ANUCHA CHUKWUKA BETHEL

THE DEVELOPMENT AND OPTIMIZATION OF CHEMICAL AND BIOLOGICAL PROCESSES FOR THE TREATMENT OF SULPHATE RICH AND HEAVY METAL BEARING WASTEWATER FROM ACID MINE DRAINAGE (AMD) BY SULPHATE REDUCING BACTERIA (SRB)

UNIVERSIDADE DO ALGARVE
Faculdade de Ciências e Tecnologias

2016

UNIVERSITY OF ALGARVE

THE DEVELOPMENT AND OPTIMIZATION OF CHEMICAL AND BIOLOGICAL PROCESSES FOR THE TREATMENT OF SULPHATE RICH AND HEAVY METAL BEARING WASTEWATER FROM ACID MINE DRAINAGE (AMD) BY SULPHATE REDUCING BACTERIA (SRB)

ANUCHA CHUKWUKA BETHEL

Dissertação / Dissertation

Mestrado Erasmus Mundus em Inovação Química e Regulamentação

(Erasmus Mundus Master in Chemical Innovation and Regulation)

Trabalho efetuado sob a orientação de:

Work supervised by:

Dr. Maria Clara Costa

Co – orientação:

Co- supervised by:

Dr. Jorge Dias Carlier

2016

THE DEVELOPMENT AND OPTIMIZATION OF CHEMICAL AND BIOLOGICAL PROCESSES FOR THE TREATMENT OF SULPHATE RICH AND HEAVY METAL BEARING WASTEWATER FROM ACID MINE DRAINAGE (AMD) BY SULPHATE REDUCING BACTERIA (SRB)

Declaration of authorship:

I declare that I am the author of this work, which is original and unpublished.

The work cites other authors and work, which are adequately referred in the text and are listed in the bibliography

(Anucha Chukwuka Bethel)

Acknowledgments

With a joyful heart, am grateful to The Almighty God, who made this work possible**.**

A group of people have contributed immensely to the success of this work and their impact cannot go unnoticed. My special gratitude goes to my supervisor, Dr. Maria Clara Costa for all her wonderful support during this work. I will like to thank also in a special way Dr. Jorge Dias Carlier, my co- supervisor who has been very instrumental in making sure things worked out. Big thanks to Dr. Ana Luis and Ana Benedito (PhD student), Luis Miguel Babau Alexandre (MSc.) for their patience and accommodating personalities helping me with many questions I asked.

Profound gratitude to my heartthrob Carla (Mrs.), your support has been extraordinary throughout the period of this work; you gave everything to make it happen; a big thank you my love.

To my fellow students, you all have been part of this success, your outstanding support will ever be remembered.

For the prayers they said, words of encouragement rendered and their unalloyed support, a very big thank you to my parents and siblings.

Resumo

O tratamento biológico de água de drenagem ácida de mina (“Acid Mine Drainage” - AMD) contaminada com sulfato e metais pesados utilizando bactérias sulfato-redutoras (“Sulphate Reducing Bacteria” – SRB) continua a ser foco de grande atenção. O posicionamento desta estratégia como alternativa viável deve-se às suas vantagens do ponto de vista económico e de sustentabilidade ambiental, comparativamente aos métodos químicos clássicos. Eficiente remoção de sulfato, baixa produção de lamas e maior estabilidade dos precipitados de sulfuretos metálicos obtidos em comparação com precipitados de hidróxidos metálicos nos métodos clássicos numa faixa de pH mais ampla tornam o método de tratamento biológico competitivo. Esta abordagem utiliza sulfureto de hidrogénio, gerado biologicamente a parir do sulfato da AMD pelas SRB no processo de aceitação de eletrões durante a oxidação de compostos de carbono, o qual se liga aos metais pesados da AMD precipitando como sulfuretos metálicos.

A falta de fontes de carbono/dadores de eletrões na AMD torna necessária adição de compostos orgânicos para uma eficiente redução de sulfato a sulfureto no processo de biorremediação desta água. Esta importância da adição de fontes de carbono/dadores de eletrões nos processos de biorremediação com SRB tem recebido a atenção de muitos investigadores, tendo já sido testados para esse fim vários compostos orgânicos. A maior parte dos produtos investigados foram subprodutos de alimentos agrícolas processados, bem como resíduos orgânicos. No entanto, fontes de carbono bem conhecidas, a maioria das quais compostos orgânicos puros como lactato, etanol, metanol e ácidos gordos voláteis, continuam a ser usados porque produzem melhores resultados em termos de eficiência de redução de sulfato. A limitação de utilizar estas fontes de carbono sintéticas é o seu custo. Na bancada de laboratório são viáveis, mas a sua utilização à escala industrial continua a enfrentar desafios de custo, tornando-se quase impossível de implementar. Além de considerações de custo, há outros fatores críticos na escolha adequada de fonte de fontes de carbono/dadores de eletrões para SRB como disponibilidade, degradabilidade, eficiência em termos de redução de sulfato com pouco ou nenhum poluente associado no efluente de descarga, bem como considerações cinéticas e termodinâmicas para favorecer as SRB na inerente competição com outras bactérias, principalmente as metalogénicas.

Tendo em conta todos estes fatores, neste trabalho foram testados como fontes de carbono/dadores de eletrões alternativas para as SRB dois sub-produtos industriais conhecidos por serem ricos em açúcares: um melaço de laranja produzido por evaporação de um licor extraído de cascas de laranja pela uma indústria de processamento de sumo laranja e um melaço

de beterraba produzido pela indústria de processamento de açúcar a partir de beterraba. Estes dois melaços foram obtidos, respetivamente, numa fábrica de sumo de laranja no sul de Portugal e numa fábrica de açúcar no sul de Espanha.

Tendo em consideração a importância do rácio CQO/[SO_4^{2-}] no uso de fontes de carbono em processos de biorremediação com SRB e tendo em conta o elevado conteúdo de CQO destes melaços, tinham sido previamente testadas no Laboratório de Tecnologias Ambientais (LTA) do Centro de Ciências do Mar (CCMAR) da Faculdade de Ciências e Tecnologia da Universidade do Algarve, várias diluições dos mesmos em meio Postgate B sem lactato em reatores em descontínuo tentando-se obter rácios próximos de 1.5, o valor teórico estimado de CQO/[SO_4^{2-}] do meio Postgate B original (com lactato). As diluições 1:200, entre várias outras testadas, deram melhores resultados na redução de sulfato com ambos os melaços testados.

Com base nestes estudos anteriores estabeleceu-se que neste trabalho experimental para avaliar a viabilidade do uso melaço de laranja e melaço de beterraba como fontes de carbono/dadores de eletrões no tratamento de AMD fossem utilizadas diluições 1:200.

A utilização de diluições de 1:200 de ambos os melaços em AMD com SRB em reatores em descontínuo, resultou numa eficiente redução de sulfato e simultânea remoção de metais pesados em apenas cerca de 14 dias, chegando-se a concentrações em conformidade com as normas legislativas nacionais para as águas de rega em Portugal (Decreto-Lei nº: 236/98, anexo XVI, 1998).

Com estes resultados, a experiencias estenderam-se para sistemas em contínuo com 1 mL/h de fluxo usando dois biorreatores de fluxo ascendente anaeróbios de leito fixo (“upflow anaerobic packed bed” - UAPB), um com melaço de laranja e outro com melaço de beterraba, ambos começando com a mesma diluição (1: 200) de melaço em AMD. Depois de otimizar as condições, aumentando o fornecimento de melaço para uma diluição de 1: 133 em AMD, conseguiu-se uma redução de sulfato eficiente e remoção de metais pesados para concentrações abaixo do limite regulamentado para águas de rega em Portugal (Decreto-Lei n.º: 236/98, anexo XVI, 1998), com um tempo de retenção aproximado de cerca de 13 dias em ambos os biorreatores.

Devido à complexidade dos melaços utilizados, analisou-se durante os ensaios de biorremediação a presença de açúcares e ácidos orgânicos. As SRB tiraram proveito em sintrofia de outros membros do consórcio de bactérias que degradaram os compostos de carbono dos melaços em formas mais simples viáveis para a redução biológica de sulfato. Nos

reatores em descontínuo e em contínuo alguns açúcares e ácidos orgânicos apareceram, estabilizaram e desapareceram dos meios testados revelando tendências que sugerem estar associados ao crescimento e atividade de SRB.

Substratos orgânicos complexos, como o melaço, não são completamente degradados em processos com SRB, como tal geram efluentes com níveis relativamente altos de CQO. Os valores de CQO dos efluentes dos biorreatores de SRB alimentados com melaço de laranja e de beterraba foram tão elevadas como 2284mgO_2/L e 3948mgO_2/L, respetivamente. Embora não haja limite de CQO definido para águas de irrigação em Portugal, o nível CQO destes efluentes é elevado em comparação com o limite de 150mgO_2/L de CQO para descargas de águas residuais. No futuro, já fora do âmbito do presente trabalho, a integração duma coluna de aerificação poderá aperfeiçoar ainda mais os efluentes dos sistemas com SRB testados neste trabalho, reduzindo a CQO para valores toleráveis.

Em suma, apesar de tudo, os melaços de laranja e beterraba suportaram o processo de biorremediação de AMD baseado em SRB, tal como indicado pela eficiente redução de sulfato e remoção de metais pesados, com um tempo de retenção aproximado de cerca de 13 dias tanto em reatores em descontínuo como de fluxo contínuo.

Palavras-chave: drenagem ácida de mina (AMD), bactérias sulfato redutoras (SRB), biorremediação, metais pesados, fontes de carbono, biorreatores anaeróbios de fluxo ascendente em leito fixo (UAPB)

Abstract

The biological method of treating high acidic sulphate and heavy metal contaminated acid mine drainage (AMD) effluents from mining industries using sulphate reducing bacteria (SRB) continue to receive wide attention. This is due to its huge advantages ranging from economical point of view to environmental sustainability, positioning it as an alternative bioremediation strategy over the classical chemical techniques. Efficient sulphate reduction, low sludge production and stability of metal sulphide precipitates in comparison to their hydroxide counter parts over wide range of pH continue to make the biological treatment method competitive. This approach uses biologically generated hydrogen sulphide from the AMD sulphates, reduced by SRB in their process of accepting electrons during the oxidation of organic matter, which precipitates the AMD heavy metals as sulphides.

Deficiency of AMD in carbon source/electron donor makes the biological sulphate reduction an electron demanding process. In order to compensate the electron insufficiency of this process, an external organic matter as a carbon source/electron donor needs to be added to achieve efficient sulphate reduction. The importance of carbon sources/electron donors for SRB mediated biological sulphate reduction is very high and for this have received attention from many researchers and several sources have been investigated. Most carbon sources investigated include many agriculturally processed food bi-products as well as organic wastes. However, well known carbon sources, most of which are pure organic compounds like lactate, ethanol, methanol and many other volatile fatty acids (VFA), continue to be used as they deliver better results in terms of sulphate reduction efficiency. The limitation of using these well-known synthetic carbon sources is cost. On the bench, results have been good but scaling up industrially continues to face cost challenges as it becomes almost impossible to implement. Apart from cost considerations, critical in making choice for SRB suitable carbon source/electron donor are factors as availability, degradability, efficiency in terms of sulphate reduction or removal with little or no associated pollutant in discharge effluent as well as kinetic and thermodynamic considerations to envisage any inherent SRB competition with methanogens.

Taking into account all these factors, in this work we tested two different types of molasses, an orange molasses produced by an orange juice processing industry through the evaporation of a liquour extracted from orange peels and a beetroot molasses produced by a sugar processing industry, as alternate carbon sources/electron donors for the sulphate reducing bacteria (SRB). This two different molasses were obtained from beetroot and orange juice factories from

Southern Spain and Southern Portugal respectively as industrial sub-products known to be rich in sugars.

Taking into consideration, the importance of COD/SO_4^{2-} ratio in the use of organic matter carbon sources for wastewater treatment by SRB and bearing in mind the high COD content of these molasses confirmed in a previous initial characterization at the Laboratory of Environmental Technologies (LET) of the Centre of Marine Sciences (CCMAR) at the Faculty of Science and Technology in the University of Algarve, several dilutions had been tested in batch, each of the molasses diluted in postgate B medium without lactate, trying to obtain COD/SO_4^{2-} ratios surrounding 1.5 in Postgate B medium. The tested batch dilution of 1:200, amongst several other tested dilutions, gave the best efficient sulphate reduction for both molasses.

Based on these previous studies it was established that for this work, experiment to evaluate the feasibility of using the orange and beetroot molasses as carbon sources/electron donors in the treatment of AMD would be performed at 1:200 dilutions.

The application of the 1:200 dilutions of both molasses in AMD with SRB in batch tests produced efficient sulphate reduction and simultaneous heavy metals removal in just about 14 days with results in compliance with the national legislative standards for sulphate and heavy metal contaminants for irrigation waters in Portugal (Decree Law no: 236/98, Annex XVI, 1998).

With these achieved batch results, we extended our experiments to continuous systems with 1mL/hr flow rate using two upflow anaerobic packed bed (UAPB) bioreactors, one with orange molasses and another with beetroot molasses, both starting with the same dilution (1:200) of molasses in AMD. After optimizing the conditions by increasing the molasses supply for a dilution of 1:133 in AMD, we achieved efficient sulphate reduction and heavy metal removal below standard limit of regulation for irrigation waters in Portugal (Decree Law no: 236/98, Annex XVI, 1998) with an approximate retention time of about 13 days in both bioreactors.

Due to the complexity of the used molasses, we analized during the bioremediation assays for the presence of sugars and organic acids. The sulphate reducers took advantage of other members of the bacteria consortia who made the molasses carbon substrates available in simpler forms for the SRB syntrophically. Analyzed batch and continuous system effluent samples showed fashions where the sugars and organic acid appeared in tested media, stabilized and disappeared subsequently suggesting an association with the SRB growth and activity.

Complex organic substrates like molasses are not completely utilized by SRB processes and as such generate relatively high COD levels in effluents. The COD in the SRB bioreactor effluents were as high as 2284mgO_2/L and 3948mgO_2/L for bioreactors fed with orange and beetroot molasses respectively. Although there is no COD limit set for irrigation waters in Portugal, the COD level of this effluents are high compared to COD limit for wastewater discharge at 150mgO_2/L. Though outside the scope of this work, in future, integrating an aeration column will further meliorate the effluents of the tested systems, reducing the COD content to bearable values.

In summary, after all, the investigated orange and beetroot molasses supported the AMD SRB based bioremediation process, as indicated by the efficient sulphate reduction and heavy metal removal with an approximate retention time of about 13 days in both the batch and the continuous flow systems.

Key-words: acid mine drainage (AMD), sulphate reducing bacteria (SRB), bioremediation, heavy metals, carbon sources, upflow anaerobic packed bed (UAPB) bioreactors

INDEXES

ABBREVIATION INDEX

AMD	Acid mine drainage
ARD	Acid rock drainage
COD	Chemical oxygen demand
DAPB	Down flow anaerobic packed bed
DO	Dissolved oxygen
EAS	Easily available substance
EC	Electrical conductivity
Eh	Redox potential
ELSD	Evaporative light scattering detector
FAAS	Flame atomic absorption spectrometer
HPLC	High performance liquid chromatography
HRT	Hydration retention time
K_{sp}	Solubility product constant
LOD	Limit of detection
MA	Methanogen Archaea
MAV	Maximum admissible value
MBR	Membrane bioreactor
MP-AES	Microwave plasma atomic emission spectrometer
MRV	Maximum recommended value
PRB	Permeable reactive barrier
SAPS	Sequencing alkalinity producing systems
SRB	Sulphate reducing bacteria
UAPB	Upflow anaerobic packed bed
UASB	Upflow anaerobic sludge blanket
UV	Ultra-violet
VFA	Volatile fatty acid

FIGURE INDEX

TABLE INDEX

1. Objectives

This work focused on the treatment of acid mine drainage (AMD) wastewater from Mina de São Domingo with a system integrating an SRB based process, decontaminating it of high sulphate and heavy metal contents, aiming to acieve concentrations within compliance limits for irrigation waters in Portugal (Decree law no: 236/98, Annex XVI, 1998).

The SRB activity is highly dependent on the carbon compound/electron donor used as source of energy. Sugar cane molasses is an efficient source of carbon compound for SRB in bioprocess removing metals from water (Geets et al.,2006). Taking this into account, in this work two other type of molasses were tested and to our knowledge, for the first time: Orange molasses and Beetroot molasses.

2. Introduction

2.1- What is acid mine drainage (AMD)

Water pollution is a threat and big concern to everyone. Understanding sources of water pollution, interactions and effects is quiet important in helping control pollutants in an environmentally safe and economically acceptable way. Majority of pollution encountered in the environment is due to human activities. One such activity that has left the water environment heavily polluted with wide impact is mining and other related economic activities like construction, civil engineering, quarrying etc. These operations have resulted in the production of acid mine drainage (AMD) or what is also referred to as acid rock drainage (ARD). Acid mine drainage (AMD) are sulphate and heavy metal bearing wastewaters from mining activities and processing of metal ores and coal. The metal composition of this mine water can include mainly iron, copper, zinc, aluminum, manganese and some metalloids like arsenic as well as radionuclides like Uranium as well traces of chromium (Johnson and Hallberg, 2005). These acidic Sulphate and metal –rich wastewaters are also bi-products of different kinds of industrial operations like galvanic processing and scrubbing of flue gases at power plants (Johnson, 2000). They are consequences of accelerated oxidation of iron pyrite and other sulphur-containing minerals upon exposure to both oxygen and water (Rios et al., 2008). The search for an efficient, economically viable method for the treatment of waste from mine sites whether active, exhausted or abandoned has been receiving attention from researchers over the last decade and whether these methods are sustainable or not sustainable remains vague and continue to gather attention from school of thought.

2.2- The chemistry of AMD generation

AMD forms through the oxidation of metal sulphides, mainly pyrites and marcasite following their oxidation by air and water during mining operations as well as mine waste dumps of inactive mine sites. The steps involved in the generation of AMD has been reported by most researchers as: Iron sulphide oxidation (1), ferrous iron oxidation (2), ferric iron hydrolysis (3) and enhanced oxidation of ferric sulphide (4) (Evangelou, 1995; Johnson & Hallberg,2005; Akcil & Koldas, 2006; Kalin et al; 2006). These are stepwise oxidation processes leading to the generation of acid mine drainage (AMD):

$$FeS_2 + 7/2O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \qquad (1)$$

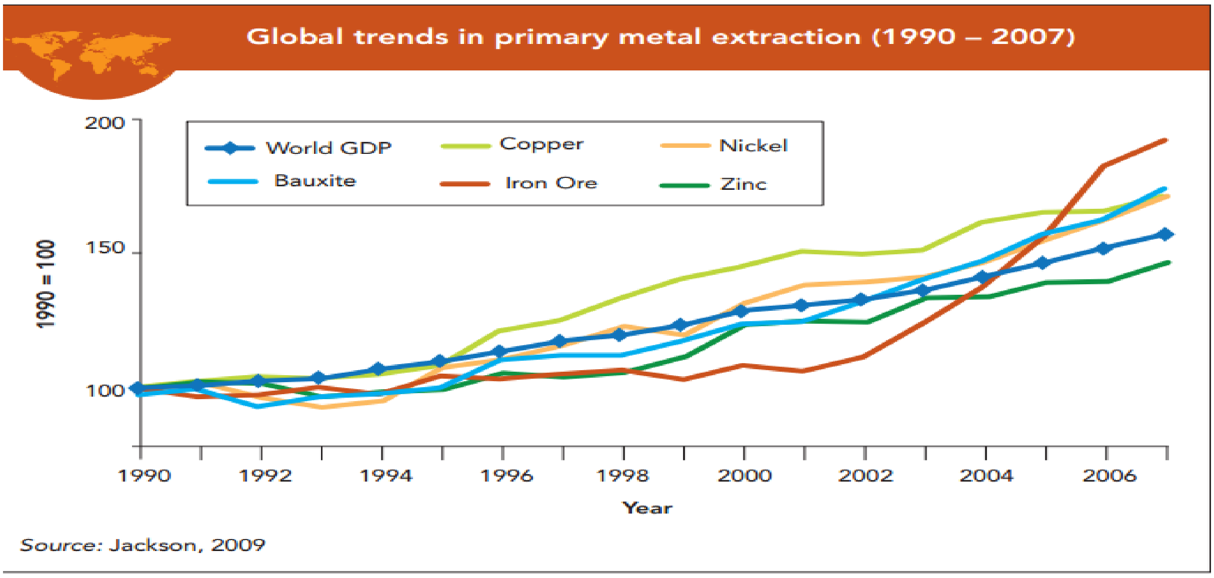
$$Fe^{2+} + 1/4O_2 + H^+ \rightarrow Fe^{3+} + 1/2H_2O \qquad (2)$$

$$Fe^{3+} + 3H2O \rightarrow Fe(OH)_3\,(s) + 3H^+ \qquad (3)$$

$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$ (4)

2.3- The nature and gravity of the problem

The demand and exploitation of ores and metals (like iron, copper, zinc, etc.) for economic development in the history of humankind dates back to pre-historic times. No doubt, that huge economic values derived from these precious elements have so far benefited human existence and development; however, it comes with a price (Costa & Duarte, 2005). Past and present mining activities are the major sources of metal contamination in soil and water environments (Johnson and Hallberg, 2005). Mining industries have produced acidic wastewaters with immense concentrations of sulphates, heavy metals, metalloids and traces of radionuclides (Johnson and Hallberg, 2005). These waters known as acid mine drainage (AMD), are one of the environmental problems faced by mining industries and their host communities (Chockalingam and Subramanian, 2009). In 1989, an estimated 19,300 Km of streams and rivers; 72,000 ha of lakes and reservoirs were reported to be heavily impacted by mine effluents globally (Johnson and Hallberg, 2005). Extending this data in comparative study and analysis since the last two decades, more environmental waters and biodiversity stand already contaminated by mining operations and activities we witnessed to this day.



Source: Jackson, 2009

Fig 2.1 –Global trends in metal extraction (1990-2007): extracted from Trends in Sustainable Development- Chemicals, mining, transport and waste management, United Nations,

New York, 2010.

Mining and mining support services can contribute significantlty to a country's value added. However, global trends in primary metal extraction (Fig 2.1) have shown little evidence of decoupling resource use from economic growth even outside environmental impacts.

Mineral extraction has a strong place in the civilization and economic history of humanity. The international Council on Mining and Metals (ICMM) in their InBrief- Trends in the mining and metals industry, October 2012, revealed that before the 19th century, Europe saw a dramatic increase in mining activities which later declined as economic and political power shifted to North America. One can say that, mining has come to stay and will continue to play a pivotal role in everyday life and economic development even as the trend shifts from developed to developing countries including China, Russia, Australia and Canada since mid-20th century.

In Portugal, mining activities has a long history. Until 2010, circa 56 mining sites were operational leading to the production of about 5 million tons of minerals meaning an economic value of about 800 million euros per annum (Vitor et al., 2012).

This however, did not come without a price as mining activities and operations is a major risk to the environment due to huge contamination of underground waters owed to infiltration of sulphate-rich heavy mineral metal extracts (Coelho & Teixeira, 2011).

With 175 abandoned mine sites (Table 2.1) in Portugal in 2009, 10 were polymetallic mine sites, with Mina de São Domingos mine (Mertola, Beja) part of this list.

Table 2.1- Total Portuguese abandoned mines since 2009 and their respective exploited minerals (Adapted from Mineral Development Company, Portugal, 2009)

Mineral	**Number of mines**	**Principal group**
Tin & Tungsten	40	Metallic minerals
Metallic Bases (e.g: Copper, Zinc, Nickel, Lead)	28	Metallic minerals
Gold	12	
Iron & Manganese	16	
Polymetallic Sulphides e.g: FeS, ZnS, PbS)	10	Polymetallic sulphides
Radioactive (e.g Ra & U)	61	Radioactive
Charcoal	3	-
Asbestos	1	-
Others	4	-
Total	175	

2.3.1- Mina de São Domingo's mine

The mine in Mina de São Domingo is located some 17 Kilometers from the village of Mertola, in the Alentejo region of Southern Portugal lying in the heart of the Iberian Pyrite Belt- a vast geographical area with unqiue geological features and well renowed for its mining activities, about 250Km long and 30-50Km wide stretching much along the South of the Iberian Peninsula, from Portugal to Spain.

The mine has worked since the Chalcolithic Age (Copper Age) over 4000 years ago and unfortunately abandoned in 1966 owing to the depletion of it's Copper deposits (Institute of Geology and Mining, 2000). The second excavation period was carried out by the Romans who intensified copper production on a large scale. Their excavation activities lasted for 385 years, from 12 to 397 A.D. and extensive investigations made based on the amount of slag that was found, revealed that they went as deep as 40 meters and obtained circa 750,000 tons of pyrite and copper over that period (Institute of Geology and Mining, 2000).

In the 19th century, the third and last period of intensive mineral extraction took place and the mining company Sabina haven obtained a mining claim of 800km^2 removed over 25 million tons of ore from the ground in the São Domingos region (Institute of Geology and Mining, 2000).

Owed to these excavation activities and with existing underground and open-abandoned mines creating complex gallery systems, ruined buildings and infrastructures, open slag dumps and artificial lagoons to settle slag runoffs from mine; an environmental problem has been created not only for the landscape and vegetation but also the ecosystem of the entire region.

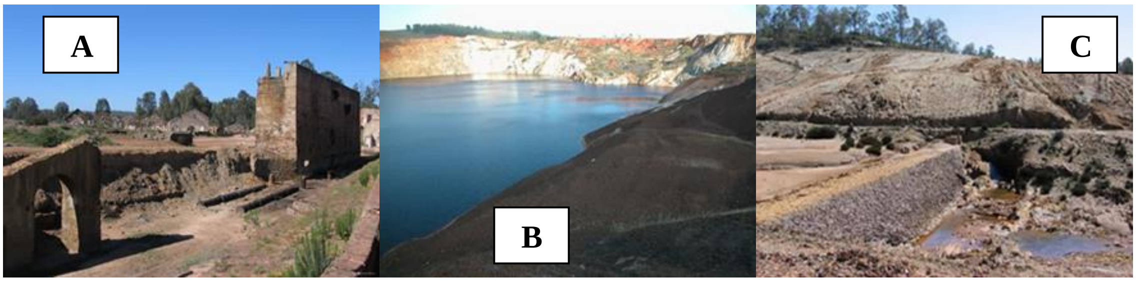


Fig 2.2- **A**: an abandoned building infrastructure in ruin since 1966, **B**- The open pit filled with AMD water and **C**- AMD water runoff

The water from the open mine pits (Figure 2.2 B) has degenerated into acid mine drainage (AMD) with high acidity and high concentration of heavy metals and sulphate. Not only noxious for the earth, the combination of these open pit AMD waters with water held by the dam near Chanca River used for consumption and irrigational purposes and underground water infiltrations makes it pose an alarming threat to animals and the people (Alvarez-Valero et al., 2008).

2.4– Sulphate reducing bacteria (SRB) and their role in metal removal

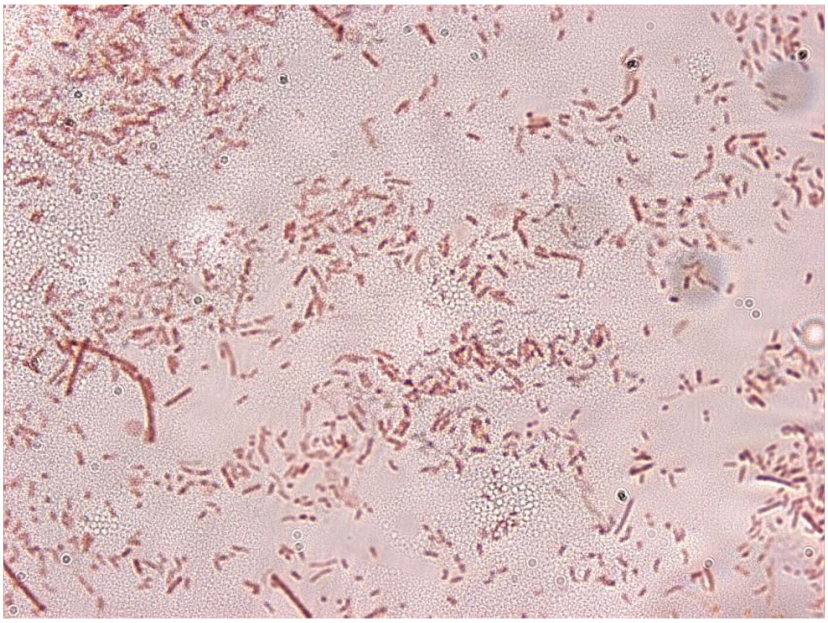


Fig 2.3 – Photomicrograph of Sulphate Reducing Bacteria (SRB). Amplification: 1000X (taken from M.S.F; Martins Neves, 2010).

Sulphate reducing bacteria (SRB) are generic heterotroph obligate anaerobes that uses sulphate as electron acceptors in the metabolism of organic matter generating sulphides, which in turn combines with metals to form their insoluble precipitate.

$$2H^{+} + SO_4^{2-} + 2C_{(Organic)} \rightarrow H_2S + 2CO_2 \quad (5)$$

$$M^{2+} + H_2S \rightarrow MS_{(s)} + 2H^{+} \quad (6)$$

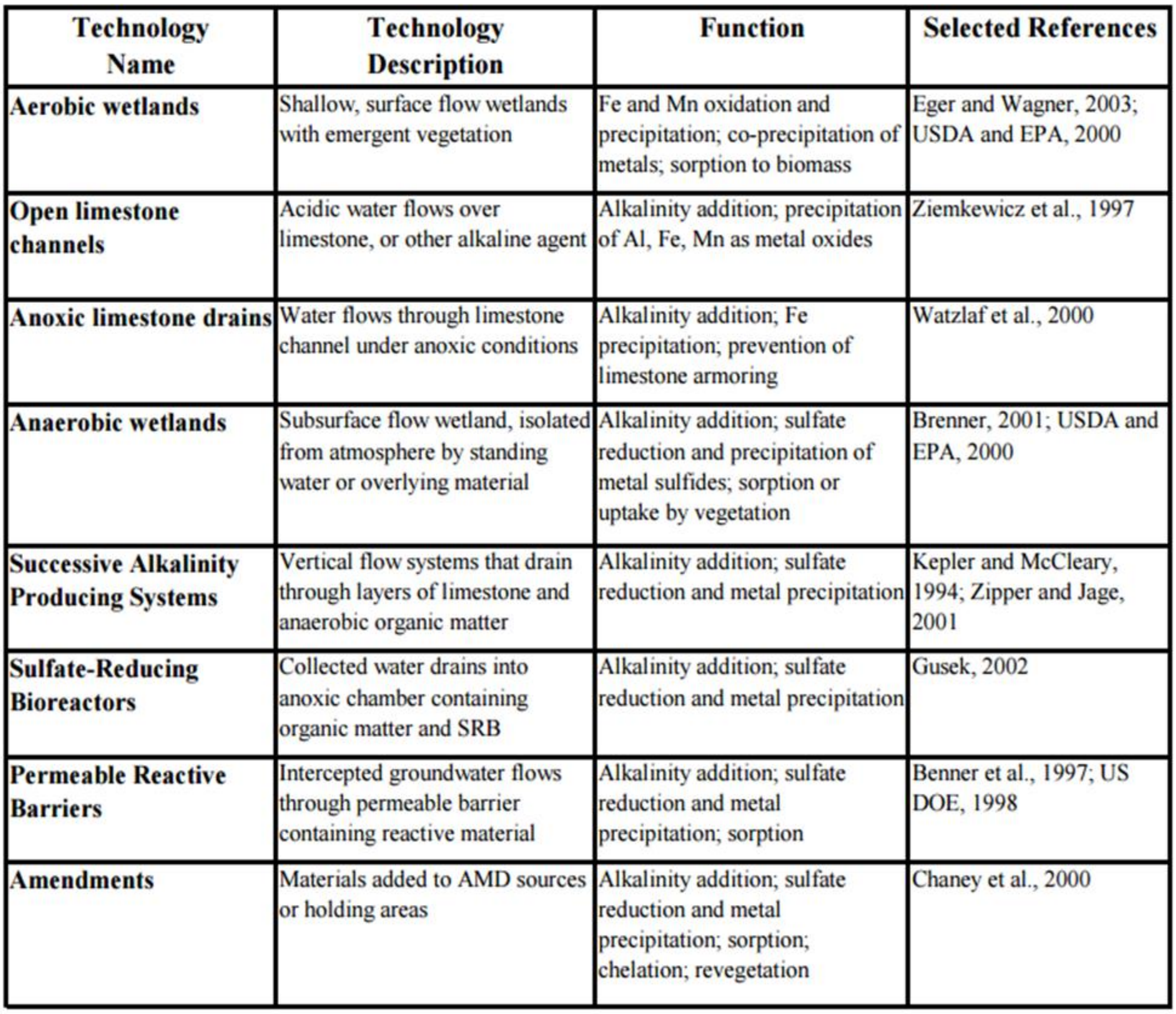
Various parameters such as pH, temperature, sulphide and metal concentrations in the AMD undergoing active biological treatment have been investigated with reports showing that they affect the growth and activity of SRB. Hard et al., (1997), reported that sulphate reducing bacteria (SRB) are sensitive to acidic waters. Garcia et al., (2001), reported that negative redox potential provides suitable environment to grow these bacteria appropriately. Postgate, (1984), said that generally, SRB can tolerate temperatures from -5°C to 75°C. Their ability to establish microenvironment in the presence of a solid support (sand or gravel) for survival in harsh environments of low pH or high oxygen concentrations has been reported by Lyew and Sheppard, (1997). Though with information on sulphide toxicity and its mechanism vague, it has been reported that sulphide when absorbed into their cells, destroys the proteins thereby making the cell inactive (Postgate, 1984). Due to the sensitivity of SRB to a lot of these factors which tend to limit their efficiency during biological treatment processes, care must be taken to ensure that, selected SRB community: are tolerant to metal concentrations, have sufficient sulphate concentration for SRB growth and activity, have an established pH media range of 5.5 to 8.5, are able to be supported for growth by the SRB electron donor system, are supplied with SRB metabolic activity essential ingredients like P, N, etc, and are functioning in a media of COD/SO_4^{2-} ratio of about 0.7-1.7.

Once sulphate reducing conditions are established, sulphide precipitation (equations 5 and 6) by SRB (Fig 2.3), becomes the predominant mechanism for metals like (Fe, Cu, Zn, Al, Mn etc) to be removed from AMD (Machemer et al., 1993; Bechard et al., 1994; Song, 2003).

