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Anaerobic digestion of sludge from marine recirculation aquaculture systems

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Abstract

Marine recirculation aquaculture systems (marine RAS) are considered as the most economical and sustainable solution to meet the increasing seafood demand. However, a considerable amount of organic-rich and salty sludge is produced and needs to be managed.

The emission minimization of marine aquaculture recirculation systems (EM-MARES) project aims to minimize the emission of marine RAS and also to achieve recuperation of bio-energy (methane) and phosphorus (struvite). This project includes three phases: coagulation of the sludge from marine RAS; anaerobic digestion in continuously stirred tank reactor (CSTR); and struvite recovery from the digested.

This research was part of the second phase of the EM-MARES project – anaerobic digestion of sludge from marine RAS in CSTR. The aim was twofold: first, to investigate the performance of CSTR inoculated with microorganisms already adapted to saline conditions and to compare the results with previous investigations about anaerobic digestion of sludge from marine RAS and typical values from anaerobic digestion of sewage sludge; second, to study the effect of salinity, potassium, compatible solutes and ferric chloride (FeCl_3) on specific methanogenic activity (SMA), phosphatase activity (PA), phosphate (PO_4^{3-}) release and extracellular polymeric substances (EPS).

A steady state operation of CSTR inoculated with microorganisms already adapted to saline conditions has been achieved. COD and VS removal efficiencies were 39.7-62.1% and 45.2-70.9%, respectively, and methane percentage in the biogas and production were 46.1-65.6% and 0.142-0.244 l methane/g COD added, respectively. The results of the present study were better than those reported by previous investigations, in terms of the obtainment of a stable operation and higher methane yields. Moreover, the volumetric methane production rate was higher than that typically found for anaerobic digestion of sewage sludge.

Batch studies showed that an increase of salinity decreased SMA, PA and PO_4^{3-} release and also affected EPS production. The two compatible solutes tested, betaine and trehalose, improved SMA, PA and PO_4^{3-} release and decreased the amount of bound EPS (bEPS) produced by the cells. Potassium did not show apparent effect on SMA, however, it improved PA and PO_4^{3-} release and increased the amount of bEPS. FeCl_3 , already tested as coagulant in the first part of the EM-MARES project, did not present any negative effect on PA and EPS, and increased PO_4^{3-} release.

This research demonstrates the anaerobic digestion in CSTR as a feasible solution for the management of sludge from marine RAS, increasing the sustainability of these systems which are the major solution to keep satisfying the increasing demand of seafood.

Keywords: marine recirculation aquaculture systems; sludge; salinity; anaerobic digestion; methane; phosphorus; ferric chloride; potassium; compatible solutes; specific methanogenic activity; phosphatase; phosphate; extracellular polymeric substances.

Resumo

Os sistemas de aquacultura marinha com recirculação (*marine recirculation aquaculture systems* (*marine RAS*)) são considerados a solução mais econômica e sustentável para satisfazer o crescente consumo de espécies marinhas. Porém, uma quantidade considerável de lamas salgadas e ricas em matéria orgânica é produzida e deve ser gerida.

O projeto “minimização da emissão dos sistemas de aquacultura marinha com recirculação” (*emission minimization of marine aquaculture recirculation systems* (EM-MARES)) tem como objetivo minimizar as emissões dos *marine RAS* mas também recuperar bio-energia (metano) e fósforo (estruvite). Este projeto inclui três fases: coagulação das lamas provenientes dos *marine RAS*; digestão anaeróbia num reator perfeitamente agitado (*continuously stirred tank reactor* (CSTR)); e recuperação de estruvite das lamas digeridas.

Este trabalho insere-se na segunda fase do projeto EM-MARES – digestão anaeróbia das lamas provenientes dos *marine RAS* num CSTR. Os objetivos foram: primeiro, investigar o desempenho de CSTR inoculado com microrganismos adaptados a elevada salinidade e comparar os resultados com investigações anteriores relacionadas com a digestão anaeróbia de lamas dos *marine RAS* e valores típicos da digestão anaeróbia de lamas de esgotos municipais; segundo, estudar o efeito da salinidade, do potássio, de solutos compatíveis e do cloreto de ferro (FeCl_3), na atividade metanogénica específica (*specific methanogenic activity* (SMA)), na atividade da fosfatase (*phosphatase activity* (PA)), na libertação de fosfato (PO_4^{3-}) e nas substâncias poliméricas extracelulares (*extracellular polymeric substances* (EPS)).

Na digestão anaeróbia das lamas dos *marine RAS* em dois CSTRs inoculados com microrganismos adaptados a elevada salinidade, uma operação estável foi conseguida. As reduções da carência química de oxigénio (*chemical oxygen demand* (COD)) e dos sólidos voláteis (*volatile solids* (VS)) foram 39.7-62.1% e 45.2-70.9%, respetivamente. A percentagem de metano no biogás e a sua produção foram 46.1-65.6% e 0.142-0.244 l metano/g COD introduzido, respetivamente. Os resultados do presente trabalho superaram os de investigações anteriores, principalmente devido à obtenção de uma operação estável e uma maior produção de metano. A taxa de produção volumétrica de metano foi superior à tipicamente observada na digestão anaeróbia de lamas de esgotos municipais.

Estudos “*bacth*” mostraram que um aumento na salinidade diminuiu a SMA, a PA e a libertação de PO_4^{3-} , e também afetou a produção de EPS. Os dois solutos compatíveis testados (betaína e trealose) aumentaram a SMA, a PA e a libertação de PO_4^{3-} , e diminuíram a quantidade produzida de EPS ligadas às células (*bound EPS* (bEPS)). A adição de potássio não afetou a SMA, mas aumentou a PA, a libertação de PO_4^{3-} e a produção de bEPS. O FeCl_3 , testado como coagulante na primeira fase do projeto EM-MARES, não mostrou nenhum efeito negativo na PA e na produção de EPS, e aumentou a libertação de PO_4^{3-} .

Esta investigação demonstra a digestão anaeróbia usando um CSTR como uma solução viável para a gestão de lamas provenientes dos *marine RAS*, contribuindo para o aumento da sustentabilidade destes sistemas que são considerados como a melhor solução para satisfazer o crescente consumo de espécies marinhas.

Palavras-chave: Sistemas de aquacultura marinha com recirculação; lamas; salinidade; digestão anaeróbia; metano; fósforo; cloreto de ferro; potássio; solutos compatíveis; atividade metanogénica específica; fosfatase; fosfato; substâncias poliméricas extracelulares.

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List of abbreviations

AMPTS II - Automatic Methane Potential Test System II

AnMBR - Anaerobic Membrane Bioreactor

ASBR - Anaerobic Sequential Batch Reactor

bEPS - bound Extracellular Polymeric Substances

BFBR - Buoyant Filter Bio-Reactor

BSA - Bovine Serum Albumin

COD - Chemical Oxygen Demand

CSTR - Continuous Stirred Tank Reactor

DIRB - Dissimilatory Iron-Reducing Bacteria

EC - Electrical Conductivity

EM-MARES - Emission Minimization of Marine Aquaculture Systems

EPS - Extracellular Polymeric Substances

FBR - Fed Batch Reactor

LCFA - Long Chain Fatty Acids

MLSS - Mixed Liquor Suspend Solids

OLR - Organic Loading Rate

PA - Phosphatase Activity

p-NP - p-nitrophenol

p-NPP - p-nitrophenylphosphate

RAS - Recirculation Aquaculture Systems

SAMBR - Submerged Anaerobic Membrane Bioreactor

sCOD - soluble Chemical Oxygen Demand

sEPS - soluble Extracellular Polymeric Substances

SMA - Specific Methanogenic Activity

SMP - Soluble Microbial Products

SRB - Sulfate Reducing Bacteria

SRT - Solids Retention Time

STP - Standard conditions of Temperature and Pressure

tCOD - total Chemical Oxygen Demand

TN - Total Nitrogen

TP - Total Phosphorus

TS - Total Solids

TSS - Total Suspended Solids

UASB - Upflow Anaerobic Sludge Blanket

VFA - Volatile Fatty Acids

VS - Volatile Solids

VSS - Volatile Suspended Solids

WSP - Water Stabilization pond

WWTP - Wastewater Treatment Plant

1. Introduction

1.1. Marine recirculation aquaculture systems (marine RAS) and environmental threats

The demand for fish and seafood has been increasing and will continue to grow with expanding population, increasing income and urban growth in the developing world. Figure 1 shows that not only the world population but also the supply of fish per capita increased between 1950 and 2008, which intensified the raise in the world consumption of fish in this period.

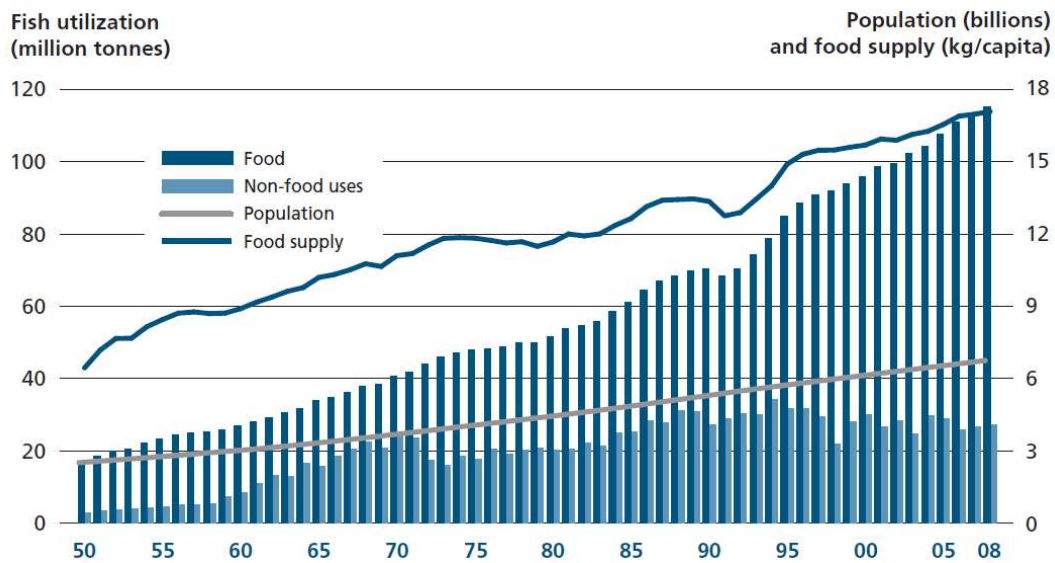


Figure 1. World fish utilization and supply (The state of world fisheries and aquaculture 2010).

Marine resources are finite and full exploration and over-exploration occur in some regions in the world. Furthermore, the regulations on discharge of aquaculture systems are becoming more stringent. Thus, a sustainable and efficient production of seafood needs to be developed.

Marine recirculation aquaculture systems (marine RAS) are considered the most economical and environmental friendly aquaculture systems capable to meet the future demand for seafood (Martins et al., 2010). Typically, in a marine RAS (Figure 2), water flows from an aquaculture tank through a series of treatment processes, and then back to the same tank. This kind of system allows to minimize water replacement, to control over most water quality constituents, to compensate for an insufficient water supply and to improve waste management and nutrient recycling (Martins et al., 2010; Mirzoyan et al., 2010).

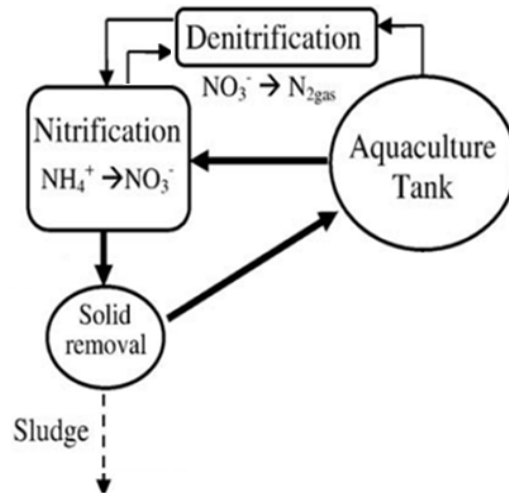


Figure 2. Scheme of a marine RAS (adapted from (Mirzoyan et al., 2010)).

The advantages of marine RAS result at a super-intensive culture, and consequently a considerable quantity of waste is produced and must be managed. One common characteristic of marine RAS is the concentration of solid waste into smaller flows that are easier to handle. The most common solids-removal units used for marine RAS are (Mirzoyan et al., 2010):

- Settling basins that are based on separation by gravity;
- Hydrocyclones or swirl separators in which centrifugal sedimentation allows for more rapid separation of the particles from the liquid;
- Microscreen filters that are based on screening particles that are larger than the screen's mesh size;
- Granular/porous media filters that are based on the passage of water through a medium on which the solids are deposited/strained.

The solids from marine RAS contain mainly fish excretions and a small amount of uneaten feed, it has a great fraction of volatiles (organics) that ranges from 50 to 92%, and high salinity levels (Mirzoyan et al., 2010).

The sludge from marine RAS has usually the following destinations: receiving water bodies, local sewer system and decentralized treatment unit, being waste-stabilization ponds (WSPs) the more used (Mirzoyan et al., 2010). Problematic is the sludge that is discharged into water-receiving bodies, which directly pollute local environments. Disposal of marine RAS sludge into municipal wastewater-treatment systems is often not allowed because it contains great amounts of organic matter and high salinities that may interfere with the treatment process. In the WSPs a considerable part of the influent organic carbon (17-30%) is transformed into methane (CH_4) but collecting this gas from large areas such as those of WSPs is expensive or inefficient (Mirzoyan et al., 2010). This leads into the liberation of this greenhouse gas into the atmosphere which contributes to climate changes. Furthermore, the resulting effluent from WSPs is often used for irrigation which, due to the high salinity of sludge from marine RAS, leads to soil and groundwater salinization.

New approaches to manage the waste of marine RAS need to be investigated. This could not only mitigate the pollution caused by marine RAS but also increase the sustainability of these systems that are the better solution to meet the increasing demand of seafood.

1.2. Emission minimization of marine aquaculture recirculation systems (EM-MARES) project

The emission minimization of marine aquaculture recirculation systems (EM-MARES) project is a PhD research carried out in the University of Technology of Delft that aims to develop an approach to waste management not only to minimize the emission of marine RAS but also to achieve recuperation of bio-energy (methane) and phosphorus (struvite).

The treatment method for backwash water originated from marine RAS, proposed in the EM-MARES project, is shown in Figure 3.

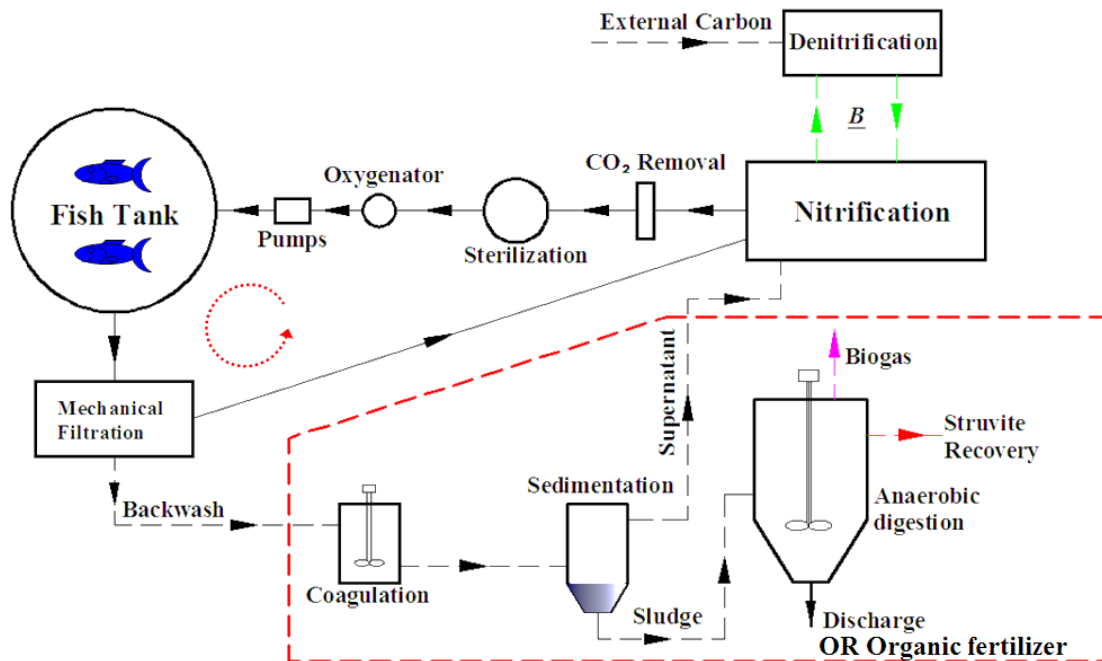


Figure 3. Scheme of marine RAS and proposed treatment method for the backwash water and sludge in the EM-MARES project (EM-MARES project).

The contents of EM-MARES project include three parts:

- 1) Selection of an appropriate coagulant with a good performance in concentration of solids and phosphorus at saline conditions and with a minimal negative effect on biodegradability of material coagulated and settled by the coagulant;
- 2) Anaerobic digestion of the sludge from marine RAS in a continuous stirred tank reactor (CSTR) inoculated by seeds cultured in saline conditions and;
- 3) Struvite recovery from the digested.

1.3. Anaerobic digestion

1.3.1. Reasons for anaerobic digestion

The interest on anaerobic digestion can be explained by considering its advantages and disadvantages, and it can be best indicated by comparing this process with aerobic digestion. In the aerobic digestion the waste is mixed with large quantities of microorganisms and air. Microorganisms use the organic matter for substrate, and use the oxygen in the air to oxidize a portion of this substrate to carbon dioxide and water for energy (McCarty, 1964a). In anaerobic digestion, the waste is also mixed with large quantities of microorganisms but without addition of air. Under these conditions, bacteria grow which are capable of converting the organic waste into carbon dioxide (CO₂) and methane gases (biogas).

In the aerobic digestion, since the microorganisms obtain more energy from the use of oxygen, their growth is fast and a great portion of the organic waste is converted into new cells. The waste converted into cells is not fully stabilized, but is only changed in form (McCarty, 1964a). These cells can be removed from the waste stream but the biological sludge still presents a significant disposal problem. In fact, anaerobic digestion is one of the most used disposal routes for the biological sludge produced by the aerobic digestion in municipal wastewater treatment plants (WWTPs) (Appels et al., 2008). In anaerobic digestion, conversion to methane gas requires relatively little energy to the microorganisms. Thus their rate of growth is slow and only a small portion of the waste is converted into new cells, being the major portion of the degradable waste converted into methane and, since this gas is insoluble and escapes from the waste, this conversion really represents waste stabilization. As much as 80 to 90% of the degradable organic matter for the waste can be removed in anaerobic digestion in contrast to aerobic digestion, where only about 50% is actually removed (McCarty, 1964a).

Due to the slow growth of the cells in the anaerobic digestion, the sludge produced in the anaerobic digestion is 6 to 8 times less than that produced in the aerobic digestion, which greatly reduces its processing and disposal costs (Metcalf & Eddy, 2003). In addition, since less biomass is produced, less nutrients are needed for the anaerobic digestion (Metcalf & Eddy, 2003). This is especially important in the treatment of industrial wastes which lack sufficient nutrients.

Since anaerobic digestion does not require oxygen, treatment rates are not limited by oxygen transfer. This reduces power requirements for digestion because there is no need for aeration. Besides that, the methane produced can be collected and burned to carbon dioxide and water for heat. This fuel is frequently used for heating buildings, running engines and producing electricity (McCarty, 1964a). The no need of oxygen and methane production outweigh the need of relatively high temperatures (at least 30 °C) for optimum operation in the anaerobic digestion, as indicated in Table 1.

Table 1. Energy balance for aerobic and anaerobic processes for the treatment of a wastewater (wastewater flow rate = 100 m³/d, wastewater strength = 10 kg COD/m³ and temperature = 20 °C) (Metcalf & Eddy, 2003).

	Energy (kJ/d)	
	Anaerobic	Aerobic
Aeration ^{a,b}		-1.9 x 10 ⁶
Methane produced ^{c,d}	12.5 x 10 ⁶	
Increase wastewater temperature to 30 °C	-2.1 x 10 ⁶	
Net energy	10.4 x 10 ⁶	-1.9 x 10 ⁶

^aOxygen required = 0.8 kg/kg COD (Chemical oxygen demand) removed

^bAeration efficiency = 1.52 kg O₂/kWh and 3600 kJ= 1 kWh

^cMethane production = 0.35 m³/kg COD removed

^dEnergy content of methane = 35 846 kJ/m³ (at 0 °C and 1 atm)

The collection of the biogas produced in the anaerobic digestion, not only allows use methane as fuel, but also prevent the release of this gas plus carbon dioxide to the atmosphere, which are greenhouses gases that can cause climate changes. In contrast, in the aerobic digestion, from the process itself and as result of the high consumption of electricity mainly generated from fossil fuels, large quantities of carbon dioxide are release into the atmosphere.

Anaerobic process generally can have higher volumetric organic loading rates (OLRs) than aerobic process, so smaller reactor volume and less space are required for treatment. OLR of 3.2 to 32 kg COD/m³/d may be used for anaerobic processes, compared to 0.5 to 3.2 kg COD/m³/d for aerobic processes (Metcalf & Eddy, 2003).

Regarding the disadvantages of the anaerobic digestion, the major concern is its longer start-up time (months for anaerobic versus days for aerobic digestion), their sensitivity to possible toxic compounds, possible operational instability, the potential for odors production, and corrosiveness of the digester gas. However, with proper waste characterization and process design these problems can be avoided and/or managed (Metcalf & Eddy, 2003).

The principal advantages and disadvantages of the anaerobic digestion are summarized in the Table 2.

Table 2. Advantages and disadvantages of anaerobic digestion compared to aerobic digestion.

Anaerobic digestion	
Advantages	Disadvantages
<ul style="list-style-type: none"> • A high degree of waste stabilization is possible • Low biological sludge production • Low nutrients requirement • Methane production, a potential energy source • Low energy required • Elimination of off-gas air pollution • Smaller reactor volume required • Rapid response after long periods without feeding 	<ul style="list-style-type: none"> • Longer start-up time to develop necessary biomass inventory • More sensitive to the effect of lower temperatures and toxic substances • Potential for production of odors and corrosive gases

The advantages of anaerobic digestion are quite significant, while the disadvantages are relatively few and can be successfully managed. The advantages normally overcome even more the disadvantages for industrial waste with high biodegradable chemical oxygen demand (COD) and/or elevated temperatures where anaerobic digestion may be a very economical solution (McCarty, 1964a; Metcalf & Eddy, 2003).

1.3.2. Chemistry and microbiology

Anaerobic digestion is a complex process which requires strict anaerobic conditions to proceed, and depends on the coordinated activity of a complex microbial association to transform organic material into methane. The chemical and microbiological aspects of anaerobic digestion are dealt with more deeply with in specialized literature (Gerardi, 2003; Gujer and Zehnder, 1983; Khanal, 2008; McCarty, 1964a).

The anaerobic digestion of organic matter basically has 5 stages: hydrolysis, fermentation, anaerobic oxidation of fatty acids and alcohols, anaerobic oxidation of intermediary products, and methanogenesis, as shown in Figure 4.

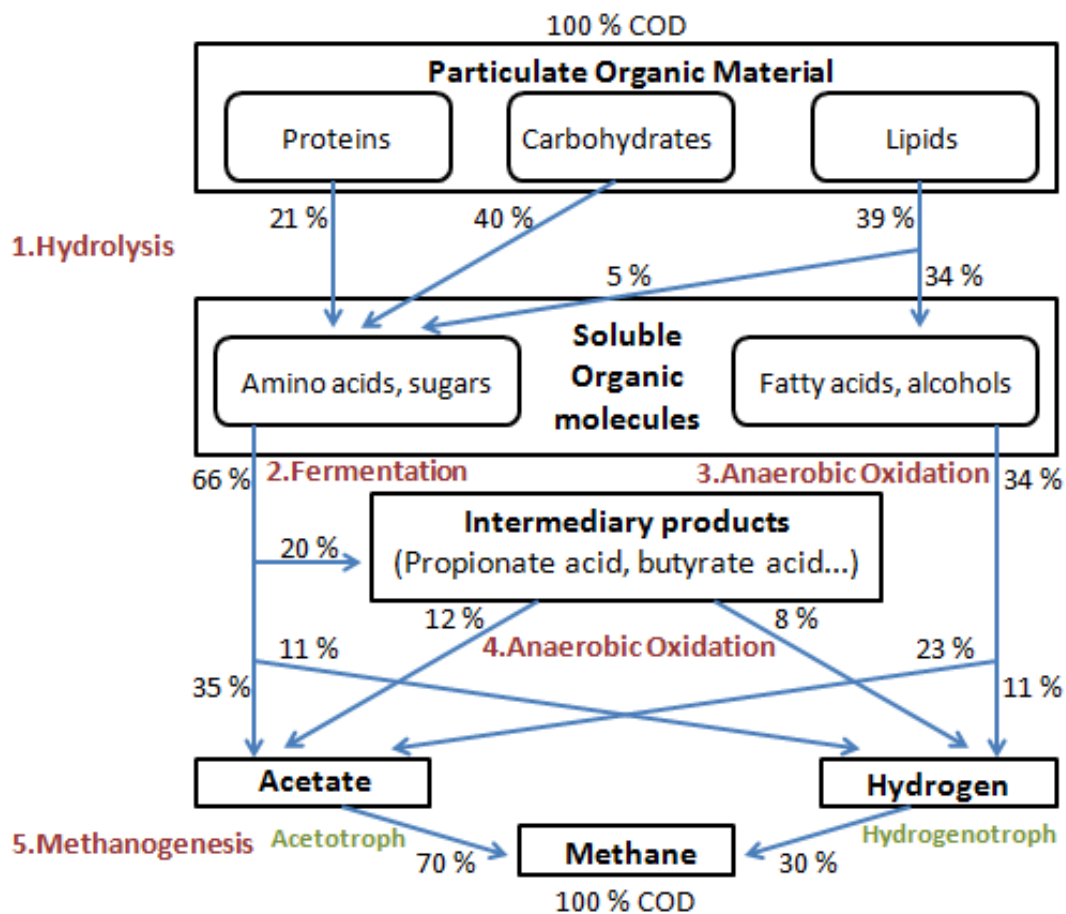
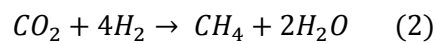


Figure 4. Stages of anaerobic digestion. Adapted from (Gujer and Zehnder, 1983). Percentages indicate stoichiometric flow in form of COD or CH₄ equivalents. In red are indicated the stages of the anaerobic digestion.

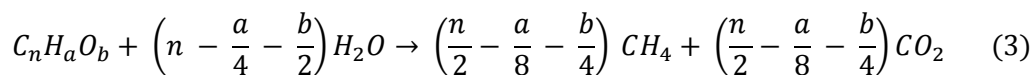
In general bacteria are unable to take up particulate organic material. In the hydrolysis stage the complex and insoluble organic material and high molecular weight compounds such as proteins, carbohydrates and lipids are converted into soluble organic molecules like amino acids, sugars and fatty acids (Gujer and Zehnder, 1983). The components formed during hydrolysis are further decomposed through fermentation and anaerobic oxidation (Gujer and Zehnder, 1983). Fermentation is a process in which organic compounds serve both as electron donors and electron acceptors (Gujer and Zehnder, 1983). The substrates for fermentation are amino acids and sugars, and the products are biomass, intermediary degradation products (propionate, butyrate and other acids) and the methane precursors: acetate and hydrogen (Gujer and Zehnder, 1983). In the anaerobic oxidation of fatty acids molecular hydrogen is the

main sink for electrons. In this process fatty acids are converted into hydrogen and, in contrast to the fermentation, elevated partial pressures of it can inhibit this process. The fourth stage of anaerobic digestion is the anaerobic oxidation of the intermediary products into acetate and hydrogen. No waste stabilization occurs during the firsts four stages of the anaerobic digestion but they are required to convert the organic matter into a form suitable for the last stage of digestion. Because the main products of these first stages are acids, the group of bacteria responsible for these processes are commonly called "acid formers" (McCarty, 1964a). It is in last stage of the anaerobic digestion, methanogenesis, that real waste stabilization occurs, and it is directly related to methane production. Acetate and hydrogen are used by a special group of bacteria termed "methanogens" to produce methane. The methanogenesis from acetate, which is responsible for about 70% of the methane produced, called acetotrophic methanogenesis, and from hydrogen, which is called hydrogenotrophic methanogenesis, follow the reactions, respectively (Gerardi, 2003):

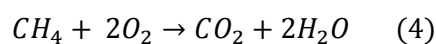


The most important methanogens, those which use acetate as substrate, grow quite slowly. Their slow growth and low rate of acid utilization normally represents the limiting step of the anaerobic digestion (McCarty, 1964a). The rate at which these methanogens utilize their substrates to produce methane is called specific methanogenic activity (SMA) (Isa et al., 1993). The assessment of SMA can be used to investigate the anaerobic reactors performance and the effect of different variables like stimulants/toxicants and environmental conditions on anaerobic bacteria, and to test the biodegradability and adaptability of particular wastes (Isa et al., 1993).

