* and *Coccochloris* may dominate and decrease brine evaporation in the high salinity ponds and crystallisers. Sodium chloride crystal habit is affected with the formation of hollow hopper crystals which incorporate contaminants and reduced salt quality(Davis, 1980).

In the intermediate ponds brine shrimp occur and feed on the planktonic microorganisms including the mucilage producing algae, debris and minerals. Faeces are excreted in membrane-bound faecal pellets which deposit into the bottom mats. Accumulation of excess organic material which promote growth of mucilage-producing algae is prevented and mat formation serves to seal the ponds. Growth of these mats removes minerals from the water and photosynthetic activity serves to oxygenate the brine resulting in the oxidation of contaminating organic matter.

In the high salinity ponds fewer species occur. The main organisms in these ponds are the red halophilic bacteria and the salt tolerant *Dunaliella*. The planktonic animals such as the brine shrimp and the brine-fly larva disintegrate and the organic matter released may be used by the red halobacteria which colour the brine, thereby promoting solar evaporation.

1.4. SUA PAN SALT-WORKS

Botswana Ash, a salt and soda ash producing industry is situated at Sua Pan, north-east Botswana. Sua Pan is the most easterly of a group of large, flat unvegetated areas in the Makgadikgadi, a large basin of internal drainage in north-east Botswana (Figure 1.1) and is approximately 50km

from east to west, and 100km from north to south (Gould, 1986). Soda ash and salt produced at Sua Pan (Botswana) are derived from an ancient brine resource thought to have originated from the drying up of what was once the largest lake in Africa, Makgadikgadi. The Makgadikgadi lies at the centre of a basin of inland drainage and the lowest point is located at the north-east corner of Sua Pan, 890m above sea level. Geological fault shifts resulted in the separation of the Makgadikgadi and Okavango systems around 100 000 years ago due to a sequence of arid and pluvial periods (Gould, 1986). The arid periods with strong east-south-easterly winds caused the formation of an extensive field of dunes whereas the pluvial periods resulted in the superposition of stream channels, forming a large lake in the Makgadikgadi.

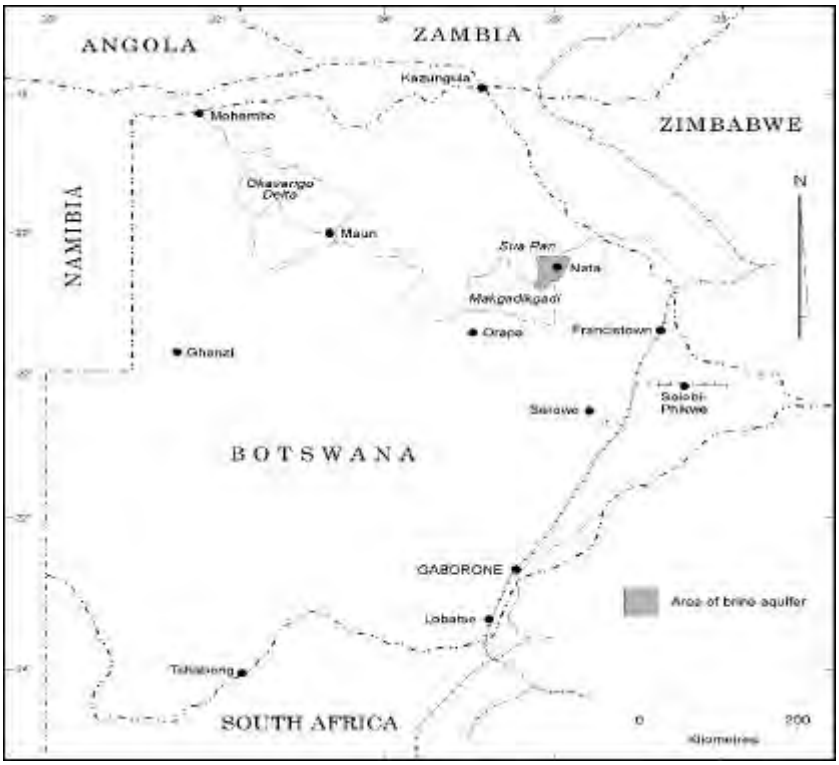

Figure 1.1. Map of Botswana showing the location of Makgadikgadi and Sua Pan (Gould, 1986).

1.5 HISTORY OF SUA PAN

The discovery of mineral deposits at Sua Pan began when salt deposits were found at the mouth of Nata river, the main river draining Sua Pan. This salt was exploited by the local inhabitants and was used for both domestic purposes and for livestock feeds. Analysis of the brines by Van Straten (1958) revealed the presence of sodium carbonate and bicarbonate as major components of the brine. Mc Carthy *et al*. (1986) have undertaken a detailed study of the mineral deposits in the Okavango Delta. These authors found that during evaporation of swamp water, efflorescent crusts consisting predominantly of sodium carbonate minerals, trona and thermonatrite, and with minor KCl are formed. Calcite crystals which are brought to the surface by ants for termitaria construction are also formed during evaporation. The composition of brines found at Sua Pan has been discussed by Gould (1986) who reported that brines taken from different parts of the pan are not identical but are similar in chemical composition as shown in Table 1.1.

Table 1.1. Chemical analyses of Sua Pan brines.

Chemical	Concentration ($g.l^{-1}$)
Na^+	67.4
K^+	2.25
Cl^-	79.74
CO_3^{2-}	13.05
HCO_3^-	7.0
SO_4^{2-}	9.0
NaCl	128.0
$NaCO_3$	23.05
$NaHCO_3$	9.64
$NaSO_4$	13.76
KCl	4.30

A major exploration around the northern part of Sua Pan began with drilling of boreholes and the construction of evaporation ponds to observe the behaviour of the brine on evaporation. This led to the construction of a larger evaporation pond to produce trona saturated brine for carbonation studies which were carried out at a pilot plant at Shashe.

In 1988 a decision was made to build a soda ash and salt plant at Sua Pan to exploit the natural deposits of the pan and by 1991 the plant was in operation under the name Soda Ash Botswana. The company was restructured in 1995 as Botswana Ash (Pty) Ltd with the Botswana Government as a major shareholder (50%). Anglo American Corporation, AECI and De Beers hold 42% and the remaining 8% is held by a bank consortium. More than 80% of soda ash produced at Sua Pan is consumed by the South African market (Kelly, 1990) and soda ash reserves at Sua Pan have been reported to be adequate to keep southern Africa self-sufficient for the next century (Vorster, 1990).

1.5.1 The Sua Pan Process

1.5.1.1 Well Field

Soda ash produced at Sua Pan involves the pumping of brine from wells which have been drilled into the aquifer system. The wellfield is situated in the northern part of Sua Pan and has an installed extraction capacity of $2400 m^3.h^{-1}$. It covers an area of approximately $200 km^2$ and comprises 98 production wells distributed between 58 pumping nodes. These wells have flow rates ranging between 6 and 20 litres per second (Taylor, 1991). Figure 1.2 shows the layout and location of the wellfield. Three different ORBIT borehole pump sizes have been installed into a delivery line of 20 kilometres to the solar evaporation ponds. Solar evaporation of the wellfield

brine referred to as well-brine occurs through a series of ponds after which the carbonate concentrate is pumped to the soda ash plant situated on the Sua Pan spit.

1.5.1.2 Evaporation Ponding System

The solar evaporation ponds are situated to the north of Sua spit and cover an area of 20km^2. The brine flows into a primary evaporation pond E0 which also operates as a cooling sump for the process plant. The warmed brine from the plant is pumped into E0 where it is mixed with influent brine and cooled before being emptied into the deep CS pond. The feed forward brine further evaporates through ponds E2, E3, E4, E5 and E6 to concentrate the soluble salts to a point where salt reaches its saturation point and begins to crystallize out of solution. At this point the brine is referred to as N-brine (from N in NaCl). N-brine is retained in the N-brine storage pond NS which feeds the crystalliser ponds (X1, X2, X3, X4). Deposition of salt occur in the crystalliser floors while further enrichment of the brine in other sodium salts occurs. Eventually a point is reached where it becomes necessary to pump the concentrated brine off to avoid excessive deposition of brine saturated secondary salts and thus contamination of the salt floor. The brine, at this point rich in sodium carbonate, is referred to as T-brine. T-brine is stored in two storage ponds ST1 and ST2. The average residence time for the brine through the ponds is 50 days with more than one million m^3 of brine inventory at any time. Figure 1.3 shows the arrangement of ponds. The area of exposure within the solar ponds can be reduced or enlarged by the controlled removal or addition of extra areas to accommodate seasonal changes in climate.

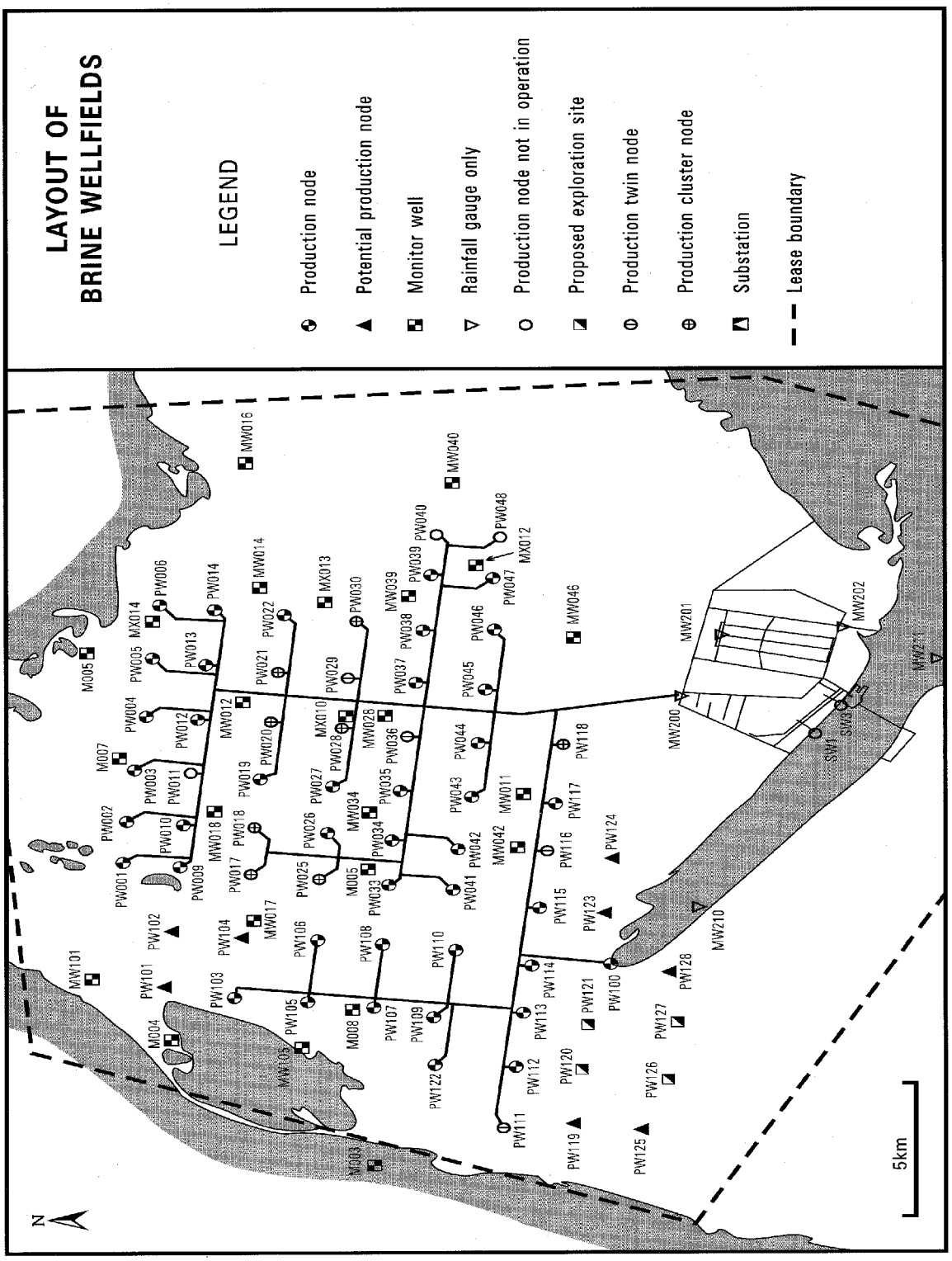

Figure 1.2. Diagram showing the layout of the wellfield.

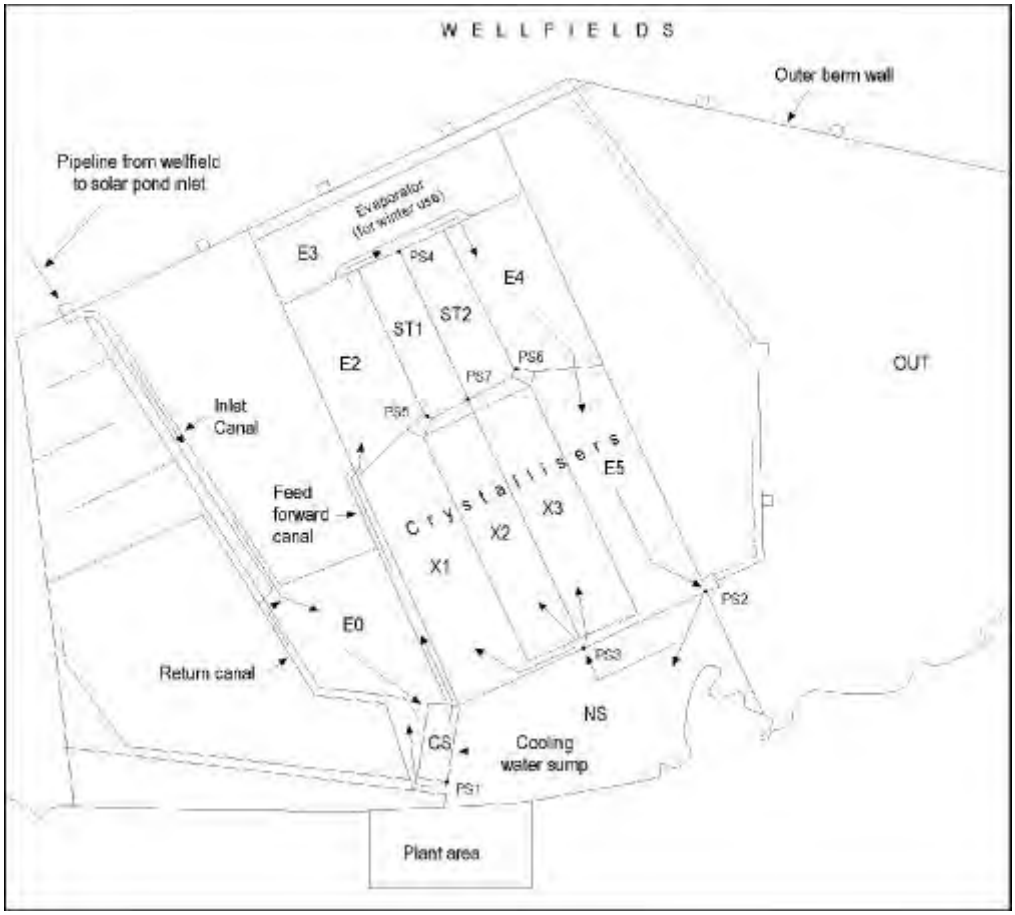

Figure 1.3. Arrangement of solar ponds.

1.5.1.3 Soda Ash Plant

The T-brine is the feed stock for the soda ash plant. To recover highly soluble sodium carbonate, the soda ash is carbonated to a sparingly soluble sodium bicarbonate, which is then purified and converted back to ash by heating at 220^0C (see chemical reactions). The ash if finally compacted into dense granules for easy handling. The flow diagram of the process is shown in Figure 1.4.

Na_2CO_3 (solution) + H_2O + CO_2 ÿ $2NaHCO_3$ + heat (1)

$2NaHCO_3$ + heat ÿ Na_2CO_3 + H_2O + CO_2 (2)

The services plant provides all of the energy, water, carbon dioxide (CO_2) and air requirements of the operation. Two fluidised bed coal-fired boilers produce the process steam requirements at a temperature of 470^0C and the steam is also converted into electricity. Flue gas obtained from the boilers is processed to produce relatively pure CO_2 for use by the soda ash plant. Flue gas contains about 10% (v/v) CO_2. 30% of this flue gas is scrubbed with a soda ash-rich brine solution to remove sulphur dioxide which reacts with monoethanolamine (MEA) used in the subsequent purification process. The scrubbed flue gas together with recycled carbonator off-gas is passed through a packed absorption column where it comes into contact with a solution containing 8-10% MEA. The CO_2 rich MEA solution is heated to 90^0C by counter-current heat exchange with the lean MEA solution flowing to the absorber. The hot MEA solution is pumped to the top of a column containing 20 trays where CO_2 is stripped by boiling the solution at 112^0C. The CO_2 leaving the stripper is cooled to condensate most of the water vapour, thus increasing the gas purity to about 96%. The gas is compressed and supplied to the process plant, excess CO_2 is liquefied to provide a buffer supply for plant start-up.

1.6 PROCESS PERFORMANCE

Soon after the commissioning of the soda ash plant, several problems were experienced, which, following a preliminary evaluation undertaken by Rhodes University, were identified as being caused by a complex interaction of biological, organic and physical factors (Rose, 1993). These problems included:

1. Fouling and blockage of well gravel packs resulting in reduced pumping capacity. This was attributed to aeration of brine during pumping which resulted in the precipitation of humic substances and bacterial growth in the gravel packs. This was overcome by reducing pumping rates and hence draw-down aeration of the brine in the well gravel packs.
2. Brine leakage from evaporation ponds. Inability to establish balanced biological systems in the ponds resulted in the inadequate development of a benthic community and mat formation on the bottom of the ponds.
3. Microbial growth in the ponds. The incoming well brine contained sufficient nutrients to support profuse blooms of *Dunaliella spp*. which were responsible for massive production of extracellular organic polymers.
4. Discolouration of the final product. Possible compounds included â-carotene produced by *Dunaliella spp*., bacterial pigments and polysaccharide.

Biological problems experienced resulted in poor salt production and contamination of soda ash. Hopper crystals and large accumulations of drift salt were observed which led to reduced evaporation rates, poor salt crystal quality and high levels of organics in the salt. High levels of halobacterial growth resulted in the production of red coloured salt. The discolouration of the soda ash product was thought to be due to a Maillard type reaction caused by heating of organic compounds during the calcining operation. These problems raised serious commercial issues for the company as the market requires high quality salt for the manufacture of chemicals and the colour in soda ash is undesirable in the detergent and glass industries.

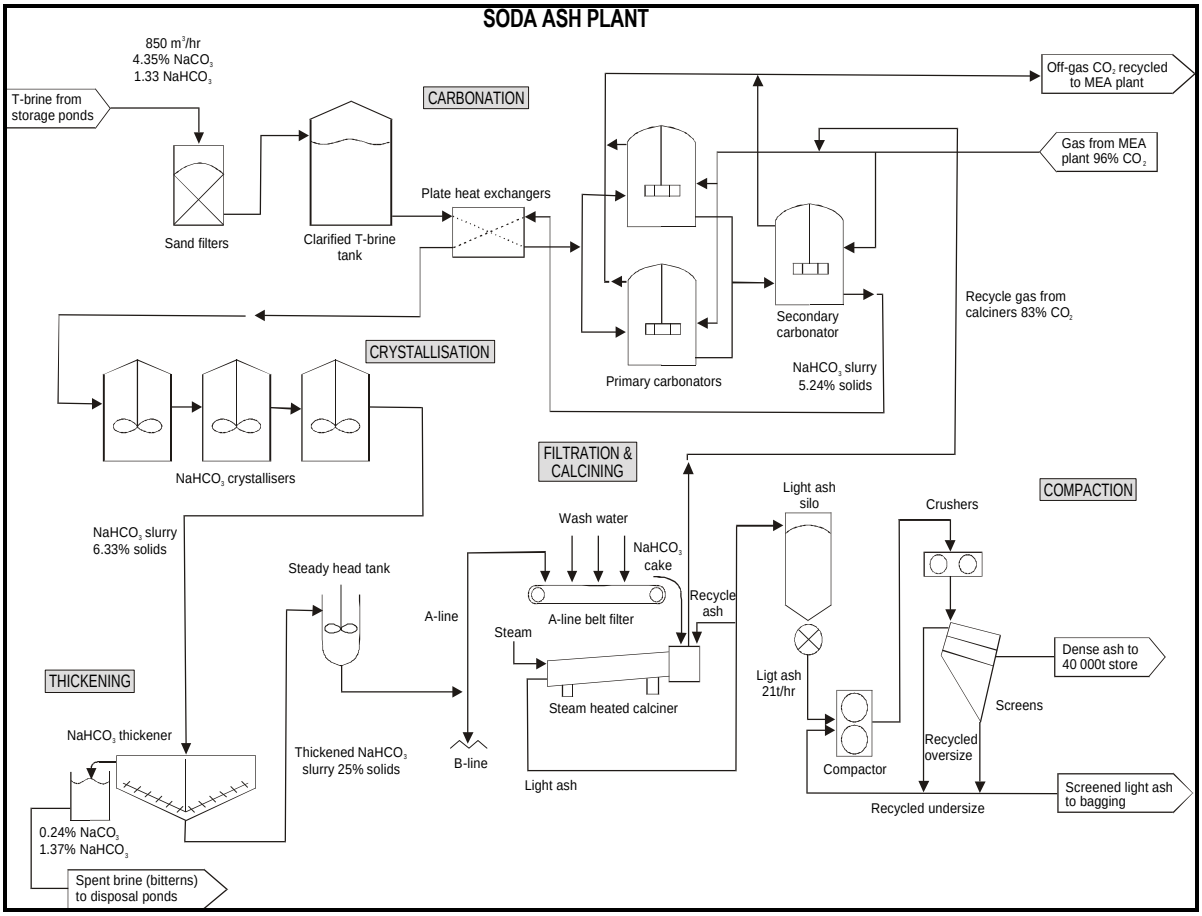

Figure 1.4. Flow diagram of the soda ash plant.

1.7 OBJECTIVES

This research project was undertaken to follow up on certain of the observations made in the original Rhodes University studies with the overall objective of conceptualising a remediation strategy and basic elements required for the development of a Biological Management Process for the soda ash production operation. The following specific objectives were identified.

, To establish the nature of the organic contaminants in the salt and soda ash products.

- To determine the origin of the individual components and their fate within the system.
- To determine whether either the algal or bacterial populations may be used in a biotechnological remediation process development exercise to remove inorganic nutrients and organic compounds from the brine.
- To evaluate ancillary physico-chemical approaches to the removal of organic compounds which could compliment biotechnological approaches to the problem.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF ORGANIC COMPOUNDS

2.1 INTRODUCTION

Organic contamination is a problem encountered in most natural soda ash. While the carbonation process appears to be the simplest method for recovering soda ash from complex brines, the sodium bicarbonate formed during carbonation is an excellent adsorbent for organic compounds (Garrett, 1995). The ash produced is usually grey in colour due to its high organic content and foaming may also occur due to the presence of organics (Garrett, 1995). Problems of this type have been reported, examples being at Searles Lake, Green Wyoming, Owens Lake and Sosa Texcoco, Mexico (Garrett, 1995). In Searles Lake, humic acids were found to be the major contributor to the organic contamination of the soda ash product.

Operation procedures usually employed to deal with the organic contamination of soda ash have been reviewed by Garrett (1995). Calcining temperatures above 400^0C, in the presence or absence of an oxidising agent such as sodium nitrate, are used for the removal of organics in the final soda ash product. Considerable organic matter is burned off at temperatures between 350^0C and 400^0C and a white ash is produced. On the other hand, the formation of sodium silicate becomes a problem at high temperatures, requiring a careful balance to be maintained between desirable removal of organics and the addition of soluble silicates and organics. Chemicals such as bauxite, magnesia, sodium aluminate and aluminium hydroxide have been used to avoid silicate formation.

Addition of oxidising agents such as chlorine and peroxides to the brine may be limited by economic constraints. Activated carbon has also been used to treat organic-contaminated liquor where soda ash is leached from trona.

2.1.1 Organic contamination of Sua Pan soda ash

The alkaline well-brine, the raw material for the soda ash produced at Sua Pan is yellowish in colour and has total organic carbon (TOC) levels of 150 ppm. The brines contain dissolved hydrogen sulphide which is associated with biologically derived carbonate deposits. Routine TOC measurements in solar ponds, however, also shows accumulation of organic carbon during brine concentration. This occurs as a result of evaporation but microbial growth observed in the evaporation ponds also contributes to the organic load of the brine (Rose, 1998a). Despite these contributions by the humic substances and microbial production to the organic load of brine, Figure 2.1 shows that the TOC levels measured in the feed-stock T-brine are consistently lower than would be anticipated by the evaporation concentration factor applied to the well-brine TOC only. This indicates that a complex interaction of organic matter production, degradation and consumption occurs during the brine concentration process. Although some organic components undergo degradation as the brine proceeds through the ponding system, there are certain components which probably remain unaltered and concentrate up during evaporation, finally reporting to the soda ash product.

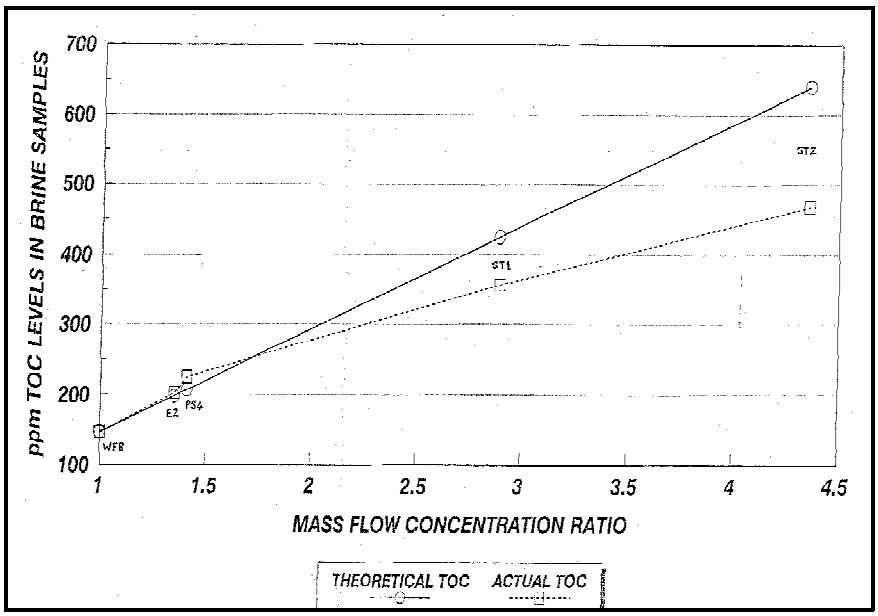

Figure 2.1. Comparison of actual and theoretical TOC levels in brine through the solar pond system. Theoretical TOC represents well-brine TOC multiplied by a concentration factor obtained by following the NaCl concentration throughout the ponds.

2.1.2 Humic substances

Humic compounds are associated with many soda ash deposits. They are refractory organic polymers of high molecular weight, naturally occurring in soils and waters with a characteristic yellow to black colour (Hayes and Swift, 1978; Aiken *et al*., 1985; Hayes *et al*., 1989). Humic substances may be formed during microbial and chemical transformation of organic matter or when small organic chemicals released during metabolic processes interact to form macromolecules. These compounds are, therefore, found wherever there is organic matter being decomposed such as in soils, sediments, and waters (Aiken *et al*., 1985; Hayes *et al*., 1989). Although humic substances are not generally considered harmful to health, they can present problems in potable

waters by complexing with heavy metals and solubilizing them, and they can also act as carriers for anthropogenic chemicals (Aiken *et al*., 1985).

Humic substances are generally classified into humic acids, fulvic acids and humin based on their solubility. Humic acids are insoluble below pH 2, fulvic acids are soluble at all pH values and humins are insoluble at all pH values (Hayes *et al*., 1989).

2.1.3 Isolation of aquatic humic substances

It is important when isolating aquatic humic substances to employ a variety of methods to yield a high quality product free of inorganic salts and low molecular weight organic acids, and in a form that will resist degradation (Aiken *et. al*.,1985). The extraction scheme normally involves the following steps:

1. Filtration

This separates dissolved from particulate organic carbon and colloidal clays which interact with humic substances.

2. Concentration

Numerous concentration methods are available and include vacuum distillation, freeze-drying, ultrafiltration, reverse osmosis, solvent extraction and, sorption methods.

3. Isolation

This step allows isolation of humic substances from inorganic salts and other organic solutes. Most methods which have been used for isolation of humic substances involves the use of adsorption and ion-exchange chromatography.

4. Preservation

Freeze-drying yields stable, easy to handle product with good properties and is a gentle method for concentrating humic substances. However, it is not suitable for preserving large volumes and all solutes except volatile organics are also concentrated, therefore, necessitating further isolation from freeze-dried residue which is difficult.

Humic substances can also be isolated by solvent extraction. The solvent used should have the following properties: a high polarity, ability to disrupt existing hydrogen bonds and provide alternative groups to form humic-solvent hydrogen bonds, a small molecular size to penetrate through the polymers, and ability to immobilize metallic cations.

Each fraction of humic or fulvic acid or humin consists of a very complicated and heterogeneous mixture of organic compounds and even after fractionation, the fractions are very heterogeneous (Hayes, 1985). This makes molecular structural determination difficult and, therefore, information regarding molecular structure of these compounds is limited. However, spectroscopic procedures; infra-red (IR) and nuclear magnetic resonance (NMR) can be used to determine the functional groups of these compounds.

2.1.4 Objectives

This study was undertaken to isolate and characterize organic compounds contaminating the brine and, therefore, also possibly the soda ash produced at Sua Pan. The investigation was confined to:

(a) Well-brine (the raw material and hence a potential source of contamination).

(b) T-brine (soda ash plant feed).

(c) Bicarbonate filter cake (product).

The final soda ash product was excluded since the high temperatures of calcining would be expected to alter the nature of organic compounds.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of Humic compounds

2.2.1.1 Freeze-dried extracts

To isolate organic compounds from brine samples (well-brine and T-brine), 100ml of the brine sample was dialysed by Pleated SnakeSkin dialysis tubing (molecular weight cut off (MWCO) of 3500D) (Pierce, Ilinois, U.S.A.) against Milli-Q water in a beaker stirred by a magnetic stirrer for two days to remove the salts. The water was changed twice daily and the solution monitored to prevent bursting of the tube. The dialysate was pooled and transferred to a round-bottomed flask and concentrated on a rotary evaporator (J. Bibby Science products, Staffordshire, UK). The temperature of the water was kept at 40^0C and below to avoid any modification of the organic compounds which might occur due to high temperatures. The concentrate was freeze-dried in an Edwards freeze dryer (Edwards, Sussex, UK).

The bicarbonate filter cake (100g) was mixed with deionised water to make a slurry which was then transferred to a dialysis tubing (MWCO 3500D) and also dialysed against Milli-Q water for two days. The dialysate was concentrated on a rotary evaporator and then freeze-dried.

2.2.1.2 Organic solvent extracts

2.2.1.2.1 Chloroform extracts

100ml of alkaline well-brine was extracted with an equal volume of chloroform (BDH Laboratory Supplies, Poole, UK) in a separating funnel (250ml). The solutions were left on an orbital shaker in conical flasks and gently shaken for about six hours at room temperature before being transferred into a separating funnel. The flasks were covered with aluminium foil to prevent solvent evaporation. It was important to avoid vigorous shaking as this caused an emulsion to form. Once in the separating funnel, the chloroform layer (bottom layer) was collected, dried in anhydrous $MgSO_4$ (Saarchem, Krugersdorp, South Africa) for approximately an hour and filtered through fluted Whatman No.1 filter paper (Whatman, Maidstone, UK). The filtrate was concentrated to about 2 ml in a rotary evaporator, keeping the temperature of the water below 40^0C.

Acidified extracts were prepared by acidifying 100ml of brine to pH 1 (Cyberscan 2000 pH meter, Wirsam Scientific) with nitric acid (BDH Laboratory Supplies, Poole, UK) and extracted with an equal volume of chloroform in a similar way to the alkaline extracts above. Acidification caused considerable foaming and the brine was allowed to settle before extracting into chloroform.

2.2.1.2.2 Ethyl acetate extracts

One litre of alkaline well-brine was extracted with an equal volume of ethyl acetate (BDH Laboratory Supplies, Poole, UK) as described. After concentration of the sample by rotary evaporation, the round-bottomed flask was fitted with a glass tap which was connected to a vacuum pump (Busch, Germany). The first trap of the vacuum pump was put in an ice bath to avoid the solvent being sucked into the pump. The tap was opened and the solvent evaporated by

vacuum. For acidified extracts, one litre of brine was acidified to pH 1 before extraction by ethyl acetate. Yellow extracts were obtained with acidified brine and an oily residue was formed after the solvent was evaporated.

2.2.2 Analyses

2.2.2.1 ^{1}H NMR Spectroscopy

Freeze-dried extracts were dissolved in D_2O (Sigma Chemical Co., St. Louis, Mo, U.S.A.) and ^{1}H NMR analyses done in a 400mHz AMX NMR spectrometer (Bruker, Germany) . Ethyl acetate extracts were dissolved in deuterated acetone (acetone-*d*) (Sigma Chemical Co., St. Louis, Mo, USA.) before analysis by NMR spectrometer.

2.2.2.2 Infra-red spectrophotometry

About 4mg of the freeze-dried extract was ground with Nujol (mulling agent) and the sample applied to NaCl discs. The sample was scanned in the MidIR region using a FTIR (Spectrum 2000) spectrophotometer (Perkin Elmer). Air was used as a background scan. Chloroform extracts were analysed in solution using dry chloroform as the background scan. These samples were applied to a KBr cell using a pasteur pipette and scanned.

2.2.2.3 Ultra-Violet spectrophotometry

The absorption spectrum of chloroform extracts was recorded in the UV region (200-400 nm) using a Shimadzu UV-160A spectrophotometer (Shimadzu, Japan). The samples were scanned in quartz cuvettes using dry chloroform as a blank. Samples were diluted so that the maximum absorption did not exceed 1.0.

2.2.2.4 Mass spectroscopy

The freeze-dried well-brine and bicarbonate extracts were sent to Stellenbosch University for analysis by Electrospray Mass Spectrometry (EMS). Samples were analysed in acetonitrile:water (50:50) containing 1% formic acid.

2.2.2.5 Carbohydrate assay

The phenol-sulphuric acid method was used for determining carbohydrate concentration in the samples. 1ml of 5% phenol (Saarchem- Krugersdorp, South Africa) and 5ml concentrated sulphuric acid (BDH Laboratory Supplies- Poole, UK) were added to 1 ml of sample solutions in 10 ml Pyrex test tubes and the samples left to stand for 10 minutes for colour development. Preparation of solutions is given in Appendix I. The colour intensity was determined spectrophotometrically at 488 nm using a Shimadzu spectrophotometer and concentrations calculated referring to the standard curve prepared between $10\mu g.ml^{-1}$-$100\mu g.ml^{-1}$ (Appendix II).

2.2.2.6 Protein assay

The Lowry method was used for determining the protein concentration in the samples. A standard curve was prepared using bovine serum albumin (BSA) (Boehringer Manheim, Germany) as described in Appendix II.

2.3 RESULTS AND DISCUSSION

During sample preparation of dialysed extracts, bicarbonate dialysates were found to contain a substantial amount of insoluble material which settled out rapidly. The soluble and insoluble fractions were separated by centrifugation and the pellet obtained re-suspended in deionized water before determining the mass balance by freeze-drying a known volume of a fraction in a glass vial of predetermined mass. The results are shown in Table 2.1.

Table 2.1. Mass balance determination in different fractions of the bicarbonate cake dialysate.