2.4.1- Sulphate reducing bacteria (SRB) active and passive bioremediation process systems

There are off-line sulphidogenic bioreactor remediation systems used in the decontamination of AMD waters (Johnson, 2000). These are active biotechnologically-engineered and controlled systems that can have different configuration designs, ranging from batch reactors, sequencing reactors, up-flow anaerobic packed bed (UAPB), down-flow anaerobic packed bed (DAPB), membrane bioreactors (MBR) to fluidized bioreactors. The off-line biosulphide active system has two components- one biological and one chemical; independently operating from each other (Rowley et al., 1997). In set-up, raw AMD enters the chemical circuit where it is exposed to hydrogen sulphide generated in the biological circuit. Through careful manipulation of the sulphide concentration as well pH, selective separation of a particular metal sulphide is possible which may then be removed from the system prior to further treatment of the effluent. Some of the treated AMD enters once again the biological circuit providing sulphate source for the bacteria in the reactor. Additional alkali to bolster already achieved alkalinity in the reactor by SRB is feasible to ensure optimized conditions (Johnson & Hallberg, 2005). The off-line sulphidogenic active bioreactors relies on the generated hydrogen sulphide gas (H_2S) by the sulphate reducing bacteria (SRB) in their system to help offer the much needed alkalinity condition as well as form sulphide precipitates with the metals present in the AMD. Thus, the off-line sulphidogenic bioreactors are usually constructed and operated in a way as to optimize the production of hydrogen sulphide generated from the reduction of the sulphate from the AMD and in turn combine this gas with the AMD to decontaminate this mine water of metals. Some of their advantages over the passive biological remediation option include: Predictable performance and readiness of control, ability of removing heavy metals such as copper and zinc present in AMD and reuse them, significant lowering of the sulphate content in treated water, complying with maximum acceptable value (MAV) concentration (Johnson & Hallberg, 2005). These processes similarly do occur in the passive sulphidogenic processes such as compost bioreactors and permeable reactive barriers (PRBs), iron oxidizing bioreactors, aerobic wetlands, etc (Johnson & Hallberg, 2005). The summarized description of the different passive biological treatment methods is outlined in Table 2.2 and Figure 2.4 below.

Table 2.2- Summary of passive treatment technologies (accessed from National Network for Environmental Management Studies (NNEMS), 2006)



Technology Name	Technology Description	Function	Selected References
Aerobic wetlands	Shallow, surface flow wetlands with emergent vegetation	Fe and Mn oxidation and precipitation; co-precipitation of metals; sorption to biomass	Eger and Wagner, 2003; USDA and EPA, 2000
Open limestone channels	Acidic water flows over limestone, or other alkaline agent	Alkalinity addition; precipitation of Al, Fe, Mn as metal oxides	Ziemkewicz et al., 1997
Anoxic limestone drains	Water flows through limestone channel under anoxic conditions	Alkalinity addition; Fe precipitation; prevention of limestone armoring	Watzlaf et al., 2000
Anaerobic wetlands	Subsurface flow wetland, isolated from atmosphere by standing water or overlying material	Alkalinity addition; sulfate reduction and precipitation of metal sulfides; sorption or uptake by vegetation	Brenner, 2001; USDA and EPA, 2000
Successive Alkalinity Producing Systems	Vertical flow systems that drain through layers of limestone and anaerobic organic matter	Alkalinity addition; sulfate reduction and metal precipitation	Kepler and McCleary, 1994; Zipper and Jage, 2001
Sulfate-Reducing Bioreactors	Collected water drains into anoxic chamber containing organic matter and SRB	Alkalinity addition; sulfate reduction and metal precipitation	Gusek, 2002
Permeable Reactive Barriers	Intercepted groundwater flows through permeable barrier containing reactive material	Alkalinity addition; sulfate reduction and metal precipitation; sorption	Benner et al., 1997; US DOE, 1998
Amendments	Materials added to AMD sources or holding areas	Alkalinity addition; sulfate reduction and metal precipitation; sorption; chelation; revegetation	Chaney et al., 2000

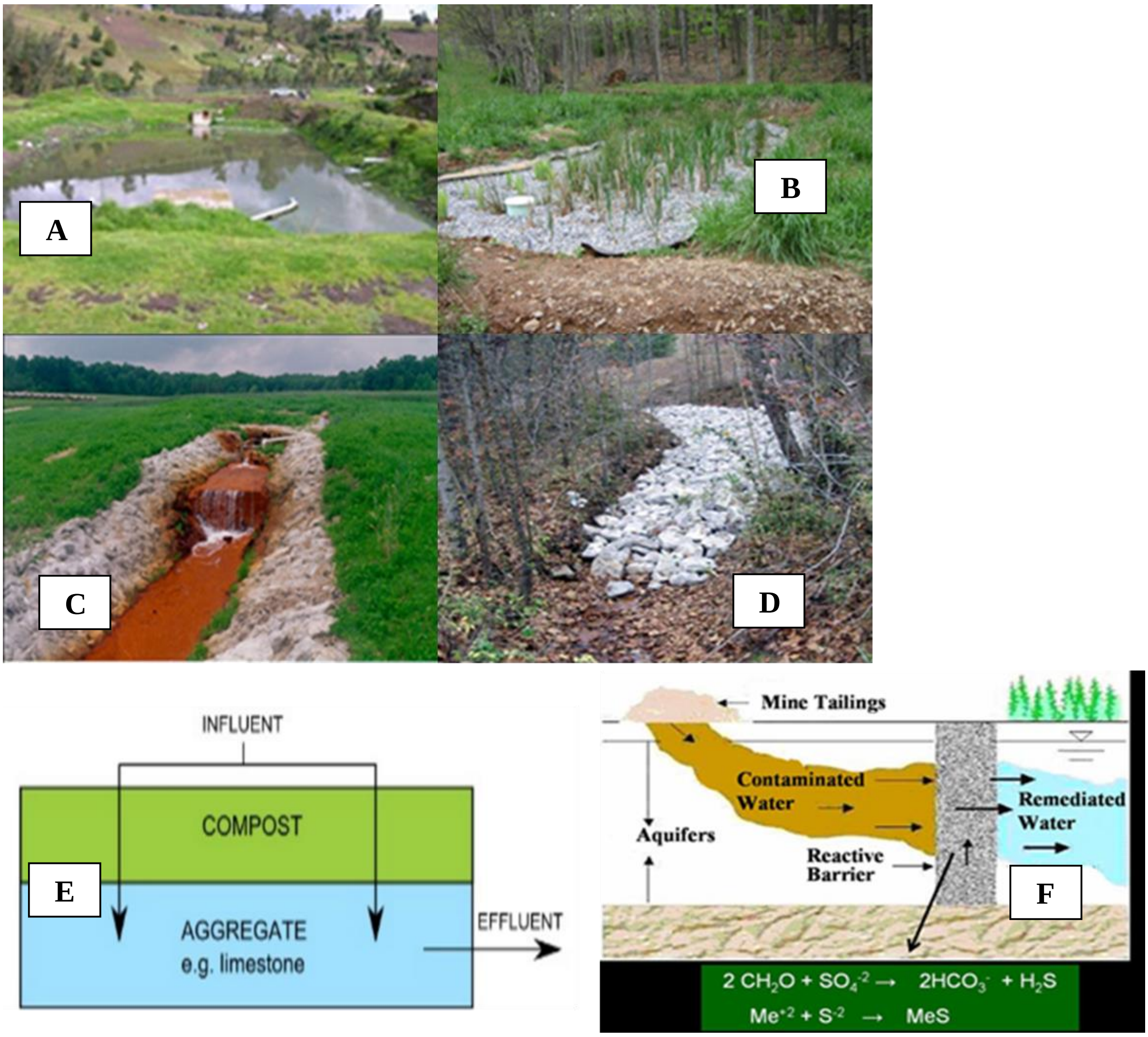


Fig 2.4- A: Aerobic wetland, **B:** Anaerobic wetland, **C:** Successive alkalinity producing systems, **D:** Open limestone channel, **E:** Compost bioreactor, **F:** Permeable reactive barrier

There are also biotechnologically-engineered and controlled systems in which the biological sulphate reduction as well as the metal precipitation occurs in a single unit. These can be considered active systems due to the possibility of controlling key parameters; however, they combine characteristics of both the active and the passive systems. One stage and two-stage systems examples are schematized in Figure 2.5.

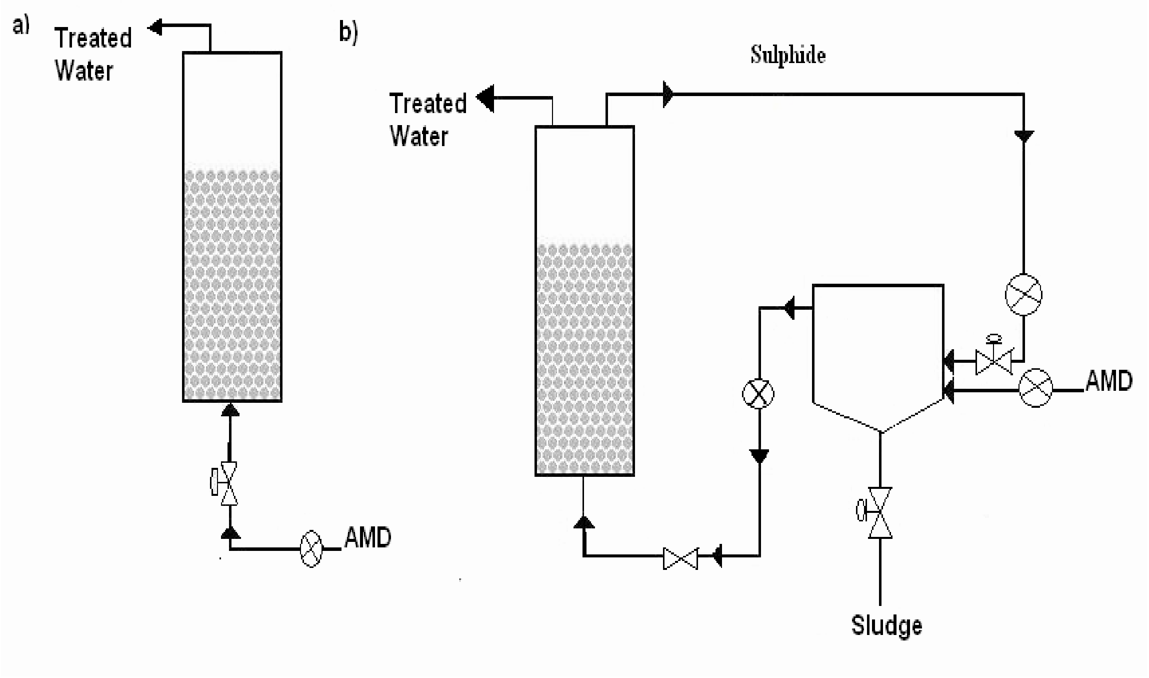


Fig 2.5 – Schemes of two different AMD sulphate reduction active based systems: a) Single process (Upflow anaerobic packed base). b) Two-stage process with sulphide re-circulation (Upflow anaerobic packed bed with a settling tank) (extracted from Martins et al., 2010)

2.5- Carbon/ electron donor for sulphate reducing bacteria (SRB) in biological remediation systems

The anaerobic respiration of sulphate reducing bacteria (SRB) through biological sulphate rduction process use some organic compounds as electron donors. Thus, this group of heterotroph bacteria rely on these organic compounds for their energy metabolism driving the sulphate to sulphide reduction mechanism in biogenic-sulphur systems. The energy requirement for this process is high, as the reduction of sulphate ($S^{+\,6}$) to sulphide (S^{2-}) needs about eight electrons to be accomplished (Choi & Rim, 1991): -

$$8H^{+} + 8e^{-} + SO_4^{2-}\ S^{2-} + 4H_2O \qquad (7)$$

Haven observed that sulphate reduction is an energy intensive process Barnes (1998); there is an obvious need for an energy rich reductant.

The issue has always been the right choice of organic substrate to make as to provide carbon/energy source for the bacteria activity. Recently, this has been more demanding taking into consideration, the need of a carbon source that offer high bio treatment efficiency and economic viability for the bio metal and sulphate remediation process (Gilbert et al, 2004). Availability, degradability, organic matter uptake to sulphate reduction ratio of the carbon source are key factors attended to when deciding on what to feed the bacteria with to enhance their activity performance.

Several studies have reported on the suitability of variety of organic substrates as carbon/energy sources. Sources like molasses, bagasse, sewage sludge, leaf mulch, wood chips, animal manure, vegetable compost, saw dust, mushroom compost, whey and other agricultural waste (Costa & Duarte; 2005, Annachhatre & Suktrakoolvait; 2001), Hay and straw carbon sources (Bechard et al., (1994), potato waste (Fyson et al., (1995); used mushroom compost (Machemer & Wildeman, (1992), lactate and cheese wey (Herrera et al., (1991), sugar cane molasses (Maree & Hill, (1989), animal waste slurries (Ueki et al., (1998) have all been reported.

Refined organic substances like ethanol and methanol (Jones & Gusek, (2004) are all well known.

However, careful selection of suitable carbon source is of great importance to ensure efficient performance and longevity in biological AMD treatment (Zagury et al., 2006).

Treatment systems such as anaerobic wetlands- Wieder (1993), Sequential Alkalinity Producing Systems, Kepler & McCleary, (1997), Biorecators (Jones & Gusek, (2004); Permeable Reactive Barriers (Blowes et al; (2000), all need the addition of biodegradable organic substances for the provision of carbon required in anaerobic alkalinity generating processes of SRB mediated AMD sulphate and heavy metals bioremediation techniques. The co-existence of sulphate reducing bacteria (SRB) along other fermentative bacteria mixed consortia communities triggers a syntrophic relationship, which influences the degradation and utilization of these complex organic substrates as energy sources. It has been reported by (Zhao et.al, (2010), that, the syntrophic relationship seen amongst various microorganisms supported the degradation of complex molecules like sucrose and glucose into simpler molecules that can be used by sulphate reducing bacteria (SRB).

The scheme below (Figure 2.5) depicts anaerobic microbial degradation of organic compounds in the presence of sulphate.

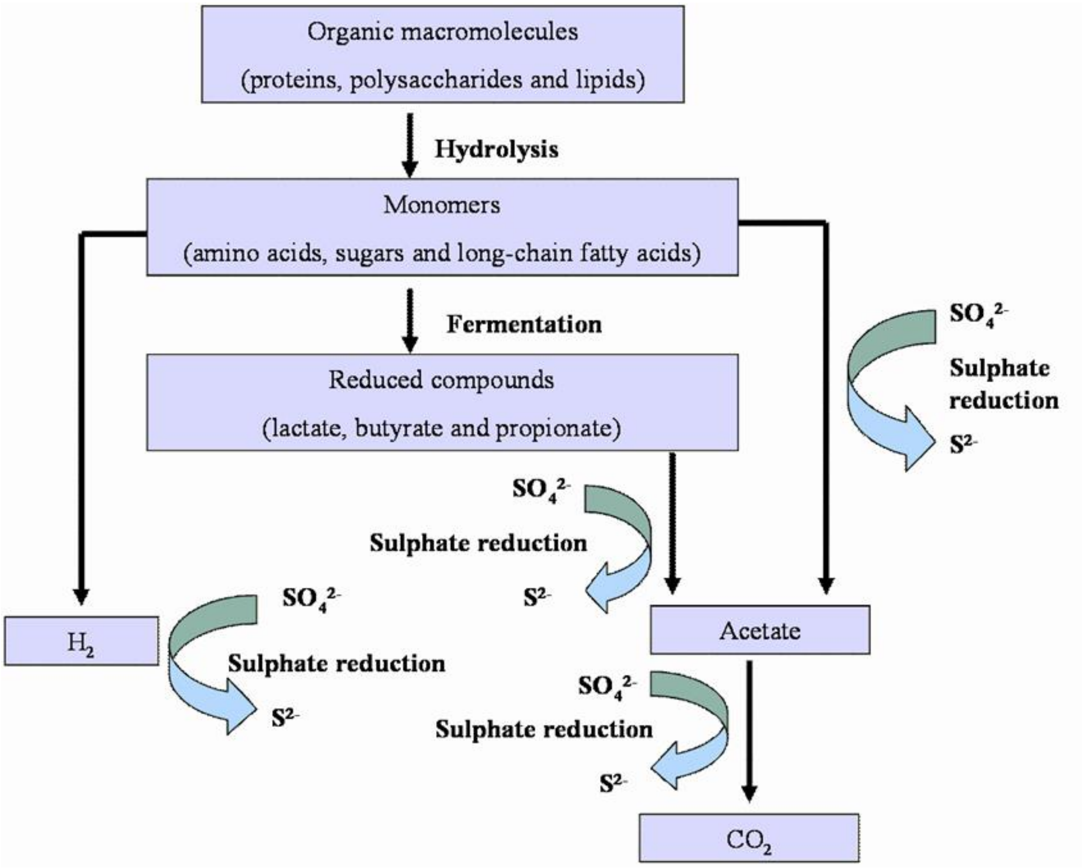


Fig 2.6- Schematic diagram showing anaerobic microbial degradation of organic compounds in the presence of sulphate (taken from Muyzer & Stams, 2008)

The selection of a suitable electron/carbon source for biological sulphate reduction depends on major considerations:

- Efficiency of treatment process or the ability of the electron donor to completely reduce or remove sulphate while minimizing the occurrence of other pollutants in the discharged effluents (environmental friendliness/sustainability of process).
- Cost of electron donor per unit of sulphate converted to sulphide (availability) (van Houten et al., 1994).
- Thermodynamic and Kinetic parameters remain important as well in the selection of electron/carbon sources as they affect the competition between SRB and MA (methanol utilizing microorganisms), hence, the remediation system efficiency (Liamleam & Annachhatre, 2007).

2.6- Molasses in the biological reduction of sulphate

The wide availability of molasses from sugar producing processes, Hilton & Archer, (1988), and its low cost makes it stand out as one of the most cost effective electron donors for SRB based processes (Liamleam & Annachhatre, 2007).

It's main component being sugar, when used as an electron donor in sulphate reduction, it undergoes fermentation by microorganisms like lactobaccili to products that are then available for SRB consumption as electron and carbon source (Maree et al., 1986, Maree et al., 1987).

The degradation of molasses molecules mainly involves its fermentation by hydrolysis to lactate (Liamleam & Annachhatre, 2007).

A typical reaction example:

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 4CH_3CHOHCOOH \quad (8)$$

Satisfactory biological sulphate reduction applying molasses as electron donor and carbon sources in Upflow Anaerobic Sludge Blanket (UASB) has been reported as a success when chemical Oxygen Demand (COD) to Sulphate ratios are < 2 (Liamleam & Annachhatre, 2007). Higher COD to SO_4^{2-} ratios of above 2 results to the accumulation of non –biodegradable portion of the molasses due to decreased COD removal (Annachhatre & Suktrakoolvait, 2001a, b). Another important aspect is that the generation of volatile fatty acid, acetate, propionate and butyrate in the reactors often creates souring problem (acidity) impacting the growth of both methanogens and sulphate reducers (Lo et al., 1990). The addition of NaOH or $NaHCO_3$ in order to maintain neutral pH creates better conditions for the fermentative conversion of molasses to lactate and for the use of it as electron donor and carbon source for SRB, making molasses a good candidate for the much needed environmentally sustained and cost effective carbon source/electron donor in AMD remediation systems using SRB.

Table 2.3- References of already studied biological sulphate reduction using molasses as carbon source

Type of molasses	Water	Reference
Cane sugar molasses	Sulphate & Sulphite rich industrial wastewater effluent	Maree & Strydom (1985)
Cane sugar molasses	Gold mine effluents supplemented with cyanide. Contaminated with cobalt, copper, iron, manganese, nickel, zinc and selenium	Maree et al (1987)
Baker's yeast factory molasses	Molasses wastewater with- low concentration of metals.	Hilton and Archer (1988)
Cane sugar molasses	Synthetic wastewater with- low concentration of metals.	Annachhatre and Suktrakoolvait (2001)
Cane sugar molasses	Sulphate & Sulphite rich industrial wastewater effluent	Maree & Hill (1989)
Cane sugar molasses	Sulphate & Sulphite rich industrial wastewater effluent	Lo et al (1990)
Hydrolyzed cane sugar molasses	Artificial municipal wastewater	Quan et al (2005)
Cane sugar molasses	Aquifer and groundwater at the precincts of a non-ferrous metal works company. Contaminated with Zn, Cd, Ni and Co, and a naturally high Fe concentration.	Geets et al (2006)
Cane sugar molasses	Synthetic wastewater with low metal concentration	Teclu et al (2009)
Cane sugar molasses	Sulphite rich pulp mill industrial effluent	Silva et al (2009)

2.7- General work outline

This work focuses on using beetroot and orange molasses schematized below in Figure 2.6 as carbon source/electron donor to support the activity of sulphate reducers in the the remediation of AMD water of Mina de São Domingo’s mine.

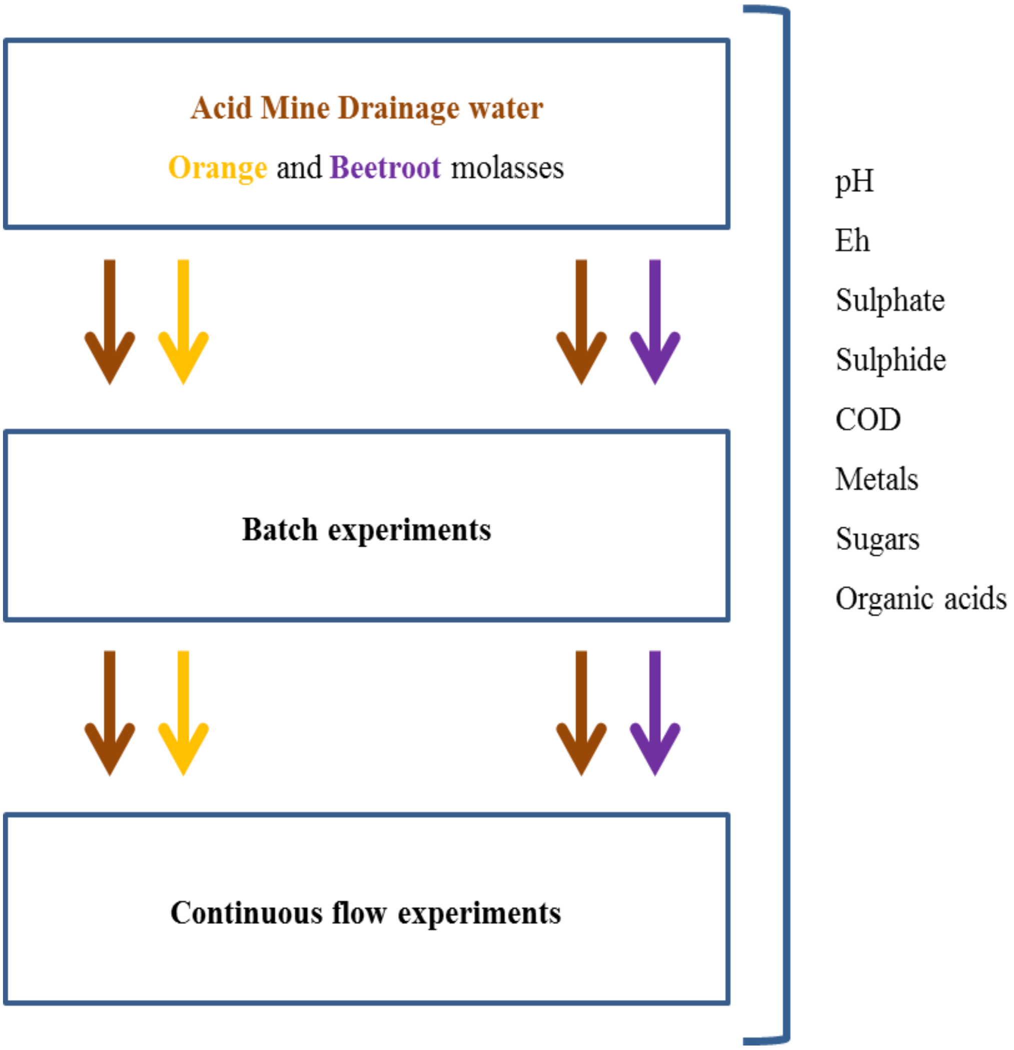


Fig 2.7- Depicting the various work stages performed in this thesis

3. Materials and methods

3.1-Sample collection

3.1.1- AMD sample

Acid mine drainage water was collected from the mine at Mina de São Domingo in Alentejo, Southern Portugal. Samples were taken from one of the AMD lagoons for use in batch test experiments. 1000ml of the collected AMD water was neutralized with 1kg of limestone chippings (1±0.5 cm diameter) to creat optimal pH conditions for SRB. Fresh samples were collected from the same lagoon for the continuous flow process test in bioreactor. The pH, sulphate concentration (mg/L), conductivity (mS/cm), redox potential (mV) as well as metal concentrations were measured as part of the AMD characterization prior to this work (Table 4.1).

3.1.2- Beetroot molasses and orange molasses

The beetroot molasses was from the sugar processing factory Azucarera, located in Jerez de La Frontera in Southern Spain while the orange molasses was from the orange juice factory Lara, located in Silves in Southern Portugal as an industrial sub-product obtained from evaporation of liquor extracted from orange peels.

The characterization of these two molasses courtesy of the contact persons in both factories is detailed below in Table 3.1.

Table 3.1- Composition of beetroot and orange molasses according to the respective supplying factories.

Beetroot molasses			Orange molasses		
Parameter	**Unit**	**Values**	**Parameter**	**Unit**	**Values**
Sucrose	%	45.55	Protein	%	<3
Glucose	%	0.14	Fats	%	<1
Fructose	%	0.45	Ash	%	2.56
Glutamine	%	0.07	Crude fibre	%	0.4
Rafinose	%	1.31	Sugars	%	26.2
pH		6.79	Calcium	mg/L	6228
Lactic acid	mg/L	31449			
Formic acid	mg/L	5259.3			
Acetic acid	mg/L	6889			
Propionic acid	mg/L	25320			
Butyric acid	mg/L	838.8			

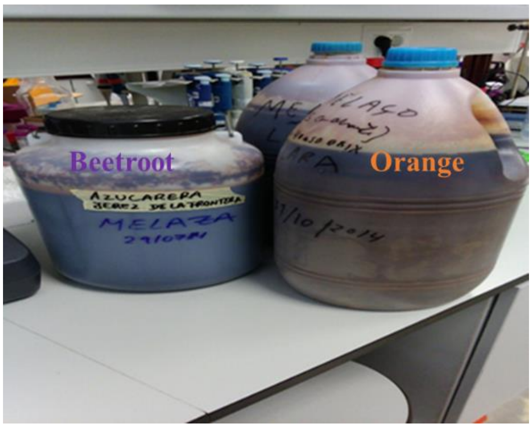


Fig 3.1- Beetroot and Orange molasses industrial sub-products

3.2- SRB inoculum preparation

The Sulphate reducing bacteria (SRB) consortia used in this work was collected from sewage sludge of a wastewater treatment plant located in Faro-Olhão, Southern Portugal and enriched with Postgate E medium without agar (Postgate, 1966), (Table 3.2). Best activity conditions of the bacteria community were ensured by transferring and growing 1% (v/v) of bacteria culture medium to Postgate B medium, Postgate, (1984) under room temperature and anaerobic condition in sealed glass bottle containing sterilized paraffin.

Table 3.2- Composition of different Postgate medium with concentration expressed in g/L (Postgate 1984).

Chemical composition	Postgate E medium medium	Original Postgate B medium	Modified Postgate B medium
KH_2PO_4	0.50	0.50	0.50
NH_4Cl	1.00	1.00	1.00
$CaCl_2.6H_2O$	1.00	-	-
$MgCl_2.7H_2O$	2.00	-	-
$CaSO_4$	-	1.00	1.00
Yeast extract	1.00	1.00	1.00
$MgSO_4.7H_2O$	-	1.20	1.20
Sodium lactate	3.50	3.50	-
Ascorbic acid	0.10	0.10	0.10
Thioglycolic acid	0.10	0.10	0.10
$FeSO_4.7H_2O$	0.50	0.50	0.50
Agar	15	-	-

3.3- Dosing molasses to support SRB activity in AMD bioremediation tests

Carbon source is quiet an important factor for the growth and activity of SRB, and the quantity added do highly influence the COD to SO_4^{2-} ratio (Das et al., 2009). The theoretical COD/SO_4^{2-} ratio for postgate B medium based on its concentration of lactate and sulphate is 1.5, which is actually in line with a reported theoretical COD/SO_4^{2-} value ratio of 1.7 required by SRB in overcoming carbon competing attempts from methanogens and other bacteria communities (Wolicka & Borkowski, 2009). Series of tests done at the Laboratory of environmental Technology (LET) to determine the adequate dilutions to make of the molasses carbon sources in Postgate B medium to guarantee best COD/SO_4^{2-} ratio needed for SRB growth and activity reported a dilution ratio of 1: 200 as the best (Alexandre, 2016). See tables 1, 2 and 3 in annex 1 for the initial characterizations of both molasses and for the results reported by Alexandre (2016). In this work, 1:200 dilutions in AMD were used for the batch tests and then were the initial doses supplied in the continuous flow experiments.

3.4- Batch experiment

Batch experiments were conducted using 100mL glass bottles at room temperature in triplicate and under anaerobic conditions by adding 10% (v/v) of liquid paraffin and sealing bottles with butyl rubber stoppers and aluminum crimp seals. The anaerobic experimental condition of the samples was monitored through visual observation of the disappearance of pink coloured resazzurin organic indicator.

The AMD used in batch experiments was previously neutralized using 1Kg/L limestone chippings (1±0.5 cm diameter) in an overnight bath. Moreover, 10% (w/v) of a powder residue from marble stone processing was used as chemical adjuvant for the biologic treatment of AMD (Barros et al., 2009). Tests were made with mixtures of beetroot molasses and of orange molasses diluted 1:200 (v: v) in neutralized AMD inoculated with 10% (v/v) of SRB cultures maintained in Postgate B medium. Two types of positive controls inoculated the same way were made. One with original Postgate B and the other with molasses diluted 1:200 (v: v) in Postgate B without lactate. The negative controls were in the conditions described for the tests, but without bacterial inoculations.

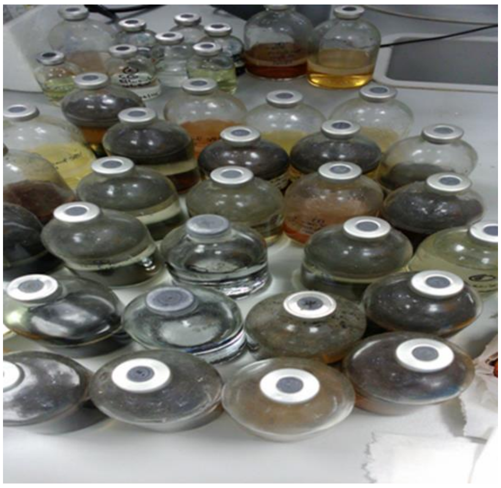


Fig 3.2- Anaerobic batch flasks used in work.

3.5- Continuous flow experiments

Haven tried the molasses in batch tests as carbon sources /electron donors for SRB activity and observed (results below) that they actively supported the reduction of sulphate as well as simultaneously decontaminated the heavy metals in the AMD effluents to concentrations below the maximum recommended values (MRV) for irrigation waters in Portugal, we extended it's application to continuous flow system where we fed the SRB consortia community in bioreactor columns with beetroot and orange molasses diluted in AMD previously neutralized also continuously in neutralization tanks.

3.5.1- Bioreactor system specifications /components

The Upflow Anaerobic Packed Bed (UAPB) bioreactors used in this work are cylindrically shaped with the following dimensions- 35cm height and 5.5cm diameter. They are filled with a mixture of 1:1 (v:v) coarse sand of about 0.2 cm diameter and limestone chippings within the range 0.5 to 1.5 cm diameters. The working volume of the filled reactor column is ~300 mL. The neutralization tanks are 22cm high, 15cm wide and 7cm deep in dimension with a working volume of 800mL containing about 1000mg of limestones chippings (0.5-1.5cm) as a neutralization agent packed in a way as to allow the AMD raw water pass in between the limestone grains before being pumped up into the reactors. Peristaltic pumps were used to move AMD to the neutralization tank and from this to the bioreactors until it comes out in a settling tank. The settling tank is 22cm high,15cm wide and 7cm deep in dimension with a working volume of about 2000mL. Syringe pumps were used to deliver molasses through tubing up into the bireactors entries.

Figure 3.3 depicts the schematic arrangement of the continuous flow system and Figure 3.4 shows the bioreactors packing material and the system modules.

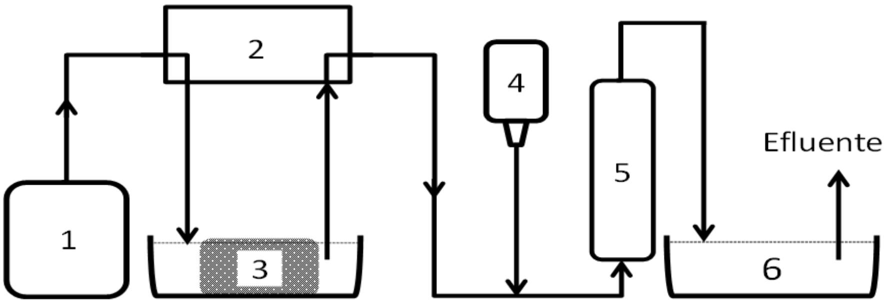


Fig 3.3- Schematic representation of the systems in use for the experiments using SRB based processes to treat AMD in continuous flow. 1 – AMD supply tank; 2 – Multichannel pump; 3 – pH neutralizing tank; 4 – organic substrate supply tank; 5 – Upflow Anaerobic Packed Bed (UAPB) bioreactor; 6 –Settling Tank.

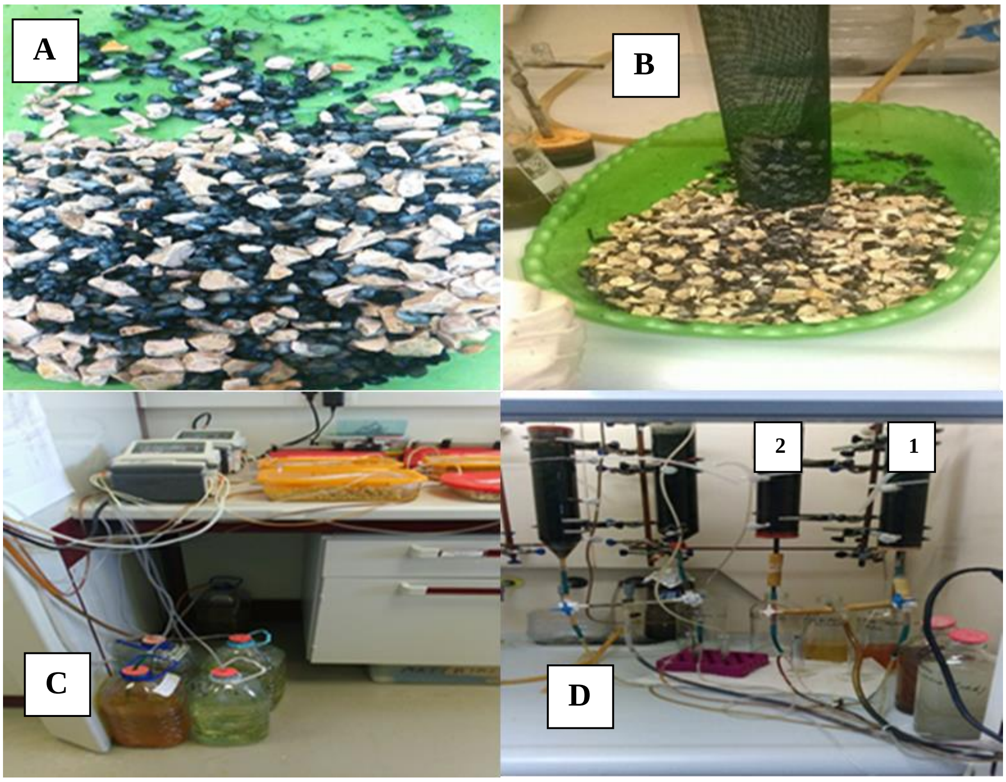


Fig 3.4- Showing: (A)- core sand mix with limestone in a 50:50 weight ratio. (B)- filling of the bioreactor mesh column with the core sand/limestone mixture. (C) - Neutralization tanks with limestones to neutralize incoming AMD water, pumping-systems for nutrient supply to bioreactor & peristaltic pumps for the upflow of the AMD raw water into the reactor. (D)- Bioreactor columns – 1: fed with orange molasses and 2: fed with beetroot molasses with upstream and downstream tubing connections from and to neutralization tanks and settling tanks respectively

3.5.2- Operating the bioreactors

The two continuous flow systems integrating SRB bioreactors used in this work had been in operation with other carbon sources/electron donors being supplied. Due to this, there was no start up phase.