The quantity of methane produced in the anaerobic digestion can be predicted through two different ways. If the composition of the substrate is known and the entire substrate is converted to gas, the theoretical methane yield can be calculated from the following equation (Gujer and Zehnder, 1983):



From this formula, it can be shown that the ultimate oxygen demand of the waste being degraded is equal to the ultimate oxygen demand of the methane gas produced. This fact allows prediction of methane production in another way, that is, from an estimate of waste stabilization (COD removal). The ultimate oxygen demand of methane gas is as follows (McCarty, 1964a):



This equation shows that one mol of methane is equivalent with two moles of oxygen. Converting to liters of methane per gram of oxygen, the relationship between waste stabilization and methane production, in the standard conditions of temperature and pressure (STP), is:

$$1 \text{ g COD removed} = 0.35 \text{ l methane (STP)} \quad (5)$$

The prediction of the methane production it is very important for the analysis of the feasibility of anaerobic digestion. It can also be used to give a rapid measurement of actual waste stabilization and to permit closely following the efficiency of digestion and the collection of biogas (McCarty, 1964a).

1.3.3. Affecting parameters

The methanogens, which are responsible for the waste stabilization in the anaerobic digestion, grow quite slowly compared to aerobic organisms and so a longer time is required for them to adjust to changes in their environmental. For this reason, there are some environmental requirements for optimum operation of anaerobic digestion in order to obtain a more efficient process. The most important affecting parameters to the anaerobic digestion are discussed below.

1.3.3.1. Temperature

Commonly, the anaerobic digestion is practiced at one of three temperature regimes, shown in Table 3.

Table 3. Temperature regimes practiced in the anaerobic digestion (Mirzoyan et al., 2010).

Psychrophilic	10-25 °C
Mesophilic	25-45 °C
Thermophilic	45-65 °C

An increasing temperature has several benefits, including an increasing solubility of the organic compounds, enhanced biological and chemical reaction rates and increasing death rate of pathogens (Appels et al., 2008). However, the application of high temperatures (thermophilic) has also negative effects: more energy is needed to heat the digester and there will be an increase of the fraction of free ammonia, which may inhibit the microorganisms (Appels et al., 2008). The anaerobic digestion can be applied at psychrophilic regime but, at these temperatures, slower reaction rates occur and longer solids retention times (SRTs), larger reactor volumes, and lower organic COD loadings are need (Metcalf & Eddy, 2003). In general, mesophilic digestion temperatures are preferred to support more optimal biological reaction rates and to provide more stable treatment (Metcalf & Eddy, 2003).

1.3.3.2. pH

Anaerobic treatment can proceed quite well with a pH varying from about 6.6 to 7.6, with an optimum range of about 7.0 to 7.2 (McCarty, 1964b). Beyond these limits, digestion can proceed, but with less efficiency. At pH below 6.2 the efficiency drops off rapidly, and the acidic conditions produced can become toxic to the methanogens.

1.3.3.3. Oxygen

Another environmental requirement for anaerobic digestion is that anaerobic conditions be maintained. Small quantities of oxygen can be quite detrimental for the methanogens and other organisms involved. This requirement necessitates a closed digestion tank, which is also desirable so the methane gas can be collected and escape of odors can be prevented (McCarty, 1964b).

1.3.3.4. Nutrients

The anaerobic process is dependent upon bacteria, which require nitrogen, phosphorus and other materials in trace quantities for optimum growth. Domestic waste normally contains a variety of these materials, and thus usually provides an ideal environment for the microorganisms. However, some industrial wastes are frequently more specific on composition and biological nutrients may lack, thus nutrients must be added for the optimum operation.

1.3.3.5. Solids retention time (SRT)

The solids retention time (SRT) is the average time the solids spend in the digester. The subsequent stages of the digestion process are directly related to the SRT. A decrease in the SRT decreases the extent of the reactions, and vice versa. Each time the sludge is discharged, a fraction of the bacterial population is removed thus implying that the cell growth must at least compensate the cell removal to ensure steady state and avoid process failure (Appels et al., 2008). In general, SRT range for effective anaerobic digestion is 10 to 60 days, being the higher SRTs required for lower temperatures (Appels et al., 2008).

1.3.3.6. Organic loading rate (OLR)

Organic loading rate (OLR) is defined as the application of soluble and particulate organic matter. It is typically expressed in mass of COD per volume of the reactor and per day. With very high OLRs the methanogens in the digester may not be able to degrade all the acids formed in the previous stages of anaerobic digestion, the acids formed will accumulate and the pH will drop to lower levels which cause a failure of the process. With very low OLRs only a small volume of methane per volume of reactor is produced making the process economically not feasible. OLRs of 3.2 to 32 kg COD/m³.d may be used for anaerobic process (Metcalf & Eddy, 2003).

1.3.3.7. Toxic materials

There are many materials, both organic and inorganic, which may be toxic to the anaerobic digestion. The concentration at which a material becomes toxic may vary from a fraction of mg/l to several thousand mg/l. Some of these materials, as salts, at some very low concentration stimulate the biological activity, but at very high concentrations are toxic for the microorganisms (Figure 5). Tables 4 and 5 present some toxic inorganic and organic compounds of concern for anaerobic digestion, respectively.

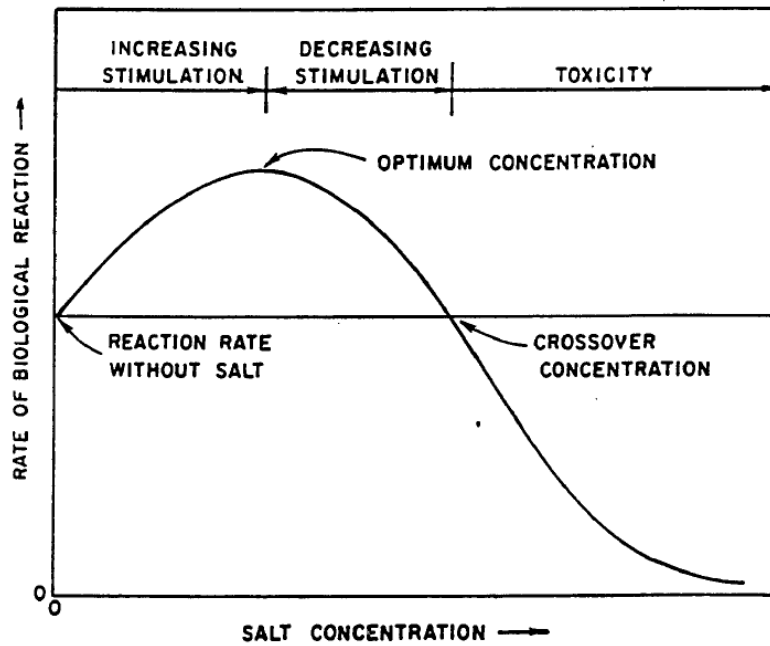


Figure 5. General effect of salts on biological reactions (McCarty, 1964c).

Table 4. Toxic inorganic compounds for the anaerobic digestion (Appels et al., 2008; McCarty, 1964c; Metcalf & Eddy, 2003).

Compound	Stimulating concentration (mg/l)	Moderately toxic concentration (mg/l)	Strongly toxic concentration (mg/l)
Sodium (Na ⁺)	100-200	3500-5500	8000
Potassium (K ⁺)	200-400	2500-4500	12000
Calcium (Ca ²⁺)	100-200	2500-4000	8000
Magnesium (Mg ²⁺)	75-150	1000-1500	3000
Ammonia (NH ₄ ⁺)		1500-3000	3000
Sulfide (S ²⁻)		200	200
Copper (Cu ²⁺)			0.5 (soluble)
			50-70 (total)
Chromium (Cr ⁶⁺)		10	3.0 (soluble)
			200-250 (total)
Chromium (Cr ³⁺)		10	2.0 (soluble)
			180-420 (total)
Nickel (Ni ²⁺)			30 (total)
Zinc (Zn ²⁺)			1.0 (soluble)
Arsenate and arsenite		>0.7	
Cyanide		1-2	
Lead-containing compounds		5	
Iron-containing compounds		>35	
Copper-containing compounds		1	
Potassium chloride (KCl)		>10000	
Chloride (Cl ⁻)		6000	

Table 5. Toxic organic compounds for the anaerobic digestion (Appels et al., 2008).

Compound	Concentration resulting in 50 % reduction in activity (mM)
1-Chloropropene	0.1
Nitrobenzene	0.1
Acrolei	0.2
1-Chloropropene	1.9
Formaldehyde	2.4
Lauric acid	2.6
Ethyl Benzene	3.2
Acrylonitrile	4
3-Chlorol-1,2-propanedial	6
Crotonaldehyde	6.5
2-Chloropropionic acid	8
Vinyl acetate	8
Acetaldehyde	10
Ethyl acetate	11
Acrylic acid	12
Catechol	24
Phenol	26
Aniline	26
Resorcinol	29
Propanol	90

1.3.4. Types of anaerobic reactors

There are mainly four different reactor types used for anaerobic digestion of sludge: the continuous stirred tank reactor (CSTR); the upflow anaerobic sludge blanket reactor (UASB); the anaerobic membrane bioreactor (AnMBR); and the anaerobic sequencing batch reactor (ASBR).

In the CSTR (Figure 6), substrate is introduced into a tank and mixed (with an impeller or biogas recirculation). This reactor has a simple operation and is very efficient in treating different types of organic-rich sludges but operational costs are high (Mirzoyan et al., 2010). The CSTR can be attached to a settler that separates the treated liquid from the solids (biomass), and the latter is returned to the CSTR.

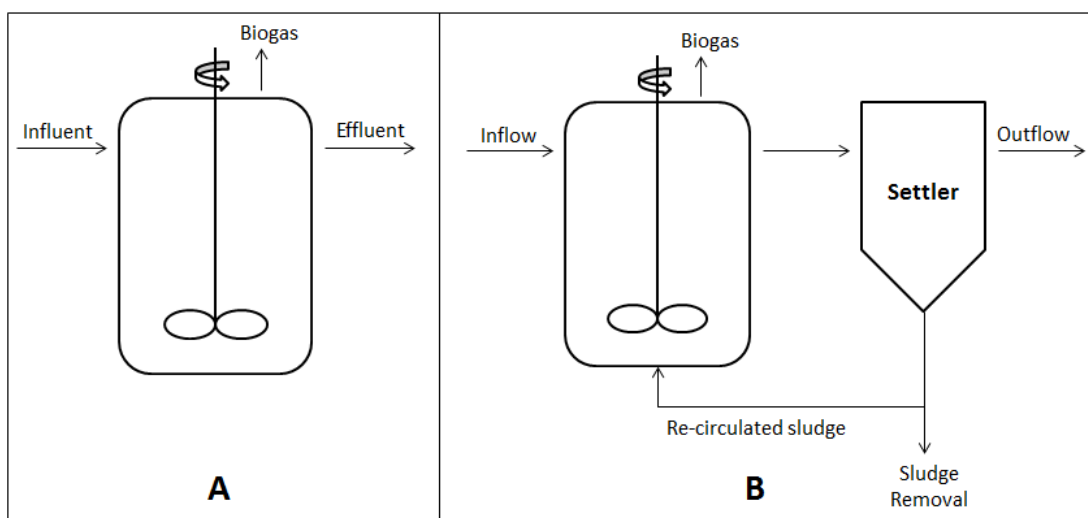


Figure 6. Schemes of CSTR with (B) and without (A) settler.

In the UASB reactor (Figure 7) the influent flows upward through a blanket of granular sludge which is suspended in the tank and is degraded by the anaerobic microorganisms.

Advantages for the UASB process are the high loadings and relatively low detention times possible for anaerobic digestion (Metcalf & Eddy, 2003). Limitations of the process are related to those wastes that are high in solids content or where their nature prevents the development of the dense granulated sludge (Metcalf & Eddy, 2003).

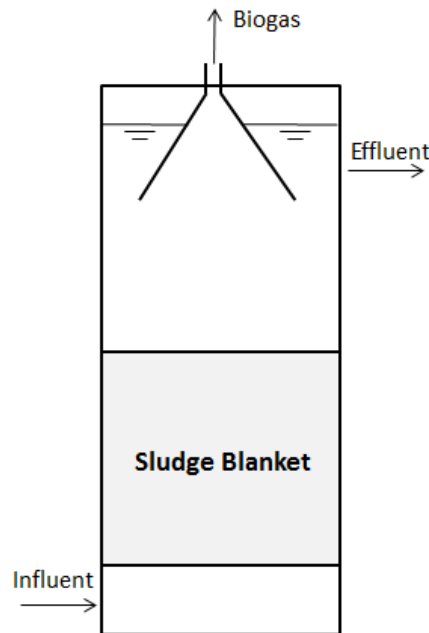


Figure 7. Scheme of a UASB reactor.

The AnMBR (Figure 8) combines the anaerobic treatment with membrane filtration. The membrane filters the particulate waste constituents from the mixed liquor solution. The clean permeate (effluent) leaves the system while the concentrate returns to the reactor. The main advantages of the AnMBR are the high effluent quality, small footprint and the applicability for retrofitting and upgrading of old wastewater-treatment plants, while high operational costs and biofouling are the major disadvantages (Mirzoyan et al., 2010).

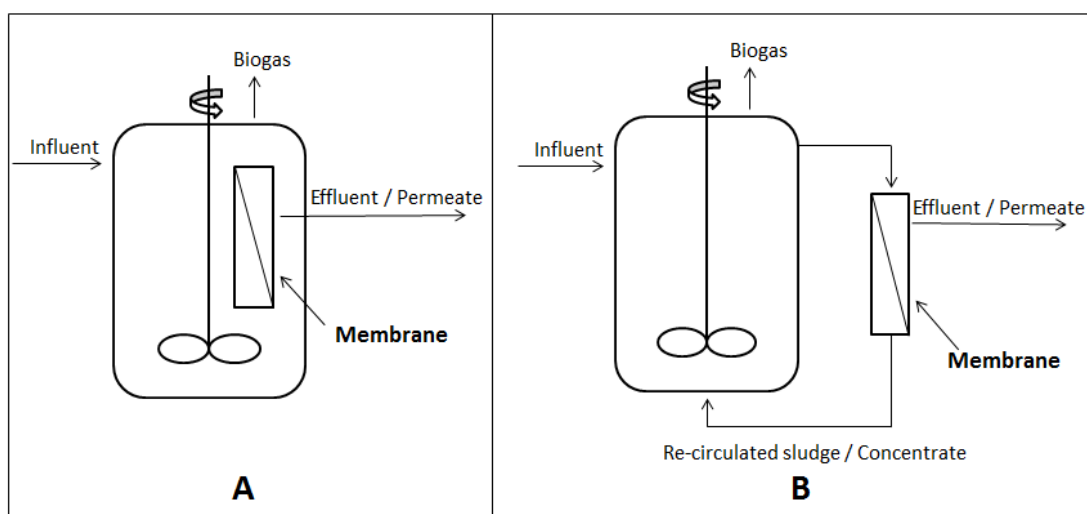


Figure 8. Schemes of AnMBR with submerge membrane (A) and with external membrane (B).

In an ASBR the influent is not continuously added into the reactor. In this kind of configuration reaction and solids-liquid separation occurs in the same vessel. The operation of

an ASBR consists of four steps (Figure 9): feeding, reaction, settling and discharging. During the reaction period, intermittent mixing for a few minutes each hour is done to provide uniform distribution of substrate (influent) and solids (biomass) (Mirzoyan et al., 2010). The control of batch reactors involves complex control functionality beyond the well-established norms for continuous processes, and it constitutes a challenging area (Berber, 1996). The main advantages are its ability to maintain high performance during periods of shock loads and relatively small footprint compared to digesters with a settler (Wilderer et al., 2000).

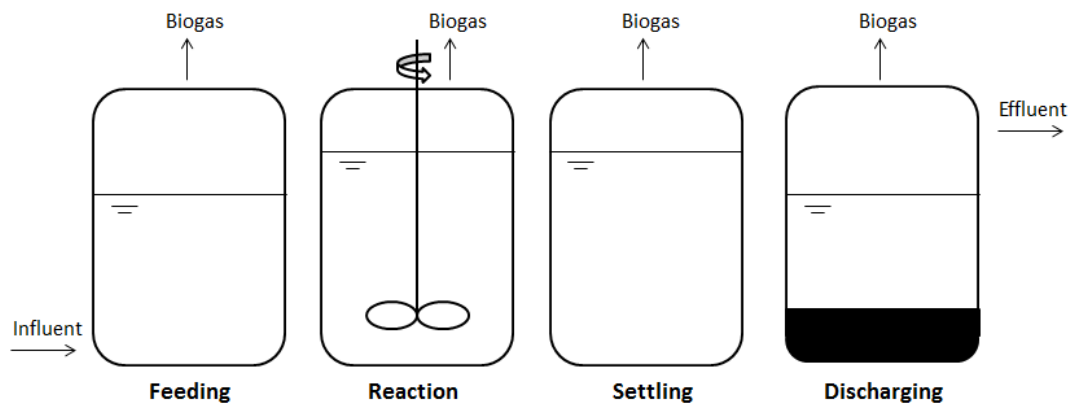


Figure 9. Four steps of a ASBR operation.

1.3.5. Feasibility of anaerobic digestion to treat sludge from marine RAS in CSTR, to recover phosphorus as struvite, and its bigger challenge

The sludge from marine RAS is mainly constituted of fish excretions and uneaten food (Mirzoyan et al., 2010) resulting in a high concentration of biodegradable organic material and nutrients. Thus, a high volume of methane per volume of sludge can be produced and no addition of nutrients is needed, making the use of anaerobic digestion a very economical solution. Since the up-concentration of solids is part of marine RAS, the resulting sludge has high solids content which limits the use of UASB and may increase biofouling in the membrane of an AnMBR. Due to the complex operation of an ASBR, CSTR, with its simple operation and high efficiency in treating organic-rich sludges, seems the most feasible option.

Not only the production of bio-energy as methane is feasible, but also recuperation of phosphorus as struvite is very promising. Struvite (magnesium ammonium phosphate (MAP) hexahydrate ($MgNH_4PO_4 \cdot 6H_2O$)) is a white inorganic crystalline mineral, density 1.71 g/cm^3 , soluble in acid, not in water, alkali and ethanol, and considered as a slow release fertilizer (Zhang et al., 2012). In sludges from marine RAS, phosphorus content in the dry mass (1.3-3.1%) is much higher than in domestic sludges (0.7%). Meanwhile, there is a considerable amount of magnesium (due to the high salinity of sludge from marine RAS) which could serve as a part of the magnesium source for the production of struvite, reducing the required addition of magnesium salts. Furthermore, ammonia is generated in the anaerobic digestion process (Möller and Müller, 2012).

In marine RAS, brackish and saline waters are used to produce seafood, with salinities of 0.5-30 g/l and 30-50 g/l, respectively. As is shown in Table 4, these levels of salt are generally considered toxic for the anaerobic digestion. There so, the biggest challenge for the anaerobic digestion of sludge from marine RAS is its high salinity level.

1.4. Adaptation of anaerobic biomass to saline conditions

Although the cations of salts in solution must be associated with the anions, the toxicity of salts was found to be predominantly determined by the cation. They are required for microbial growth and, consequently, affect specific growth rate like any other nutrient (Chen et al., 2008). The problem of high salt level is that it causes bacterial cells to dehydrate due to osmotic pressure (Chen et al., 2008). However, there are two fundamental strategies for microorganisms to survive under osmotic stress (Figure 10):

- Cells increase the intracellular ion concentration (mainly potassium (K^+)) with the aim to balance the external osmotic pressure. In this case all intracellular enzymes have to adapt to the new conditions. This so called “salt in strategy” is used by anaerobic halophilic microorganisms, whose entire physiology has been adapted to high saline environments (Vyrides et al., 2010; Vyrides and Stuckey, 2009). *Haloanaerobacter chitinovorans*, *Haloanaerobium congolense*, *Halanaerobium lacusrosei*, *Haloanaerobium praevalens* and *Haloanaerobium alcaliphilum* are examples of anaerobic halophilic organisms (Kapdan and Erten, 2007).
- Accumulation of compatible solutes by the microorganisms. The high external osmotic pressure is balanced within the cytoplasm by high molecular weight organic compatible solutes without the need for special adaptation of the intracellular enzymes. These compounds also serve as protein stabilizers in the presence of high ionic strength inside the cell. Compatible solutes can be synthesized by the cell, or be provided by the medium. For most species uptake from the medium is energetic more favorable than biosynthesis (Vyrides et al., 2010; Vyrides and Stuckey, 2009). Besides allowing cells to live in saline conditions, compatible solutes can provide beneficial effects on membrane integrity, protein folding and stability (Oh et al., 2008). These osmoprotectants are a chemically diverse group of compounds, including amino acids such as proline, quaternary ammonium compounds such as glycine betaine, sugar alcohols and sugars (Oh et al., 2008).

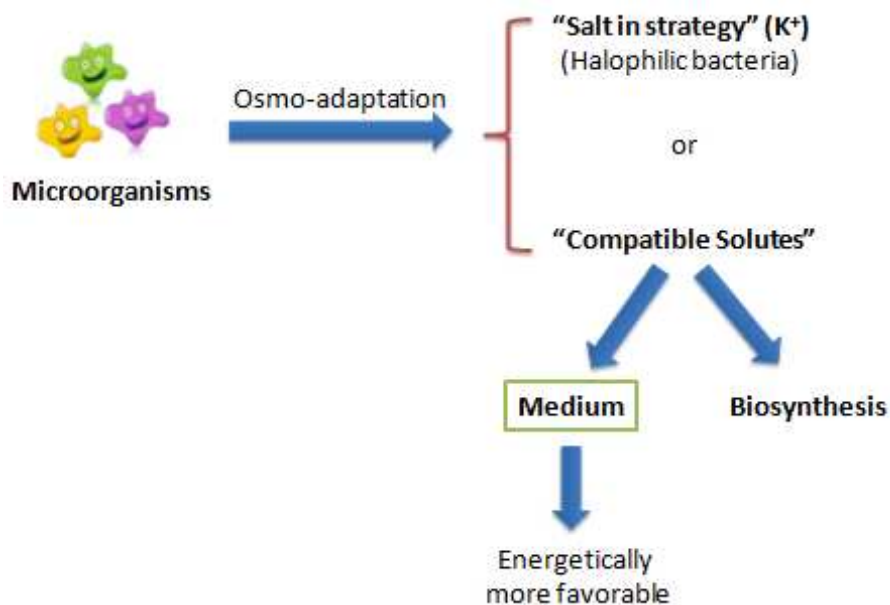


Figure 10. Strategies used for microorganisms to survive under high osmotic pressure.

1.5. Extracellular polymeric substances (EPS)

Apart from compatible solutes, it was also reported that cells produce extracellular polymeric substances (EPS) to cope with high salinity (Vyrides et al., 2010). EPS are compounds produced by the microorganisms which protect them against dewatering and extreme conditions. They can be products of cellular lyses and hydrolysis of macromolecules (Sheng et al., 2010). These substances can also be used as a carbon and energy source during starvation (Liu and H.P. Fang, 2002).

Proteins and carbohydrates are usually found to be the major components of EPS (Sheng et al., 2010). Humic substances may also be a key component of the EPS, accounting for approximately 20% of the total amount (Sheng et al., 2010). Lipids, uronic acid and DNA have also been found in EPS (Liu and H.P. Fang, 2002). The EPS can exist in two different forms: bound EPS (bEPS) and soluble EPS (sEPS) (Figure 11) (Sheng et al., 2010). Bound EPS are attached into the cells wall, while soluble EPS are dissolved into the solution. The latter are also referred to as soluble microbial products (SMP) (Ferreira, 2011). The structure of bound EPS is generally depicted by two layer model (Figure 11) (Sheng et al., 2010). The inner layer consists in tightly bound EPS (TB-EPS), which has a certain shape and is bound stably with the cell surface, and the outer layer, which consists of loosely bound EPS (LB-EPS) with dispersible slime layer without an obvious edge.

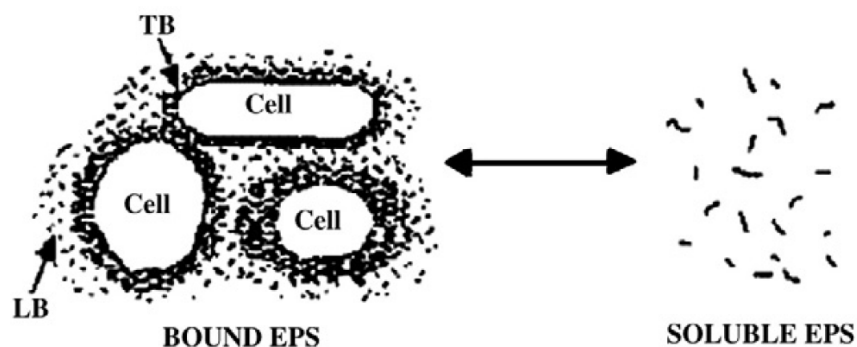


Figure 11. Different forms of EPS (Sheng et al., 2010).

The study concerning SMP is limited, and normally the EPS mentioned in the literature without being specified are bEPS. The latter have a significant influence on the physicochemical properties of microbial aggregates, including structure, surface charge, flocculation, settling properties, dewatering properties, and adsorption ability (Sheng et al., 2010). However, previous studies showed that SMP also have a crucial effect on the microbial activity and surface characteristics of sludge (Sheng and Yu, 2007). Besides that, it has been shown that in well operated anaerobic systems the majority of the effluent COD originates from SMP produced by the system itself (Aquino et al., 2002). Thus, the in-depth study of both forms of EPS is a matter of great interest not only in terms of improving the comprehension of biological treatment, but also improving the efficiency of such treatment through the optimization of operational parameters, even more when dealing with salty wastes, as in the case of the sludge from marine RAS.

1.6. Phosphatase

Phosphatase is an extracellular and hydrolytic enzyme which catalyzes reactions leading to inorganic PO_4^{3-} release from organic bound form of phosphorus (Anupama et al., 2008) (Figure 12). There are two main varieties of phosphatase, those with optimum activity under acid conditions and those with their optima under alkaline conditions (Ashley and Hurst, 1981). It can be produced by bacteria, fungi and yeast, and is responsible for phosphorus cycling in environment (Anupama et al., 2008). Phosphatase activity (PA) is related with the population of the acid formers (Ashley and Hurst, 1981; Bull et al., 1984).

Stringent environmental regulations make it mandatory to remove organic phosphorus in wastewater and phosphatase plays a fundamental role in this field. It can be used as a parameter to measure the activity of the acid formers (Bull et al., 1984) and, since it leads to PO_4^{3-} release in anaerobic reactors, it is also related with struvite formation (Figure 12).

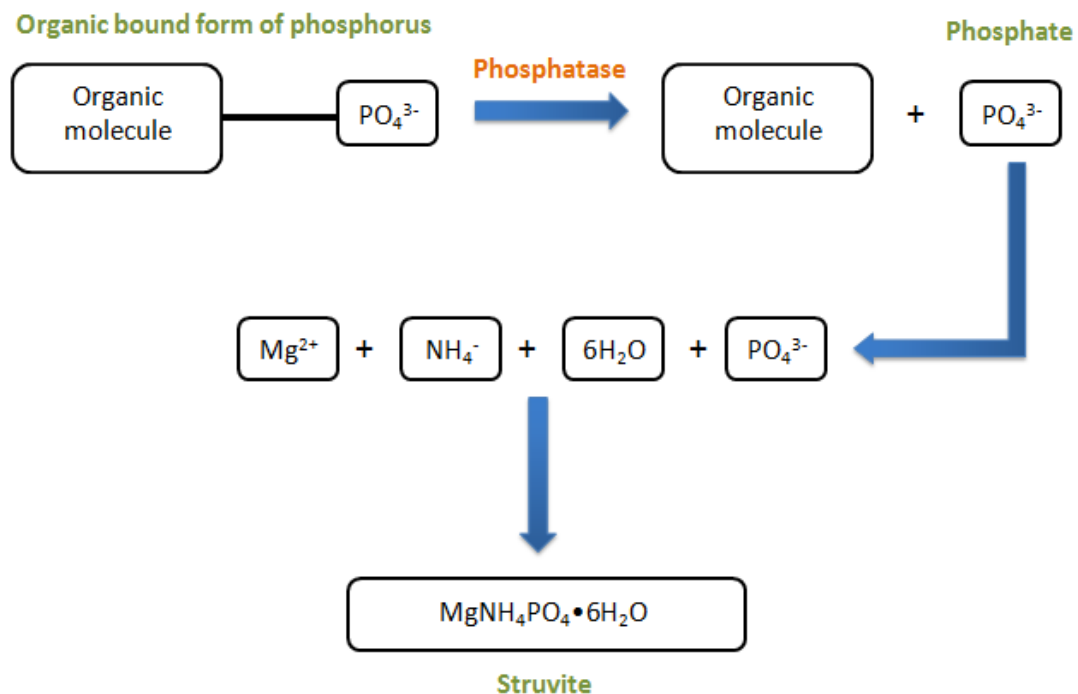


Figure 12. Scheme evidencing the relation between phosphatase and struvite formation.