Sample	Solid Mass ($mg.ml^{-1}$)
Total dialysate	1.4
Supernatant	1.2
Pellet	0.1

2.3.1 Carbohydrate and Protein determination

Total carbohydrate and protein determinations in dialysates of T-brine and bicarbonate filter cake are shown in Table 2.2. A considerable amount of carbohydrate was measured in both T-brine and bicarbonate cake with the latter having a higher concentration, and only trace amounts of protein were measured in both samples. Carbohydrate concentration was also determined in different fractions of the bicarbonate dialysate. Due to the rapid separation of the soluble and insoluble fractions of the dialysate, accurate determination could only be achieved in the supernatant and pellet fractions, not the total dialysate (Table 2.3). Despite these erroneous results, there was a

clear indication that soluble carbohydrates constituted the largest fraction of the carbohydrate concentration in the bicarbonate cake.

Table 2.2. Results showing carbohydrate and protein concentrations of T-brine and bicarbonate filter cake dialysates.

Sample	Concentration (Fg.ml^{-1})	
	Carbohydrate	Protein
T-brine	53	2
Bicarbonate	114	1.78

Table 2.3. Carbohydrate determination in different fractions of the bicarbonate cake dialysate.

Sample	Carbohydrate (Fg.ml^{-1})
Total dialysate	114
Supernatant	111
Pellet	26

Concentration by evaporation may have resulted in high carbohydrate concentrations measured in the bicarbonate extract. On the other hand, this possibly indicated the production of organic matter of carbohydrate nature in the ponds. Production of extracellular polysaccharides by algae is a well documented phenomenon and, considering the massive blooms of *Dunaliella spp*. that occur in the evaporation ponds, the occurrence of these compounds is to be anticipated.

2.3.2 Humic Compounds

The presence of humic substances in both freeze-dried and solvent extracts was determined by spectral analysis; UV, IR, NMR and MS. Freeze-drying had the disadvantage of producing a product which could not be resolubilized. Sampling was done such that organic contamination could be followed from the source, through the ponds and to the final product.

2.3.2.1 Fractionation based on solubility differences

Fractionation of humic substances is essentially based on differences in solubilities, under acidic and alkaline conditions, of humic acids, fulvic acids and humin. Humin is insoluble at all pH values and therefore acidification of the samples in this study, was aimed at the fractionation of humic acids from fulvic acids. Humic acids are precipitated out in acid solution (Hayes *et al*., 1989), however, there was no precipitation observed when the samples were acidified. A greater proportion of extract was obtained in acidified extracts of ethyl acetate and the extracts obtained were yellow compared to the colourless alkaline extracts. This was a clear indication that the fulvic acids which are soluble at all pH values, constituted the major proportion of the humic substances in brine samples.

Accumulation of masses of a black precipitate, resulting in well blockage, hence reduced brine output, was one of the problems identified earlier at Sua Pan by Rose (1993). It was proposed that brine foaming caused precipitation of humic substances with iron (Fe) into a tar-like humic-iron chelate. Masses of this precipitate have also been observed in the channel feeding the evaporators. It was speculated that by the time the brine reaches the primary evaporators, almost all the humic

acids have been precipitated out, explaining why no precipitation of humic acid components was achieved during acidification.

2.3.2.2 Infra-Red Spectrophotometry

Samples were analysed by IR to determine the nature of functional groups and to characterize the organic compounds isolated. For dialysed extracts, well-brine and bicarbonate were analysed but, due to the impracticality of extracting bicarbonate, well-brine and T-brine solvent extracts were analysed. Similar spectra, implicating the presence of similar functional groups, were obtained for both well-brine and bicarbonate freeze-dried extracts (Figures 2.2 and 2.3 respectively). Absorption bands in the region 1250-1000 cm^{-1} may indicate C-O stretching bands in phenols or in esters, but due to the appearance of two bands in this region, the bands were interpreted as the presence of an ester group.

Figures 2.4 and 2.5 show IR spectra of chloroform extracts of well-brine and T-brine respectively. Similar functional groups were also observed in both well-brine and T-brine extracts. Although dialysed and solvent extracts gave different spectra, the same type of functional groups were implicated in both extracts as shown in interpretation Tables 2.4 to 2.6. A greater proportion of extract was obtained by solvent extraction and peaks observed had greater intensities compared to dialysed extracts. The IR spectrum recorded for the chloroform was a typical infra-red spectrum that has been described by Hayes and Swift (1978) for humic substances.

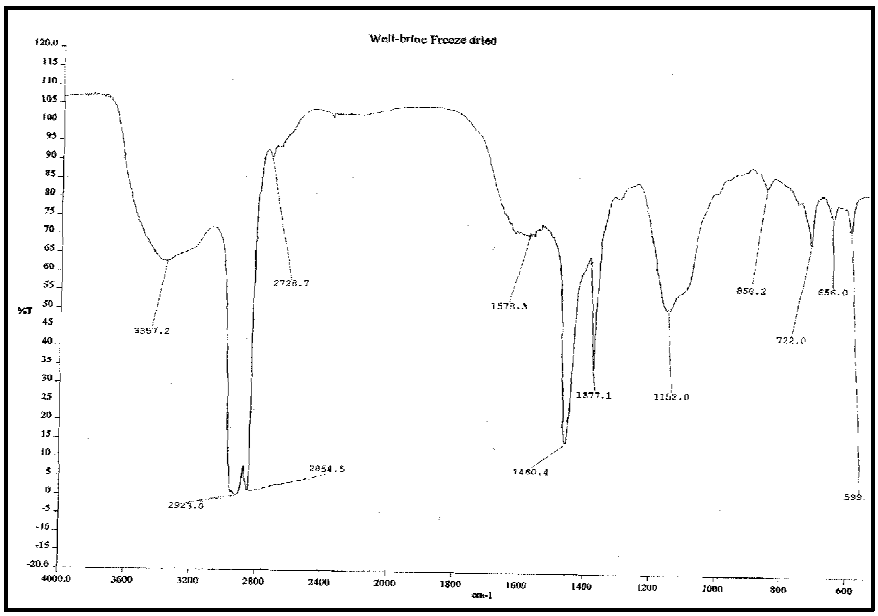

Figure 2.2. IR spectrum of well-brine freeze-dried extract. The sample was prepared in Nujol mulling agent and scanned in the Mid-IR region (600-4000cm^{-1}).

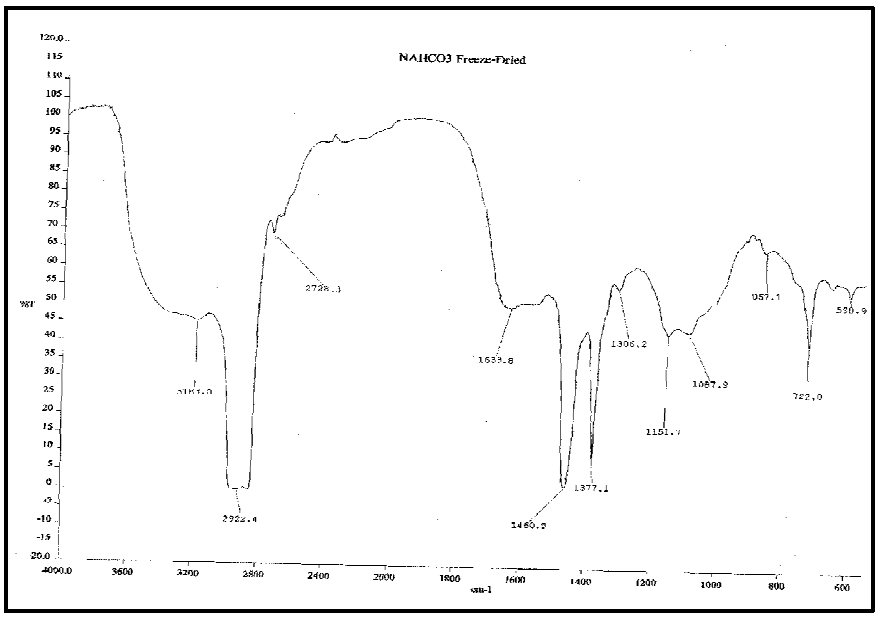

Figure 2.3. IR spectrum of the bicarbonate cake freeze-dried extract. The sample was prepared in Nujol mulling agent and scanned in the Mid-IR region (600-4000cm^{-1}).

Table 2.4. Interpretation of IR spectrum of well-brine extract.

IR Absorption (nm)	Implication
3357.2	O-H
2923 2854	Nujol
2728.7	C-H
1578.3	Aromatic (C=C)
1460.4 1377.1	Nujol
1306	C-N
1152	Ester group
858 722 656	Aromatic

Table 2.5. Interpretation of IR spectrum of bicarbonate extract.

Absorption (nm)	Implication
3183	O-H
2922	Nujol
2728	C-H
1633.8	Aromatic (C=C)
1460.9 1377.1	Nujol
1306.2	C-N
1151.7 1087.9	Ester group
857.1 722 656	Aromatic

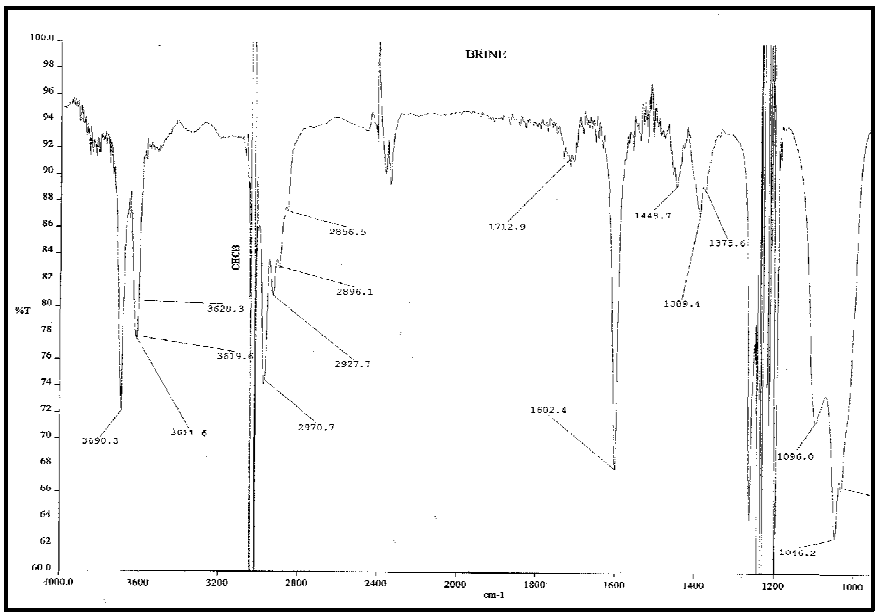

Figure 2.4. IR spectrum of chloroform extract of alkaline well-brine. Liquid samples in chloroform were scanned in the Mid-IR region using chloroform as the background scan.

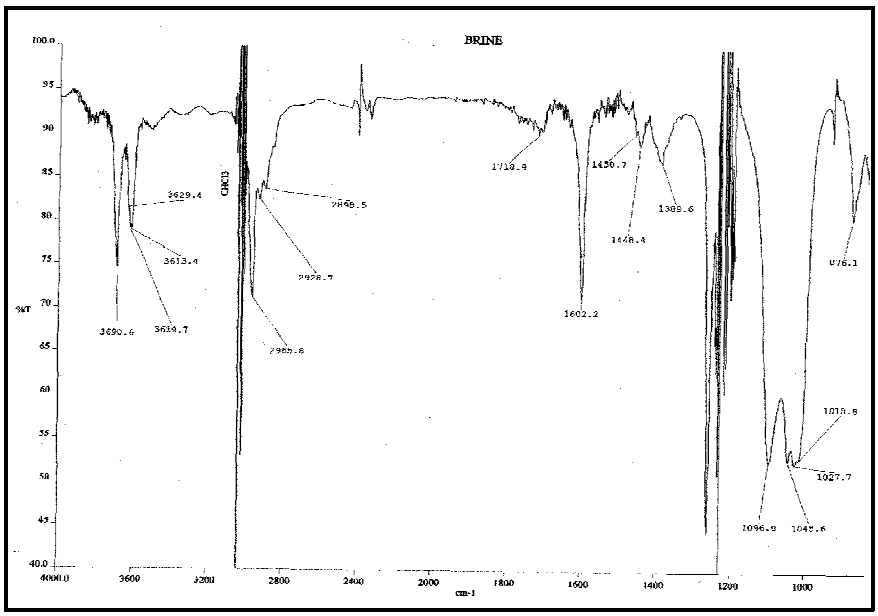

Figure 2.5. IR spectrum of chloroform extract of alkaline T-brine. Liquid samples in chloroform were scanned in the Mid-IR region using chloroform as the background scan.

Table 2.6. Interpretation of IR spectra of well-brine and

T-brine chloroform extracts.

IR absorption (nm)	Implication
3690 3611	OH
2970 2927.7 2899.1 2856.5	Aliphatic C-H
1712.9	Carboxyl and carbonyl C=O
1602.4	Aromatic
1389	C-N

It was interesting to note that the intensity of the absorption peak indicating the presence of aromatic components was less in the T-brine extract compared to well-brine (Figures 2.4 and 2.5). An increase in concentrations of the organic compounds should be expected in T-brine due to the evaporation concentration factor. It is therefore possible that a fraction of the aromatic component is degraded in the ponding system. Although the information derived from these analyses was essentially compositional rather than structural, the presence of the same functional groups in well-brine, T-brine and bicarbonate may have indicated a carryover of some organic compounds from the incoming well-brine through the system to the bicarbonate product.

2.3.2.3 UV Spectrophotometry

Chloroform extracts were scanned in the UV/Visible region and ëmax of the spectra recorded (Table 2.7). The absorption bands indicated the presence of an aromatic chromophore and the difference in ëmax between the extracts may have implied variation on the extent of alkylation on the aromatic rings (Brown *et al*., 1988). Humic substances contain phenolic and other aromatic functional groups which act as chromophores in the UV/VIS region (Hayes and Swift, 1978). It

is possible, however, that the presence of other chromophores may have been masked by overlapping of absorption bands.

Table 2.7. UV spectroscopic analysis of chloroform extracts.

Sample	Peak (nm)	Implication
Alkaline well-brine	266	Aromatic
Alkaline T-brine	262	

2.3.2.4 ^{1}H NMR Spectroscopy

Proton NMR spectra of freeze-dried extracts are shown in Figures 2.6 to 2.9. The freeze-dried extracts had very low solubility in D_2O and the spectra consisted of broader lines which are characteristic of solid samples. The spectra were interpreted by dividing each spectrum into regions as outlined in Table 2.8. The interpretations have been influenced by Wershaw's (1985) summary of chemical shift assignments for underivatized humic substances and the work of Norwood and Christman (1987).

Table 2.8. ^{1}H NMR chemical shift regions used for interpretation of NMR spectra.

Region	Proton type
1 - 2 ppm	Aliphatic protons
2 - 3.2 ppm	Aliphatic protons on carbons á to aromatic rings or carbonyl groups
3.5 - 4.2 ppm	Methoxyl protons (protons on carbons á to oxygen)
6.5 - 8.5 ppm	Aromatic protons

The spectrum of the well-brine freeze-dried extract (Figure 2.6) had bands in the regions 1-3 ppm, 3.5 - 4 ppm and a single sharp band at approximately 8.5 ppm. According to the above table, this indicated the presence of aliphatic protons, including protons on carbons á to aromatic rings and carbonyl groups, methoxyl and aromatic protons respectively. An intense DOH band was observed around 5 ppm.

A similar spectrum for the bicarbonate extract (Figure 2.8) was observed. However, the bicarbonate extract constituted a higher proportion of aliphatic, methoxyl and aromatic protons compared to the well-brine extract. This may have indicated that there were compounds present in bicarbonate, which were not present in well-brine. The presence of a higher proportion of aromatic protons in the bicarbonate extract, may, on the other hand have implied that aromatic rings of humic compounds in bicarbonate were less substituted compared to the aromatic rings in well-brine (Norwood and Christman, 1987).

An interesting observation made was that the same type of bands were observed in the region 3.5 -3.9 ppm in both well-brine and bicarbonate extracts (Figures 2.7 and 2.9). This may be an indication of the presence of a similar component in both well-brine and bicarbonate.

Generally ^{1}H NMR spectra of solvent extracts gave a better resolution and a higher proportion of aromatic protons was observed (Figures 2.10 to 2.13) compared to freeze-dried extracts. It should be noted that larger volumes of brine samples were extracted in this case. Well-brine spectrum (Figure 2.10) showed bands in the regions of aliphatic, methoxyl and aromatic protons whereas no aliphatic and methoxyl protons were observed in the T-brine extract (Figure 2.12).

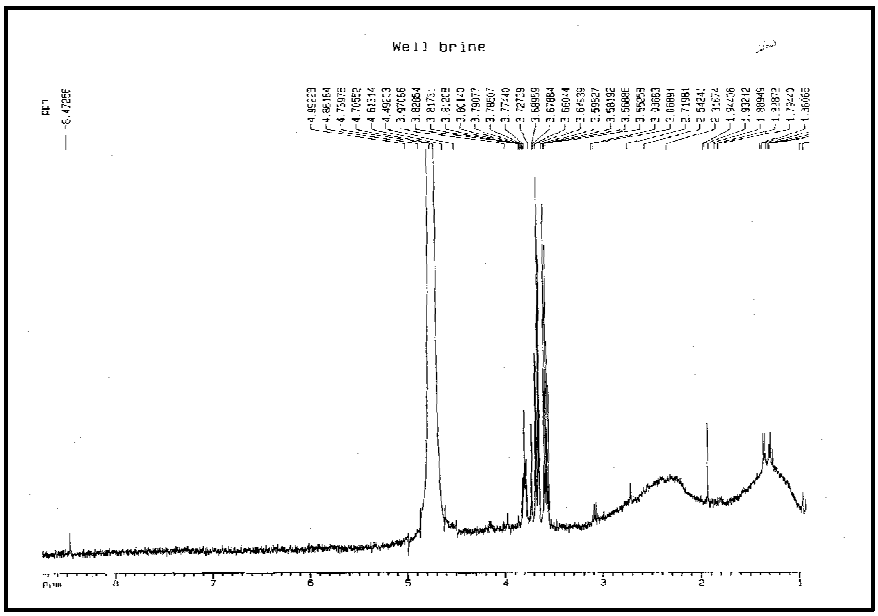

Figure 2.6. ^{1}H NMR spectrum of well-brine freeze-dried extract. Samples were dissolved in D_2O and scanned.

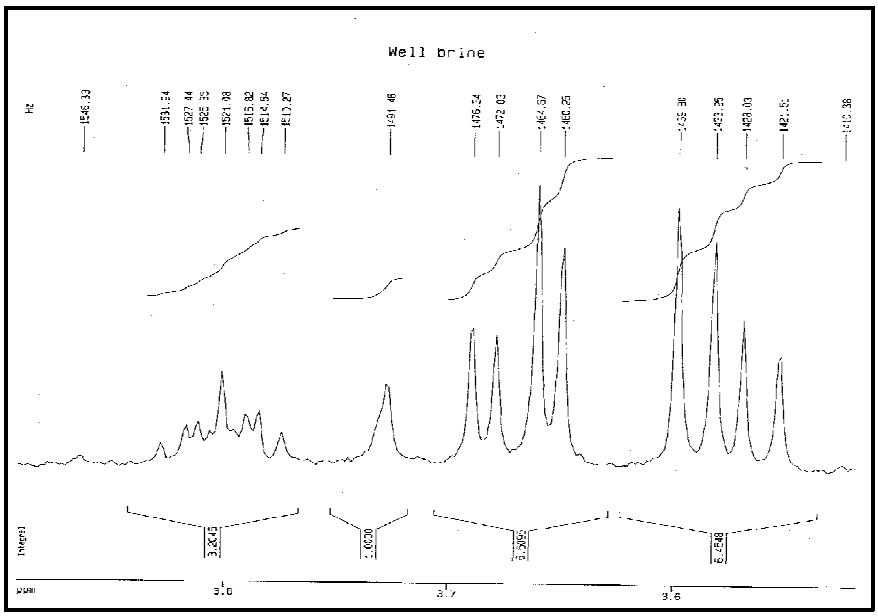

Figure 2.7. ^{1}H NMR spectrum of well-brine freeze-dried extract. Peaks in the region 3.5-3.9 ppm were expanded.

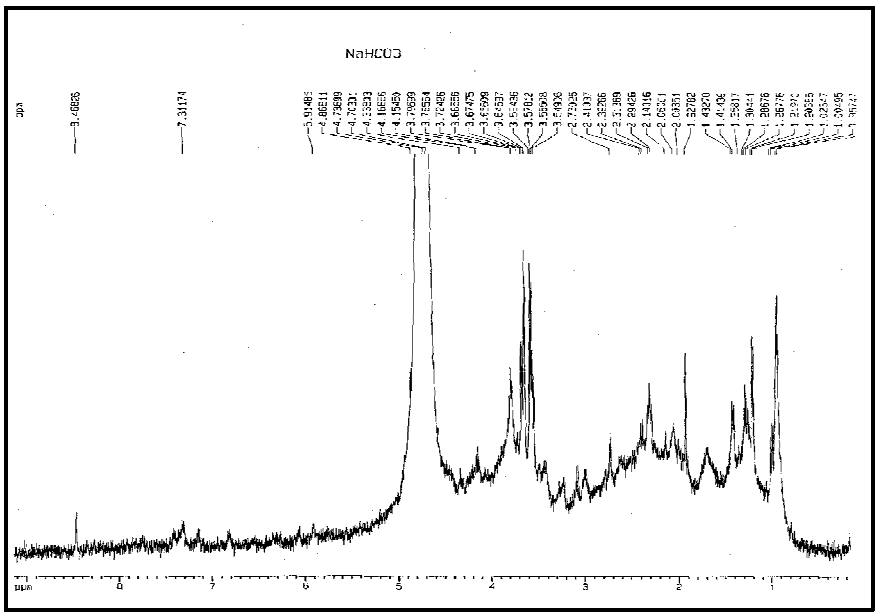

Figure 2.8. ^{1}H NMR spectrum of bicarbonate cake freeze-dried extract. Sample was dissolved in D_2O.

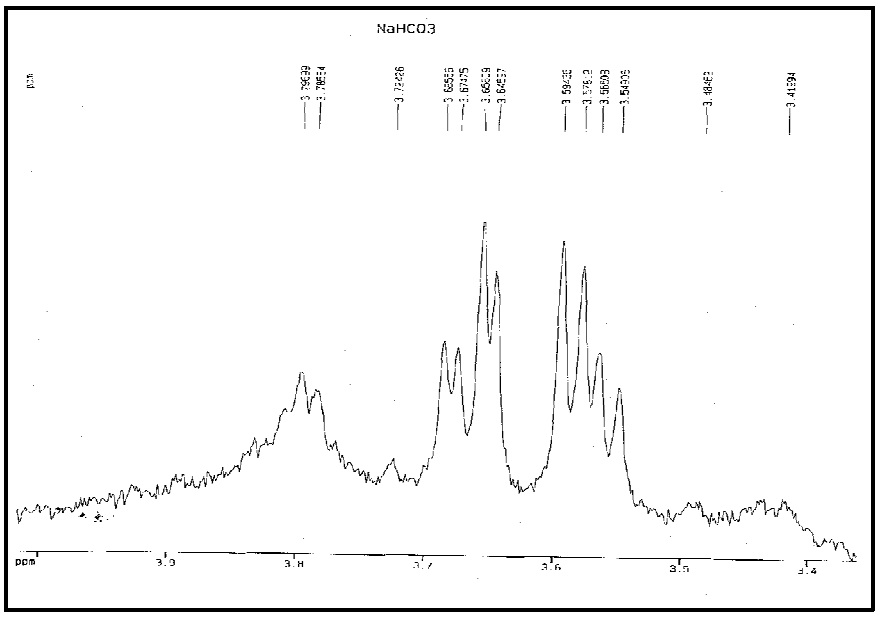

Figure 2.9. ^{1}H NMR spectrum of bicarbonate cake freeze-dried extract. Peaks in the region 3.5-3.9 ppm were expanded.

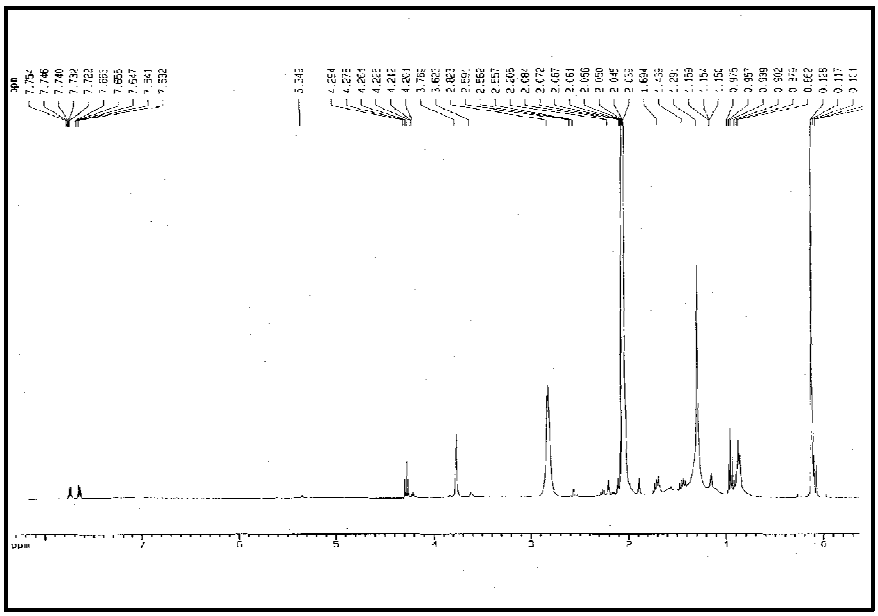

Figure 2.10. ^{1}H NMR spectrum of ethyl acetate extract of alkaline well-brine dissolved in deuterated acetone.

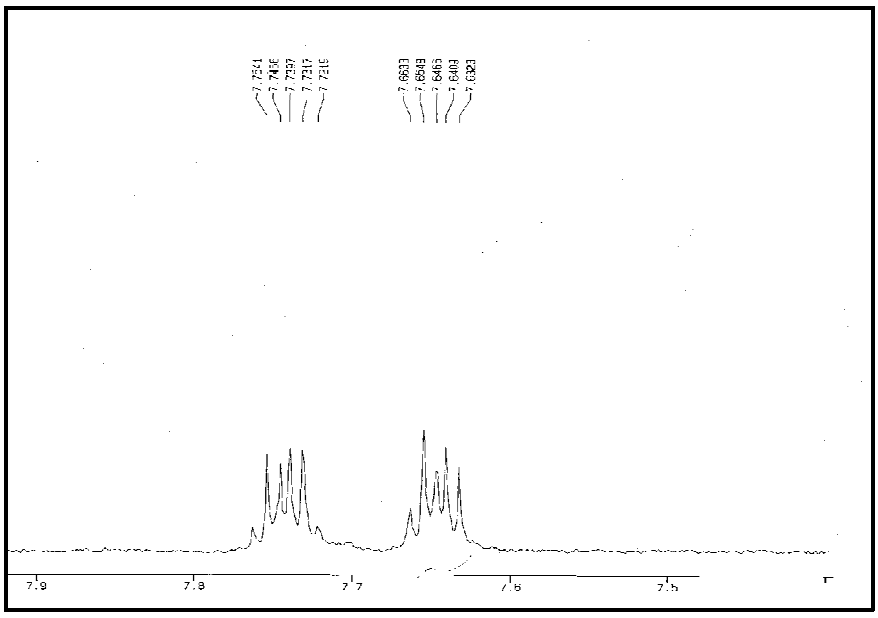

Figure 2.11. ^{1}H NMR spectrum of ethyl acetate extract of alkaline well-brine. Peaks in the region 7.6-7.8 were expanded.

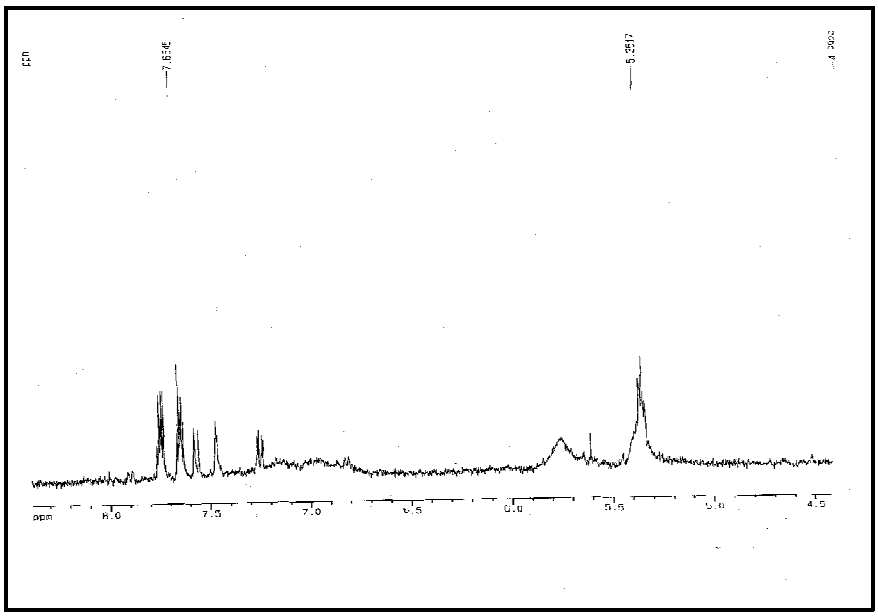

Figure 2.12. ^{1}H NMR spectrum of ethyl acetate extract of T-brine dissolved in deuterated acetone.

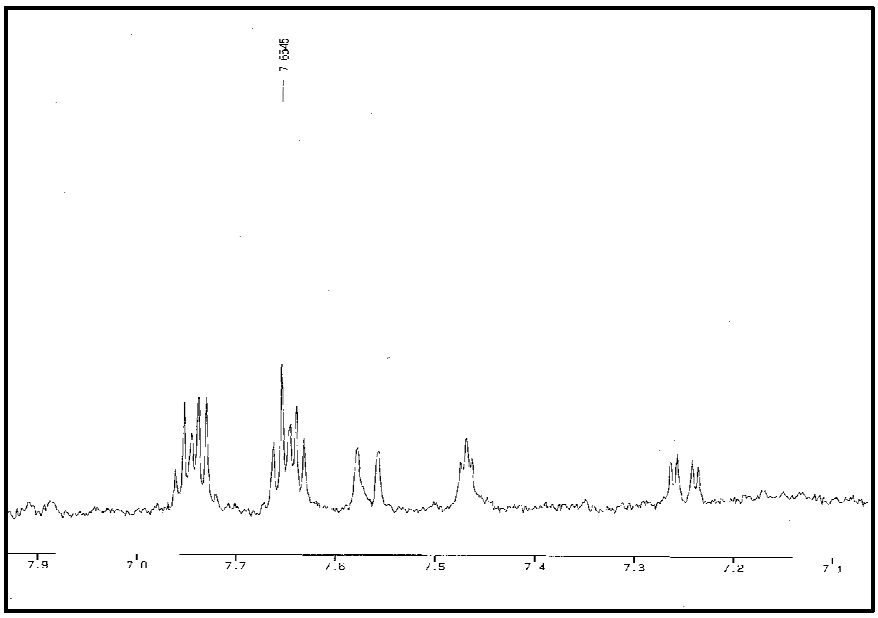

Figure 2.13. ^{1}H NMR spectrum of ethyl acetate extract of T-brine. Peaks in the region 7.2-7.8 were expanded.

Bands were observed in the regions 5.2 - 5.9 ppm and 6.5 - 8 ppm in the T-brine extract. According to Wershaw (1985), bands in the region 4 - 5.5 ppm can be assigned to exchangeable protons but Brown *et al*. (1988) assigned these to alkene protons.

The results obtained were comparable to the spectra that have been described for fulvic acids (Wershaw, 1985; Norwood and Christman, 1987). No fractionation of extracts into humic and fulvic acids was achieved through acidification. Ion- exchange resins are widely used to achieve this fractionation, however, these were not applicable in this case due to the high salt content of the brine samples since the resin would be forced to take up the unwanted ions instead of the organic material.

The results shown by proton NMR spectroscopy were also compositional rather than structural and further work is required for identification purposes. The use of phenolic standards such as lignin, a precursor for humic substances, and sample derivatization coupled with sample degradation would be required. Nonetheless, the results confirm the findings of IR and UV spectroscopy that well-brine contains aliphatic and aromatic compounds in a mixture of fulvic acids and a certain proportion of these compounds are carried over through the system to T-brine, finally reporting to the bicarbonate, and the soda ash product.

A complex interaction of production and consumption of organic compounds, which has been observed in the system, could also be reflected by NMR results due to the variation in the proportions of certain protons between samples of well-brine, T-brine and bicarbonate.

2.3.2.5 Mass Spectrometry

MS analysis of the freeze-dried extracts was done by the University of Stellenbosch and the results are shown in Figure 2.14. Sample D was the spectrum of well-brine and sample B, bicarbonate. The fragment masses obtained in both spectra are presented in Tables 2.9 to 2.11 and in each case the relative proportion of each peak to the base peak (most intense peak, assigned a value of 100%), indicated.

The most prominent peaks (mass 118.21, 260.01, 401.76 and 543.62) were further fragmented and the spectra are shown in Figure 2.15.

Table 2.9. Presentation of MS spectrum of well-brine and bicarbonate extracts.

Well-brine		Bicarbonate	
m/z	**%**	**m/z**	**%**
118.21	47	118.21	100
216.03	17	140.02	41
260.01	100	153.98	30
401.76	34	223.63	34
543.62	16	254.15	85
		264.37	85
		281.81	37

Table 2.10. Fragmentation of peaks of mass 543.62 and 401.76.

Daughters of 543.62		Daughters of 401.76	
m/z	**%**	**m/z**	**%**
259.89	100	259.48	100
400.48	38	401.4	50
543.44	36		

Table 2.11. Fragmentation of peaks of mass 260.01 and 118.21.

Daughters of 260.01		Daughters of 118.21	
m/z	**%**	**m/z**	**%**
118.39	100	79.86	58
259.98	78	95.73	100
		118.61	80

Most naturally occurring polymers such as humic substances have a wide range of molecular weight since the processes involved in their synthesis often give rise to different numbers of monomer units in the molecule. In addition, contamination by low molecular weight impurities may complicate data interpretation. Although it was not possible to identify the molecular peak in both samples, the fragments observed confirmed the observations made in IR and NMR analyses.