The experiments in continuous flow here described, started by substituting the previously supplied organic substrates with the molasses in the conditions described in table 3.3 below. However, an aclimatization period was expected , because changing the types of organic substrates supplied to the bioreactors alter the dynamics of their communities.

The starting flow rates were in line with the dilution factors successfully tested in the batch experiments, where 1:200 dilutions of each of the molasses (orange and beetroot) in AMD gave an efficient sulphate reduction after approximately 14 days.

Detailed results from bioreactors are dicussed later.

Table 3.3- Operating conditions of the two Upflow Anaerobic Packed Bed (UAPB) bioreactors.

Parameter	UAPB Reactor 1	UAPB Reactor 2
AMD Flow rate (ml/hr)	1.00	1.00
Hydraulic residence time (HRT) (d)	~13	~13
Initial molasses flow rate (μl/hr)	5	5
Optimized molasses flow rate (μL/hr)	7.5	7.5

3.6- Analytical methods

3.6.1- Sulphate and sulphide concentrations

The concentration of the sulphate and sulphide ions in sample solutions were measured with UV-Visible Spectrophotometer (DR 2800, Hach –Lange). Sulphide concentration was determined using the methylene blue method (665nm, Hach-Lange) while the sulphate concentration was by sulfaVer4 method (Hach-Lange) at 450nm.

3.6.2- pH and Eh values

The pH and E_h values were determined using potentiometric pH/E_h Meter (GLP 21, Crison).

3.6.3- Metal concentrations

The Iron (Fe), Copper (Cu) and Zinc (Zn) metal concentrations were determined by Flame - Atomic Absorption Spectrometer (FAAS) model nova 350 Analytikjena. Aluminium (Al) concentration was analyzed using Microwave plasma atomic emission spectrometer (MP-AES), Agilent Technologies, 4200 model.

3.6.4- Sugar molasses analysis

Sugar analysis was carried out by HPLC with a Knauer Advanced Scientific Instrument using an Evaporative Light Scattering Detector (ELSD) Varian380-LC model with a column Rezex RPM –Monosaccharide Pb, Size 300*7.8mm, model OOH-O135-KO.

3.6.5- Organic acid analysis

Organic acids were analyzed by HPLC with a Knauer Advanced Scientific Instrument with a UV-Detector module, using a Rezex RFQ-Fast Acid H^+[8%] –Size: 100*7.80mm.

3.6.6- Experimental standard curves and limits of detection

The calibration curves for the analysed metals, sugars and organic acids were determined with series of standard concentrations. The limits of detection for the metals were determined using the calibration curve method. For FAAS, the equipment software (Aspect LS) calculates it automatically. For the MWP-AES, it was calculated according to the next mathematical relationship:

$$LOD = \frac{3S_{y/x}}{m}$$

Where $S_{y/x}$ is the estimation of the standard deviation of the regression line and m= slope of the calibration curve.

For the sugars and organic acids, any concentration generating a visible peak was considered. See Tables 1, 2 and 3 in annex 4 for details of batch and continuous flow systems experimental analysis limits of detection.

3.6.7- Chemical oxygen demand (COD) analysis

The COD was measured with the LCK 514 method (Hach-Lange) using a UV-Visible Spectrophotometer (Hach-Lange DR 2800) at 605nm following the the procedures of supplier with the option of heating at 148°C for 2hrs (using a digital dry bath (ACCUBLOCK^{TM})).

4. Results and discussion

4.1-AMD initial characterization

The results generated from the initial characterization of the physico-chemical parameters of the Mina de São Domingo's mine AMD water is detailed in the table below. Analysis were performed at Laboratory of Environmental Technology (LET) unit of the Centre of Marine Sciences (CCMAR) in the Department of Chemistry and Pharmacy (DCP), Faculty of Science and Technology, University of Algarve, Faro, Portugal.

Table 4.1- Characterization of AMD water from Mina de São Domingo's mine depicting measured parametric values of heavy metals, sulphate, pH and Eh in two (2) different occasions

Sample	**Unit**	**AMD 1-6**	**AMD 1-6**
Date of collection		31-01-15	17-02-16
Laboratory of analysis		LTA	LTA
Iron (Fe)	mg/L	44.70	172.20
Copper(Cu)	mg/L	30.09	40.54
Zinc(Zn)	mg/L	28.15	43.26
Aluminum(Al)	mg/L	138.26	205.40
Sulphate (SO_4^{2-})	mg/L	1848.00	2170.00
pH	Sorensen scale	2.48	2.49
Eh	mV	401.00	607.00
EC	mS	2.90	3.00

n/b: AMD_{1-6} collected 31-01-15 was used for the batch experiments while AMD_{1-6} collected 17-02-16 was used for the continuous flow experiment.

4.2- Batch experiments - SRB activity with beetroot and orange molasses

The growth and activity of SRB in this experiment using beetroot and orange molasses as carbon sources/electron donors was investigated using the described conditions in sections 3.3 and 3.4 and monitored as detailed in section 3.7 of materials and methods.

The efficiency of sugar molasses as carbon source for SRB metal-remediation of the AMD was determined by measuring parametric evolutions of effluents over a 21-day routine monitoring. Determination of the evolving values of pH and Eh was paramount as these two factors are important in ensuring continuous growth and activity of SRB. Hence, pH value of above 5 and Eh value of around -200mV is recommended for optimal SRB reducing ability in an anaerobic environment (Cohen, 2006). Reducing sulphate concentration values and corresponding sulphide concentration build up were indicators of SRB activity at this point.

4.2.1- Positive control 1: Testing sulphate reduction as SRB activity indicator

The positive control media was a content of the sulphate reducing bacteria (SRB) in postgate B medium with lactate as the carbon source. This very medium served to monitor the response of the SRB consortia used in this work with their activity response indicating their viability. Evolutions of pH, Eh, sulphate concentration reduction and the corresponding biogenic sulphide generation were monitored as benchmark indicators for the bacteria activity over 14-day period. Revelations from pH and Eh monitored values indicated favourable pH and Eh range values supportive of SRB growth and activity for bioremediation processes. pH values of this media lied within the range of 6.8, 7.08 and 7.0 for day 0, day 7 and day 14 of our parametric monitoring maintaining already investigated conducive pH range of 5.5-8.0 for SRB activity (Elliot et.al; 1998). On the other hand, Eh values were supportive too; achieving well above the limit value of -100mV needed for SRB sustainability (Postgate, 1984) and above the -200mV recommended by Cohen (2006). Following SRB inoculation on day 0; day 7 and day 14 witnessed Eh values of around -348mV in media, showing an established anaerobic environment for SRB activity and sulphate concentration shift to sulphide. With pH values within promoting range for SRB activity as well as Eh values, sulphate concentrations dropped with corresponding surge in biogenic sulphide. Before day 14, sulphate concentration had already fallen from 1011mg/L on day 0 to 148.67mg/L by day 7. Sulphide concentration grew in media to a maximum value of 329.66mg/L on day 7. The viability of our SRB inoculum remains high as sulphate reduction and biogenic sulphide generation from our investigations showed up concurrently.

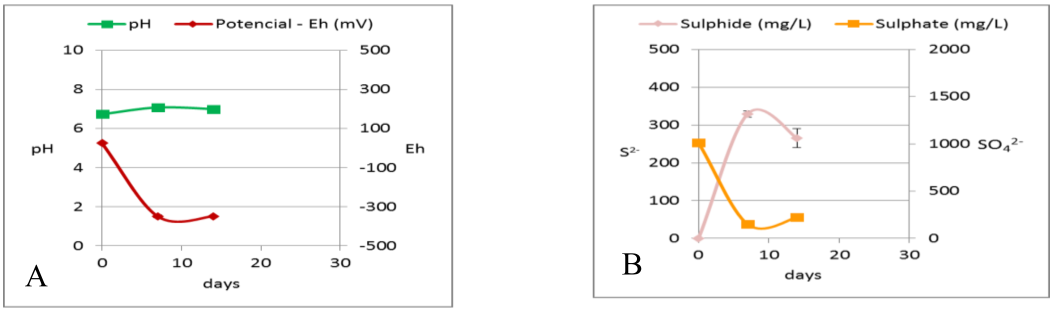


Fig 4.1- Positive control 1 monitoring with Postgate B original (i.e with lactate) **with** SRB and 10% calcite (w/v) : (A)- pH and Eh values.
(B)- Concentrations of sulphide and sulphate. Data are average of triplicates and error bars indicate standard deviations of average values.

4.2.2- Positive control 2: Beetroot molasses diluted 1:200 in Postgate B medium (without lactate), with SRB and 10% calcite tailings

Haven tested the viability of our SRB consortia used in this work by their relative responses of growth and activity in original postgate B medium, we introduced beetroot molasses as carbon source in lieu of lactate, the most commonly used energy source for SRB in research. Data values from our investigations showed that the SRB consortia reduced the sulphate concentration of the AMD media in an efficient way over the monitoring period. Removal of sulphate ion from the media resumed following a decreasing fashion post inoculation with a value of 1427mg/L on day 0. By day 7, we saw sulphate removed with values of 1662mg/L though a little high from previous day 0; however, no significant difference. Reasons to this might have been adjustment of the bacteria community in their bid trying to break down the sugar components into easily available substances (EAS). By day 14, concentration of sulphate reduced to around 1337mg/L and even went down by almost half the following day 21 with a value around 768mg/L. Correspondingly, we observed that while sulphate was being removed, sulphide concentration rose. From an undetectable value the initial day of inoculation, sulphide concentration rose to around 38mg/L by day 7. By day 14, sulphide concentration had already gone up to almost 5 times the previous day 7 with a value around 189mg/L. Day 21, saw a further increase to around 195mg/L. From the ongoing trend, the SRB consortia are to grow supported on beetroot molasses as an alternate carbon source to lactate; removing the sulphate concentration content of the media and generating biogenic sulphide simultaneously. The pH and Eh values were within values promoting bacteria activity from our data revelations. With pH values of around 5 to 7 and Eh values maintained below -100mV, an anaerobic environment was in place for the SRB consortia to thrive triggering off a dynamic shift from sulphate to sulphide.

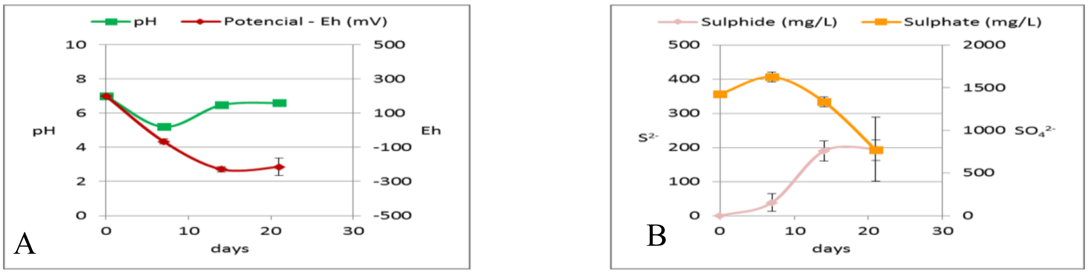


Fig 4.2- Positive control 2 monitoring of beetroot molasses diluted 1:200 in Postgate B medium (without Lactate), **with** SRB and 10% calcite (w/v) : (A)- pH and Eh values (B)– Concentrations of sulphide and sulphate. Data are average of triplicates and error bars indicate standard deviations of average values.

4.2.3- Positive control 3: Orange molasses diluted 1:200 in Postgate B medium(without lactate), with SRB and 10% calcite tailings

Similarity in evolution trend showed up as well with the alternating of lactate in postgate B medium with orange molasses as carbon source. The pH and Eh values were within favourable range for SRB growth and activity. pH values of range around 5 to 7 were obtained and maintained by the calcite tailing material acting as a buffering agent. Eh values were below the -100mV limit value for efficient SRB mediated bioremediation process (Postgate, 1984). With stabilized pH and Eh media values, sulphate concentration assumed more or less a similar trend just like in the case of beetroot molasses; by day 21, about 561mg/L of sulphate was detected in media. Sulphide concentration simultaneously did rise as we witnessed drop in sulphate concentration. Revelations from both beetroot molasses and orange molasses data values showed evident sustainability of SRB growth and activity as observed from sulphate concentration reduction and sulphide build up relatively.

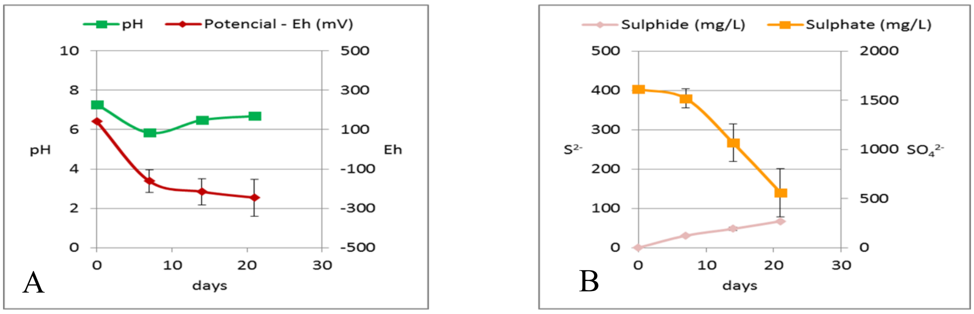


Fig 4.3- Positive control 3 monitoring of orange molasses diluted 1:200 in Postgate B medium without Lactate, **with** SRB and 10% calcite (w/v) : (A)- pH and E_h values. (B)- Concentrations of sulphide and sulphate. Data are average of triplicates and error bars indicate standard deviations of average values.

4.3 - Tested molasses results - sulphate and heavy metal removal

4.3.1-Beetroot molasses diluted 1:200 in AMD with SRB and 10% calcite tailings

Following convincing results from the positive controls of our SRB activity, we extended our investigation of maintaining them in AMD medium using beetroot molasses and orange molasses as carbon source support.

Using beetroot molasses as alternate carbon source substrate, we monitored sample effluents controlling physical and chemical parametric values. pH, Eh, SO_4^{2-}, S^{2-} and heavy metal concentrations of zinc (Zn), iron (Fe) and copper (Cu) were monitored of diluted 1:200 beetroot molasses in AMD with SRB.

Evolution indications of pH reveal around 6 point something over the 21 days monitoring period. pH values were stabilized more or less within the same range being the fact that the local calcite tailings material used buffered the media.

Redox potential (Eh) values for the media was maintained within favourable limits for SRB growth and activity. Postgate (1984) stated that the minimum required redox potential value to achieve an oxidation-reduction environment for anaerobic SRB culture medium is -100mV.

Apart from day 0, where there was a record of positive Eh value at 266mV; being the fact that this day the media was just inoculated with SRB hence a shift to anaerobic conditions was not yet in place. However, Eh values showed negative values well below -100mv at around -131mV, -271mV and around -302mV for day 7, day 14 and day 21 respectively. Indications from our pH and Eh values for this media reveals a less acidic but improved alkalinity system as well as a favourable anaerobic environment respectively. This therefore, implied that SRB activity was imperative to make for a shift of sulphate to sulphide ion, which combines with the heavy metals removing them as metal sulphides precipitates (equations 5 and 6).

The heavy metal contaminants associated with the AMD (Zn, Fe and Cu) still in solution after pH neutralization (day 0) were almost totally removed at day 7 as metal sulphides following their direct chemical combination reaction with the generated biogenic sulphide. At the very first day of the SRB media inoculation being day 0, zinc metal concentration was 3.76mg/L; a value above the less than 2mg/L maximum recommended value (MRV) for this metal set by Portuguese national law on irrigation waters. Zinc metal by the following day 7, was below limit of detection of 0.0808 mg/L (Table 1 of annex 4) and removed significantly out of solution. By day 14, zinc metal was still undetectable. Iron (Fe) concentration was already very low at day 0, due to its precipitation with neutralizing pH. Day 0 concentration of about 0.477mg/L reduced to an insignificant value by day 7. However, by day 14, there was a detection of about 1.31mg/L of iron, though still below the less than 5mg/L maximum recommended value (MRV) of iron concentration limit in Portuguese national legislation for irrigation waters. This little surge might have been due to reduction of some less soluble Fe^{3+} (precipitated during AMD neutralization) to the more soluble Fe^{2+} at such neutral pH. However, the following day 21, iron concentration reduced again to around 0.56mg/L, putatively due to formation of metal sulphides. In the instances of iron removal from solution over the monitored days, though, there were traces of iron still in solution for day 14 and 21 after SRB inoculation, their concentration in any case, remained quiet below the maximum recommended value (MRV) national legislation for irrigation waters in Portugal.

Copper metal on the other hand after the initial day 0 SRB inoculation was removed out of solution to a point of undetectable limit of 0.3559 mg/L (Table 1 of Annex 4) for days 7, 14 and 21.

The sharp trend fall in zinc and copper concentrations might be attributed to formation of metal sulphides. Though, biogenic sulphide was not produced but this trend was observed. The trend so far shown by the SRB consortia subjected to beetroot molasses as carbon source indicates the sustainability of the growth of the bacteria community triggering sulphate reduction and sulphide generation simultaneously which combines to remove the heavy metals in form of metallic sulphides out of solution. Refer to equation 5 and 6 in section 2.4.

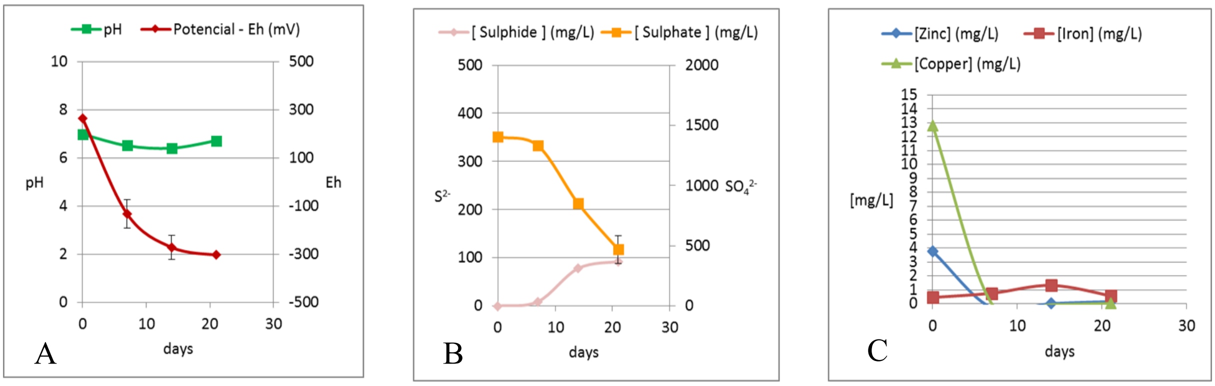


Fig 4.4- Test monitoring of beetroot molasses diluted 1:200 in AMD **with** SRB and 10% calcite (w/v): (A)- pH and E_h values. (B)- Concentrations of sulphide and sulphate (C)- Zn, Fe and Cu metal concentrations over a period of 21-days. Data are average of triplicates and error bars indicate standard deviations of average values.

4.3.2- Negative control: Beetroot molasses diluted 1:200 in AMD without SRB and with 10% calcite tailings

Without SRB community consortia inoculants, the media physical and chemical parameters showed no evident proof of sulphate concentration reduction neither biogenic sulphide generation. The pH values were on the favourable range for bacteria growth and activity needed for efficient biological sulphate reduction. Values within the range of 6 point something was recorded for the pH of the media. Redox potential remained on the positive side above 100mV down the line for days 7, 14 and 21 accordingly. The implication of this remains that since there were no SRB inoculants, no established reduction oxidation anaerobic environment triggering SRB sulphate shift to sulphide was envisaged. Indeed, there were no detectable sulphide concentrations in media over the days of monitoring. However, though there was no major reduction of sulphate over the monitoring period, sulphate concentration in media in the third week dropped from the initial surrounding 1500mg/L to about 1000mg/L. One could expeculate that this might have resulted from some microbiological population in media coming from the molasses (not sterile), initiating some kind of sulphate removal. Nevertheless, since there is no evidence of biogenic sulphide generation, other than SRB must have been ivolved in this removal. Other explanation could be chemical changes in the medium resulting from reactions between its components.

For the metal concentrations, zinc was still detected in solution at appreciable quantity over the days of monitoring, exceededing the less than 2mg/L maximum recommended value (MRV). Concentration on day 0 was 3.76mg/L and 5.4mg/L, 3.75mg/L and 6.67mg/L for days 7, 14 and 21 respectively. There were instances of rise and fall fashion in zinc concentration over these days. This can be also putatively attributed to dynamic changes in the media due to reactions between its components or due to some microbial population evolution initiated from the non sterile molasses.

Copper ion concentration slowly descended and went to almost undetectable value by day 21. From 12.8mg/L on day 0 to around 9.84mg/L, 6.03mg/L and 0.46mg/L for days 7, 14 and 21 respectively. The low solubility constant (K_{sp}) of the metal ion could explain it's higher removal probably by precipitation promoted by changes in the media as suggested above.

On the contrary, iron showed a raising trend. Day 0 concentration of about 0.45mg/L increased to 0.49mg/L by day 7, to 1mg/L by day 14 and 1.82mg/L by day 21. As suggested before in section 4.3.1, this little surge might have been due to reduction of some less soluble Fe^{3+} (precipitated during AMD neutralization) to the more soluble Fe^{2+} at such neutral pH.

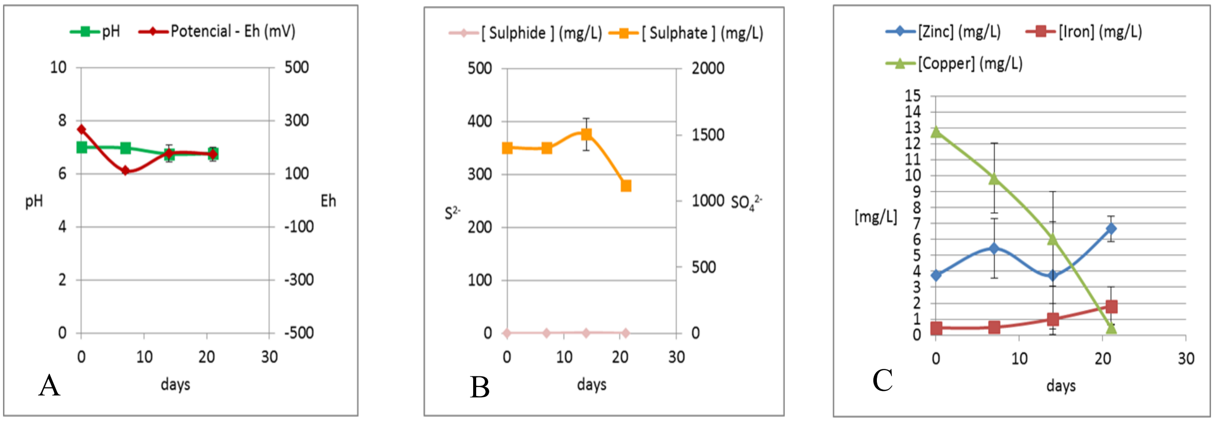


Fig 4.5- Test monitoring of beetroot molasses diluted 1:200 in AMD **without** SRB and with 10% calcite (w/v): (A) - pH and E_h values.

(B)- Concentrations of sulphide and sulphate.

(C)- Zn, Fe and Cu metal concentrations.

Data are average of triplicates and error bars indicate standard deviations of average values

4.3.3- Orange molasses diluted 1:200 in AMD with SRB and 10% calcite tailings

We also tested the suitability of orange juice molasses as a source of carbon for sulphate reducing bacteria (SRB).

Similarly, sample effluents were monitored controlling physical and chemical parametric values like the test media with beetroot molasses. pH, Eh, SO_4^{2-} and S^{2-} and heavy metal concentrations of zinc, iron and copper were monitored of diluted 1:200 orange molasses in

AMD with SRB. pH and Eh values during the 21-day monitoring lied within the range of 6.30 to 6.52 and -256mV to -128mV respectively haven progressed from initial pH value of 6.79 and Eh value of 236mV on day 0 of inoculation. As the bacteria adjust, the redox potential being a very important factor in initiating bacteria sulphate reduction; Postgate (1984), established an anaerobic condition guaranteeing sulphate reduction shift to sulphide generation in media following day 7, 14 and 21 data evolutions.

Sulphate reduction concentration to not a much significant difference value from day 0 appreciated to 1343.67mg/L by day 7. Explanations to this, borders on the fact that some oxidation reaction was still going on as orange molasses was still not available yet to the SRB consortia community for growth. By day 14, sulphate concentration had gone down by almost half of what it was the previous day 7 with a value of 713.67mg/L. The steep sided descending fashion of sulphate continued up to day 21 with concentration value of 418.33mg/L already now below the 575mg/L maximum recommended value (MRV) for irrigation waters in Portugal.

With the exhibition of sliding sulphate concentration trend, sulphide concentration surged the days after inoculation. Day 7, 14, and 21 saw a rise in sulphide concentration with values of 11.93mg/L, 122.55mg/L and 418.33mg/L respectively.

The generated biogenic sulphide (refer to equations 5 & 6 in section 2.4) in a way of addition reaction, reacts with the AMD mobilized heavy metal contaminated water forming insoluble precipitate thereby reducing sulphate and metal concentration (Costa & Duarte 2005).

About 4.2 mg/L of zinc ion in the system reduced to 0.49mg/L on day 7. By day 14, 0.33mg/L of zinc was only available. The following day 21, concentration appreciated to 0.52 mg/L from the previous day 14. These values were well below the 2 mg/L maximum recommended value of zinc in irrigation waters in Portugal.

Iron (Fe) concentration on day 0 was 0.456 mg/L and had a steep rise to about 0.81 mg/L on day 7. This slight increase might have been due to similar reasons as had already been reported in section 4.3.1. However by day 14, iron (Fe) concentration had gone down to about 0.38 mg/L from the previous day value. By day 21, concentration of iron (Fe) increased again to 0.54 mg/L as explanation to this rests on the near neutral pH values aiding the neutralization of less soluble Fe^{3+} precipitating to more soluble Fe^{2+} in solution. Though, concentrations fail below the maximum recommended value of 5 mg/L for irrigation waters in Portugal.

We observed a sharp copper reduction from 10.7mg/L on day 0 to 0.24 mg/L on day 7. On day 14, it reduced further to 0.05 mg/L and by day 21 copper concentration was 0.15 mg/L.

Overall, copper removal from the effluents for this 21 day evolution, showed values below the set limit of 0.2 mg/L for irrigation waters in Portugal.

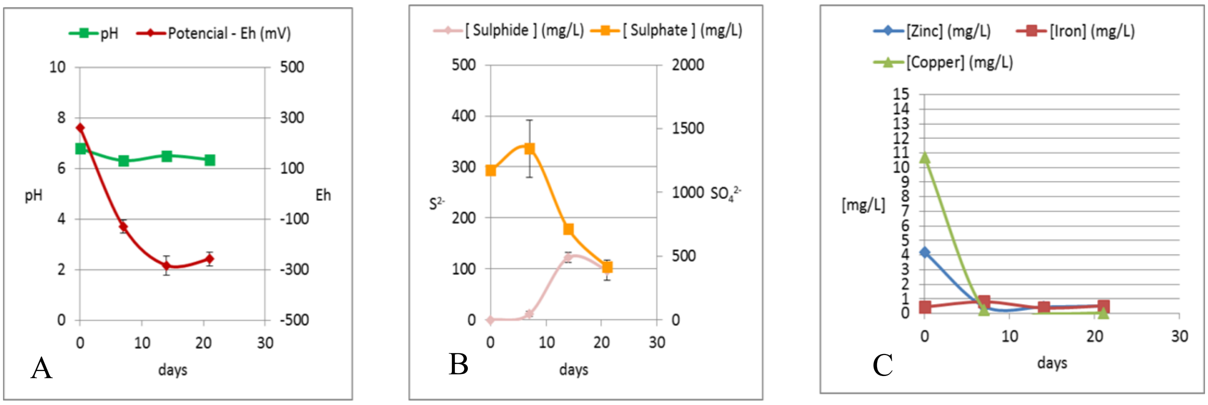


Fig 4.6- Test monitoring of orange molasses diluted 1:200 in AMD with SRB and 10% calcite (w/v): (A) - pH and E_h values.

(B) - Concentrations of sulphide and sulphate.

(C) - Zn, Fe and Cu metal concentrations.

Data are average of triplicates and error bars indicate standard deviations of average values.

4.3.4- Negative control: Orange molasses diluted 1:200 in AMD without SRB and with 10% calcite tailings

Investigations in media without SRB consortia evolved in physical and chemical parametric values revealing no reduction in sulphate concentration and non removal of heavy metal contaminants of the AMD. Generated values of sulphate and heavy metal concentrations remained far above the maximum recommended value (MRV) limits of both parameters defined in Portuguese national decree law for irrigation waters. Our results, indicated that pH values were 6.79, 6.96, 6.92 and 6.58 on the average for days 0, 7, 14 and 21 respectively. These values were favourable for SRB activity and lied within the recommended 5-9 value already reported by Elliot et.al; (1998). Redox potential values of 263mV, 164.33mV, 225.67mV and 227.33mV for day 0, 7, 14 and 21 on average showed lack of SRB activity as a redox potential of around -200mV must be met to trigger sulphate-reducing activity (Cohen, 2006).

With the absence of SRB consortia in the media, sulphate concentration right from day 0 to the 21-day routine check, saw no significant reduction with values of 1177 mg/L, 1374.33mg/L, 1400.33mg/L, 1029mg/L for day 0, 7, 14 and 21 recorded respectively. Concentrations for days 7 and 14 were a bit high, though not significantly different from the others and this could be an attribute of some other microorganisms present in the orange molasses degrading the substrate as sulphate contents filters more into the media. However, the insignificant sulphate reduction and corresponding little or no sulphide generation in this media establishes the fact that there were no SRB inoculants. Hence, no activity was traced down the line.

Zinc ion concentration remains on the build with values of 4.2mg/L, 4.3mg/L for days 0 and 7 respectively. By day 14, it stepped up once small to 5.47 mg/L from the previous concentration on day 7 and day 21 concentration was more or less the same as day 14, with a value of 5.39mg/L.

However the case, these values were out of recommended limit for zinc concentration defined as 2mg/L for irrigation waters in Portugal.

Iron concentration remains more or the same at 0.4mg/L over the 21 days evolution monitoring and did not drop lower than this value.

However, with values below the 5mg/L recommended as the defined limit in Portuguese national decree for irrigation waters(Decree Law no: 236/98, Annex XVI, 1998). Explainations borders on the fact that Iron metal must have been removed out of solution as the pH values were above the minimum 4.5 value required to precipitate the metal.

Copper concentration of 10.7 mg/L on day 0 dropped to 8.85mg/L by day 7. This trend continued with Cu metal concentration dropping further to 6.6mg/L and 0.13mg/L for day 14 and 21 respectively.

Though with no detectable generated biogenic sulphide, explaination borders on the fact that copper metal can precipitate easily at the least available concentration of sulphide present in media as the metal has a very low solubility constant (K_{sp}).

There could not have been any SRB growth nor activity based on the fact that ,the media was not inoculated and the Eh value was not conducive for SRB anaerobic sustainable environment.

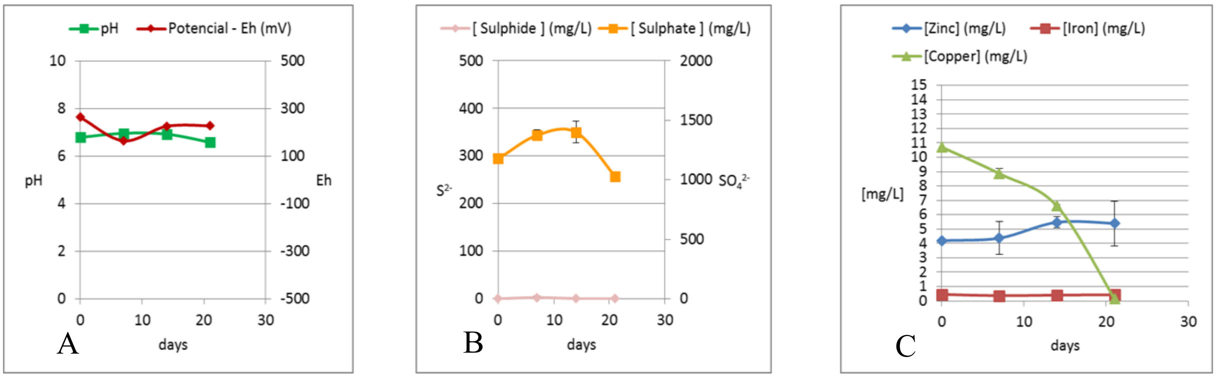


Fig 4.7- Test monitoring of orange molasses diluted 1:200 in AMD **without** SRB and with 10% calcite (w/v): (A) - pH and E_h values.

(B)- Concentrations of sulphide and sulphate

(C)- Zn, Fe and Cu metal concentrations

Data are average of triplicates and error bars indicate standard deviations of average values.

4.4- Organic components of molasses

Having molasses in large quantities from sugar processing is one of the criterion in the choice of using it as a source of carbon for SRB growth and on the account of its economic feasibility (Hilton & Archer ,1988).

Comprising mainly of sugars, it is fermented by microorganisms like lactobacilli (equation 8) when used as carbon source/electron donor in sulphate reduction to products that are available in less complex forms for sulphate reducing bacteria (SRB) (Maree et al., 1986 , Maree et al., 1987).

For this reason, we analysed the presence of some sugar molecules known to be present in molasses. Presence of the following sugar products were analysed: sucrose, glucose and fructose. Because various low molecular weight compounds produced from anaerobic fermentation like organic acids, volatile fatty acids (VFA), alcohols can serve as electron donor for SRB (Liamleam and Annachhatre, 2007), we decided to analyze the presence of organic acid fermentation end products in this work. The following organic acids were investigated: acetic acid, formic acid, propionic acid, butyric acid and lactic acid. See Figure 2.6 in section 2.5 for detailed chemical reaction metabolic pathways for the various types of carbon sources and Table 3.1 in section 3.1.2 for the chemical composition of the used molasses industrial sub-products in this work.