1.7. State of the art

1.7.1. Anaerobic digestion of sludge from marine RAS in CSTR

The results of a literature review on the anaerobic digestion of sludge from marine RAS in CSTR are presented in Table 6.

Table 6. Operational conditions of anaerobic digestion systems used to treat sludge from marine RAS in CSTR.

Reference	Fish culture	Water Salinity (g/l)	Temperature (°C)	SRT (days)	Digester volume (l)
(Gebauer, 2004)	Salmon	Saline (35)	35	24-65	15
(Gebauer and Eikebrokk, 2006)	Salmon smolt	Brackish (14)	35	55-60	15

Gebauer (2004) used CSTR-type system for anaerobic digestion of saline sludge from a salmon farm (TS = 8.2-10.2 wt% and COD = 60-74 g/l) under mesophilic conditions. The inoculum of the reactor was a mixture of digested municipal sewage sludge and cow manure. Two scenarios were studied: anaerobic digestion of undiluted and diluted sludge. For the first scenario, because the inoculum was taken from a low salinity environment, the salinity in the digester was increased gradually. However, stabilization of the process was not achieved. The authors explained it by the inhibition of sodium (10.2 g/l). To overcome it the sludge was diluted 2 times in the second scenario and a stable process was achieved. Even in the unstable first scenario, high reduction of organic load (VS and COD) and methane production were achieved (Table 7).

A similar setup was used by Gebauer and Eikebrokk (2006) to treat brackish sludge from salmon smolt hatching (TS = 6.3-12.3 wt% and COD = 160.1-183.4 g/l). The inoculum was taken from the experimental digester used in the second scenario of the previous study. Complete stabilization of the process was also not achieved for all period of the experiment because of the steady decrease of the COD removal and the increasing of volatile fatty acids (VFA) concentrations. It was explained by inhibition of high content of ammonium (NH₃) in the sludge and high concentration of long-chain fatty acids (LCFA) originating from the fish feed. The results (for shorter periods between destabilization) were very close to the previous publication (Table 7). The fertilizing value of the treated sludge was estimated to be 3.4-6.8 kg N and 1.2-2.4 kg P per ton. However, because of its high VFA content it would necessitate special means of application. Furthermore, it was calculated that the energy from the methane that was produced would be sufficient to cover about 2-4% of the energy demands of a flow-through hatchery.

Table 7. Performance the CSTRs on the anaerobic digestion to treat sludge from marine RAS.

Reference	Stabilization (% removed)		Methane production		Inhibition
	VS	COD	% of biogas	l methane /g COD added	
(Gebauer, 2004)	47-62 ^a 58 ^b	37-55 ^a 60 ^b	49-54 ^a 57.6 ^b	0.114-0.184 ^a -	Na ^a -
(Gebauer and Eikebrokk, 2006)	74-79	54-45	59-61	0.140-0.154	NH ₃ and LCFA

^aundiluted sludge

^bdiluted sludge

The previous results showed that both attempts to treat the sludge from marine RAS using anaerobic digestion in CSTR did not reach a stable process. The first study (Gebauer, 2004) confirmed that the high salinity level of sludge from marine RAS can cause the failure of the anaerobic process. However, for both studies, for periods between destabilization, high

reduction of organic load (COD and VS) and methane production were achieved which makes promising the use anaerobic digestion in CSTR to treat sludge from marine RAS, case the toxicity caused by the high salinity of the sludge could be overcome.

1.7.2. Potassium and compatible solutes in the improvement of anaerobic digestion under saline conditions

Compatible solutes were tested by Oh et al. (2008), Vyrides and Stuckey (2009) and Vyrides et al. (2010) to enhance the anaerobic digestion at high salinity, being potassium only addressed in the latter study.

Oh et al. (2008), using batch tests, studied the effect of compatibles solutes (glycine betaine, choline, carnitine and trehalose) on the anaerobic digestion of salt-containing food wastes (17.5 g sodium chloride (NaCl)/l). The anaerobic seed sludge used was taken from an anaerobic digester in a municipal wastewater treatment plant. First, to test the inhibition of anaerobic digestion by NaCl, the sludge was washed with distilled water and the NaCl concentration was adjusted to 10, 35, 60, 75 and 100 g/L. The methane production decreased 50% with 10 g/l NaCl, 80% with 35 g/L NaCl and for higher concentrations no methane was produced. To overcome NaCl inhibition, 1 g/l of compatible solutes was added separately to the washed sludge with 10 and 35 g/L NaCl and to the non-washed sludge with 11.6 g/L NaCl (original salt-containing food waste diluted 80:20 (distilled water:food waste)). For the sludge with 10 g/l NaCl, glycine betaine and choline increased the methane about twofold compared to the control. The same result was achieved for the sludge with 35 g/l NaCl but only with the addition of glycine betaine. For the non-washed sludge, betaine and choline increased methanogenic activity about five to sixfold. For glycine betaine, it was found that the optimal concentration was 1.5 g/l. It was also observed that the addition of betaine in the beginning of the batch tests and only after 7 days increased the methane production in the same proportion. The addition of betaine after only 14 days of incubation did not improve the methane production, which could not be explained. Finally, the accumulation of intracellular glycine betaine in the anaerobic biomass was reported, and it started to occur after 5 days of incubation.

Vyrides and Stuckey (2009) used two batch reactors (5 l) inoculated with anaerobic sludge from a wastewater treatment plant and operated under stable OLR of 2 g COD/l day. The substrate was glucose and nutrients according to (Owen et al., 1979). One of the reactors was operated for 6 months under the conditions above mentioned while the other, after 5 month of operation as above, was subjected for about 1 month to 30 g NaCl/l. Addition of 1 mM of Glycine betaine, α -glutamate and β -glutamate, on batch tests with a medium with 35 g NaCl/l, using the anaerobic biomass not acclimated to sodium, resulted in an increase in cumulative methane production, being the glycine betaine the most effective. Under no salinity, compatible solutes did not result in any excess production of methane. The study showed that the methanogens were severely affected by sodium toxicity and that glycine betaine was found to be more beneficial for the methanogens than for the propionic acid utilisers. When comparing the addition of 1 mM of glycine betaine to non-adapted biomass and biomass previously expose to salinity, both in a medium with 35 g NaCl/l, the authors found that the methane production by the biomass previously exposed to salinity was only slightly higher compared to the biomass that was not exposed to salinity. Different feeding strategies

revealed that the no replacement of the medium, instead of removing and adding a new medium with the same concentration of substrate, was more beneficial to the biomass. It was explained by the removal of compatible solutes which are used as a strategy to regulate solute concentrations. According to this, the authors suggested that this is the reason why continuous reactors have better performance than batch reactors, because in the continuous reactors the equilibrium between cells and compatible solutes is not changing as abruptly as in the case of batch reactors.

Batch and continuous studies related to the enhancement of saline waste treatment using anaerobic digestion were made by Vyrides et al. (2010). The inoculum used on both batch and continuous tests was taken from the batch reactor from the previous study. Batch tests showed that trehalose was the dominant solute that accumulated during exposure to a salt stress environment (20 and 40 g NaCl/l) with concentrations in the range of 0.027-0.175 $\mu\text{mol/mg}$ dry weight. 1 mM of compatible solutes (trehalose, glycine betaine and N-acetyl- β -lysine) and potassium were separately added to batch tests under 35 g NaCl/l. Trehalose, N-acetyl- β -lysine and potassium slightly decreased sodium inhibition while betaine dramatically improved the adaptation of the anaerobic biomass. It was found that the methane production with a concentration as low as 0.1 mM was very close to that obtained with 1 mM of glycine betaine, 41.2 ml and 45.6 ml after 360 hours, respectively. The prolonged effect of glycine betaine on anaerobic biomass was also shown. After the first batch containing 0.1 and 1 mM of glycine betaine, the medium was removed and no addition of glycine betaine was done for the following four batch feedings. The results showed that the activity of the biomass continued to increase even when glycine betaine was not added continuously. This was explained by the fact that the cells maintained the betaine in their inside so they can act as osmoprotectants over time. Also no significant shift in the archaeal microbial community with high salinity was also reported. Two submerged anaerobic membrane bioreactors (SAMBRs) (volume of 3 L and HRT of 12 h) were used in order to investigate different strategies of addition of glycine betaine under high fluctuations in salinity (0 and 35 g NaCl/l) during continuous operation. The results showed that the addition of 1 mM glycine betaine slightly alleviates sodium inhibition, and the strategy of adding 0.1 mM glycine betaine for 10 days slightly improved the performance of a continuous SAMBR. On the other hand, the addition of 5 mM glycine betaine and operation in batch mode for 2 days significantly enhanced organic degradation in the SAMBRs. Another strategy that resulted in high performance was the injection of 1 mM glycine betaine for 5 days. Despite the positive effects of adding glycine betaine to anaerobic biomass under batch saline conditions, several betaine addition strategies were not so effective under continuous operation. The authors explained it by the time required for the glycine betaine to be taken up by the anaerobic biomass because some of the glycine betaine may be biodegraded by anaerobic bacteria.

The results of the latter studies showed that potassium and compatible solutes can enhance the anaerobic digestion at high salinity, being compatible solutes more efficient. Between the compatible solutes tested, for all the studies glycine betaine presented the best results. The use of potassium and compatible solutes was mainly focused in the acclimatization of biomass non-adapted to saline conditions to a medium with high salinity. However, no studies were found where these compounds are added into a system where the biomass is already acclimated to a high salinity level in order to test if the process can be improved.

1.7.3. EPS in the anaerobic digestion under saline conditions

Vyrides and Stuckey (2009) investigated the production of EPS by anaerobic biomass under high salinity (20 g NaCl/l and 40 g NaCl/l). Through batch tests, it was found that the higher the salinity, the higher the amount of EPS produced. It was also observed that the composition of EPS changed under high salinities and over time. At high levels of salt, the main constituents of the EPS were low molecular weight compounds, while in low levels were high molecular weight compounds. The levels of EPS at 20 g NaCl/l decreased over time (between 24 and 72h of incubation), while at 40 g NaCl/l the levels increased. The increase of the concentration of EPS with high salinity was explained as a response of biomass to counteract the sodium toxicity. The decrease of EPS levels at 20 g NaCl/l over time was justified by the acclimatization of the cells to the initial stress conditions, so after 72h less EPS was produced than in 24h, being part of EPS released into the medium, while part of it was biodegraded. In the biomass exposed to 40 g NaCl/l the cells could not acclimatize and continued to produce EPS. The analysis of particle size distribution at biomass exposed to normal conditions and 40 g NaCl/l showed higher mean flock size at high salinity and it was attributed to the higher production of bound EPS that forms the outer surface of the cell, and as a result cells attached more easily to each other.

Comparing the production of EPS of two SAMBRs operating at high salinity levels with and without addition of glycine betaine, Vyrides et al. (2010) reported that the concentration of EPS in the SAMBR where glycine betaine was added was almost 2 times less than that from the SAMBR without glycine betaine. It was explained that the presence of glycine betaine allowed the rapid adaptation of anaerobic biomass to saline conditions.

These studies showed that EPS have an important role in the adaptation of biomass into environments with high salinity. The research on EPS in the anaerobic digestion at saline conditions is quite limited and more research is needed to better understand its role in the process.

1.7.4. Phosphatase activity (PA) in the anaerobic digestion

The first studies of PA specifically related with anaerobic digestion were on using it as a predictor of digester failure (Ashley and Hurst, 1981; Wang et al., 1990). In both studies, an increase in acid and alkaline PA was found in anaerobic digesters overfed 10 days before higher concentrations of VFAs and lower pH values were detected. Apart from that, Ashley and Hurst (1981) also reported four groups of PA, with optimum pH of 4.2, 6.6, 9.4 and 11.4. Both studies also reported that PA was related to the population of the acid forming bacteria. This was addressed by Bull et al. (1984), which used alkaline PA as a parameter to investigate the activity of the acid formers in an anaerobic reactor.

An overall analysis of PA in four different anaerobic reactors (Table 8) was made by Anupama et al. (2008). PA was higher in continuously fed reactors (880-2632 $\mu\text{M/h}$), compared to a fed-batch reactor (FBR) (540-1249 $\mu\text{M/h}$). The higher PA was explained by the higher biomass concentration (MLSS) in the continuous reactors. The PA was found in all the reactors, but exhibited a 10-30% variation even at steady state reactor conditions. Alkaline phosphatase dominated in BFBR and UASB-1 while acid phosphatase showed higher activity in FBR and UASB-2, which indicated that the composition of the substrate seems to determine the kind of phosphatase (either alkaline or acid) in the reactor. The PA could not be related with the

inorganic PO_4^{3-} in the reactors. However, batch studies with anaerobic sludge showed a negative correlation between added inorganic phosphorus and PA which was explained on the basis of competitive repression of phosphatase by inorganic phosphorus ion. This enzyme activity was also found in both flocculated and suspended cells, and 60-65% of it was cell bound, and the remaining was entrapped to EPS and in cell-free form. An increase in PA was observed under starvation and higher salinity (above 15 g/l). In the first case, the increase was explained by a response to stress caused by the lack of simple organics which made the cells produce more enzymes (including phosphatase) to try to hydrolyze more complex materials. The increase of phosphatase activity under saline conditions occurred because of the lysis of the cells due to sodium toxicity, which releases phosphatase into the medium. The same study was also reported that Archaea and Bacteria contributed 45% and 55%, respectively, to the total PA in the anaerobic sludge.

Table 8. Characteristics of the anaerobic reactors used for PA study (Anupama et al., 2008).

Digester type	Digester volume (l)	Feed waste	OLR (kg COD/m ³ /d)	MLSS ^a (g/l)
BFBR ^b	11.9	Synthetic sewage	1.923	4.894
UASB-1	10	Fish process discharge effluent	5.72	2.54
UASB-2	10000	Leachate from agro-waste digester	0.322	0.362
FBR ^c	1	Fish process discharge effluent	0.5	0.22

^a Buoyant Filter Bio-Reactor (Haridas et al., 2005)

^b Mixed liquor suspend solids

^c fed-batch reactor

No studies were found on phosphatase in the anaerobic digestion of marine RAS and other salty wastewaters and, therefore, research is needed in this area to increase the knowledge about phosphatase and anaerobic digestion under saline conditions.

1.8. Incentives

1.8.1. Use of an inoculum already adapted to saline conditions to overcome the toxicity caused by the high salinity of sludge from marine RAS

Gebauer (2004) results showed that the high salinity level of sludge from marine RAS, as expected, can impede the use of anaerobic digestion to treat sludge from marine RAS. It has also been shown that acclimatization of an inoculum taken from a non-saline environment was not possible. These results motivated the attempt of using an inoculum already adapted to saline conditions to try to overcome the toxicity caused by the high salinity of sludge from marine RAS.

1.8.2. Study on effect of salinity, potassium, compatible solutes and ferric chloride (FeCl_3) on the anaerobic digestion of sludge from marine RAS

High salinity level is the biggest challenge in using anaerobic digestion to treat the sludge from marine RAS. Thus, an in-depth study of the effect of salinity on the anaerobic digestion of sludge from marine RAS is very important. Salinity in the marine RAS may vary due to the fact that many fish farms use directly salty groundwater with not adjustment of salinity for their cultures, and knowledge about the effect of this change in the anaerobic digestion of sludge from RAS is crucial to preventing the process failure.

The literature review on the use of potassium and compatibles solutes to enhance the anaerobic digestion under saline conditions showed that the use of potassium and compatible solutes can improve the process. This was an incentive to test whether these compounds can enhance the anaerobic digestion of the salty sludge from marine RAS.

In the first part of the EM-MARES project, ferric chloride (FeCl_3) presented a good performance as a coagulant in the up-concentration of solids and phosphorus from the backwash water from marine RAS. However, it is important to investigate what is the effect of this chemical on the anaerobic digestion of sludge from marine RAS in order to assess the feasibility of using coagulation using FeCl_3 before anaerobic digestion, as proposed by the EM-MARES project.

1.8.3. SMA, PA, PO_4^{3-} release and EPS as the key parameters to study the effect of salinity, potassium, compatible solutes and FeCl_3 on the anaerobic digestion of sludge from marine RAS, according to the goals of the EM-MARES project

The EM-MARES project aims at recovering energy in the form of methane and phosphorus in the form of struvite from the anaerobic digestion of sludge from marine RAS. As was already discussed, the SMA assessment can be used to study the effect of toxicants/stimulants in methane production. PA and PO_4^{3-} release are related with the aim of the EM-MARES project of recovering phosphorus as struvite from the digested. The literature review showed that EPS play an important role on the anaerobic digestion under saline conditions. This was an incentive to study EPS, since these substances might also have an important role in the anaerobic digestion of sludge from marine RAS.

Based on the discussion in the previous paragraph, it was considered that SMA, PA, PO_4^{3-} release and EPS are the key parameters to study the effect of salinity, FeCl_3 , potassium and compatible solutes in the anaerobic digestion of sludge from marine RAS, taking into account the objectives of the EM-MARES project.

1.9. Objectives

The EM-MARES project aims to minimize the waste from marine RAS, by combining coagulation/sedimentation of backwash water and anaerobic digestion of sludge from marine RAS in CSTR, with methane and struvite recovery.

This research was included on the second part of the EM-MARES project – anaerobic digestion of the sludge from marine RAS in CSTR. This thesis can be divided in two parts, with the follow objectives:

- Anaerobic digestion of sludge from marine RAS in CSTR – In this part the aim was to investigate the performance of CSTR inoculated with microorganisms already adapted to saline conditions to treat the sludge from marine RAS and to compare the results with the previous studies and with typical values from anaerobic digestion of sewage sludge;
- Batch studies – In this part of research batch studies were conducted in order to better understand and improve the anaerobic digestion of sludge from marine RAS. For that, the batch studies focused on the effect of salinity, potassium, compatible solutes and FeCl_3 on SMA, PA, PO_4^{3-} release and EPS.

1.10. Thesis Structure

This thesis is composed of four chapters. The main subjects in each chapter are presented below.

Chapter 1 presents the marine RAS and the associated environmental problems, the introduction and literature review of the concepts addressed as well as the incentives, context and main objectives of this thesis.

Chapter 2 presents the materials and methods used both in the experimental work and in the processing of the results.

Chapter 3 presents the results and their discussion.

Chapter 4 presents the main conclusions taken from the obtained results, their social and scientific relevance and recommendations for further research.

2. Materials and Methods

This research was conducted in the Water Lab of the Faculty of Civil Engineering and Geosciences, Delft University of Technology, The Netherlands.

2.1. Analyses

2.1.1. PA assay

2.1.1.1. Mechanism

PA assay was conducted based in the description by Anupama et al. (2008). In Figure 13 the mechanism behind of the method applied is represented. Phosphatase removes the phosphate group of *p*-nitrophenylphosphate (*p*-NPP) to generate *p*-nitrophenol (*p*-NP), which is deprotonated under alkaline conditions to produce *p*-nitrophenolate, which is yellow and has strong absorption at 405 nm (G-Biosciences, 2011).

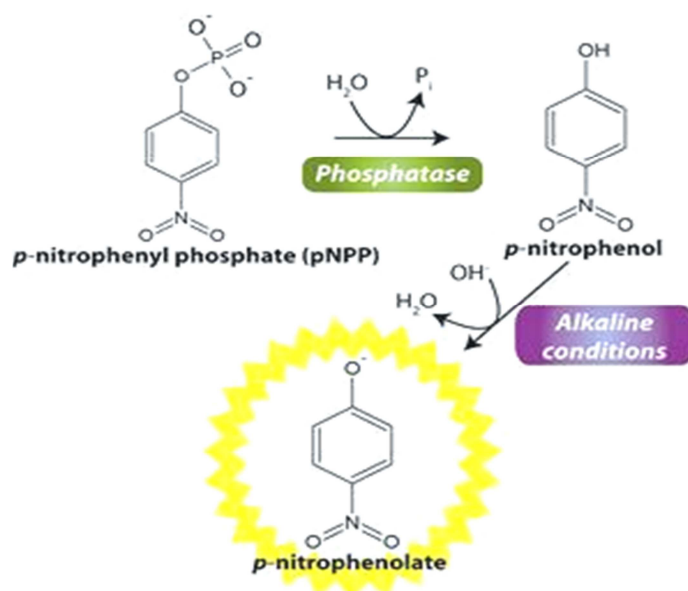


Figure 13. Mechanism of the method used for PA determination (G-Biosciences, 2011).

2.1.1.2. Procedure

A volume of 2 ml of sludge sample was made up to 8 ml of 0.2 M sodium acetate-acetic acid (CH₃COONa·3H₂O-CH₃COOH) buffer (pH = 4.8) or 0.2 M sodium carbonate-sodium bicarbonate (Na₂CO₃-NaHCO₃) buffer (pH = 9.4) for acid and alkaline PA analysis, respectively. The mixture was sonicated with an ultrasonic cleaner (Cole Parmer® EW-08895-01) for 1 min at 35 °C. To the mixture, 2 mL of 1 g/l p-NPP solution as substrate was added, the solution was mixed, deoxygenated by bubbling nitrogen (N₂) for 1 min, and incubated (Innova® 44 Incubator Shaker) at 130 rpm and 35.5 °C for 1h. After incubation, 2 ml of 1M NaOH were added to the solution to stop the reaction and to create alkaline conditions. The sample was then centrifuged (Sorvall® ST 16R) at 9000 rpm for 20 min and the supernatant's absorbance was read at 405 nm with a spectrophotometer (GENESYS® 6) in 1 cm cuvette using demineralized water as reference. A blank was prepared using 2 ml of demineralized water instead of sludge and 2 ml of 0.2 M Tris-HCl (pH = 7.6) instead of p-NPP solution. The absorbance was converted into mM through a calibration curve previously prepared (see annex A) and the latter was

divided by the incubation time (1h) to obtain the PA ($\mu\text{M}/\text{h}$). The total PA was obtained by the sum of acid and alkaline PA. All the samples (sludge and blank) were done in triplicate. Figure 14 summarizes the procedure for the PA assay.

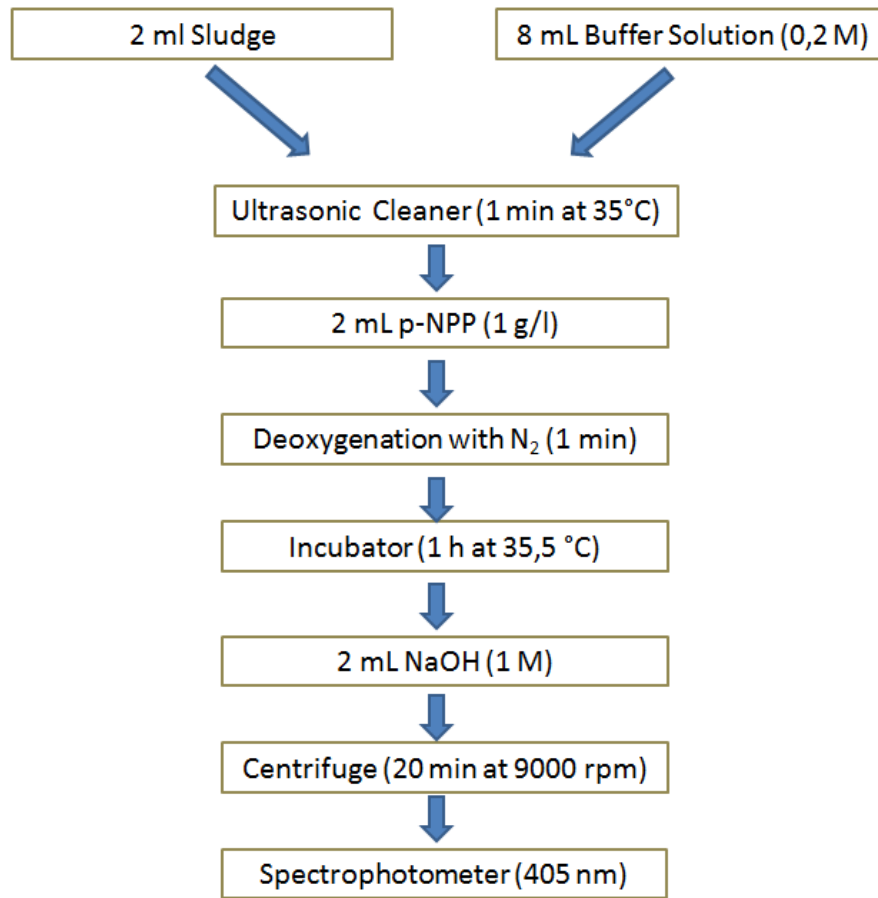


Figure 14. Scheme of PA assay.

2.1.2. Specific methanogenic activity (SMA) assay

2.1.2.1. Mechanism

In the SMA test, activity is not determined directly as the substrate utilization rate, rather, the methane production rate (which reflects the rate of substrate utilization) is noted. The higher the methane production rate, the higher the activity. The methane production rate is further converted into substrate utilization rate through its relation with COD removed (present in equation 5).

2.1.2.2. Automatic Methane Potential Test System II (AMPTS II)

The SMA assay was carried out in an Automatic Methane Potential Test System II (AMPTS II), Bioprocess Control Sweden AB. The instrument setup is divided into three units: A, B and C. In unit A (sample incubation unit), up to 15 vials containing a sample with anaerobic inoculum are incubated at desired temperature. The media in each vial is mixed by a slow rotating agitator. In unit B (CO₂-fixing unit), the biogas produced in each vial passes through an individual vial containing an alkaline solution. Several acid gas fractions, such CO₂ and hydrogen sulfide (H₂S), are retained by chemical interaction with NaOH, only allowing methane

to pass through to the methane gas monitoring unit. A pH indicator is added into each vial for verifying the acid binding capacity of the solution. In unit C (gas volume measuring device), the volume of methane released from unit B is measured using a wet gas flow measuring device with a multi-flow cell arrangement (15 cells). This measuring device works according to the principle of liquid displacement & buoyancy and can monitor ultra-low gas flows; a digital pulse is generated when a defined volume of gas flows through the device. An integrated embedded data acquisition is used to record, display and analyze the results. Figure 15 displays a photo of the instrument setup for the SMA assay installed in the Water Lab with the identification of the three different units.

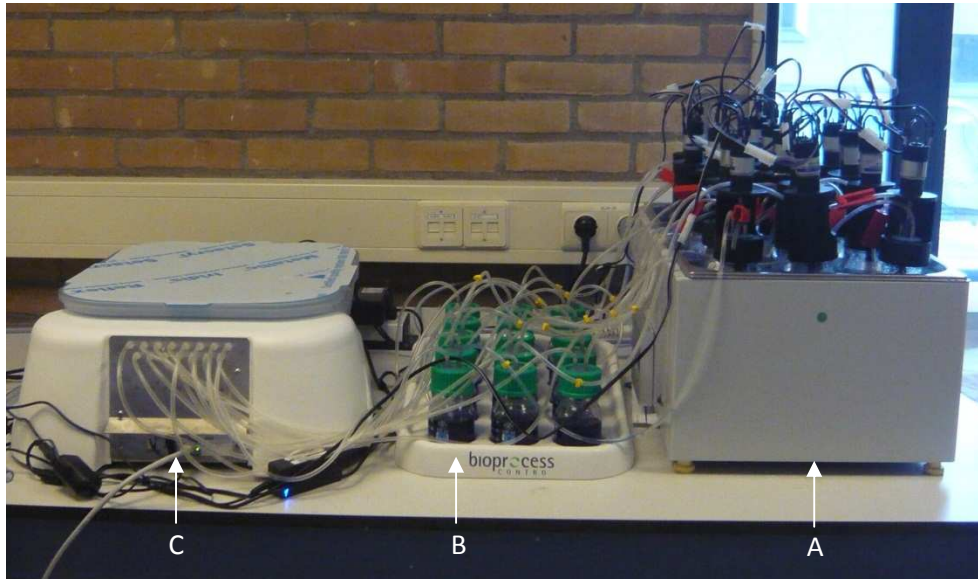


Figure 15. Instrument setup for the SMA assay. A - Sample incubation unit; B - CO₂-fixing unit; C - Gas volume measuring device.

2.1.2.3. Procedure

Sodium acetate (CH₃COONa·3H₂O) was used as substrate, with a concentration of 2.0 g COD/l. To keep the saline conditions as the substrate used the reactors, the sodium acetate solution was prepared with brackish water collected in the same fish farm as the substrate of the reactors. A ratio of 2:1 between inoculum (based on VSS) and substrate (based on COD) was used in the test. Through this ratio and the total volume used, 200 ml, the volume of sludge and substrate were calculated:

$$\frac{V_{sludge} \times VSS_{sludge}}{V_{substrate} \times COD_{substrate}} = 2 \quad (6)$$

$$V_{sludge} + V_{substrate} = 200 \text{ ml} \quad (7)$$

The volume of substrate and sludge previously calculated were added in 650 ml SCHOTT bottles, along with phosphate buffer, macronutrients and micronutrients (trace elements) with dosages and constituents listed in Table 9.

Table 9. Dosages and constituents of phosphate buffer, macronutrients and trace elements used on the SMA assay (according to (Owen et al., 1979)).