The results showed a range of peaks, both in well-brine and bicarbonate extracts, ranging from 118 to 852 m/z. The most prominent peaks, i.e. peaks of mass 118, 260 were observed in both samples. Although peaks of mass 401 and 544 were observed in both samples, they were less prominent in the bicarbonate sample. MS analysis clearly showed the presence of polymeric

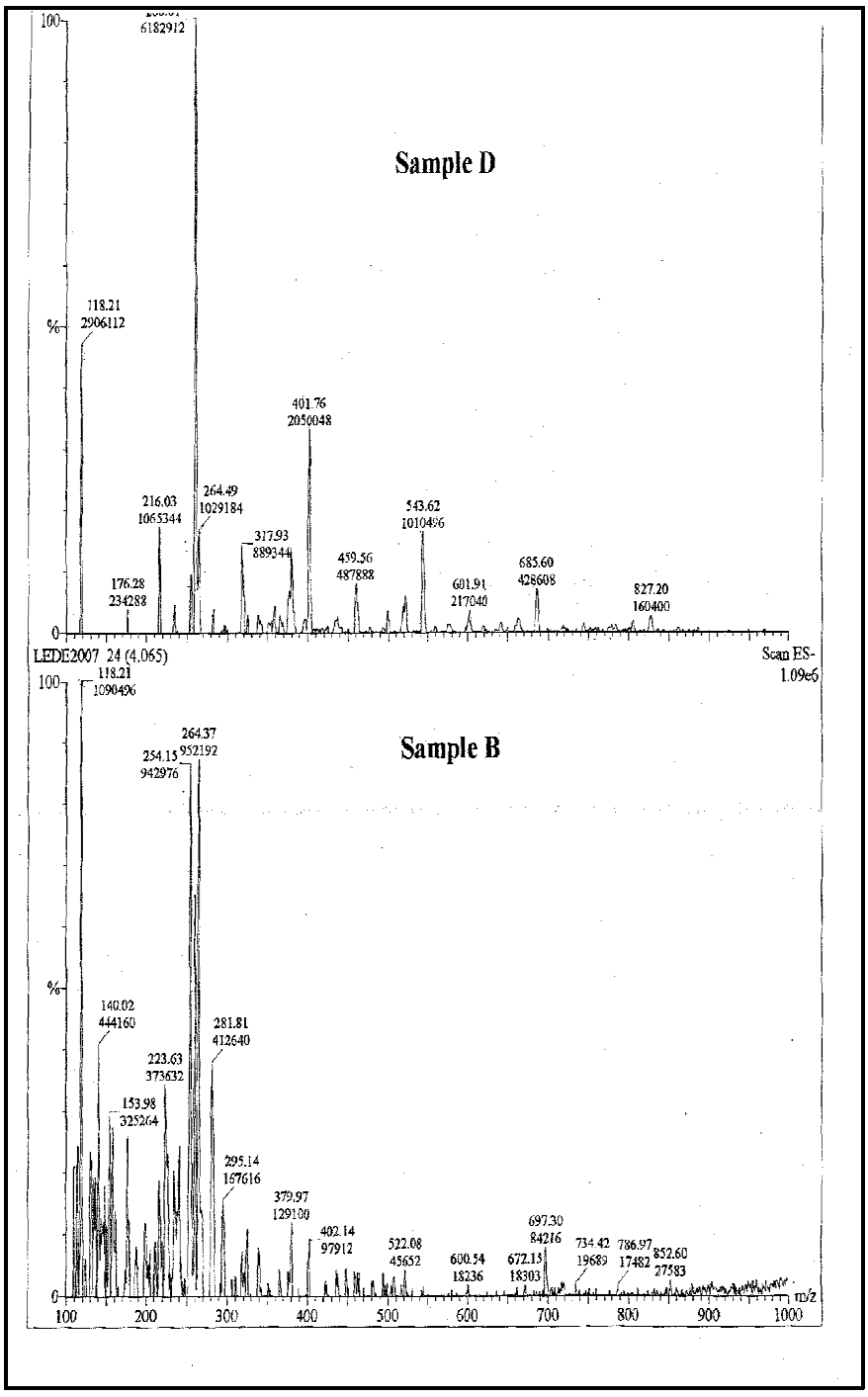

Figure 2.14. EMS analysis of freeze-dried extracts of well-brine (Sample D) and bicarbonate cake (Sample B). Samples were dissolved in acetonitrile:water, 50:50 (v/v) containing 1% formic acid.

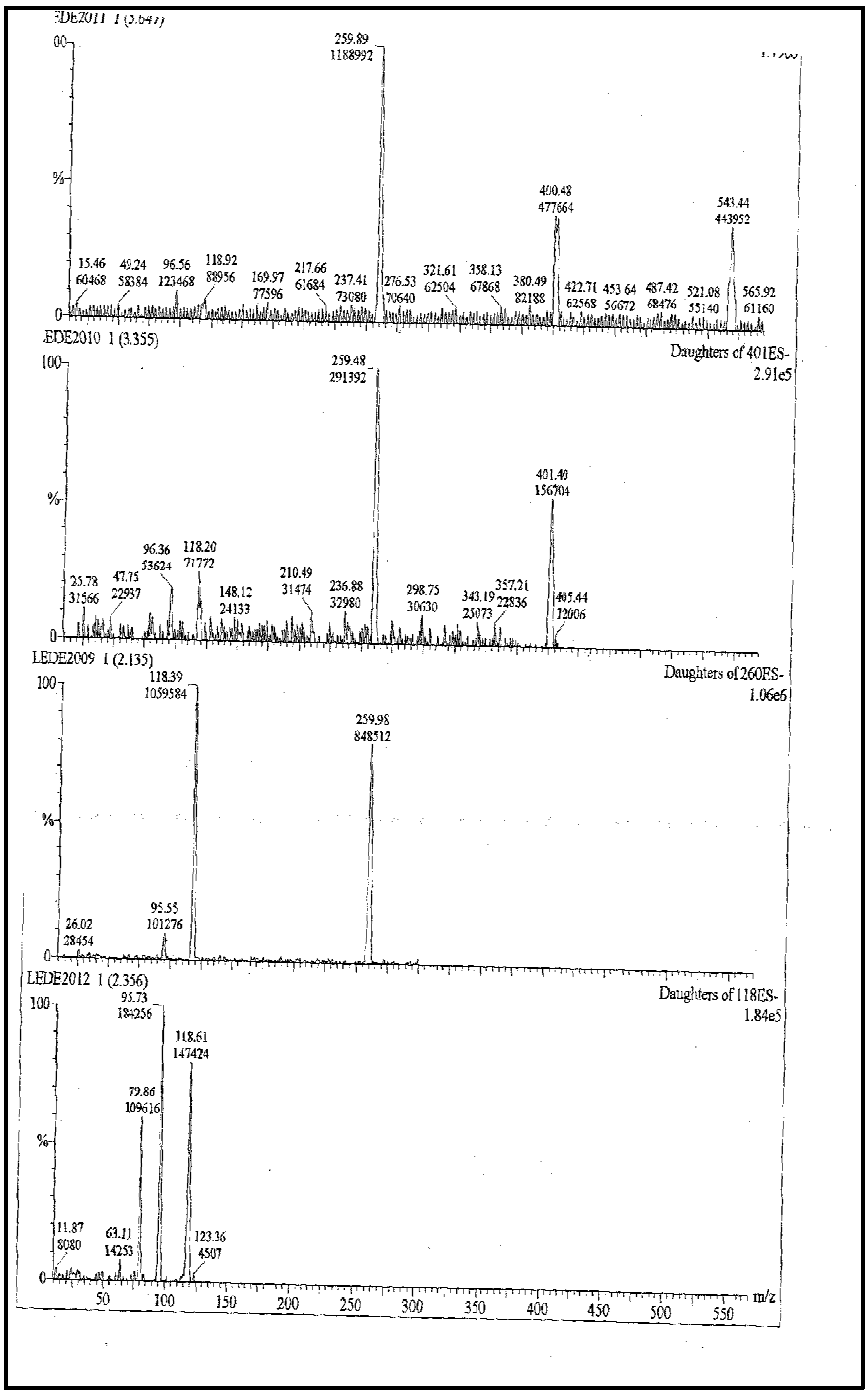

Figure 2.15. Analysis of daughter peaks of well-brine and bicarbonate EMS spectra.

compounds in both well-brine and bicarbonate. This confirmed the results obtained by IR and NMR spectroscopies suggesting certain well-brine organic components also existed in bicarbonate, indicating a carry-over through the system.

Structural identification of these components was not determined, however, MS analysis showed that on further fragmentation of these fragments, new fragments, each differing by 142 mass units, were obtained. This suggested the presence of a polymerised compound with repeating monomers of 142 mass units in both well-brine and bicarbonate.

2.4 CONCLUSION

Information derived from this study was mainly compositional rather than structural and provides a basis of understanding the nature of the organic contamination encountered in the soda ash production at Sua Pan. The results of this study provide a very strong indication that a component of the organic contamination in the final product derives directly from organics present in the influent well-brine. Whether these organic compounds consists of the polymerised molecule of mass 142, reflected in MS analysis, still needs to be determined. The results have also indicated that the concentration of the aromatic component may be reduced through the ponding process. This implies a possibility for practical implementation of physico-chemical or biological remediation strategies for dealing with the organics problem.

CHAPTER 3

CONTRIBUTION OF ALGAE TO ORGANIC CONTAMINATION

3.1 INTRODUCTION

Production of organic compounds by photosynthetic algae is reported to be a major source of organic matter utilised by bacteria in aquatic systems (Brock and Clyne, 1984; Sondergaard *et al*., 1985; Norrman and Zweifel, 1995). In solar saltworks, this production serves as an important energy and organic carbon source, mainly for bacteria, since with increasing salinity, the brine ecosystem changes from a nutritionally-independent to a nutritionally-dependent system (Davis, 1980).

Primary producing photosynthetic microorganisms, growing in the preliminary low salinity ponds, die with increasing salinity and, together with other trophic groups, release nutrients and organic carbon to a population of chemoorganotrophic organisms which develop in the high salinity ponds (Davis, 1980; Larsen, 1980). Other sources of organic matter may include runoff from land and defecation by aquatic animals and birds such as flamingos. Accumulation of organic matter in high salinity ponds occurs due to concentration by evaporation and the inability of bacteria growing in these ponds to breakdown the full organic load. (Davis, 1980).

Failure to maintain a balanced ecosystem may allow photosynthetic species to predominate, resulting in overproduction of organic matter.

3.1.1 Production of Extracellular Polysaccharides by algae

Production of organic carbon by algae and its bacterial assimilation is an interaction which has been extensively studied (Brock and Clyne, 1984; Sondergaard *et al*., 1985; Bjornsen, 1988; Giordano *et al*., 1994). The zone of organic enrichment around algal cells was recognized by Bell and Mitchell (1972) who termed this zone the phycosphere (analogous to the rhizosphere of plants). Since then the issue of whether release of organic matter occurs in healthy cells has been the subject of some controversy. Sharp (1977) suggested that release could be an experimental artifact arising during measurement. Most studies have since confirmed that release does in fact occur in healthy cells (Mague *et al*., 1980; Brock and Clyne, 1984; Bjornsen, 1988; Giordano *et al*., 1994; Myklestad, 1995; Malinsky-Rushansky and Legrand, 1996).

Production of extracellular polysaccharides by algae has received wide attention as these compounds have a number of applications in the food, pharmaceutical, and other industries. These include their use as gelling agents, stabilizers and additives to improve textures of some food products (Margaritis and Pace, 1985; Kaplan *et al*., 1987). Other potential applications include the use of these polymers in the preparation of aqueous driving fluids in enhanced oil recovery (EOR), and as natural metal chelators for reducing metal toxicity (Kaplan *et al*., 1987). Sulphated polysaccharides have shown activity against various retroviral reverse transcriptases and may be potentially used against the HIV (Sudo *et al*., 1995).

The release of organic matter by algae has been suggested to be a response to extreme environmental stress such as changes in the quantity and quality of light (Claus, 1988; Zlotnik and Dubinsky, 1989), salinity (Sudo *et al*., 1995), temperature (Claus, 1988) or nutrient limitation (Myklestad, 1995). Conditions favouring release also seem to be growth-phase dependent with more release observed during stationary phase (Guillard and Wangersky, 1958; Huntsman, 1972). Carbohydrates, especially polysaccharides, sometimes constitute 80-90% of the total extracellular release (Myklestad, 1995).

Release of organic matter by *Dunaliella* species has been reported both in laboratory cultures (Guillard and Wangersky, 1958; Huntsman, 1972; Bell, 1983; Giordano *et al*., 1994) and in natural hypersaline environments (Oren and Shilo, 1985). Extracellular polysacharides may exist as cell-bound capsular material or cell-free slime which on release into the surrounding medium causes increased viscosity (Vincenzini *et al*., 1990). The ecology of a solar salt-works and conditions prevailing in the ponds, therefore, plays a crucial role in the performance of any particular system.

3.1.2 Objectives

The objectives of this study were to:

1. Describe the main characteristics of the biology of the solar evaporation ponds at Sua Pan.
2. Investigate the factors influencing organic carbon release by algae.
3. Determine the nature of the organic compounds released.
4. Determine whether organic compounds produced by microbial growth in the ponds contribute to the TOC of the final soda ash product.

3.2 MATERIALS AND METHODS

3.2.1 Algal culture and growth

A culture of *Dunaliella spp*. was isolated from brine samples obtained from evaporation pond E2 at Sua Pan. Although earlier investigations of this system had involved the study of pure cultures of the two main *Dunaliella* strains growing in Sua Pan solar ponds i.e. *D. salina* and *D. viridis* (Rose, 1998a), in this study, the experimental work involved the use of a mixed culture. The laboratory studies in this case would, therefore, reflect what happens in the ponds without excluding the interaction between the two algal strains. This culture was routinely grown in BAAM medium, the composition of which is given in Appendix I. Solid media were solidified by adding 2% agar-agar (Merck, Darmstadt, Germany).

3.2.2 Growth studies

A 10% inoculum of *Dunaliella* culture in logarithmic phase was used in all growth studies. 500ml of liquid medium, in flasks stoppered with cotton wool was inoculated and incubated in a shaker (Labcon, Johannesburg, South Africa) at 140rpm, and placed in a controlled environment chamber at 25^0C under continuous illumination provided by cool white flourescent lamps. For light stress, cultures were grown under high pressure sodium lamps (1500µmol.m^{-2}.s^{-1}) under a 12hour light:12hour dark cycle.

3.2.3 Analyses

3.2.3.1 Cell growth

Cell growth was determined by cell counts done in an improved Neubauer Haemocytometer (bright line) (Marienfield, Germany). Counts were done in triplicates and the results reported represent the mean of triplicates. Growth was also determined by measuring the chlorophyll *a* content in the culture. Chlorophyll *a* was extracted into 100% acetone and the concentration determined spectrophotometrically in a Shimadzu UV/VIS 160A spectrophotometer (Shimadzu, Japan) according to Lichtenhaler (1987). 10ml of the sample was filtered through a GF/A filter (Whatman, Maidstone, UK) and the filter paper transferred to a centrifuge tube. 10ml of 100% acetone (BDH Laboratory Supplies, Poole, UK) was added and a glass rod used to crush the filter paper to ensure complete extraction of the chlorophyll. The tubes were covered in foil and stored at 4^0C overnight. The extracted solutions were centrifuged at 5000 rpm for 15 minutes on a Labofuge Ae bench centrifuge (Haraeus Sepatech), and the absorbance of the acetone extracts read at 661.6nm and 644.8nm. Chlorophyll *a* concentration was calculated according to the following equation:

Chl *a* ($mg.l^{-1}$) = (11.24 $A_{661.6}$ - 2.04 $A_{644.8}$) × acetone volume(ml)/sample volume (ml).

3.2.3.2 Nutrient concentrations

Analysis of ammonium, phosphorus and nitrate concentrations was determined using ammonium, phosphorus (PMB) and nitrate spectroquant cell tests supplied by Merck (Darmstadt, Germany). Samples were filtered through 0.45Fm cellulose acetate syringe filters (Micron Separations Inc., Westboro, MA, U.S.A.) and diluted accordingly before analysis. Readings were taken from the spectroquant SQ-118 (Merck, Darmsatdt, Germany), see Appendix II for detailed procedures.

3.2.3.3 Quantitative determination of extracellular polysaccharides

The anthrone method for determination of total carbohydrates was used to quantify the extracellular polysaccharides. *Dunaliella* cultures were centrifuged in a bench centrifuge (5000 rpm for 15 minutes) to separate the cells before analysis. Filtration of the samples was avoided due to the tendency of some filter papers to adsorb some organic materials. 1ml of the supernatant (uninoculated medium for the blank) was added to pyrex tubes in triplicates. The samples and anthrone reagent were chilled in ice-water. 5ml of anthrone reagent was added and the contents mixed in a vortex mixer. The tubes were transferred to a boiling water bath for 10 minutes and then to the ice-water before the absorbance was read at 625 nm in a Shimadzu spectrophotometer. The concentration of total carbohydrate ($mg.l^{-1}$ glucose) was calculated using the standard curve prepared using glucose standards. For reagent and standard curve preparation see Appendix I and II respectively.

Extracellular polysaccharides were also quantified by measuring the Total Organic Carbon (TOC) of the medium. 10ml of the supernatant above was acidified to pH 2-3 with nitric acid (BDH Laboratory Supplies, Poole, UK) and sparged with air (Afrox, Port Elizabeth, South Africa) for approximately 6 minutes to remove inorganic carbon. 40Fl of the sample was injected into the Dorhmann DC-180 TOC analyser (Rosemount Analytical Division, Santa Clara, CA, U.S.A.). Preparation of standards and calibration of the TOC machine were done according to the manufacturer's instructions.

3.2.3.4 Isolation of extracellular polysaccharide

Algal cultures were centrifuged using a Beckman JA-21 centrifuge (10 000 rpm, 10^0C, 20 minutes). Two volumes of 2-propanol (BDH Laboratory Supplies, Poole, UK) was added to

depleted medium to precipitate the polysaccharide according to Smith and Pace (1982). After decanting the liquid, the precipitate was dialysed (MWCO 3500D) against deionized water and freeze-dried. Monosaccharide composition of the polysaccharide was determined.

3.2.3.5 Monosaccharide composition

The samples were hydrolysed and volatile derivatives of constituent monosaccharides to be analysed by GLC were prepared according to Mc Ginnis (1982). 3-4g of sample was hydrolysed with 4M triflouroacetic acid (Fluka Chemie, Buchs, Switzerland) in a Reactitherm heating block (Pierce, Ilinois, U.S.A.) for one hour at 125^0C. The hydrolysate was dried in a rotary evaporator at 40^0C. 2ml of deionised water was added and dried to remove the acid, this was repeated three times and the sample dissolved in 0.2ml of water followed by oximation in 0.4ml hydroxylamine (BDH Laboratory Supplies, Poole, UK) in N-methylimidazole (Fluka Chemie, Buchs, Switzerland) (0.5g hydroxylamine.HCl in 20ml N-methylimidazole). The solution was heated in an oil bath for 10 minutes at 80^0C, cooled in ice and acetylated by adding 1ml acetic anhydride (BDH Laboratory Supplies, Poole, UK), keeping the sample in ice. The sample was transferred to a separating funnel and extracted with chloroform. The chloroform layer was sequentially washed with 10% H_2SO_4, water, saturated sodium bicarbonate and water. The sample was dried with anhydrous sodium sulphate (Saarchem, Krugersdorp, South Africa), filtered, concentrated on the rotary evaporator and then analysed using gas/liquid chromatography (Model 6890, Hewlett Packard).

3.3 RESULTS

3.3.1 Pond Ecology of Sua Pan salt-works

The microbial ecology of the evaporation ponding system was studied in order to identify biological processes and factors playing a major role on the performance of the system. The influent brine is highly alkaline (>pH 8) and saline (12% (w/v)) with sodium being the predominant ion. This brine is pumped into pond E0 and is concentrated through evaporation ponds E2 to E6 before the sodium chloride-rich brine is pumped into the N-Brine storage pond which feeds the crystallizing ponds (see Figure 1.3). As a result of evaporation, the chemistry of the brine changes as the brine proceeds through the ponding system as shown in Table 3.1.

Table 3.1. Major ions in the solar ponds (Monthly averages, July 1992).

Location	S.G	Temperature (^{0}C)	NaCl (%)	KCl (%)	Na_2SO_4 (%)	Na_2CO_3 (%)	$NaHCO_3$ (%)
Inlet	1.13	21.03	12.51	0.26	1.44	2.23	0.7
CS	1.15	17.19	14.25	0.3	1.55	2.39	0.98
E0	1.16	21.71	15.43	0.38	1.49	2.8	1.01
NS	1.23	15.42	21.46	0.52	2.78	3.66	1.06
X2	1.25	18.45	21.54	0.69	3.36	4.23	0.99
T-brine	1.25	18.13	20.9	0.82	3.74	4.25	0.99

The brine also contain phosphorus, nitrogen and dissolved sulphide concentrations of 50mg.l^{-1}, 7mg.l^{-1} and 60 ppm respectively. Nitrogen in the brine occurs almost entirely as ammonium. Repeated analyses of the brine in different laboratories have failed to show the presence of nitrate in the brine.

Temperatures in the Sua Pan area varies from 18^{0}C to 32^{0}C in the summer months and 5^{0}C to 23^{0}C during winter. A temperature gradient is also observed through the ponding cascade as

shown in Table 3.1. In addition, the pond operation allows shallow and stagnant zones to occur within ponds and as a result, both temperature and salinity gradients occur within a pond as shown in Figure 3.1. The isotherm plots shown in Figure 3.1 were obtained by measuring the temperature of the brine across the pond, following a grid pattern. Under conditions prevailing in the ponds, the biological community is, therefore, continuously exposed to conditions of increased salinity and light intensity, and fluctuating temperatures.

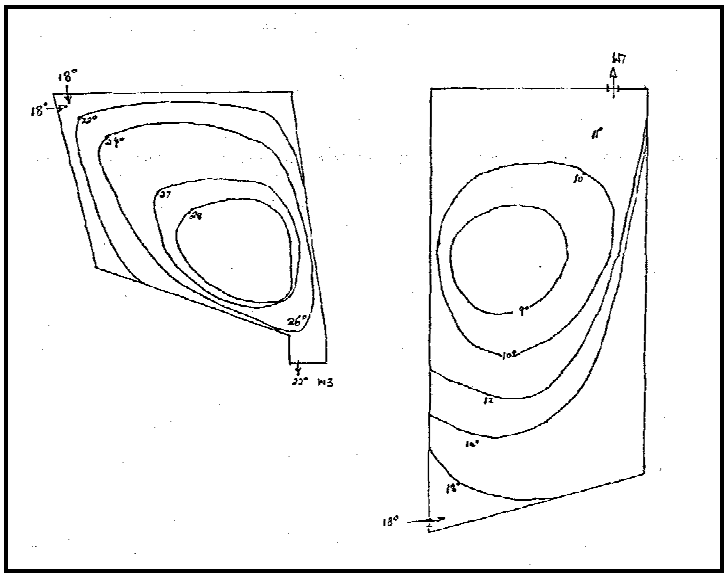

Figure 3.1. Isotherm plots showing brine temperature distribution profile in (a) Pond E0 and (b) Pond E2.

3.3.1.1 Planktonic population

Dunaliella spp. dominate the planktonic population in the evaporation series (E0-E6) (Figure 1.3). A high biomass of this alga occurring in the preliminary evaporation ponds colour the brine greenish. Two different strains occur in the system, the carotenoid accumulating strain (*D. salina*) and another strain (*D. viridis*) which remains green at all salinity ranges. Measurements made in September 1997 showed that the biomass of *Dunaliella spp*. increased from pond E0 (12-33 $\times 10^4$ cells.ml^{-1}) to pond E2 (68-95 $\times 10^4$ cells.ml^{-1}) but decreased as the brine became more concentrated in pond E6 (45 $\times 10^4$ cells.ml^{-1}). A layer of slime accumulated on the pond floors and the occurrence of this slime varied through the ponding cascade. This slime is polysaccharide in nature and is probably a result of overproduction of EPS by *Dunaliella spp*. in the water column (Rose,1998a).

The brine appears khakhi to orange along the salinity gradient as the carotenogenic strain, *D. salina* becomes predominant. As the brine enters the N-Brine storage, it becomes more pink and the intensity of this colour increases in the crystalliser and T-brine storage ponds. This is caused by the proliferation of halobacteria which constitute the major plankton in these ponds. Figure 3.2 shows brine samples collected from the ponds in the order of increasing salinity. Halobacteria growing in the downstream ponds utilise organic matter produced in the upstream ponds and the slime layer occurring in the upstream ponds is found to decrease in these ponds. Inclusions of these bacteria in salt crystals lead to the production of pink salt with a high moisture content. Overproduction of organic matter in the preliminary evaporation ponds also interferes with salt crystal formation and both hopper and small crystals are formed. This results in crystallisers with marshy salt peripheries.

Figure 3.2. Picture showing brine samples collected from: **A**- Inlet; **B**- E2; **C**- E5; **D**- X2. Reflection in sample A occurred due to the clarity of the brine.

3.3.1.2 Benthic Population

The slime settling out of the water column is adsorbed on the pond sediment, forming a compact layer in the form of a benthic mat. Consolidated mats are, however, rare in the system. Of the ponds studied, Pond E0 had the best developed bottom community. The dissolved sulphide occurring in the influent brine supports a rich growth of purple sulphur bacteria observed at the point of brine discharge into Pond E0. In other sections of the ponds, the benthic mat consists of an uppermost orange layer. Microscopic examination of this layer has shown the presence of bacteria which have not yet been identified. However the presence of sulphide oxidisers was indicated by sulphur globules observed in the bacterial cells. This is followed by a green slimy layer in which algae and blue-green algae entrapped in the slime, are observed and then a deep purple layer of anaerobic photosynthetic bacteria above the black layer of sulphate-reducing bacteria.

3.3.2 Growth of *Dunaliella spp.*

Growth of *Dunaliella spp*. in the alkaline-type brine of Sua Pan was evaluated in flask studies to determine conditions favourable to EPS production by a mixed culture of *Dunaliella spp*. collected from the ponds at Sua Pan. *Dunaliella spp*. was also cultured in defined inorganic medium for comparative studies.

Growth of cultures growing in well-brine and BAAM medium was monitored by measuring cell counts and chlorophyll *a* concentration as shown in Figures 3.3 and 3.4 respectively. Cells grown in well-brine entered a long (4 days) lag phase before growth which increased with time was observed. The long lag phase observed in cultures grown in well-brine may have been due to the inhibitory effect of the dissolved sulphide in well-brine. High sulphide concentrations have been shown to have adverse effects on algal growth (Almasi and Pescod, 1996).

A higher growth rate was observed when cells were grown in BAAM medium. Cells immediately entered the log phase until day 8 when the cells entered a death phase directly. Unlike well-brine, BAAM contained trace elements essential for *Dunaliella* growth.

3.3.3 The effect of light intensity on EPS production

Dunaliella spp. growing in the ponds are constantly exposed to high light intensities and therefore the effect of light intensity on extracellular release of polysaccharides was investigated. Figures 3.5 and 3.6 show production of polysaccharide as measured by the concentration of glucose and TOC in the medium under normal light and high light intensities respectively.

The culture grown under normal light intensity reached the death phase by day 8. There was a sharp increase in carbohydrate concentration in the medium and this increase continued through all growth phases. An increase in TOC level of the medium was also observed even though this was slow compared to the carbohydrate increase. A high growth rate was observed under high light intensity but by day 4 the cells had reached the death phase probably due to light stress. Carbohydrate and TOC concentrations increased throughout the culture period and leakage from senescent cells probably contributed to this increase after the death phase had been reached. A greater carbohydrate and TOC concentration in the medium was observed in cultures grown under high light intensity compared to cultures grown under normal light intensity.

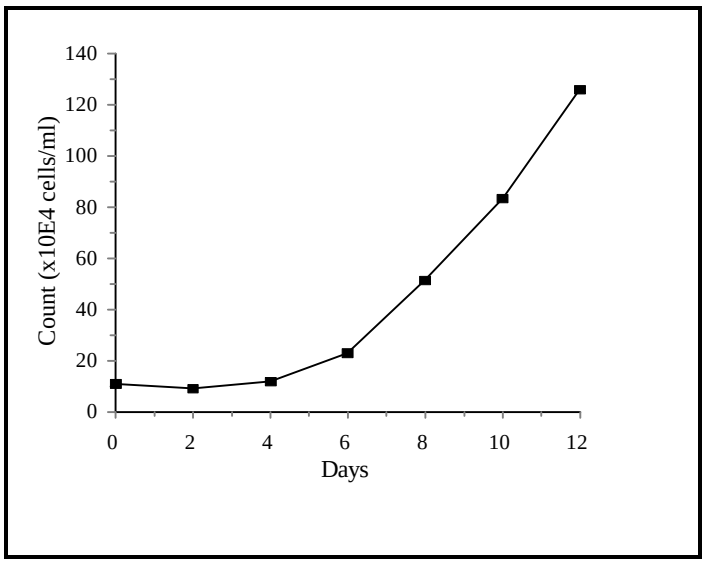

Figure 3.3. Growth of *Dunaliella spp*. in well-brine showing biomass determined by cell count.

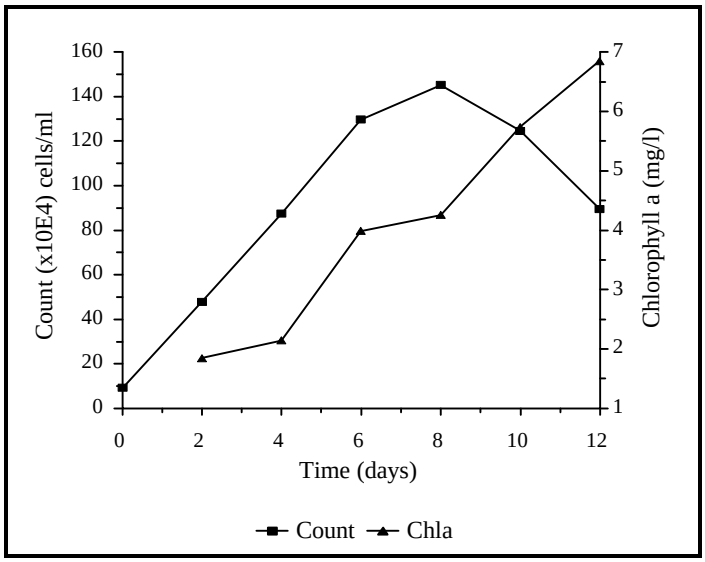

Figure 3.4. Growth of *Dunaliella spp*. in BAAM medium showing biomass determined by cell count and chlorophyll*a* concentration.

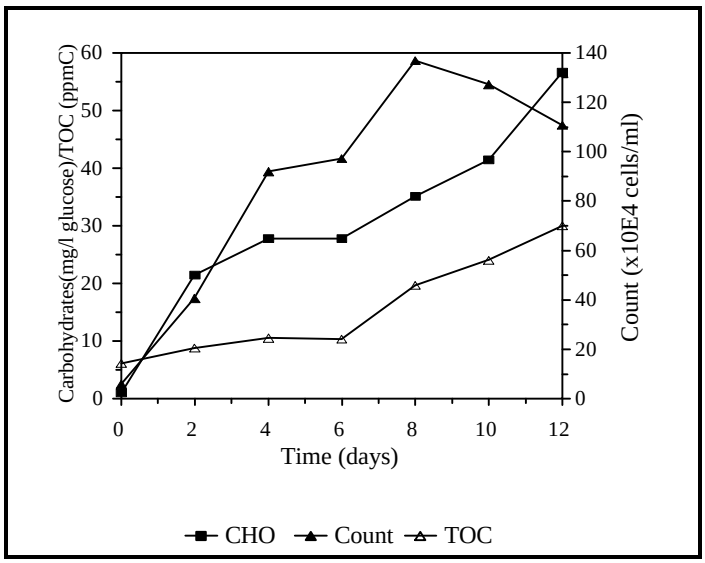

Figure 3.5. Production of extracellular polysaccharide in cultures grown in BAAM medium under normal light intensity.

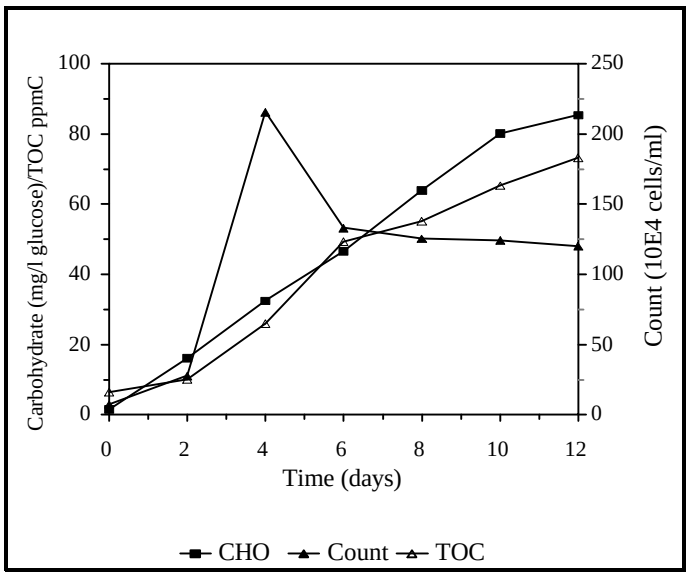

Figure 3.6. Production of extracellular polysaccharide in cultures grown in BAAM medium under high light intensity (1500Fmol.m^{-2}.s^{-1}).

No correlation between the extracellular production and the growth phase was observed. EPS seemed to be produced throughout all growth phases.

3.3.4 The effect of temperature on EPS production

Growth of *Dunaliella spp*. at normal light intensity, was influenced by temperature as shown in Figure 3.7. The highest cell counts were maintained at 35^0C whereas growth was limited at 38^0C. The amount of extracellular polysaccharide produced at different temperatures was measured as accumulation of organic carbon (TOC) in the culture medium. Figure 3.8 shows that accumulation of organic carbon was highest at 35^0C and lowest at 38^0C indicating a correlation between biomass and EPS production.

3.3.5 Effect of salinity on EPS production

Figure 3.9 shows that as salinity increases, the cell numbers and growth rate of *Dunaliella* decreases. This observation has been made in the ponds where the number of cells is seen to decline as the brine proceeds through the ponding cascade. The effect salinity has on the amount of TOC produced is shown in Figure 3.10. Accumulation of organic carbon was found to be salinity-dependent over a wide range of salinity. High TOC levels were obtained in cultures grown at high salinity. These results indicate that although proliferation of algae may occur in the first evaporating ponds, accumulation of organic matter occurs downstream where algal growth becomes limited.

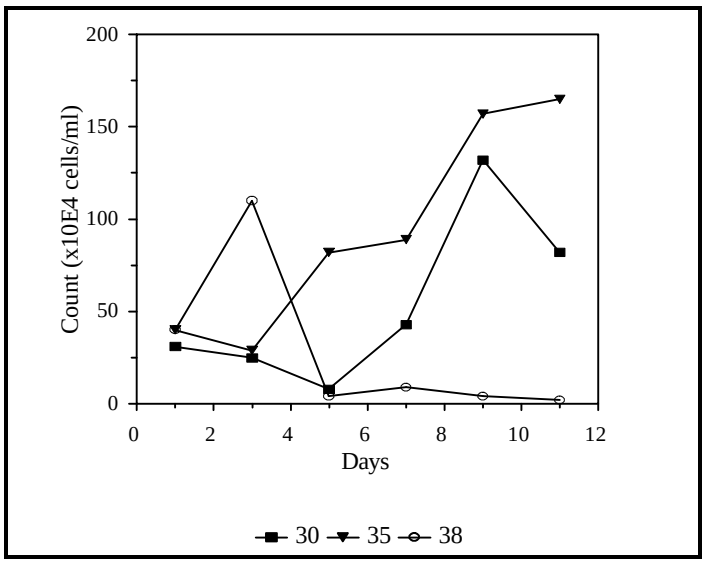

Figure 3.7. The effect of temperature on growth of *Dunaliella spp*. under normal light intensity. Growth determined at 30, 35 and 38^{0}C.

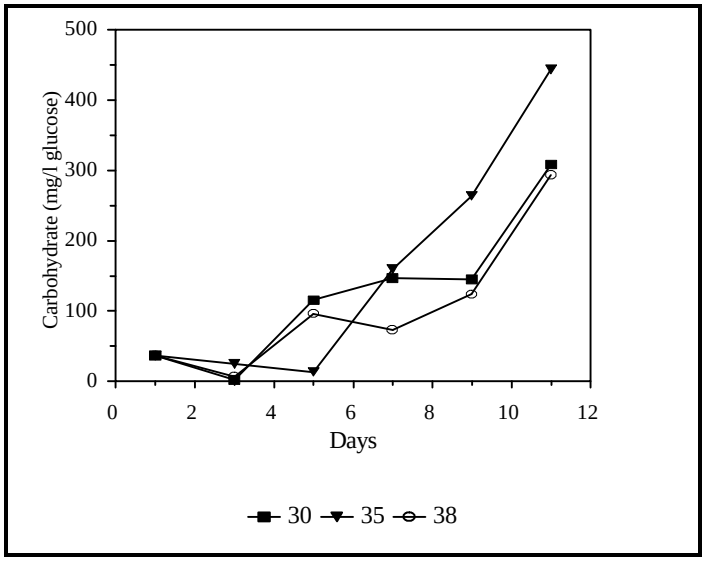

Figure 3.8. The effect of temperature on EPS release by *Dunaliella spp*. grown at 30^{0}C, 35^{0}C and 38^{0}C under normal light intensity.