4.4.1- Carbon compounds in batch tests with beetroot molasses: sugars and organic-acids

4.4.1.1-Sugars: beetroot molasses diluted 1:200 in AMD with SRB and 10 % calcite tailings

a. Sucrose

We identified the presence of sucrose in the media containing beetroot molasses as organic carbon substrate source. In the media with SRB innoculum, close to a 4406mg/L of sucrose component was measured as at day 0 of innoculation. Taking into acount the beetroot molasses characterization given by the supplier (45.55% (or 455.5g/L) of sucrose) (Table 3.1 of section 3.1.1) and the dilution tested (1:200 (v:v)), the expected content of sucrose at day 0 would be 2277.5mg/L; that is, about half the value measured. Having no other justification, we are left with the hypothesis of an error in the preparation of the tested dilution of molasses in AMD. Sucrose declined a bit to an average of 3894.33mg/L by day 7 as the bacteria community starts to initiate break down of the sugar components. Evolution monitoring by day 14 and 21 witnessed a drastic uptake of the sucrose component of this media with concentration of the remaining sucrose droping to 473.33mg/L and 440.67mg/L respectively (Figure 4.8) and almost 10 times lower than the previous day 7 value. This stregthens the fact that syntrophic relationship amongst the microbial consortia members provided metabolic pathway where the sucrose from beetroot molasses was degraded into simplest molecules then available to be assimilated by the SRB.

b. Glucose

As expected, this media had the glucose sugar below detection limit at day 0 (Figure 4.8 and Table 2 , annex 4). Considering the beetroot molasses characterization given by the supplier (0.14% (or 1.4g/L) of glucose) and the dilution tested (1:200 (v:v)), the expected content of glucose at day 0 would be 7mg/L; that is, below the lowest detected standard concentration (Table 2 of annex 4). At days 7 and 14 though sucrose started to be degraded, glucose was still undetected. This might have occurred due to a shift in sucrose concentration to its monomeric sugar components of glucose and fructose brought by it's microbial fermentative degradation and immediately taken up by the bacteria community consortia. Suporting this idea, by day 21 we noticed a little remain of glucose; again probably as a result of the shift in bacteria population taking up glucose for metabolism haven declined as amount trickles down the line.

c. Fructose

With this media, we detected fructose on day 0 at a value of around 1365mg/L and then declined to about 267mg/L on average by day 7. By day 14 and day 21, this has been consumed off this media by the bacteria consortia community as it was not detected. Fructose, such like glucose, most probably resulted from sucrose degradation and then some members of the microbial community must have dwelt on this sugar for it's growth.

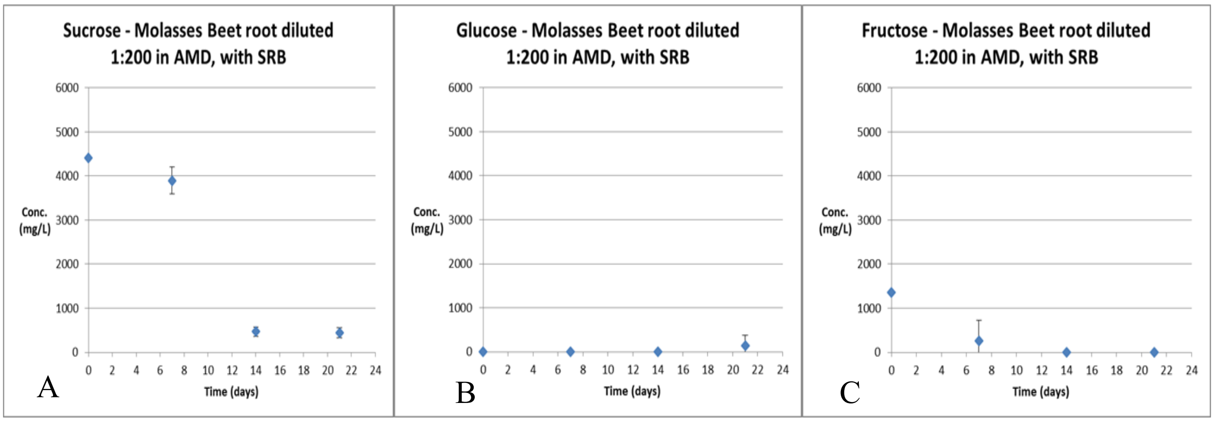


Fig 4.8- Depicting sugar compounds: (A)- Sucrose (B)- Glucose (C)- Fructose from beetroot molasses diluted 1:200 in AMD **with** SRB and 10% calcite tailings.

Data are average of triplicates and error bars indicates standard deviation of average value.

4.4.1.2-Sugars: Negative control - beetroot molasses diluted 1:200 in AMD without SRB and with 10 % calcite tailaings

a. Sucrose

Sucrose concentration in media the first day, remained the same with little difference with respect to day 7. However, by day 14 and 21, the concentration had gone downhill with values stood at 1324.33mg/L and 1230.67 on the average respectively. Though, with no innoculants, sucrose component was removed off the media but not as evidently seen as in the media with SRB innoculants. This might have occurred due to the presence of some microorganims in the media coming from the non sterile molasses or sucrose degradation due to chemical reactions. Overall, there was no much significant difference in concentration values of sucrose between day 0 and 7 in this media and day 14 and 21 as well.

b. Glucose

Without SRB innoculum, the media showed detectable quantities of glucose sugar components. Day 14 and day 21 witnessed a reflection of glucose sugar in media : 2334.33mg/Land 713.67 mg/L, respectively (Figure 4.9). This period corresponds to the days when sucrose decreased dramatically, supporting the idea of degradation of sucrose in its monomeric components. On other hand, the quantity of glucose by day 21 started to drop. This might be attributed to fermentative microorganisms present in media, coming from the molasses, which might as well be assimilating some quantity of glucose for their metabolic activities. However, contrary to the media with SRB innoculants, in wich glucose was almost totally consumed (Figure 4.8), here in the negative control this was not the case.

c. Fructose

Fructose concentration profile for day 0 and day 7 were at about 1365mg/L and 1716mg/L respectively. The difference between these concentration values were not significant and as such pointing to the fact that there has not been any major uptake on the average ; hence, media was without SRB innoculants. By day 14, it had already dropped to around 638mg/L and the following day 21, we were still having some quantity left in the media at around 115.26mg/L representing over 10 times less of what was available by day 0 on the average (Figure 4.9C). Again, though without SRB innoculants, fructose consumption in this media might have been due to microorganisms present in media, probably coming from the non sterile molasses used. However, in comparison of the concentration values of fructose revealed in this media (Figure 4.9C) and in the media with SRB innoculants (Figura 4.8C), there was an evident difference indicating indicating a greater and faster consumption in the test with inoculum..

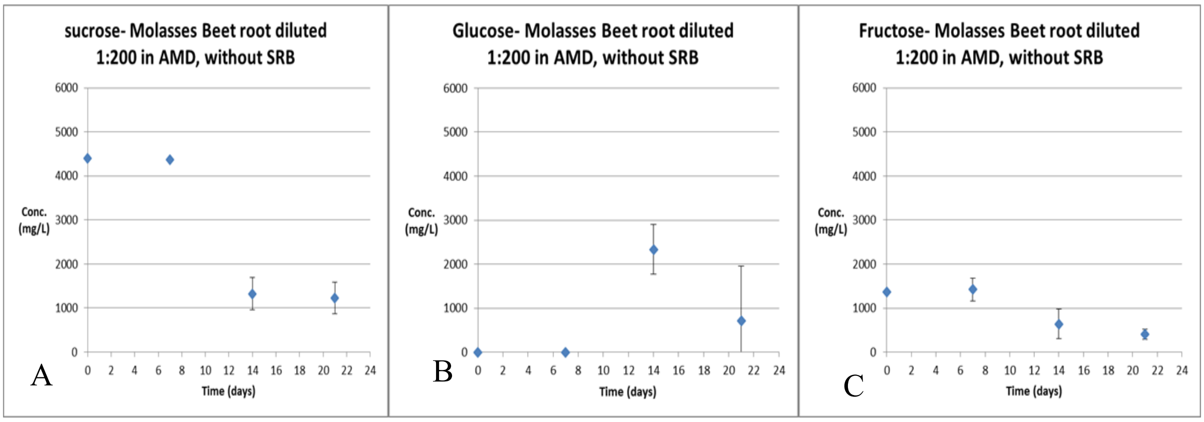


Fig 4.9- Depicting sugar compounds: (A)- Sucrose (B)- Glucose (C)- Fructose from negative control: beetroot diluted 1:200 in AMD **without** SRB and with 10% calcite tailings. Data are average of triplicates and error bars indicates standard deviation of average values.

4.4.1.3 - Organic acids: beetroot molasses diluted 1:200 in AMD with SRB and 10 % calcite tailings

a. Acetic acid

Acetic acid salt form of acetate is a key intermediate in the breakdown of organic substances in anaerobic processes (Liamleam & Annachhatre ,2007).

As a volatile fatty acid (VFA), it is available for use as a carbon source /energy donor for some SRB in the sulphate reduction process (Liamleam & Annachhatre ,2007). However, some sulphate reducing bacteria (SRB) community do not have the ability to oxidize acetate, even with excess sulphate concentration levels (Lens et al., 2002).

We noticed that acetic acid was generated right from the day 0 of inoculation upto the day 21, suggesting it was not completely consumed (Figure 4.10A). This further confirms the fact that in our media, some microorganisms as part of our bacterial consortia did actually degrade the sugar beetroot molasses to acetate and there were very little assimilation of it, if at all by members.

b. Formic acid

There was no detection of formic acid in this tested media, hence, it was below of the lowest detected of the prepared standard. See Table 3 of annex 4.

c. Propionic acid

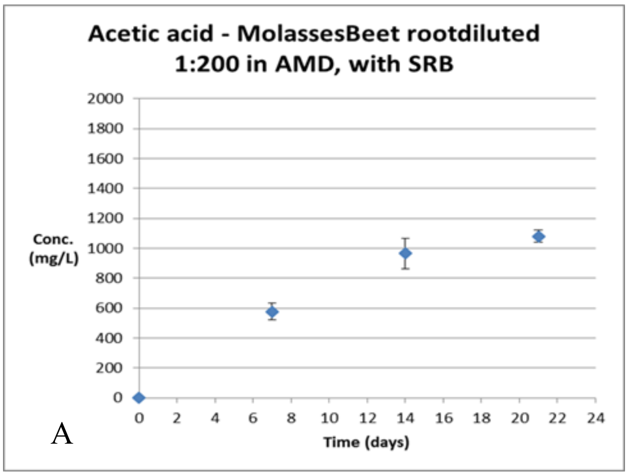
The degradation of the salt form of propionic acid, propionate is substancially enhanced by the presence of SRB (Speece, 1996). They do this either by direct utilization or through interspecies transfer (Liamleam & Annachhatre, 2007). A propionate degrading SRB, has been reported to effectively breakdown propionate to acetate with more effeciency at high sulphate concentration (Harada et al., 1994). This work further strengthens this hypothesis as we observed the acid form of propionate and acetate in our SRB inoculated media over this 21 days (Figure 4.10.B).

d. Butyric acid

Just like propionate, the salt form of butyric acid ,butyrate is an important product of anaerobic sulphate reduction process (Speece, 1996). As a volatile fatty acid, its pathway of mineralization involves syntrophy between hydrogen-acetate consuming sulphate reducing bacteria and hyrogen acetogens as well as direct consumption by SRB (Widdel ,1988). Our results suggest that in the tested media with SRB inoculum, butyric acid was generated on day 14 and consumed by the consortium the following day 21 (Figure 4.10.C).

e. Lactic acid

Lactate, the conjugate base of lactic acid, has been assessed as an organic substrate for enrichment of sulphate reducers (Widdel, 1988). Several species of sulphate reducers can use lactic acid as an electron donor and carbon source (Liamleam & Annachhatre, 2007). Lactate in its synthetic form, is the most widely used SRB carbon source (Postgate 1984; Barnes 1998). Despite it’s high biomass yield and high alkalinity production, cost issues plays it down being used mostly at industrial scale as a carbon source (Liamleam & Annachhatre, 2007). We observed lactic acid geneneration on day 7 and day 14 and consumed off by our SRB consortia by day 21 (Figure 4.10.D). This further strengthens the view that, lactic acid was produced by some members of the microbial consortia during the degradation of sugars and then consumed off by the SRB consortia members to sustain activity.



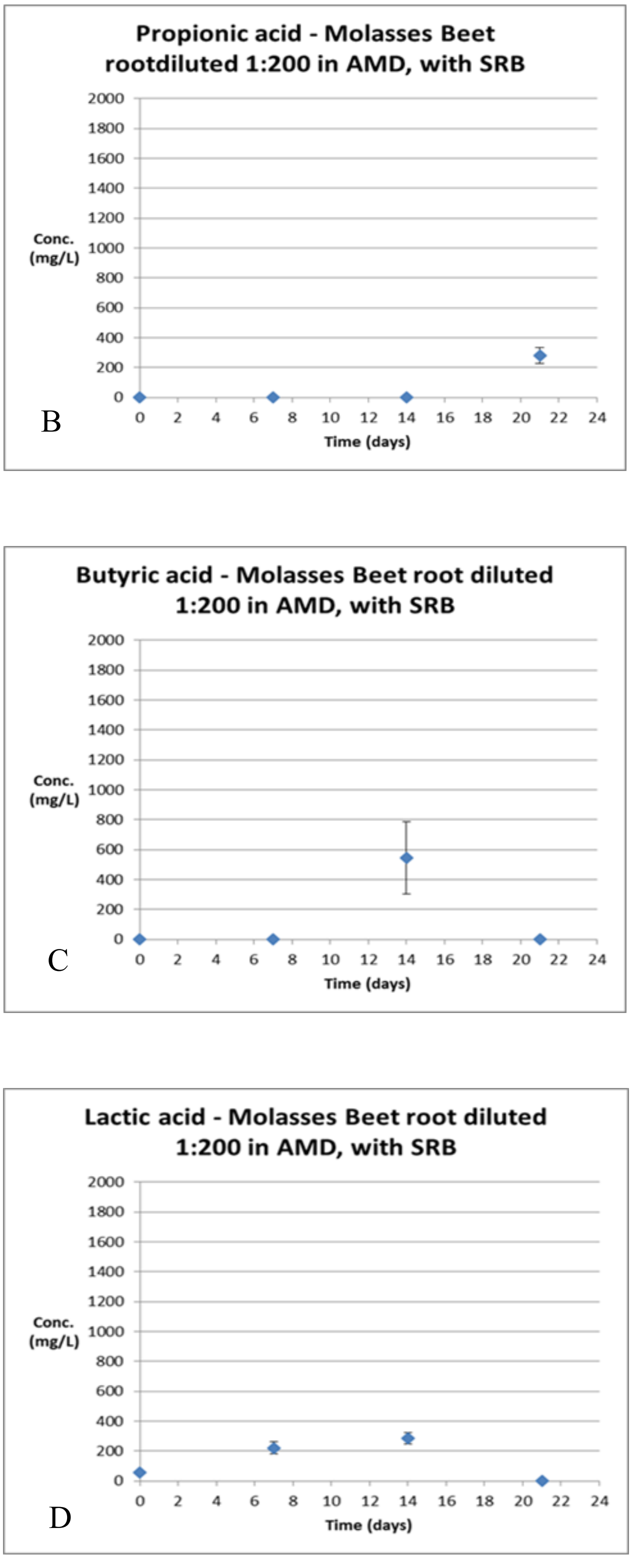


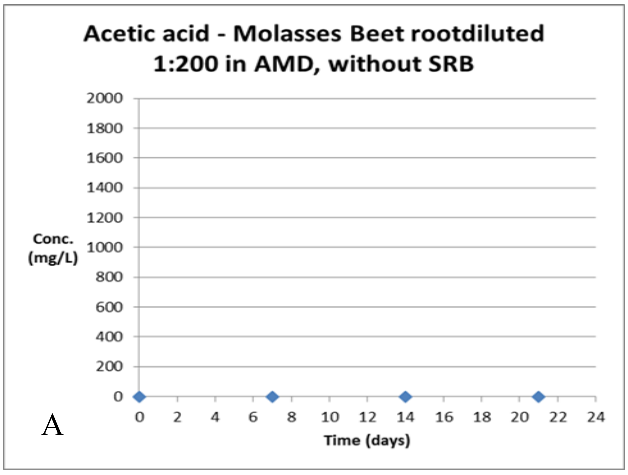
Fig 4.10- Depicting organic acids: (A)- Acetic acid (B)- Propionic acid (C)- Butyric acid (D)- Lactic acid in the test with beetroot molasses diluted 1:200 in AMD **with** SRB and 10% calcite tailings. Data are average of triplicates and error bars indicates standard deviation of average values.

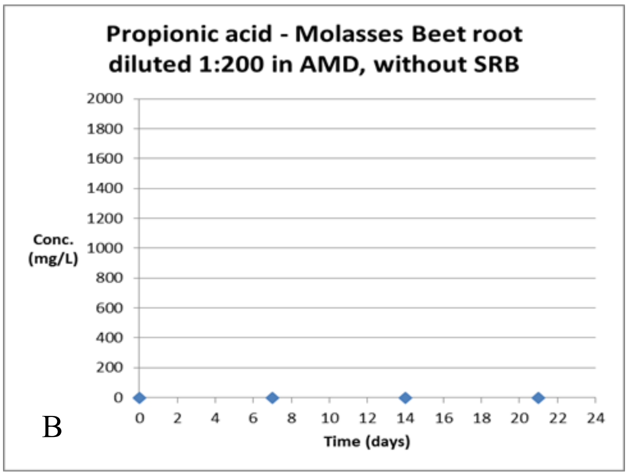
4.4.1.4 - Organic acids: beetroot molasses diluted 1:200 in AMD without SRB and with 10 % calcite tailings

Acetic acid, formic acid, propionic acid and butyric acid were below limit of detection for their tested standards(Table 3, annex 4) in this medium without SRB innoculum (Figures 4.11 A,B and C).

However, the case for lactic acid aicd showed some traces of it's concentration (55.47mg/L) in media on day 7 but was not significantly different across all the other days of monitoring (Figure 4.11D).

We could basically put forward that, for this medium without microbial inoculum, the fermentation of sugars generating organic acid products remained unrevealed as there were no fermentative organisms that would have sparked that evolution.





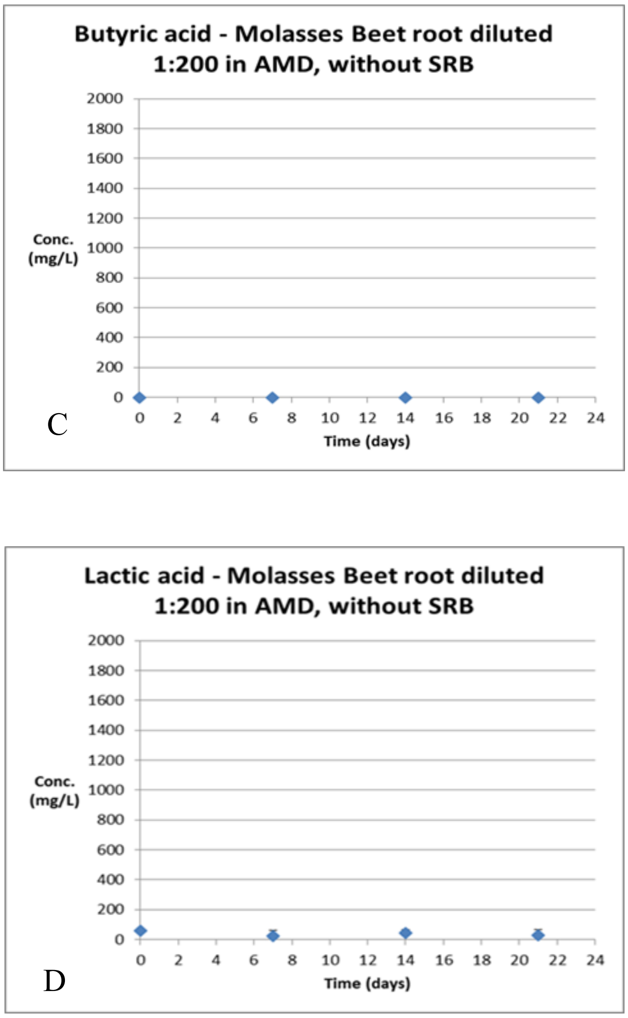


Fig 4.11- Depicting organic acids: (A)- Acetic acid (B)- Propionic acid (C)- Butyric acid (D)- Lactic acid in the negative control – beetroot molasses diluted 1:200 in AMD **without** SRB and with 10% calcite tailings. Data are average of triplicates and error bars indicates standard deviation of average values.

4.4.2- Carbon compounds in batch tests with orange molasses: sugars and organic acids

4.4.2.1- Sugars: orange molasses diluted 1:200 in AMD with SRB and 10 % calcite tailings

a. Sucrose

The amount of sucrose detected on day 0 in this media was about 1303mg/L and this appreciated a little above this by day 7 to something around 1407.42mg/L as the bacteria community initiate activity on the sugar components to have it more easily available.

However, taking into account again orange molasses characterization given by the supplier (table 3.1 of section 3.1.1) as 26.2% (or 262g/L) sugar components, the expected sugar content of the 1:200 dilutions should be about 1310mg/L and this justifies what already had been obtained by day 0 monitoring in sucrose concentration knowing that sucrose is a dissacharide molecule of glucose and fructose monomeric units. By day 14, the available sucrose had reduced to 318.33mg/L about 5 times it's amount the previous day 7.

Following day 21 evolution, about 3/4 the amount on previous day 14 remained in media with a revealation of 247mg/L concentration value. Again, this showes that the orange molasses degraded to more simpler sugar components and was taken up by SRB. See evolution of sucrose in Figure 4.12A below.

b. Glucose

With glucose, the evolution trend in the media with SRB innoculants, showed a sharp disapperance of the available glucose concentration 1192mg/L detected as at day 0 of innoculation. The SRB consortia consumed off the glucose molecules in a quick fashion leaving behind little or nothing which was not so significant upon detection during the monitoring by days 7 and 14. See Table 2of annex 4. However, the amount of glucose that was still seen in the media system as at day 21 of about 331.33mg/L might have been due to a shift of the SRB community population as the previous days 7 and 14 before now, showed evolution below limit of tested glucose standard further strengthening the point that members of the SRB community couldn't have survived beyond day 14. Hence extension of the activity of other syntrophic members lead to insignificant amount of more glucose remaining as could be seen by day 21 (Fig 4.12C).

c. Fructose

The evolution monitoring trend showed by this media, revealed fructose concentration level by day 0 and day 7 at about 141mg/L and 178mg/L respectively and was below limit of detection (Table 2, annex 4) again the following day 14 and 21. Reasons being that SRB community consortia might have consumed them off.

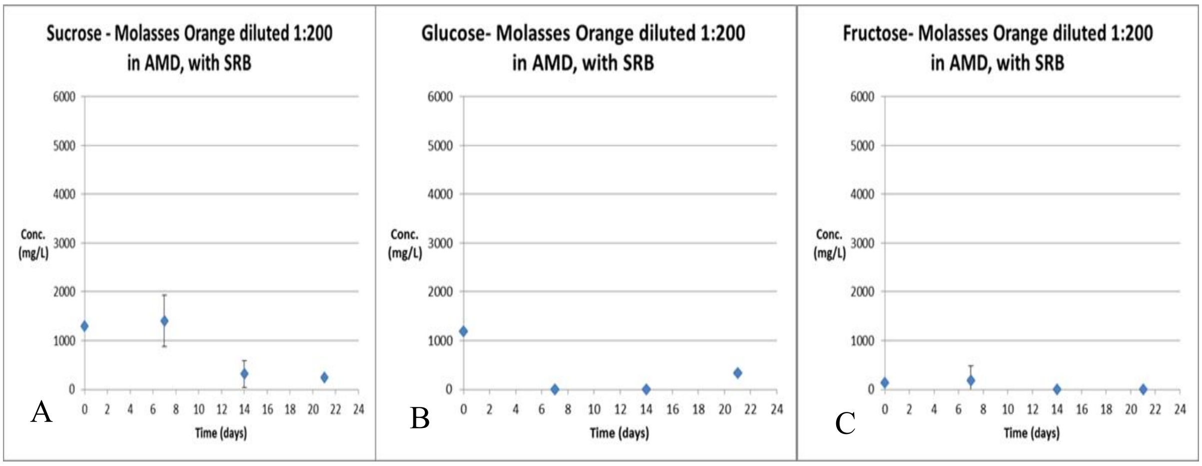


Fig 4.12- Depicting sugar compounds: (A)- Sucrose (B)- Glucose (C)- Fructose from orange molasses diluted 1:200 in AMD **with** SRB and 10% calcite tailings.

Data are average of triplicates and error bars indicates standard deviation of average values.

4.4.2.2- Sugars: Negative control- orange molasses diluted 1:200 in AMD without SRB and with 10 % calcite tailings

a. Sucrose

Without SRB innoculants, by day 7 the amount of sucrose hiked to almost 2000mg/L value from what it was the very first day 0 of innoculation at 1303mg/L; probably due to fermentation by microbes present in the orange molasses. By day 14, the trend was on the dwindling side with majority of generated sucrose already consumed by other microorganisms and similar reasons as indicated in the case of beetroot molasses. Day 21 showed almost the same level in sucrose concentration as at day 14 of 762.83mg/L with a value of 741.67mg/L. However, from the evolved values and in comparison to the media with SRB inoculants, concentration of sucrose revealation still made a difference though not very significant due to other sugar dependent microorganisms present in the orange molasses (non sterile). In any case, media with SRB showed a concentration reduction profile of sucrose which was not outrightly the case with this particular media without SRB; proofing that the SRB depended on sucrose.

b. Glucose

Glucose concentration on day 7 was undetected in media following a concentration value of about 1192mg/L the previous day 0 innoculation.Reasons to this, might have been due to the presence of microorganisms in the non sterile orange molasses, taking up the degraded glucose molecules. Day 14 revealed a concentration of 1462mg/L and this resurge could be attributed to other fermetative micoorganisms present in the orange molasses degrading the sugars.

By day 21, though without SRB innoculants, concentration declined a little from previous day 14 to a value of 1100mg/L. Noteworthy, is the traceable level of glucose consumption witnessed in this media without SRB. Much of this might have been attibuted to the presence of other fermentative microorganisms from the non sterile orange molasses. In real sense, glucose concentration was significantly different with respect to the media with SRB innoculants over the days of monitoring; strengthening our point that simpler sugar molecules like glucose were supportive of SRB.

c. Fructose

Fructose concentration after innoculation by day 0 remained more or the less the same as day 7; but surging up a little by day 14 and ended up declining by day 21. Concentration values of 141mg/L, 298.67mg/L, 928.33mg/L, 469.33mg/L were recorded for days 0, 7, 14 and 21 respectively.Though with a revealation of uptake of fructose; media did not significantly represent evolution declining trend fashion showed by the SRB innoculated media as has been previously reported which had more greater and faster consumption.

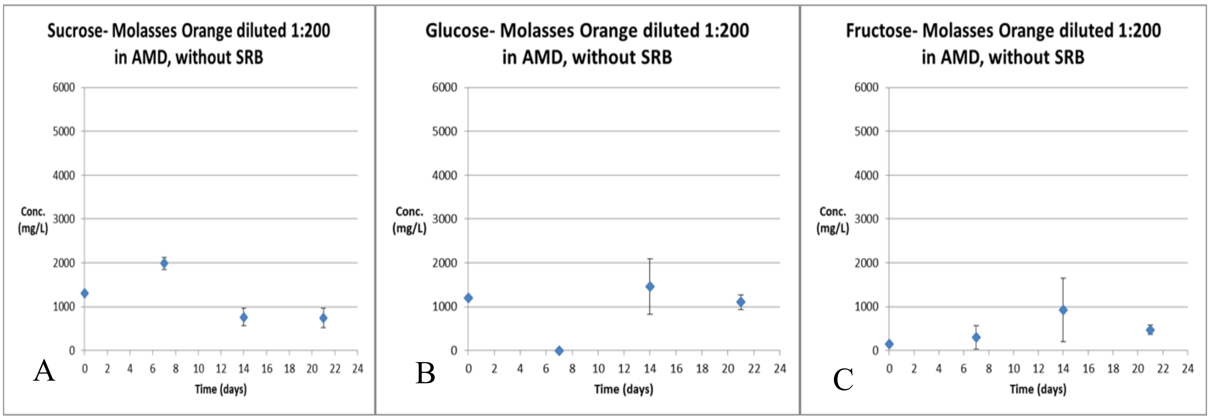


Fig 4.13-Depicting sugar compounds: (A)- Sucrose (B)- Glucose

(C)- Fructose from orange molasses diluted 1:200 in AMD **without** SRB and with 10% calcite tailings.

Data are average of triplicates and error bars indicates standard deviation of average values.

4.4.2.3- Organic acids: orange molasses diluted 1:200 in AMD with SRB and 10 % calcite tailings

a. Acetic acid

We observed the generation of acetic acid by day 7 stabilizing more or less at the same concentration the following days 14 and 21(Figure 4.14A).This verifies that in the orange molasses, some SRB consortia members were responsible of degrading the orange molasses to yield acetic acid, which the other members couldn't completely consume.

The reason being that acetic acid is an intermediate in organic substrate fermentation not completely utilized by most SRB's (Lens et al.,2002).

b. Lactic acid

Lactic acid had a slight decrease in concentration by day 7, stablized more or less at the same concentration on day 14 and completely consumed off the media by day 21. See Figure 4.14B. This reveals that, it was generated and used up by the SRB community consortia during the course of their activity.

Other volatile fatty acids (VFA) like formic acid, propionic acid and butyric acid were not detected as their concentrations were below the concentrations for their respective test standards used for the analysis. See Table 3 of annex 4.

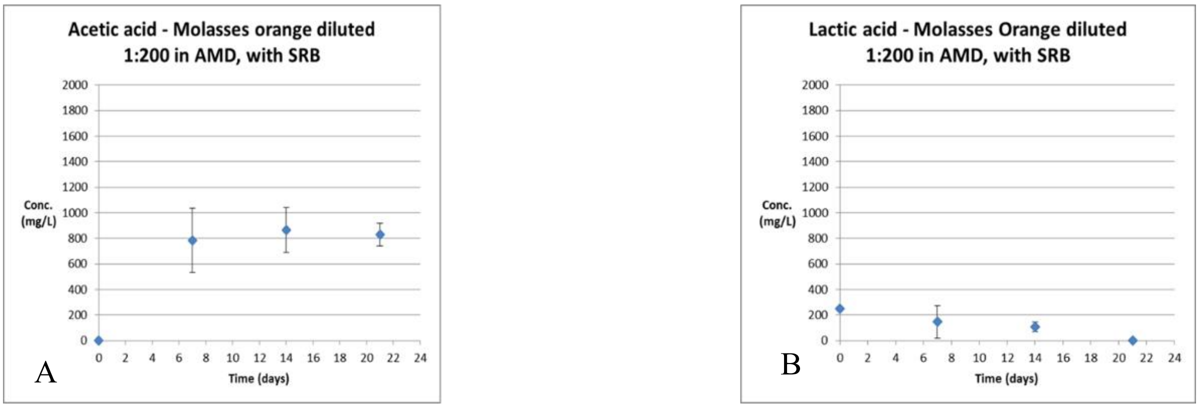


Fig 4.14- Depicting organic acids: (A)- Acetic acid (B)-Lactic acid in the test with orange molasses diluted 1:200 in AMD **with** SRB and 10% calcite tailings. Data are average of triplicates and error bars indicates standard deviation of average values.

4.4.2.4- Organic acids: orange molasses diluted 1:200 in AMD without SRB and with 10 % calcite tailings

a. Acetic acid

Acetic acid was below detection in this media as the concentration was below the limit of detection of the standard used in analysis (Figure 4.14A). See Table 3 of annex 4.

b. Lactic acid

Lactic acid concentration remained more or less in media upto day 14 from previous day 0 and 7 and then a little decline by day 21. This might have been taken up by other microorganisms present in the orange molasses. However, there were no significant difference between the lactic acid concentrations for the various day of evolution monitoring where more or less stabilized amount remained in solution till day 21. This was the opposite of the trend we saw in media with SRB where concentrations declined in a more steadily fashion to support SRB. See Figure 4.15B.

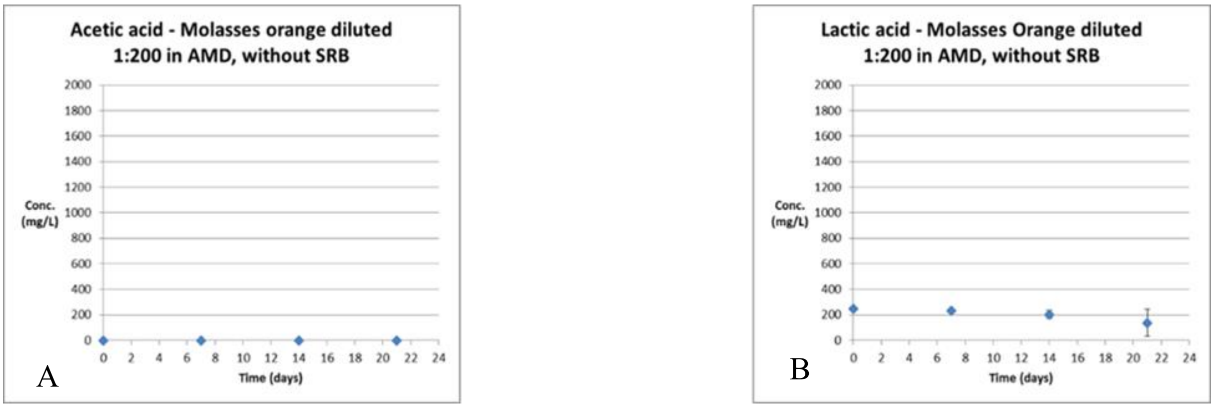


Fig 4.15- Depicting organic compounds: (A)- Acetic acid (B)-Lactic acid in the negative control – orange molasses diluted 1:200 in AMD **without** SRB and with 10% calcite tailings.

Data are average of triplicates and error bars indicates standard deviation of average values.

5. Continuos flow experiments

5.1- Effluent from the upflow anaerobic packed bed (UAPB) bioreactor fed with orange molasses diluted in AMD – reactor 1

Dosement of bioreactor 1 with orange molasses as the carbon source/electron donors started on day 935 of reactor lifetime since its setup. Previously, the organic compound being used was ethanol. Just before starting to feed bioreactor 1 with orange molasses limestone chippings were added to the bioreactor as described in the materials and methods, aiming to prevent acidification caused by fermentation of sugars. Two days after, by day 937, we monitored effluent discharge from the reactor as to see the evolution of the parametric factors indicative of SRB activity in the simultaneous sulphate reduction and heavy metal removal remediation purposes of this work. At this time of the reactor 1 effluent monitoring, we did actually accommodate the fact of not expecting anything extraordinary in terms of the SRB activity. Hence, we bored in mind that the reactor system needs time to adjust and re-shape its microbial dynamics haven just switched carbon source/electron donor supplement.

5.1.1- pH, redox potential (Eh) and electrical conductivity (EC)

The pH value of reactor 1 effluent was 8.84 on day 937 and redox potential value was -317mV, reflecting favourable conditions for SRB activity, hence establishment of an anaerobic environment supportive of shift in sulphate to sulphide. The trend of the pH and Eh parametric evolution assumed values respectively around 6.5 and -250mV for days 943, 950, 957, 970 and 978 (Figure 5.1A and 5.1B). Beyond these days, as the reactor system progressed following the breakdown of the orange molasses, pH values took a slight leap from 6.37 on day 985 to 6.58 on day 999. By day 1003, pH value stabilized at 6 point something and maintained this value until day 1012 at a value of 6.77. Exceptionally to this trend again was on day 1019 where the pH surged around neutral value at 7.4 and then dropped again on day 1027 at 6.84. Thus, pH never dropped below 5.5 reported to be critical for efficient SRB metal bioremediation (Elliot, et al., 1998). See Figure 5.1A.