Solution	Dosage	Constituents	
Phosphate buffer	50 ml/l	K ₂ HPO ₄ ·3H ₂ O	0.2 M
		NaH ₂ PO ₄ ·2H ₂ O	0.2 M
		NH ₄ Cl	170 g/l
Macronutrients	6 ml/l	CaCl ₂ ·2H ₂ O	8 g/l
		MgSO ₄ ·7H ₂ O	9 g/l
		FeCl ₃ ·4H ₂ O	2 g/l
		CoCl ₂ ·6H ₂ O	2 g/l
		MnCl ₂ ·4H ₂ O	0.5 g/l
		CuCl ₂ ·2H ₂ O	30 g/l
		ZnCl ₂	50 g/l
		HBO ₃	50 g/l
		Trace elements	0.6 ml/l
Resazurine	0.5 g/l		
Yeast extract	2 g/l		
(NH ₄) ₆ Mo ₇ O ₂ ·4H ₂ O	90 mg/l		
Na ₂ SeO ₃ ·5H ₂ O	100 mg/l		
NiCl ₂ ·6H ₂ O	50 mg/l		
HCl 36%	1 ml/l		

The bottles were closed and the agitators were installed. The content in each bottle was purged with N₂ during 2 minutes and the bottles were put in the thermostatic water bath previously heated at 35.0 °C (unit A) and the motor connections were made. The bottles were then connected with the correspondent bottles of the unit B, using plastic tubes (Tygon® tubing). In this unit, each bottle contained 100 mL of NaOH solution (3 M) and 0.5 ml of Thymolphthlein pH-indicator (0.4%). The latter bottles were connected in the corresponding cells of the gas volume measuring device (unit C). Finally, using a laptop connected to AMPTS II, the motors were switched on and the experiment was started. During the experiment, the cumulative volume and flow of methane were recorded and graphically displayed. The experiment was finished when no significant methane production was observed, the data recorded was downloaded and the SMA was calculated. A blank was prepared using only brackish water instead of substrate solution. All the samples were analyzed in duplicate.

2.1.2.4. SMA calculation

For the SMA calculation the slope (maximum) of the cumulative methane production per hour was obtained. Taken into account the initial amount of sludge (as VSS) in each bottle (considered constant during the assay), the relation between methane production and the COD removed and considering that the methane was measured at room temperature (20°C), SMA was calculated as follows:

$$SMA (g COD_{CH_4}/g VSS/d) = \frac{Slope (ml CH_4/h) \times 24 h/d}{VSS (g) \times 350 ml CH_4/g COD} \times \frac{273.15 + 20^\circ C}{273.15} \quad (8)$$

2.1.3. EPS assay

Both bEPS and SMP were measured. Proteins and carbohydrates were considered to represent both forms of EPS, since normally they are their dominant components. Generally, EPS are presented in terms of mg (proteins or carbohydrates)/g VSS, being VSS representative of the biomass in the sludge. In this research, when studying the effect of salinity on EPS, the unit used was mg (proteins or carbohydrates)/l. The reason for this was that salinity is an inhibitor of anaerobic digestion, therefore, it can cause the decrease of the activity of biomass. This means that VSS of the substrate might not be consumed by the cells and it will be part of the VSS measure, making it not representative of biomass.

2.1.3.1. Extraction of EPS

The extraction method of EPS used (Figure 16) consists in a modification of ultracentrifugation/heating method described by Zhang et al. (1999). Samples were centrifuged at 12000 G during 15 min. The supernatant was filtered (0.45 μ m) and the SMP were measured from the filtrate. The biomass was re-suspended in a 0.9% NaCl solution and heated in a water bath (JULABO® TW B20) at 80°C during 1 h. The hot tubes were then centrifuged at 12000 G during 15 min. The supernatant was filtered (0.45 μ m) and the bEPS were measured from the filtrated.

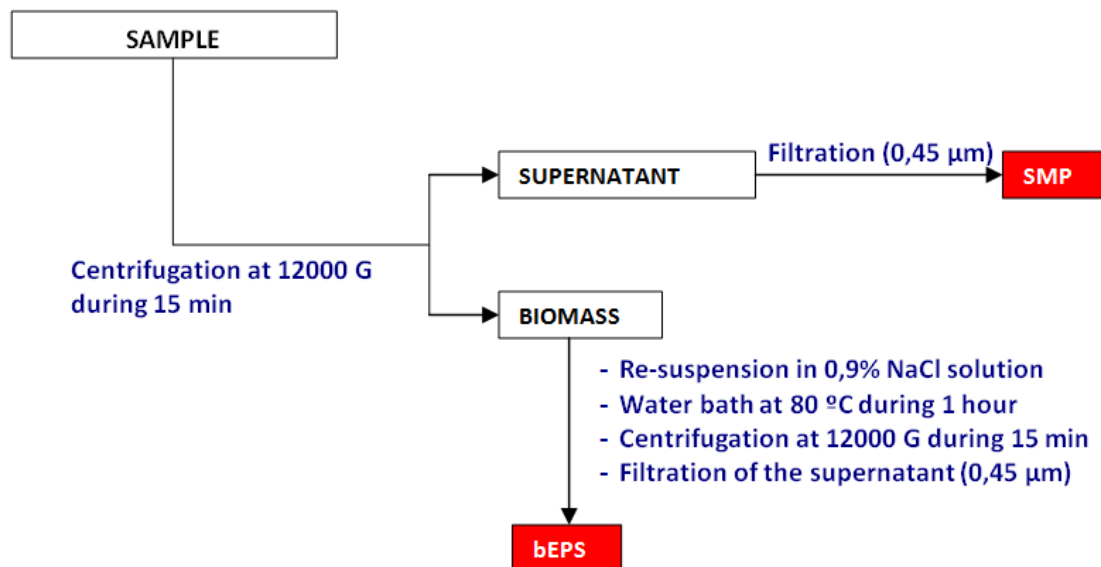


Figure 16. Procedure for bEPS and SMP extraction.

2.1.3.2. Proteins measurement

2.1.3.2.1. Mechanism

Proteins were measured by the method of Lowry et al. (1951). This method is based on a color forming reaction between peptide bonds and copper. The formed copper bounds are reduced by Folin-Ciocalteu phenol reagent to a blue color. By this method all proteins with two or more peptide bonds are analyzed.

2.1.3.2.2. Procedure

Table 10 depicts the reagents used for the proteins measurement, and the procedure is described below.

Table 10. Reagent used for proteins and humic substances determination.

Reagent A	143 mM NaOH and 270 mM Na ₂ CO ₃
Reagent B	57 mM Cupric Sulphate (CuSO ₄)
Reagent C	124 mM Potassium Sodium Tartrate (KNaC ₄ H ₄ O ₆ ·4H ₂ O)
Reagent D	Mixture of the reagents A,B and C in proportion 100:1:1
Reagent E	Mixture of Folin-Ciocalteu solution with demineralized water in proportion 1:2

A volume of 5 ml of extracted sample was made up with 7 ml of reagent D and left at room temperature during 10 min. Then 1 ml of reagent E was added and the mixture was kept during 45 min at room temperature. The absorbance of the sample was then measured in the spectrophotometer at 750 nm in 4 cm quartz cuvettes using demineralized water as reference, and the results were obtained using with a calibration curve previously prepared with Bovine Serum Albumin (BSA). The calibration curve used for the proteins measurements is in the annex B.

2.1.3.3. Carbohydrates measurement

2.1.3.3.1. Mechanism

The method for the carbohydrates measurement was based on the description by DuBois et al. (1956). By this method, the polymers are hydrolyzed with concentrated sulphuric acid and the monomers formed are dehydrated to aromatic compounds. The aromatic compounds are determined after coloring with phenol.

2.1.3.3.2. Procedure

A volume of 4 ml of extracted sample was made up with 2 ml of 5% phenol solution and left at room temperature during 10 min. Then 10 ml sulfuric acid were added and the mixture was kept during 30 min at room temperature. The absorbance of the sample was then measured in the spectrophotometer at 487 nm in 4 cm quartz cuvettes using demineralized water as reference, and the results were obtained using a calibration curve previously prepared with standard glucose (D-glucose monohydrated). The calibration curve used for the carbohydrates measurement is in annex B.

2.1.4. Other analyses

pH was measured using a WTW InoLab® Multi 720 meter equipped with a WTW pH electrode SenTix® 41. Salinity and electrical conductivity (EC) were measured through a WTW LF 325 microprocessor conductivity meter equipped with a TetraCon® 325 standard-conductivity cell. Total solids (TS), volatile solids (VS), total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed by standard methods according to APHA (2005). Total and soluble chemical oxygen demand (tCOD and sCOD, respectively), total phosphorus (TP), total nitrogen (TN), phosphate (PO₄³⁻) and ammonia (NH₄⁺) were measured using Merck Spectroquant® cell test kits. All the analyses, except pH, salinity and EC, were performed in triplicate. Ions composition was measured by ion chromatography in an external laboratory.

2.2. Anaerobic digestion of sludge from marine RAS in CSTR

2.2.1. Inoculum and substrate sources

The inoculum was sludge taken from a full-scale anaerobic digester at the fish processing factory A. van de Groep & zonen BV, located in Spakenburg, The Netherlands. The digester was in operation for more than 4 years at salinity of 17 g/l (EM-MARES project).

The substrate was sludge collected from a pilot-scale sieve installed in the marine fish farm GrovisCo®, located in Stavenisse, The Netherlands. This farm, which used a recirculation system, cultivates Turbot (70000 kg per year) and salty vegetables called “Sea Lavender” and “Glasswort”. After being collected, the sludge was analyzed and stored at -25°C before use. Table 11 presents the characterization of the sludge used as substrate, the range and the mean value. Annex 3 presents all the results from the characterization of the substrate as well the period when it was used.

Table 11. Characteristics of the substrate used in this research.

Parameter	Range	Mean value
pH	5.90 - 6.10	6,00 ± 0,11
Salinity (g/l)	13.5 - 13.9	13,7 ± 0,17
EC (mS/cm)	22.2 - 23.1	22,6 ± 0,42
TS (g/l)	94.8 ± 0.72 - 126 ± 2.04	110 ± 15,1
VS (g/l)	58.7 ± 2.90 - 86.2 ± 2.05	72,6 ± 11,4
TSS (g/l)	67.8 ± 1.38 - 97.7 ± 2.90	78,7 ± 13,6
VSS (g/l)	49.4 ± 11.6 - 69.6 ± 2.59	58,0 ± 8,95
tCOD (g/l)	84.3 ± 1.84 - 123 ± 6.46	103 ± 18,7
sCOD (g/l)	9.10 ± 0.30 - 12.6 ± 0.16	11,0 ± 1,64
TN (g/l)	3.10 ± 0.12 - 4.70 ± 0.07	3,80 ± 0,65
TP (g/l)	1.00 ± 0.16 - 2.90 ± 0.32	1,90 ± 1,03
NH ₄ ⁺ (mg/l)	242 ± 5.90 - 424 ± 3.61	316 ± 77,3
PO ₄ ³⁻ (mg/l)	168 ± 5.66 - 251 ± 1.41	218 ± 35,6
Ions composition (mg/l) ^a	Value	
B	5.0	
Br	27.4	
Ca	229.6	
Cl	8841.4	
Fe	0.3	
K	151.7	
Mg	284.0	
Na	4595.4	
SO ₄	978.4	
Sr	6.8	
Si	5.2	

^aAnalysis made at brackish water used in the fish production process of the fish farm where the substrate was collected. It was considered to have also approximately the same ions composition as the substrate since they have approximately the same salinity (salinity of the brackish water = 14 g/l). This analysis was made once.

2.2.2. Experimental setup

The experimental setup was constituted by two glass lab-scale CSTRs: one reactor with 4 l of working volume (R1) and another reactor with an operating volume of 5.3 l (R2). The experimental setup can be divided into two sub-experimental setups: one for reactor R1 (Figure 17) another for reactor R2 (Figure 18). In the experimental setup of reactor R1, a water bath (1) (Tamson® TC16) was used to heat the water inside of a “jacket” (6) with the purpose of keep the temperature of the reactor (5) at 35°C. The substrate (2) was added using a peristaltic sludge pump (3) (Watson Marlow® 120U) and the reactor was discharged from the

bottom (4). The reactor content was mixed by means of an impeller (7) equipped with a motor (9) (Micromotors® series HL149) and a speed controller (9) (Delta Elektronika® E 015-2). The biogas produced went through a bottle containing water (10) and its volume was recorded through a gas counter (11) (Ritter® MiliGascounter MGC-1 PMMA). The biogas was subsequently conducted into a another bottle (12) containing 200 ml of 3 M NaOH solution and 0,5 ml of 0,4% Thymolphthlein pH indicator in order to retain CO₂ and H₂S, passing only methane for the second gas meter (13). The volume of biogas and methane produced were continuously recorded using DASyLab® 11 software installed on a laptop (9). In the experimental setup of reactor R2, instead of using an impeller, biogas recirculation was used to mixed the reactor content, using a gas pump (7) (KNF® Neuberger PM25370-86). This reactor was also equipped with a pH and temperature meter. All the data (pH, temperature, volume and rate of biogas and methane produced) were continuously recorded using the LabVIEW™ 2010-Version 10.0.1. Figures 19 and 20 display photos of the entire experimental setup and both sub-experimental setups installed in the Water Lab, respectively.

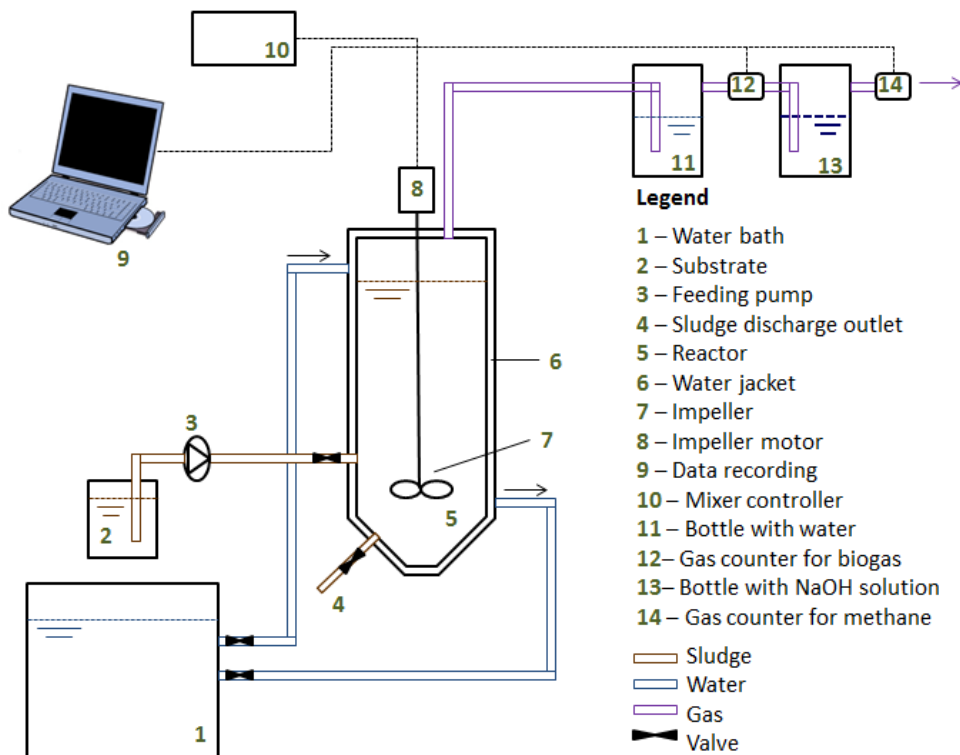


Figure 17. Scheme of the experimental setup of the reactor R1.

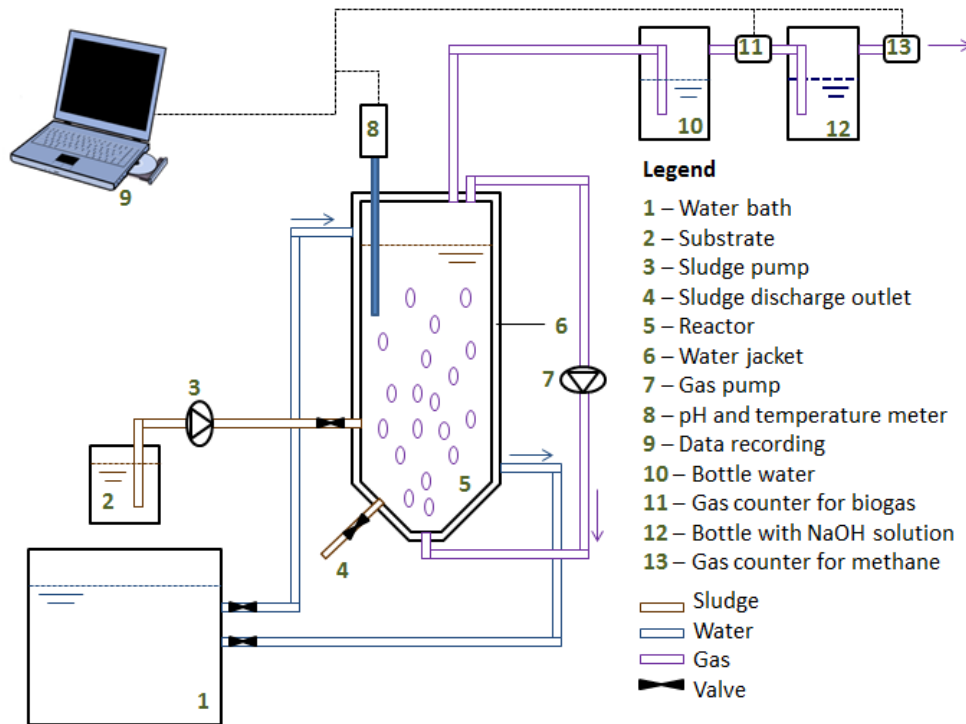


Figure 18. Scheme of the experimental setup of the reactor R2.

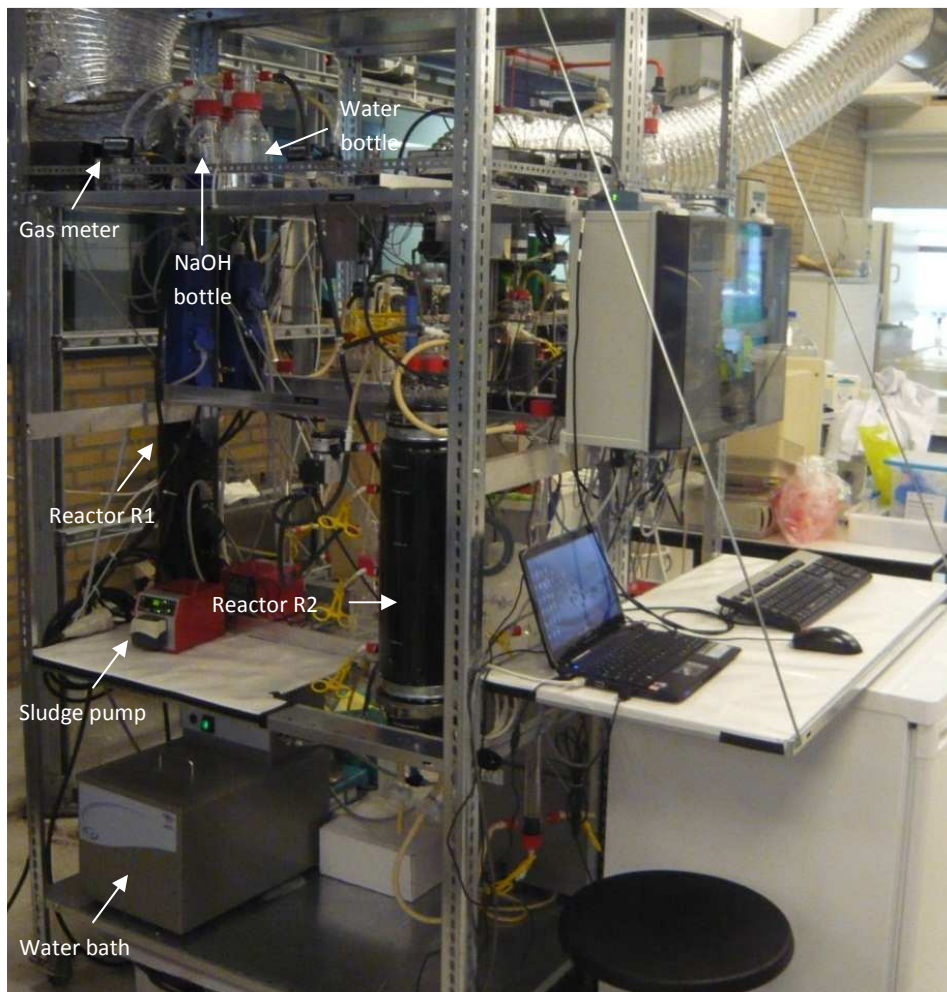


Figure 19. Photo of the experimental setup used in this research.

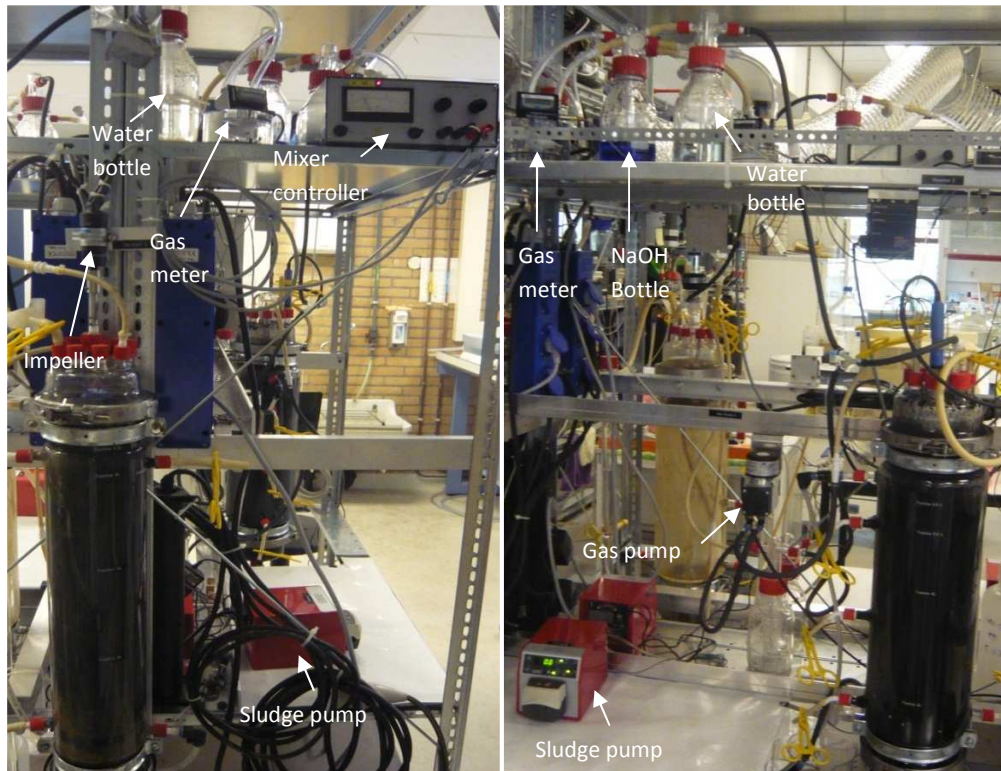


Figure 20. Photo of the reactor R1 (left) and reactor R2 (right).

2.2.3. Operational conditions

R1 was the first to be inoculated and it was in operation for 7 months while R2 was working for 4 months. Both reactors were operated with constant SRT, in a semi-continuous mode: fed with 250 ml of substrate every two days. The OLR changed according with the changes in the composition of the substrate. The performance of the reactors was investigated in the last 3 months. In this period, the discharged sludge from both reactors was analyzed weekly. The parameters analyzed were the same used to characterize the substrate from the reactors (Table 11), except from the ions composition. Due to technical reasons the data of methane production from the reactor R2 of the firsts 42 days could not be recorded. Table 12 summarizes the operation conditions of the two lab-scale reactors used in this research.

Table 12. Operational conditions of the two lab-scale reactors.

	R1	R2
Working volume (l)	4	5.3
Temperature (°C)	35	35
SRT (d)	32	42
OLR (kg COD/m ³ /d)	2.80-3.76	1.99-2.91

2.3. Batch studies

Batch studies were performed using the discharged sludge from the reactor R1 as inoculum. After discharging from the reactor, the sludge was stored at 0°C and, before it was used in the batch assays, was re-activated in the incubator at 35.5 °C and 130 rpm for 1 day.

2.3.1. Effect of salinity on SMA, PA, PO₄³⁻ release and EPS

To test the effect of salinity on SMA, the SMA assay was performed as described above with the addition of NaCl (99.8%, VWR Prolabo®) into the bottles content to adjust salinity to the desired values. The salinity was adjusted to 25, 35, 40, 45 and 50 g/l. A control group was kept without addition of NaCl.

To study the effect of salinity on PA, PO₄³⁻ release and EPS, 30 ml of sludge were added into 100 ml SCHOTT bottles and the salinity was adjusted as described previously to 25, 30, 40 and 50 g/l. The bottles were purged with N₂ for 1 min and incubated at 130 rpm and 35.5°C. PA assay and PO₄³⁻ analyses were conducted after 3 days of incubation and EPS assay was performed after 5 days of incubation. The bottles were fed with the same substrate and OLR as the reactor R1. All bottles were prepared in triplicate. A control group was also kept without addition of NaCl.

2.3.2. Effect of potassium and compatible solutes on SMA, PA, PO₄³⁻ release and EPS

The effect of potassium and compatible solutes on SMA, PO₄³⁻ release, PA and EPS was addressed with the same procedure described above to study the effect of salinity. Potassium chloride (KCl) was used as source of potassium and two compatible solutes were tested: trehalose and betaine (Figure 21). The concentration and combination of the different compounds tested are depicted in Table 13.

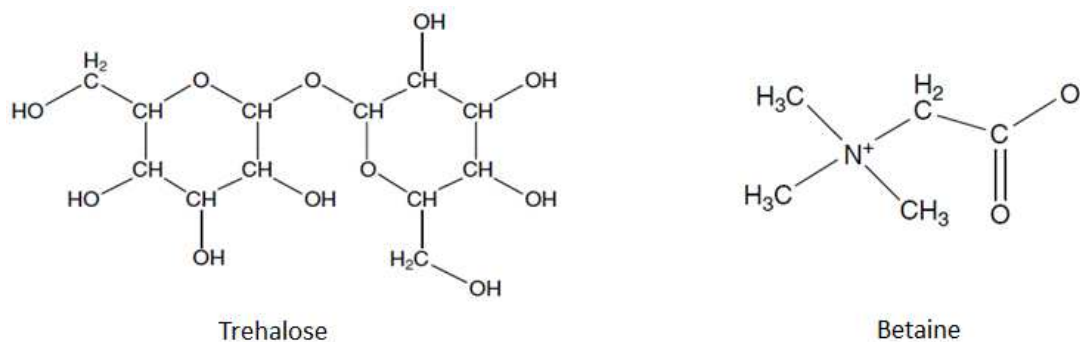


Figure 21. Chemical structures of the two compatible solutes tested.

Table 13. Concentration and combination of the different compounds tested to study the effect of potassium and compatible solutes on SMA, PO_4^{3-} release, PA and EPS.

Compounds tested	Concentration (mM)
KCl (99.5%, Merck®)	1
Trehalose (dihydrate, 99.5%, Alfa Aesar®)	0.5
	1
	2
Betaine (anhydrous, 98%, Alfa Aesar®)	0.5
	1
	2
Trehalose + KCl	1 ^a
Betaine + KCl	1 ^a

^a from each compound

Due to the fact that the instrument used for the SMA assays had only 15 cells (as was mentioned above) the SMA assays to test the effect of the two compatible solutes were done separately. In the first assay the potassium and/or betaine were tested and in the second assay was tested the effect of potassium and/or trehalose. The effect of the two compatible solutes was compared through their comparison between the controls from each assay.

2.3.3. Effect of FeCl_3 on SMA, PA, PO_4^{3-} release and EPS

To address the effect of FeCl_3 on SMA, the SMA assay was conducted as described above but the concentration of substrate was 0.5 g/l instead of 2.0 g/l and the volume in each bottle was 400 ml instead 200 ml. The concentration of FeCl_3 was adjusted to 12, 25, 50, 70 and 100 mg/l using a stock solution of FeCl_3 (27%, VWR Prolabo®).

The procedure to study the effect of FeCl_3 on PO_4^{3-} release, PA and EPS was the same as described above to study the effect of salinity, and the concentration of FeCl_3 was adjusted as it was done for the SMA assay.

2.4. Processing of data

The data was processed with Microsoft Office Excel. The mean value of duplicates and triplicates was presented with the corresponding standard deviation. In case of triplicates, in order to increase the significance of the results, when the standard deviation was higher than 5%, one of the samples was rejected, keeping only the two other samples with lower standard deviation.

3. Results and Discussion

3.1. Anaerobic digestion of sludge from marine RAS in CSTR

Figures 22 and 23 present the operational conditions of both reactors over time, with respect to OLR, SRT (Figure 22) and pH (Figure 23). Figures 24, 25 and 26 present the performance of both reactors over time related to COD removal efficiency, solids removal efficiency, and methane production, respectively. Table 14 summarizes the operational conditions and performance of the reactors in the present study together with values from the previous studies dealing with anaerobic digestion of sludge from marine RAS in CSTR, and typical values for anaerobic digestion of sewage sludge. All the results from the analyses made at the sludge from both reactors can be found on the annex D.