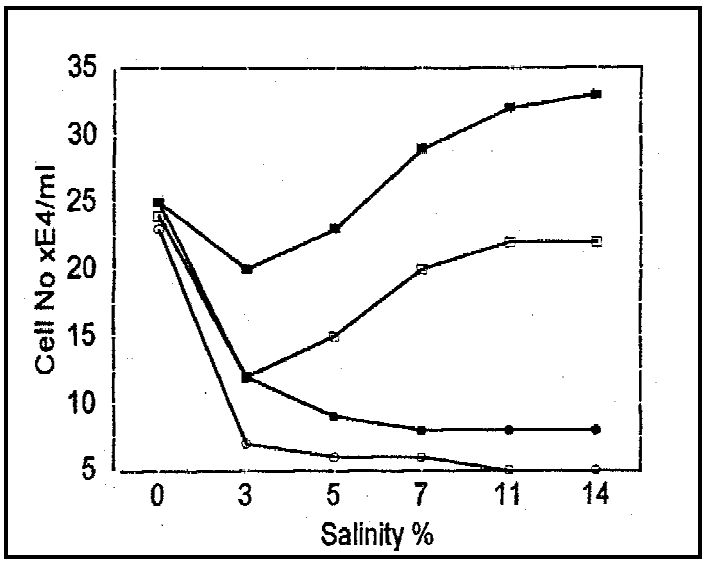

Figure 3.9. Growth of *Dunaliella spp*. at different salinities;
(■) 17%; (□) 25%; (●) 30% and (○) 35% (w/v).

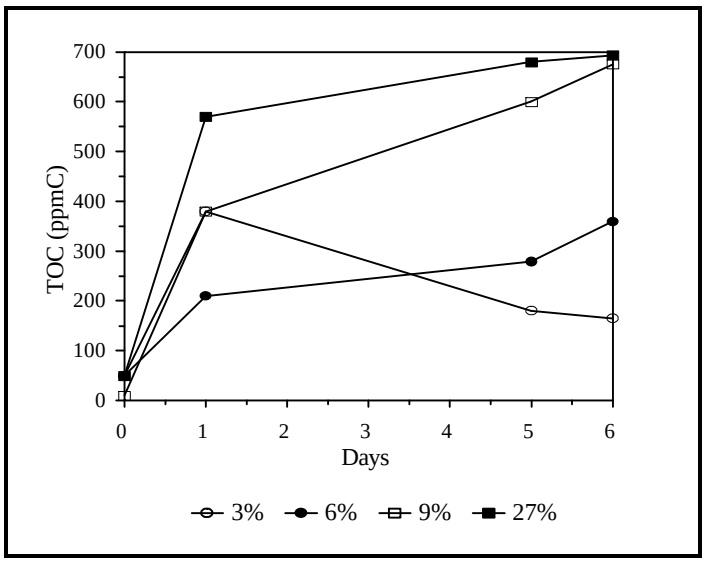

Figure 3.10. The effect of salinity on accumulation of organic carbon in the growth medium of *Dunaliella spp*.

3.3.6 Effect of nutritional status on EPS release

Algal growth depends on the nutritional status of the medium and this plays a major role in the production of EPS. Results obtained in a collaborative study have shown that nitrogen and carbon concentrations influences both growth and production of EPS by *Dunaliella* (Selepe, 1999). Studies done under conditions of nitrogen limitation have shown that while growth of algae at low N concentrations was not affected, the amount of EPS produced was enhanced. This was even more pronounced when the amount of inorganic carbon source was doubled as shown in Figure 3.11.

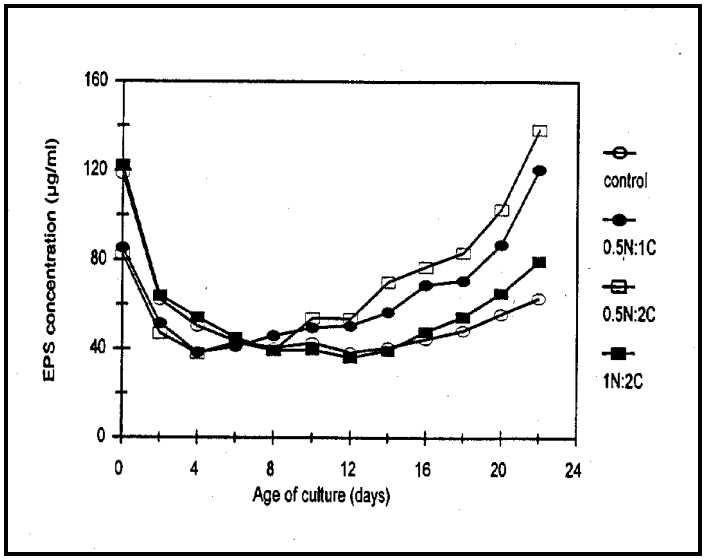

Figure 3.11. The effect of nutritional status related to C:N ratio on EPS release by *Dunaliella spp*.

3.3.7 Characterization of EPS

The EPS produced by *Dunaliella spp*. in shake flask studies was characterised in order to determine the significance of this production on organic contamination occurring in the ponds and in the final product. This was achieved by characterising freeze-dried extracts of T-brine and bicarbonate filter cake (see section 2.2.1.1 for sample preparation) in a similar manner and comparing the results with *Dunaliella* EPS extract.

The polysaccharide in the culture medium was isolated by alcohol precipitation and purified by dialysis before determining the constituent monosaccharides. Monosaccharide constituents were analysed by gas chromatography and the relative peak height used to calculate the proportions of the constituent monosaccharides relative to glucose.

The results showing characterisation of *Dunaliella spp*. EPS are given in Figures 3.12 and 3.13 and in Tables 3.2 and 3.3. The chemical composition of *Dunaliella spp*. component consisted of at least six sugars; arabinose, xylose, mannose, glucose, galactose and 2-*o*-methyl hexose, with 2-*o*-methyl hexose being the most abundant. Identification of 2-*o*-methyl hexose was obtained by comparing the spectra with monosaccharide profile of *Klebsiella spp*. exopolysaccharide (Hackland *et al*., 1988).

Monosaccharide analyses of T-brine and bicarbonate extracts are shown in Figures 3.14-3.16 and Tables 3.4-3.6. The capping sugar, 2-*o*-methyl hexose, identified as the most abundant sugar in the Dunaliella EPS extract, was not observed in either T-brine or bicarbonate extracts.

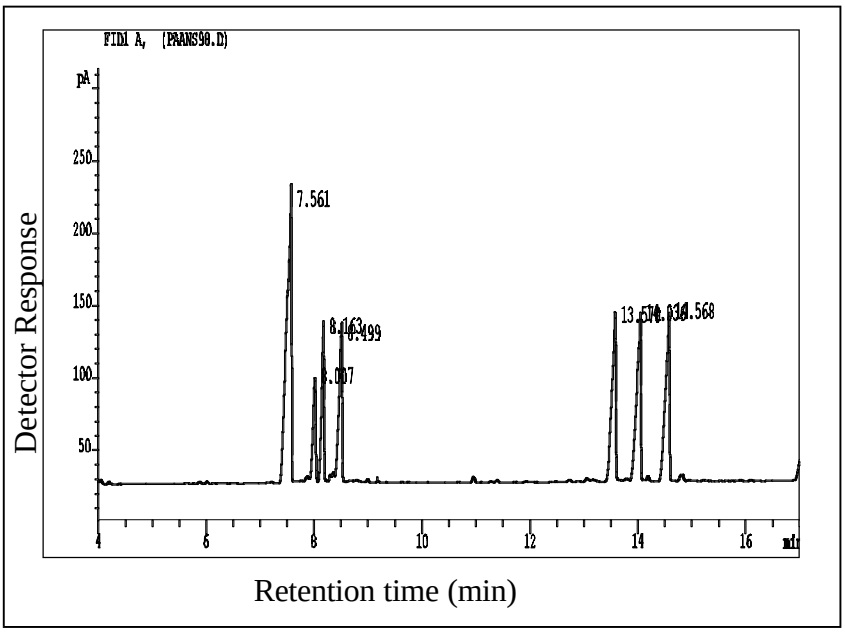

Figure 3.12. GC spectrum of commercial monosaccharide standards on a DB-17 column.

Table 3.2. Identity of commercial monosaccharide standards on a DB-17 column.

Retention time	Monosaccharide
7.561	Arabinose
8.007	Ribose
8.163	Fucose
8.499	Xylose
13.57	Mannose
14.03	Glucose
14.568	Galactose

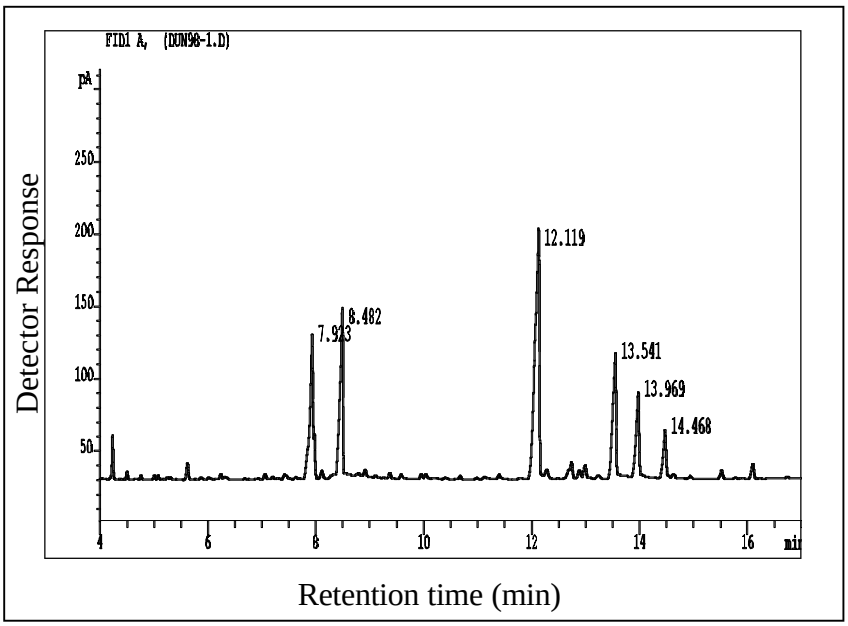

Figure 3.13. GC spectrum of *Dunaliella* EPS monosaccharides on a DB-17 column.

Table 3.3. Identity of *Dunaliella* EPS monosaccharide composition on a DB-17 column.

Retention time	Monosaccharide	Proportion
7.923	Arabinose	1.7
8.482	Xylose	1.8
12.119	2-o-methyl hexose	2.8
13.541	Mannose	1.4
3.969	Glucose	1
14.468	Galactose	0.6

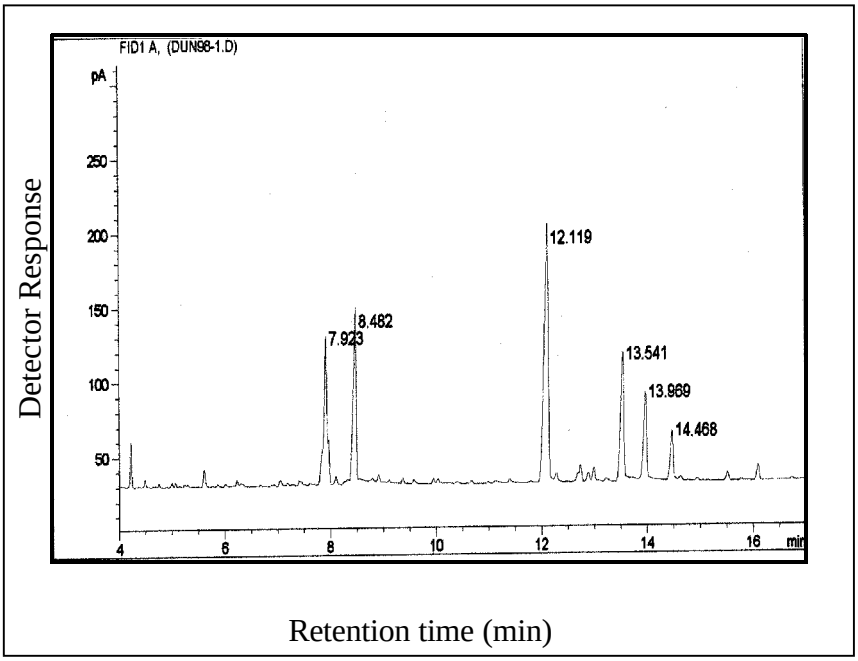

Figure 3.14. GC spectrum of monosaccharide standards on a DB-225 column.

Table 3.4. Identity of commercial monosaccharide standards on a DB-225 column.

Retention time	Monosaccharide
5.941	Rhamnose
6.938	Ribose
7.123	Fucose
7.906	Arabinose
9.144	Xylose
15.404	Mannose
18.285	Glucose
19.756	Galactose

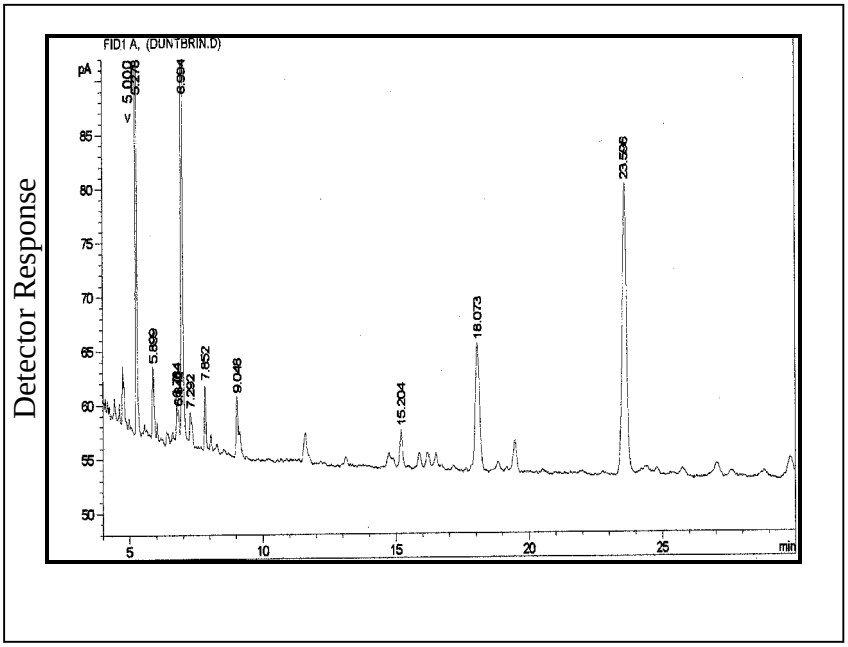

Figure 3.15. GC spectrum of T-brine extract on a DB-225 column.

Table 3.5. Identity of T-brine monosaccharide composition on a DB-225 column.

Retention time	Monosaccharide	Proportion
5.899	Rhamnose	0.6
6.994	Ribose	7.7
7.292	Fucose	0.3
7.852	Arabinose	0.5
9.048	Xylose	0.5
15.204	Mannose	0.3
18.073	Glucose	1
19.5	Galactose	0.2

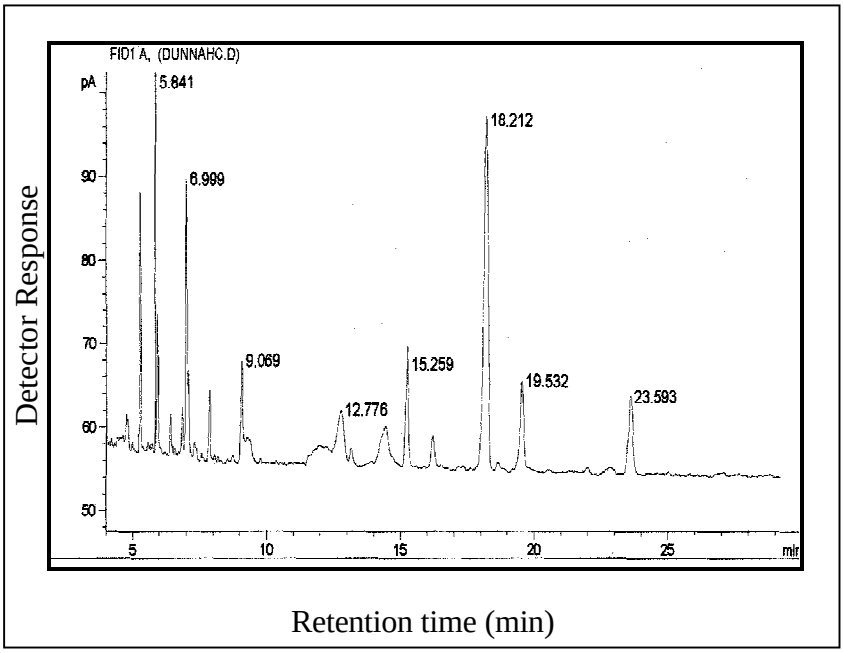

Figure 3.16. GC spectrum of bicarbonate extract on a DB-225 column.

Table 3.6. Identity of bicarbonate monosaccharide composition on a DB-225 column.

Retention time	Monosaccharide	Proportion
5.841	Rhamnose	4.7
6.999	Ribose	0.8
9.069	Xylose	0.3
15.259	Mannose	0.3
18.212	Glucose	1
19.532	Galactose	0.3

It should be noted, however, that despite the variation in proportions of sugars identified in T-brine, bicarbonate and *Dunaliella spp.*, the results reflect the presence of a similar type of polysaccharide in all the samples. The same type of monosaccharides have been reported for cyanobacterial extracellular polysaccharides except for the 2-*o*-methyl hexose (Vincenzini *et al.*, 1990; Sudo *et al*., 1995).

3.4 DISCUSSION

Excretion products of algae include a wide range of organic compounds including proteins, glycerol, polysaccharides, monosaccharides, nucleic acids, glycollic acid and amino acids (Fogg, 1983). The chemical composition of certain components of these extracellular products is not well understood (Myklestad, 1995). Since carbohydrates, especially polysaccharides, have been reported to constitute the major excretion compounds (80-90%), carbohydrate excretion was the principal area of focus in this study. It should be noted that there is a possibility that the amount of EPS produced was overestimated in these experiments since glucose was used as a standard. For accuracy, the polysaccharide or a sugar mixture containing the same percentage monosaccharide composition should have been used since glucose standards have been reported to react more efficiently than sugar mixtures (Plude *et al*., 1991).

EPS release into the culture medium, monitored by the increase in soluble carbohydrates was correlated with light and temperature stress. Although release occurred under normal light intensities, this increased when cultures were exposed to high light intensity. *Dunaliella* cells responded to light stress by increased production of polysaccharide. These results are in

accordance with the findings of Mague *et al*.(1980) and Groeger and Kimmel (1989) who found a correlation between light intensity and extracellular release of polysaccharides. Belay and Fogg (1978) have explained an increase in extracellularly produced material at high light intensities to be due to photo-inhibition of fixation of carbon into particulate matter. On the other hand, at high light intensities the algae reached death phase faster than at normal light intensities. Prolonged exposure of algae to high irradiances result in cellular photo-damage and oxidation (photo-inhibition) which reduces algal biomass.

The importance of nutritional status in the release of extracellular compounds from phytoplankton has been noted by a number of authors (Hellebust, 1974; De Philippis *et al*., 1993; Myklestad, 1995). Nitrogen limitation was found to enhance polysaccharide production by the *Dunaliella* culture. This increase was more pronounced when a high ratio of carbon source to limiting nitrogen source was used. The same trend has been observed by other authors (Zevenhuizen, 1981; De Philippis *et al*., 1993; Tavernier *et al*., 1997) but conflicts with the findings of Sudo *et al*. (1995). However, it should be noted that the pattern of excretion may be species-specific which means that the effects of nutrient limitation may be different in different situations.

When nitrogen has been fully utilized, protein synthesis and cell division stops and synthesis of EPS becomes the major photosynthetic activity (De Philippis *et al*., 1993; Myklestad, 1995). This has been confirmed by Vincenzini *et al*. (1990) who found that magnesium deficiency enhanced EPS production in *Cyanospira capsulata* by reducing protein synthesis. Inhibition of cell division helps maintain high photosynthetic activity since increase of shading among cells, typical of batch cultures, does not occur (De Philippis *et al*., 1993). Myklestad (1995) has also found that not only

is the concentrations of nutrients important, but also the ratio of components. The nature of the nitrogen source can also affect biomass formation and EPS production (Tavernier *et al.*, 1997). In the case of *D. salina*, more EPS was seen to be produced when nitrogen was supplied as ammonia as opposed to nitrate (Giordano *et al.*, 1994).

Cultures subjected to salinity stress produced more EPS than cultures grown at low salinity. Growth was also limited at high salinities. Although glycerol is accumulated for osmotic balance, maintenance of high intracellular glycerol concentrations may require large amounts of energy hence limiting energy available for synthetic purposes thus inhibiting certain enzymes.

There is conflicting evidence regarding the growth phase at which high rates of EPS release occurs. High release has been observed in both the stationary phase (Hellebust, 1974) and in log phase (Myklestad *et al.*, 1989). In this study an increase in both TOC and carbohydrate concentrations were observed throughout the culture period indicating production at all growth phases. This is in accordance with the observation of Myklestad *et al.* (1989) that excretion occurs during all phases of growth with the rate of release in exponential phase as high or higher than that of stationary phase. Although excretion seems to occur during all growth phases, senescent cells probably contributed to the major accumulation of EPS in cultures grown under high light intensity since the cells entered death phase within a short period compared to the cells grown under normal light intensity.

Acetylated aldonitrile derivatives of the EPS revealed the presence of a polysaccharide consisting of glucose, arabinose, xylose, mannose, galactose and 2-*o*-methyl hexose. These derivatives have

an advantage over the widely used trimethylsilyl ether since each sugar gives only one peak and has good chromatographic properties (McGinnis, 1982). *Dunaliella* growing in solar ponds is continuously exposed to a combination of light, temperature and salinity stresses which would be expected to be resolved by EPS production. Production of EPS in the ponds may actually be higher than in laboratory observations since the presence of an external sink for extracellular products (such as heterotrophic bacteria) has been proposed to increase EPS release by algae (Jones and Cannon, 1986). Release of organic compounds by algae plays a role in natural environments by providing an organic carbon source for the heterotrophic bacteria.

2-*o*-methyl hexose which acts as capping sugar, constituted a major proportion of the *Dunaliella* EPS. This sugar is unusual in algal EPS. Although a fingerprint of the *Dunaliella* EPS was observed in both T-brine and the bicarbonate filter cake extracts, 2-*o*-methyl hexose was not observed in either of these extracts. The results, therefore, indicate that while some of the algal EPS is degraded to some degree as brine flows through the pond system, there is a portion that remains in the system and is incorporated into the bicarbonate during the crystallization process, finally reporting to the soda ash product.

This study has confirmed the contribution of algae growing in the ponds to the accumulation of organic matter in the ponds, hence organic contamination of soda ash. Conditions appropriate for the production of EPS by *Dunaliella spp*. prevail in the ponding system.

The study was undertaken to determine processes involved in the accumulation and the fate of organic carbon in the ponding system in order to design a remediation strategy for dealing with

this problem. Based on this study, it can be concluded that remediation strategies for reducing the organic load in the system should aim at:

(a) reducing the total algal biomass, hence primary production in the primary evaporation ponds. This could be achieved in a pre-treatment operation to remove the nutrients influent to the system. A thorough investigation of this operation would be required since nutrient limitation would be expected to be resolved by increased EPS production. However, the amount of EPS produced is dependent on the size of the standing crop in the system and a relatively small amount of EPS would be expected if these stress factors are imposed on a very dilute culture.

(b) operating the downstream ponds (NS, X and ST) such that the oxidising capabilities of the red halophilic bacteria would be favoured to breakdown organic matter produced in the upstream ponds. This would require a detailed study of the growth characteristics of these bacteria.

(c) utilising the adsorption capacity of the pond floor material which was seen to bind EPS material successfully in the sediment layers.

3.5 CONCLUSIONS

Conclusions that can be drawn from this study are summarised below:

1. High nutrient levels in the influent well-brine result in the establishment of halophilic algae in the low salinity ponds. Failure of a benthic community to develop, hence reducing the nutrient load, and lack of the brine shrimp causes the proliferation and overproduction by *Dunaliella spp*. This results in the proliferation of heterotrophic bacteria in the high salinity ponds.

2. Stress conditions of high illumination, increasing salinity and temperature, and nitrogen limitation all interact to enhance organic carbon release by *Dunaliella spp*. These conditions all prevail in Sua Pan solar ponds. A direct correlation between algal biomass and the amount of organic carbon released was observed.

3. *Dunaliella spp*. produce an EPS constituting at least six sugars; arabinose, xylose, 2-*o*-methyl hexose, mannose, glucose and galactose (1.7:1.8:2.8:1.4:1:0.6).

4. Although some of the organic matter produced in the upstream ponds is oxidised in the ponds downstream where growth of heterotrophic bacteria occurs, a portion of these algal organics still occur in the T-brine storage ponds and is incorporated into the bicarbonate during crystallization, causing high TOC levels in the final soda ash product.

CHAPTER 4

REMEDIATION POTENTIAL OF NUTRIENT STRIPPING SYSTEMS

4.1 INTRODUCTION

Nutrients influent to the system has been identified as a major cause of algal blooms observed in the primary evaporation ponds at Sua Pan. The production of polysaccharides by *Dunaliella spp*. in the ponds, when exposed to stress conditions such as salinity, temperature and light, has been demonstrated. Although a portion of the organic matter produced is degraded by bacteria during the course of brine concentration, it is evident that these polysaccharides may constitute a major proportion of the dissolved organic matter contaminating the soda ash produced at Sua Pan.

As already noted, management of algal growth in the preliminary ponds is one of the strategies that could be employed to reduce the organic load contributed by algal production. This can be achieved either by elimination of algae growing in the ponds or by utilising nutrient stripping systems to remove the contaminating phosphorus and nitrogen. The elimination option is usually applied when immediate algal control is required. Algal separation techniques such as filtration may be employed (Truax and Shindala, 1994) but algicides such as chlorine, bromine and copper sulphate are also widely used. The application of compounds such as copper sulphate and alum,

however, is limited in brines containing sodium carbonate since insoluble metal carbonates may precipitate from solution (Seshadri and Buch, 1958).

Nutrient stripping systems has been extensively studied since inorganic nutrients cause eutrophication in coastal waters. Treatment methods include the utilisation of plants (Tsuno *et al*., 1990), microalgae (Craggs *et al*., 1995; Nurdogan and Oswald, 1995; Lau *et al*., 1996; Craggs *et al*., 1997; Lau *et al*., 1997; Sawayama *et al*., 1998) and chemicals, for phosphorus precipitation (Hartley *et al*., 1997). Plants and algae have advantages of renewability and utilization of solar energy (Sawayama *et al*., 1998). Microalgae have a considerable potential since under suitable conditions microalgae can grow at higher rates than plants and their role in nutrient removal in sewage and industrial wastewaters has been well demonstrated (Craggs *et al*., 1995; Nurdogan and Oswald, 1995; Cromar *et al*., 1996; Lau *et al*., 1996; Craggs *et al*., 1997; Lau *et al*., 1997; Sawayama *et al*., 1998).

4.1.1 Objectives

This study was undertaken to evaluate the feasibility of a *Dunaliella* - based algal ponding system for nutrient removal in the influent well-brine. Both laboratory flask studies and scale-up evaluation of the process in a pilot-scale high rate algal ponding (HRAP) system was undertaken. The performance of a pilot-scale (HRAP) plant was monitored to evaluate the potential of the algal polishing model for the management of microbial growth and production in the ponds. The potential of chemical precipitation of phosphorus for nutrient removal, was also evaluated.

4.2 MATERIALS AND METHODS

4.2.1 Algal culture and growth medium

A culture of *Dunaliella spp*. was obtained from a sample of brine from the cooling pond (CS) (see Figure 1.2) where a dense culture of *Dunaliella spp.* is found. This culture was used for both shake flask studies and for inoculating two high rate algal ponds (A and B). Shake flask studies involved the use of both a mixed culture to represent what happens in the system and pure cultures of *D. salina* and *D. viridis* prepared from the above sample. This culture was grown in well-brine for both shake flask and scaled-up studies. The culture was maintained in BAAM medium (Appendix I) which was also used for comparative studies in shake flasks studies.

4.2.2 Growth studies

Growth studies in shake flasks were done in 500ml flasks containing 250ml of the medium inoculated with a 10% culture growing in BAAM medium. Cultures were incubated in a controlled environment chamber at 25^0C on a shaker.

4.2.3 Pond Design and operation

Two pilot-scale algal raceway ponds constructed from concrete and lined with PVC were used for the scale-up studies. The design of these ponds is shown in Figure 4.1. Mass culture of *Dunaliella spp.* was carried out in these ponds which are situated on-site at Sua Pan. Each pond has an area of $20m^2$ and was operated at a depth of approximately 30 cm. Two ponds (A and B) were

operated in parallel. After inoculation of the ponds with the above culture, the ponds were operated in batch mode for a week to allow accumulation of biomass. Tap water was added to compensate for medium loss by evaporation. Samples were taken twice a day in the morning (09h00) and afternoon (16h00) for phosphorus, ammonium, chlorophyll a, and cell count analysis. After a week the operation was changed to a continuous mode. Every day 10% of the contents of Pond A were emptied and replaced with fresh well-brine, similarly 20% of Pond B was replaced. The algal culture was monitored for a period of 40 days.

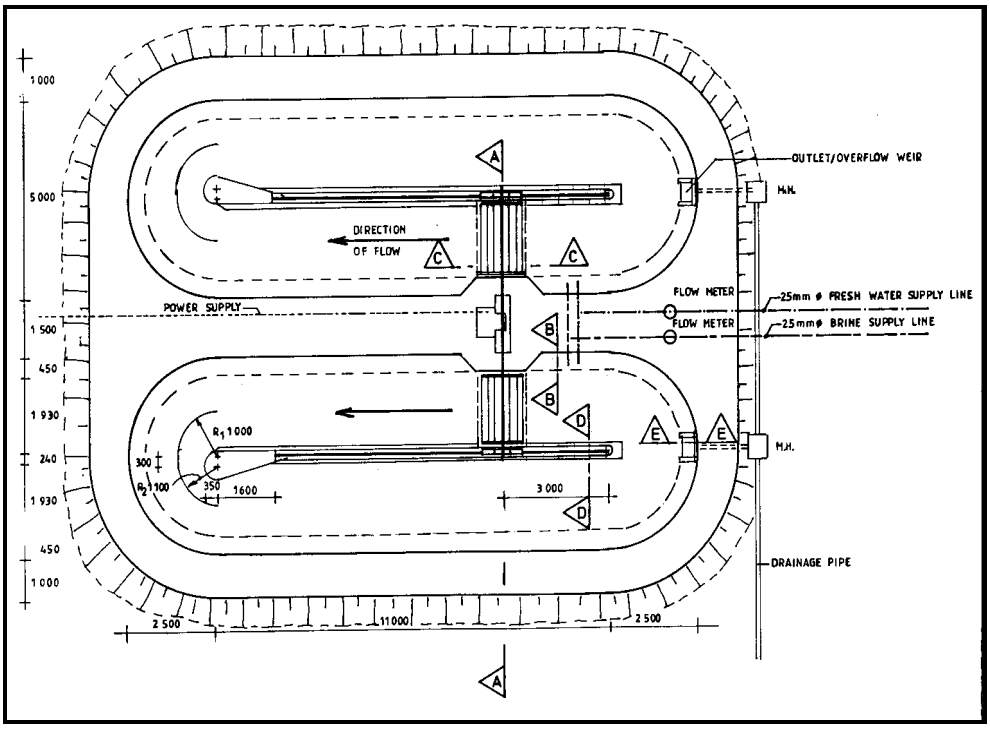

Figure 4.1. Design of the pilot-scale HRAPs

4.2.4 Analyses

Nutrient concentrations were measured against deionized water blanks using ammonium, phosphorus (PMB), and nitrate Spectroquant cell tests supplied by Merck (Darmstadt, Germany). Samples were filtered through a 0.45Fm cellulose acetate syringe filter to remove particulate material and then diluted accordingly with deionized water. The improved Neubaer Haemocytometer was used for the cell count and chlorophyll was extracted as described in section 3.2.3.1.

4.2.5 Chemical Phosphate precipitation

To compare nutrient removal by algae to that of conventional chemical precipitation operations, three precipitants were evaluated; $FeCl_3.6H_2O$, $FeSO_4$, $AlCl_3.6H_2O$ and $CaCO_3$ (Saarchem, Krugersdorp, South Africa). 100ml of well-brine collected from the inlet was added to 250ml conical flasks. Different dosages of these chemicals were added to the brine (2, 4, 6, 8 and 10mg.ml^{-1}) and the flasks well- mixed briefly. The precipitates formed were allowed to settle for approximately 10 minutes and the supernatant liquid carefully withdrawn and filtered through 0.45Fm cellulose acetate syringe filters (Micron Separations Inc.) to remove particulates. Samples were then analysed for phosphorus using the phosphorus cell kit (Merck, Darmstadt, Germany).

4.3 RESULTS

4.3.1 Shake Flask studies

Figures 4.2 and 4.3 show growth of the mixed *Dunaliella spp*. culture in the alkaline well-brine indicating removal of phosphorus and ammonium ions. Nitrate concentrations were not determined in these cultures since repeated analyses have shown that well-brine does not contain nitrate. All the nitrogen occurs almost entirely as ammonium. Phosphorus and ammonium were taken up during algal growth and by the end of the culture period, phosphorus concentration in the brine was reduced from 45 $mg.l^{-1}$ to 23 $mg.l^{-1}$ (49% reduction) (Figure 4.2). Ammonium was reduced to undetectable levels by day 10 (Figure 4.3). Although algal utilisation appears to account for the depletion of ammonium, aeration of cultures could have caused an amount of ammonium to be released as ammonia gas.

Higher growth rates were observed when *Dunaliella spp*. was grown BAAM medium (Figures 4.4 and 4.5). These high growth rates were associated with high removal rates of phosphorus and nitrate concentrations. By the end the culture period, 93% phosphorus and 45% nitrate removal from the medium was observed. The rate of nitrate utilisation was lower compared to phosphorus. Sawayama *et al*. (1998) have reported that different removal profiles of nitrate and phosphorus is due to different cell storage systems for nitrogen and phosphate.

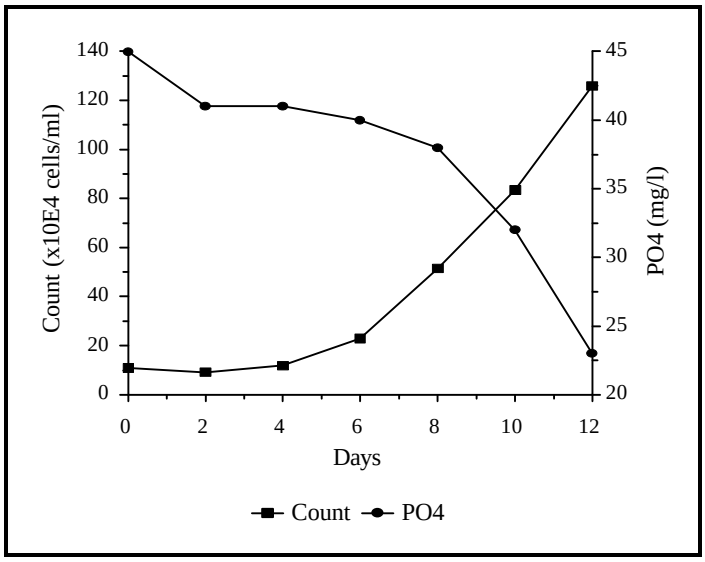

Figure 4.2. Phosphate utilisation by *Dunaliella spp*. grown in well-brine.

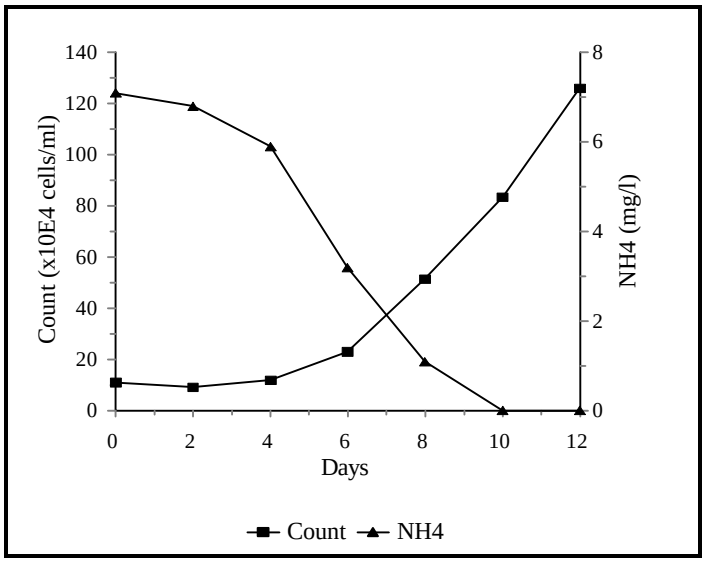

Figure 4.3. Ammonium utilisation by *Dunaliella spp*. grown in well-brine.