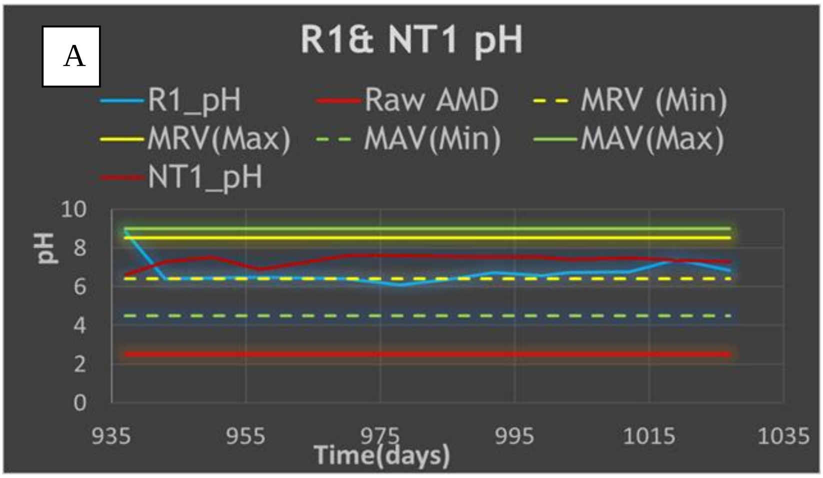
Up until day 985, Eh was below -200mV. However, by day 992, Eh switched a little higher to -198mV. In any case, this was still favourable as the required anaerobic environment for SRB activity of below -100mV reported by Postgate (1984) was still maintained. By day 999, the system adjusted again to previous Eh values below -200mV (Figure 5.1B).

With pH trending fashion near neutral values between 6 and 8 and Eh values maintained below -200mV, the system was in good alkalinity conditions with a favourable anaerobic environment to promote SRB growth and activity.

The conductivity just after the start of dosing with orange molasses, maintained values of around 3 point something mS (Figure 5.1C). By day 957, conductivity showed a spike at 4.18mS but then dropped a little to 4.06mS and 3.86mS by days 970 and 978 respectively. Down the line as the system progressed, revelations from days 985, 992, 999 showed conductivity values profiling at more or less the same values of 3.85mS, 3.77mS and 3.92mS respectively. Days 999 and 1003 saw a little rise in EC values of 3.92mS, 4.92mS respectively and steadied at the same level up to day 1012 at 4.32mS. By day 1019, it dropped a little to 4.13mS relatively to the previous day and surged again to 4.31mS on day 1027 (Figure 5.1C).

There was an evident significant difference in values of conductivity recorded of the influent neutralization tank to reactor 1 system with EC values below 3.5mS while EC effluents values of reactor1 were at 3.5mS or even higher above 4mS with days 937 and 943 the only exception. In as much as the evolved EC values from reactor system 1 did not interfere with the biological functions of the SRB communities, a probable explanation of the hike in EC values of the effluents stems from the applied limestone neutralizing agents used as the buffering agent of the media. Hence, some calcium and magnesium ions released from limstones to AMD must have played some role in the conductivity rise.

From the recorded values of the pH, Eh and conductivity following the introduction of orange molasses into reactor 1, we can make up that the SRB consortia were favoured as the system conditions of the controlled parameters revealed optimized values for their efficient growth and activity.



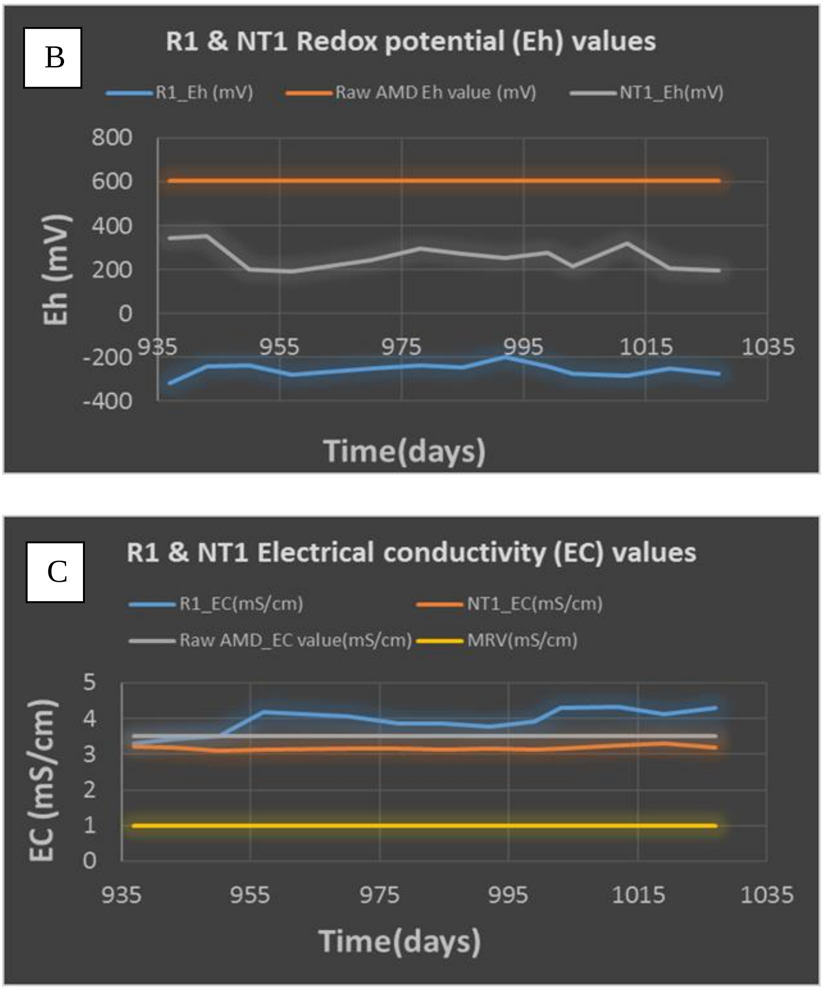


Fig 5.1- Depicting: (A) pH, (B) redox potential (Eh) and (C) conductivity (EC) evolution monitoring of bioreactor fed with orange molasses diluted in AMD – reactor 1 and neutralization tank 1.

Legends- R1- Reactor 1; NT1- Neutralization tank 1; AMD- Acid mine drainage; MRV- Maximum recommended value; MAV- Maximum admissible value.

5.1.2- Biological sulphate reduction

Although pH, Eh and electric conductivity values favour SRB activity; sulphate concentration following the introduction of orange molasses into reactor 1 surged up and simultaneously biogenic sulphide generation dropped considerably. Immediately after starting the dosing of orange molasses into reactor 1, on day 937, sulphate concentration was much lower (295mg/L) than in raw AMD (Figure 5.2). This was because, the reactor had been efficiently operational with SRB fed on ethanol previously. This is further evident looking at the sulphate concentration evolution profile of this reactor pre day-935, which was below the maximum recommended value (MRV) of 575mg/L as issued by the Portuguese national legislation decree no: 236/98 for irrigation waters. Then, until day 943 we recorded a sharp shoot in sulphate concentration with a peak value of 2543mg/L (Figure 5.2). This might have occurred as the reactor microbial community did not had time yet to respond and adjust to introduction of a new substrate as carbon source/electron donor. By day 950, the sulphate concentration took a sliding reduction from the previous week establishing a value of 1070mg/L and by day 957 an almost 2-fold drop was observed. However, after that, there was appreciation once again in sulphate concentration to concentrations around 1000mg/L Thus, since enough time had passed to acclimatize the bacterial community to the new source of nutrients and efficiency in the sulphate reduction did not improve, by day 993 the dosage of orange molasses supplied to the reactor was changed by a 50 % increase: from 0.005 to 0.0075 ml per hour. See Table 3.1 on the operating conditions of the upflow anaerobic packed bed (UAPB) bioreactors used in this work. Effluent evolution monitoring immediately after augmenting the amount of orange molasses availability for the bacteria community per hour reflected an improved sulphate reduction subsequently (Figure 5.2). By day 999, we recorded a sulphate concentration of 789mg/L, almost 40% in value of the previous week. Day 1003 witnessed further drop to around 500mg/L and stayed like that until the end of this work. This concentration is below the maximum recommended value (MRV) for irrigation waters set by decree no- 236/98, annex XVI of Portuguese national legislation. As concentration of sulphate begin to drop after nutrient augmentation, sulphide concentration surged simultaneously. Sulphide concentration had values around 50mg/L before nutrient augmentation on day 993, and then went rising to 119.40mg/L and 194.10mg/L for days 999 and 1003 respectively, haven a peaked at day 1012 with a sulphide concentration of 362.73mg/L but then dropped to values between 100 and 200mg/L.

In summary, we have noticed that post period of the orange molasses injection showed an increase in sulphate concentration as the dynamics of the microbial community consortia tried to adjust a shift in balance due to nutrient substrate change. It was necessary to allow time for the fermentative organisms to begin degrade molasses sugars, producing smaller molecules viable to feed SRB. However, as the orange molasses becomes more readily available and with an increase of nutrient quantity by 50%, sulphate concentration starts to drop reflecting once again an enhanced sulphate reduction activity of the SRB members.

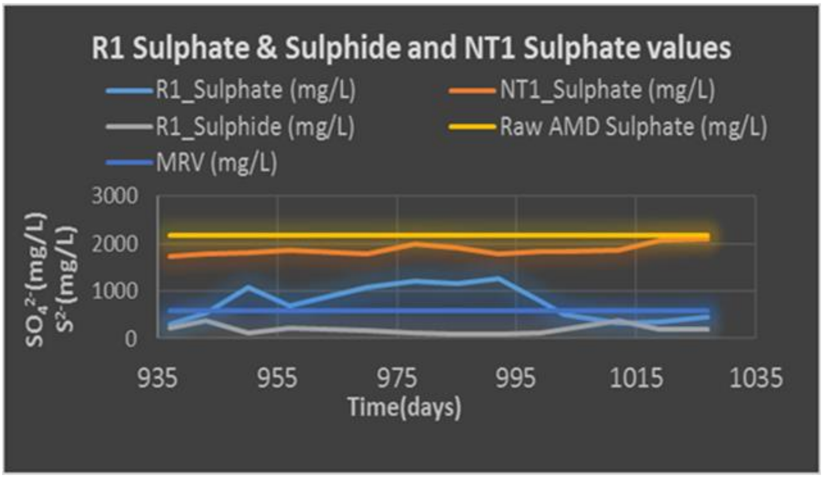


Fig 5.2- Depicting sulphate and sulphide evolution monitoring of bioreactor fed with orange molasses diluted in AMD – reactor 1 and Neutralization tank 1.

Legend: R1-Reactor 1, NT1- Neutralization tank 1, AMD- Acid mine drainage, MRV- Maximum recommended value.

5.2- Effluent from the upflow anaerobic packed bed (UAPB) bioreactor fed with beetroot molasses diluted in AMD – reactor 2

Dosement of bioreactor 2 with beetroot molasses as the carbon source/electron donors started on day 940 of reactor lifetime since its setup. Reactor 2 had previously been on wastewater treat plant (WWTP) sewage water as carbon source/ electron donors. Just before the start of feeding bioreactor 2 with beetroot molasses, limestone chippings were added to the bioreactor as described in the materials and methods, aiming to prevent acidification caused by fermentation of sugars. Three days after, by day 943, we monitored effluent discharge from the reactor as to see the evolution of the parametric factors indicative of SRB activity in the simultaneous sulphate reduction and heavy metal removal remediation purposes of this work. At this time of the reactor 2 effluent monitoring, we did actually accommodate the fact of not expecting anything extraordinary in terms of the SRB activity. Hence, we bored in mind that the reactor system needs time to adjust and re-shape its microbial dynamics haven just switched carbon source/electron donors supplement.

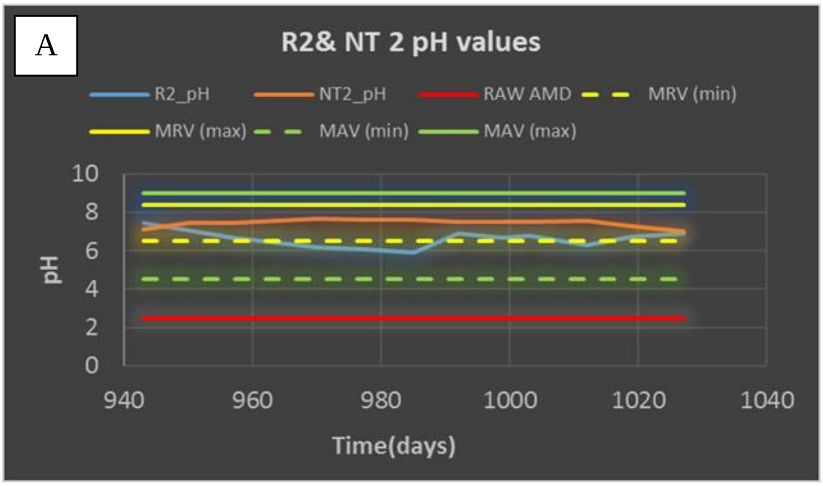
5.2.1- pH, redox potential (Eh) and conductivity (EC)

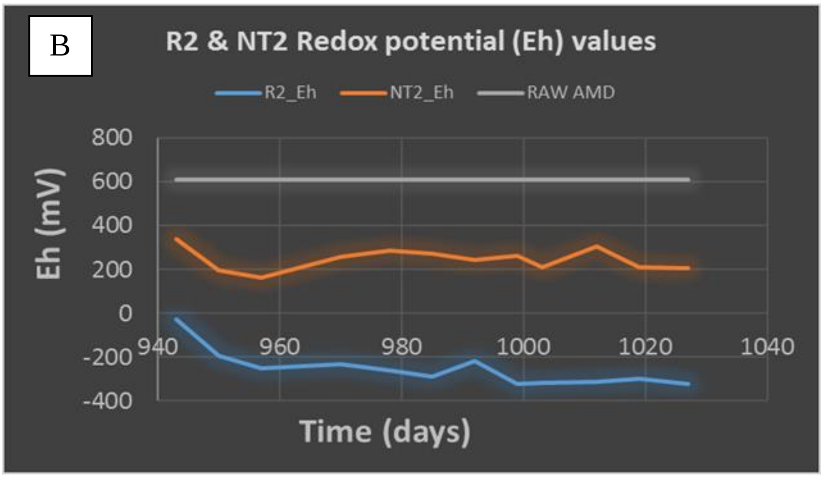
pH values evolving from our parametric monitoring since the commencement of beetroot molasses dosing of this reactor, showed a sliding drop fashion in values. By day 943 and just 3 days after the initial dosement of beetroot molasses into reactor 2, pH value was 7.47. By day 950, it had dropped to 7.05 and continued with values in a land sliding fashion of 6.7, 6.19 and even as low as 6.06 for day 957, 970 and 978 respectively. By day 985, pH value had dipped as low as 5.93 and was heading towards a critical point value for SRB efficiency (Figure 5.3A). In order to avoid the reactor creeping more into low pH values the coming days which might affect the efficiency of the SRB in sulphate reduction due to high medium acidity, we discharged reactor 2 of it's AMD and beetroot molasses content. The medium matrix alkalinity upgrading was with fresh limestones making a portion of the gravel mixture and already contained limestones in the reactor. Afterwards, our results from the monitored pH value by day 992 revealed an improvement in pH value recording at 6.92, which is conducive for SRB (Figure 5.3A). Days 999 and 1003, revealed pH of values a little below the range of 7 than recorded the previous week with values of 6.69 and 6.81 respectively. pH continued to improve with values of 6.29, 6.75 and 6.90 for days 1012, 1019 and 1027 respectively. At this point, the system had re-adjusted to the pH range of near neutral values after the limestone addition. These values are quiet conducive for SRB mediated remediation anaerobic environments. See figure 5.3A.

The redox potential (Eh), three days after the commencement of beetroot molasses feeding into reactor 2, witnessed an Eh value of -28mV possibly due to system re-adjustment. The Eh saw further reduction by day 950 at a value -195mV. By day 957, 970, 977, 984, and 992, Eh values dropped to -253mV, -232mV, -260mV, -290mV, -218mV respectively.

With more control of the pH value, which was heading towards critical point the previous week and then improved through fresh limestone addition, bioreactor 2 further showed up with Eh values of around -300mV on the average for days 1003, 1012, 1019 and 1027 respectively. Apart from the value for day 984 of -218mV, all the other days except for day 943 were we recorded the highest value of -28mV, the rest days showed favourable Eh values for SRB activity. A minimum Eh value of -100mV is needed for the establishment of an anaerobic environment needed for SRB maintenance (Postgate, 1984). With the values already revealed from the bioreactor matrix below -100mV except -28mV witnessed for day 943 at the very beginning; the Eh parametric condition of bioreactor 2 is comfortable to initiate a shift in sulphate.

The conductivity of the medium following bioreactor dose commencement of molasses beetroot was 2.55mS by day 943. Conductivity value rose again to 4.65mS by day 950. By day 957, it saw a drop once small to 3.98 as the system continues to re-adjust. By day 970, 977, 985 and 992, conductivity values appreciated on the average to 4.39, 4.48, 4.45, and 4.85(mS) respectively (Figure 5.3 C). EC values beyond day 992 after the introduction of fresh limestone neutralizers into the bioreactor matrix surged to values between the range of 5 and near 6mS. This could have resulted due to the migration of Ca^{2+}ions into the matrix medium. However, the conductivity of the treated media effluents from the reactor did surged in the coming days after dosing with beetroot molasses and saw further increase following the pH control of the system on day 992. Presence of particulate ions in media as indicated by the conductivity parameter and with their values around 4 point something on the average were not considered limiting to bacteria activity so long as factors of pH and Eh remain favourable for SRB maintenance.





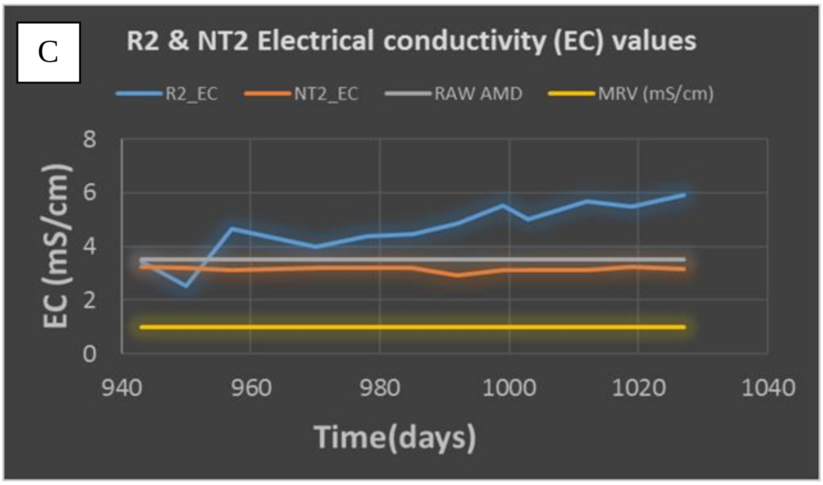


Fig 5.3- Depicting: (A) pH, (B) redox potential (Eh) and (C) conductivity (EC) evolution monitoring of bioreactor fed with beetroot molasses diluted in AMD – reactor 2 and neutralization tank 2.

Legends- R2- Reactor 2; NT2- Neutralization tank 2; AMD- Acid mine drainage; MRV- Maximum recommended value; MAV- Maximum admissible value.

5.2.2- Biological sulphate reduction

We had a response of high sulphate concentration value of 1158mg/L from bioreactor 2 by day 943 haven dosed the bioreactor with beetroot molasses on day 940. By day 950, the sulphate concentration had already gone up to 1328mg/L as the bioreactor begin to re-adjust to the change of the new carbon source nutrient. Biologically generated sulphide from the analyzed bioreactor effluents were significantly low at 2.1mg/L and 11.87mg/L respectively as the sulphate concentration remains considerably high (Figure 5.4). By day 957, sulphate concentration had gone a low to 1043mg/L and sulphide concentration appreciating to 89.6mg/L. Day 970 revealed a further rise in sulphate concentration at a value of 1186mg/L with a corresponding sulphide concentration of 109.20mg/L. By day 977 and 984, sulphate concentration re-initiated a downside trend fashion with 1111mg/L and 1026mg/L respectively. Correspondingly, sulphide concentration rose to 128.28mg/L for day 977 and dropped again by 984 with a value of 71.31mg/L setting a sort of rise and fall trend in sulphate concentration and corresponding sulphide generation with reasons probably due to the re-adjustment of bioreactor in response to new carbon source, the dip in pH value and fresh limestone addition response. By day 992, our result showed a hike of sulphate concentration to 1546mg/L and further reduction in biogenic sulphide to a low of 25.65mg/L after the introduction of fresh limestone to condition acidity. As the bioreactor tends to be more stabilized,1003 revealed a reduction in sulphate following an augmented beetroot molasses dosement of 50% of it’s 0.005ml/hr nutrient flow rate to 0.0075ml/hr nutrient flow rate on day 999. With sulphate still in the range of over a 1000mg/L, sulphide concentration relatively improved from values of the previous week with concentration values of 90.24mg/Land 67.80mg/L respectively. By days 1012 and 1019, sulphate concentrations declined to values in the range of hundred (Figure 5.4) approaching the 575mg/L limit standard for irrigation waters in Portugal (Decree law no: 236/98, Annex XVI, 1998). Biogenic sulphide concentration increased correspondingly as more sulphates are reduced on these days. From 827mg/L on day 1012 to 727mg/L on day 1019, sulphate concentration dropped to below 500mg/L with a value of 336mg/L on day 1027 complying with the set standard limit in irrigation waters in Portugal. Sulphide concentration relatively has been on the rise over the days 1012, 1019 and 1027 with values above 100mg/L respectively. With the optimization of the pH value, conducive Eh value and improved carbon source supply for the SRB by 50%, we have seen from our investigation that bioreactor 2 needed some time for the fermentative microorganisms to degrade the sugar molecules into more easily available components for SRB as it responded in a faster and steadied way of sulphate reduction.

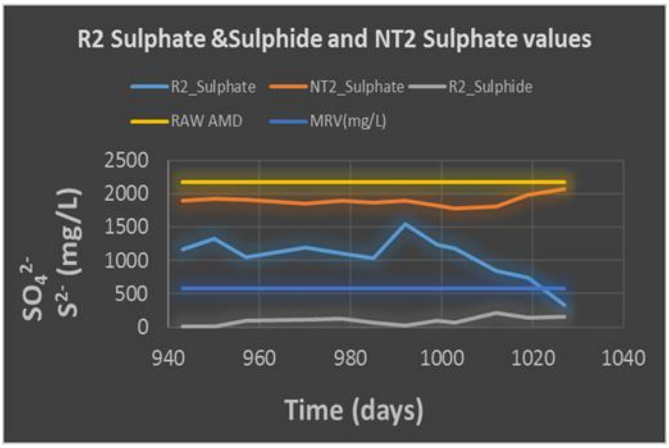


Fig 5.4- Depicting sulphate and sulphide evolution monitoring of bioreactor fed with beetroot molasses diluted in AMD – reactor 2 and Neutralization tank 2.

Legend: R2-Reactor 2, NT2- Neutralization tank 2, AMD- Acid mine drainage, MRV- Maximum recommended value.

5.3 - Neutralization tank

The bioremediation stage considered in the system used in this work is preceded by a stage where the pH of the raw AMD is neutralized prior to finding way up to the bioreactor columns. The potential adavantages of metal sulphide precipitation include generation of lower sludge volume and lower solubility of the metal sulphides generated compared with hydroxides or carbonates (Table 5.1) (Bayrakdar et al., 2009; Gonzalez Silva et al., 2009). Besides, a high degree of selective metal precipitation is possible with sulphide in contrast to hydroxide precipitation (Figure 5.5) (Huisman et al., 2006). In the neutralization stage pH values of the raw AMD water are adjusted to values already investigated by other researchers and seen to be optimal SRB activity and growth. Keeping pH values above 6.5 and below 8.5 steadily as reported by Barton and Hamilton (2007), guarantees ideal condition for SRB. Besides, prior to the upflow of the AMD raw water into the reactor, selective precipitation of metals by pH control is possible hence, optimal pH values for the precipitation of several metals have been suggested by Hammack et al., (1994), Govind et al., (1997) and Tabak et al. (2003): ZnS precipitate at pH values between 2 and 5. Fe (II) do not precipitate as Fes until pH gets to value above 4.5 while copper can precipitate at extremely low pH values (≤ 1.0) as CuS without the precipitation of other metals. With all these possibilities, selective precipitation of metals as sulphides via pH control has been exploited in this work

Table 5.1- Solubility constants of some metals in their various forms of hydroxides, carbonates and sulphides at 25°C (Extracted from Sillen and Martell, 1964).

		K_{SP}	
Metal	OH^-	CO_3^{2-}	S^{2-}
Cu^{2+}	$2x10^{-19}$	$1.3x10^{-10}$	$1.0x10^{-36}$
Fe^{2+}	$2.0x10^{-15}$	$5.0x10^{-11}$	$1.0x10^{-18}$
Zn^{2+}	$5.0x10^{-17}$	$1.0x10^{-7}$	$1.0x10^{-23}$
Cd^{2+}	$2.0x10^{-14}$	$2.5x10^{-14}$	$1.0x10^{-28}$
Pb^{2+}	$4.0x10^{-15}$	$1.6x10^{-13}$	$1.0x10^{-28}$
Ni^{2+}	$2.0x10^{-16}$	$1.3x10^{-7}$	$1.0x10^{-22}$

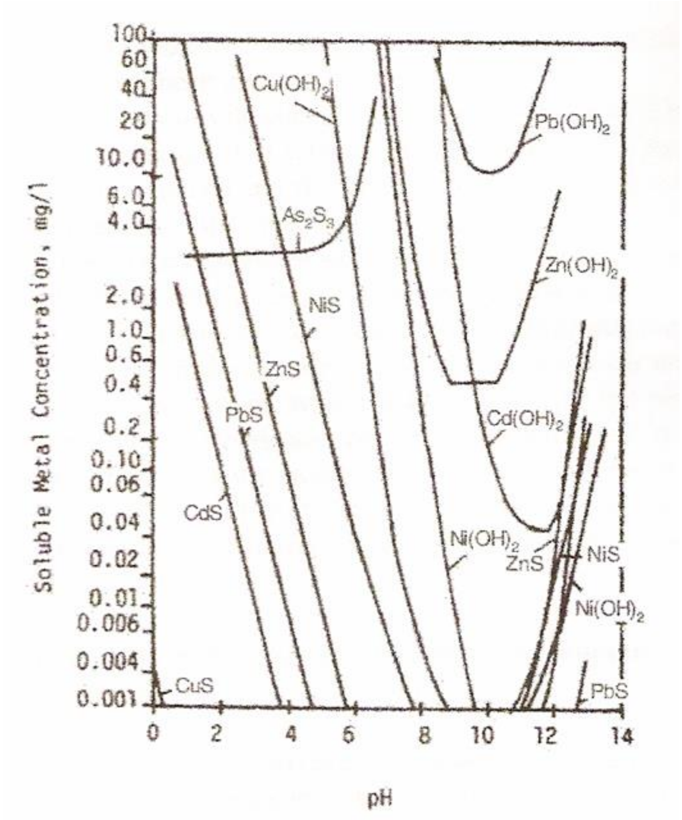


Fig 5.5- Solubility of metal hydroxides and sulphides as a function of pH (Extracted from Huisman et al., 2006)

5.3.1- Neutralization tank preceding the UAPB fed with orange molasses – neutralization tank 1

In the neutralization tank 1, pH values rose from the 2.49 in raw AMD (Table 4.1) to values between 6.5 and 7.5 (Figure 5.1A). Dwelling on this, the pH values were optimal and maintained a favourable range required for SRB sustenance.

Beside favourable pH values at this stage of the bioremediation process, redox potential (Eh) and conductivity remain more or less at steady points which do not influence what is expected in the bioreactor in terms of kinetic shift of sulphate concentration to sulphide generation.

The Eh evolution values in the neutralization tank fluctuated within the range of 300mV to 198mV. Values were still on the positive side of the scale for the Eh at this level as there was no established anaerobic environment. The AMD, even though conditioned to neutralize pH, is expected to still mobilize sulphates. This was confirmed by the level of sulphate concentration at this point which was still more or less around 2000mg/L for the days of investigation (Figure 5.2). In what concerns the monitored metals (Zn, Fe, Cu and Al), their concentrations dropped drastically in the neutralization tank (Figures 5.7A, 5.8A, 5.9A and 5.10A) as they precipitate due to pH neutralization. Al, Fe and Cu are removed to values below their respective maximum recommended limites for irrigation waters in Portugal. However, in the case of Zn, concentrations in the neutralization tank most times were above 2 mg/L, the recommended limit for irrigation water. This will be discussed more in detail in section 5.4- Heavy metal removal. Electrical conductivity (EC) values in the neutralization tank were resonating around 3point something or more less 4mS for the evolution monitoring days (Figure 5.1C), values that are higer than around 3mS measured in the raw AMD (Table 4.1). Reasons attached to this might have been the removal of some metal concentrates at this point.

5.3.2- Neutralization tank preceding the UAPB fed with beetroot molasses – neutralization tank 2

Similarly, in the neutralization tank 2, pH values rose from the 2.49 in raw AMD (Table 4.1) to values between 6.5 and 7.5 (Figure 5.3A). Dwelling on this, the pH values were optimal and maintained a favourable range required for SRB sustenance.

Beside favourable pH values at this stage of the bioremediation process, redox potential (Eh) and conductivity remain more or less at steady points which do not influence what is expected in the bioreactor in terms of kinetic shift of sulphate concentration to sulphide generation.

The Eh evolution values in the neutralization tank fluctuated within the range of 300mV to 200mV. Values were still on the positive side of the scale for the Eh at this level as there was no established anaerobic environment. The AMD, even though conditioned to neutralized pH, still mobilize sulphates. This was confirmed by the level of sulphate concentration at this point which was still more or less around 2000mg/L for the days of investigation (Figure 5.4). With respect to the the monitored metals (Zn, Fe, Cu and Al), their concentrations dropped drastically in the neutralization tank (Figures 5.11A, 5.12A, 5.13A and 5.14A) as they precipitate due to pH neutralization. Again, Al, Fe and Cu are removed to values below their respective maximum recommended limites for irrigation waters in Portugal. However, for Zn, concentrations in the neutralization tank most times were above 2 mg/L, the recommended limit for irrigation water. This will be discussed more in detail in section 5.4- Heavy metal removal.

Electrical conductivity (EC) values in the neutralization tank were resonating around 4point something for the evolution monitoring days (Figure 5.3C), values that are higher than the 3mS measured in the raw AMD (Table 4.1). Reasons attached to this might have been similar case already reported for neutralization tank 1

5.4- Heavy metal removal

With the availability of the generated sulphide from the biological sulphate reduction by sulphate reducing bacteria (SRB), some metals still in solution after the neutralization stage can be removed by precipitation as metal sulphides. Potential advantages of metal sulphide over their corresponding hydroxides or carbonates have been investigated and reported by Bayrakdar et al., (2009) (Table 5.1). Their lower sludge volume and lower solubility makes them preferable to their carbonates or hydroxide counterparts.

5.4.1 Metal removal in system 1- Neutralization tank 1 and Bioreactor 1 metal removal

I. Zinc (Zn)

With zinc concentration in raw AMD as 43.26mg/L (Table 4.1); from the neutralization tank 1, zinc metal evolved at a high value of about 13.47mg/L by day 937. This could be due to little change in pH balance that dropped a little with a value of around 6.6 as we added fresh limestone to the neutralization tank. 7 days after and by day 943, zinc concentration dropped low to about 3 times what it was the previous week at 4.38mg/L (Figure 5.7A).

By this day, there was an improvement in pH value to a point of around 7.3. Zinc concentration continue to drop even lower than the set limit of 2mg/L of maximum recommended value in Portuguese national legislation for irrigation waters. By day 950, as pH continue to stabilize around neutral point with a value of 7.54, zinc value recorded 0.74mg/L (Figure 5.7A).

By day 957, as pH drop in the tank, to a value of 6.9, zinc concentration rose to 1.03mg/L. This appreciative trend in zinc concentration continued as the pH remains more or less stable around neutral point with values of 1.47mg/L, 2.07mg/L, 2.32mg/L, 2.26mg/L, 2.39mg/L, 5.44mg/L, 5.33mg/L and 6.29mg/L for days 970, 978, 985, 992, 999, 1003, 1012, 1019 and 1027 respectively (Figure 5.7A).

From our investigation, we observed relative large variations in zinc concentrations to pH change. On the average, pH value of 7.5 and above gave a considerate reduction in zinc concentration in relative terms with other values over the days.

However, the level of zinc metal at this decontamination point was still most of the days above the maximum recommended value (MRV) of Portuguese national decree for irrigation waters. We did not dwell much on these values as the reactor takes off the remaining metal concentration on a more efficient level. The rise and fall trend in zinc removal pattern in connection with pH was expected as the chemical balance diagram of zinc can further explain the unsteady rise and fall in zinc metal concentration at pH above 7.5 (Fig 5.6).

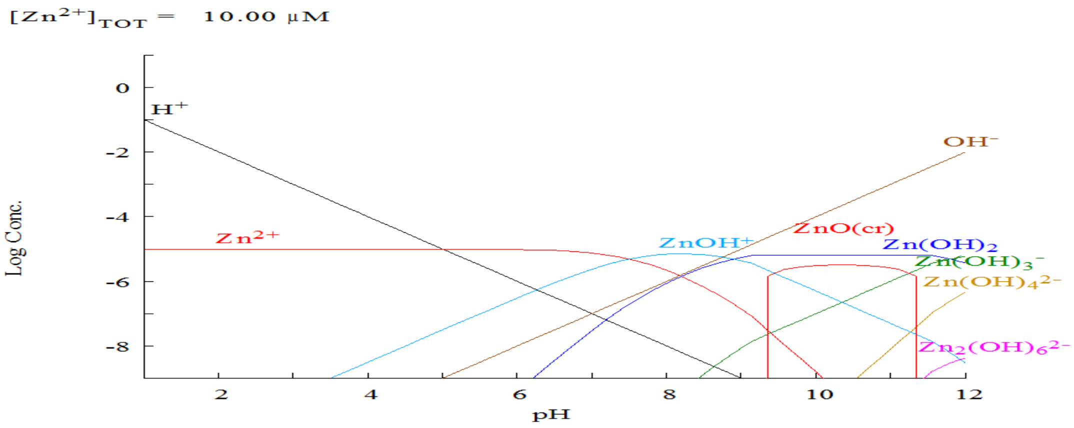


Fig 5.6 – Chemical balance diagram for zinc, done with Medusa-Hydra software (Ignasi Puigdomenech, updated in 2015).

Upstream system 1, the reactor effluent evolution revealed zinc concentration below limit of detection of it's tested standard (Table 1, annex 4). Apart from day 937, 943, 950 and 957 (Figure 5.7B) where almost nothing was seen of zinc concentration and going by the fact that the reactor was still adjusting and acclimatizing to the new orange molasses carbon source (Figure 5.7B). From day 970 and down the line after the 50% feed increment of reactor 1, biogenic sulphide evolutions from sulphate reduction appreciated and in corresponding terms, the zinc metals combined directly with the hydrogen sulphide to form metal sulphides precipitates out of solution. Besides, bacteria sulphate reduction removing the zinc from solution via sphalerite formation, Younger et al., (2002); it might have as well be removed as it complexes with organic ligands in the reactor (Machemer and Wildman, 1992). From our evolutions, though there were level of zinc removal at the neutralization stage but not in an outright fashion that meets the standard limit of zinc metal according to Portuguese national legislation of irrigation waters.

Removal below limit of standards of zinc majorly occurred in the reactor and was primarily due to the sulphate reducing bacteria (SRB) activities.