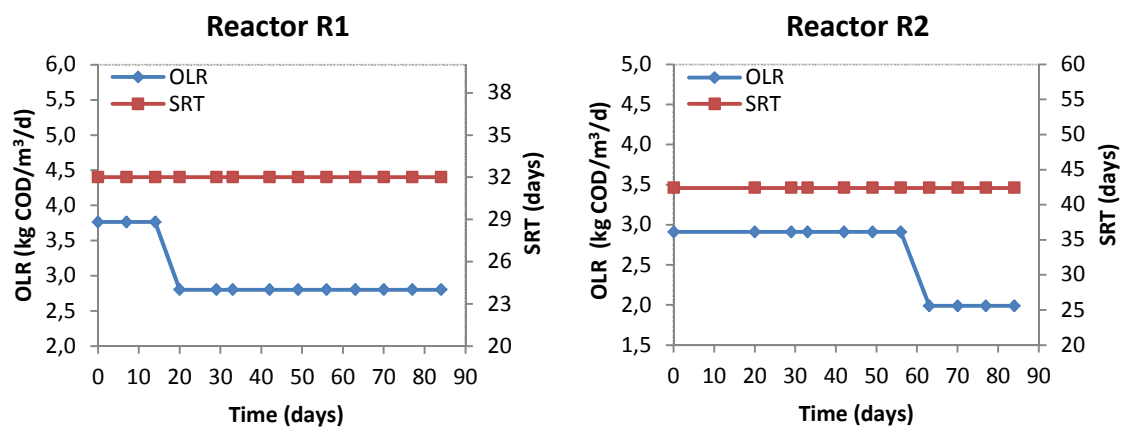


Figure 22. OLR and SRT of the two anaerobic CSTRs used to treat the sludge from marine RAS during the experimental period.

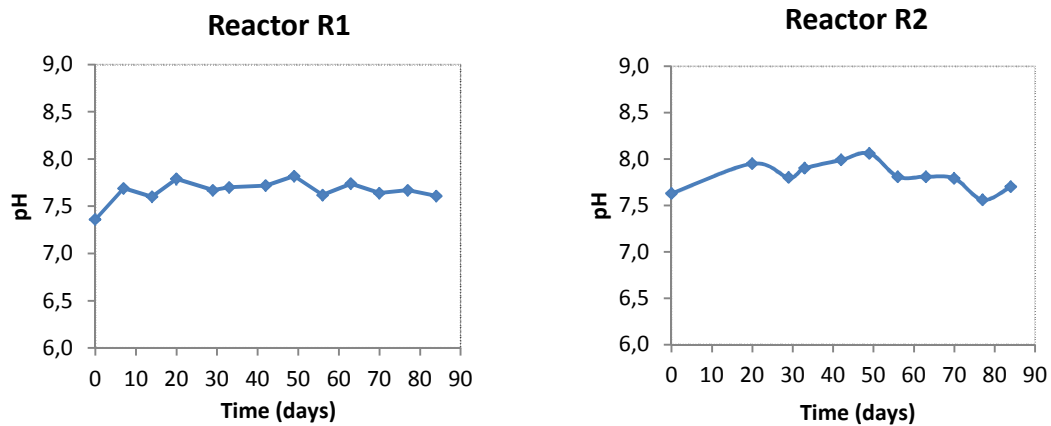


Figure 23. pH of the two anaerobic CSTRs used to treat the sludge from marine RAS during the experimental period.

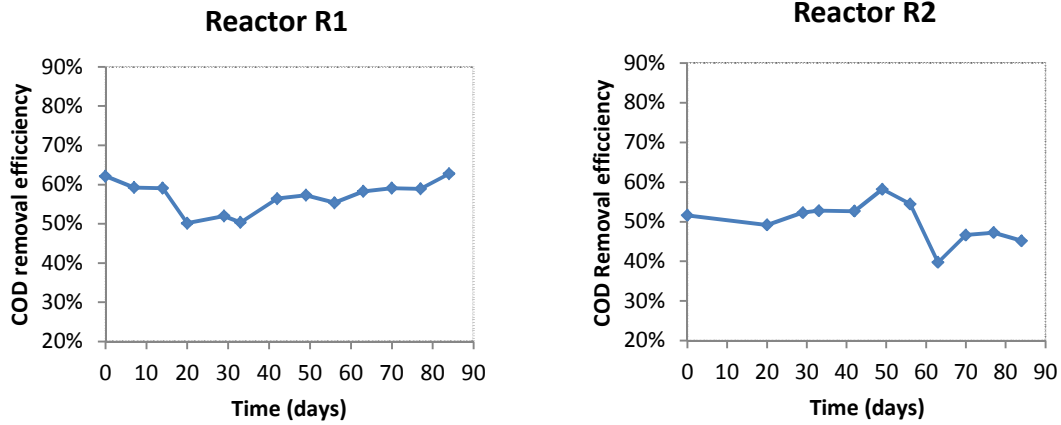


Figure 24. COD removal efficiency of the two anaerobic CSTRs used to treat the sludge from marine RAS during the experimental period.

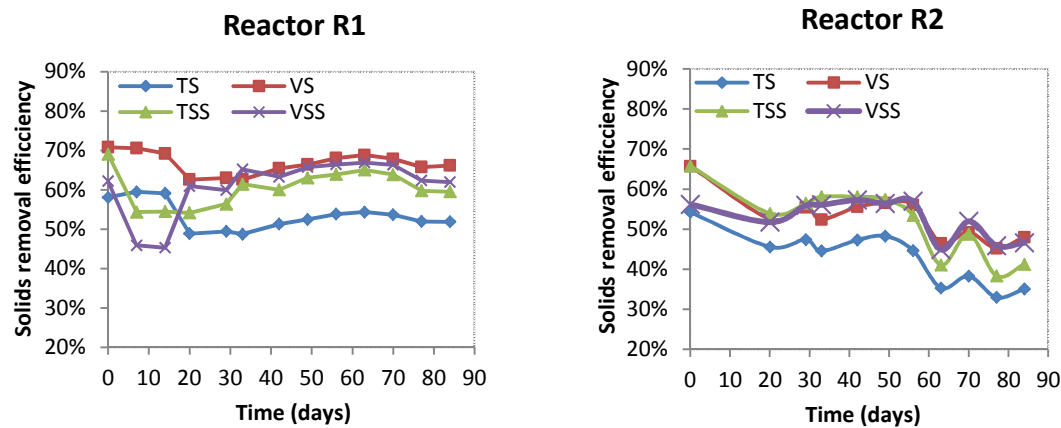


Figure 25. Solids removal efficiency of the two anaerobic CSTRs used to treat the sludge from marine RAS during the experimental period.

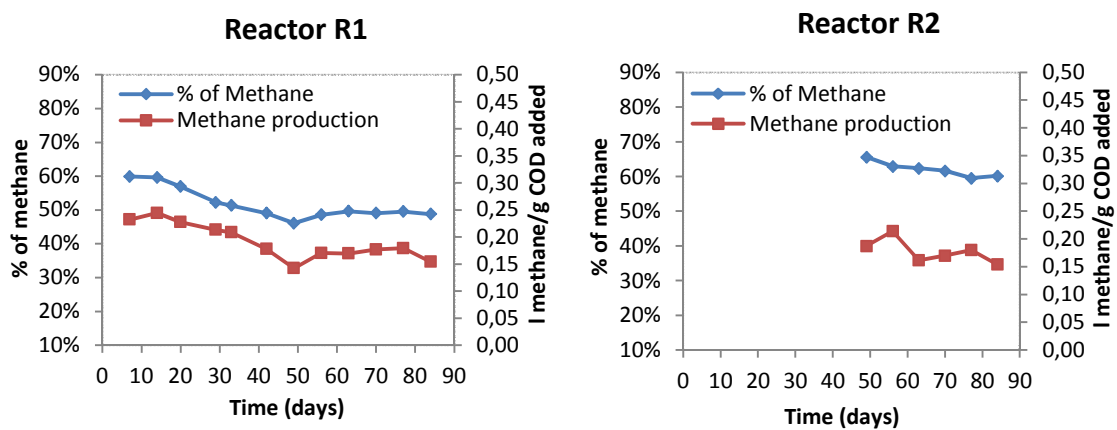


Figure 26. Methane percentage in biogas and production of the two anaerobic CSTRs used to treat the sludge from marine RAS during the experimental period.

Table 14. Operational conditions and performance of the reactors in the present study together with values from previous studies dealing with anaerobic digestion of sludge from marine RAS in CSTR and typical values for anaerobic digestion of sewage sludge in CSTR.

	Marine RAS sludge in CSTR				Gebauer and Eikebrokk (2006)	Sewage sludge in CSTR ^a
	Present study		Gebauer (2004)			
	R1	R2	Undiluted	Diluted		
Operational conditions						
SRT (days)	32	42	24-60	30	55-60	10-20
OLR (Kg COD/m ³ /d)	2.80-3.76	1.99-2.91	1.24-3.12	1.10	2.9-3.0	2.2-6.7
Salinity (g/l)	14	14	35	17.5	14	0-5 ^b
Removal efficiency (%)						
COD	50.2-62.1	39.7-58.2	36.7-55.2	60	44.8-53.5	
VS	60.1-70.9	45.2-65.7	47.4-61.9	58	74.1-77.5	45-60
Methane production (STD)						
Methane in biogas (%)	46.1-59.9	59.5-65.6	48.9-54.1	57.6	59.4-60.5	55-70
l methane/ g COD added	0.142-0.244	0.154-0.214	0.114-0.184	0.154	0.140-0.154	
l methane/g VS added	0.126-0.281	0.221-0.350	0.160-0.241	0.220	0.260-0.281	0.20-0.34
l methane/L sludge added	25.5-56.7	26.0-52.8	8.0-13.7	5.2	24.8-25.7	
l methane/L digester/d	0.797-1.772	0.812-1.650	0.228-0.414	0.174	0.428-0.444	0.35-1.4
l methane/g VS digester/d	0.017-0.031	0.017-0.019	0.010-0.020	0.019	0.020-0.024	0.018-0.100

^a Data from (Gebauer and Eikebrokk, 2006)

^b Salinity of fresh water

Steady state conditions were achieved in the present investigation for both reactors as indicated by the constant pH (Figure 23), COD and solids removal efficiencies (Figures 24 and 25, respectively), and methane percentage and production (Figure 26) during the experimental period. The decrease observed in COD and solids removal efficiencies after the days 18 and 63 for reactors R1 and R2, respectively, is due to the change in their OLRs at these days (Figure 22). However, for reactor R1, COD and solids stabilization returned to those verified before the change in the OLR and for reactor R2 is expected to observe the same result.

The reactors of the present investigation were operated basically in the same OLRs compared with those from the previous studies dealing with anaerobic digestion of sludge from marine RAS in a CSRT (Table 14). The SRT of reactor R1 was close to that used by Gebauer (2004) when treating diluted sludge from marine RAS, whereas the SRT used by Gebauer (2004) when treating undiluted sludge and that used by Gebauer and Eikebrokk (2006) were higher than those of the both reactors from the present investigation. COD and VS removal efficiencies of the present study were in the range of the previous investigations, except for a lower VS removal efficiency compared to that obtained by Gebauer and Eikebrokk (2006), which may be due to the higher SRT in the latter study. Methane production, except of methane content in the biogas, in terms of liters of methane/g VS added and specific methane production rate (liters of methane/VS in the digester/d) which were in the same range of those at the previous investigations, was considerably higher in the present investigation, especially compared with that achieved by Gebauer (2004) treating diluted sludge. Moreover, Gebauer (2004), when treating undiluted sludge, and Gebauer and Eikebrokk (2006) found inhibition of the process and steady state conditions were not achieved, contrarily with the results from the present study.

During the experimental period both reactors were operated within the range of OLRs used in sewage CSRTs, but at 1.5 and 2.0 times (for reactor R1 and R2, respectively) longer SRTs. The range of the VS removal efficiency, methane content in biogas and methane production in

terms of liters of methane/g VS added were as expected as those of sewage CSRTs. The range of the volumetric methane production rate (liters of methane/liters of digester/d) was higher than that observed in the sewage CSRTs, which was due to the higher COD of the sludge from the marine RAS. The range of specific methane production rate was in a slightly lower range than that observed from sewage CSRTs, which may be explained by the longer SRTs of the reactors from the present study. However, the difference is very small and acceptable, taking into account the high salinity of the sludge from marine RAS. The investigation of the performance of a CSRT with a lower SRT, in the range of those observed in the sewage CSRTs, might be an interesting research topic, since for a CSRT, lower SRTs results into smaller reactors and consequently economic advantages. However, it is necessary to take into account that a lower SRT also makes the reactor more sensitive to changes in its inflow, which may be an important issue in marine RAS due to changes in the salinity and/or feed composition used in the cultivation process.

3.2. Batch Studies

3.2.1. Effect of salinity on SMA, PA, PO_4^{3-} release and EPS

3.2.1.1. SMA

Figure 27 presents the effect of salinity on the SMA of the sludge harvested from the reactor R1. Annex E contains the graph of cumulative methane production as well as the time interval from which the slope to calculate SMA was derived.

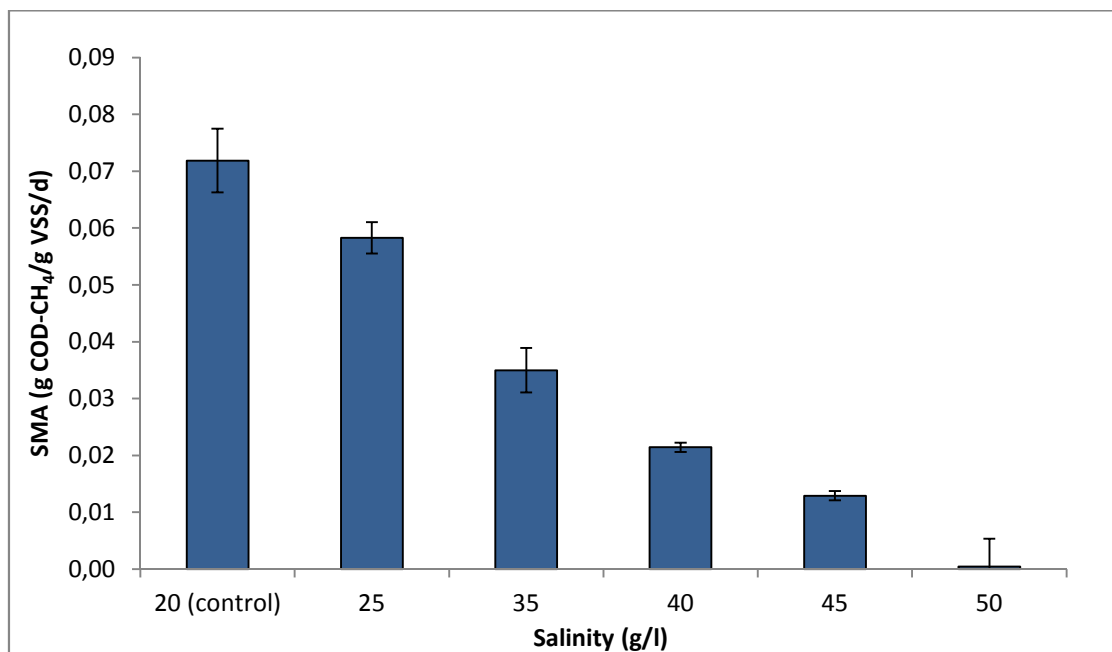


Figure 27. Effect of salinity of SMA.

Figure 27 shows that SMA decreased as the salinity increased. The salinity of 35 g/l caused 50% inhibition in the SMA while at salinity of 50 g/l SMA was virtually ceased.

Soto et al. (1993) reported 50% inhibition of SMA when the salinity was increased from 0 to 12 g/l with NaCl, whereas Oh et al. (2008) reported 50% of inhibition when the salinity was increased from 0 to 10 g/l, also with NaCl. In the present study, only when the salinity was

increased by 15 g/l (20 to 35 g/l), SMA was inhibited 50%. This higher tolerance might be explained by an antagonistic effect caused by ions present in the brackish water used in the SMA assay (see Table 11). Another reason may be that the inoculum used in the present study, contrarily with the studies mentioned above, was taken from a saline environment, and it could present a higher tolerance to high salinity levels. Nevertheless, it was evident that the methanogens, and hence methane production, were severely affected by the increase in salinity.

3.2.1.2. PA

Figure 28 presents the results of the effect of salinity on PA.

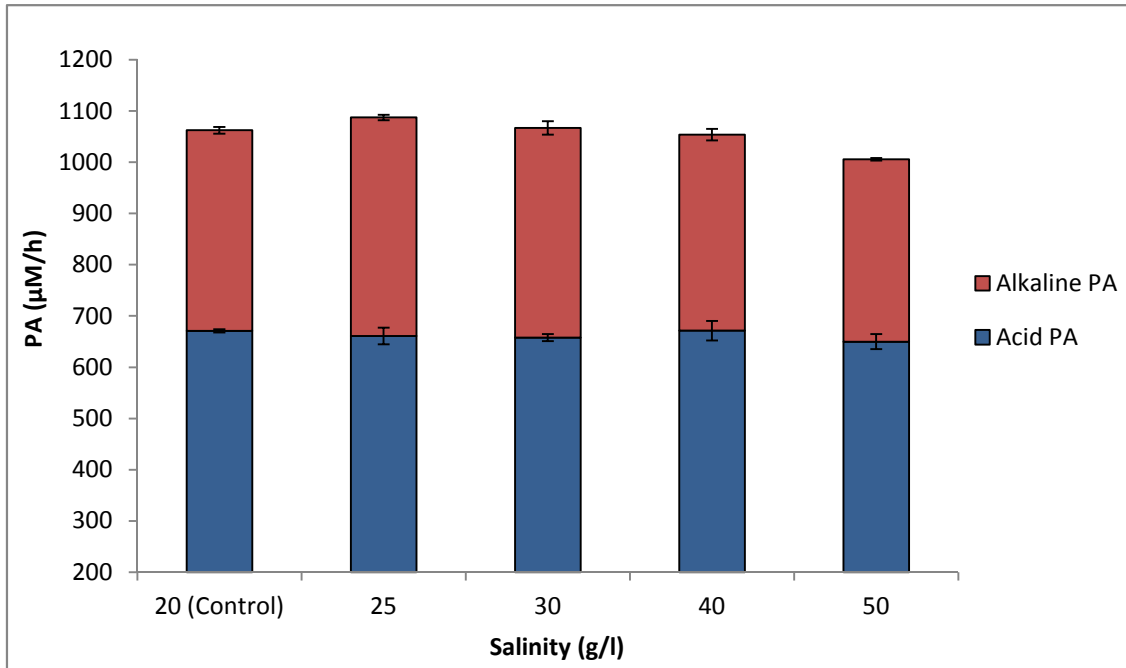


Figure 28. Effect of salinity on PA.

Figure 28 shows a slight decrease in PA with the increase of salinity. The lowest PA, at salinity of 50 g/l, was 4% lower than the control. Acid PA was higher than the alkaline PA, which is in agreement with the results reported by Anupama et al. (2008) where was analyzed the PA of anaerobic digesters fed with fish processing industry discharge. The PA of the control, $1064 \pm 10 \mu\text{M/h}$, was close to that obtained by Anupama et al. (2008), around $1000 \mu\text{M/h}$.

Anupama et al. (2008), when increasing the salinity of an inoculum from a non-saline source found, in contrast to the results of the present study, that PA increased with the increase of salinity. The authors attributed this to the lysis of the cells that caused the release of all intracellular material to the medium (including enzymes like phosphatase). Taking into account that PA is related with the population of acid formers, the reason for the results of the present study may be due to that the population of acid formers taken from a saline environment is able to deal with the increase of salinity. Hence, the increase in salinity decreased their activity, but did not result into their lyses.

The results, together with the previous results from the effect of salinity on SMA, also show that, as expected, the increase of salinity was much more detrimental for the population of methanogens than for the population of acid formers.

3.2.1.3. PO_4^{3-} release

Figure 29 presents the effect of salinity on PO_4^{3-} release.

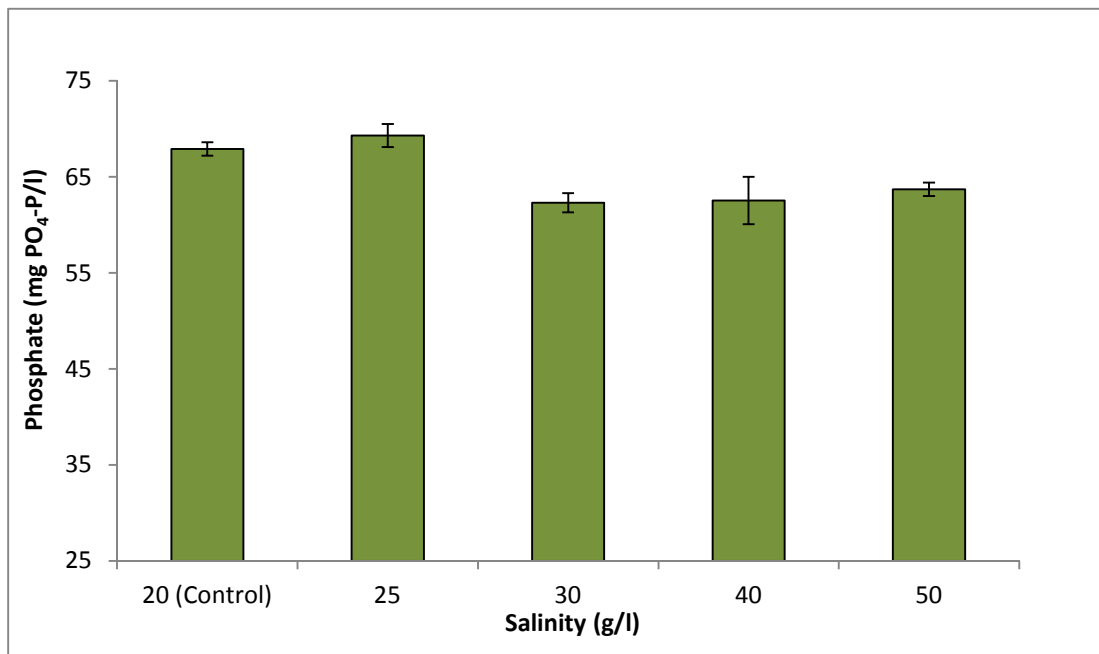


Figure 29. Effect of salinity on PO_4^{3-} release.

Figure 29 shows that the increase of salinity to more than 25 g/l reduced the concentration of PO_4^{3-} with 8% for salinities of 30 and 40 g/l and 6% for salinity of 50 g/l, compared with the control.

These results are not completely in agreement with the results for PA. PO_4^{3-} release was more affected by salinity than PA and they could not be correlated. However, these results agree with those reported by Anupama et al. (2008) in which PA and PO_4^{3-} concentration were analyzed from four different reactors and the two parameters could not also be related. In the same study, when adding external PO_4^{3-} , PA decreased, and the authors suggested that high PO_4^{3-} levels inhibited PA. The previous results show that the relation between PA and PO_4^{3-} is complex and might be based on inhibition/stimulation by PO_4^{3-} concentration. Thus, PA cannot be used to predict the PO_4^{3-} content in the anaerobic digestion.

3.2.1.4. EPS

The effect of salinity on SMP and bEPS is presented in Figures 30 and 31, respectively.

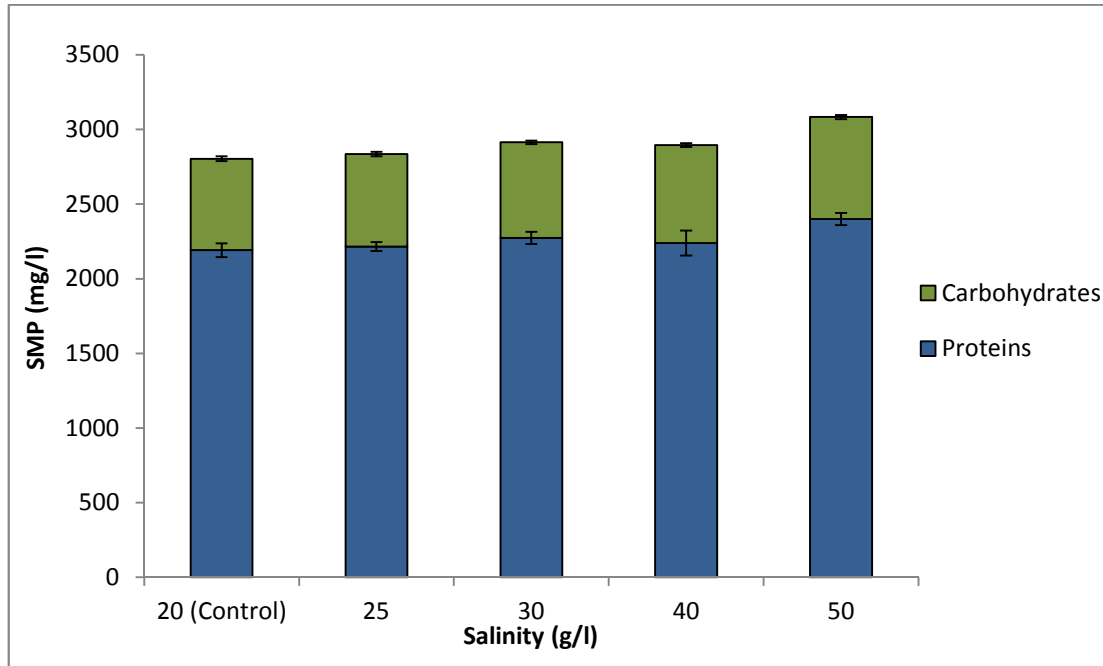


Figure 30. Effect of salinity on SMP.

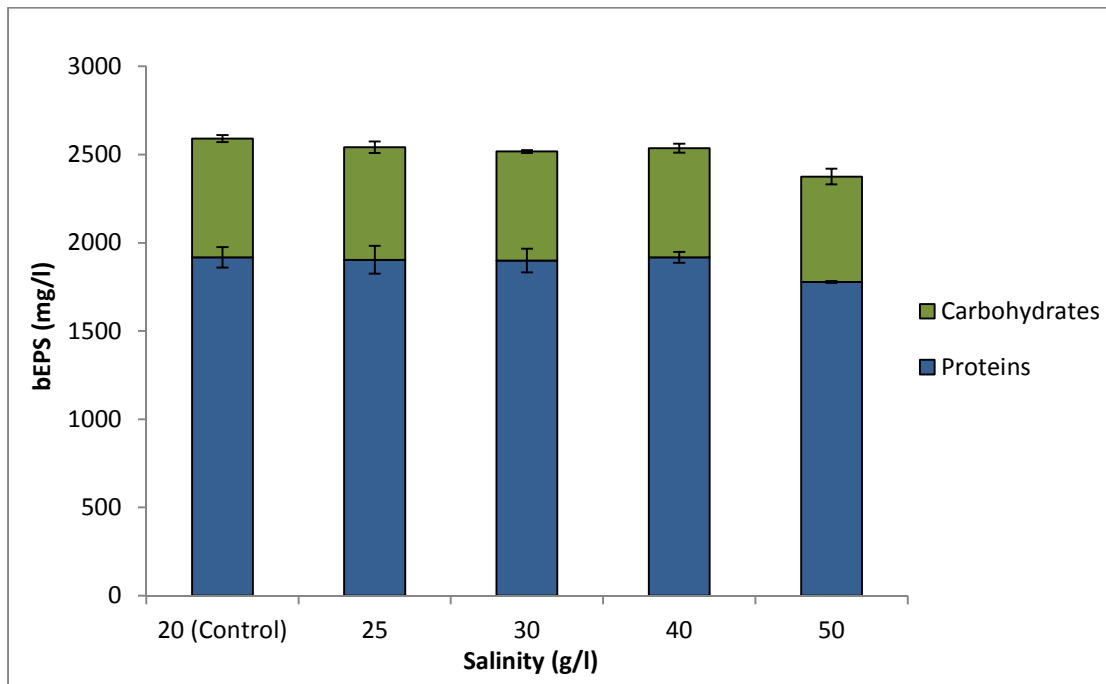


Figure 31. Effect of salinity on bEPS.

Figure 30 shows that SMP increased with the increase of salinity. Contrarily, bEPS decreased as salinity increased, as shown in Figure 31.

Ismail et al. (2010), when studying the granule strength in a UASB reactor operated under high salinity conditions, suggested that a high concentration of Na^+ reduces the granule strength, producing a weak dispersed sludge. Since in the present study salinity was also increased by adding NaCl, the Na^+ ions might also have interacted with the bEPS, weakening

their connection with the cells. This may have caused the release of bEPS into the medium, and hence their transfer to the SMP, which explains the results of the present research.

3.2.2. Effect of potassium and compatible solutes on SMA, PA, PO₄³⁻ release and EPS

3.2.2.1. SMA

Figures 32 and 33 present the effect of potassium and/or compatible solutes on SMA. Figure 32 shows the results of the SMA assay when betaine was tested and Figure 33 when trehalose was tested. Annex E contains the graph of cumulative methane production as well as the time interval from which the slope to calculate SMA was derived.