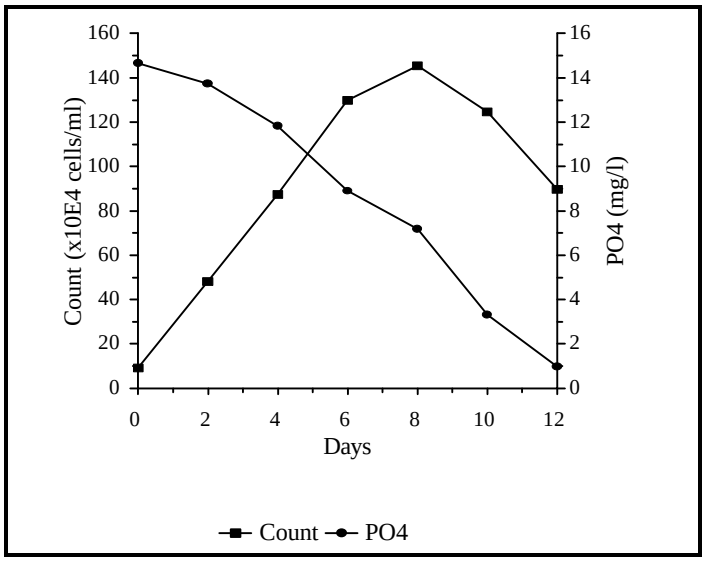

Figure 4.4. Phosphate utilisation by *Dunaliella spp*. grown in BAAM medium.

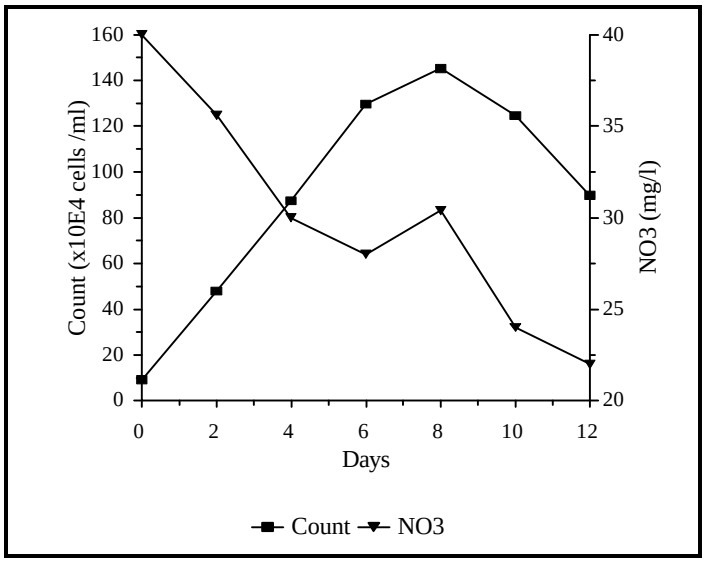

Figure 4.5. Nitrate utilisation by *Dunaliella spp*. grown in BAAM medium.

To investigate the significance of nutrient concentration in the brine on growth of pure cultures of *D. salina* and *D. viridis*, nitrogen and phosphate were supplemented to the brine as KNO_3 and KH_2PO_4 to make up nutrient levels comparable to those of the defined inorganic medium. The results are shown in Figures 4.6 and 4.7. Higher cell densities were obtained in cultures grown in well-brine supplemented with nutrients. This indicated that growth of both *D. salina* and *D. viridis* in ponds is nutrient limited.

Phosphorus and nitrogen removal rates by actively growing *Dunaliella spp*. demonstrated in shake flask studies were found to depend on the biomass attained with high cell densities resulting in high nutrient removal. Although shake flask studies were carried out under controlled conditions in a constant environment chamber, it is possible that the results obtained may be a representation of trends to be anticipated in a scaled-up system.

From these results, it could be postulated that the removal of the contaminating nutrients (P and N) could be achieved in a pre-treatment operation by manipulating a dense culture of *Dunaliella spp*. growing in well-brine. This would reduce algal blooms, hence primary production that occurs in the preliminary evaporation ponds. Rose (1998a) therefore proposed investigation of a strategy whereby microbial growth and resultant organic production in the ponds could be prevented by nutrient starvation of the cooling pond system. The construction of a pilot-scale HRAP was undertaken for this purpose.

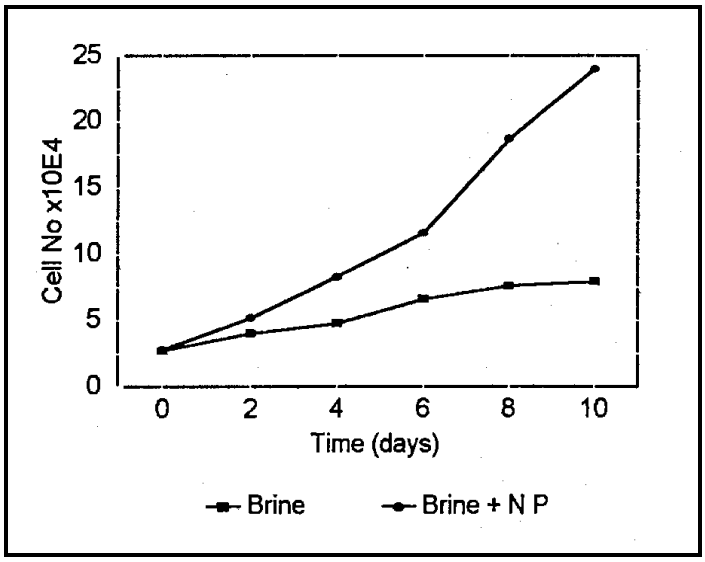

Figure 4.6. Comparison of growth of *D. viridis* in well-brine and well-brine supplemented with nitrogen and phosphorus.

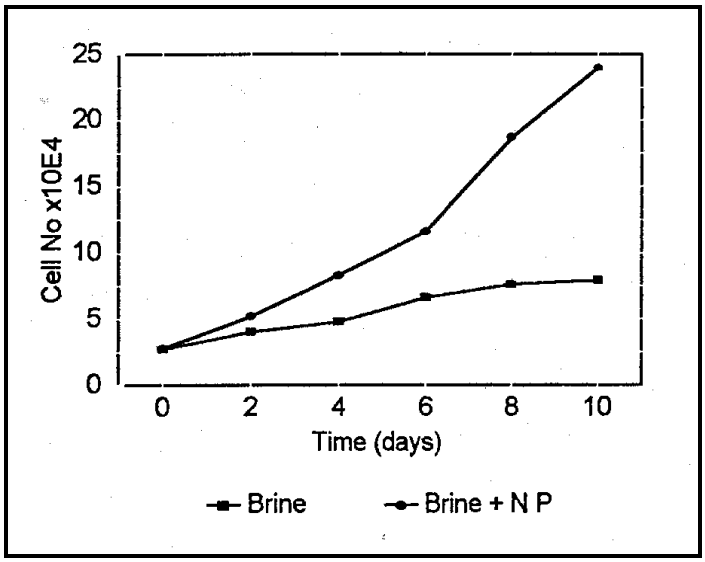

Figure 4.7. Comparison of growth of *D. salina* in well-brine and well-brine supplemented with nitrogen and phosphorus.

4.3.2 HRAP Scale-up Studies

Two HRAPs were operated, Pond A and B. Both Pond A and B were run in batch mode for 7 days for biomass production before the operation was switched to a continuous mode using fresh well-brine as the feed. Pond A was operated at a dilution of 10% and Pond B at 20%. During batch operation of the ponds, the cell density in Pond A reached 5 $\times 10^5$cells.ml^{-1} which corresponded to a 10% phosphorus utilisation (Figure 4.8). Ammonium was completely removed from the brine by day 2 (Figure 4.9). In Pond B the biomass reached 4.6 $\times 10^5$cells.ml^{-1} and phosphorus utilisation of 9.5% was achieved during this period (Figure 4.10). Ammonium was also reduced to undetectable levels within two days (Figure 4.11).

Just after the continuous mode of operation was initiated, a decrease in cell biomass was observed followed by a progressive increase, in a time dependent manner, with the highest cell count in Pond A reaching 320.4 $\times 10^4$cells.ml^{-1} and 174.9$\times 10^4$cells.ml^{-1} in Pond B by the end of the 40-day study period. Phosphorus removal rates also increased during the logarithmic growth of algae and after a cell count of 10^6 cells.ml^{-1} had been attained, an average removal rate of 12 ± 2 mg.l^{-1} phosphorus per day was maintained in both ponds. Residual phosphorus concentrations of 27 mg.l^{-1} and 32 mg.l^{-1} were measured in ponds A and B respectively by the end of the study period. This corresponded to 47% and 37% phosphorus reduction in ponds A and B respectively. In both ponds ammonium was completely removed within one day.

The results obtained in the scale-up studies were in accordance with the shake flask studies implicating a relationship between cell density and the rate of nutrient removal. These results

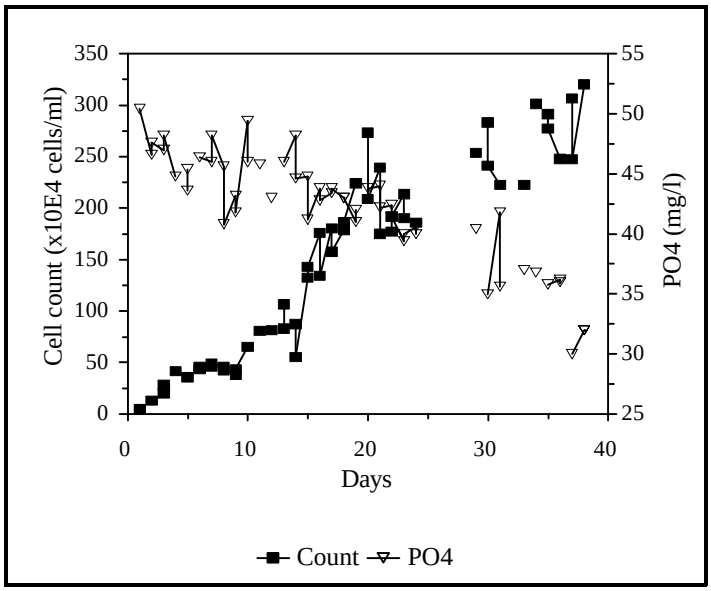

Figure 4.8. *Dunaliella spp*. growth and phosphorus utilisation in Pond A.

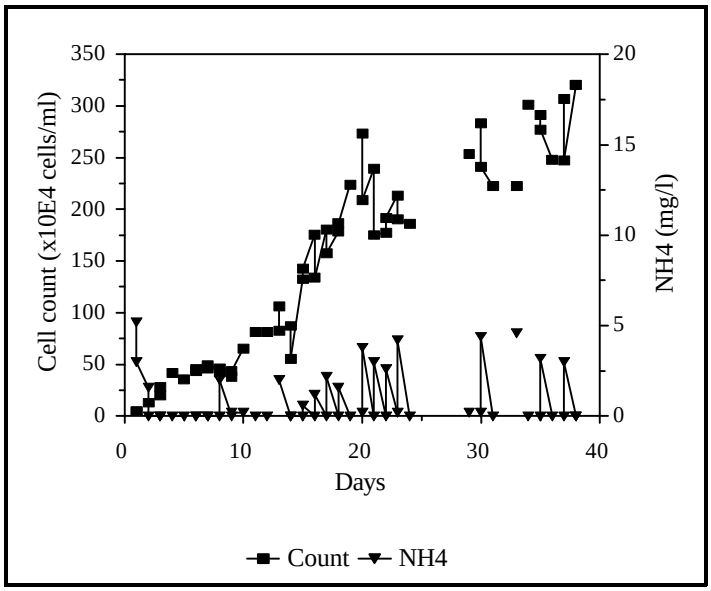

Figure 4.9. *Dunaliella spp*. growth and ammonium utilisation in Pond A.

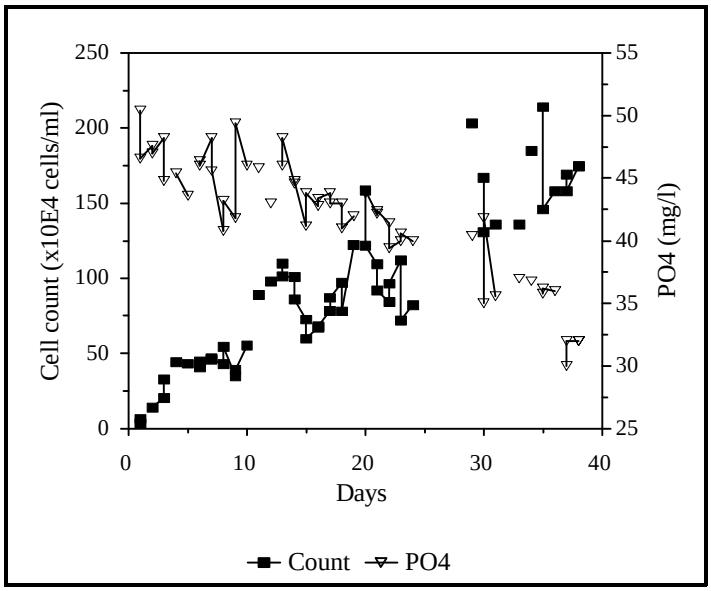

Figure 4.10. *Dunaliella spp*. growth and phosphorus utilisation in Pond B.

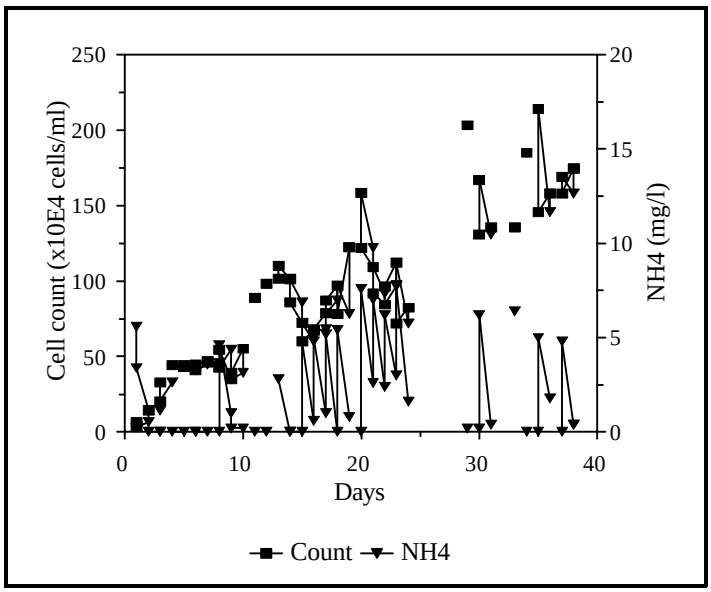

Figure 4.11. *Dunaliella spp*. growth and ammonium utilisation in Pond B.

also showed that while the continuously operated system could sustain dilutions of 10 to 20%, above 20% washout would be expected to occur and this would have an impact on the nutrient removal efficiency of the system. To assess if post HRAP brine would be able to sustain growth of *Dunaliella spp*., growth of actively growing *Dunaliella spp*. in fresh well-brine and filtered brine from Pond A were compared and the results are shown in Figure 4.12. The results showed that while treated brine would still sustain growth of *Dunaliella* to a certain degree, growth would be severely limited by the nutrient stripping operation.

The results obtained in this study were preliminary, to prove the concept of the feasibility of the application of algal biotechnology for the remediation of nutrient contaminated brine. A detailed study on the performance of the HRAP and the impact this treatment will have on the brines of the receiving ponds still needs to be undertaken before a full-scale HRAP pre-treatment operation can be implemented.

4.3.3 Chemical phosphorus precipitation

The potential of four different chemicals for reducing phosphorus concentration in well-brine was evaluated. Phosphorus concentration in well-brine was reduced by all the chemicals tested; $FeCl_3.6H_2O$, $FeSO_4$, $AlCl_3.6H_2O$ and $CaCO_3$ with the highest precipitation obtained with $FeCl_3.6H_2O$ (Figure 4.13). However, very high dosages were required to achieve this precipitation. The amount of phosphorus precipitated increased with dosage up to 10mg.ml^{-1} which decreased the concentration from 44.6mg.l^{-1} in well-brine to 1.7 mg.l^{-1}, accounting for a 96.1% removal when ferric chloride was used. The maximum percentage precipitation obtained with $FeSO_4$, $AlCl_3.6H_2O$ and $CaCO_3$ was 63.2% (dosage:10 mg.ml^{-1}), 69.2% (dosage:8 mg.ml^{-1})

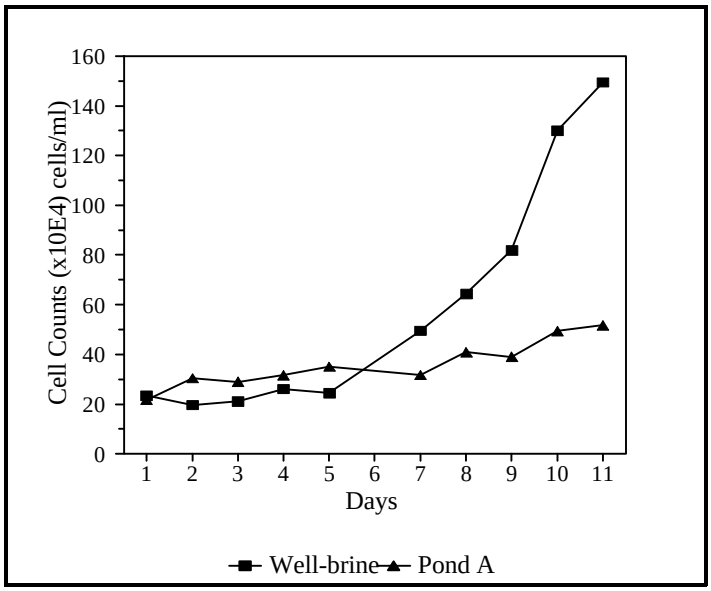

Figure 4.12. Comparison of *Dunaliella spp*. growth in well-brine and depleted medium from Pond A.

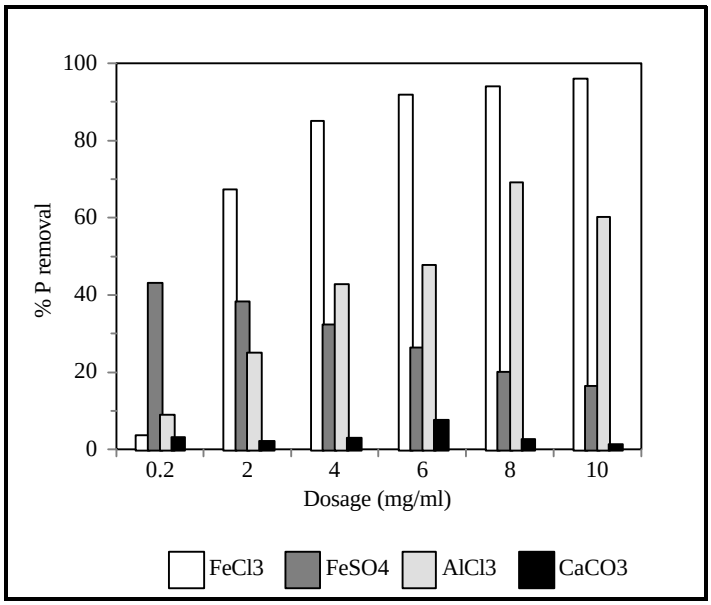

Figure 4.13. Chemical phosphorus precipitation in well-brine.

and 7.6% (dosage:6 $mg.ml^{-1}$) respectively. This corresponded to residual phosphorus concentrations of 16.4 $mg.l^{-1}$, 13.7 $mg.l^{-1}$ and 41.2 $mg.l^{-1}$. None of the chemicals evaluated had any impact on the TOC levels of well-brine during phosphorus precipitation (results not reported).

4.4 DISCUSSION

Growth studies of *Dunaliella spp.* in shake flasks have demonstrated the ability of algal systems to strip inorganic nutrients (P and N) from the alkaline brines found in Sua Pan ponds. Both P and N were simultaneously utilised during algal growth although different removal profiles of phosphorus and ammonium were observed. Overall values for P and N removal from well-brine during the 12 day culture period were 49% and 100% respectively. Shake flask studies clearly demonstrated the feasibility of a HRAP for the pre-treatment of well-brine.

Although higher initial phosphorus and ammonium concentrations were recorded in the scale-up studies, the results observed in shake flask studies provided an indication of what may be anticipated in the scaled-up system. It should be noted that there is a possibility that an amount of phosphorus could have been precipitated out and ammonium may have escaped as ammonia during sample transportation to the laboratory and storage. Nutrient reduction effected by algal assimilation was also observed in HRAPs. Phosphorus uptake was also lower than ammonium uptake in this case. High rates of nutrient removal were observed during the logarithmic growth of the culture and high removal efficiencies corresponding to the standing crop of a pond were

observed. After the 40 day study period, 47% and 37% P removal in ponds A and B respectively was obtained and this corresponded to dilutions of 10% and 20% respectively. Ammonium was completely removed in both ponds within a few hours. These values are within the range that has been reported in algal ponds used for the treatment of sewage wastewater (Craggs *et al*., 1995; Cromar *et al*., 1996; Craggs *et al*., 1997). Phosphorus and nitrogen removal efficiencies in the range 34-100% and 46-100% respectively have been reported by these authors.

The trend of nutrient removal observed in this study, showing rapid removal of nitrogen compared to phosphorus, has also been observed in other studies (Weissman *et al*., 1978; Nurdogan and Oswald, 1995; Craggs *et al*., 1997; Sawanyama *et al*., 1998) and has been reported to be typical of high rate ponds which function as nitrogen-limited systems (Weissman *et al*., 1978). Nitrogen content of algae is approximately ten times more than phosphorus content which may explain the differences in the uptake of these nutrients (Nurdogan and Oswald, 1995). On the other hand, Mc Carthy *et al*. (1977) has demonstrated that phytoplankton preferentially use nitrogen in the form of ammonium.

In a HRAP algae grow in continuously mixed ponds which ensure prevailing aerobic conditions suitable for bacterial oxidation of waste (Oswald, 1988; ; Nurdogan and Oswald, 1995; Almasi and Pescod, 1996). However, bacterial counts and organic carbon profiles of the system were not determined in this study and, therefore, the impact of heterotrophic growth on the system is not known. This could be essential in determining the overall performance of such a system. Algal

heterotrophic growth, which contribute to a certain degree to the organic load reduction in organic wastes, has also been noted in saline algal systems (Rose, 1992; Dunn, 1998).

The assimilation of nutrients by microalgae was correlated to the productivity of the pond. Cromar and Fallowfield (1997) have observed that increasing the retention time of the HRAP increased the algal biomass which in turn resulted in increased P removal. However, an optimum standing crop, above which further productivity or nutrient removal can not be achieved, exists for each pond due to self-shading which occurs when high cell densities are attained (Cromar *et al.*, 1996).

Although complete ammonium removal was achieved in this system, the lowest residual phosphorus concentration was 27 $mg.l^{-1}$ which theoretically is still within the concentration range required for *Dunaliella* growth (Borowitzka and Borowitzka, 1988; Ben-Amotz and Avron, 1989). However, the results have shown that subsequent growth of a fresh inoculum of *Dunaliella* cells in brine treated in a HRAP was severely limited. Ryther and Dunstan (1971) have indicated that nitrogen is in most cases the limiting nutrient for algal growth in coastal marine waters. It has been shown in chapter 3 that where conditions of high light intensity, salinity and temperature prevail, nitrogen limitation would be anticipated to be resolved by EPS production which may lead to accumulation of organic matter.

For comparative studies, chemical precipitation of phosphorus was evaluated. Phosphorus precipitation was achieved with all the chemicals tested with ferric chloride giving the highest

removal efficiency and $CaCO_3$ the lowest. The percentage phosphorus removal was in the order $FeCl_3.6H_2O$>$AlCl_3.6H_2O$>$FeSO_4$>$CaCO_3$. Ferric chloride dosages of $10g.l^{-1}$ were required to achieve phosphorus removal efficiencies of more than 90%. Doses of ferric chloride could, therefore, be used for nutrient removal in the system. This has an advantage of precipitating out the dissolved hydrogen sulphide which is inhibitory to algal growth. However, high dosages required may impose economical limitations on the application of ferric chloride. Nurdogan and Oswald (1995) have demonstrated that enhanced nutrient and algal removal in HRAPs can be achieved by the addition of calcium and magnesium ions to the system.

It should be noted that this study was designed as a proof-of-concept investigation of the feasibility of algal systems for nutrient removal in the alkaline well-brine found at Sua Pan. The results reported are, therefore, essentially preliminary and comprehensive follow-up studies would still need to be undertaken. The value of scale-up studies in the design of large-scale operations have been highlighted by Borowitzka and Borowitzka (1989).

4.5 CONCLUSIONS

Although the results obtained in this study requires further substantiation, it has been demonstrated that algal systems have a potential in the removal of nutrients in well-brine. By manipulating a dense culture of *Dunaliella spp*. ahead of the cooling system, complete removal of ammonium can be anticipated, while phosphorus removal efficiencies of approximately 50% may be attained during unoptimised operation. This study was undertaken to evaluate the potential of nutrient

stripping systems for brine remediation and it was clearly shown that algal growth in treated brine is substantially reduced. Similar effects were shown for ferric chloride but large dosages were required, indicating some economic constraints. The further investigation of the HRAP approach would be needed to establish its application for the biological management of the ponding system at Sua Pan.

CHAPTER 5

REMEDIATION POTENTIAL OF BACTERIAL SYSTEMS CONSUMING ORGANIC COMPOUNDS

5.1 INTRODUCTION

There is relatively little known about the potential of halophilic bacteria to degrade pollutants at high salt concentrations (Oren *et al.*, 1992). Several authors have reported the ability of some halophilic bacteria to degrade easily degradable substrates such as simple sugars and amino acids (Oren, 1988; Oren *et al.*,1992), complex substrates such as aromatic compounds (Harwood and Gibson, 1988; Bertrand *et al.*, 1990; Kamal and Wyndham, 1990; Yanase *et al.*, 1992; Woolard and Irvine, 1994; 1995a; 1995b) and halocarboxylic acids (McGrath and Harfoot, 1997). However, for most of these studies, eubacteria were mainly responsible for degradation of these compounds since degradation occurred at moderate salinities.

Most of the labile organic matter is remineralised aerobically at the sediment-water interface where organic matter is initially deposited (Arnosti *et al.*, 1994; Kristensen *et al.*, 1995). Two major physiological groups of aerobic heterotrophic bacteria found in hypersaline environments are the extremely halophilic archaebacteria of the genera *Halobacterium*, *Halococcus*, *Haloferax* and *Haloarcula*, and the halophilic or halotolerant eubacteria (Oren *et al.*, 1992). Eubacteria maintain

low intracellular salt concentrations, hence their metabolic diversity is greater than in archaebacteria (Oren *et al*., 1992).

Anaerobic mineralisation of organic matter involves a consortium of microorganisms (Arnosti and Repeta, 1994; Canfield, 1994; Ollivier *et al*., 1994; Arnosti *et al*., 1994; Kristensen *et al*., 1995). Sulphate is an important electron acceptor involved in the mineralisation of organic matter in hypersaline sediments (Oren, 1988; Ollivier *et al*., 1994). Biological treatment of saline wastewater usually results in reductions in COD removal rate and efficiency (De Baere *et al*.,1984; Belkin *et al*., 1993; Shipin *et al*., 1994; Kargi and Dincer, 1996). Sodium, chloride and sulphate ions found in these waters impose inhibition or toxicity effects during biological treatment (De Baere *et al*., 1984; Rinzema *et al*.,1988; Liu and Boone, 1991; Veiga *et al*., 1992a; 1994). Efficient anaerobic digestion of saline wastewaters, however, can be achieved by adapting the sludges to high salinity levels as has been done in the fish-canning industry (Mendez *et al*., 1992; Veiga *et al*., 1992b; 1994). Halophilic anaerobic bacteria have been reviewed (Oren, 1988; Oren *et al*., 1992; Lowe *et al*., 1993; Ollivier *et al*., 1994) but the significance of these bacteria on organic remineralisation is still not known.

Measurements of brine TOC in the ponds have shown that levels lower than would be anticipated are maintained throughout the ponding system (see Figure 2.1). This is a clear indication that organic matter remineralisation does occur to some degree as the brine proceeds through the ponding system, implying the potential of bacterial systems for remediation purposes.

5.1.1 Objectives

This study was undertaken to isolate bacteria growing in the solar ponds and evaluate the feasibility of biological treatment of brine containing a high load of organic matter using these organisms. Different bacterial systems were evaluated under both aerobic and anaerobic conditions for the remediation of T-brine.

5.2 MATERIALS AND METHODS

5.2.1 Enrichment cultures

5.2.1.1 Anaerobic cultures

Anaerobic cultures were enriched in 5l flasks containing about 3l of enrichment medium of the following composition ($g.l^{-1}$): K_2SO_4, 2; $MgCl_2$, 1; Na_2S, 0.5; NH_4Cl, 1; K_2HPO_4, 1; vitamin solution, 10ml and trace element solution, 10ml (Appendix I). This medium was inoculated with sediment samples from ponds E0, E2 and ST2. NaCl was used to adjust salinity of the culture medium to 11,18, 23,25% and saturated NaCl. The flasks were left in a controlled environment chamber at 30^0C until activity indicated by blackening of the medium was observed. These cultures were used to feed gravel packed filters which were used to investigate the ability of these organisms to degrade organic compounds in the brine (see Figure 5.1).

5.2.1.2 Aerobic cultures

Enrichment of the aerobic halobacteria was achieved by supplementing T-brine with 1% (w/v) yeast extract powder (Biolab, Johannesburg, South Africa). The enriched cultures were incubated in a controlled environment chamber at 37^0C, on an orbital shaker (170rpm). The culture developed an intense pink to red colour after a 7-day incubation period. To isolate the organisms, this culture was streaked on solid halophilic medium (see Appendix I for composition) and yeast extract medium. To prepare yeast extract medium, T-brine was supplemented with 1% (w/v) yeast extract and solidified with 2% (w/v) agar-agar (Merck, Darmstadt, Germany). The brine was diluted to 25% salinity with deionized water. The agar was dissolved in deionized water and autoclaved separately from the brine and yeast extract. A brown precipitate was formed and the agar did not solidify when all the components were autoclaved together. Colonies were formed on the yeast extract medium within five days whereas it took at least two weeks for growth to appear on the halophilic medium.

5.2.2 Degradation experiments

5.2.2.1 Aerobic/Anaerobic Filters

Glass columns, 1m long with an internal diameter of 6cm and packed with quarried chip stone to provide an attachment medium for the bacteria (Figure 5.1) were set up. These columns were seeded with anaerobic cultures grown in flasks. Two columns were set up, one operated aerobically by continuously sparging with air and the other anaerobically. The columns were irradiated by high light intensity. This was to allow development of photosynthetic bacteria. Two litres of T-brine was fed into these columns at a pumping rate of 100ml.min^{-1}. No growth was observed in the aerobic column whereas a purple-pigmented bacterium developed in the anaerobic

column. The feed was then switched from T-brine in the aerobic column to well-brine. Three columns were operated according to the table below.

Filter	Feed
Aerobic	Well-brine
Anaerobic 1	T-brine
Anaerobic 2	Well-brine

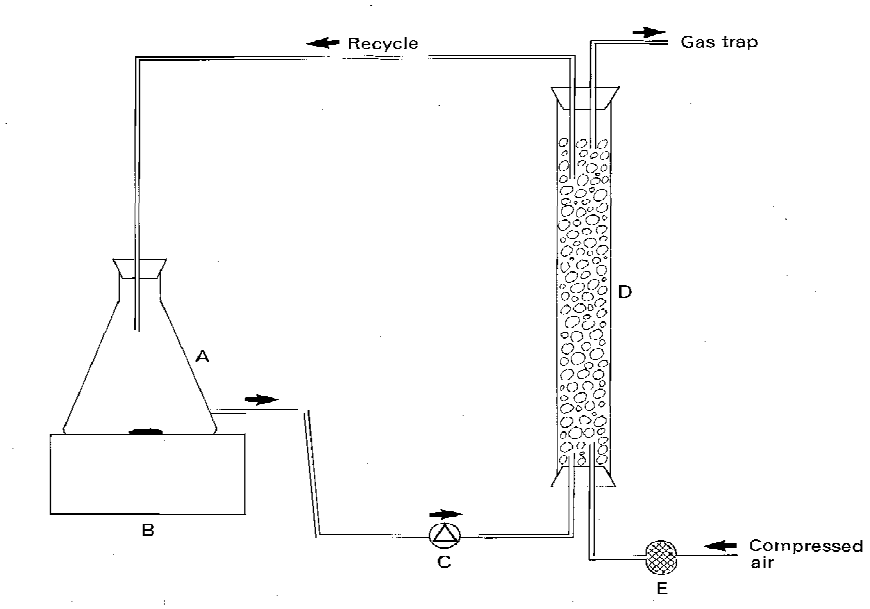

Figure 5.1. Schematic diagram showing the design of the gravel packed filters. **A**-2l Flask containing the feed; **B**-Magnetic stirrer; **C**-Peristaltic pump; **D**-Glass column packed with gravel; **E**-Air filter.

5.2.2.2 Shake flask studies

Aerobic bacteria isolated above were used as inocula for shake flask studies. 250ml of T-brine was inoculated with a 5% inoculum of a five-day culture in 500ml flasks and incubated in a rotary

absence of light by covering the flasks in foil. Every two days samples were drawn, filtered through 0.22Fm cellulose acetate filter (Micron Separations Inc.) before TOC analysis.

To produce soda ash from the brine samples after treatment, the following procedure was followed:

1. Brine (700ml) was heated to 55^0C.
2. Carbon dioxide gas was sparged through and an electric blender used to achieve vigorous mixing.
3. The reaction was allowed to continue until a pH of 7.4 was recorded and then CO_2 gas and mixing were immediately stopped.
4. The solution was allowed to cool to approximately 32^0C before decanting most of the liquor.
5. The bicarbonate formed was filtered through Whatman No.1 filter paper in a Buchner funnel and washed with 200ml deionized water.
6. The bicarbonate was air dried before calcined at 210^0C in an oven and the ash formed analysed for TOC by the coulometry method (Appendix II).

5.2.2.3 Algal organics

The ability of halobacteria to degrade algal polysaccharide was investigated. *Dunaliella* grown under high light intensity (30^0C) was centrifuged (1000 rpm, 10^0C, 15 minutes) using a JA-20 Beckman centrifuge and the depleted medium enriched with the following salts ($g.l^{-1}$): NH4Cl, 1; MgSO4.7H2O, 5; KCl, 2. 100ml of this medium was inoculated with halobacteria growing in log phase. The halobacteria culture was centrifuged at 15000 rpm, 20^0C, washed with salts medium

(halophilic medium without casamino acids, yeast extract and proteose peptone) and suspended in the same medium. 5% of this inoculum was used to inoculate algal depleted medium. The flasks were incubated at 37^0C under aerobic/light, aerobic/dark, anaerobic/light and anaerobic/dark conditions. Control samples were not inoculated.

5.2.3 Immobilization experiments

5.2.3.1 Attachment

Attachment of halobacteria to the following solid support materials was investigated: porous glass beads, wood chips, polystyrene beads and soda glass chips. These materials were added to a flask containing 100ml of T-brine enriched with 1% (w/v) yeast extract. The flasks were incubated on a shaker at 37^0C and any attachment observed microscopically. To select for bacteria able to stick to glass beads, as soon as growth was observed, the glass beads were washed several times in sterile yeast extract medium and then incubated with inoculated medium. This was repeated several times and growth observed under scanning electron microscopy (SEM).