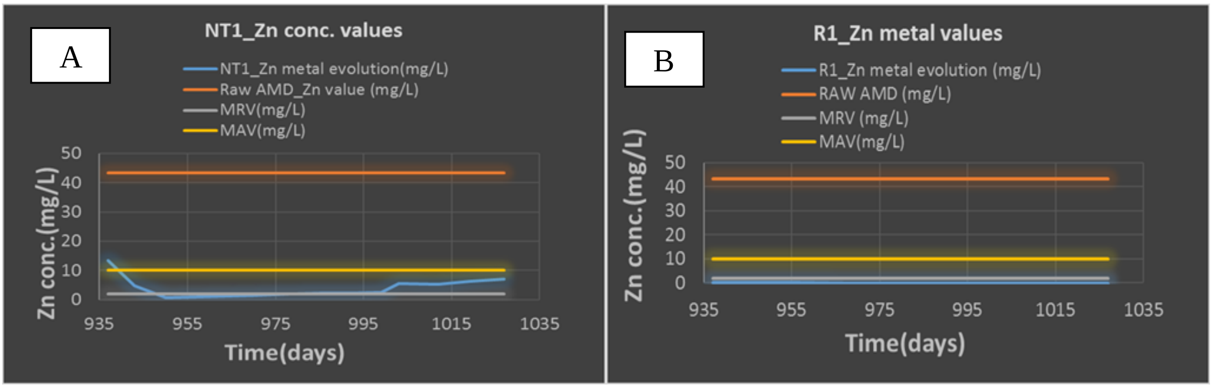


Fig 5.7- Depicting zinc metal removal from: (A)- neutralization tank 1 and (B)- reactor 1

Legend: NT1- Neutralization tank 1, R1- Reactor 1, AMD- Acid mine drainage,
MRV- Maximum recommended value, MAV- Maximum admissible value

II. Iron (Fe)

Iron concentration in the raw AMD initial characterization was very high with a value of 172.20mg/L as indicated in table 4.1. However, values evolving in the neutralization tank 1 revealed iron concentration falling drastically way below 5 mg/L, the maximum recommended value (MRV) as set by the Portuguese national legislation for irrigation waters (Decree law no: 236/98, 1998). Haven achieved pH ranged value of 6.6 by day 937, 2 days after introducing fresh limestones into neutralization tank 1, iron concentration was at 0.48mg/L (Figure 5.8 A). This steadied up until day 957 and then, as pH value continued to rise, iron concentration continued to drop, with days 970, 978, 985 and 992 emerging with more or less the same values of 0.19mg/L, 0.24mg/L, 0.13mg/L, 0.15mg/L respectively. Days 999, 1003, 1012 and 1019 tilted trend fashion a bit with 0.36mg/L, 0.38mg/L, 0.41mg/L, 0.48mg/L, as pH started to drop a little, and day 1027 with a 0.87mg/L which seem to be the highest in that order (Figure 5.8A).

At this neutralization stage, iron removal must have been initiated and driven by pH, being at values > 4 (Younger et al., 2002). Possible iron metal removal must have occurred as the influent aqueous trivalent iron of the AMD at pH as low as 2.49 from the initial characterization might have precipitated as oxyhydroxide iron (iii) complex once the pH was raised (Kirby and Cravotta, 2005). By visual inspection, reddish brown deposits were visible in the neutralization tanks; this further strengthens the proof that majority of the iron was already taken off at this point.

Upstream, with the influent from the neutralization tank 1 haven majority of the iron concentrates removed, evolutions of iron concentrations from reactor 1 effluents were almost within the same range as was the outcome from the neutralization stage (Figure 5.8 A & B). However, unexpectedly we recorded a little bit of higher values in iron concentration of the effluents from the reactor. However still, values were always below 1mg/L, much lower than the maximum recommended value (MRV) of 5mg/L as defined by the Portuguese national regulation on irrigation waters (Decree law no: 236/98, 1998).

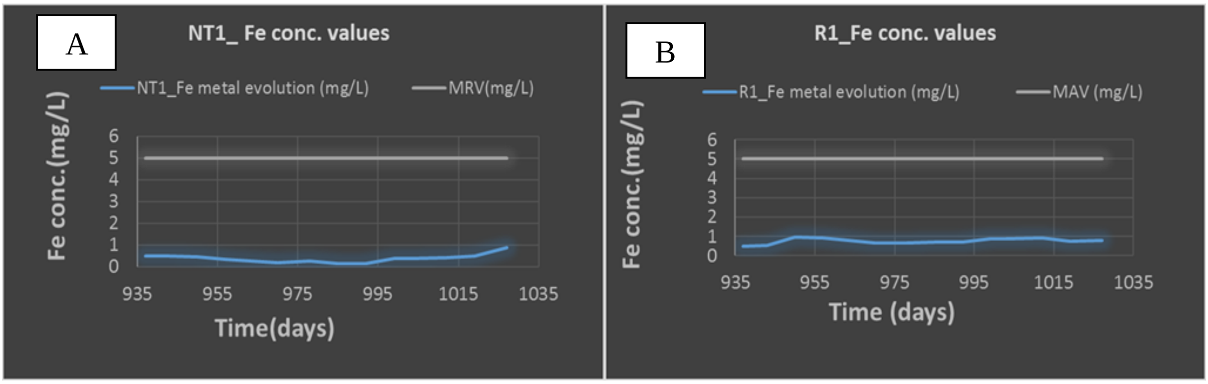


Fig 5.8- Depicting iron metal removal from: (A)- neutralization tank 1 and (B)- reactor 1

Legend: NT1- Neutralization tank 1, R1- Reactor 1, AMD- Acid mine drainage,
MRV- Maximum recommended value

III. Copper (CU)

In the neutralization tank copper evolution (Figure 5.9A) was below 0.3559mg/L, the limit of detection used of the calibration standard in the AAS analysis (Table 1 annex 4). Exception to this was on day 937, when the neutralization tank was set up with fresh limestones with value on this day at 0.73mg/L (Figure 5.9A). This value is quiet negligible in comparison to copper concentrate value of 40.54mg/L in the initial AMD characterization (Table 4.1). pH value by day 937 was 6.6, the lowest scored, suggesting that copper removel was related to pH neutralization, as expected. Notwithstanding this revelation, copper concentration was below maximum recommended value (MRV) of 0.2mg/L as defined by the Portuguese national legislation in irrigation waters (Decree law, no: 236/98, 1998). In summary, the system proved effective in copper removal.

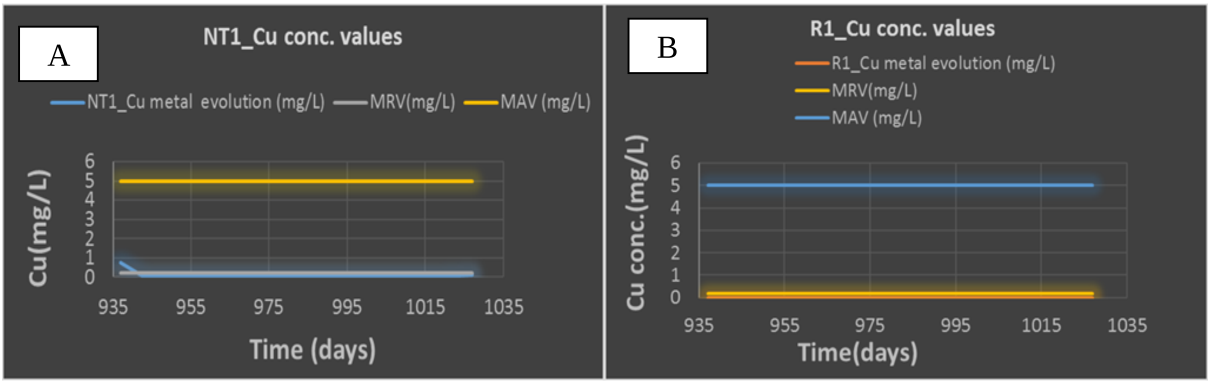


Fig 5.9- Depicting copper metal removal from: (A)- neutralization tank 1 and (B)- reactor 1

Legend: NT1- Neutralization tank 1, R1- Reactor 1, AMD- Acid mine drainage,
MRV- Maximum recommended value, MAV- Maximum admissible value

IV. Aluminum (Al)

Aluminum concentration in the raw AMD was very high with a value of 205.4mg/L (Table 4.1). In the neutralization tank 1 aluminum was removed all along the days showing very minute concentrations with values lower than 1mg/L (Figure 5.10 A). Thus, always below the maximum recommended value (MRV) of the Portuguese national regulation defined for irrigation waters at 5mg/L (Decree law, no: 236/98, 1998). At this stage of the process, aluminum metal must have been chemically precipitated out of solution as concentrations have been reported to decrease to less than 1mg/L at pH between 5 and 8 (Hedin et al., 1994). At the prevailing pH conditions of the neutralization tank 1, aluminum could have equally precipitated as alunite [$KAl_3(OH)_6(SO_4)_2$] as well $Al(OH)_3$ which exist as $Al(H_2O)_3(OH)_3$, a neutral compound of low solubility (Sheoran et al., 2010).

In the reactor, aluminum concentrations were always still below 1mg/L as in the neutralization tank. Thus also below the defined maximum recommended value for irrigation waters. Minute concentrations were recorded on days 970, 978, 985, 992, 999, 1012, 1019 and 1027 as 0.32mg/L, 0.20mg/L, 0.41mg/L, 0.35mg/L, 0.44mg/L, 0.49mg/L, 0.55mg/L, 0.13mg/L and 0.42mg/L respectively (Figure 5.10B).

Aluminum must have been removed in forms of hydroxides and sulphate precipitates (Sheoran et al., 2010). The alunite [$KAl_3(OH)_6(SO_4)_2$] form of aluminum might as well possibly have combined with the organic carbon compounds of the used orange molasses in complex precipitated forms.

In conclusion, the system was efficient in removing the majority of the aluminum in the raw AMD to concentrations in conformity with national legislation for irrigation waters in Portugal.

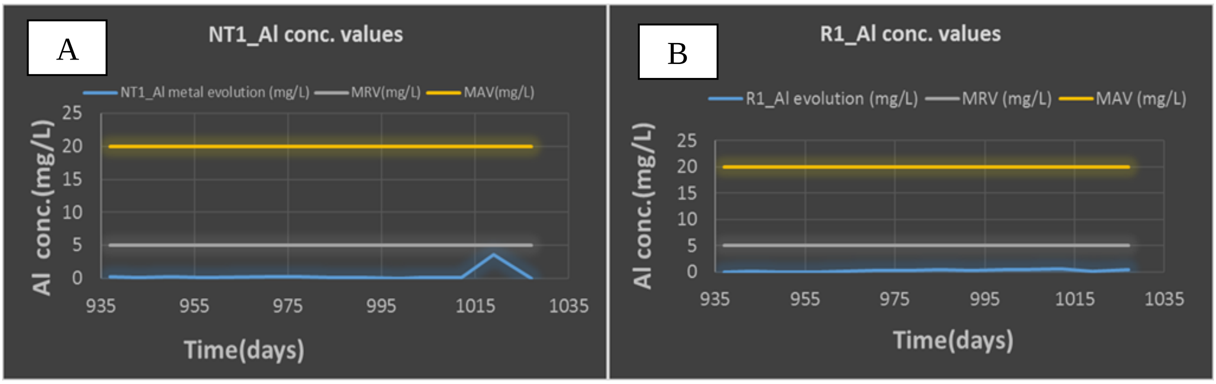


Fig 5.10- Depicting aluminum metal removal from: (A)-neutralization tank 1 and (B)-reactor1

Legend: NT1- Neutralization tank 1, R1- Reactor 1, AMD- Acid mine drainage,
MRV- Maximum recommended value, MAV- Maximum admissible value

5.4.2- Metal removal in system 2 - Neutralization tank 2 and Bioreactor 2

I. Zinc (Zn)

Again, with zinc concentration in raw AMD as 43.26mg/L (Table 4.1); from the neutralization tank 2, zinc metal evolved at a value of about 5.8mg/L by day 943. This could be due to little change in pH balance with a value of around 7.14 as we added fresh limestone to the neutralization tank prompting such response. 7 days after and by day 950, zinc concentration dropped low to about 3.42mg/L (Figure 5.11A).

By this day, there was an improvement in pH value to a point of around 7.44. Zinc concentration continue to drop and edge closer to the set limit of 2mg/L of maximum recommended value in Portuguese national legislation for irrigation waters. By day 957, as pH continue to revolve around neutral point with a value of 7.46, zinc value recorded 2.58mg/L (Figure 5.11A).

By day 970, as pH continue to remain stabilized around neutral point range in the tank, with value 7.68, zinc concentration rose to 5.53mg/L. This appreciative trend in zinc concentration continued as the pH remains more or less stable around neutral point with values of 7.5mg/L, 9.63mg/L, 7.09mg/L, 7.56mg/L, 7.64mg/L, 13mg/L, and 16.56mg/L for days 978, 985, 992, 999, 1003, 1012, 1019 and 1027 respectively (Figure 5.11A).

From our investigation, we observed relative large variations in zinc concentrations to pH change. On the average, pH value of about 7.44 gave a considerate reduction in zinc concentration in relative terms with other values over the days.

However, the level of zinc metal at this decontamination point was still most of the days above the maximum recommended value (MRV) of Portuguese national decree for irrigation waters. Little attention was paid to these values as the reactor takes off the remaining metal concentration on a more efficient level. The rise and fall trend in zinc removal pattern in connection with pH was expected as the chemical balance diagram of zinc can further explain the undulating pattern in zinc metal concentration reduction at pH above 7.5 (Fig 5.6).

Upstream system 2, the reactor effluent evolution showed no detectable presence in zinc concentration. Apart from day 943, 950 (Figure 5.11B) where almost nothing was seen of zinc concentration and going by the fact that the reactor was still adjusting and acclimatizing to the new beetroot molasses carbon source (Figure 5.11B). From day 970 and down the line after the 50% feed increment of reactor 2, biogenic sulphide evolutions from sulphate reduction appreciated and in corresponding terms, the zinc metals combined directly with the hydrogen sulphide to form metal sulphides precipitates out of solution. Besides, bacteria sulphate reduction removing the zinc from solution via sphalerite formation, Younger et al., (2002); it might have as well be removed as it complexes with organic ligands in the reactor (Machemer and Wildman, 1992). From our evolutions, though there were level of zinc removal at the neutralization stage but not in an outright fashion that meets the standard limit of zinc metal according to Portuguese national legislation of irrigation waters. Though an exception to this might be seen on day 1019 and 1027 where zinc concentration rose to 0.9mg/L and 2.63mg/L respectively due to probably the chemical imbalance behaviour of Zn at different higher pH values as other reported.

In any case, removal below limit of standards of zinc majorly occurred in the reactor and was primarily due to the sulphate reducing bacteria (SRB) activities.

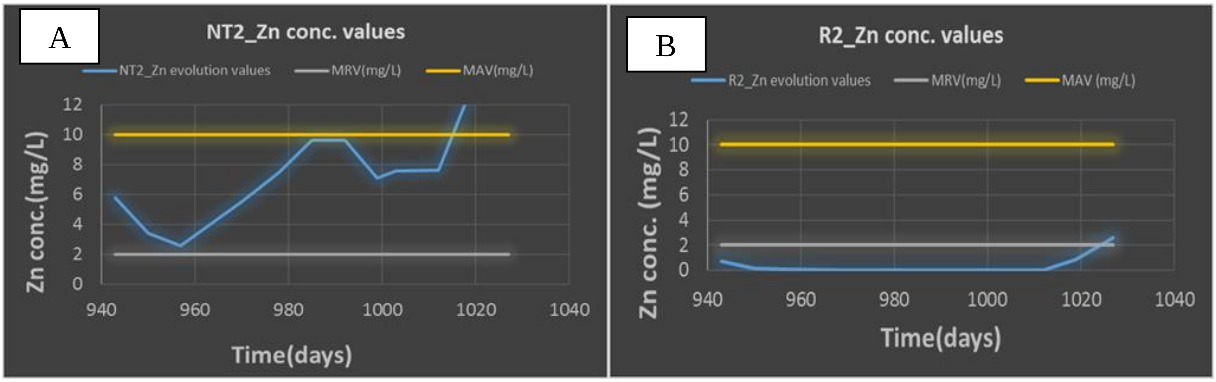


Fig 5.11- Depicting zinc metal removal from: (A)- neutralization tank 2 and (B)- reactor 2

Legend: NT2- Neutralization tank 2, R2- Reactor 2, AMD- Acid mine drainage,
MRV- Maximum recommended value, MAV- Maximum admissible value

II. Iron (Fe)

Iron concentration in the raw AMD initial characterization was very high with a value of 172.20mg/L as indicated in table 4.1 However, values evolving in the neutralization tank 2 revealed iron concentration falling drastically way below 5 mg/L, the maximum recommended value (MRV) as set by the Portuguese national legislation for irrigation waters (Decree law no: 236/98, 1998). Haven achieved pH ranged value of 7.14 by day 943, 3 days after introducing fresh limestones into neutralization tank 2, iron concentration was at 0.54mg/L (Figure 5.12A). As pH value continued to rise, iron concentration continued to drop, with days 950, 957, 970, 978 and 985 emerging with declining values of 0.37mg/L, 0.32mg/L, 0.17mg/L, 0.16mg/L and 0.18mg/L respectively. Days 992, 999, 1003, 1012, 1019 and 1027 tilted trend fashion a bit with 0.49mg/L, 0.42mg/L, 0.46mg/L, 0.49mg/L, 0.34mg/L and 0.41mg/L respectively as pH started to drop a little. (Figure 5.12A).

Similarly, as already reported, at this neutralization stage, iron removal must have been initiated and driven by pH, being at values > 4 (Younger et al., 2002). Possible iron metal removal must have occurred as the influent aqueous trivalent iron of the AMD at pH as low as 2.49 from the initial characterization might have precipitated as oxyhydroxide iron (iii) complex once the pH was raised (Kirby and Cravotta, 2005). By visual inspection, reddish brown deposits were visible in the neutralization tanks; this further strengthens the proof that majority of the iron was already taken off at this point.

Upstream, with the influent from the neutralization tank 2 haven majority of the iron concentrates removed, evolutions of iron concentrations from bioreactor 2 effluents were almost within the same range as was the outcome from the neutralization stage (Figure 5.12A & B). Although, unexpectedly we recorded a little bit of higher values in iron concentration of the effluents from the reactor. However still, values were always below 1mg/L, except on few occasions earlier on when bioreactor started to be fed with beetroot molasses with values on day 950 and 957 emerging as 3.57mg/L and 1.02 mg/L respectively. Later on also, days 1019 and 1027 showed up with iron concentration values a little bit above 1mg/L as 1.19mg/L and 1.27mg/L respectively. Though, these exceptional revealations showed values a little bit above 1mg/L, iron concentrations remained much lower than the maximum recommended value (MRV) of 5mg/L as defined by the Portuguese national regulation on irrigation waters (Decree law no: 236/98, 1998).

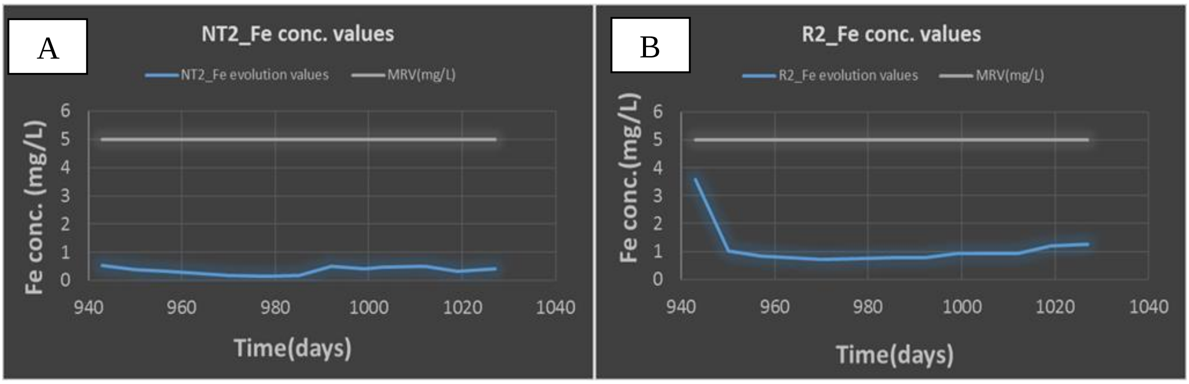


Fig 5.12- Depicting iron metal removal from: (A)- neutralization tank 2 and (B)- reactor 2

Legend: NT2- Neutralization tank 2, R2- Reactor 2, AMD- Acid mine drainage,
MRV- Maximum recommended value

III. Copper (CU)

In the neutralization tank copper evolution (Figure 5.13A) was below 0.3559mg/L, the limit of detection used of the calibration standard in the AAS analysis (Table 1 annex 4). Copper concentration was below maximum recommended value (MRV) of 0.2mg/L as defined by the Portuguese national legislation in irrigation waters (Decree law, no: 236/98, 1998).

In summary, the system proved effective in copper removal (Figure 5.13 A & B).

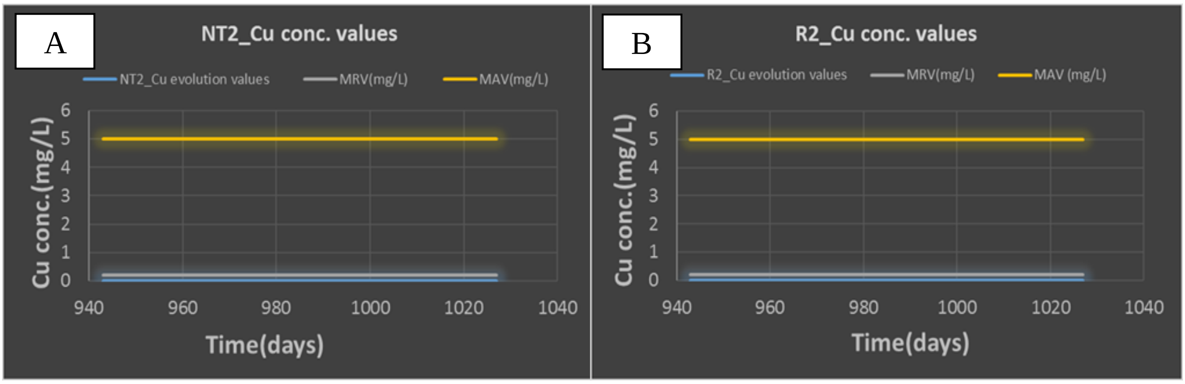


Fig 5.13- Depicting copper metal removal from: (A)- neutralization tank 2 and (B)- reactor 2

Legend: NT2- Neutralization tank 2, R2- Reactor 2, AMD- Acid mine drainage,
MRV- Maximum recommended value, MAV- Maximum admissible value

IV. Aluminum (Al)

As already reported, aluminum concentration in the raw AMD was very high with a value of 205.4mg/L (Table 4.1). In the neutralization tank 2, aluminum was removed and all along the days showing very minute concentrations with values more or less or even lower 1mg/L (Figure 5.14A). Thus, always below the maximum recommended value (MRV) of the Portuguese national regulation defined for irrigation waters at 5mg/L (Decree law, no: 236/98, 1998). At this stage of the process, aluminum metal must have been chemically precipitated out of solution as concentrations have been reported to decrease to less than 1mg/L at pH between 5 and 8 (Hedin et al., 1994). At the prevailing pH conditions of the neutralization tank 1, aluminum could have precipitated as alunite [$KAl_3(OH)_6(SO_4)_2$] as well $Al(OH)_3$ which exist as $Al(H_2O)_3(OH)_3$, a neutral compound of low solubility (Sheoran et al., 2010).

In the reactor, aluminum concentrations were always still below 1mg/L as in the neutralization tank. The only exception to this was aluminum concentration of 2.62mg/L on day 957 and this could have been issues of sample contamination. Besides, values were below the defined maximum recommended value for irrigation waters. Minute concentrations were recorded on days 970, 978, 985, 992, 999, 1012, 1019 and 1027 as 0.34mg/L, 0.33mg/L, 0.49mg/L, 0.26mg/L, 0.85mg/L, 0.68mg/L, 0.93mg/L, 0.13mg/L and 0.34mg/L respectively (Figure 5.14B).

Again as already reported, Aluminum must have been removed in forms of hydroxides and sulphate precipitates (Sheoran et al., 2010). The alunite [$KAl_3(OH)_6(SO_4)_2$] form of aluminum might as well possibly have combined with the organic carbon compounds of the used orange molasses in complex precipitated forms.

In conclusion, the system was efficient in removing the majority of the aluminum in the raw AMD to concentrations in conformity with national legislation for irrigation waters in Portugal.

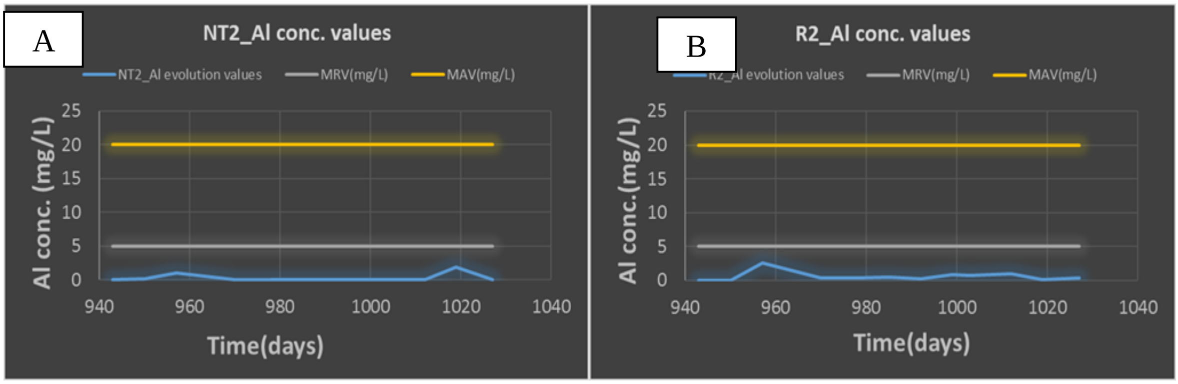


Fig 5.14- Depicting aluminum metal removal from: (A)-neutralization tank 2 and (B)-reactor2

Legend: NT2- Neutralization tank 2, R2- Reactor 2, AMD- Acid mine drainage,

MRV- Maximum recommended value, MAV- Maximum admissible value

5.5- Carbon compounds in the continuous flow experiments: sugars and organic acids

Molasses as an indirect or complex substrate require complex microbial community to degrade the organic matter and support SRB growth (Sheoran et al., 2010). By fermentation and hydrolysis, sugars as sucrose, glucose, fructose need to be degraded into simpler compounds, such as ethanol and various organic acid products, becoming available in a symbiotic manner for the SRB community growth and activity (Lebel et al., 1985).

For this reason, we analyzed for the presence of molasses products in our reactor effluents such as sucrose, glucose and fructose and also for organic acids like acetic acid, formic acid, propionic acid, lactic acid, citric acid and butyric acid were analyzed in the effluents from the two bioreactors tested with the two molasses as carbon sources/electron donors (orange molasses and beetroot molasses). Table 5 of annex 3 summarizes the results for organic compounds analysed in the continuos flow experiments and in the sections below are discussed the results of those that appear to be involved in the bioremediation process.

5.5.1- Sugars in the bioreactor tested with orange molasses – reactor 1

I. Sucrose

With initial molasses regime flow rate of 0.005ml/hr, reactor 1 effluent sucrose concentration started at 1653.36mg/L by day 978. Then the downstream sucrose effluent concentration starts to decrease as the reactor continued to adjust for better dynamics (Figure 5.15). With improved molasses administration to 0.0075mL/hr since day 993, despite the consequent 50% rise in the supplement of sugars, sucrose concentration on reactor 1 effluent continued to decrease. By day 1003 the effluent had about 955.71mg/L of sucrose, stabilizing at the same concentration level up to day 1027. At this time the inlet sucrose concentration was 3010.51mg/L, thus 68 % of this sugar was degraded in the bioreactor. The fact that this evolution in the degradation of sucrose was simultaneous with an improvement on sulphate reduction indicates that this sugar had a major role in the symbiotic dynamics supporting of the SRB in the bioreactror feed with orange molasses.

Glucose and fructose concentrations of reactor 1 effluents were below limits of detection (Table 2, annex 4).

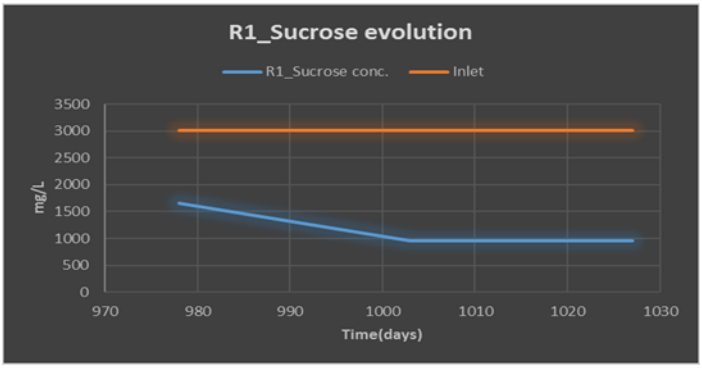


Fig 5.15- Depicting sucrose evolution on the effluent of reactor 1

Legend: R1- Reactor 1, Inlet- Initial sucrose concentration

5.5.2- Sugars in the bioreactor tested with beetroot molasses- reactor 2

I. Sucrose

Again, with an initial molasses regime flow rate of 0.005ml/hr, reactor 2 effluent sucrose concentration started at 1495.51mg/L by day 978. Then the downstream sucrose effluent concentration starts to decrease as the reactor continued to adjust for better dynamics (Figure 5.16). With improved molasses administration to 0.0075mL/hr since day 999, despite the consequent 50% rise in the supplement of sugars, sucrose concentration on reactor 2 effluent continued to decrease. By day 1003 the effluent had about 1450.04mg/L of sucrose, though not so significant from previous day 978 value. By day 1027, effluent sucrose concentration had gone down side to a low of 955.71mg/L.

At this time the inlet sucrose concentration was 5273.42mg/L, thus over 80% of this sugar was degraded in the bioreactor. The fact that this evolution in the degradation of sucrose was simultaneous with an improvement on sulphate reduction indicates that this sugar had a major role in the symbiotic dynamics supporting of the SRB in the bioreactror fed with beetroot molasses.

Again, glucose and fructose were below limit of detection (Table 2, annex 4) on the effluent of reactor 2 as was equally the case with reactor 1.

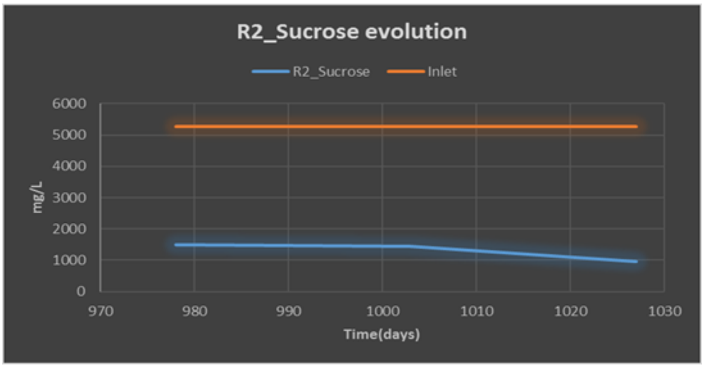


Fig 5.16- Depicting sucrose evolution on the effluent of reactor 2

Legend: R2- Reactor 2, Inlet- Initial sucrose concentration

5.5.3- Organic acids in the bioreactor tested with orange molasses – reactor 1

We analyzed the following acids- acetic acid, formic acid, propionic acid, lactic acid, citric acid and butyric acid. Amongst these, only acetic acid and butyric acid for both were detected on the bioreactors effluents (Table 5 of annex 3).

I. Acetic acid

SRB took advantage of other microbiological groups to make available simpler end-products from complex substrates like the tested orange molasses and beetroot molasses used in this work. Acetic acid is reported as a very common intermediate in the fermentation and hydrolysis of complex organic compounds by several species, like lactobacilli for example, and it is not utilized by all SRB (Lens et al., 2002).

Thus, since the bioreactor started to be fed with orange molasses and with the resident SRB community unable to completely utilize acetic acid, a gradual increase of this compound was expected. However, the concentration of acetic acid in reactor 1 effluent was relatively high in the beginning and then descended and stabilized during the first nutrient flow rate feeding regime of 0.005ml/hr until day 992 (Figure 5.17). This initial high amount certainly happened because of a problem that occurred on the first night of supply of orange molasses where a large amount of molasses rushed all at once into the reactor. Following the augmentation of reactor 1 nutrient flow rate to 0.0075mL/h, the microbial activity enhanced thereby reinvigorating more acetic acid generation. This surge in acetic acid climaxed around day 999, then declined a little, stabilized for a week and re-initiated the trend in rise. All of this points to the conclusion that acetic acid was generated and significant amount remained because the microbial community did not consume it and if at all, not completely. Nevertheless, the fluctuations observed in the concentration of acetic acid after the start of feeding the bioreactor with orange molasses and after increasing the quantity supplied are a sign of the elasticity of bioreactors with mixed microbial communities in their capacity of restructuring their symbiotic relationships to adapt to new conditions.

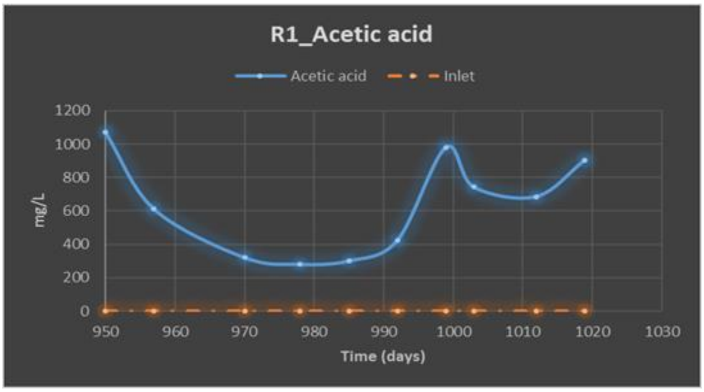


Fig 5.17 - Depicting acetic acid evolution on the effluent of reactor 1.

Legend: R1- Reactor 1, Inlet- Initial acetic acid concentration

II. Butyric acid

Butyric acid as a volatile fatty acid (VFA) is a fermentation-degraded end-product of organic substrates, important in anaerobic sulphate reduction (Speece, 1996). Butyric acid in it's salt form has been reported by Widdel, (1998), to be mineralized and consumed by hydrogen producing acetogens during sulphate reduction. We analyzed for the presence of butyric acid on the bioreactor effluent and discovered in it's evolution an upsurge in concentration level as we initiated first feeding regiment of orange molasses and subsequent adjustment until it was residual or below limit of detection (Table 3, annex 4).

By day 957 the reactor peaked in it's surge with butyric acid concentration of around 485.56mg/L from reactor 1 downstream effluents. As the system continue to stabilize with more quantifiable sulphate reduction suggesting enhanced SRB growth, the concentration level of the butyric acid from of the effluents began to drop. By day 978, it had already slided to as low as around 168mg/L less by more than half of it's climax concentration point on day 957. By day 985, it rose again hitting a second peak point concentration level; this time around a little lower than the first concentration-level peak point on this day 992. The difference in concentration-level peak point between day 957 and 992, points to the fact that enhanced SRB activity was online as reactor 1 sulphate reduction dynamics sets in to a more stabilized trend. Following our 50% nutrient augmentation, from 0.005 to 0.0075 mL/h, subsequent evolutions reveal a sharp uptake of butyric acid to residual or below limit of detection concentrations for day 999 and beyond. This again shows that the system once subjected to the new orange molasses carbon source, generated degraded organic acids with SRB coming in to utilize them for growth and activity.