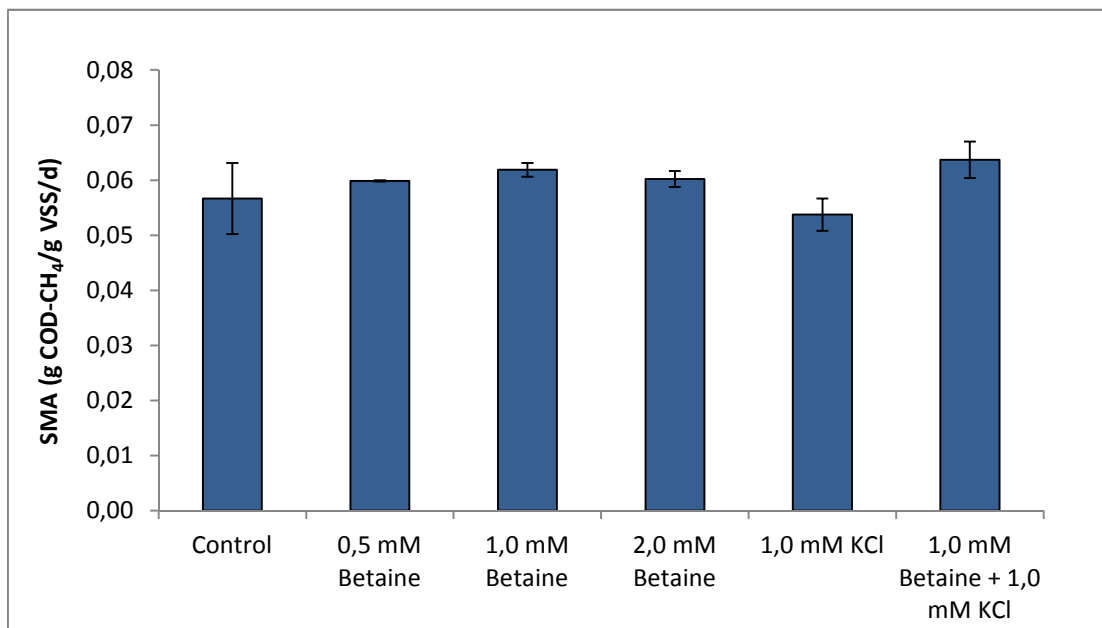


Figure 32. Effect of potassium and betaine on SMA.

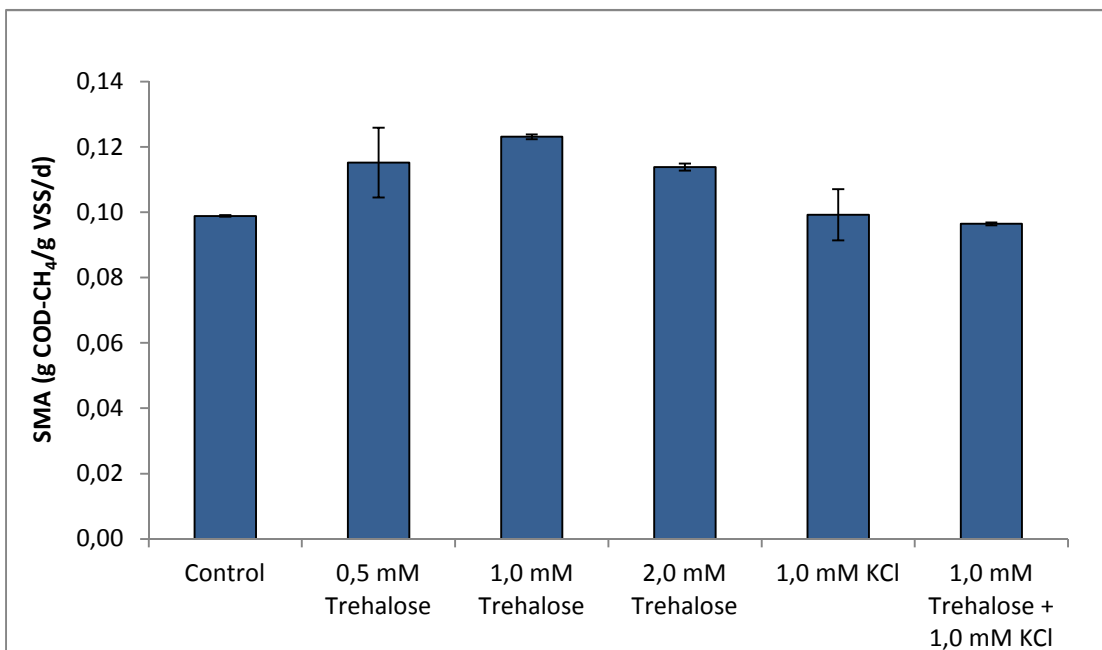


Figure 33. Effect of potassium and betaine on SMA.

Figure 32 shows that betaine increased the SMA, with an optimum concentration value of 1 mM. At this concentration, SMA was about 9% higher compared with the control. The addition of potassium did not improve SMA, which was slightly lower than the control (5%). The combination of betaine and potassium yielded a 12% increase of SMA.

Figure 33 shows that trehalose also increased SMA with an optimum concentration of 1 mM, but in this case the increase was 25% compared to the control. Also here, potassium addition did not result into any significant change of SMA. Contrarily to the combination of betaine and potassium, the combination of trehalose and potassium presented a negative effect compared with only trehalose.

The importance of compatible solutes for the adaptation of the cells to high salinity was shown by Oh et al. (2008), Vyrides and Stuckey (2009) and Vyrides et al. (2010). However, in the present study, the inoculum was already adapted to saline conditions and compatible solutes were added without changing the salinity. The results of the present study showed that anaerobic digestion under saline conditions could be improved by the addition of compatible solutes to the medium, even when the toxicity was already overcome and a stable process was achieved, as was shown previously through the performance of the reactors. The reason for that may be that, even after the cells overcome the toxicity of high salinity, they may not achieve their maximum activity (compared to fresh conditions) due to the fact that they need to keep the external osmotic pressure balanced, and for that they produce compatible solutes themselves, using part of the energy obtained from the substrate instead of producing methane. External addition of compatible solutes made that the cells no longer needed to produce compatible solutes so that and they could use the substrate to produce methane.

Contrarily to the results of Oh et al. (2008) and Vyrides et al. (2010), where betaine was more effective than trehalose, in the present study trehalose was the most effective. Vyrides et al. (2010) reported that trehalose was the dominant solute accumulated by the cells, when exposed to high salinity. The inoculum used in the present investigation was already adapted to high salinity levels and, in order to achieve their adaptation, the cells needed to produce compatible solutes which, according to the latter study, was mainly trehalose. Therefore, since the cells were already dealing with the high salinity of the medium, probably using mainly trehalose as compatible solute, it might have been easier for them to take up this compatible solute, which explains why in the present investigation trehalose was the most effective compatible solute.

No effect of potassium on SMA, as was observed, may be due to the fact that there were no (or very few) halophilic bacteria in the population of the methanogens that use this compound to cope with high salinity. The explanation for the observed performance when potassium and compatible solutes were added simultaneously might be because potassium could interact chemically with the compatible solutes, changing their properties and their "ability" to be absorbed by the cells. In case of betaine this resulted into a positive effect and in case of trehalose into a negative effect.

3.2.2.2. PA

Figures 34, 35 and 36 present the effect of betaine, trehalose and potassium on PA, respectively.

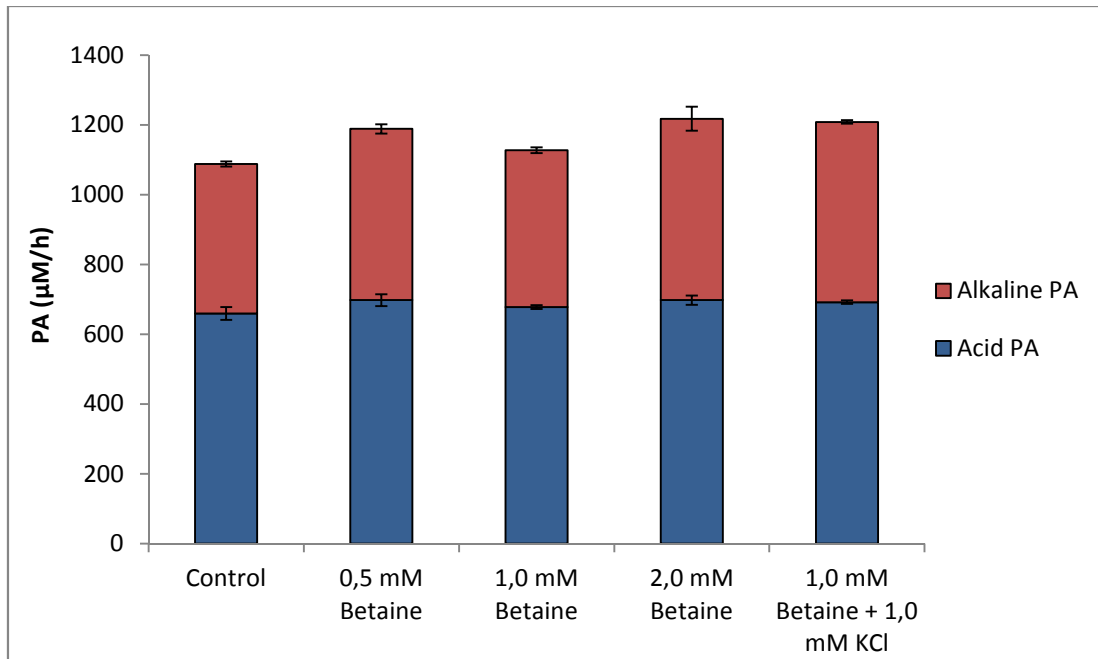


Figure 34. Effect of betaine on PA.

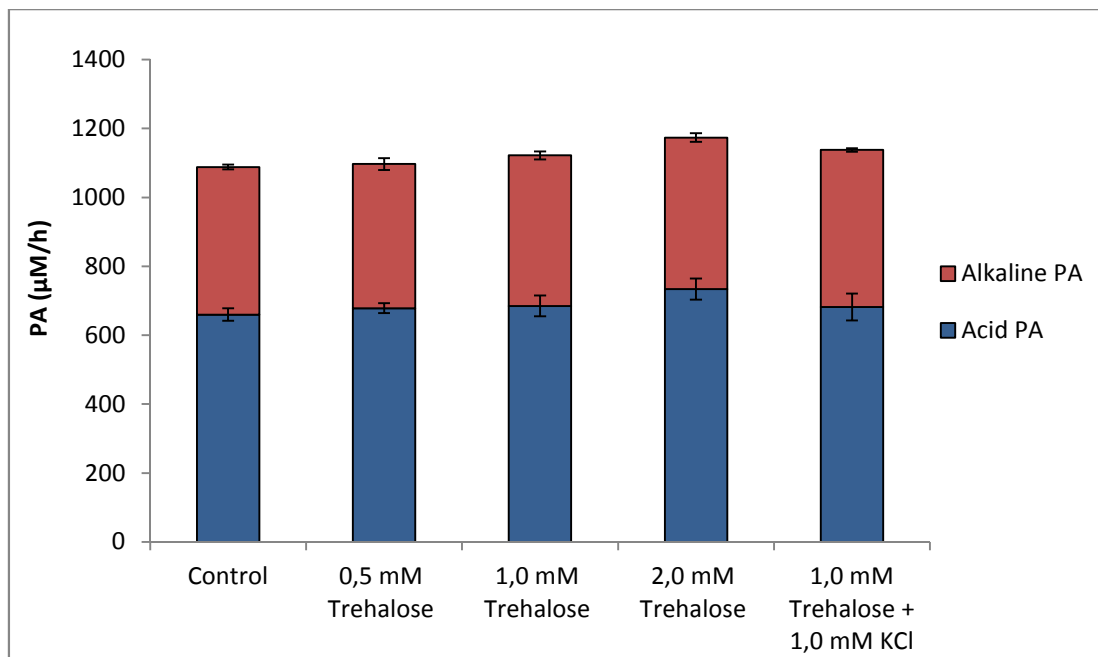


Figure 35. Effect of trehalose on PA.

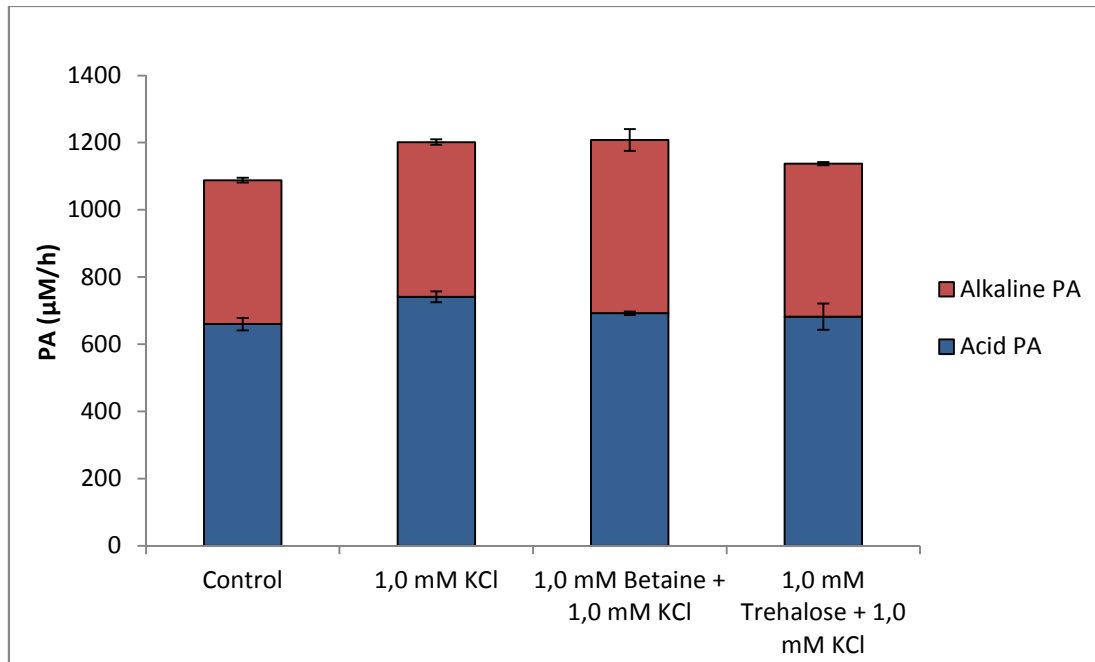


Figure 36. Effect of potassium on PA.

Betaine improved PA, as is shown in the Figure 34. The optimum concentration was 2 mM, where PA was 11% higher than the control. The addition of potassium improved the performance of betaine alone. The addition of 1 mM of betaine improved PA by 3% whereas the combination of betaine and potassium improved PA by 5%.

Figure 35 shows that trehalose also improved PA. The optimum concentration was also 2 mM, where the PA was 7% higher than the control. The addition of potassium together with trehalose improved the performance of this compatible solute. The addition of 1 mM of trehalose improved PA by 3% whereas the combination of trehalose and potassium improved PA about by 4%.

Potassium also had a positive effect on PA (Figure 36). It improved PA about 9% compared with the control. The addition of compatible solutes together with potassium did not show any substantially effect compared with the addition of only this compound. This compound was more effective than the two compatible solutes tested at the same molar concentration, since 1 mM of trehalose and betaine only increase PA by 3 and 4%, respectively.

The results show that the addition of compatible solutes had also a positive effect in the population of the acid formers as occurred with the population of the methanogens. However, the effect of these compounds in these different populations did not follow the same trend. For the acid formers, it appears that higher the concentration of compatible solutes, higher their activity, instead of having an optimum concentration of 1 mM as was the case for the methanogens. Another difference was that betaine was the most effective compatible solute instead of trehalose. Moreover, the addition of potassium was also effective and even better than the compatible solutes. With respect to the compatible solutes, these results may be explained by supposing that acid formers have different ways of interacting with these compounds than methanogens. The positive effect of potassium might be explained by the presence of a considerable fraction of halophilic bacteria in the acid formers population.

3.2.2.3. PO_4^{3-} release

Figures 37, 38 and 39 display the effect of betaine, trehalose and potassium on PO_4^{3-} release, respectively.

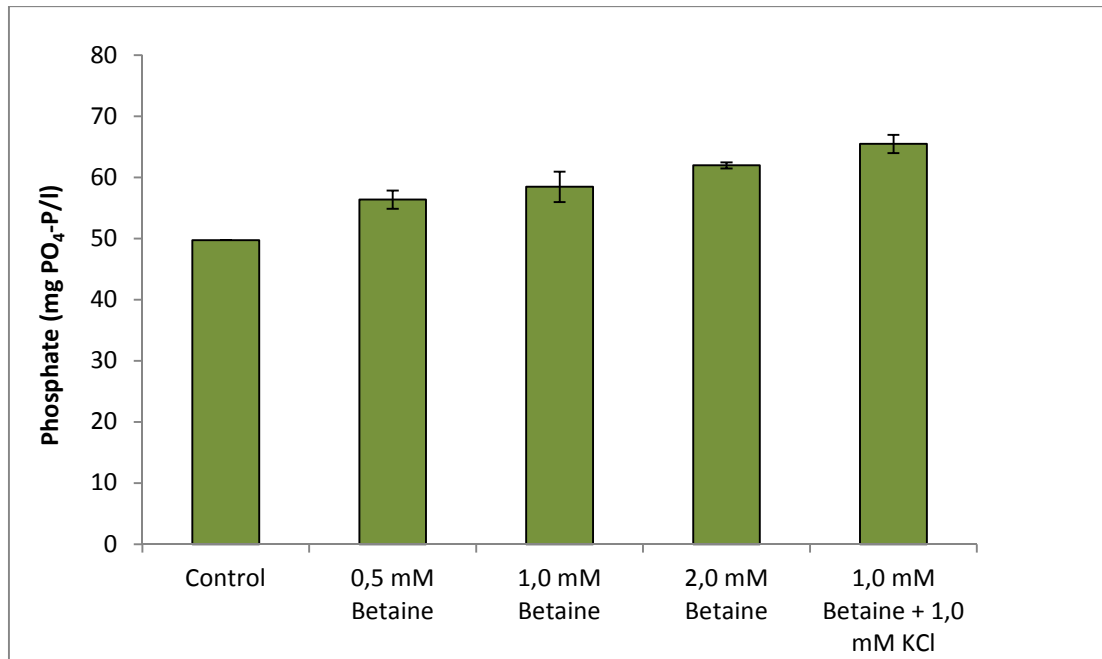


Figure 37. Effect of betaine on PO_4^{3-} release.

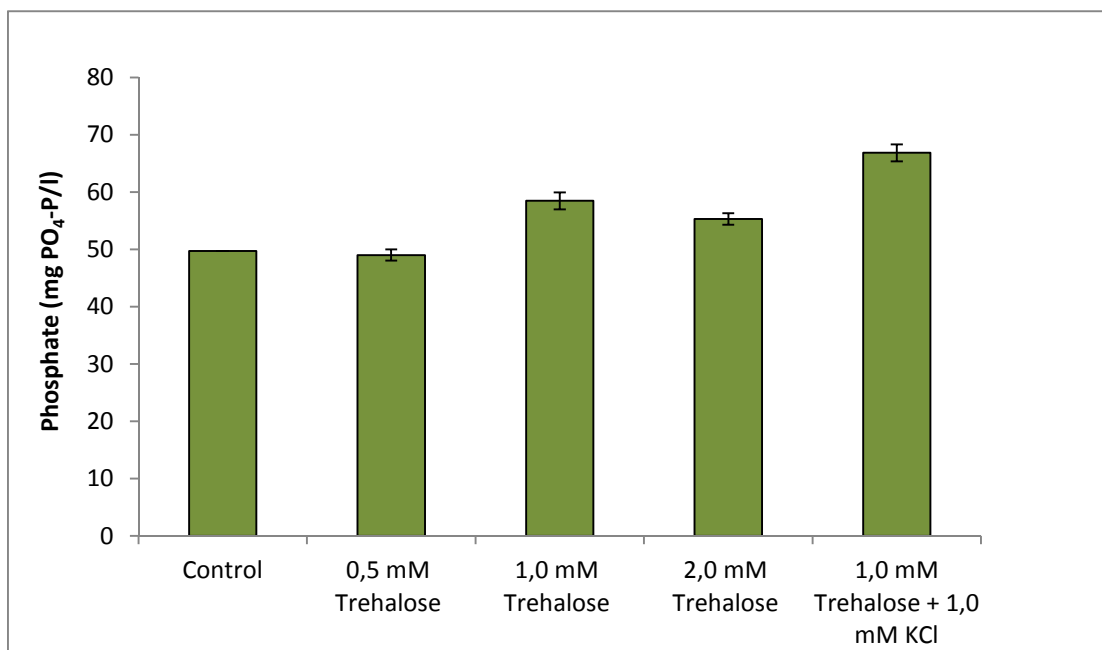


Figure 38. Effect of trehalose on PO_4^{3-} release.

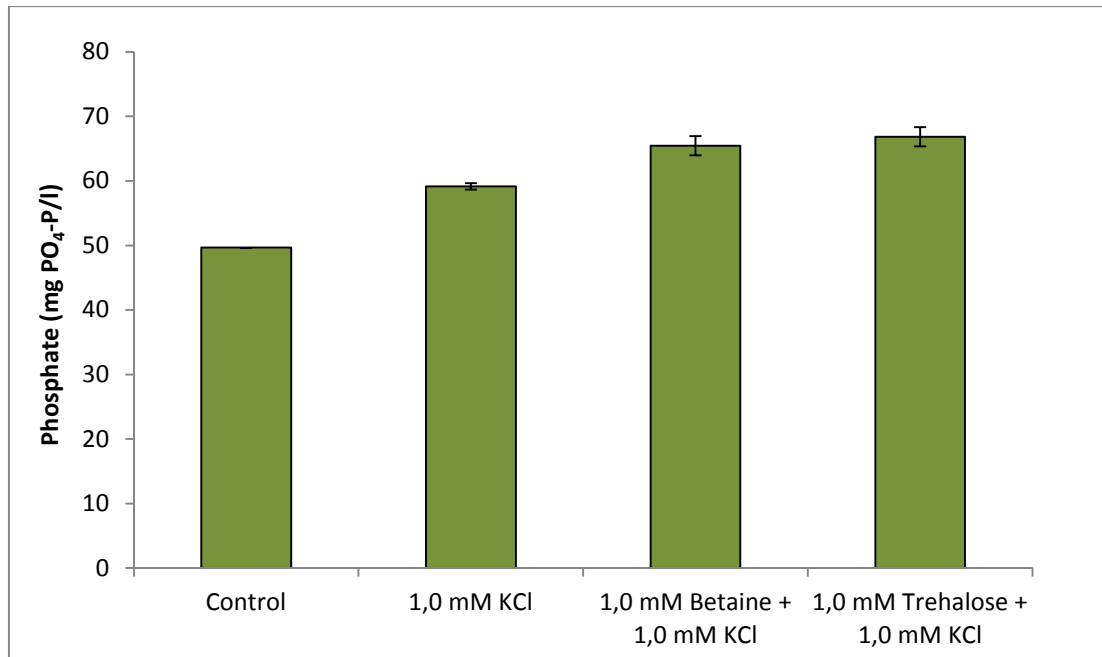


Figure 39. Effect of potassium on PO₄³⁻ release.

Figure 37 shows that betaine improved PO₄³⁻ release. The optimum concentration was 2 mM, where PO₄³⁻ was 25% higher than the control. The addition of potassium together with betaine improved the performance of this compatible solute. The addition of 1 mM of betaine improved PO₄³⁻ concentration by 18% whereas the combination of betaine and potassium improved PO₄³⁻ concentration by 32%.

Trehalose also improved PO₄³⁻ release, as is shown in Figure 38. The optimum concentration was 1 mM, where the PO₄³⁻ concentration was 18% higher than the control. The addition of potassium together with trehalose improved the performance of this compatible solute. The combination of 1 mM of trehalose and 1 mM of potassium improved PO₄³⁻ concentration by 35%.

Potassium also had a positive effect on PO₄³⁻ release (Figure 39). It improved PO₄³⁻ release about 19% compared with the control. It was more efficient than only trehalose and betaine at the same molar concentration. The addition of compatible solutes together with potassium showed a better result compared with the addition of only potassium. The combination of potassium with trehalose and betaine improved the PO₄³⁻ release by 35 and 32%, respectively.

Once again, the results are not in agreement with the results from the effect of compatible solutes on PA. The main differences were that potassium and compatible solutes had a larger effect on the PO₄³⁻ release than PA and the combination of potassium with compatible solutes improved the performance of these compounds. The lack of relationship between PA and PO₄³⁻ was already discussed above. Nevertheless, it is evident that compatible solutes and/or potassium improved PO₄³⁻ release.

3.2.2.4. EPS

Figures 40, 41 and 42 present the effect of betaine, trehalose and potassium on SMP. Figures 43, 44 and 45 present the effect of betaine, trehalose and potassium on bEPS.

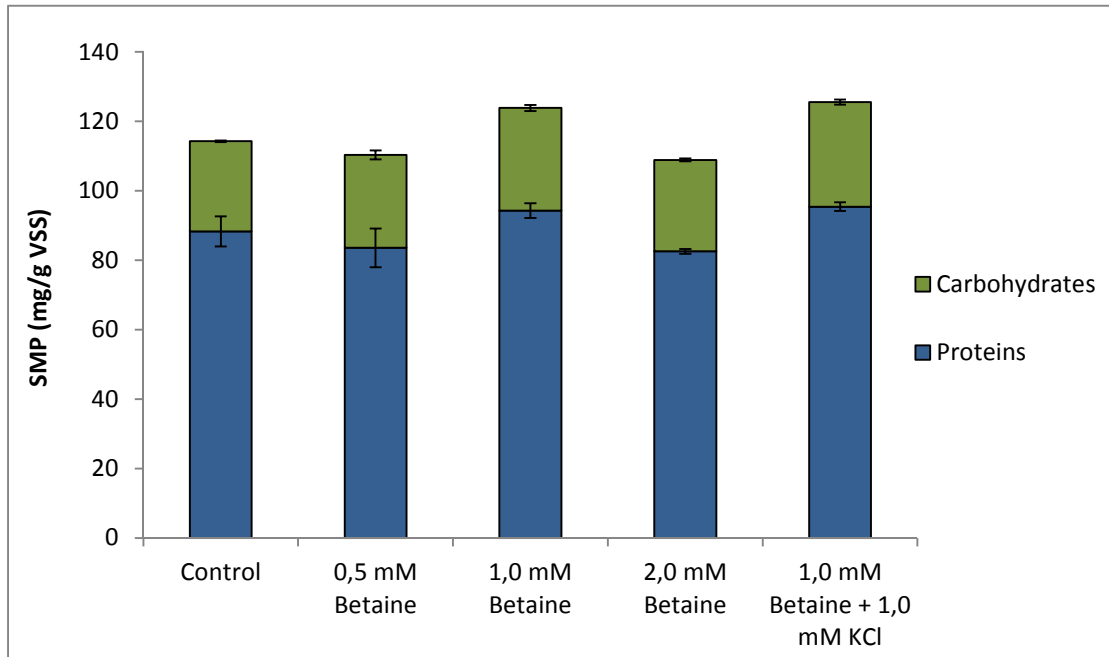


Figure 40. Effect of betaine on SMP.

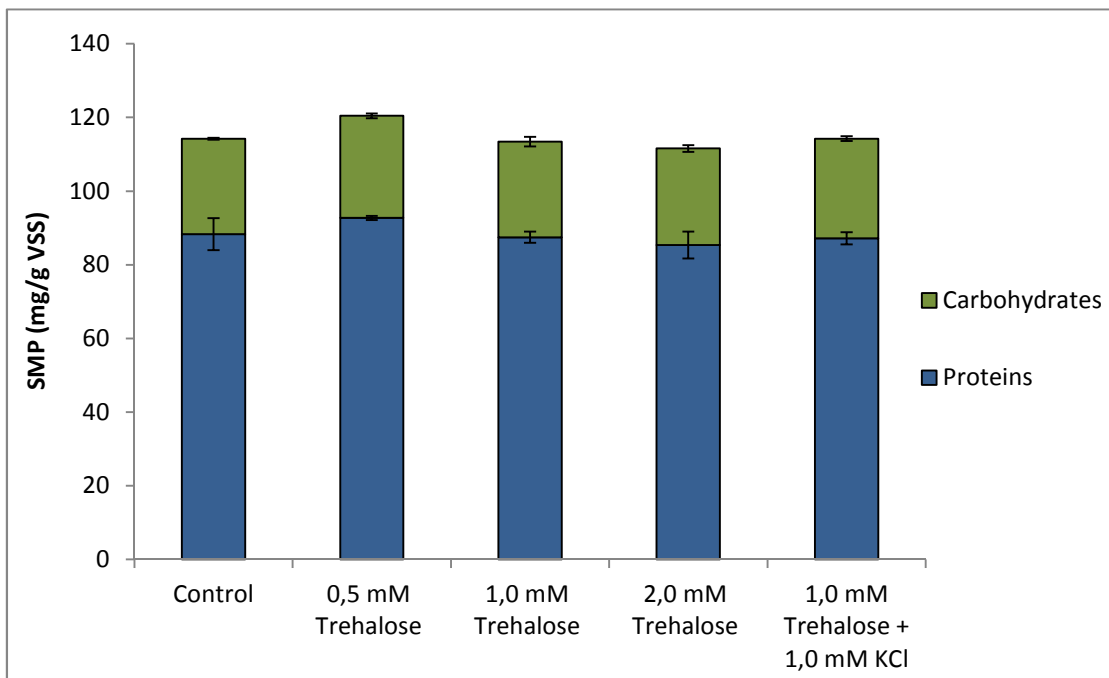


Figure 41. Effect of trehalose on SMP.