Two types of reactors were evaluated for the immobilisation of halobacteria. A sequencing batch biofilm reactor (SBBR) was set up according to Woolard and Irvine (1994) to assess the ability of bacteria to form a biofilm on silicone tubing. The reactor consisted of coils of silicone tubing suspended in a glass reaction vessel. Air was supplied and allowed to diffuse through the silicone tubing, which also provided a surface for biofilm formation. The reactor design is shown in Figure 5.2. The reactor was fed with T-brine supplemented with 1% (w/v) yeast extract. This reactor was run for two weeks before a biofilm was formed on the silicone rubber tubing.

A transverse-flow capillary membrane bioreactor described by Burton *et al*. (1998), was also evaluated for the remediation of T-brine (Figure 5.3). In such a reactor, the membrane function as an immobilization matrix, therefore, allowing the reaction to occur simultaneously with a separation function. The reactor was inoculated with a culture of halobacteria suspended in salts medium and then fed with T-brine. The TOC of the permeate was monitored.

5.2.3.2 Entrapment

5.2.3.2.1 Alginate

Halobacteria growing in yeast extract medium were centrifuged (10 000 rpm for 25 minutes) and washed with salts medium. A 3.5% (w/v) solution of alginic acid was autoclaved and cooled to approximately 40^0C before the cells suspended in salts medium were added a final concentration of 1.75% (w/v) alginate. The mixture was then extruded dropwise from a pipette into a gently stirred solution of cold 2% (w/v) $CaCl_2.2H_2O$ to form beads with cells entrapped inside. The beads were left to harden in the calcium chloride solution for one hour before being washed with salts medium. These beads were used to inoculate T-brine and TOC was then monitored.

5.2.3.2.2 Agar

A culture of halobacteria was centrifuged as above and added to a solution of 3% (w/v) agar-agar (50^0C-40^0C). The agar was allowed to solidify and fragmented using a sieve. The agar with cells entrapped were washed in salts medium.

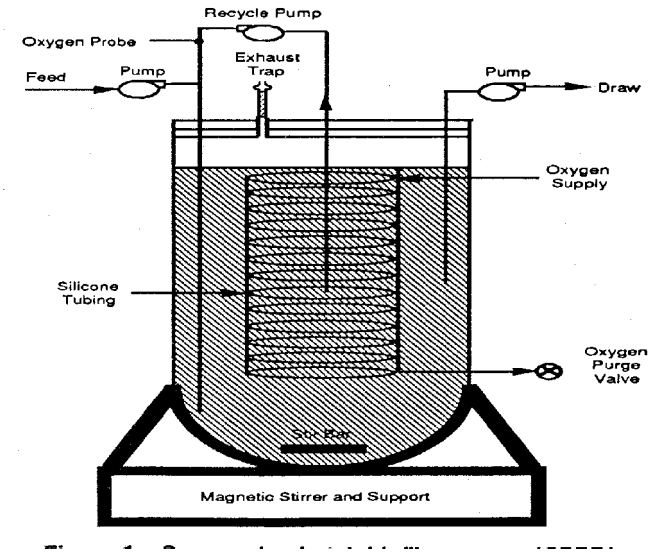

Figure 5.2. Diagram showing the design of the SBBR (Woolard and Irvine, 1994).

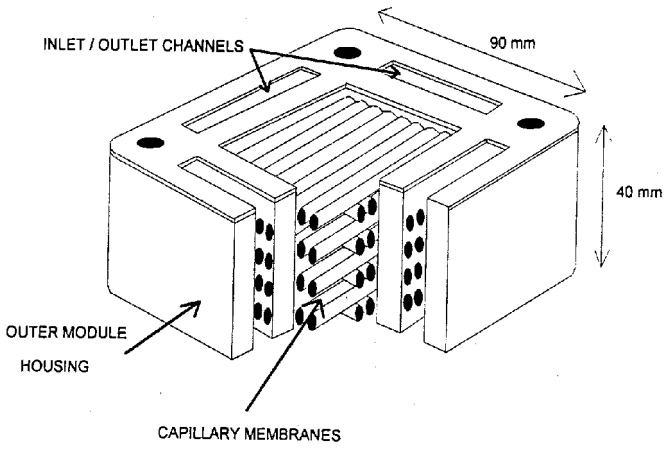

Figure 5.3. Diagram showing the transverse flow capillary membrane bioreactor module (Burton *et al*., 1998).

5.2.4 Analyses

5.2.4.1 Growth

Growth was monitored by measuring the optical density of the culture at 660nm using a UV-160 Shimadzu spectrophotometer.

5.2.4.2 Electron Microscopy

Preparation of samples for Scanning Electron Microscopy (SEM) were done according to Cross (1979). Samples were fixed overnight in 2.5% (v/v) glutaraldehyde in 0.1M sodium phosphate buffer and then washed in 0.1M sodium phosphate buffer. After two buffer washes, the samples were dehydrated by transfer through a series of ascending concentrations of ethanol (30-100%). This was followed by critical point drying using liquid carbon dioxide and the samples were mounted on specimen stubs and sputter coated with a thin layer of gold. Samples were examined on a JEOL JSM 840 Scanning Electron microscope.

5.2.4.3 TOC

Brine samples were filtered through a 0.22Fm cellulose acetate syringe filter (Micron Separations Inc., Westboro, MA, U.S.A.), acidified with nitric acid to pH 2-3 and sparged with air for approximately 6 minutes to remove the inorganic carbon. 40Fl of the sample was analysed by the Total Organic Carbon analyser (Dohrmann DC-180).

5.2.3.4 Organic extraction

After the filters had been run for two weeks, 100 ml of brine from the columns was extracted with the same volume of chloroform and one litre extracted with ethyl acetate by following the same

procedure as in section 2.2.1.2.2. Chloroform extracts were analysed by IR and ethyl acetate extracts by ^{1}H NMR.

5.2.3.5 Nutrient analysis

Phosphorus, ammonium and nitrate were analysed using Spectroquant cell test kits. Samples were filtered through 0.22Fm cellulose acetate syringe filters and diluted accordingly before analysis for nutrients.

5.3 RESULTS

5.3.1 Isolation of bacteria and characterisation of growth

Three aerobic isolates were obtained from T-brine and these were classified as either pigmented or non-pigmented. One non-pigmented strain was isolated and this isolate formed yellowish colonies whereas the other two formed deep red and pink colonies. Although it was possible that the red and pink isolates belonged to the same species, their growth was characterised separately in this study. The non-pigmented strain was a moderate halophile, growing optimally at 10% salinity although growth was observed over a wide range of salinity (Figure 5.4). Both the pigmented strains showed optimal growth at 15% but the overall biomass attained at the end of the culture period was as high at 25% salinity and highest at 32% for the pink isolate (Figures 5.5 and 5.6).

All the isolates grew optimally at 45^0C and pH > 9 and growth was severely retarded at 25^0C (Figures 5.7 to 5.9). The presence or absence of light did not seem to have a major impact on growth of the pigmented isolates (Figures 5.10 to 5.12) but high light intensities were inhibitory to these isolates. Microscopical examination have indicated the presence of motile rods for the pigmented isolates. However, the scanning electron micrographs showed a mixture of rods and cocci for all the isolates (Figure 5.13).

5.3.2 TOC reduction by select cultures

The ability of the above isolates to degrade organic matter in T-brine was evaluated in shake flask cultures. A mixed culture was used to represent biological activities occurring in the ponds. Activity of these bacteria was investigated under both aerobic and anaerobic conditions in the presence and absence of light. It was important to evaluate these conditions since at any particular point in the water column, a halobacterial cell will be exposed to a combination of these conditions.

Halobacteria grew aerobically, reducing the organic carbon content of T-brine by 27% in eight days (Figures 5.14 and 5.15). More TOC degradation was observed in the absence of light for the first four days. On the other hand when algal extracellular organic carbon was used as a carbon source for halobacteria, rapid growth associated with rapid organic carbon degradation was observed (Figures 5.16 and 5.17). TOC reduction was also enhanced in the absence of light and by day 6, 64% reduction was observed under dark conditions compared to 54% under light conditions. No growth or TOC reduction was observed in cultures incubated in an anaerobic chamber (Figures 5.16 and 5.17).

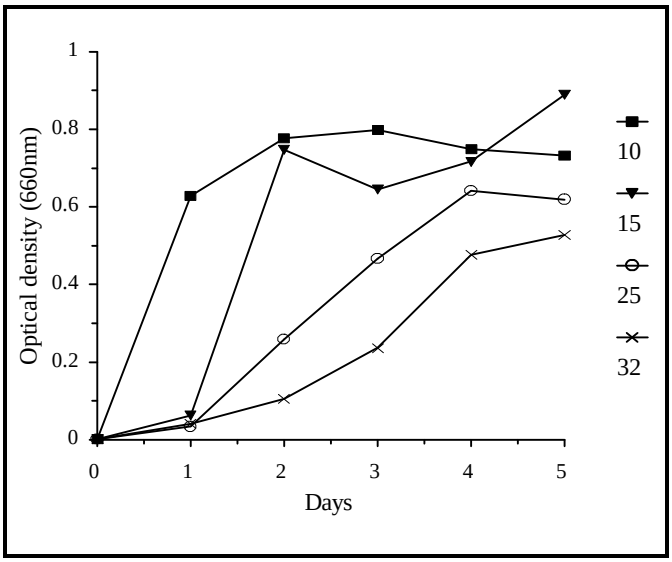

Figure 5.4. Effect of salinity on growth of the yellow isolate at salinities 10, 15, 25 and 32%; pH 9.4, temperature 37^0C.

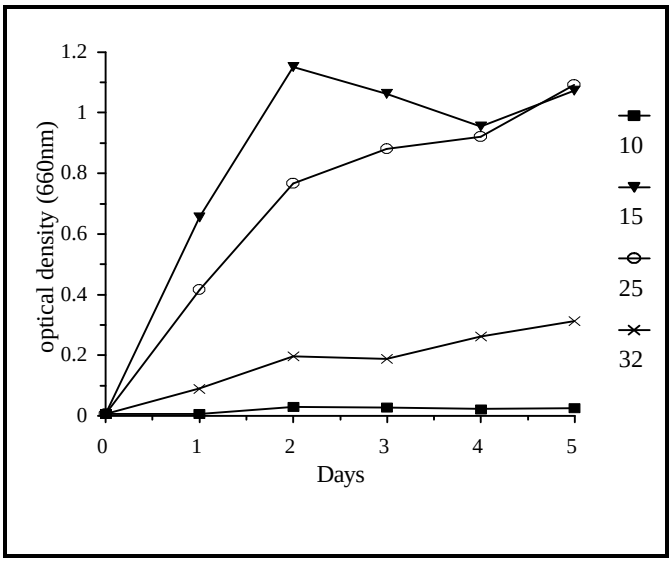

Figure 5.5. Effect of salinity on growth of the red isolate at salinities 10, 15, 25 and 32%; pH 9.4, temperature 37^0C.

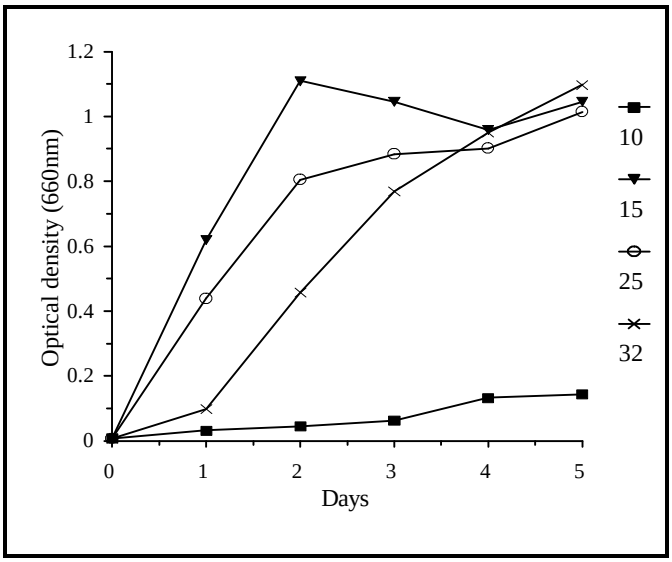

Figure 5.6. Effect of salinity on growth of the pink isolate at salinities 10, 15, 25 and 32%; pH 9.4, temperature 37^0C.

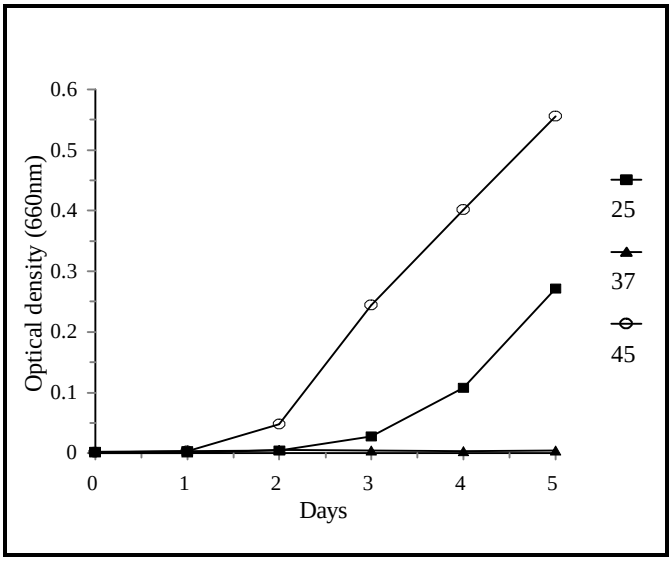

Figure 5.7. Effect of temperature on growth of the yellow isolate at 25, 37 and 45^0C. pH 9.4, salinity 25%.

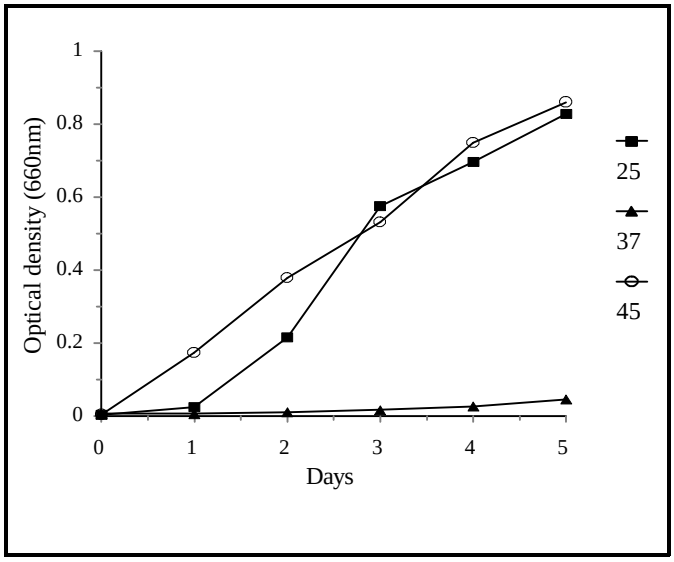

Figure 5.8. Effect of temperature on growth of the red isolate at 25, 37 and 45^{0}C. pH 9.4, salinity 25%.

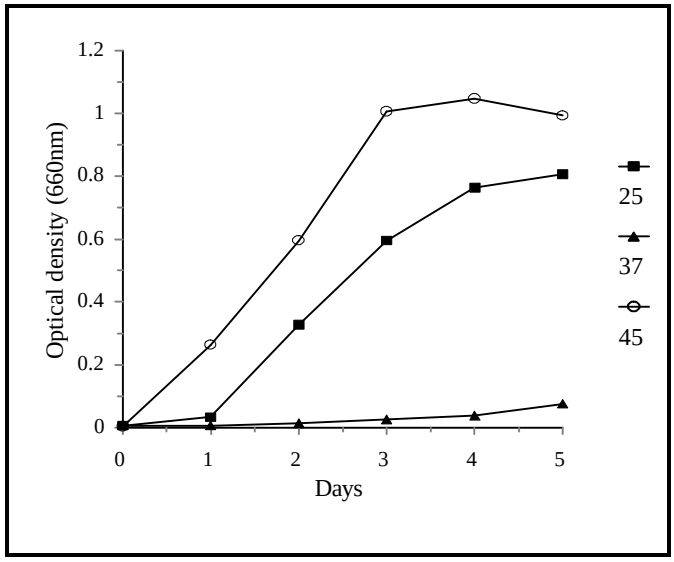

Figure 5.9. Effect of temperature on growth of the pink isolate at 25, 37 and 45^{0}C. pH 9.4, salinity 25%.

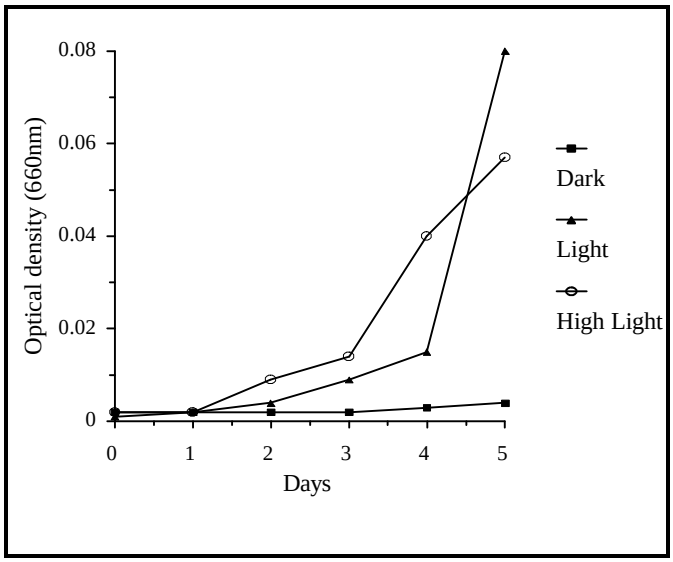

Figure 5.10. Effect of light intensity on growth of the yellow isolate; salinity 32%, pH 9.4.

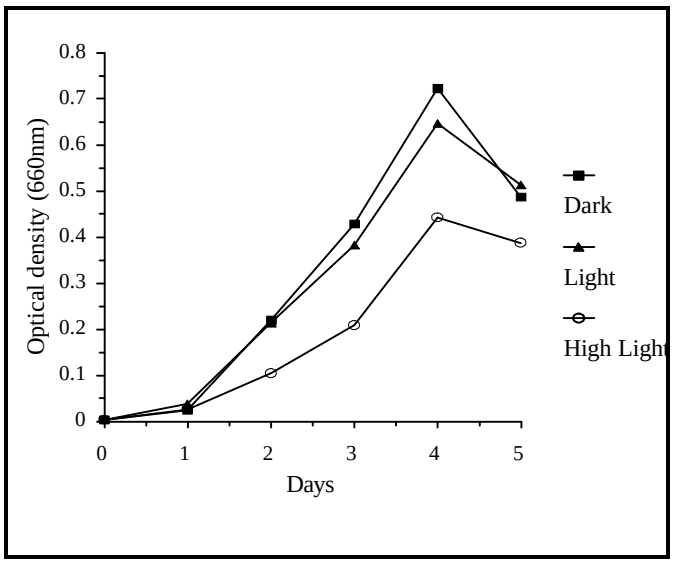

Figure 5.11. Effect of light intensity on growth of the red isolate; salinity 32%, pH 9.4.

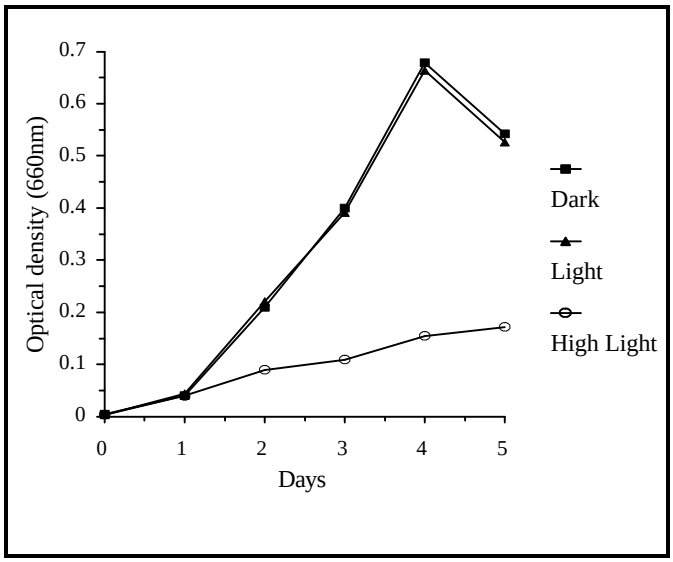

Figure 5.12. Effect of light intensity on growth of the pink isolate; salinity 32%, pH 9.4.

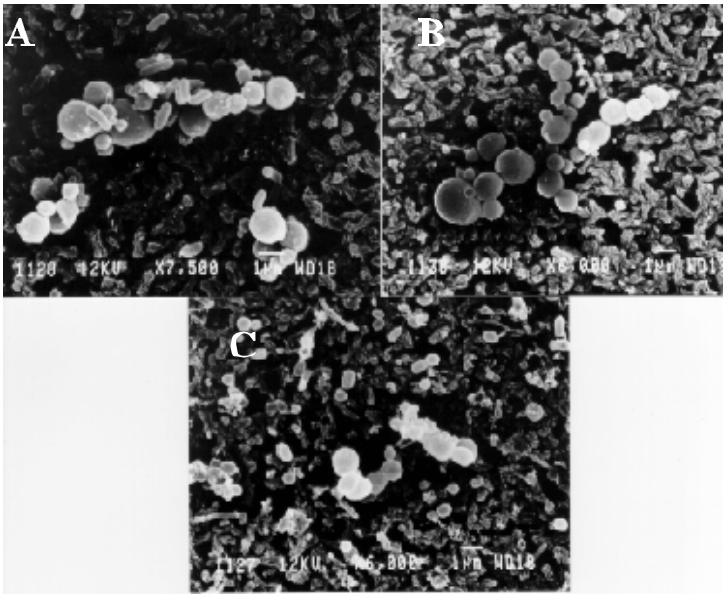

Figure 5.13. Scanning electron micrograph of **A**. Yellow isolate **B**. Red isolate **C**. Pink isolate.

Soda ash was produced from T-brine samples treated under aerobic conditions and the results are shown in Table 5.1. Halobacterial activity had very low impact on the quality of the soda ash product. No TOC reduction was observed in soda ash produced from T-brine treated under aerobic conditions in the presence of light (Table 5.1), instead an increase was observed. TOC reduction was also observed in control samples. This was expected since the brine used was not sterilized.

Table 5.1. Results showing soda ash quality produced from treated brine.

Sample	Soda ash TOC (ppmC)	% Reduction
T-brine	1868	
Control	1797	3.8
Aerobic/Light	2051	-
Aerobic/Dark	1778	4.8

5.3.3 Immobilization of halobacteria

Shake flask studies were undertaken to evaluate the potential of halobacteria for organic carbon degradation in T-brine. The results indicate that, on optimisation, bacterial systems may have a great potential for the remediation of T-brine. For scale-up studies, immobilization systems were investigated in order to design a reactor which could be applied for this type of operation. Immobilization by attachment to support materials and by entrapment in organic polymers was evaluated. Attachment to solid support materials tested was not successful (Table 5.2) except for the silicone tubing in the SBBR on which a thick biofilm developed. It was difficult to assess attachment on wood chips since coloured compounds (tannins) were released into the medium, colouring it brown to black. The results were therefore reported as negative. Although

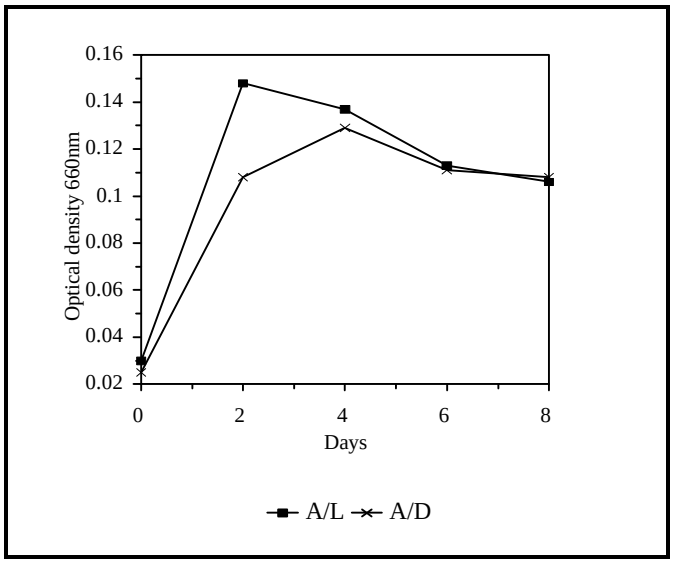

Figure 5.14. Growth of halobacteria in T-brine under aerobic/light (A/L), aerobic/dark (A/D) conditions.

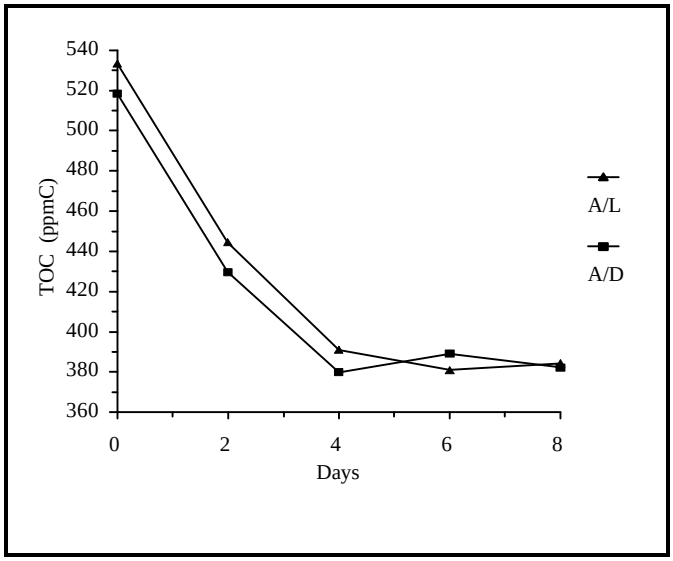

Figure 5.15. TOC measurements in T-brine treated under aerobic/light (A/L), aerobic/dark (A/D) conditions.

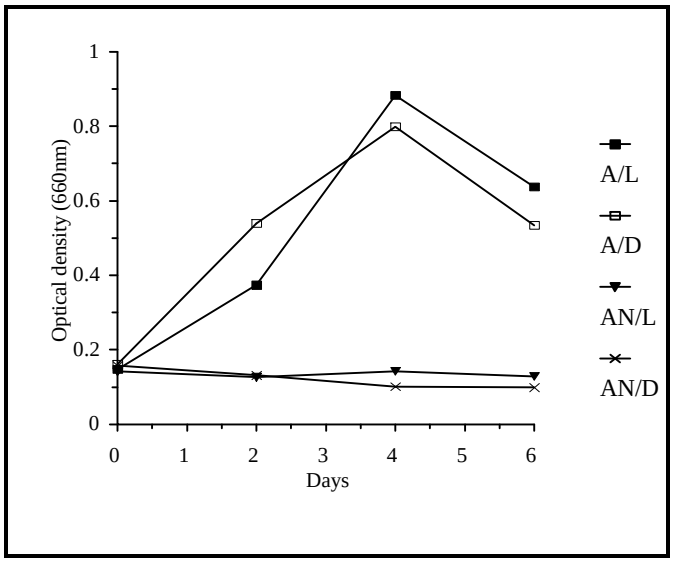

Figure 5.16. Growth of halobacteria utilising algal depleted medium as carbon source under aerobic/light (A/L), aerobic/dark (A/D), anaerobic/light (AN/L) and anaerobic dark (AN/D) conditions.

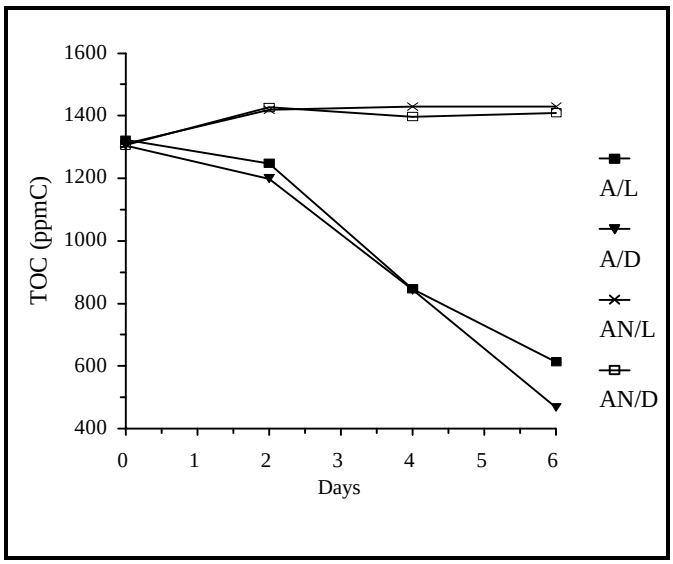

Figure 5.17. Utilisation of algal depleted medium by halobacteria grown under aerobic/light (A/L), aerobic/dark (A/D), anaerobic/light (AN/L) and anaerobic dark (AN/D) conditions.

immobilization on other support materials was not successful, selection of bacteria able to attach to the porous glass beads was achieved. The morphology of the bacteria changed to elongated rods as shown in the scanning electron micrograph in Figure 5.18.

Table 5.2. Results showing attachment of halobacteria on various support materials.

Material	Attachment
Glass beads	+
Wood chips	-
Polystyrene	-
Pumice stone	-
Silicone tubing	+++

+++ = Excellent ++ = Good + = Poor - = No attachment

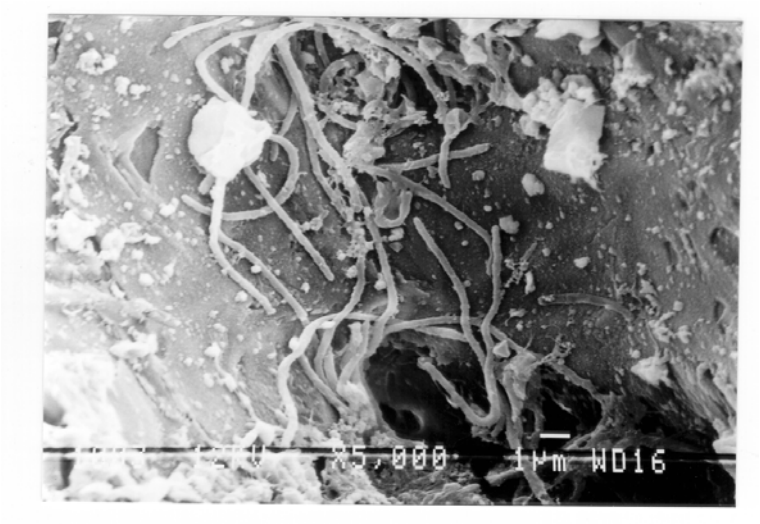

Figure 5.18. Scanning electron micrograph of glass beads after several transfers into sterile medium.

Despite the success in the selection of bacteria able to attach to the porous glass beads, the application of these beads was limited by the fact that, upon inoculation of T-brine with these beads, an increase in brine TOC was observed. A thick biofilm was formed in membrane-aeration systems such as the silicone tubing in a SBBR. However, there was no TOC reduction observed and some of the biofilm was lost when the SBBR was fed with T-brine. The characteristic pink colour of T-brine was removed when the capillary membrane system was used but the TOC of the brine remained unaltered. Another successful immobilization approach was the entrapment of these bacteria into alginate and agar polymers. However, this approach was not suitable in this case because the high pH of brine caused the dissolution of alginate and agar beads when used to inoculate the brine. This resulted in an increase in the TOC levels of the brine (results not shown).

5.3.4 Gravel Pack Filters

Attempts to immobilize halobacteria on solid support materials and by entrapment in organic polymers was not successful. A different approach was therefore investigated in reactors packed with chip stone. To allow for the development of a population able to attach to these stones, the feed brine was continuously recycled.

Anaerobic red-pigmented phototrophic bacteria immobilized on the gravel pack were observed whereas there was no growth in the aerobic column fed with T-brine. Pigments produced by these bacteria had absorption maxima at 498, 534, 359, 761 nm in methanol. This indicates the presence of bacterioruberin, and bacteriochlorophyll *a* (Britton, 1985). When the feed was switched from T-brine to well-brine, algal growth developed under both aerobic and anaerobic conditions. Both TOC and nutrient concentrations were monitored. The TOC profile of the brine shows that there was no reduction in the organic load of T-brine treated in the anaerobic filter (Table 5.3). Failure

of bacterial activity to reduce the organic load in brine may have been due to two factors; under these conditions, bacteria grew photosynthetically utilising light as an energy source or the bacteria may break down the complex organic compounds into smaller compounds without altering the total organic carbon content of the brine. On the other hand, an increase in brine TOC was observed in filters fed with well-brine.

Organic extracts of brine samples were analysed by ^{1}H NMR and IR to determine the impact of microbial activity on the organic fraction of the brine. The solvents used were found to extract the same fractions from both well-brine and T-brine and IR spectra of T-brine and well-brine were similar. There was no significant difference in the organic fractions extracted into chloroform in untreated and treated brine (Figures 5.19 to 5.22). On the other hand, NMR spectra of T-brine showed the presence of different organic compounds in the brine before and after treatment (Figures 5.23 and 5.24). Since the compounds were not identified, it may be speculated that these results indicated that organic compounds were broken down or converted into new compounds in the anaerobic filter fed with T-brine. The brine TOC level however, remained unaltered. Figures 5.25 to 5.27 show NMR spectra of extracts obtained from filters fed with well-brine and the appearance of new peaks were artefacts of treatment.

Table 5.3. TOC profile in gravel packed aerobic and anaerobic filters.

Time (days)	TOC (ppmC)		
	Aerobic	**Anaerobic 1**	**Anaerobic 2**
0	182.09	369.17	189.4
2	195.7	375.37	194.99
4	198.19	356.69	239.98
6	203.91	383.15	226.54
8	203.47	342.09	240.67
10	206	366.01	225.73

There was a reduction in nutrient concentrations in filters fed with well-brine. High phosphorus and low ammonium concentrations were measured in the filter fed with T-brine (Anaerobic 1), hence the results for this filter were plotted on the secondary y-axis (Figures 5.28 and 5.29). There was a 3.4% reduction in phosphorus concentration in this filter within ten days whereas in filters fed with well-brine a 55% and 46% phosphorus removal were obtained aerobically and anaerobically respectively (Figure 5.28). Ammonium was reduced to undetectable levels by day two (Figure 5.29). There is a possibility that although oxygen was not supplied to the anaerobic filter fed with well-brine (Anaerobic 2), the production of oxygen by algae growing in this filter must have caused aerobic conditions to occur.

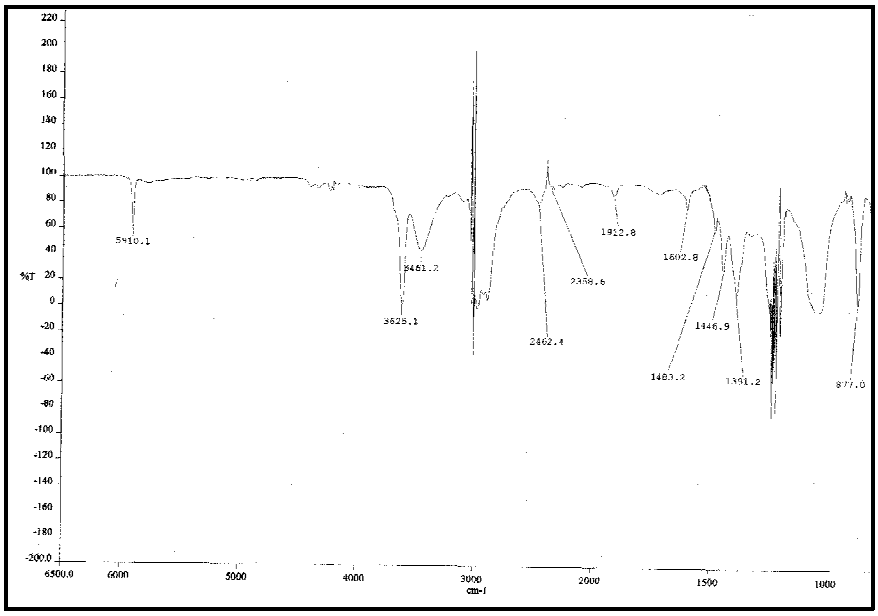

Figure 5.19. IR spectrum of chloroform extract of well-brine. Sample analysed in chloroform, using chloroform as background scan.