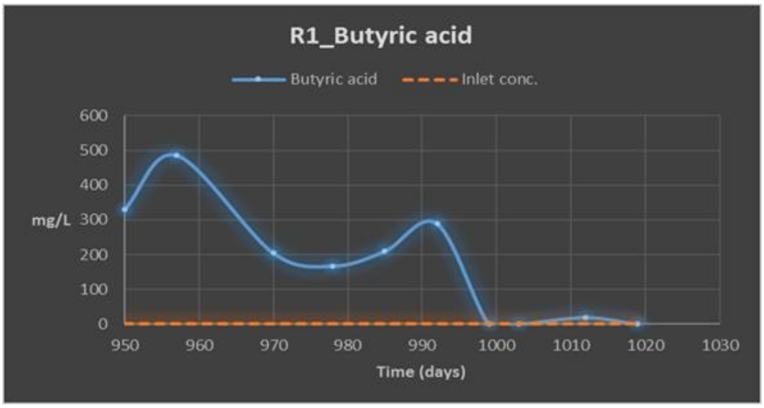


Fig 5.18 - Depicting butyric acid effluent evolution of reactor 1

Legend: R1- Reactor 1, Inlet- Initial butyric acid concentration

5.5.4- Organic acids in the bioreactor tested with beetroot molasses – reactor 2

I. Acetic acid

Again for similar reasons as already reported in section 5.5.3 with orange molasses in reactor1, we analyzed for acetic acid as well in beetroot molasses and only acetic acid and butyric acid are reported as the rest were below analysis limit of detection (Table 3 annex 4).

Once the bioreactor started to be fed with beetroot molasses, the resident SRB community was unable to completely utilize acetic acid and as such prompting a gradual increase of this compound. This could be seen right from the beginning as acetic acid concentration ascended and stabilized during the first nutrient flow rate feeding regime of 0.005ml/hr until day 985, thereafter, descended a little further reaveling that SRB might have utilized it at this point but not outrightly (Figure 5.19). Following the augmentation of reactor 2 nutrient flow rate to 0.0075mL/h, the microbial activity enhanced thereby reinvigorating more acetic acid generation. This surge in acetic acid climaxed around day 1012, then started to declined a little as some of the already generated ones in media gets more or less assimilated by some members of the SRB consortia. All of this points to the conclusion that acetic acid was generated and significant amount remained because the microbial community did not consume it and if at all, not completely. Nevertheless, the fluctuations observed in the concentration of acetic acid after the start of feeding the bioreactor with beetroot molasses and after increasing the quantity supplied are a sign of the elasticity of bioreactors with mixed microbial communities in their capacity of restructuring their symbiotic relationships to adapt to new conditions.

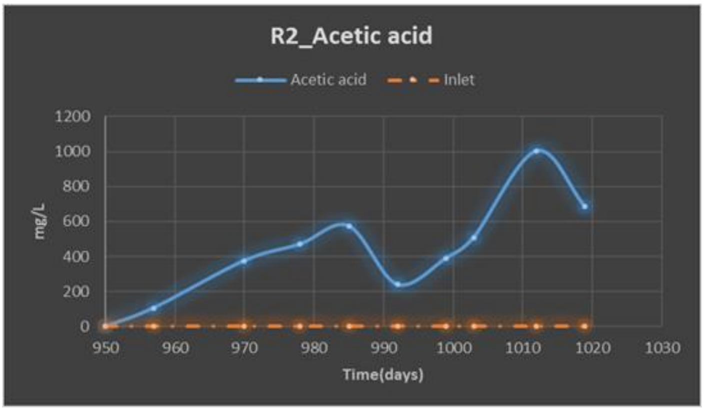


Fig 5.19 - Depicting acetic acid effluent evolution of reactor 2
Legend: R2- Reactor 2, Inlet- Initial acetic acid concentration

II. Butyric acid

For similar reasons as already reported in section 5.5.3 with orange molasses in reactor 1, we analyzed for butyric acid in beetroot molasses.

By day 970 the reactor peaked in it’s surge with butyric acid concentration of around 355.88mg/L (Fig 5.20) from reactor 2 downstream effluents. As the system continue to stabilize with more quantifiable sulphate reduction suggesting enhanced SRB growth, the concentration level of the butyric acid from the effluents began to drop. By day 978, it had already slided to around 249.65mg/L though not so significant from it’s climax concentration point on day 970. By day 985, it dropped little lower again than the concentration witnessed the previous week with value at 182.22mg/L. The difference in concentration-level peak point between day 970 and 985, points to the fact that enhanced SRB activity was online as reactor 2 sulphate reduction dynamics sets into a more stabilized trend. Concentration appreciated again to 208.02mg/L by day 992 and continued in this manner following our 50% nutrient augmentation, from 0.005 to 0.0075 mL/h, with subsequent evolution revealing a hike in butyric acid concentration for day 999 at 371.43mg/L. Post second regime periods of nutrient augementation feed into reactor 2 produced a reduction in butyric acid concentration with days 1003, 1012 and 1027 showing more or dwindled values of 340.76mg/L, 298.45mg/L and 340.24mg/L(Fig 5.20) respectively

Again, this shows that the system once subjected to the new beetroot molasses carbon source, generated degraded organic acids with SRB coming in to utilize them for growth and activity.

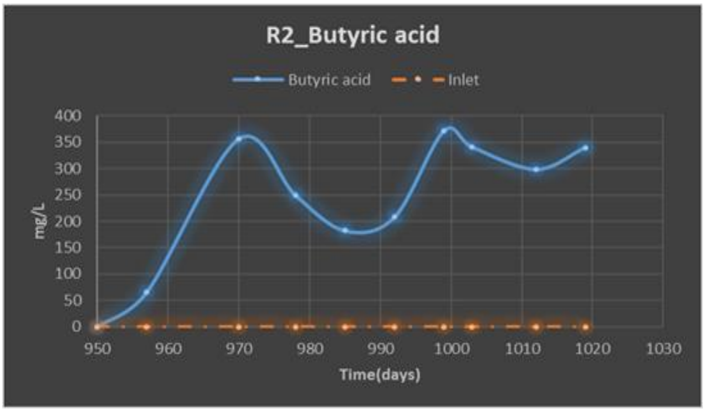


Fig 5.20 - Depicting butyric acid effluent evolution of reactor 2
Legend: R2- Reactor 2, Inlet- Initial acetic acid concentration

5.5.5- Nutrient and Effluent Flow rates for bioreactors tested with orange and beetroot molasses - reactors 1 and 2

Our continuous flow system experiments were carried out with reactor flow rate of 1mL/h for approximately 13 days of retention time and molasses flow rates of 0.005ml/hr set for both reactors as the first regime (Table 3.1). Upgrading the quantity of nutrient delivered into the reactors by 50% in a second regime (Fig 5.21 A & B), efficient sulphate reduction was achieved. By mechanical means using the nutrient delivery pump, we administered beetroot molasses into reactor 2 while reactor 1 was manually administered. The decision to use manual injection method instead of mechanical means for reactor 1 was taken after the injected orange molasses through pump formed fermented froth-like bubbles which drove all at once nutrient quantity meant for the reactor for about 10 days just in the first day. Due to this, we relaxed system 1 for about a period of 10 days without administering fresh nutrient from day 941 when this was observed after setting up the nutrient delivery pump on day 935 till day 951(Fig 5.21A). Beyond this point, we assumed the nutrient feeding regiment of 0.005ml/hr till day 992 when we improved nutrient quantity to 0.0075 mg/L (150% of the first regime) trying to enhance bacteria activity and improve kinetics of sulphate reduction.

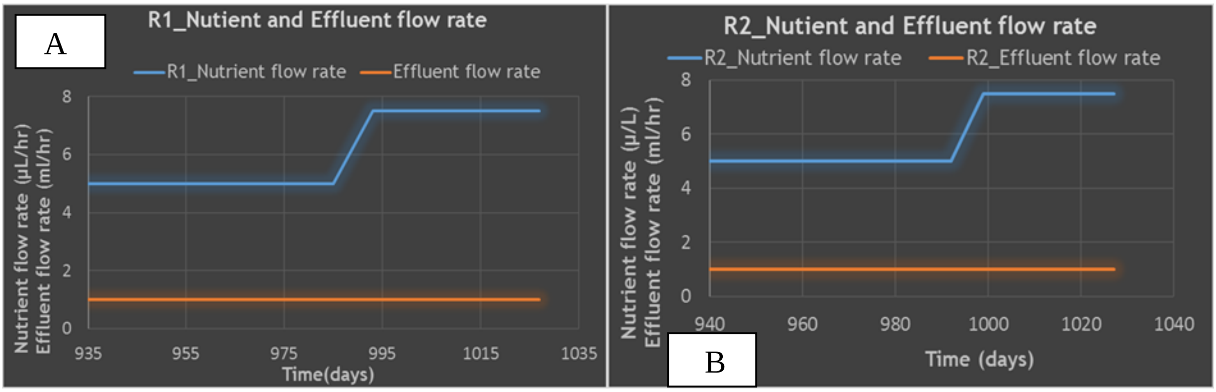


Fig 5.21 – Depicting molasses and effluents flow rates pattern evolution of –

A)- Reactor 1 B)- Reactor 2

Legend: R1- Reactor 1 and R2- Reactor 2 with Effluent flow rates

5.6- Chemical oxygen demand (COD)

Though, with no limit defined for COD in irrigation waters in Portugal, the COD limit for treated wastewater discharge is 150 mg O_2/L. The COD values measured in the biologically treated effluents from both tested bioreactors completely outweighs this limit. Effluents samples from the operational time with 0.0075mL/h molasses regime gave 2284 mg O_2/L and 2182 mg O_2/L COD values for reactor 1 and 2 respectively.

However, with the objective of this work focusing on testing the suitability of orange molasses and beetroot molasses as carbon sources/electron donors for SRB in the treatment of AMD, we did not make extra attempts of working on the panacea for the COD challenge. In any case, it is known that in tested SRB based processes feed with sugar cane molasses, with the integration of an aerobic stage, the unacceptable residual concentrations of COD can be removed from the end effluent (Maree, et al., 1987).

6. Conclusions

This work was carried out in two stages and two different types of molasses were tested as carbon sources/electron donors in SRB mediated sulphate and heavy metal contaminants removal from AMD water, while trying to optimize some important factors like pH, amount of molasses and required time, parameters seen critical in biological AMD decontamination.

The first stage involved testing a type of molasses produced by an orange juice industry and a type of molasses produced by sugar processing from beetroot in batch. The second stage used our batch experimental results to investigate the efficiency of continuous flow systems (upflow anaerobic packed bed) (UAPB) reactors.

The batch experiments confirmed that dilutions of 1:200 of any of the two tested molasses in AMD are supportive of SRB viability, allowing the high sulphate and heavy metal concentrations in AMD from Mina de São Domingo's mine to be removed below their defined legislative Portuguese standards for irrigation waters in about 14 days.

The continuous flow systems with bioreactors fed with one or the other molasses were optimized with a similar retention time (approximately 13 days), but the quantities of each molasses supplied had to be augmented (1:133 ratio (v:v) molasses:AMD) for effective sulphate reduction and heavy metal removal below defined limits set by Portuguese national legislation for irrigation waters.

7. Future prospects

The continuous flow systems are being optimized aiming at achieving lower retention times (RT).

Having economic and environmental advantage over the classical AMD treatment methods, process can be industrially scaled up and not limited only to AMD wastewater treatment but applied across other industrial sulphate rich and heavy metal mobilized effluents.

A pilot Plant will be tested at Mina de São Domingo’s mine (bioreactor with 150L/day flow rate) or 6.25L/hr.

The generated biogenic sulphide in the process can be captured and re-oxidized to elemental sulphur, a good agricultural fertilizer quality.

Conversion of the acetate generated degradation end product in a bid to reduce carbon foot print and asses it’s methane gas bioenergy potential.

9. Bibliography

Akcil, A., Koldas, S., (2006). Acid Mine Drainage (AMD): causes, treatment and case studies. Journal of Cleaner Production. 14: 1139-1145.

Alexandre, L.M.B, (2006). Batch tests for the selection of low cost carbon sources required for the growth and activity of sulphate reducing bacteria. Master thesis, University of Algarve, Faro, Portugal.

Alvarez-Valero, A.M., Perez-Lopez, R., Matos, J., Capitan, M.A., Nieto, J., Saez, J., Carballo, M., (2008). Potential environmental impact at sao Domingos mining district (Iberian Pyrite Belt, SW, Iberian Peninsula): evidence from a chemical and mineralogical characterization. Environmental Geology. 65: 117-120

Annachhatre, A.P., Suktrakoolvait S., (2001a). Biological sulphide oxidation in a fluidized bed reactor. Environmental Technology. 22: 661-72.

Annachhatre, A.P., Suktrakoolvaite, S., (2001b). Biological sulphate reduction using molasses as carbon source. Water Environment. 73: 118-126.

Barnes, L.J., (1998). Removal of heavy metals and sulphate from contaminated ground water using sulphate reducing bacteria: development of a commercial process in S.K. Skida, R.L., Irvine (eds), Bioremediation Technologies, vol.3, Technomic Publishing Company Inc., Lancaster, USA

Barros, R.J., Jesus, C., Martins, M., Costa, M.C., (2009). Marble stone processing powder residue as chemical adjuvant for the biologic treatment of acid mine drainage. Process Biochem. 44, 477–480

Barton, Hamilton, E., (2007). Sulphate reducing bacteria- Environmental and Engineering Systems. Cambridge University Press.

Bayrakdar, A., Sahinkaya, E., Gungor, M., Uyanik, S., Atasoy, A.D., (2009). Performance of sulfidogenic anaerobic baffled reactor (ABR) treating acidic and zinc containing wastewater. Bioresource Technology. 100: 4354-4360

Bechard, G., Yamakazi, H., Gould, W.D., Berdad, P., (1994). Use of cellulosic substrates for the microbial treatment of acid mine drainage. Journal of Environmental Quality. 23: 111-6.

Benner, S.G., Blowes, D.W., Ptacek, C.J., (1997). A full scale porous reactive wall for prevention of acid mine drainage. Ground Water Monitoring and Remediation, 17 (4): 99-107.

Blowes, D.W., Ptacek, C.J., Benner, S.G., McRae, CWT, Bennett T.A., Puls, R.W., (2000). Treatment of inorganic contaminant using permeable reactive barriers. Journal of Contamination Hydrology. 45: 121-37.

Brenner, F.J., (2001). Use of constructed wetlands for acid mine drainage abatement and stream restoration. Water Science and Technology. 44 (11): 449-454

Coelho, P.C., Teixeira, J.P., (2011). Mining Activities: Health Impacts. Elsevier 788-802.

Chaney, R.L., Brown, S.L., Angle, J.S., Stucznyski, T.I., Daniels, W.L., Henry, C.L., Siebielec, G., Li, Y. M., Malik, M., Ryan, J.A., Compton, H., (2000). In situ Reclamation /Reclamation/Restoration of Metals Contaminated Soils Using Tailor-Made Biosolids Mixtures. Symposium on Mining, Forest and Land Restoration. The Successful Use of Residuals /Biosolids/Organic Matter for Reclamation Activities, Denver, CO, July 17-20, 2000

Chokalingam, E., Subramanian, S., (2009). Utility of Eucalyptus tereticornis (Smith) bark and Desulfotomaculum nigrificans for the remediation of acid mine drainage. Bioresource Technology. 100: 615-621.

Choi, E., Rim, J.M., (1991). Competition and inhibition of sulphate reducers and methane producers in anaerobic treatment. Water Science Technology.

Cohen, R.R.H., (2006). Use of mircobes for cost reduction of metal removal from metal and mining industry and waste streams. Journal of Cleaner Production. 14(12-13), 1146-1157.

Costa, M.C., Duarte, J.C., (2005). Bioremediation of acid mine drainage using acidic soil and organic wastes for promoting sulphate reducing bacteria activity on a column reactor. Water Air Soil Pollution. 165: 325-345

Das, B.K., Roy, A., Koschorrek, M., Manda, S.M., Wendt-Potthoff, K., Bhattacharya, J., (2009). Occurrence and role of algae and fungi in acid mine drainage environment with special reference to metals and sulphate immobilization. Water Research. 43: 883-894.

Eager, P., Wagner, J., (2003). Wetland treatment systems- How long will they really work?

Proceedings of Sudbury, 2003: Mining and Environment, Sudbury, Ontario, May 25-28, 2003

Elliot, P., Ragusa, S., Catcheside, D., (1998). Growth of sulphate reducing bacteria under acid conditions in an upflow anaerobic bioreactor as treatment system for acid mine drainage. Water Resources. 32: 3724-3730.

Evangeluo, V.P., (1995). Pyrite Oxidation and it's Control. New York: CRC Press, pp. 275

Fyson, A., Kalin, M., Smith, M., (1995). Microbially mediated metal removal from acid mine drainage. In: Young, M.M., Anderson, W.A., Chakrabarty, A.M., editors. Environmental biotechnology: Principles and Practice. Dordrecht: Kluwer Academic Publishers. Pp. 11.

Garcia, M., Moreno, D.A., Ballester, A., Blaquez, M.L., Gozalez, F., (2001). Bioremediation of an industrial acid mine water by metal-tolerant sulphate reducing bacteria. Minerals Engineering. 14: 997-1008.

Geets, J., Vanbroehhoven, K., Borremans, B., Vangronsveld, J., Diels, L., van der Lelie, D., (2006). Column experiments to assess the effects of electron donors on the efficiency of in situ precipitation of Zn, Cd, Co and Ni in contaminated groundwater applying the biological sulphate removal technology. Environmental Science Pollution Resources. 13(6): 362-372.

Gilbert, O., de Pablo, J., Cortina, J.L., Ayora, C., (2004). Chemical characterization of natural organic substrates for biological mitigation of AMD. Water Resources 38: 4186-4196.

Gonzalez-Silva, B.M., Briones-Gallardo, R., Razo-Flores, E., Celis, L.B., (2009). Inhibition of sulphate reduction by iron, cadmium and sulphide in granular sludge. Journal of Hazardous Materials. 172:400-407

Gusek, J.J., (2002). Sulphate reducing bacteria bioreactor design and operating issues. Is this the passive treatment technology for your mine drainage? Proceedings of the National Association of Abandoned Mine Land Programmes: Reclamation 2002, Sept 15-18, 2002, Park City, UT. Accessed at: http://www.ogm.utah.gov/amr/nammlp/.

Govind, R., Kumar, U., Puligadda, R., Anita, J., Tabak, H., (1997). Biorecovery of metals from acid mine drainage. In Tedder, D.W., Pohland, F.G., (ed) Emerging Technologies in Hazardous Waste Management 7. Plenum Press, New York, USA, pp. 91-101.

Hammack, T.W., Edenborn, H.M., Dvorak, D.H., (1994). Bench scale tests to selectively recover metals from acid mine drainage using biogenic H_2S, in proc. 3rd International Conference on the Abatement of Acidic Drainage, Pittsburg, PA (USA), April 24-29: 214-222.

Harada, H.S., Uemura, S., Momonoi, K., (1994). Interaction between sulphate reducing bacteria in UASB reactors fed with low strength wastes containing different levels of sulphate. Water Resources. 28: 335-67.

Hard, B., Friednich, S., Babbel, W., (1997). Bioremediation of acid mine water using facultative methylotrophic metal-tolerant sulphate reducing bacteria. Microbiology Research. 152: 65-73.

Hedin, R.S., Nairn, R.W., Kleinmann, R.I.P., (1994). Passive treatment of coalmine drainage. Bureau of Mines Information Circular 9389, US Bureau of Mines, Pittsburg, PA, p35.

Herrera, L.J., Hernandez, J., Ruiz, P., Gantenbein, S., (1991). Desulfovibrio desulfuricans growth kinetics. Environmental Toxicological Water Quality. 6: 225-38.

Hilton, M.G., Archer, D.B., (1988). Anaerobic digestion of a sulphate rich molasses wastewater: inhibition of hydrogen sulphide production. Biotechnology Bioengineering. 31: 885-8.

Huisman, J.L., Schouten, G., Djikman, H., (2006). Biotechnological treatment of pickle liquors. In: Dutrizac, J., Riveros, P.A., (ed) Iron control technologies. Metallurgical Society Montreal, pp 805-814.

Ignasi Puigdomenech (updated 2015) Medusa-Hydra Chemical Equilibrium Software (Medusa: 32-bit version 16 Dec. 2010; Hydra: 32-bit version 18 Aug. 2009 – database update 01-01-2015) KTH Royal Institute of Technology, 100 44 Stockholm, Sweden.

International Council on Mining and Metals. (2012). Trends in the mining and metals industry. In Mining's contribution to sustainable development. In Brief.

Institute of Geology and Mining. (2000). Environmental State in the Portuguese Test Site: Sao Domingos Mine: Past and Present. Mineral Report, Institute of Geology and Mining.

Jones, J., Gusek, J., (2004). Scaling up design challenges for large scale sulphate reducing bioreactors. Proceedings of the 2004 Ontario MEND Workshop, Sludge Management and Treatment of Weak Acid or Neutral pH drainage. May 26- 27, Sudbury, Ontario, Canada. Pp. E7.

Johnson, D.B., (2000). Biological removal of sulfurous compounds from inorganic wastewaters. In: Lens P, Hulshoff Pol L, editors. Environmental Technologies to Treat Sulphur Pollution: Principles and Engineering: International Association on Water Quality. 175-206.

Jonhson, D.B., Hallberg, K.B., (2005). Acid mine drainage remediation options: a review. Science of the Total Environment. 338: 3-14.

Kalin, M., Fyson, A., Wheeler, W.N., (2006). The chemistry of conventional and alternative treatment systems for the neutralization of acid mine drainage. Science of the Total Environment. 366: 395-408.

Kepler, D.A., McCleary, E.C., (1994). Successive alkalinity producing systems (SAPS) for the treatment of acid mine drainage. Proceedings, international land reclamation and mine drainage conference. April 24-29, Pittsburg, PA, USDI, Bureau of Mines SP 06A-94; 195-204.

Kepler, D.A., McCleary, E.C., (1997). Passive aluminum treatment processes. Proceedings, 18th West Virginia surface mine drainage task force symposium, April 15-16, Morgen town, WV.

Kirby, C.S., Cravotta, C.A., (2005). Net alkalinity and net acidity 2: Practical considerations. Applied Geochemistry. 20: 1941-1964

Lebel, A., Do Nascimento, H.G.C., Yen, T.F., (1985). Molasses promoted biological sulphur recovery from high sulphate wastes. Proceedings 40th Industrial Waste Conference, West Lafayette.

Lens, P., Vallero, M., Esposito, G., Zandvoort, M., (2002). Perspectives of sulphate reducing bioreactors in environmental biotechnology. Revised Environmental Science Biotechnology. 1: 311-25.

Liamleam, W., Annachhatre, A.P., (2007). Electron donors for biological sulphate reduction. Biotechnological Advancement. 25: 452-463.

Lo, K.V., Chen, A., Liao, P.H., (1990). Anaerobic treatment of baker's yeast wastewater: II. Sulphate removal. Biomass. 23: 25-37.

Lyew, D., Sheppard, J.D., (1997). Effects of physical parameters of a gravel bed on the activity of sulphate reducing bacteria in the presence of acid mine drainage. Journal of Chemical Technology and Biotechnology. 70: 223-230.

Machemer, S.D, Wildeman, T.R., (1992). Adsorption compared with sulphide precipitation as metal removal processes from acid mine drainage in a constructed wetland. Journal of Contamination Hydrology. 9: 115-113.

Machemer, S.D., Reynolds, J.S., Lavdon, L.S., Wilderman, T.R., (1993). Balance of sulphide in a constructed wetland built to treat acid mine drainage. Idaho Springs, Colorado. Journal of applied Geochemistry. 8 (6): 587-603

Maree, J.P., Strydom, W.F., (1985). Biological sulphate removal in an upflow packed bed reactor. Water Resource. 19: 1101-6

Maree, J.P., Gerber, A., Strydom, W.F., (1986). A biological process for sulphate removal from industrial effluents. Water SA. 12(3): 139-44.

Maree, J.P., Gerber, A., Hill, E., (1987). An integrated process for biological treatment of sulphate containing industrial effluents. J WPCF. 59: 1069-74.

Maree, J.P., Hill, E., (1989). Biological removal of sulphate from industrial effluents and concomitant production of sulphur. Water Science and Technology. 21: 256-276

Martins, M., Santos, E.S., Pires, C., Barros, R.J., Costa, M.C., (2010). Production of irrigation water from bioremediation of acid mine drainage: Comparing the performance of two representative systems. Journal of Cleaner Production. 18: 248-253.

Martins, M.S.F., (2010). Bio-removal of toxic metals by metal resistant anaerobic bacteria: molecular characterization and performance studies. Doctoral Thesis: “Biotechnological Sciences”. University of Algarve, Faro, Portugal.

Mineral Development Company, Portugal ,2009.

Muyzer, G., Stams, A.J.M., (2008). The ecology and biotechnology of sulphate reducing bacteria. Microbiology. 6: 441-454.

National Network for Environmental Management studies (NNEMS), 2006.

Portuguese Decree-Law 236/98, 1998. ANNEX XVI - Decreto-Lei n. º 236/98 de 1 de Agosto - DIÁRIO DA REPÚBLICA — I SÉRIE-A N. º 176 - 1-8-1998.

Postgate, J.R., (ed) (1984). The sulphate reducing bacteria. Cambridge University Press, New York, pp. 208.

Postgate, J., (1966). Media for sulphur bacteria. Laboratory Practice. 15(11): 1239-1244.

Quan, Z., Jin, Y., Yin, C., Lee, J., Lee, S., (2005). Hydrolysed molasses as an external carbon source in biological nitrogen removal. Bioresource Tecnology. 96: 1690-1695.

Rios, C.A., Williams, C.D., Roberts, C.L., (2008). Removal of heavy metals from acid mine drainage (AMD) using coal fly ash, natural clinker and synthetic zeolites. Journal of Hazardous Materials.156: 23-25.

Rowley, M., Warkentin, D.D., Sciotte, V., (1997). Site demonstration of the biosulphide process at the former Britannia mine. Proceedings of the Fourth International Conference on Acid Rock Drainage, May 30- June 6, Vancouver, BC, vol. IV, 1531-48.

Sheoran, A.S., Sheoran, V., Choudhary, R.P., (2010). Bioremediation of acid-rock drainage by sulphate reducing prokaryotes: A review. Minerals Engineering. 23: 1073-1100

Sillen, L.G., Martell, A.E., (1964). Stability constants of metal ion complexes. The Chemical Society, London.

Silva, F., Nadias, H., Prates, A., Arroja, L., Capella, I., (2009). Molasses as an external carbon source for anaerobic treatment of sulphite evaporator condensate. Bioresource Technology. 100: 1943-1950

Song, Y., (2003). Mechanisms of lead and zinc removal from lead mine drainage in constructed wetland. Ph.D. Dissertation, Civil Engineering Dep., Faculty of Graduate School, University of Missouri- Rolla, MO

Speece, R.E., (1996). Anaerobic biotechnology for industrial wastewaters. Tennessee: Archae Press.

Tabak, H., Scharp, R., Burckle, J., Kawahara, F., Govind, R., (2003). Advances in treatment of acid mine drainage and biorecovery of metals: 1: Metal precipitation for recovery and recycle. Biodegradation. 14: 423-436.

Teclu, D.G., Liang, M.D., Wallis, F.M., (2009). Bioremediation of contaminated waters sources with sulphate reducing bacteria. Proceedings of International Mine water conference, (pp.606-613). Pretoria, South Africa, 19-23 October.

Trends in Sustainable Development- Chemicals, mining, transport and waste management

United Nations, New York, 2010

Ueki, K., Kotaka, K., Itoh, K., Ueki, A., (1998). Potential availability of anaerobic treatment with digester slurry of animal waste for the reclamation of acid mine water containing sulphate and heavy metals. Journal of Fermentation Technology. 66: 43-50.

U.S. Department of Agriculture (USDA) and EPA Region III, (2000). A handbook of constructed Wetlands, Vol. 4: Coal Mine Drainage. Report# 843F00003

U.S. Department of Energy (US DOE), (1998). Research and Application of Permeable Reactive Barriers. Document# K0002000

Van Houten, R.T., Hulshoff Pol, L.W., Lettinga, G., (1994). Biological sulphate reduction using gas lift reactors fed with hydrogen and carbon dioxide as energy and carbon source. Biotechnology Bioengineering. 44: 586-594.

Vitor, M.S., Rocha, A.O., Reis, J.M., (2012). Portugal Mining and Minerals Legal Regime, Incentives and Taxation in Portugal. Obtained January 22nd, 2016 from http://www.worldservicesgroup.com/publications.asp? action=article&artid=4904

Watzlaf, G.R., Schroeder, K.T., Kairies, C., (2000). Long term performance of alkalinity producing passive systems for the treatment of mine drainage. Presented at the American Society for Surface Mining and Reclamation,17th Annual Meeting, Tampa, Florida, June 11-15, 2000.

Widdel, F., (1998). Microbiology and Ecology of sulphate reducing bacteria. In: Zehnder, A.B.J., (ed) Biology of anaerobic organisms. Wiley, New York, 469-585.

Weider, R.K., (1993). Ion input/output budgets for five wetlands constructed for acid mine drainage treatment. Water Soil Air Pollution. 71: 231-270.

Wolicka, D., Borkowski, .A., (2009). Phosphogypsum biotransformation in cultures of sulphate reducing bacteria in whey. International Biodeterioration & Biodegradation. 63: 322–327.

Younger, P., Banwart, S.A., Hedin, R.S., (2002). Mine water: Hydrology, pollution, remediation. Kluwer Academic Publishing, Boston.

Zagury, G.J., Kulnieks, V.I., Neculite, C.M., (2006). Characterization and reactivity assessment of organic substrates for sulphate reducing bacteria in acid mine drainage treatment. Chemosphere. 69: 944-954.

Zhao, Y.G., Wang, A.J., Ren, N.Q., (2004). Effect of carbon sources on sulphidogenic bacteria communities during the starting up of acidogenic sulphate reducing bioreactors. Bioresource Technology. 101: 2952-2959

Ziemkiewicz, P.F., Skousen, J.G., Brant, D.L., Sterner, P.L., Lovett, R.J., (1997). Acid mine drainage treatment with armoured limestone in open limestone channels. Journal of Environmental Quality. 26: 1017-1024

Zipper, C., Jage, C., (2001). Passive Treatment of Acid- Mine Drainage with Vertical- Flow Systems. Virginia Cooperative Extension Publication # 460-133.

Annex 1

Results of previous studies that helped plan this work

Table1- Initial characterization of tested sub-products in terms of their pH, SO_4^{2-} COD, N and P content values (adapted from Alexandre, 2016).

Parameter	**Sub- products** **Sugar beetroot molasses**	**Orange-juice molasses without conservative**
pH	7.64	5.35
SO_4^{2-}	2290	985
COD (mgO_2/L)	1192000	707000
N(mgN/L)	51136	21364
P(mgP/L)	17379	399
COD/SO_4^{2-}	596	353.5

Table 2- Initial values for monitored parameters of pH, E_h, COD, SO_4^{2-} and their respective COD/SO_4^{2-} ratio, in the previous tests to select alternative carbon sources for sulphate reducing bacteria (adapted from Alexandre, 2016).

	Dilutions tested in Postgate B medium without lactate	pH	Redox potential (Eh) (mV)	COD(mgO_2/L)	SO_4^{2-} (mg/L)	COD/SO_4^{2-}
Beetroot molasses+10% calcite tailings (w/v)	1:400	7.46	86	4035	1273	3.17
	1:200	7.36	29	6580	1348	4.88
	1:100	7.37	-71	11920	1398	8.53
	1:50	7.31	-165	23840	1493	15.97
Orange molasses +10% calcite tailings (w/v)	1:400	7.63	53	448	1202	0.37
	1:200	7.57	64	741	1222	0.61
	1:100	7.30	57	1062	1190	0.89

Table 3- Final pH and Eh values and maximum sulphide concentration as well asmaximum sulphate uptake and respective required time (adapted from Alexandre, 2016).

Sub-products	Dilutions tested in Postgate B medium without lactate	Final pH	Final Eh (mV)	Max. $[S^{2-}]$ (mg/L)	Max. $\%SO_4^{2-}$ Uptake (mg/L)	Time for Max.SO_4^{2-} Uptake (days)	Time for at least 90% SO_4^{2-} Uptake (days)
Beetroot molasses+10% calcite tailings (w/v)	1:400	6.96	-344	138	71	26	14
	1:200	7.00	-301	192	85	40	26
	1:100	6.51	-262	46	30	40	40
	1:50	6.46	-114	4		-	-
Orange molasses+10% calcite tailings (w/v)	1:400	6.95	-303	49	51	28	28
	1:200	8.84	-344	106	68	28	28
	1:100	6.42	-233	83	46	28	21

Annexe 2

Detailed results obtained in the bacth experiments

Table 1- Depicting measured values of pH, E_h(mV) ,SO_4^{2-} (mg/L) ,S^{2-}(mg/L) in average of triplicates of beetroot molasses diluted 1:200 in Postgate B medium without lactate, with 10% calcite and with SRB inoculum

Test media	**Parameters monitored**			
Beetroot molasses diluted 1:200 in Postgate B medium (without lactate) + SRB + 10% calcite tailings	pH	Eh(mV)	SO_4^{2-}(mg/L)	S^{2-}(mg/L)
Day 0: 17/11/15				
Initial sample values	6.98	201	1427	
Average				
Standard deviation				
Day 1: 24/11/15				
R1	5.30	-67	1681	5.72
R2	5.23	-81	1632	5.02
R3	5.08	-50	1567	0.86
Average	5.20	-66	1626.67	3.87
Standard deviation	0.11	15.52	57.19	2.63
% Sulphate reduction				
Day 2: 01/12/15				
R1	6.95	-215	1308	0
R2	6.55	-244	1301	0.94
R3	6.72	-227	1403	2.6
Average	6.74	-228.67	1337.33	1.18
Standard deviation	0.20	14.57	56.98	1.32
% Sulphate reduction			6.28	
Day 3: 08/12/15				
R1	6.59	-219	828	11.51
R2	6.66	-262	850	29.89
R3	6.59	-161	628	17.19
Average	6.59	-214	768.67	19.53
Standard deviation	0.01	50.69	122.32	9.41
% Sulphate reduction			46.13	

Table 2- - Depicting measured values of pH, Eh (mV) ,SO_4^{2-} (mg/L) ,S^{2-}(mg/L) in average of triplicates of orange molasses diluted 1:200 in Postgate B medium without lactate, with 10% calcite and with SRB inoculum

Test media	**Parameters monitored**			
Orange molasses diluted 1:200 in Postgate B medium (without lactate) + SRB + 10% calcite tailings	pH	Eh(mV)	SO_4^{2-}(mg/L)	S^{2-}(mg/L)
Day 0: 17/11/15				
Initial sample values	7.28	142	1614	
Average				
Standard deviation				
Day 1: 24/11/15				
R1	5.81	-109	1462	29.27
R2	5.94	-224	1464	32.1
R3	5.77	-152	1634	4.06
Average	5.84	-161.67	1520	21.81
Standard deviation	0.09	58.11	98.73	15.44
% Sulphate reduction			5.82	
Day 2: 01/12/15				
R1	6.36	-281	1011	45.25
R2	6.61	-218	910	50.85
R3	6.5	-148	1280	13.71
Average	6.49	-215.67	1067	36.6
Standard deviation	0.13	66.53	191.25	20.02
% Sulphate reduction			33.89	
Day 3: 08/12/15				
R1	6.75	-295	426	66.64
R2	6.77	-305	412	68.21
R3	6.54	-137	845	3.55
Average	6.69	-245.67	561	46.13
Standard deviation	0.13	94.24	246.05	36.89
% Sulphate reduction			65.24	

Table 3- Depicting measured values of pH, E_h(mV) ,SO_4^{2-} (mg/L) ,S^{2-}(mg/L) in average of triplicates of original Postgate B medium, with 10% calcite and with SRB inoculum

Test media	**Parameters monitored**			
Postgate B medium (Original) + SRB + 10% calcite tailings	pH	Eh(mV)	SO_4^{2-}(mg/L)	S^{2-}(mg/L)
Day 0: 17/11/15				
Initial sample values	6.74	25	1011	
Average				
Standard deviation				
Day 1: 24/11/15				
R1	7.05	-342	125	321.42
R2	7.09	-349	175	338.06
R3	7.12	-355	146	329.5
Average	7.09	-348.67	148.67	329.66
Standard deviation	0.04	6.51	25.11	8.32
% Sulphate reduction			85.3	
Day 2: 01/12/15				
R1	7.03	-348	202	290.52
R2	7	-346	255	265.5
R3	6.98	-351	210	240.56
Average	7	-348.33	222.33	265.53
Standard deviation	0.03	2.52	28.57	24.98
% Sulphate reduction			78.01	
Day 3: 08/12/15				
R1	7.18	-346	139	193.95
R2	7.13	-348	142	259.22
R3	7.09	-347	208	230.28
Average				
Standard deviation				
% Sulphate reduction				

Table 4- Depicting measured values of pH, E_h (mV) ,SO_4^{2-} (mg/L) ,S^{2-}(mg/L) in average of triplicates of beetroot molasses diluted 1:200 in AMD, with 10% calcite and with SRB inoculum.