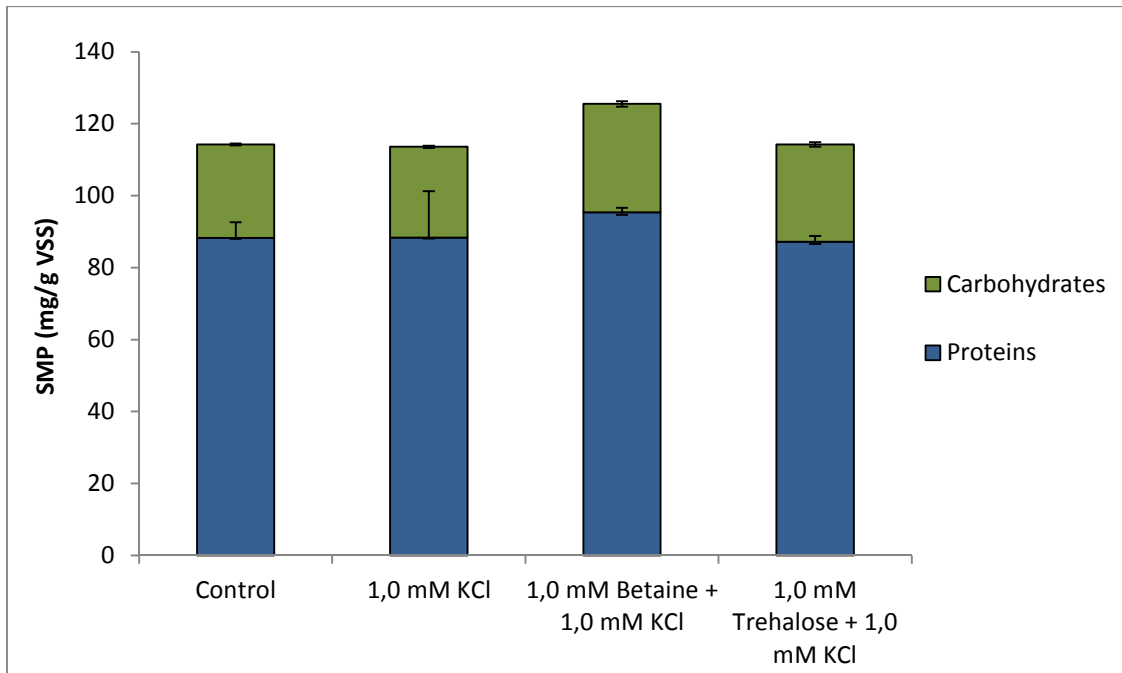


Figure 42. Effect of potassium on SMP.

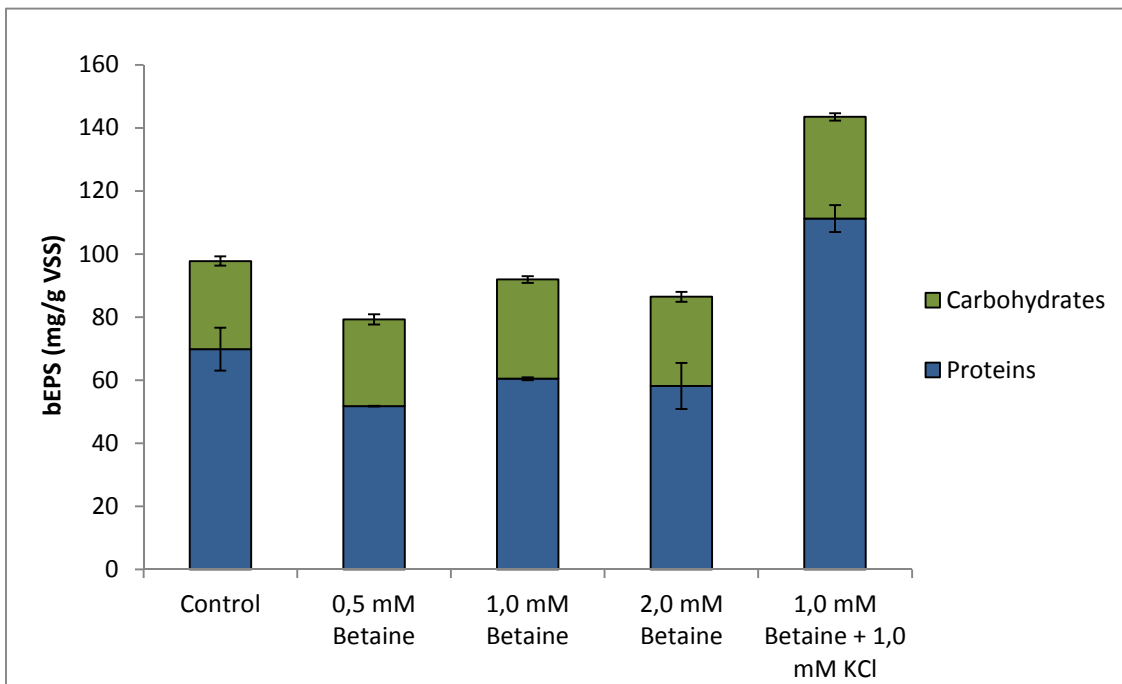


Figure 43. Effect of betaine on bEPS.

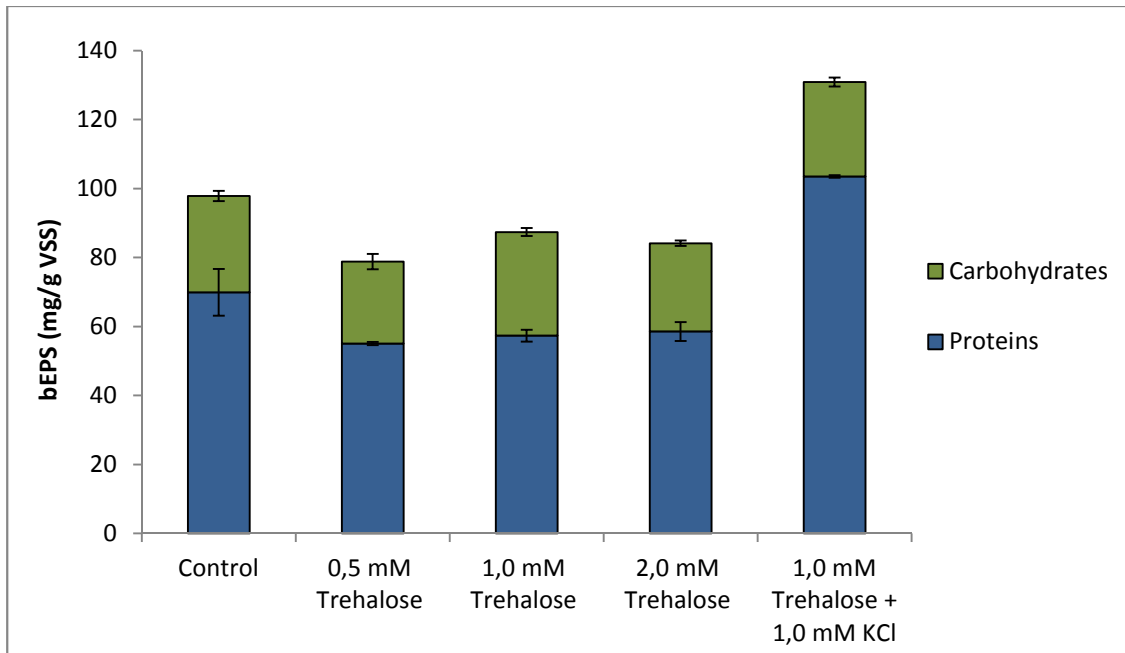


Figure 44. Effect of trehalose on bEPS.

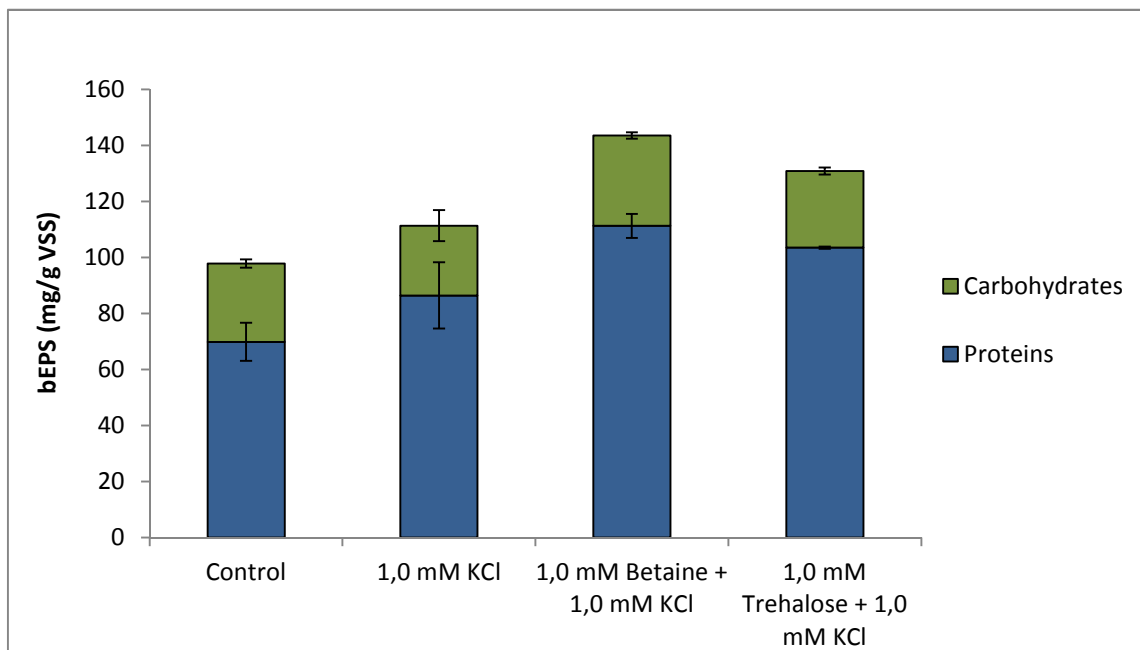


Figure 45. Effect of potassium on bEPS.

Figures 40, 41 and 42 show that potassium and compatible solutes did not substantially affect the production of SMP by the microorganisms. However, for bEPS, compatible solutes decreased the amount of bEPS produced, as shown in Figures 43 and 44, whereas the addition of potassium increased the amount of bEPS produced, mainly because of the increase of the amount of proteins, as shown in Figure 45.

The decrease of bEPS production when compatible solutes were added can be explained by the fact that these compounds, as was already shown above through SMA, PA and PO_4^{3-} release, helped the cells to alleviate the osmotic stress caused by the high salinity and, therefore, less bEPS were needed to be produced. These results were also reported by Vyrides et al. (2010) who found lower EPS production when glycine betaine was added to alleviate salt

toxicity. The reason that only the bEPS production was affected by the presence of compatible solutes may be because this form of EPS is the main responsible to help the cells to deal with high salinity levels.

The increase of bEPS proteins production with potassium may be because of the interaction of this ion with bEPS. bEPS are bound with cells mainly through ion bridging (Sheng et al., 2010), hence ions concentration may also influence bEPS content. Moreover, as in the present study, Murthy (1998) also reported an increase in proteins from bEPS with an increase in potassium concentration, when studying the effect of this compound on activated sludge properties.

3.2.3. Effect of FeCl₃ on SMA, PA, PO₄³⁻ release and EPS

3.2.3.1. SMA

Figure 46 presents the effect of different dosages of FeCl₃ on SMA. Annex E contains the graph of cumulative methane production as well as the time interval from which the slope to calculate SMA was derived.

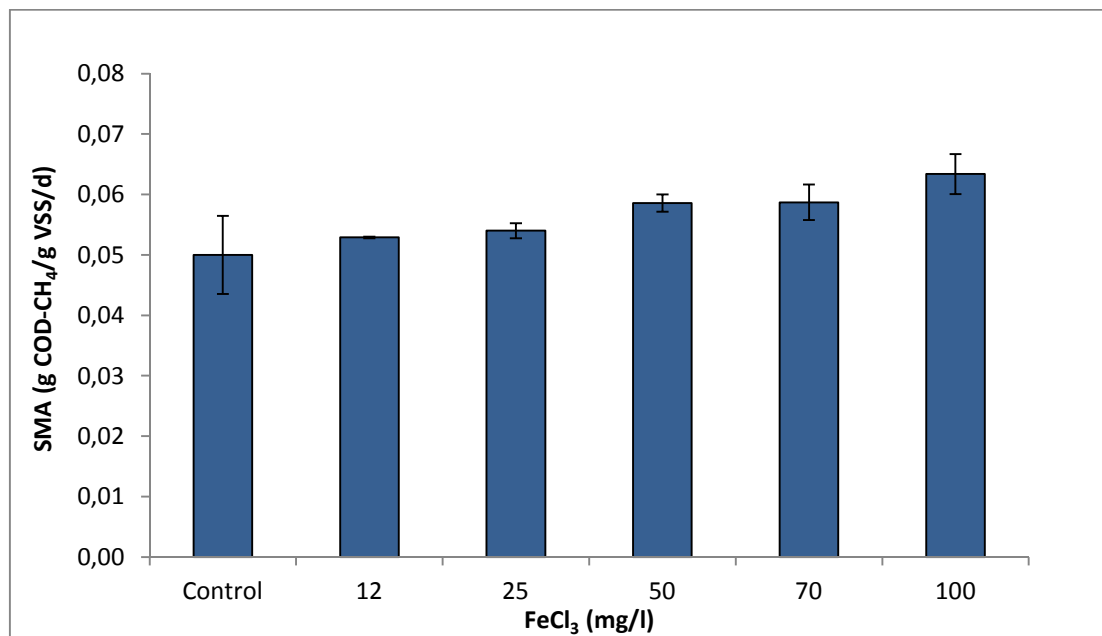


Figure 46. Effect of FeCl₃ on SMA.

Figure 46 shows that the higher the dosage of FeCl₃ the higher the SMA. At dosage of 100 mg FeCl₃/l, the SMA was 27% higher than the SMA of the control.

The brackish water used for the SMA assay had a sulfate (SO₄²⁻) concentration of 978.4 mg/l (Table 11). In the anaerobic process sulfate is reduced to sulfide (S²⁻) by the sulfate reducing bacteria (SRB). This process causes inhibition of methanogenesis because of the competition between SRB and methanogens for the substrate and the toxicity of the sulfide formed (mainly in form of H₂S (Khanal, 2008)) to methanogens (Chen et al., 2008). Choi and Rim (1991) reported that methanogens predominate with COD/SO₄²⁻ ratio higher than 2.7 while SRB predominate when COD/SO₄²⁻ ratio is lower than 1.7. Another study indicated that methanogens only predominate when COD/SO₄²⁻ is higher than 10 (Mirzoyan et al., 2008). In the present study, the COD/SO₄²⁻ ratio for the SMA assay to study the effect of FeCl₃ was 0.5

(COD = 500 mg/l and SO_4^{2-} = 978.4 mg/l). This low ratio indicates that the inhibition of the methanogens may have occurred. The addition of FeCl_3 released Fe^{3+} into the medium which can be reduced to Fe^{2+} by the dissimilatory iron-reducing bacteria (DIRB) (Fredrickson and Gorby, 1996). The latter form of iron may have interacted with sulfide and precipitated as FeS , decreasing the amount of sulfide in the medium. Therefore, the presence of FeCl_3 may have alleviated the inhibition caused by the lower $\text{COD}/\text{SO}_4^{2-}$, which explained why SMA increased with addition of this compound.

Due to this unexpected effect observed in this SMA assay, the results could not be used to predict what the effect of FeCl_3 on the methanogens in the reactors of the present investigation. The competition between the methanogens and SRB was not observed in the reactors due to the fact that their $\text{COD}/\text{SO}_4^{2-}$ were much higher than that in the SMA assay, because the higher COD of the substrate (Table 11). However, the results showed that it may be interesting to investigate the use of FeCl_3 to overcome sulfide inhibition in the anaerobic digestion.

3.2.3.2. PA

Figure 47 presents the effect of different dosages of FeCl_3 on PA.

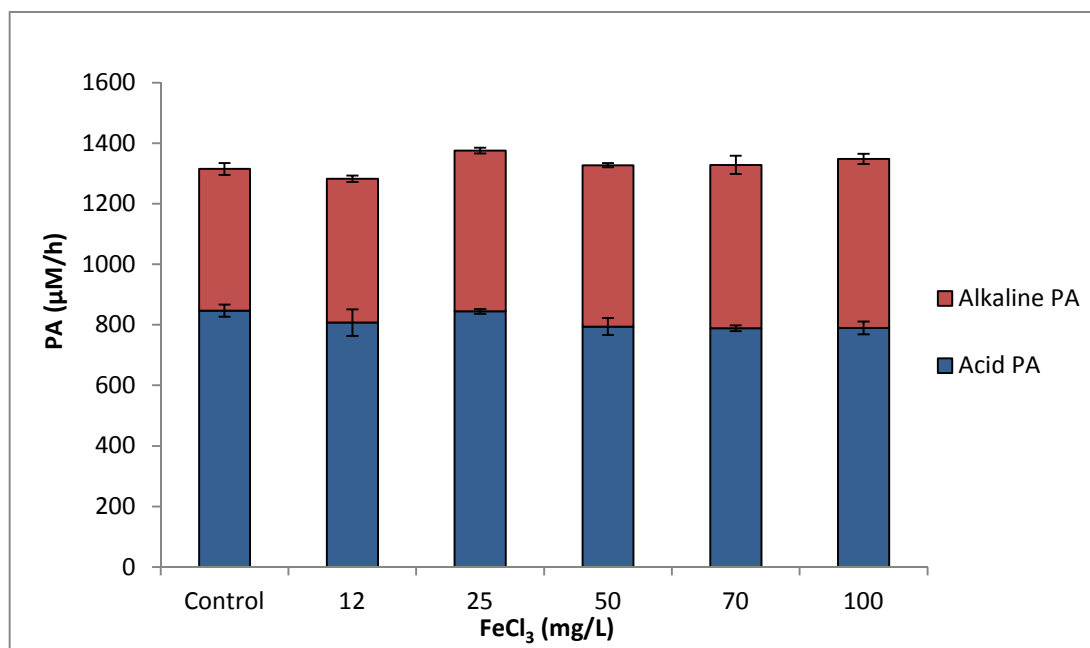


Figure 47. Effect of FeCl_3 on PA.

Figure 47 shows that the addition of FeCl_3 did not result into significant changes of PA. The results show that the population of the acid formers was not affected by the addition of FeCl_3 .

3.2.3.3. PO_4^{3-} release

Figure 48 presents the effect of FeCl_3 on PO_4^{3-} release.

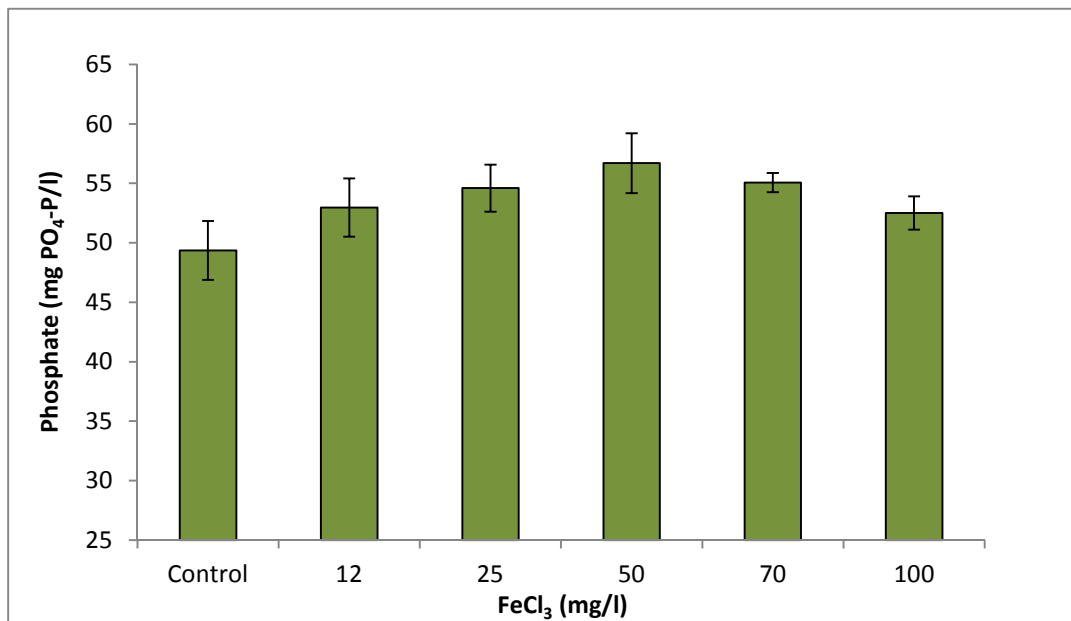


Figure 48. Effect of FeCl_3 on PO_4^{3-} release.

Figure 48 shows that the addition of FeCl_3 increased the PO_4^{3-} release, being 50 mg FeCl_3/l the optimum concentration, where PO_4^{3-} concentration was 15% higher than in the control.

Iron is a micronutrient required for an optimum anaerobic digestion. It is present in iron-sulphur clusters, various enzymes and cytochromes and it is responsible for electron transport (Zandvoort et al., 2003). The concentration of iron in the substrate was 0,3 mg/l (Table 11), lower than that used in the SMA assay (3 mg/l), which is considered to be the minimum need for the growth of anaerobic microorganisms, according to (Owen et al., 1979). This lack of iron in the substrate may explain why the addition of FeCl_3 increased the PO_4^{3-} concentration. The added iron may have been used by the cells, increasing their activity and consequently the PO_4^{3-} released in to the medium. The existence of an optimum concentration of FeCl_3 at 50 mg/l may be related with the precipitation of PO_4^{3-} as ferric phosphate (FePO_4) for higher concentrations of FeCl_3 . This may have caused the decrease of dissolved PO_4^{3-} concentration in the medium. Furthermore, also less Fe^{3+} was available for being used by the cells to release PO_4^{3-} . However, even at these higher dosages of FeCl_3 the PO_4^{3-} concentrations were higher than the control (12 and 6% for 70 and 100 mg/l of FeCl_3 , respectively). The optimum dosage of FeCl_3 for concentration of solids and phosphorus found in the first part of the EM-MARES project was 70-80 mg/l. This was not the optimum for PO_4^{3-} release, but it increased the PO_4^{3-} release compared with the control, where no FeCl_3 was added.

3.2.3.4. EPS

Figures 49 and 50 present the effect of FeCl₃ on SMP and bEPS, respectively.

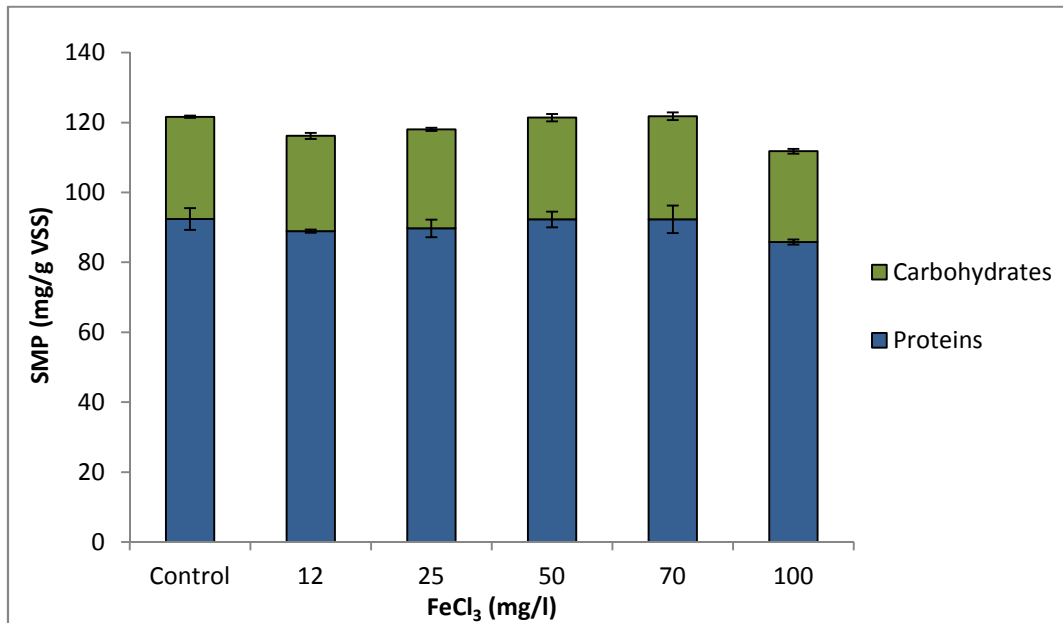


Figure 49. Effect of FeCl₃ on SMP.

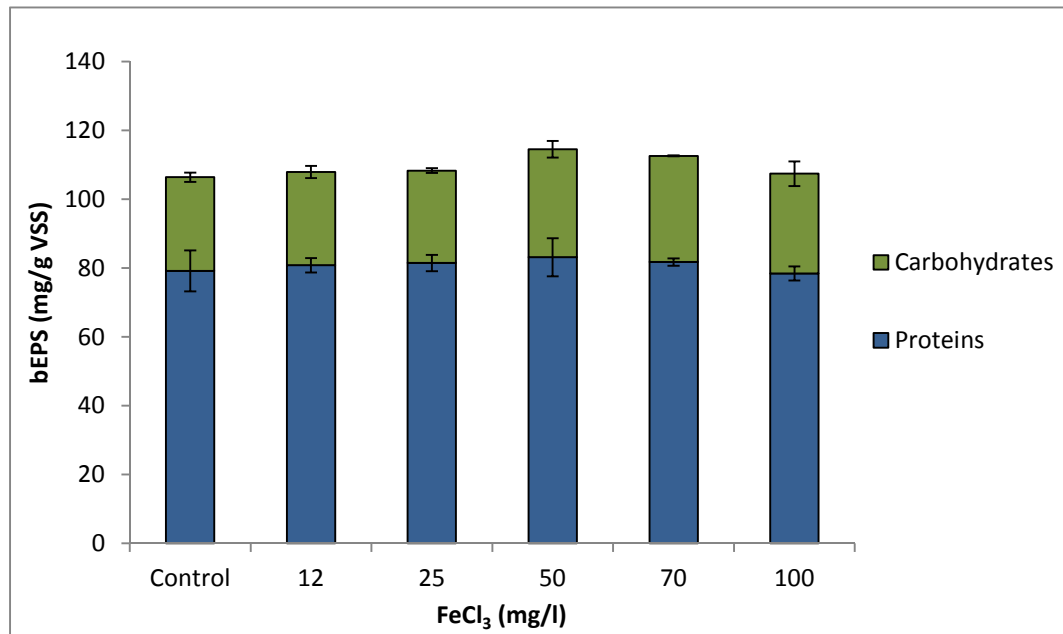


Figure 50. Effect of FeCl₃ on bEPS.

Figures 49 and 50 show that the addition of FeCl₃ did not result into any substantial effect on SMP and bEPS, respectively. These results indicate that FeCl₃ did not cause any stress to the microorganisms. Besides that, the Fe³⁺ from FeCl₃ did not interact with the bEPS, as was observed with sodium and potassium.

4. Conclusions

4.1. Anaerobic digestion of sludge from marine RAS in CSTR

Anaerobic digestion of sludge from marine RAS in CSTR using an inoculum already adapted to saline conditions presented a steady state operation, even with changes in their OLRs. COD and VS removal efficiencies of 50.2-62.1% and 60.1-70.9% for reactor R1 were achieved, respectively. For reactor R2, COD and VS removal efficiencies were 39.7-58.2% and 45.2-65.7%, respectively. Methane content in the biogas of 46.1-59.9% and 59.5-65.6%, for reactor R1 and R2, respectively, could be achieved. Methane yield of reactor R1 was 0.142-0.244 l/g COD added and for reactor R2 that was 0.154-0.214 l/g COD added.

The results of the present study were better than the results of the previous investigations dealing with anaerobic digestion of sludge from marine RAS in CSTR, in terms of a stable operation and higher methane yields. A considerably higher volumetric methane production rate was obtained, compared with the range of typical values from anaerobic digestion of sewage sludge.

4.2. Batch studies

4.2.1. Effect of salinity on SMA, PA, PO_4^{3-} release and EPS

The salinity increase severely affected SMA. PA was slightly affected by the increase of salinity. PO_4^{3-} release decreased with high salinity. PA could not be used to predicted PO_4^{3-} concentration. Regarding EPS, bEPS decreased while SMP increased when the salinity increased.