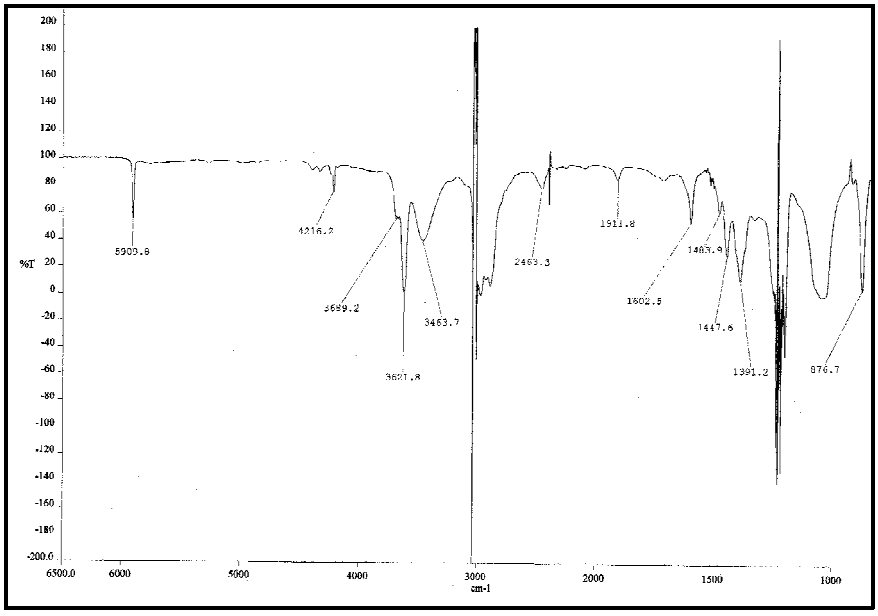

Figure 5.20. IR spectrum of chloroform extract of T-brine from anaerobic filter 1.

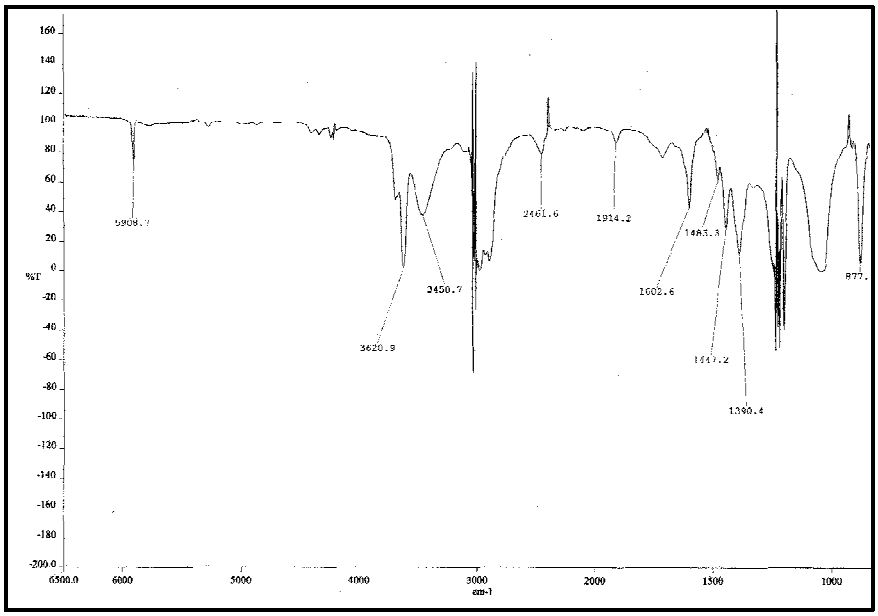

Figure 5.21. IR spectrum of chloroform extract of well-brine from anaerobic filter 2.

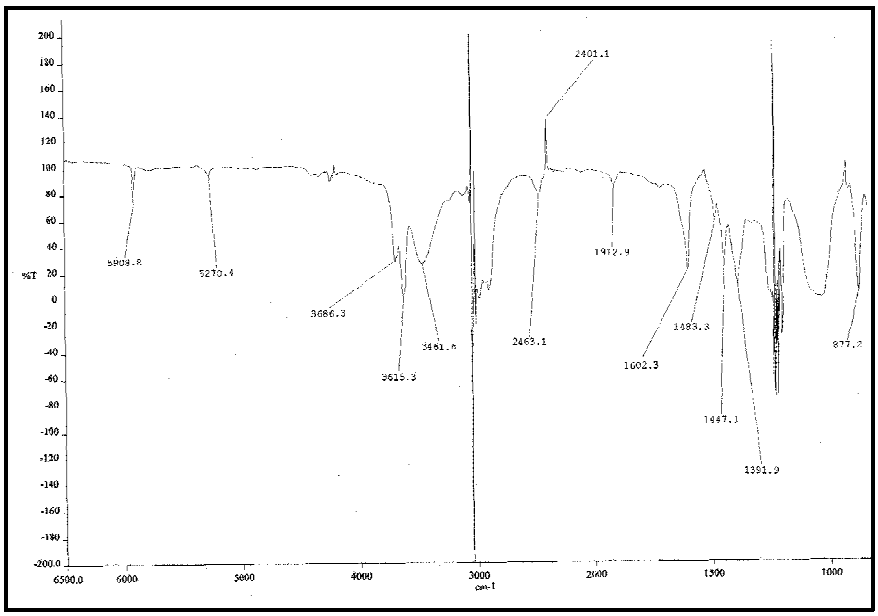

Figure 5.22. IR spectrum of chloroform extract of well-brine from aerobic filter.

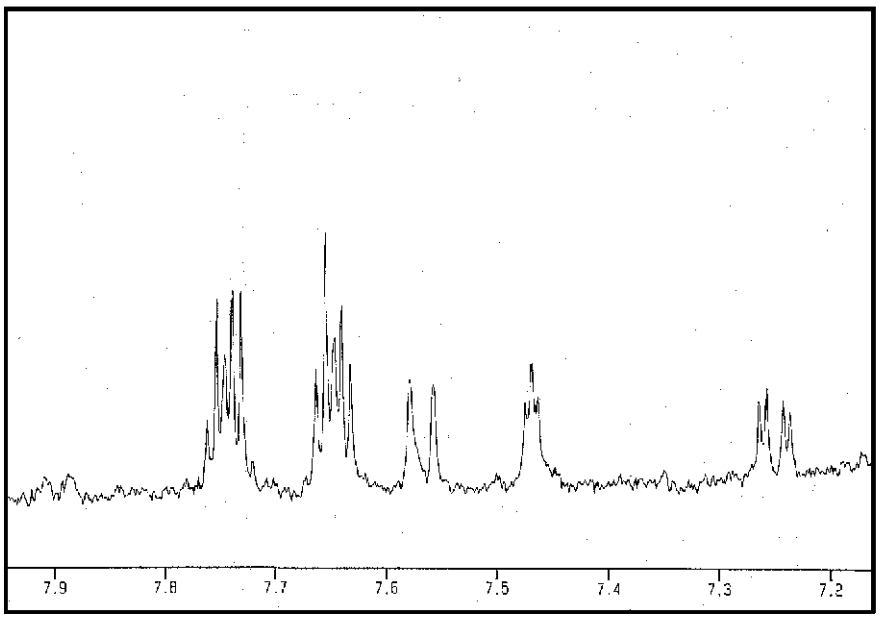

Figure 5.23. ^{1}H NMR spectrum of ethyl acetate extract of T-brine.

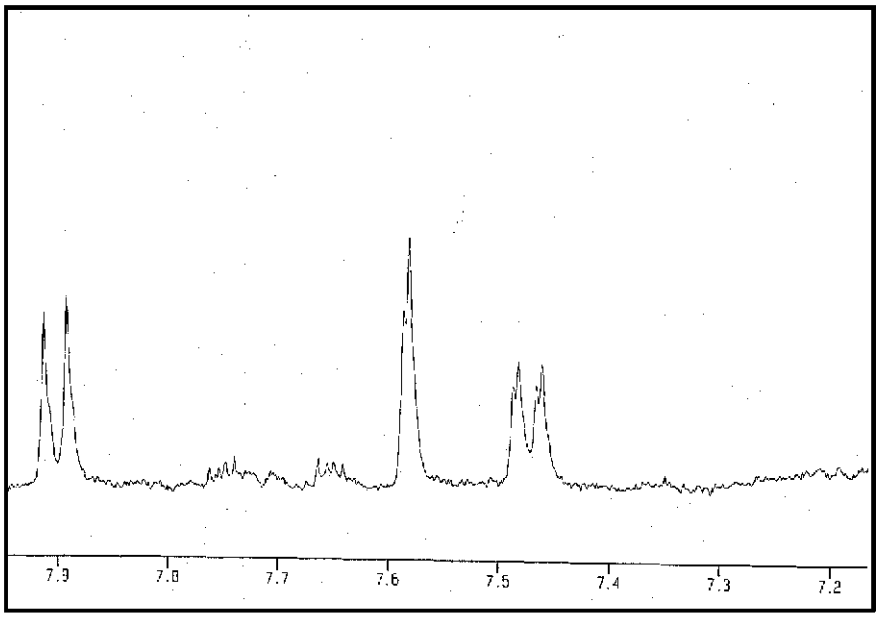

Figure 5.24. ^{1}H NMR spectrum of ethyl acetate extract of T-brine from anaerobic filter 1.

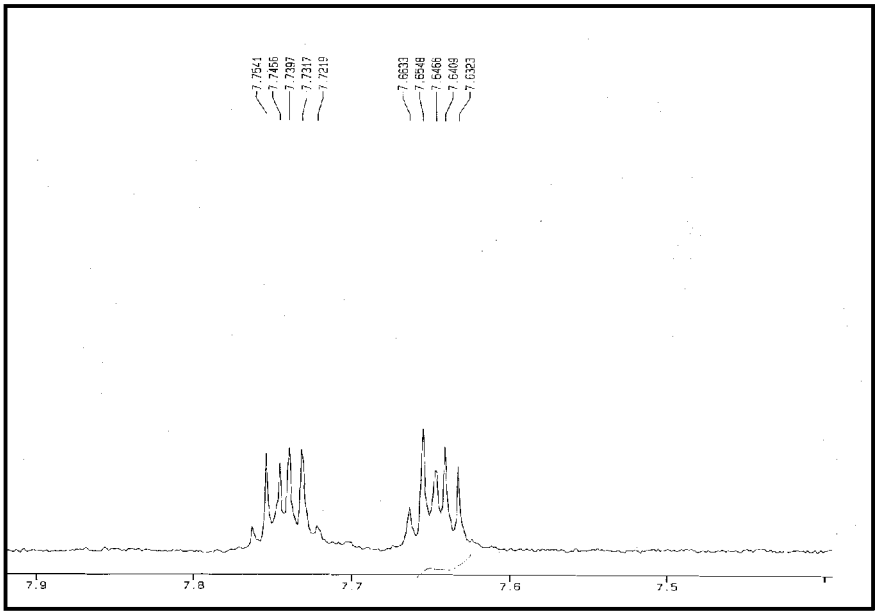

Figure 5.25. ^{1}H NMR spectrum of ethyl acetate extract of well-brine.

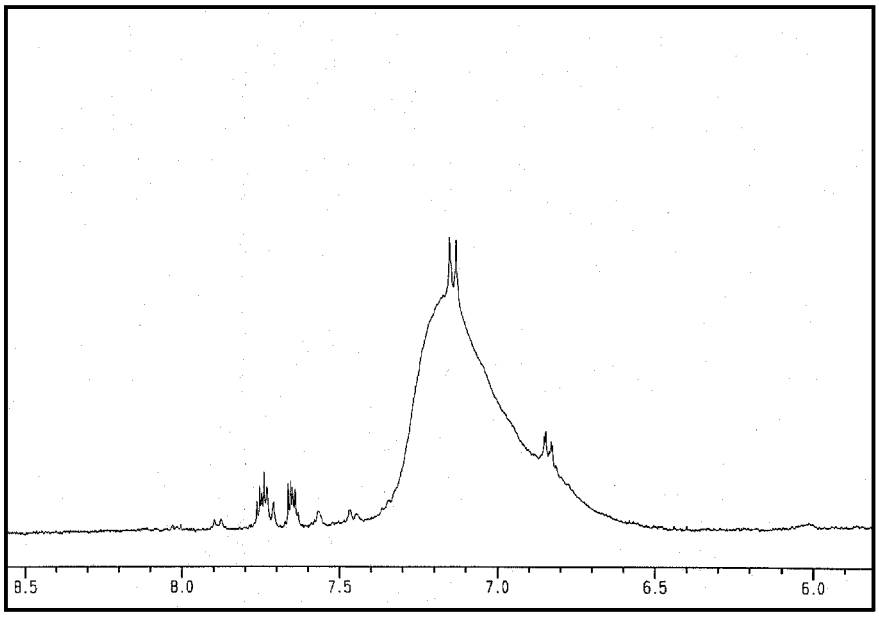

Figure 5.26. ^{1}H NMR spectrum of ethyl acetate extract of well-brine from anaerobic filter 2.

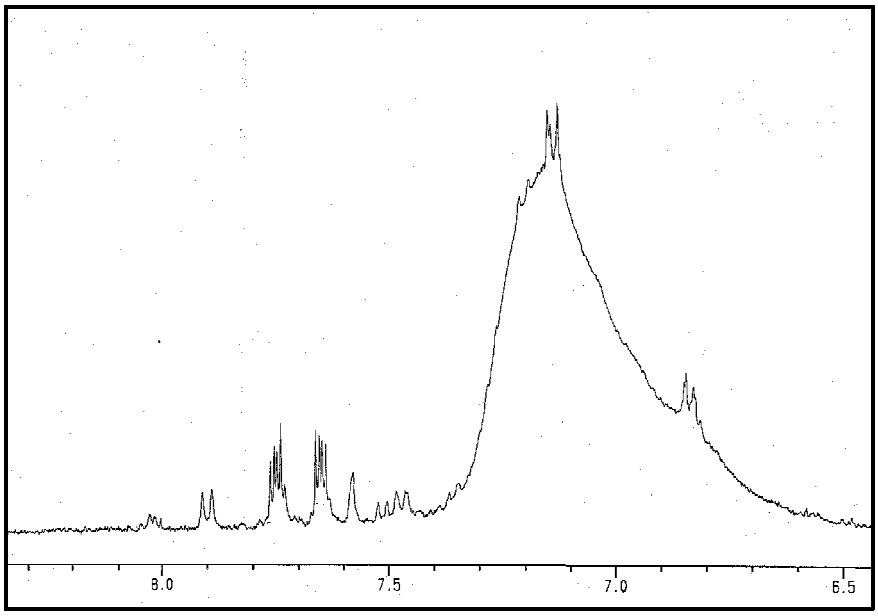

Figure 5.27. ^{1}H NMR spectrum of ethyl acetate extract of well-brine from the aerobic filter.

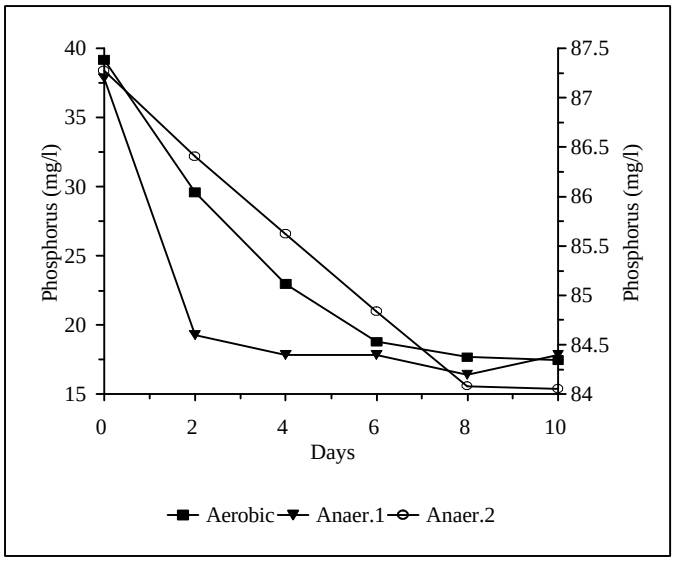

Figure 5.28. Phosphorus profile in filters.

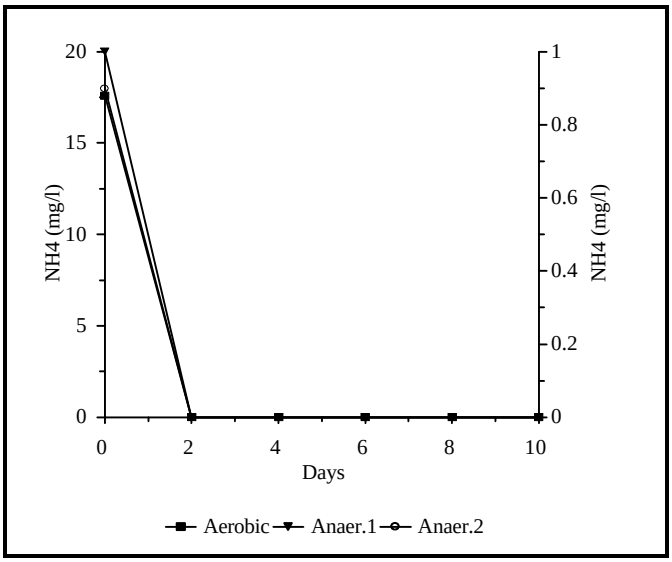

Figure 5.29. Ammonium profile in filters.

5.4 DISCUSSION

Research on the degradation of complex organic compounds by halophilic bacteria or their application in wastewater treatment is limited despite the salinization issue which is a pollution problem facing many water systems today. Wastewaters containing high concentrations of salt are produced by many industries and biological removal of organics from these brines could minimize the environmental impact by reducing toxicity of the waste. To achieve this without costly dilution, halophilic heterotrophic organisms able to tolerate hypersaline environments should be used (Woolard and Irvine, 1994). On the other hand, the application of algal biotechnology for the treatment of saline effluent wastes and the beneficiation potential for these wastes have been demonstrated (Rose, 1992).

This study was undertaken to evaluate the degradative ability of the extreme halophilic bacteria for application in the remediation of T-brine. Few studies have been done on the degradation of environmentally significant organic compounds at high salinities (Oren *et al*., 1992) and the degradation of organic compounds so far reported have implicated activity by halotolerant eubacteria rather than archaebacteria (Yanase *et al*., 1992; Woolard and Irvine, 1994; 1995a, 1995b).

There is evidence that organic remineralisation occurs during the course of evaporation in Sua Pan solar ponds since TOC levels in T-brine are consistently lower than would be anticipated by the evaporation concentration factor applied to well-brine. Black sediments in the evaporating ponds also show anaerobic activity of by sulphate reducing bacteria. This has not been demonstrated under laboratory conditions. However, it was speculated that the breakdown of polymers into smaller compounds without altering the overall brine TOC level occurred during treatment. Although it has been reported that activity at high salinities is attributed to the extremely halophilic archaebacteria, halotolerant eubacteria may also appear to play a role in the remineralisation of organic matter at these salinities. These studies have also implicated the production of organic compounds which may explain the increase in TOC levels observed in filters fed with well-brine. Algae that grew in these filters are capable of producing extracellular polysaccharide which is released into the medium causing organic carbon levels to increase.

Failure to degrade non-specific organic compounds as measured by the total TOC under anaerobic conditions indicate that heterotrophic activity was hindered under these conditions. Under anaerobic conditions in the presence of light, halobacteria derive energy from light through photophosphorylation. Although these bacteria are able to grow under conditions of low oxygen availability, they cannot grow under fastidious anaerobic conditions (Hartmann *et al*., 1980) unless

the inoculating cells already contain large amounts of bacteriorhodopsin. This explains why no growth or activity in shake flask studies was observed under anaerobic conditions since cultures were incubated in an anaerobic chamber. The utilisation of light as an alternative energy source explains why degradation of organic carbon was reduced in cultures grown in the presence of light and enhanced in cultures incubated in the absence of light.

In order to develop a system for biological treatment, knowledge on both the degradative ability and the performance of these bacteria in a biological reactor was required. For this purpose, immobilization of bacteria was investigated but scale-up of this work was not successful. Immobilization of halophilic bacteria onto support materials was found to be difficult. A Sequencing biofilm batch reactor (SBBR) which has been reported to maintain slow growing and poor settling microbes in saline and hazardous waste-water treatment (Herzbrun *et al*., 1985; Woolard and Irvine, 1994) failed to maintain an active biofilm when applied to this study.

Although the gravel pack filters did not indicate organic carbon degradation, the potential of these filters to sequester nutrients contaminating the incoming well-brine has been demonstrated. This suggests the possibilities for their use in the pre-treatment of well-brine. The manipulation of these filters in such a way that both photosynthetic and heterotrophic organisms growth is achieved, may result in both nutrient and organic carbon reduction in well-brine.

5.5 CONCLUSIONS

The possible application of aerobic and anaerobic bacterial processes for the remediation of brine at Sua Pan has been demonstrated. Although heterotrophic activity was inhibited under anaerobic conditions, the breakdown of complex polymers to smaller compounds could be possibly be achieved. The feasibility of T-brine remediation in an aerobic bioreactor operated in the absence of light was also demonstrated and the study suggested the possibility of using a system which can simulate the gravel packed filters for pre-treatment of well-brine to reduce the nutrient load. The purpose of this investigation was to evaluate the preliminary feasibility and the results indicate the advisability of more detailed follow-up studies. The results are promising and demonstrates a great potential for the remediation of T-brine utilising bacterial systems degrading organic compounds. Detailed work is, however, needed on immobilization.

CHAPTER 6

REMEDIATION POTENTIAL OF TOC ADSORPTION

6.1 INTRODUCTION

It has been shown that upon optimisation, biological systems may have great potential in the remediation of organic contamination occurring in the ponds and in the final product. A possibility for the application of physico-chemical processes for the removal of organic compounds, however, was also noted and this study evaluated the feasibility of adsorption of TOC in the system.

Conventionally, industrial applications for the removal of organic compounds involve the use of ion-exchange resins and activated carbon. Activated carbon, for instance, is used in the food and water treatment industries for the removal of refractory organics such as phenols (Daifullah and Girgis, 1998). Economic constraints may, however, limit the application of these conventional processes. On the other hand, ion-exchange resins can not be applicable in the presence of a high solute content as in the case of brines. In such a situation the resin would preferably pick up the ions instead of the organic compounds. The study therefore was aimed at evaluating an economical approach for the adsorption of TOC in the brine.

Observations made in the system revealed that the EPS slime that settles out of the water column to the pond floor is adsorbed onto the pond sediments to form a compact layer. This was found to play a role in the formation of bottom mats. This certainly indicated that clay-type soils occurring on the pond floors have sorption characteristics related to the production of organic

matter by algae. The same type of phenomenon can be anticipated in the pan. The pan is seasonally flooded with water and during this time algal growth occurs before the pan dries out. Sorption characteristics could therefore be anticipated in the clay-type soils found on the pan surface.

Sorption of organic compounds onto soils has been a subject of wide interest as this plays an important role in the transport, fate and remediation of organic pollutants in the subsurface. Several workers have reported the ability of soils to adsorb a wide range of organic compounds (Ong and Lion, 1991; Lin *et al*., 1994; Piatt and Brusseau, 1998; Ruiz *et al*., 1998; Webber Jr. *et al*., 1998). These soils owe their sorptive capabilities to the organic matter and minerals contained in a particular soil. Sorption of organic compounds on sediments has also been demonstrated by Karickhoff *et al*. (1979) who reported that the sorptive capacity of the sediment depends on the particle-size distribution and organic matter determined by the dynamics of the water body.

6.1.1 Objectives

This study was undertaken to determine the potential of physico-chemical processes for the adsorption of organic compounds for the remediation of T-brine. The clay-type soil collected from the pan, referred to as pan-top in this study, was evaluated and activated carbon was used for comparative studies. The removal of both the dissolved TOC and particulate fractions was investigated.

6.2 MATERIALS AND METHODS

6.2.1 Adsorption experiments

Two adsorbents were investigated in this study, pan-top and activated carbon (GAC and PAC) for comparative studies.

6.2.1.1 Pan-top

Adsorption capacity

Clay (Pan-top) material was collected from the pond surface at Sua Pan. To test the ability of this material to adsorb organic compounds contaminating T-brine, 200g of pan-top per one litre of T-brine was used.

Up-flow system

200g of pan top material was packed in a glass column positioned upright. One litre of T-brine was then pumped in an up-flow direction using a peristaltic pump and samples removed for TOC analysis periodically. Samples collected contained some pan-top and were filtered through Whatman No.1 filter paper using a buchner funnel, prior to analysis.

Down-flow system

200g of pan top material was packed in a buchner funnel lined with a Whatman No.1 filter paper. One litre of T-brine was added and allowed to filter through, either by gravity or by applying vacuum. Samples were withdrawn at 30 minute intervals for TOC analysis. Wet samples of pan-top were dried overnight in an oven at 100^0C and ground prior to use. Each experiment was done in triplicate and control samples were filtered through Whatman No.1 filter paper without pan-top.

Optimisation of adsorption capacity

Pan-top weight: To investigate whether the amount of TOC reduced depended on the amount of pan-top used, different amounts of pan-top per litre of T-brine were used, in this case, 100g.l^{-1}, 200g.l^{-1} and 400g.l^{-1}. The TOC content in both brine samples and soda ash samples was measured.

Sequential filtration: Three litres of T-brine were filtered through pan-top and 600ml of the filtrate used for soda ash production. The rest of the filtrate was re-filtered through fresh pan-top material and the procedure repeated until a total of three filtrates were produced. The TOC content of soda ash was measured in each stage.

Regeneration of pan-top

Soda ash was produced from T-brine, filtered through fresh pan-top, and its TOC measured. The same pan-top was then acid-washed three times with nitric acid, pH 2 and rinsed with T-brine before repeating the filtration procedure and soda ash produced from the filtered brine samples before and after acid-wash. The TOC content in soda ash before and after the acid-wash treatment was compared.

Mechanism of adsorption

To investigate if the pan-top acted as an ion-exchanger, T-brine and well-brine samples were filtered through the pan-top material and samples withdrawn at 30 minute intervals. The composition of ions in the samples was determined.

6.2.1.2 Activated carbon

Adsorption capacity

Granular Activated Carbon (GAC)

Initial studies were carried out using GAC. The influence that residence time and stirring had on the amount of organic carbon adsorbed were investigated. 200g of GAC was added to one litre of T-brine and the mixture continuously stirred. Samples were withdrawn at 10-minute intervals, filtered through a GF/A filter paper and the filtrate analysed for TOC. Control samples were also filtered through a GF/A filter paper. To investigate if stirring had any impact on the amount of organic carbon adsorbed, GAC was added to one litre of T-brine and one set of samples were continuously stirred on a magnetic stirrer and the other left standing for one hour. The mixtures were filtered and the filtrates carbonated to produce soda ash.

Powdered Activated Carbon (PAC)

Powdered activated carbon was obtained from Merck. Experiments were performed to evaluate the adsorption capacity of PAC. 100 ml aliquots of T-brine were mixed with different amounts of PAC; 1g, 2.5g, 5g, 10g and 20g in 200ml beakers. A magnetic stirrer was used to form a uniform slurry. The mixture was then allowed to stand for 1 hour and 2 hours. Each mixture was filtered through GF/A filters to get rid of the activated carbon particles and the clear filtrate obtained was analysed for TOC and colour. The amount of PAC considered the dosage required for maximum TOC removal based on the above experiment, was used to treat one litre of T-brine as above. The filtrate obtained was used to produce soda ash and analysed for TOC.

6.2.2 Analyses

6.2.2.1 TOC

Brine samples: 25ml aliquots of brine samples were acidified to pH 2-3 with nitric acid. The acidified samples were stored at 4^0C until analysed. Samples were sparged with air for about 10 minutes before injecting 40Fl of sample into a Dohrman DC-180 TOC Analyser.

Soda ash samples: A sample of soda ash was dried in the oven at 220^0C for 15 minutes to remove the bicarbonate. Approximately 150-180 mg of this sample was weighed on a platinum boat and analysed in a CO_2 Coulometer fitted with the Model 5012 cell. Combustion of the sample was carried out at 530^0C.

Soda ash production

For soda ash production, 600ml of brine (treated or untreated) was heated to 55 ± 2^0C. The heated brine was transferred to a one litre plastic beaker fitted with a gas sparger and a baffle. The brine was carbonated, continuously stirring the brine with an overhead stirrer at 1750 rpm. The pH of the solution was monitored and the reaction was stopped (switching off the gas and the stirrer) when the pH reached 7.4. The mixture was allowed to cool to 32 ± 3^0C before decanting most of the liquor and then filtering the bicarbonate through a Whatman No.1 filter paper. The bicarbonate cake was washed with 200ml of deionized water. The cake was air dried and then calcined in an oven at 220^0C for 3 hours.

6.2.2.2 Colour

The colour of the brine samples was determined spectrophotometrically by recording the absorbance in the range 400-800nm in a GBC UV/VIS 911A spectrophotometer. Photometric measurement was also undertaken by measuring the absorbance of the brine samples using a

Merck Spectroquant SQ 118. The absorbance of the solutions was recorded as absorbance per metre (m^{-1}). Deionized water was used as a blank in both cases.

6.3 RESULTS

6.3.1 Adsorption capacity of pan-top

6.3.1.1 Total Organic Carbon

The ability of pan-top to adsorb TOC from T-brine was monitored by measuring TOC in the brine after filtration through a bed of pan-top. Brine samples were analysed for TOC using the Dohrmann TOC analyser. The impact of this type of treatment on the final soda ash quality was determined by producing soda ash from treated brine and measuring the TOC in soda ash samples using the Coulometry method.

The adsorption capacity of pan-top was evaluated in both the up-flow and down-flow systems and the results are shown in Figures 6.1 and 6.2 respectively. A reduction in the TOC of brine was observed in both systems. However, limitations on the application of the up-flow system due to the instability of the pan-top bed during brine flow, was observed. Subsequent studies were then undertaken using the down-flow system.

Table 6.1 and 6.2 show the effects of pan-top treatment on the TOC of brine samples and soda ash produced from these samples respectively. Filtration in this case was carried out by gravity. This resulted in very long filtration runs and to reduce the duration of these runs, vacuum was applied and the results compared. The results for vacuum filtration are shown in Tables 6.3 and

6.4. The results shown in Tables 6.1-6.4 represent three different filtration runs, each utilising fresh pan-top.

It was shown that during treatment of T-brine with pan-top, a reduction in TOC of the brine, not exceeding 10%, occurred. When soda ash was produced from the treated brine samples and TOC determined by the Coulometry method, a significant reduction was observed both in samples filtered by gravity and vacuum. A 35% soda ash TOC reduction was achieved when brine was filtered using gravity whereas the application of vacuum resulted in a 42% soda ash TOC reduction (Tables 6.2 and 6.4). In addition to the removal of organic components in T-brine, the characteristic pink colour of T-brine was completely removed during this treatment.

Table 6.1. TOC determination of T-brine samples treated by pan-top using gravity filtration.

Sample	Brine TOC(ppmC)	% Reduction
Control	555.6	
1	507.2	8.7
2	521.1	6.2
3	508.2	8.5
Average	512.2 ± 7.8	7.8 ± 1.4

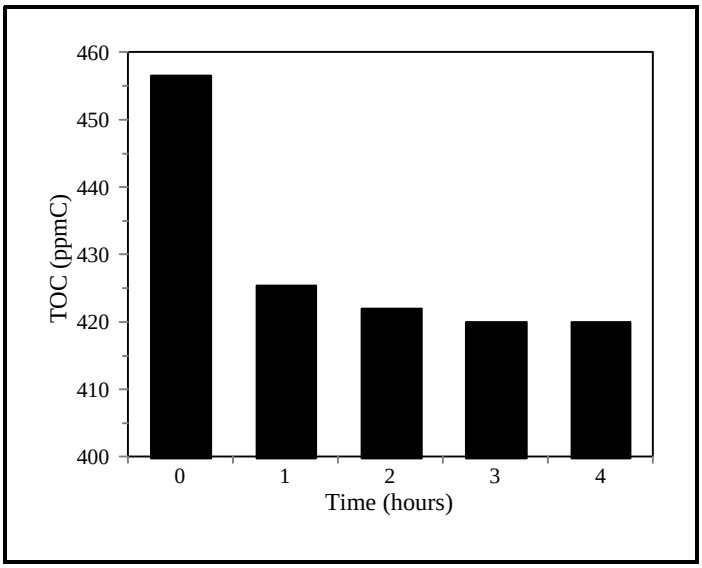

Figure 6.1. TOC profile of T-brine treated in an up-flow system. Samples analysed by Dohrmann TOC analyser.

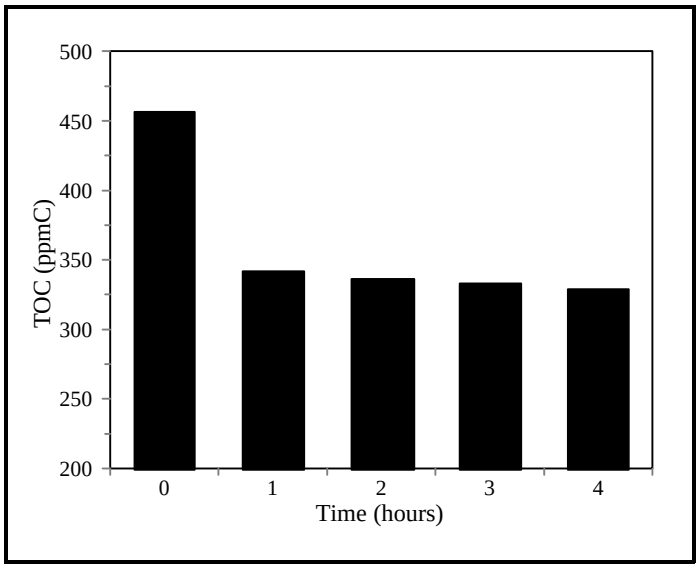

Figure 6.2. TOC profile of T-brine treated in a down-flow system. Samples analysed by Dohrmann TOC analyser.

Table 6.2. TOC determination of soda ash produced from T-brine treated by pan-top using gravity filtration.

Sample	Soda ash TOC (ppmC)	% reduction
Control	1625.7	
1	1060.7	34.8
2	1108.5	31.9
3	965	40.7
Average	1044.7 ± 72.9	35.7± 4.5

Table 6.3. TOC determination of T-brine samples treated by pan-top using vacuum filtration.

Sample	Brine TOC(ppmC)	% Reduction
Control	613.3	
1	578.5	5.7
2	563.6	8.1
3	577.4	5.9
Average	573.1 ± 8.3	6.6 ± 1.4

Table 6.4. TOC determination of soda ash produced from T-brine treated by pan-top using vacuum filtration.

Sample	Soda ash TOC (ppmC)	% reduction
Control	1809.4	
1	1089.5	39.8
2	1152.9	36.3
3	885	51.1
Average	1042.3 ± 139.9	42.4 ± 7.7

6.3.1.2 Colour Removal

The removal of colour in brine samples was quantified spectrophotometrically and photometrically using the Merck SQ 118 Spectroquant. Visual inspection of the treated brine showed that T-brine was clarified during treatment by pan-top as shown in Figure 6.3. This was confirmed by the absorption spectra of control and filtered T-brine samples as shown in Figure 6.4. Photometric colour measurement of these samples is shown in Table 6.5.

Figure 6.3. Picture showing colour removal in T-brine
A: Before treatment and **B**: After treatment with pan-top.