Test media	**Parameters monitored**			
Beetroot molasses diluted 1:200 in AMD + SRB + 10% calcite tailings	pH	Eh(mV)	SO_4^{2-}(mg/L)	S^{2-}(mg/L)
Day 0: 17/11/15				
Initial sample values	6.99	266	1406	
Average				
Standard deviation				
Day 1: 24/11/15				
R1	6.24	-197	1322	11.79
R2	6.57	-85	1293	9.61
R3	6.57	-113	1375	6.24
Average	6.51	-113.67	1330	9.21
Standard deviation	0.1	58.29	41.58	2.8
% Sulphate reduction			5.41	
Day 2: 01/12/15				
R1	6.42	-300	846	79.59
R2	6.38	-300	834	88.12
R3	6.43	-213	874	66.75
Average	6.41	-271	851.33	78.15
Standard deviation	0.03	50.23	20.53	10.76
% Sulphate reduction			39.45	
Day 3: 08/12/15				
R1	6.73	-301	343	89.7
R2	6.69	-302	564	105.89
R3	6.75	-305	501	91.99
Average	6.72	-302.67	469.33	12.91
Standard deviation	0.03	2.08	113.85	
% Sulphate reduction			66.62	

Table 5- Depicting measured values of pH, E_h(mV) ,SO_4^{2-} (mg/L) ,S^{2-}(mg/L) in average of triplicates of beetroot molasses diluted 1:200 in AMD, with 10% calcite and without SRB inoculum.

Test media	**Parameters monitored**			
Beetroot molasses diluted 1:200 in AMD - SRB + 10% calcite tailings	pH	Eh(mV)	SO_4^{2-}(mg/L)	S^{2-}(mg/L)
Day 0: 17/11/15				
Initial sample values	6.99	266	1406	
Average				
Standard deviation				
Day 1: 24/11/15				
R1	7.01	101	1384	
R2	6.72	120	1430	
R3	7.18	113	1398	
Average	6.97	113.33	1404	
Standard deviation	0.23	9.61	23.58	
% Sulphate reduction			0.14	
Day 2: 01/12/15				
R1	6.95	203	1390	79.59
R2	6.55	184	1633	88.12
R3	6.72	142	1490	66.75
Average	6.74	176.33	1504.33	78.15
Standard deviation	0.20	31.22	122.13	10.76
% Sulphate reduction				
Day 3: 08/12/15				
R1	6.59	203	1082	89.7
R2	7.03	152	1144	105.89
R3	6.67	165	1135	91.99
Average	6.76	173.33	1120.33	12.91
Standard deviation	0.23	26.5	33.5	
% Sulphate reduction			20.32	

Table 6- Depicting measured values of pH, E_h(mV) ,SO_4^{2-} (mg/L) ,S^{2-}(mg/L) in average of triplicates orange molasses diluted 1:200 in AMD, with 10% calcite and with SRB inoculum

Test media		**Parameters monitored**		
Orange molasses diluted 1:200 in AMD + SRB + 10% calcite tailings	pH	Eh(mV)	SO_4^{2-}(mg/L)	S^{2-}(mg/L)
Day 0: 17/11/15				
Initial sample values	6.79	263	1177	
Average				
Standard deviation				
Day 1: 24/11/15				
R1	6.33	-158	1174	17.46
R2	6.31	-111	1259	9.7
R3	6.34	-115	1598	8.63
Average	6.33	-128	1343.67	11.93
Standard deviation	0.02	26.06	224.33	4.82
% Sulphate reduction				
Day 2: 01/12/15				
R1	6.53	-238	746	115.88
R2	6.47	-300	685	133.98
R3	6.55	-307	710	117.78
Average	6.52	-281.67	713.67	122.55
Standard deviation	0.04	37.98	30.66	9.95
% Sulphate reduction			39.37	
Day 3: 08/12/15				
R1	6.51	-281	454	76.91
R2	6.51	-227	391	99.25
R3	6.06	-261	410	115.3
Average	6.36	-256.33	418.33	97.15
Standard deviation	0.26	27.3	32.32	19.28
% Sulphate reduction			64.46	

Table 7- Depicting measured values of pH, E_h(mV) ,SO_4^{2-} (mg/L) ,S^{2-}(mg/L) in average of triplicates of orange molasses diluted 1:200 in AMD, with 10% calcite and without SRB inoculum

Test media	**Parameters monitored**			
Orange molasses diluted 1:200 in AMD - SRB + 10% calcite tailings	pH	Eh(mV)	SO_4^{2-}(mg/L)	S^{2-}(mg/L)
Day 0: 17/11/15				
Initial sample values	6.99	263	1177	
Average				
Standard deviation				
Day 1: 24/11/15				
R1	6.96	164	1322	1.18
R2	6.93	163	1426	1.56
R3	6.99	166	1365	5.71
Average	6.96	164.33	1374.33	2.82
Standard deviation	0.03	1.53	47.69	2.51
% Sulphate reduction				
Day 2: 01/12/15				
R1	6.89	234	1490	-
R2	6.97	225	1305	0.72
R3	6.91	218	1406	0.06
Average	6.92	225.67	1400.33	0.39
Standard deviation	0.04	8.02	92.63	0.46
% Sulphate reduction			39.37	
Day 3: 08/12/15				
R1	6.28	234	1073	-
R2	6.26	224	1001	-
R3	7.2	224	1013	-
Average	6.58	227.33	1029	-
Standard deviation	0.54	5.77	38.57	-
% Sulphate reduction			12.57	-

Table 8- Depicting measured values of pH, E_h(mV), Fe (mg/L), Cu (mg/L) & Zn (mg/L) in average of triplicates of beetroot molasses diluted 1:200 in AMD, with 10% calcite and with SRB inoculum

Test media	**Parameters monitored**				
Beetroot molasses diluted 1:200 in AMD + SRB + 10% calcite tailings	pH	Eh(mV)	Iron*(mg/L)	Copper* (mg/L	Zinc*(mg/L)
Day 0: 17/11/15					
Initial sample values	6.99	266	0.45	12.8	3.76
Average					
Standard deviation					
Day 1: 24/11/15					
R1	6.40	-197	0.58	< LOD	< LOD
R2	6.57	-85	0.81	< LOD	< LOD
R3	6.57	-113	0.87	< LOD	< LOD
Average	6.51	-131.67	0.75	0.01	0
Standard deviation	0.1	58.29	0.16	0.06	0.18
% metal removal				100	100
Day 2: 01/12/15					
R1	6.42	-300	1.31	< LOD	< LOD
R2	6.38	-300	1.01	< LOD	< LOD
R3	6.43	-213	1.63	< LOD	< LOD
Average	6.41	-271	1.32	0	0
Standard deviation	0.03	50.23	0.31	0.04	0
% metal removal				100	100
Day 3: 08/12/15					
R1	6.73	-301	0.54	< LOD	0.21
R2	6.69	-302	0.55	< LOD	< LOD
R3	6.75	-305	0.6	< LOD	0.18
Average	6.72	-302.67	0.56	0.01	0.15
Standard deviation	0.03	2.08	0.03	0.07	0.08
% metal removal				100	96.12

*- Average, standard deviations and % metal removal was calculated with all the measured values including those below limits of detection (LOD).

Limit of detection values:

Fe: 0.1668mg/L, Cu: 0.3559mg/L, Zn: 0.0808mg/L

Table 9- Depicting measured values of pH, E_h(mV), Fe (mg/L), Cu (mg/L) & Zn (mg/L) in average of triplicates of beetroot molasses diluted 1:200 in AMD, with 10% calcite and without SRB inoculum.

Test media	**Parameters monitored**				
Beetroot molasses diluted 1:200 in AMD - SRB + 10% calcite tailings	pH	Eh(mV)	Iron*(mg/L)	Copper* (mg/L)	Zinc*(mg/L)
Day 0: 17/11/15					
Initial sample values	6.99	266	0.45	12.8	3.76
Average					
Standard deviation					
Day 1: 24/11/15					
R1	7.01	101	0.22	11.15	7.36
R2	6.72	120	0.96	7.31	5.31
R3	7.18	113	0.28	11.08	3.64
Average	6.97	111.33	0.49	9.85	5.44
Standard deviation	0.23	9.61	0.41	2.2	1.86
% metal removal				23.08	
Day 2: 01/12/15					
R1	6.95	203	0.26	8.6	4.81
R2	6.55	184	2.1	2.79	< LOD
R3	6.72	142	0.64	6.72	6.43
Average	6.74	176.33	1	6.03	3.75
Standard deviation	0.20	31.21	0.97	2.96	3.34
% metal removal				52.85	0.35
Day 3: 08/12/15					
R1	6.59	203	2.97	0.26	6.02
R2	7.03	152	0.56	0.71	7.59
R3	6.67	165	1.93	0.43	6.4
Average	6.76	173.33	1.82	0.46	6.67
Standard deviation	0.23	26.5	1.21	0.23	0.82
% metal removal				96.38	96.12

*- Average, standard deviations and % metal removal was calculated with all the measured values including those below limits of detection (LOD).

Limit of detection values:

Fe: 0.1668mg/L, Cu: 0.3559mg/L, Zn: 0.0808mg/L

Table 10- Depicting measured values of pH, E_h(mV), Fe (mg/L), Cu (mg/L) & Zn(mg/L) in average of triplicates of orange molasses diluted 1:200 in AMD, with 10% calcite and with SRB inoculum.

Test media	**Parameters monitored**				
Orange molasses diluted 1:200 in AMD + SRB + 10% calcite tailings	pH	Eh(mV)	Iron*(mg/L)	Copper* (mg/L)	Zinc*(mg/L)
Day 0: 17/11/15					
Initial sample values	6.79	263	0.46	10.7	4.2
Average					
Standard deviation					
Day 1: 24/11/15					
R1	6.33	-158	0.59	0.35	0.69
R2	6.31	-111	1.14	< LOD	0.44
R3	6.34	-115	0.7	< LOD	0.32
Average	6.33	-128	0.81	0.24	0.49
Standard deviation	0.02	26.06	0.29	0.09	0.19
% metal removal				97.76	88.43
Day 2: 01/12/15					
R1	6.53	-238	0.32	< LOD	0.79
R2	6.47	-300	0.42	< LOD	0.46
R3	6.55	-307	0.42	< LOD	0.13
Average	6.52	-281.67	0.38	0.01	0.46
Standard deviation	0.04	37.98	0.06	0.05	0.33
% metal removal			15.54	100	89.05
Day 3: 08/12/15					
R1	6.51	-281	0.69	< LOD	0.54
R2	6.51	-227	0.46	< LOD	0.5
R3	6.06	-261	0.46	< LOD	0.52
Average	6.36	-256.33	0.54	0.05	0.52
Standard deviation	0.26	27.3	0.14	0.15	0.02
% metal removal				99.55	96.12

*- Average, standard deviations and % metal removal was calculated with all the measured values including those below limits of detection (LOD).

Limit of detection values:

Fe: 0.1668mg/L, Cu: 0.3559mg/L, Zn: 0.0808mg/L

Table 11- Depicting measured values of pH, E_h(mV), Fe (mg/L), Cu (mg/L) & Zn(mg/L) in average of triplicates of orange molasses diluted 1:200 in AMD, with 10% calcite and without SRB inoculum.

Test media	**Parameters monitored**				
Orange molasses diluted 1:200 in AMD - SRB + 10% calcite tailings	pH	Eh(mV)	Iron*(mg/L)	Copper* (mg/L)	Zinc*(mg/L)
Day 0: 17/11/15					
Initial sample values	6.79	263	0.46	10.7	4.2
Average					
Standard deviation					
Day 1: 24/11/15					
R1	6.96	164	0.35	8.62	5.64
R2	6.93	163	0.39	9.3	3.44
R3	6.99	166	0.36	8.71	4.04
Average	6.96	164.33	0.37	8.86	4.37
Standard deviation	0.03	153	0.02	0.34	1.14
% metal removal			19.55	17.2	
Day 2: 01/12/15					
R1	6.89	234	0.39	6.61	5.91
R2	6.97	225	0.41	6.67	5.33
R3	6.91	218	0.41	6.53	5.18
Average	6.92	225.67	0.41	6.6	5.47
Standard deviation	0.04	8.02	0.01	0.07	0.38
% metal removal			10.85	38.3	
Day 3: 08/12/15					
R1	6.28	234	0.43	< LOD	4.91
R2	6.26	224	0.4	< LOD	7.13
R3	7.2	224	0.45	0.42	4.14
Average	6.58	227.33	0.43	0.14	5.39
Standard deviation	0.54	5.77	0.02	0.25	1.55
% metal removal			5.98	98.72	

*- Average, standard deviations and % metal removal was calculated with all the measured values including those below limits of detection (LOD).

Limit of detection values:

Fe: 0.1668mg/L, Cu: 0.3559mg/L, Zn: 0.0808mg/L

Table 12- Depicting measured sugar compounds: Sucrose, Glucose and Fructose in beetroot molasses diluted 1:200 in AMD, with 10% calcite and with SRB inoculum.

BEETROOT MOLASSES DILUTED 1:200 IN AMD + SRB +10% CALCITE TAILINGS				
SUCROSE				
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	4406	4406	0
1ª week- R1	7	4226	3894	305
1ª week- R2		3625		
1ª week- R3		3832		
2ª week- R1	14	569	473	107
2ª week- R2		493		
2ª week- R3		358		
3ª week- R1	21	560	441	119
3ª week- R2		323		
3ª week- R3		439		
GLUCOSE				
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	0	0	0
1ª week- R1	7	0	0	0
1ª week- R2		0		
1ª week- R3		0		
2ª week- R1	14	0	0	0
2ª week- R2		0		
2ª week- R3		0		
3ª week- R1	21	413	138	239
3ª week- R2		0		
3ª week- R3		0		
FRUCTOSE				
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	1365	1365	0
1ª week- R1	7	0	267	463
1ª week- R2		0		
1ª week- R3		801		
2ª week- R1	14	0	0	0
2ª week- R2		0		
2ª week- R3		0		
3ª week- R1	21	0	0	0
3ª week- R2		0		
3ª week- R3		0		

Table 13- Depicting measured sugar compounds: Sucrose, Glucose and Fructose in beetroot molasses diluted 1:200 in AMD, with 10% calcite and without SRB inoculum

BEETROOT MOLASSES DILUTED 1:200 IN AMD - SRB +10% CALCITE TAILINGS				
		SUCROSE		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	4406	4406	0
1^{a} week- R1	7	0	4373	1
1^{a} week- R2		4374		
1^{a} week- R3		4372		
2^{a} week- R1	14	1721	1324	362
2^{a} week- R2		1012		
2^{a} week- R3		1240		
3^{a} week- R1	21	863	1231	356
3^{a} week- R2		1256		
3^{a} week- R3		1573		
		GLUCOSE		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	0	0	0
1^{a} week- R1	7	0	0	0
1^{a} week- R2		0		
1^{a} week- R3		0		
2^{a} week- R1	14	2967	2334	565
2^{a} week- R2		1880		
2^{a} week- R3		2156		
3^{a} week- R1	21	2141	714	1236
3^{a} week- R2		0		
3^{a} week- R3		0		
		FRUCTOSE		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	1365	1365	0
1^{a} week- R1	7	1716	1420	261
1^{a} week- R2		1323		
1^{a} week- R3		1222		
2^{a} week- R1	14	1014	638	337
2^{a} week- R2		538		
2^{a} week- R3		362		
3^{a} week- R1	21	0	115	0
3^{a} week- R2		322		
3^{a} week- R3		485		

Table 14- Depicting measured sugar compounds: Sucrose, Glucose and Fructose in orange molasses diluted 1:200 in AMD, with 10% calcite and with SRB inoculum.

ORANGE MOLASSES DILUTED 1:200 IN AMD + SRB +10% CALCITE TAILINGS				
		SUCROSE		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	1303	1303	0
1^a week- R1	7	1481	1407	527
1^a week- R2		1893		
1^a week- R3		848		
2^a week- R1	14	0	318	276
2^a week- R2		491		
2^a week- R3		464		
3^a week- R1	21	250	247	5
3^a week- R2		250		
3^a week- R3		241		
		GLUCOSE		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	1192	1192	0
1^a week- R1	7	0	0	0
1^a week- R2		0		
1^a week- R3		0		
2^a week- R1	14	0	0	0
2^a week- R2		0		
2^a week- R3		0		
3^a week- R1	21	324	331	41
3^a week- R2		375		
3^a week- R3		295		
		FRUCTOSE		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	141	141	0
1^a week- R1	7	534	178	308
1^a week- R2		0		
1^a week- R3		0		
2^a week- R1	14	0	0	0
2^a week- R2		0		
2^a week- R3		0		
3^a week- R1	21	0	0	0
3^a week- R2		0		
3^a week- R3		0		

Table 15- Depicting sugar compounds: Sucrose, Glucose and Fructose in orange molasses diluted 1:200 in AMD, with 10% calcite and without SRB inoculum

ORANGE MOLASSES DILUTED 1:200 IN AMD - SRB +10% CALCITE TAILINGS				
		SUCROSE		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	1303	1303	0
1ª week- R1	7	0	1987	134
1ª week- R2		1892		
1ª week- R3		2082		
2ª week- R1	14	956	763	193
2ª week- R2		763		
2ª week- R3		570		
3ª week- R1	21	649	742	224
3ª week- R2		579		
3ª week- R3		997		
		GLUCOSE		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	1192	1192	0
1ª week- R1	7	0	0	0
1ª week- R2		0		
1ª week- R3		0		
2ª week- R1	14	2148	1462	636
2ª week- R2		1347		
2ª week- R3		891		
3ª week- R1	21	909	1100	170
3ª week- R2		1235		
3ª week- R3		1156		
		FRUCTOSE		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	141	141	0
1ª week- R1	7	0	299	260
1ª week- R2		470		
1ª week- R3		426		
2ª week- R1	14	1761	928	727
2ª week- R2		605		
2ª week- R3		419		
3ª week- R1	21	360	469	108
3ª week- R2		472		
3ª week- R3		576		

Table 16- Depicting organic compounds: Acetic acid, Propionic acid, Butyric acid, and Lactic acid from beetroot molasses diluted 1:200 in AMD, with 10% calcite and with SRB inoculum.

BEETROOT MOLASSES DILUTED 1:200 IN AMD + SRB +10% CALCITE TAILINGS				
		ACETIC ACID		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	0	0	0
1ª week- R1	7	617	728	265
1ª week- R2		1029		
1ª week- R3		536		
2ª week- R1	14	883	965	101
2ª week- R2		1078		
2ª week- R3		935		
3ª week- R1	21	1307	1156	133
3ª week- R2		1109		
3ª week- R3		1053		
		PROPIONIC ACID		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	1192	1192	0
1ª week- R1	7	0	0	0
1ª week- R2		0		
1ª week- R3		0		
2ª week- R1	14	0	0	0
2ª week- R2		0		
2ª week- R3		0		
3ª week- R1	21	334	279	54
3ª week- R2		277		
3ª week- R3		226		
		BUTYRIC ACID		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	0	0	0
1ª week- R1	7	0	0	0
1ª week- R2		0		
1ª week- R3		0		
2ª week- R1	14	0	363	358
2ª week- R2		373		
2ª week- R3		715		
3ª week- R1	21	0	0	0
3ª week- R2		0		
3ª week- R3		0		
		LACTIC ACID		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	59	59	0
1ª week- R1	7	191	221	42
1ª week- R2		269		
1ª week- R3		203		
2ª week- R1	14	259	186	176
2ª week- R2		313		
2ª week- R3		0		
3ª week- R1	21	0	0	0
3ª week- R2		0		
3ª week- R3		0		

Table 17- Depicting organic compounds: Acetic acid, Propionic acid, Butyric acid and Lactic acid from beetroot molasses diluted 1:200 in AMD, with 10% calcite and without SRB inoculum.

BEETROOT MOLASSES DILUTED 1:200 IN AMD - SRB +10% CALCITE TAILINGS				
ACETIC ACID				
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	0	0	0
1ª week- R1	7	0	0	0
1ª week- R2		0		
1ª week- R3		0		
2ª week- R1	14	0	0	0
2ª week- R2		0		
2ª week- R3		0		
3ª week- R1	21	0	254	439
3ª week- R2		760		
3ª week- R3		0		
PROPIONIC ACID				
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	0	0	0
1ª week- R1	7	0	0	0
1ª week- R2		0		
1ª week- R3		0		
2ª week- R1	14	0	0	0
2ª week- R2		0		
2ª week- R3		0		
3ª week- R1	21	0	0	0
3ª week- R2		0		
3ª week- R3		0		
BUTYRIC ACID				
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	0	0	0
1ª week- R1	7	0	0	0
1ª week- R2		0		
1ª week- R3		0		
2ª week- R1	14	0	363	358
2ª week- R2		373		
2ª week- R3		715		
3ª week- R1	21	0	205	354
3ª week- R2		613		
3ª week- R3		0		
LACTIC ACID				
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	59	59	0
1ª week- R1	7	0	22	38
1ª week- R2		67		
1ª week- R3		0		
2ª week- R1	14	181	91	79
2ª week- R2		60		
2ª week- R3		32		
3ª week- R1	21	3	57	55
3ª week- R2		114		
3ª week- R3		55		

Table 18- Depicting organic compounds: Acetic acid andLactic acid from orange molasses diluted 1:200 in AMD, with 10% calcite and with SRB inoculum

ORANGE MOLASSES DILUTED 1:200 IN AMD + SRB +10% CALCITE TAILINGS				
		ACETIC ACID		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	0	0	0
1^a week- R1	7	605	522	486
1^a week- R2		0		
1^a week- R3		961		
2^a week- R1	14	708	865	177
2^a week- R2		831		
2^a week- R3		1057		
3^a week- R1	21	803	830	89
3^a week- R2		757		
3^a week- R3		929		
		LACTIC ACID		
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	248	248	0
1^a week- R1	7	234	147	128
1^a week- R2		0		
1^a week- R3		206		
2^a week- R1	14	0	58	90
2^a week- R2		81		
2^a week- R3		135		
3^a week- R1	21	0	0	0
3^a week- R2		0		
3^a week- R3		0		

Table 19- Depicting organic compounds: Acetic acid and Lactic acid from orange molasses diluted 1:200 in AMD, with 10% calcite and without SRB inoculum.

ORANGE MOLASSES DILUTED 1:200 IN AMD - SRB +10% CALCITE TAILINGS				
ACETIC ACID				
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	0	0	0
1ª week- R1	7	0	0	0
1ª week- R2		0		
1ª week- R3		0		
2ª week- R1	14	0	0	0
2ª week- R2		0		
2ª week- R3		0		
3ª week- R1	21	0	0	0
3ª week- R2		0		
3ª week- R3		0		
LACTIC ACID				
Samples	Day	Concentration(mg/L)	Average(mg/L)	Standard deviation
Initial	0	248	248	0
1ª week- R1	7	232	233	2
1ª week- R2		235		
1ª week- R3		231		
2ª week- R1	14	208	203	29
2ª week- R2		173		
2ª week- R3		230		
3ª week- R1	21	0	90	109
3ª week- R2		60		
3ª week- R3		211		

Annexes 3

Detailed results obtained in the continuous flow experiments

Table 1- Values for pH, Eh, Ec, SO_4^{2-} and heavy metals (Fe, Al, Zn and Cu) measured in the neutralization tank (NT 1) preceding the bioreactor fed with orange molasses (R1).

Date	Days								
24-02-16	935	pH	Eh(mV)	Cond.(mS)	SO_4(mg/L)	Fe(mg/L)	Al(mg/L)	Zn(mg/L)	Cu(mg/L)
26-02-16	937	6.60	344	3.23	1735	0.48	0.18	13.47	0.73
03-03-16	943	7.30	353	3.20	1782	0.48	0.12	4.82	< LOD
10-03-16	950	7.54	199	3.09	1816	0.45	0.28	0.74	< LOD
17-03-16	957	6.90	190	3.12	1855	0.32	0.06	1.03	< LOD
30-03-16	970	7.60	243	3.17	1783	0.19	0.25	1.47	< LOD
07-04-16	978	7.60	294	3.17	1999	0.24	0.27	2.07	< LOD
14-04-16	985	7.57	273	3.13	1910	< LOD	0.10	2.32	< LOD
21-04-16	992	7.51	255	3.16	1770	< LOD	0.06	2.26	< LOD
28-04-16	999	7.51	276	3.13	1835	0.36	0.01	2.39	< LOD
03-05-16	1003	7.42	217	3.17	1821	0.38	0.08	5.44	< LOD
12-05-16	1012	7.47	319	3.24	1858	0.41	0.05	5.33	< LOD
19-05-16	1019	7.38	207	3.29	2080	0.48	3.59	6.29	< LOD
27-05-16	1027	7.27	196	3.19	2101	0.87	0.02	7.05	< LOD

LOD- Limit of detection.
Fe: 0.1668mg/L, Al: 0.0219mg/L, Zn: 0.0808mg/L, Cu:0.3559mg/L

Table 2- Values for pH, Eh, Ec, SO_4^{2-} and heavy metals (Fe, Al, Zn and Cu) measured in effluent samples of the bioreactor fed with orange molasses (R1).

Dates	**Days**	**pH**	**Eh(mV)**	**Cond.(mS)**	**SO_4(mg/L)**	**S^{2-}(mg/L)**	**Fe(mg/L)**	**Al(mg/L)**	**Zn(mg/L)**	**Cu(mg/L)**
24-02-16	935	IST FEEDING REGIME NUTRIENT FLOW RATE OF 0.005ml/hr								
26-02-16	937	8.84	-317	3.30	295	208	0.49	< LOD	0.22	< LOD
03-03-16	943	6.41	-242	3.42	2543	369	0.54	0.13	0.31	< LOD
10-03-16	950	6.45	-234	3.50	1070	125	0.99	< LOD	0.26	< LOD
17-03-16	957	6.50	-279	4.18	679	222	0.94	< LOD	0.14	< LOD
30-03-16	970	6.39	-251	4.06	1079	162	0.65	0.32	< LOD	< LOD
07-04-16	978	8.07	-234	3.86	1211	122	0.67	0.20	< LOD	< LOD
14-04-16	985	6.37	-244	3.85	1158	88	0.69	0.41	< LOD	< LOD
21-04-16	992	6.72	-198	3.77	1250	77	0.70	0.35	< LOD	< LOD
2ND FEEDING REGIME INCREAMENT BY 50% OF PREVIOUS FLOW RATE: 0.0075ml/hr										
28-04-16	999	6.58	-240	3.92	789	119	0.89	0.44	< LOD	< LOD
03-05-16	1003	6.72	-272	4.29	504	194	0.90	0.49	< LOD	< LOD
12-05-16	1012	6.77	-286	4.32	324	363	0.91	0.55	< LOD	< LOD
19-05-16	1019	7.40	-249	4.13	338	187	0.74	0.13	< LOD	< LOD
27-05-16	1027	6.84	-272	4.31	444	187	0.78	0.42	< LOD	< LOD

LOD- Limit of detection.
Fe: 0.1668mg/L, Al: 0.0219mg/L, Zn: 0.0808mg/L, Cu:0.3559mg/L

Table 3- Values for pH, Eh, Ec, SO_4^{2-} and heavy metals (Fe, Al, Zn and Cu) measured in the neutralization tank (NT 2) preceding the bioreactor fed with beetroot molasses (R2).

Date	Days								
29-02-16	940	pH	Eh(mV)	Cond.(mS)	SO_4(mg/L)	Fe(mg/L)	Al(mg/L)	Zn(mg/L)	Cu(mg/L)
03-03-16	943	7.14	339	3.26	1902	0.54	0.03	5.8	< LOD
10-03-16	950	7.44	198	3.19	1919	0.37	0.22	3.42	< LOD
17-03-16	957	7.46	164	3.12	1914	0.32	1.03	2.58	< LOD
30-03-16	970	7.68	258	3.21	1844	0.17	< LOD	5.53	< LOD
07-04-16	978	7.62	286	3.21	1893	0.16	< LOD	7.5	< LOD
14-04-16	985	7.63	270	3.19	1860	0.18	< LOD	9.63	< LOD
21-04-16	992	7.53	245	2.92	1902	0.49	< LOD	9.63	< LOD
28-04-16	999	7.52	263	3.14	1817	0.42	< LOD	7.09	< LOD
03-05-16	1003	7.49	211	3.11	1773	0.46	< LOD	7.56	< LOD
12-05-16	1012	7.54	307	3.13	1811	0.49	0.05	7.64	< LOD
19-05-16	1019	7.29	209	3.26	1990	0.34	< LOD	13	< LOD
27-05-16	1027	6.99	204	3.15	2067	0.41	< LOD	16.56	< LOD

LOD- Limit of detection.
Fe: 0.1668mg/L, Al: 0.0219mg/L, Zn: 0.0808mg/L, Cu:0.3559mg/L

Table 4- Values for pH, Eh, Ec, SO_4^{2-} and heavy metals (Fe, Al, Zn and Cu)) measured in effluent samples of the bioreactor fed with beetroot molasses (R2).

		pH	**Eh(mV)**	**Cond.(mS)**	**SO_4(mg/L)**	**S^{2-}(mg/L)**	**Fe(mg/L)**	**Al(mg/L)**	**Zn(mg/L)**	**Cu(mg/L)**
29-02-16	940	IST FEEDING REGIME NUTRIENT FLOW RATE OF 0.005ml/hr								
03-03-16	943	7.47	-28	3.42	1158	2	3.57	< LOD	0.73	< LOD
10-03-16	950	7.05	-195	2.55	1328	12	1.02	< LOD	0.11	< LOD
17-03-16	957	6.70	-253	4.65	1043	90	0.84	2.62	0.08	< LOD
30-03-16	970	6.19	-232	3.98	1186	109	0.72	0.34	< LOD	< LOD
07-04-16	978	6.06	-260	4.39	1111	128	0.74	0.33	< LOD	< LOD
14-04-16	985	5.93	-290	4.45	1026	71	0.76	0.49	< LOD	< LOD
21-04-16	992	6.92	-218	4.85	1546	26	0.77	0.26	< LOD	< LOD
		2ND REGIME INREAMENT BY 50% OF PREVIOUS FLOW RATE: 0.0075ml/hr								
28-04-16	999	6.69	-320	5.51	1239	90	0.92	0.85	< LOD	< LOD
03-05-16	1003	6.81	-316	5.02	1183	68	0.93	0.68	< LOD	< LOD
12-05-16	1012	6.29	-314	5.70	837	207	0.94	0.93	< LOD	< LOD
19-05-16	1019	6.75	-298	5.48	737	137	1.19	0.13	0.90	< LOD
27-05-16	1027	6.90	-320	5.91	336	151	1.27	0.34	2.63	< LOD

LOD- Limit of detection.

Fe: 0.1668mg/L, Al: 0.0219mg/L, Zn: 0.0808mg/L, Cu:0.3559mg/L

Table 5- Organic acids and sugars monitored in samples from both bioreactors: fed with orange and beetroot molasses

Bioreactor fed with Orange juice				Bioreactor fed with Beetroot			
	Inlet(mg/L)	Outlet (values for 10 weeks tested)				Outlet (values for 10 weeks tested)	
Compound		Average(mg/L)	Standard deviation(mg/L)	Compound	Inlet(mg/L)	Average(mg/L)	Standard deviation(mg/L)
Acetic acid	nd	632	293	Acetic acid	nd	435	289
Butyric acid	nd	171	167	Butyric acid	nd	241	128
Propionic acid	nd	nd	nd	Propionic acid	nd	nd	nd
Lactic acid	nd	nd	nd	Lactic acid	nd	nd	nd
Formic acid	nd	nd	nd	Formic acid	nd	nd	nd
Citric acid	nd	nd	nd	Citic acid	nd	nd	nd
Sucrose	3011	1188	403	Sucrose	5273	1300	299
Glucose	nd	nd	nd	Glucose	nd	nd	nd
Fructose	nd	nd	nd	Fructose	nd	nd	nd

nd- not detected

Annex 4

Calibration curves and Limits of Detection

Table 1- Calibration characteristics of analyzed metals.

	Cu	**Fe**	**Zn**	**Al**
Method	**FAAS***	**FAAS***	**FAAS***	**MW-AES****
Minimum standard concentration (mg/L)	0.1	0.25	0.05	0.1
Maximum standard concentration (mg/L)	10	10	1.5	10
Correlation coefficient ($R^2_{adjusted}$)	0.9948	0.9984	0.9900	0.9999
Limit of detection (mg/L)	0.3559	0.1668	0.0808	0.0219

* Flame atomic absorption spectrometry.
** Micro Wave atomic emission spectrometry.
nc - not calculated

Table 2- Calibration characteristics of analyzed sugars.

	Sucrose	**Glucose**	**Fructose**
Method	**HPLC – ELS Detector**		
Minimum standard concentration (mg/L)	60	60	60
Maximum standard concentration (mg/L)	500	750	750
Correlation coefficient (R^2)	0.9884	0.9968	0.9994
Limit of detection	avp	avp	avp

avp – all visible peaks

Table 3- Calibration characteristics of analyzed organic acids.

	Acetic	**Lactic**	**Propionic**	**Butyric**	**Formic**	**Citric**
Method	**HPLC – UV visible Detector (210nm)**					
Minimum standard concentration (mg/L)	70	30	15	80	50	15
Maximum standard concentration (mg/L)	40000	20000	10000	5000	30000	10000
Correlation coefficient (R)	0.9937	0.9978	0.9805	0.9950	0.9917	0.9793
Limit of detection	avp	avp	avp	apv	apv	apv

avp – all visible peaks