These results show that salinity has an important effect in the anaerobic digestion and it should be monitored in the anaerobic digestion of sludge from marine RAS in order to prevent the failure of the process.

4.2.2. Effect of potassium and compatible solutes on SMA, PA, PO_4^{3-} release and EPS

Both compatible solutes tested (trehalose and betaine) improved SMA, PA and PO_4^{3-} release and decreased the amount of bEPS produced. Trehalose was the more effective on the improvement of SMA whereas betaine was the more effective in the improvement of PA and PO_4^{3-} release. Potassium did not affect SMA but it did improve PA and PO_4^{3-} release and increase bEPS.

Based on the results and the objectives of the EM-MARES project, the anaerobic digestion of sludge from marine RAS, can be improved by the addition of compatibles solutes, resulting into higher methane production and PO_4^{3-} release, which are related with the aims of the EM-MARES project: recovery bio-energy as methane and phosphorus as struvite. Regarding to potassium, it only presented a positive effect in the latter goal of the EM-MARES project.

4.2.3. Effect of FeCl₃ on SMA, PA, PO₄³⁻ release and EPS

SMA increased with the increase of FeCl₃ dosage. This result could not be used to predict FeCl₃ effect on SMA due to the probable and unexpected sulfide inhibition observed in the SMA assay, and not observed in the reactors. However, PO₄³⁻ release was improved with the addition of FeCl₃. FeCl₃ did not present any significant effect on PA and in the production of both forms of EPS.

According to the results of the present investigation, FeCl₃ did not show any negative effect in the anaerobic digestion of sludge from marine RAS in CSTR. It supports the feasibility of using FeCl₃ as a coagulant for the concentration of solids and phosphorus from backwash water from marine RAS before anaerobic digestion of sludge in CSTR, as suggested in the EM-MARES project. Due to the improvement of PO₄³⁻ release, FeCl₃ may actually have a positive effect in the recovery of phosphorus as struvite which is another aim of the EM-MARES project.

4.3. Scientific and social relevance

In the first part of this research, anaerobic digestion of sludge from marine RAS in CSTR inoculated with microorganisms already adapted to saline conditions was shown as a feasible solution to treat the sludge from marine RAS. This contributes to the increase of the sustainability of marine RAS as a major solution to meet the increasing demand of seafood. Batch studies, conducted in the second part of this research, are a great contribution for the EM-MARES project as well for the knowledge related with the anaerobic digestion at saline conditions.

4.4. Recommendations for further research

Investigation about the performance of CSTR for the anaerobic digestion of sludge from marine RAS with lower STRs of those used in this research, in the range of those typically used in the anaerobic digestion of sewage sludge, may be an interesting topic of research since it leads a smaller reactors and consequently economic advantages.

As was shown in the results from the effect of FeCl₃ on SMA, FeCl₃ might be very efficient in alleviating sulfide inhibition and, therefore, it may be attractive to study the use of FeCl₃ to mitigate sulfide inhibition in the anaerobic digestion.

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Annex A. Calibration curve for PA determination.

Figure 51 presents the calibration curve used for PA determination.

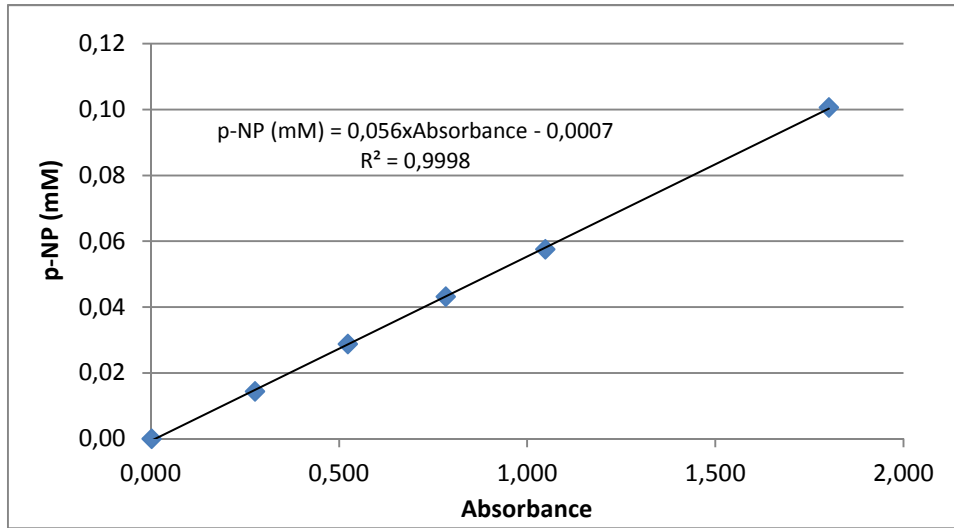


Figure 51. Calibration curve used for PA determination.

Annex B. Calibrations curves for EPS determination.

Figures 52 and 53 present the calibration curves used for proteins and carbohydrates measurements in EPS determination, respectively.

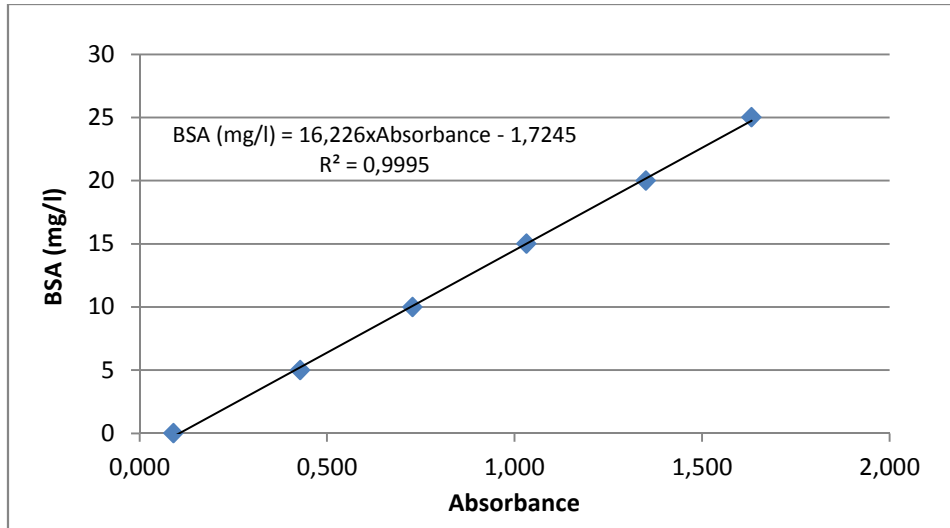


Figure 52. Calibration curve used for proteins determination.

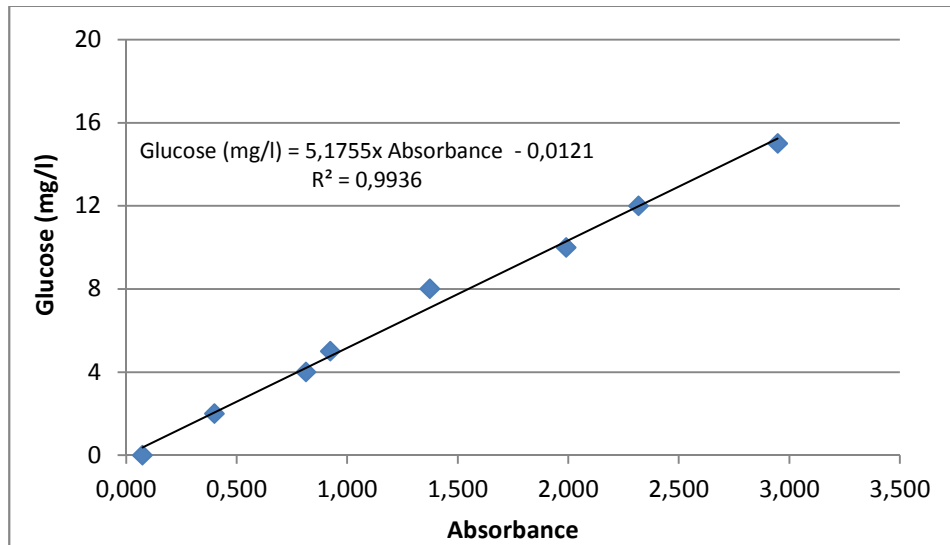


Figure 53. Calibration curve used for carbohydrates determination.

Annex C. Characterization of substrate.

Table 15 presents the characterization of the substrate in each time it was collect at the fish farm, the period when it was utilized and the reactor(s) where it was used.

Table 15. Characterization of the substrate in each time it was collect at the fish farm, the period when it was utilized and the reactor(s) where it was used.

Information				
Period of use	0 to 18 th day	18 th to 84 th day	18 th to 56 th day	56 th to 84 th day
Reactor	R1 and R2	R1	R2	R2
Characterization				
pH	5.9	6.1	6.0	6.1
Salinity (g/l)	13.6	13.6	13.5	13.9
EC (mS/cm)	23.1	22.3	22.2	22.8
TS (g/l)	126.2 ± 2.04	100.0 ± 2.16	119.6 ± 2.30	94.8 ± 0.72
VS (g/l)	86.2 ± 2.05	70.3 ± 1.50	75.4 ± 1.00	58.7 ± 2.90
TSS (g/l)	79.2 ± 18.67	69.9 ± 0.92	97.7 ± 2.90	67.8 ± 1.38
VSS (g/l)	49.4 ± 11.60	60.1 ± 0.79	69.6 ± 2.59	52.7 ± 1.38
tCOD (g/l)	113.2 ± 5.51	89.6 ± 2.83	123.4 ± 6.46	84.3 ± 1.84
sCOD (g/l)	10.0 ± 0.59	12.1 ± 0.21	12.6 ± 0.16	9.1 ± 0.30
TN (g/l)	4.7 ± 0.07	3.5 ± 0.28	4.0 ± 0.06	3.1 ± 0.12
TP (g/l)	2.9 ± 0.32	2.7 ± 0.16	2.7 ± 0.11	1.0 ± 0.25
NH ₄ ⁺ (mg/l)	309.0 ± 4.24	289.3 ± 2.31	424.0 ± 3.61	241.8 ± 5.90
PO ₄ ³⁻ (mg/l)	168.0 ± 5.66	218.7 ± 1.15	232.7 ± 3.06	251.0 ± 1.41

Annex D. Characterization of the sludge from the reactors.

Table 16. Results of all the characterization of the sludge from reactor R1.

Parameter	Day						
	0	7	14	20	29	33	42
pH	7.36	7.69	7.60	7.79	7.67	7.70	7.72
Salinity (g/l)	20.0	20.2	20.6	21.1	20.9	20.9	21.0
EC (mS/cm)	32.1	32.5	33.2	33.7	34.3	33.8	33.7
TS (g/l)	55.5 ± 0.87	53.7 ± 0.95	54.1 ± 1.15	51.1 ± 0.42	50.6 ± 2.13	51.3 ± 1.40	48.7 ± 0.33
VS (g/l)	27.8 ± 0.28	27.5 ± 0.44	28.8 ± 0.57	26.3 ± 0.17	26.0 ± 1.10	26.3 ± 1.04	24.3 ± 0.08
TSS (g/l)	24.5 ± 0.19	36.1 ± 1.84	36.1 ± 1.00	32.0 ± 1.71	30.5 ± 1.40	26.9 ± 1.20	27.9 ± 2.03
VSS (g/l)	18.7 ± 0.38	26.7 ± 1.00	27.0 ± 0.61	23.5 ± 1.88	24.1 ± 0.98	20.9 ± 0.94	22.1 ± 0.88
tCOD (g/l)	45.6 ± 0.71	49.1 ± 1.13	49.3 ± 1.42	44.7 ± 1.34	43.0 ± 1.70	44.5 ± 1.15	39.0 ± 0.86
sCOD (g/l)	8.50 ± 0.40	7.73 ± 0.28	8.42 ± 0.08	6.82 ± 0.08	6.23 ± 0.22	6.05 ± 0.14	5.81 ± 0.31
TN (g/l)	3.85 ± 0.07	4.45 ± 0.07	4.55 ± 0.07	4.90 ± 0.10	4.30 ± 0.10	4.17 ± 0.12	3.87 ± 0.12
TP (g/l)	1.53 ± 0.04	1.34 ± 0.09	1.31 ± 0.05	1.22 ± 0.04	1.23 ± 0.04	1.26 ± 0.06	1.04 ± 0.00
NH ₄ ⁺ (mg/l)	2154 ± 2.83	2420 ± 24.0	2401 ± 47.7	2389 ± 36.3	2519 ± 47.4	2445 ± 12.9	2189 ± 28.4
PO ₄ ³⁻ (mg/l)	57.0 ± 7.07	51.3 ± 1.15	55.3 ± 1.15	61.0 ± 1.41	60.7 ± 2.31	60.0 ± 0.00	62.0 ± 0.00
Parameter	Day						
	49	56	63	70	77	84	
pH	7.82	7.62	7.74	7.64	7.67	7.61	
Salinity (g/l)	21.6	21.0	21.3	21.2	21.0	20.8	
EC (mS/cm)	34.6	33.6	34.1	34.0	33.5	33.3	
TS (g/l)	47.6 ± 0.32	47.6 ± 0.62	45.7 ± 0.18	46.4 ± 0.28	48.1 ± 1.00	48.1 ± 0.51	
VS (g/l)	23.6 ± 0.15	22.4 ± 0.34	21.9 ± 0.05	22.6 ± 0.15	24.1 ± 0.58	23.8 ± 0.39	
TSS (g/l)	25.8 ± 1.02	25.2 ± 1.17	24.4 ± 1.77	25.2 ± 0.42	28.1 ± 0.97	28.3 ± 0.39	
VSS (g/l)	20.7 ± 0.76	20.2 ± 0.36	19.9 ± 0.71	20.2 ± 0.21	22.6 ± 0.68	22.9 ± 0.76	
tCOD (g/l)	38.3 ± 2.33	40.0 ± 0.57	37.4 ± 0.35	36.7 ± 0.23	36.8 ± 0.00	33.4 ± 0.85	
sCOD (g/l)	6.00 ± 0.15	6.01 ± 0.27	5.69 ± 0.09	5.84 ± 0.12	5.81 ± 0.26	4.48 ± 0.02	
TN (g/l)	3.87 ± 0.15	3.60 ± 0.00	3.70 ± 0.20	3.50 ± 0.10	3.70 ± 0.17	3.87 ± 0.15	
TP (g/l)	1.00 ± 0.00	0.99 ± 0.03	0.99 ± 0.03	0.94 ± 0.01	1.02 ± 0.04	1.03 ± 0.02	
NH ₄ ⁺ (mg/l)	2249 ± 15.1	2299 ± 0.01	2869 ± 45.8	2827 ± 10.6	2934 ± 7.80	2898 ± 34.0	
PO ₄ ³⁻ (mg/l)	72.0 ± 2.00	64.7 ± 0.00	67.9 ± 1.51	70.6 ± 2.08	75.7 ± 1.42	85.3 ± 2.55	

Table 17. Results of all the characterization of the sludge from reactor R2.

Parameter	Day						
	0	20	29	33	42	49	56
pH	7.63	7.95	7.80	7.90	7.99	8.06	7.81
Salinity (g/l)	21.3	20.9	21.5	24.4	21.9	22.6	22.0
EC (mS/cm)	33.4	33.4	34.4	34.3	34.8	36.1	35.3
TS (g/l)	60.6 ± 0.56	65.1 ± 1.42	62.9 ± 0.97	66.3 ± 0.95	63.1 ± 0.77	61.9 ± 1.07	66.2 ± 0.12
VS (g/l)	32.1 ± 0.41	35.8 ± 0.75	33.6 ± 0.44	35.9 ± 0.52	33.5 ± 0.50	32.8 ± 0.60	33.1 ± 0.27
TSS (g/l)	27.1 ± 4.10	45.0 ± 1.40	42.6 ± 1.55	40.9 ± 1.55	41.0 ± 1.85	41.6 ± 0.96	45.5 ± 1.14
VSS (g/l)	21.7 ± 4.45	33.6 ± 1.23	30.7 ± 0.69	30.6 ± 0.82	29.7 ± 0.79	30.3 ± 0.74	29.9 ± 0.43
tCOD (g/l)	58.3 ± 1.18	62.7 ± 5.66	58.9 ± 1.70	58.3 ± 1.46	58.5 ± 2.90	51.6 ± 1.27	56.2 ± 2.51
sCOD (g/l)	15.1 ± 0.32	9.30 ± 0.14	9.34 ± 0.11	9.27 ± 0.41	8.20 ± 0.28	8.62 ± 0.33	8.36 ± 0.18
TN (g/l)	4.30 ± 0.14	4.23 ± 0.21	4.60 ± 0.17	5.03 ± 0.12	4.75 ± 0.07	4.40 ± 0.00	4.90 ± 0.10
TP (g/l)	1.27 ± 0.01	1.29 ± 0.09	1.15 ± 0.07	1.53 ± 0.09	1.35 ± 0.18	1.06 ± 0.03	1.16 ± 0.03
NH ₄ ⁺ (mg/l)	3162 ± 48.1	3052 ± 107	3508 ± 86.1	3076 ± 70.1	3012 ± 111	3064 ± 52.5	2999 ± 0.06
PO ₄ ³⁻ (mg/l)	25.0 ± 7.07	100 ± 2.83	104 ± 0.00	88.7 ± 1.15	74.0 ± 2.00	84.0 ± 4.00	113 ± 0.00

Parameter	Day			
	63	70	77	84
pH	7.81	7.79	7.56	7.70
Salinity (g/l)	22.6	22.4	22.2	22.2
EC (mS/cm)	36.0	35.6	35.2	35.3
TS (g/l)	61.3 ± 0.15	58.5 ± 0.58	63.6 ± 0.64	61.6 ± 1.45
VS (g/l)	31.4 ± 0.13	29.9 ± 0.27	32.2 ± 0.38	61.6 ± 0.69
TSS (g/l)	40.0 ± 1.59	34.8 ± 0.84	41.8 ± 1.46	30.5 ± 2.21
VSS (g/l)	29.1 ± 1.06	25.3 ± 0.70	28.6 ± 1.23	39.9 ± 0.76
tCOD (g/l)	50.8 ± 1.71	45.0 ± 3.36	44.5 ± 1.30	28.2 ± 1.22
sCOD (g/l)	8.27 ± 0.16	8.16 ± 0.14	7.11 ± 0.15	46.2 ± 0.02
TN (g/l)	4.37 ± 0.15	4.27 ± 0.21	4.17 ± 0.15	6.17 ± 0.15
TP (g/l)	0.98 ± 0.17	1.12 ± 0.12	0.93 ± 0.01	4.73 ± 0.04
NH ₄ ⁺ (mg/l)	3780 ± 72.2	3599 ± 32.5	3775 ± 50.6	3609 ± 39.7
PO ₄ ³⁻ (mg/l)	76.0 ± 2.83	72.8 ± 0.28	69.5 ± 2.53	64.3 ± 0.14

Annex E. Results of the SMA tests - Cumulative methane production used to calculate SMA.

Figures 54, 55, 56 and 57 present the cumulative methane production from the SMA assays as well the period in which the slope to calculate SMA was gotten.

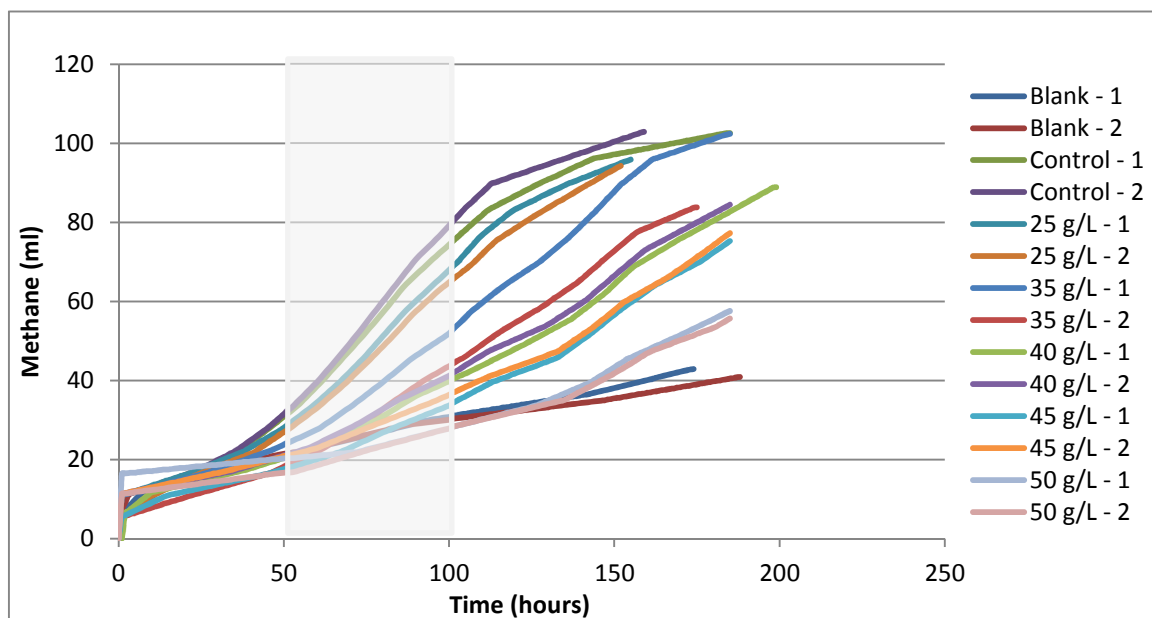


Figure 54. Cumulative methane production from the effect of salinity on SMA. The shaded area corresponds in the period where the slope was gotten to calculate the SMA.

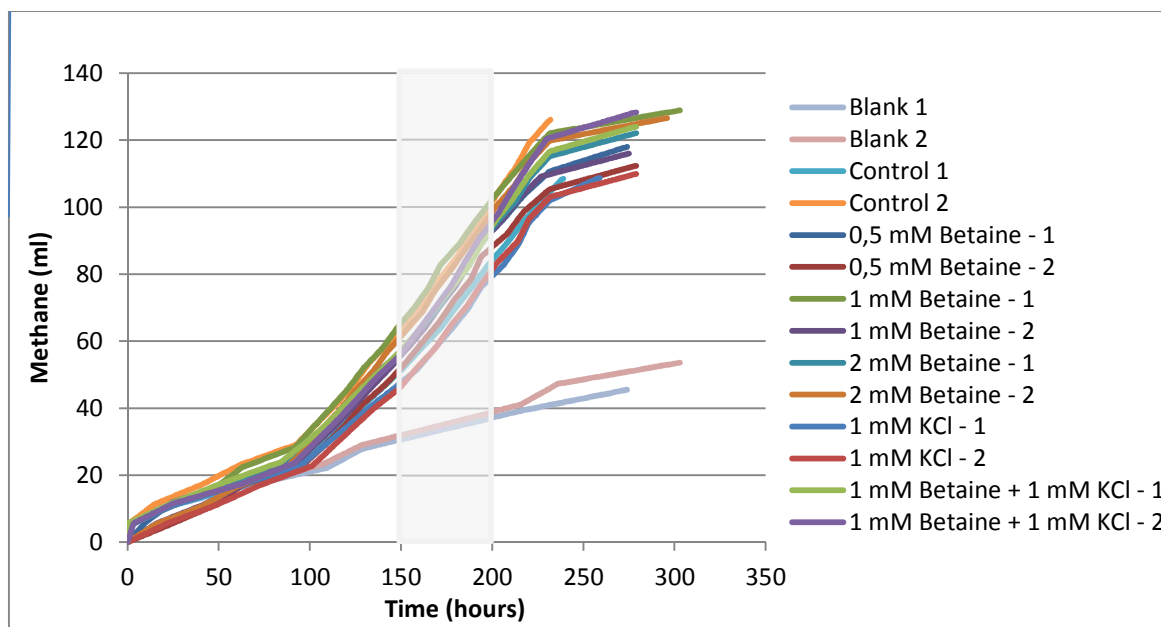


Figure 55. Cumulative methane production from the effect of betaine and/or potassium on SMA. The shaded area corresponds in the period where the slope was gotten to calculate the SMA.

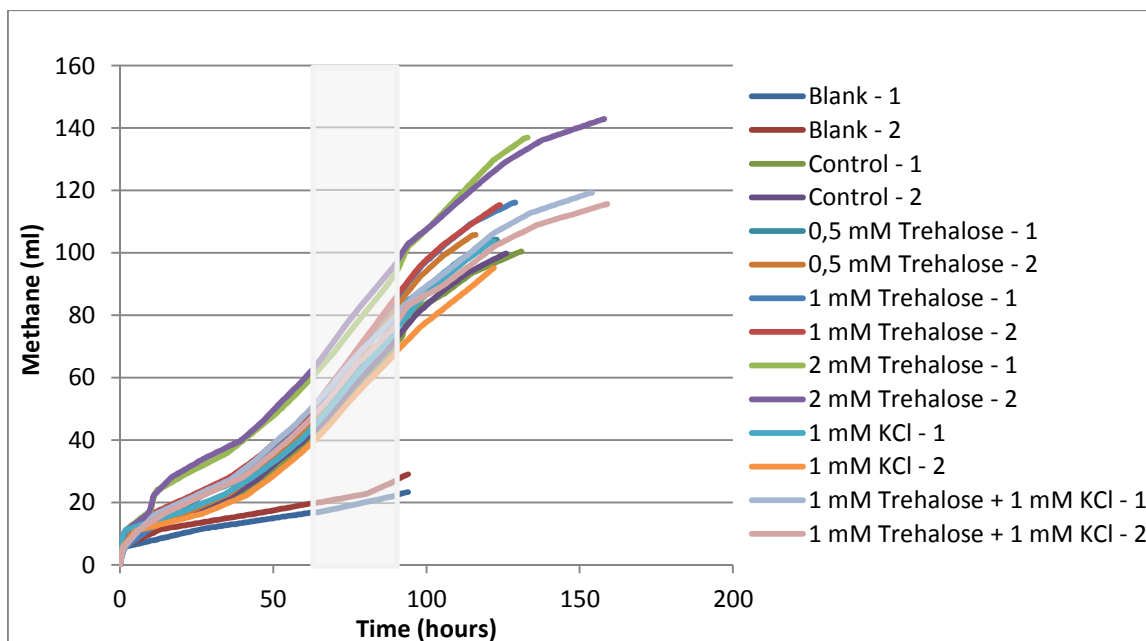


Figure 56. Cumulative methane production from the effect of trehalose and/or potassium on SMA. The shaded area corresponds in the period where the slope was gotten to calculate the SMA.

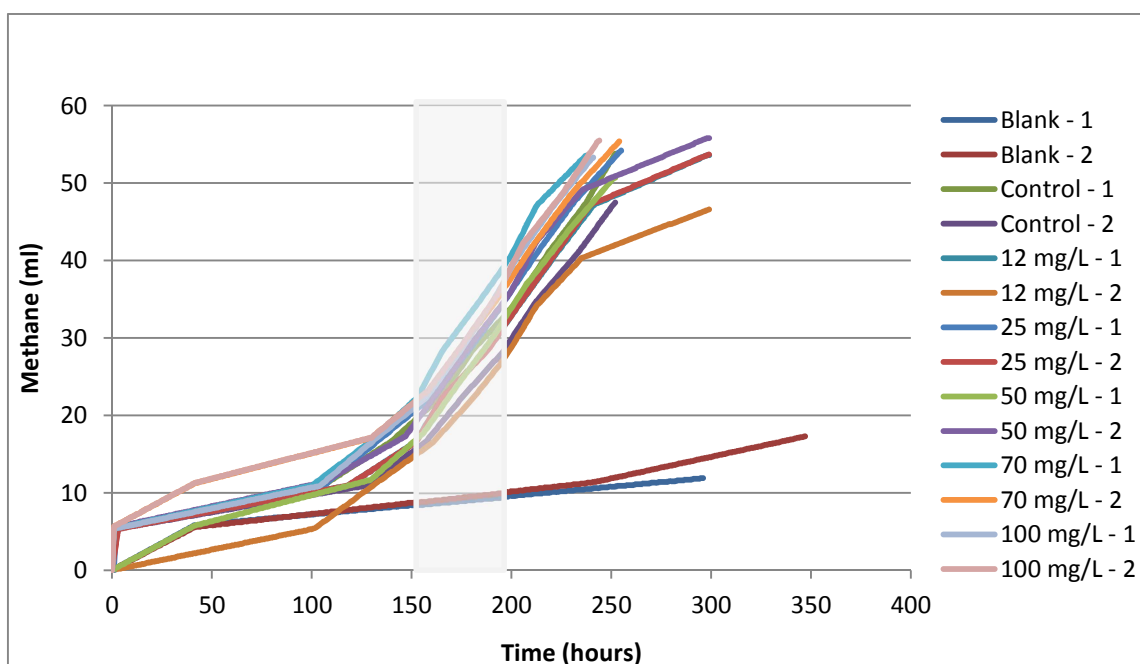


Figure 57. Cumulative methane production from the effect of FeCl_3 on SMA. The shaded area corresponds in the period where the slope was gotten to calculate the SMA.