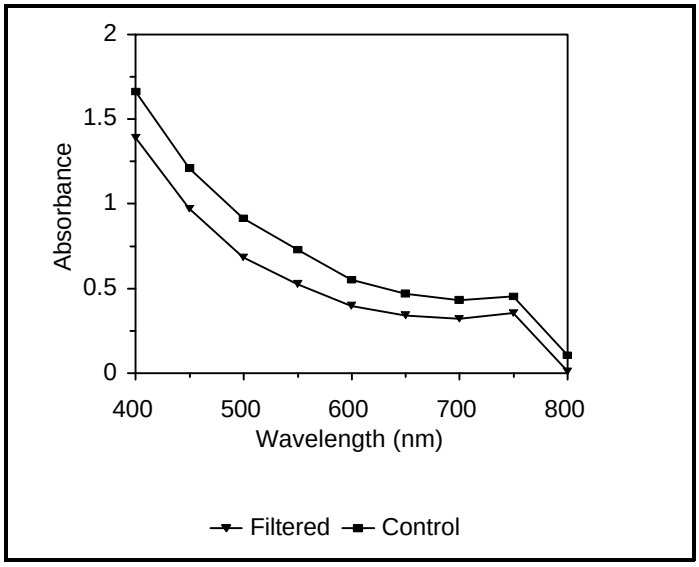

Figure 6.4. Absorption spectra showing colour removal in T-brine treated with pan-top.

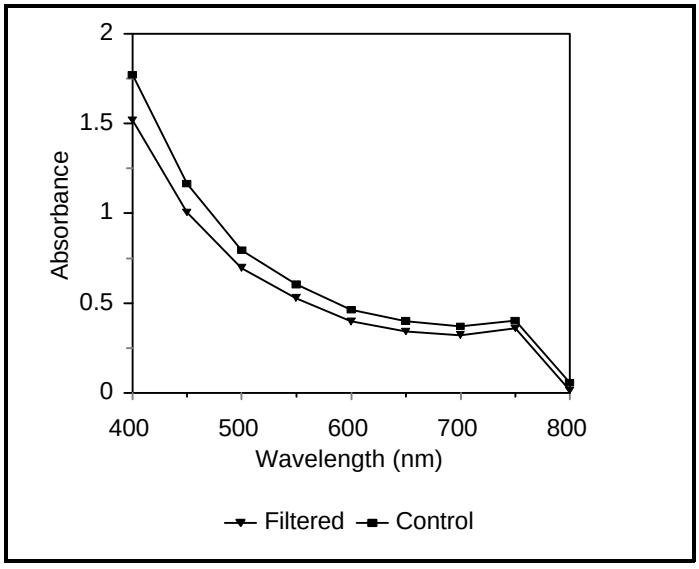

Figure 6.5. Absorption spectra showing colour removal in 20% (w/v) solutions of soda ash produced from T-brine treated with pan-top.

Table 6.5. Results showing photometric colour measurements in T-brine treated with pan-top.

Sample	Colour (m^{-1})	% Reduction
Control	36.3	
Filtered T-brine	6.8 ± 0.2	81.2 ± 0.4

During soda ash production, the bicarbonate produced from the clarified T-brine was white compared to the usual pink bicarbonate produced from untreated T-brine. This allowed the production of a white ash after calcining the bicarbonate filter cake at 220^0C. On dissolution of the soda ash samples, solutions of soda ash produced from untreated T-brine were yellowish whereas clearer solutions were obtained for soda ash produced from clarified T-brine. The colour of these solutions was quantified spectrophotometrically and the results are shown in Figure 6.5.

6.3.2 Optimisation of Adsorption capacity of pan-top

To optimise the adsorption capacity of pan-top, the effect of the amount of pan-top on TOC removal was investigated. T-brine treated with different amounts of pan-top was used to produce soda ash and the TOC of soda ash samples determined in a Coulometer (Table 6.6). Repeated filtration of T-brine through unused pan-top, referred to as sequential filtration in this study was also investigated as a means of optimising the adsorption capacity of pan-top. Soda ash was produced from the treated T-brine samples after each filtration step until three filtration steps were achieved. The results are shown in Table 6.7.

The amount of TOC reduced in soda ash was independent of the amount of pan-top used. Above 100g.l^{-1} pan-top, a 35% reduction in soda ash TOC was maintained. A similar trend was observed

when T-brine was sequentially filtered through pan-top although a higher removal (42%) was observed.

Table 6.6. The effect of pan-top weight on TOC removal in soda ash.

Pan-top ($g.l^{-1}$)	Soda ash TOC(ppmC)		
	Control	Filtered	% Reduction
100	1890.7	1215.5 ± 43.1	35.7 ± 2.3
200	1850.4	1071.0 ± 102.9	37.7 ± 6.2
400	1399.3	964.3 ± 47.6	31.1 ± 3.4

Table 6.7. The effect of sequential filtration on TOC removal in soda ash.

Sample	Soda ash TOC (ppmC)	% Reduction
Control	1825.4	
Filtrate 1	1029.0 ± 52.6	43.6 ± 2.7
Filtrate 2	1044.8 ± 48.2	42.7 ± 3.1
Filtrate 3	1074.1 ± 46.2	41.2 ± 2.8

6.3.3 Mechanism of adsorption by pan-top

The results obtained so far indicated that pan-top was acting as an adsorbent, retaining halobacterial cells responsible for the pink colouration of T-brine. However, there was a possibility that pan-top could have been acting as an ion-exchanger therefore exchanging some ions with the charged halobacterial cells. The presence of ion-exchange properties in pan-top was therefore investigated by filtering T-brine through pan-top and periodically determining the ionic composition of the brine. To eliminate the possibility of interference by a high load of organic

compounds occurring in T-brine, well-brine was also evaluated. The results are shown in Tables 6.8 and 6.9 and conclusively show that pan-top does not have ion-exchange properties.

Table 6.8. The effect of pan-top treatment on the ion composition of T-brine.

Time (min)	Cl^- (%)	CO_3^{2-} (%)	HCO_3^- (%)	K^+ (ppm)	SO_4^{2-} (%)	SG
0	20.61	5.39	1.06	8000	3.79	1.2604
30	20.14	6.01	1.02	8000	3.99	1.2684
60	20.21	5.95	1.1	8000	3.99	1.2637
90	20.21	5.82	1.07	8000	3.94	1.2667
120	20.27	5.84	0.94	8250	4.08	1.2604

Table 6.9. The effect of pan-top treatment on the ion composition of well-brine.

Time (min)	Cl^- (%)	CO_3^{2-} (%)	HCO_3^- (%)	K^+ (ppm)	SO_4^{2-} (%)
0	13.61	2.56	0.71	3000	0.96
30	13.83	2.59	0.71	2750	0.95
60	13.46	2.59	0.71	3000	0.95
90	13.71	2.51	0.78	3000	0.94
120	13.51	2.55	0.75	3000	0.96

Furthermore, the colour removal observed may have been due to the physical effects of filtration. To investigate this, T- brine was continuously stirred with the pan-top in a flask on a magnetic stirrer and then allowed to settle. The supernatant was carefully withdrawn and the colour and TOC of the brine determined. Clarification of T-brine was achieved, although repeated treatments were required to achieve complete clarification as shown in Table 6.10. The results show that pan-top does in-fact have sorptive characteristics.

Table 6.10. Colour and TOC determination in T-brine treated with pan-top by continuous stirring..

Sample	Colour (m^{-1})	TOC (ppmC)
Control	32.2	705.2
Filtration 1	19.5	695.4
Filtration 2	12	667.6

6.3.4 Regeneration of pan-top

One of the most important aspects in determining the potential of economical application of pan-top for physico-chemical removal of TOC was whether this material could be regenerated. The process of adsorption is reversible which allows the possibility of regenerating the adsorbent. The feasibility of acid-wash was evaluated for this purpose and the results are reported in Table 6.11. The results show three filtration runs done before and after acid-wash of pan-top.

The results show that although pan-top regenerated by acid-wash was less effective in reducing TOC of soda ash produced from treated brine, a significant amount of TOC reduction could still be achieved by using regenerated pan-top. The colour of treated brine was quantified photometrically. It was interesting to note that TOC reduction was directly related to the colour of brine used for producing soda ash. Less TOC reduction was achieved with clarified T-brine.

Table 6.11. Results showing TOC of soda ash samples produced from T-brine filtered through pan-top before and after acid-wash. (1A, 2A and 3A: after acid-wash).

Sample	Colour (m^{-1})	Soda ash TOC (ppmC)	% Reduction
Control	30.9	1770.8	
1	7.2	1178.4	33.5
1A	6.8	1389	21.6
2	6.2	949.5	46.4
2 A	1.2	1278.6	27.8
3	4.9	1194.3	32.6
3 A	18.8	1734.9	2.1

6.3.5 Adsorption capacity of activated carbon

6.3.5.1 Total Organic Carbon

To determine the adsorption capacity of activated carbon, the effect of residence time and the amount of activated carbon required for maximum TOC removal, was investigated. Figure 6.6 shows that TOC reduction in T-brine was dependent on the contact time between the adsorbent (GAC) and the brine. In one hour, 384 ppmC was removed when a dosage of 200g GAC per litre of T-brine was used (2ppmC/g.l^{-1}). This corresponded to 60.3% TOC reduction. Continuous stirring was also found to enhance TOC removal as shown in TOC determination of soda ash produced from brine treated with GAC (Table 6.12). This probably continuously exposed adsorption sites for organic compounds.

Based on these results, the effect of the amount of PAC on TOC removal in T-brine was investigated at contact times of one and two hours and the results are shown in Figure 6.7. With

PAC, maximum TOC removal was achieved when treatment was carried out at a dosage of 200g.l^{-1} with a contact time of 2 hours. Results obtained with dosages above 200g.l^{-1} were erroneous due to the formation of a thick slurry, making it impossible to thoroughly mix PAC with brine. A dosage of 200g.l^{-1} was, therefore applied in subsequent studies. Table 6.13 shows TOC of soda ash produced from T-brine treated with PAC.

Table 6.12. Effect of stirring on TOC removal in soda ash produced from T-brine treated with GAC.

Sample	Soda ash TOC (ppmC)	% Reduction
Control	1852.4	
Unstirred	1327.5	28.3
Stirred	1218.5	34.2

Table 6.13. Results showing TOC measurements of soda ash produced from T-brine treated with PAC.

Sample	Soda ash TOC (ppmC)	% Reduction
Control	1535.02	
Treated	917.60 ± 76.9	40.2

6.3.5.2 Colour Removal

The characteristic pink colour of T-brine was also removed when activated carbon was used. Table 6.14 shows the photometric colour measurement of brine treated with different amounts of PAC as was shown in Figure 6.7. The colour removed was dependent on the amount of

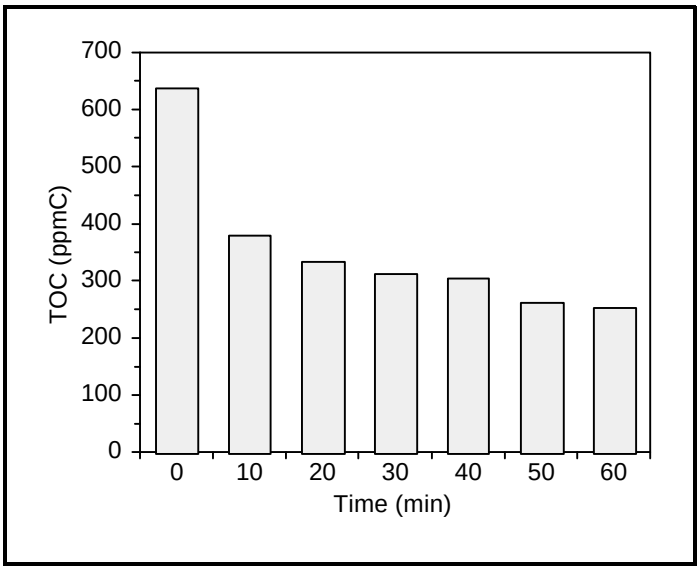

Figure 6.6. The effect of contact time on TOC adsorption in T-brine treated with GAC.

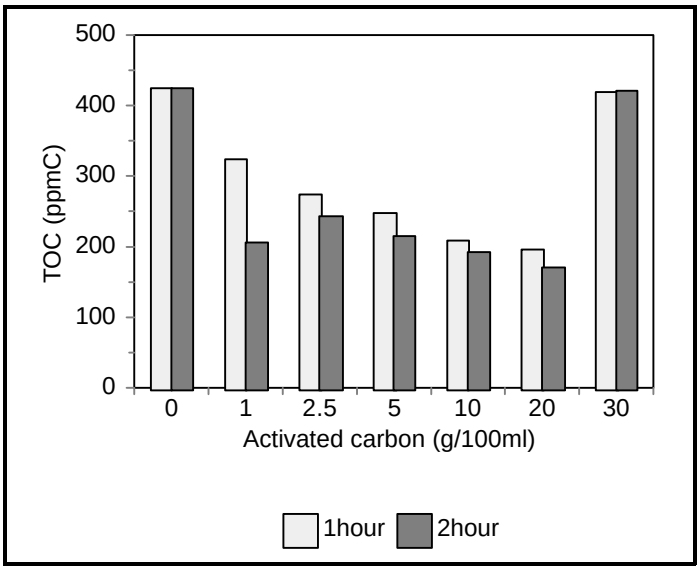

Figure 6.7. The effect of amount of PAC and contact time on TOC adsorption in T-brine treated with PAC.

activated carbon used. This brine clarification also allowed production of white bicarbonate filter cake and soda ash

Table 6.14. Photometric colour measurements of T-brine treated with different amounts of PAC for two hours.

PAC (g/100ml)	Colour (m^{-1})	% Reduction
0 (Control)	28.7	
1	10.6	63.1
2.5	2.2	92.3
5	0.8	97.2
10	0.8	97.2
20	0.8	97.2

6.4 DISCUSSION

This study demonstrated the ability of pan-top to physically adsorb a certain fraction of organic components in T-brine. It is apparent that this TOC removal relates largely to the halobacterial and algal fraction rather than the dissolved TOC. The removal of particulate material causing colour in T-brine resulted in the production of a whiter ash with 35% less TOC than untreated samples. This corresponded to only a small reduction (approximately 7%) in TOC of the brine. This indicate that only a small fraction of TOC in T-brine contributes to the colour component of the final soda ash product. Enhancement of TOC reduction in soda ash was observed when the retention time of brine was reduced by applying vacuum. This indicate that in long filtration runs, adsorption and desorption of organic components can be anticipated.

According to the results of this study, the discolouration of the soda ash product is caused by the particulates, mainly biomass, occurring in the ponds and rather than the Maillard reaction caused by heating of the product. Pan-top selectively removed cells from the brine and once the cells had been removed, exposure of more active sites did not result in further adsorption. This possibly explains failure to optimize TOC reduction in soda ash by increasing the amount of pan-top used or by sequential filtration.

In their adaptation to saline conditions, halobacteria have developed a high proportion of negatively charged acidic amino acids in their proteins and therefore cells maintain a negative charge on their cell surface. This may explain the preferential sorption of these bacteria by pan-top. Highly polar carotenoids found in halophilic bacteria reinforce the lipid membranes of these bacteria (Nakatani *et al.*, 1991) which would also be expected to enhance polarity of the cells. This opens a window for exploiting the application of pan-top in a cell separation unit since immobilization of these bacteria was identified as a major limitation for the application of bacterial systems in T-brine remediation as indicated in the previous chapter. The mechanism of interaction between the adsorption sites of pan-top and the cells is not known. However, it has been confirmed that pan-top does not have any ion-exchange properties.

The feasibility of pan-top regeneration by acid-wash was demonstrated, although the regenerated pan-top was less effective than fresh pan-top. Ruiz *et al*. (1998) have found that clay, sand and limestone could be completely regenerated by heating the soil to 403K and then cooling to 298K. Although the heating and cooling process could not be economically feasible at large scale, further work needs to be done to evaluate other regeneration procedures such as water and air wash or possibly drying of pan-top.

When the adsorption capacity of pan-top was compared to activated carbon, TOC reduction of 40% was observed in soda ash samples. High amounts of activated carbon required to achieve similar effects demonstrated by pan-top, however, indicated economic constraints on the application of activated carbon. The potential of pan-top for the pre-treatment of T-brine prior to carbonation is therefore substantiated by its economic advantage over activated carbon. The TOC of soda ash produced from T-brine treated with pan-top under laboratory conditions was maintained around 1000 ppmC. This indicate that, should this operation be successfully developed, TOC levels less than 1000 ppmC should theoretically be anticipated since carbonation is carried out under a positive pressure in the process plant.

These results coupled with the economic advantage pan-top offers, justifies further development of this study. A laboratory scale filtration unit should be designed and evaluated for the remediation of T-brine prior to carbonation. Depending on the performance of this unit, it may supplement or even replace the existing sand filters.

6.5 CONCLUSIONS

The study has shown that a potential exists for physico-chemical processes for the adsorption of TOC from T-brine. An interaction of physical and chemical processes occurring in the pan has allowed pan-top to develop sorptive characteristics able to remove TOC related to halobacterial and algal cells and not dissolved TOC. The colouration of soda ash is therefore caused by the biomass occurring in the ponds and by removing this biomass, white soda ash with approximately 35% less TOC can be produced in an unoptimised process.

CHAPTER 7

GENERAL DISCUSSION

This project was undertaken to address the organic problem experienced in the soda ash evaporite production system at Sua Pan. The aim was to establish the origin and nature of organic compounds occurring in such systems, and, to identify and evaluate practical remediation strategies for dealing with this problem.

The study has shown that the organic contamination of the final product is resolved into two fractions, the dissolved organic carbon (TOC) fraction and the colour component. The results of the study provide a strong indication that organic compounds in well-brine and to a large extent, the EPS produced by microbial growth in the ponds, comprises the major components causing organic contamination in the final product. These components constitute the dissolved TOC fraction in the final soda ash product whereas the colour component appears to derive from the bacterial and algal cells occurring in the system.

Well-brine organics consist of humic substances which separate into two fractions on discharge into the ponding process; the humic acid fraction which chelates with ferric ion to form a black precipitate and the soluble fulvic acid fraction which passes through the system, finally reporting to the soda ash product. Blooms of *Dunaliella spp*. occur in the ponds due to the high concentrations of nutrients in the incoming well-brine and this results in the production of EPS. This production is aggravated by the pond operation which permits the occurrence of conditions favourable to EPS production i.e. high temperature, illumination and salinity. Characterisation of

this EPS has made it possible to trace the algal component through the ponding system to the final product.

A reduction in the concentration of the dissolved TOC fraction was observed through the ponding process. Lower concentrations of the aromatic component were observed in T-brine compared to the influent well-brine, in spite of the evaporation concentration factor. On the other hand, binding of the algal EPS to the pond sediments, found to play a role in mat formation, was also observed. Further reduction of the algal organics occurs in ponds where halobacterial activity is high. These observations strongly indicated the possibility of implementing biological or physico-chemical remediation strategies for dealing with the organic problem.

It was against this background that feasibility studies were undertaken to evaluate the potential of nutrient stripping, bacterial and TOC adsorption systems for the removal of both the dissolved TOC, and colour fractions in the product.

The study demonstrated that nutrient stripping and bacterial consumption were viable strategies to deal with the dissolved TOC fraction in the ponding system. Bacterial systems are, however, limited by poor immobilization characteristics and this still needs further development. However, it was shown that algal systems have a definite potential for effective control of microbial growth, hence organic carbon production, in the ponds by stripping nutrients such as phosphorus and nitrogen in the influent well-brine.

The potential of the *Dunaliella*-based HRAP process was demonstrated at a pilot-scale level. The results indicated that in an unoptimised process, complete nitrogen and up to 50% phosphorus

removal could be anticipated in such a system. Subsequent growth of *Dunaliella spp*. in this nutrient-depleted brine was severely constrained. The results of this study, however, indicate that nutrient limitation would be expected to be resolved by increased EPS production, but one could anticipate that the magnitude of this production would be low for a dilute culture. While similar effects were noted with chemical phosphorus precipitation, the large quantities of precipitant required indicated economic limitations for chemical nutrient removal processes.

The application of pan surface clay (pan-top), shown to have adsorption characteristics related to the production of organic compounds by algae, failed to show any capability in the removal of the dissolved TOC. However, this material proved to have substantial potential in removing the particulate material which constitutes the colour contaminants of the final product. The sorption of bacterial and algal cells onto pan-top resulted in the clarification of the feed-stock T-brine, allowing the production of white soda ash. These results, indicate the practical opportunity available for a filtration system utilising pan-top, for removing colour in the final product.

Based on the results acquired, the following critical points for the management of the evaporite system can be identified:

Algal biotechnology appears to have real potential in dealing with processes leading to organic contamination of both salt and soda ash. With the successful operation of a HRAP, a reduction in microbial growth and production in the ponds could be anticipated. This in turn should reduce the standing crop (of both algae and bacteria) in the system resulting, in turn, in the reduction of both the dissolved TOC, and the colour fractions contaminating the product. It may be advised

that a comprehensive evaluation of the *Dunaliella*-based HRAP pilot plant be undertaken, as a long-term remediation strategy.

Modification of pond operation procedures could also have a significant impact on the overall performance of the system. Situations where stagnant and shallow zones occur in the ponds should be avoided as these function as cell and EPS generators. Continuous operation would allow constant conditions within successive and connected brine bodies to be maintained. However, the downstream ponds, N-Brine storage, crystallisers and T-brine storage, should be operated at shallow depths to allow maximum activity of halobacteria which require aerobic conditions and high temperatures for the breakdown of organic compounds.

FUTURE PERSPECTIVES

This research has demonstrated the practical application of a well-brine pre-treatment operation at a fundamental level and pilot scale-up studies. A thorough evaluation of a plant to be used in this operation, at pilot scale, was therefore proposed (Rose, 1998b). The plant will include pre-treatment operations before the brine can be discharged into the HRAP. These would include an aeration box to be dosed with ferric chloride to precipitate the contaminating phosphorus and the humic acid fraction of the well-brine organics. After the particulate material has been separated in a settler, the brine would be passed through a stone-lined raceway which will simulate the gravel packs evaluated for bacterial immobilization. The population growing in this raceway would be expected to result in nutrient cycling, and on optimisation, organic carbon remineralisation could also be anticipated. Further treatment of the brine would then be undertaken in a *Dunaliella*-based HRAP and the treated brine discharged into the evaporating pond as shown in Figure 7.1. The study would determine operational parameters such as brine loading rates, retention time, cell

recycle requirement and nutrient removal rates. A cell separation unit suitable for this operation is still to be determined.

Based on these research findings, future research work should involve further characterisation to identify organic compounds occurring in such systems. A development of immobilization techniques for halophilic bacteria and optimisation of such systems for TOC reduction should also be undertaken. The application of pan-top for colour removal in T-brine and soda ash should also be evaluated and the research should determine the properties of pan-top responsible for its sorption characteristics such as the organic carbon content and particle size.

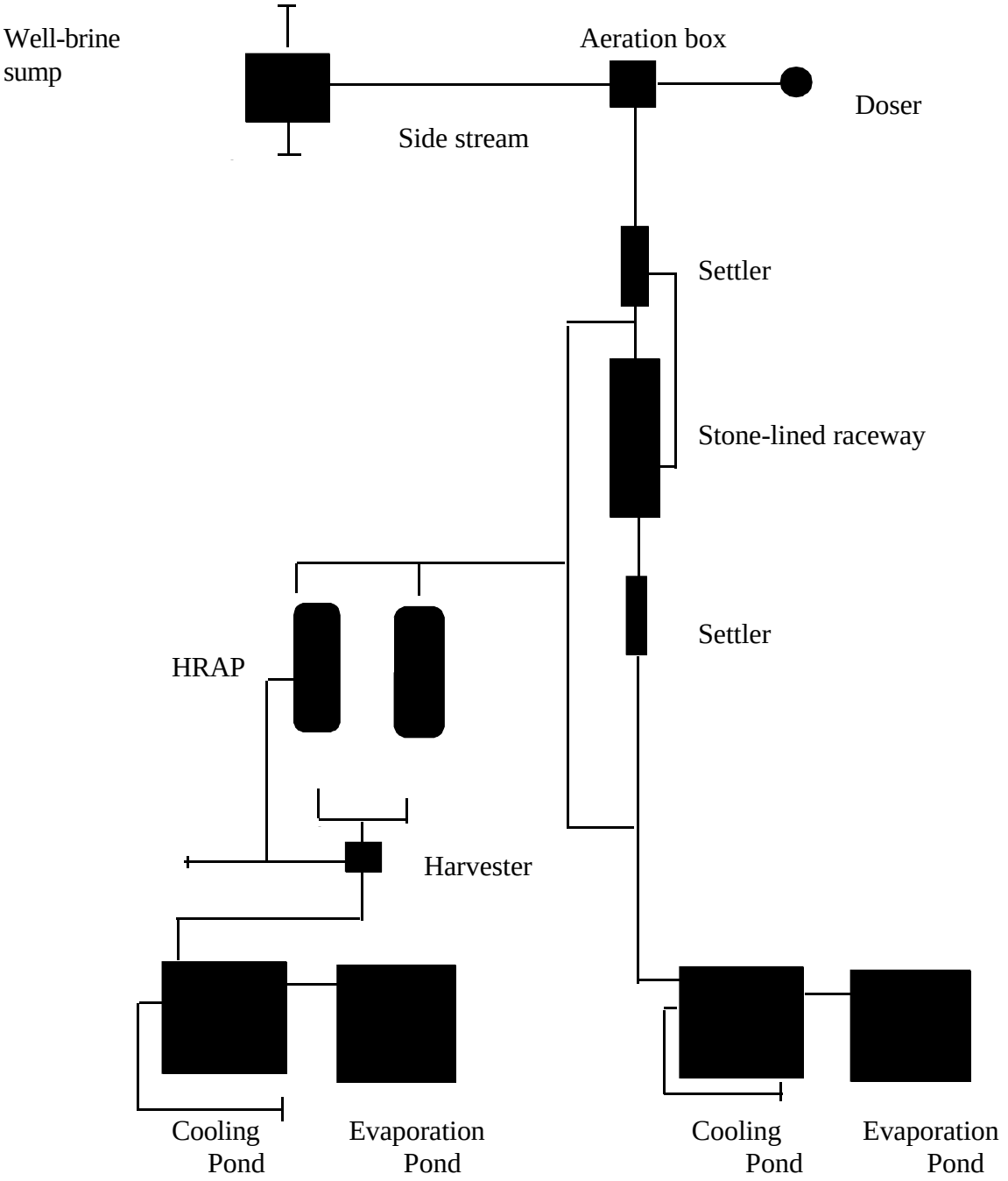

Figure 7.1. Flow diagram showing the pilot plant plan (Rose, 1998b).

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APPENDICES

APPENDIX I: REAGENTS FOR MEDIA PREPARATION

Halophilic medium

Chemical	$g.l^{-1}$
Yeast extract powder	10
Proteose peptone	5
Casamino acids	5
Trisodium citrate	3
KCl	2
$MgSO_4.7H_2O$	20
NaCl	200

BAAM medium

Chemical	$g.l^{-1}$
NaCl	120
$NaHCO_3$	4.2
KNO_3	0.51
$MgSO_4.7H_2O$	1.23
KH_2PO_4	0.027
$CaCl_2.2H_2O$	0.044
Trace element solution	2ml

Trace element solution for BAAM medium

Chemical	$g.l^{-1}$
EDTA	22.23
H_3BO_3	11.453
$MnCl_2.4H_2O$	1.382
$ZnCl_2$	0.1681
$CoCl_2$	0.00259

Enrichment solutions for anaerobic cultures

Vitamin solution		**Trace element solution**	
Chemical	$mg.l^{-1}$	Chemical	$g.l^{-1}$
Biotin	2	$FeCl_3.6H_2O$	0.2
Folic acid	2	$MnCl_2.4H_2O$	0.1
Pyridoxine.HCl	10	$CoCl_2.6H_2O$	0.17
Thiamine.HCl	5	$CaCl_2$	0.1
Riboflavin	5	$ZnCl_2$	0.1
Nicotinic acid	5	$CuCl_2.2H_2O$	0.02
Calcium pantothenate	5	H_3BO_3	0.01
Vitamin B_{12}	0.1	$NaMoO_4.2H_2O$	0.01
Lipoic acid	5	$NiCl_2.6H_2O$	0.03
p-Aminobenzoic acid	5	Na_2SeO_3	0.02

APPENDIX II: ASSAY PROCEDURES

1. Carbohydrate Assay

A. Phenol-sulphuric acid method

Stock solution: Dissolve 100mg of glucose in 100ml of 0.15% w/v benzoic acid added as a preservative in ddH2O.Dilute 1:10in dH_2O to give a 100Fg.ml^{-1} stock solution. Prepare standards ranging from 10-100 Fgml^{-1} from the diluted solution.

Sulphuric acid solution: Add 750ml of reagent grade sulphuric acid to 250ml of dH_2O to prepare a 75% v/v sulphuric acid solution.

Phenol reagent: Dissolve 5g phenol in 100ml of water.

Procedure

1. Add 1.0 ml phenol reagent and mix thoroughly and rapidly.
2. Add 5ml sulphuric acid solution, mix and let stand for 10 minutes
3. Place tubes in a water bath at 250C for 15 minutes
4. Read the absorbance at 488 nm against the blank.

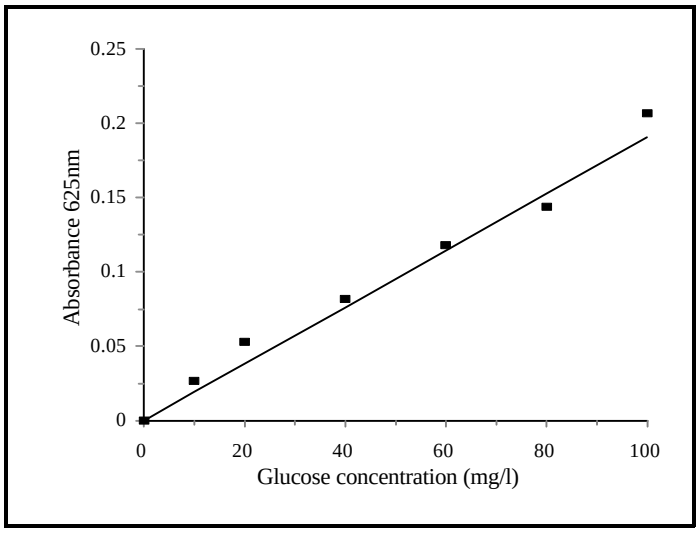
B. Anthrone method

Anthrone reagent: Add 200mg anthrone to 5ml of absolute ethanol and make up to 100ml with 75% sulphuric acid. Stir until dissolved and prepare fresh daily.

Preparation of glucose standards

Concentration ($mg.l^{-1}$)	Vol. Stock solution (ml)	Vol. ddH_2O
0	0	1
10	0.1	0.9
20	0.2	0.8
30	0.3	0.7
40	0.4	0.6
50	0.5	0.5
60	0.6	0.4
70	0.7	0.3
80	0.8	0.2
90	0.9	0.1
100	1	0

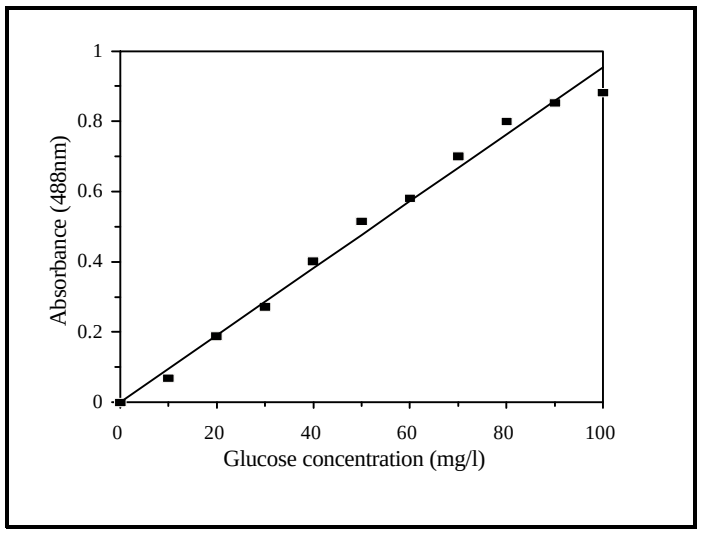
Glucose standard curve (Anthrone method)



Regression output:

Constant	0.005683
Std Err of Y Est	0.009398
R squared	0.985705
No. of observations	7
Degrees of freedom	5
X Coefficient	0.001907
Std Err of Coef	0.000103

Glucose standard curve (Phenol-sulphuric acid method)



Regression output:

Constant	0
Std Err of Y Est	0.032534
R squared	0.989383
No. of observations	11
Degrees of freedom	10
X Coefficient	0.009543
Std Err of Coef	0.000166

2. Protein determination

BSA standard: Add 0.9g BSA to 300ml of water. Gently swirl to moisten the BSA and avoid vigorous shaking which causes foaming. Let stand overnight at 4^0C and then mix gently.

Reagent A: Dissolve 20g of Na_2CO_3 in 1.0 litre of 0.1M NaOH (4.0g NaOH/litre water). Store at 4^0C in a polyethylene bottle.

Reagent B_1: Dissolve 1.0g of $CuSO_4.5H_2O$ in 100 ml of water. Store at 4^0C.

Reagent B_2: Dissolve 2.0g of sodium potassium tartrate, tetrahydrate in 100 ml of water.

Reagent C: To prepare a 3.0 mg/ml stock solution, place 10ml of reagent B_2 in a flask. With stirring, add 10ml of reagent $B_1$and then 1.0 litre of reagent A. Prepare this reagent fresh on the day of the experiment.

Reagent E: Dilute a commercial 2.0N Folin reagent to 1.0N with water. Store in a dark brown bottle.

Preparation of standards

Concentration ($mg.ml^{-1}$)	Vol. Stock solution (ml)	Vol. ddH_2O
0	0	1
0.3	0.1	0.9
0.6	0.2	0.8
0.9	0.3	0.7
1.2	0.4	0.6
1.5	0.5	0.5

Procedure

1. Add 5.0ml of reagent C to all tubes. Mix and let stand for 10-15 minutes at room temperature.

2. Add 0.5ml of reagent E to all tubes, mixing well after each addition.

3. Let the tubes stand at room temperature and measure absorbance at 540 nm.

3. Phosphorus determination

Principle:

In a solution acidified with sulphuric acid, orthophosphate ions react with molybdate ions to form molybdophosphoric acid which is reduced by ascorbic acid to phosphomolybdenum blue (PMB), the concentration of which is determined photometrically.

Procedure

1. Add 1ml of acidified sample to the reaction cell and mix thoroughly
2. Add 5 drops of reagent P-2K and mix and then add one level blue microspoon of reagent P-3K and dissolve.
3. Leave to stand for 5 minutes and read the sample on a spectroquant SQ-118 using method

number 143.

4. Ammonium determination

Principle:

In a strongly alkaline solution NH_4-N is almost entirely present as ammonia which react with a substituted phenol to form a blue indophenol which can be determined photometrically.

Procedure

1. Add 1ml of sample to the reaction vessel and mix.
2. Add one level microspoon of reagent NH4-1K and dissolve.
3. Allow to stand for 15 minutes and read on the spectroquant using method number 126.

5. Nitrate determination

Principle:

In the presence of chloride in a strong sulphuric acid solution, nitrate reacts with resorcinol to form a red-violet indophenol dye which is determined photometrically

Procedure

1. Add 1 level microspoon of reagent NO3-1K to the reaction vessel and shake briefly.
2. Add 2ml of the sample and mix.
3. Allow to stand for 15 minutes and take the reading using method number 117.

6. Soda ash TOC analysis (Coulometry method)

Principle:

Pure oxygen is passed over the sample heated in a furnace (530^0C) which decomposes all carbon compounds into volatile gases. These CO_2 rich gases pass into the coulometric cell which reacts with MEA to form an acid which changes the colour in MEA by changing the pH of the solution. This colour change is photometrically detected and the coulombs of electricity required for the release of a coulometrically generated base to bring the pH to its original state are converted to µgC.

Procedure

1. The soda ash sample was heated at 220^0C for 15 minutes to remove the bicarbonate.

2. About 150-180 mg of the sample was weighed on a platinum boat and analysed in the TOC analyser.