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Physiology of the polyextremophile *Natranaerobaculum magadiense*

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"Anything is possible
when you have the right people there to support you"
- Misty Copeland

"Success isn't just about what you accomplish in your life;
it's about what you inspire others to do."
-Unknown

"It always seems impossible until it's done."
- Nelson Mandela

"If you fell down yesterday, stand up today."
-H.G. Wells

Abstract

Concrete is a strong, durable and cheap material. Its main problem is cracking, inducing to major problems like sulphate and chloride attacks, carbonation and decalcification. Repairing concrete can be very costly. The solution is the Self Healing Concrete. A sustainable, reliable, economical, in long term, and stable concept. Based on an aerobic spore-forming bacteria introduced in concrete and whenever there was a crack and water came in, the bacteria would start precipitating $CaCO_3$. Concrete can be found also in oil wells. Due to high temperatures and pressures, suffers a lot of stresses and tensions. In Aarhus University, researchers decided to apply the Self Healing Cement in this anaerobic environment. A proper bacteria needed to be found to handle such conditions. *Natranaerobaculum magadiense (*N. magadiense*)* was the bacteria to be studied. The project is centred on the precipitation of $CaCO_3$ induced by *N. magadiense*. The main goal of this work was to study the physiology of *N. magadiense* at different conditions. It was investigated its behaviour in terms of extreme conditions, since it is a polyextremophile. The project tests involved a reduction of carbonate (HCO_3^-) from the optimum media, that conflict with microbial $CaCO_3$ precipitation. *N. magadiense*'s behaviour when the pH was changed. A carbon source replacement to measure the CO_2/CO_3^{2-} production and extreme conditions were applied and the results were evaluated. We proved that *N. magadiense* cannot grow without carbonates. It was not found a good replacement carbon source. $[HCO_3^-]$ cannot be reduced a lot, and [NaCl] cannot be a replacement compound, since $[Cl^-]$ inhibited the cells, and it is not recommended to change the pH. The iron reduction process is more noticeable at lower temperatures and the fermentative process is at higher temperatures. In conclusion, *N. magadiense* is a good candidate to be used in the oil industry but more research is needed to move to its application.

Keywords: Concrete, cracking, *N. magadiense*, self-healing, microbial, calcium, carbonate, precipitation, anaerobe, polyextremophile, oil, industry

Resumo

O betão é um material resistente, duradouro e barato. O principal problema é o *cracking*, causando grandes problemas como ataques de sulfato e cloreto, carbonatação e descalcificação. A sua reparação pode ser muito cara. A solução é a auto-regeneração do betão. Um conceito sustentável, confiável, económico, a longo prazo e estável. Baseado numa bactéria aeróbica formadora de esporos introduzida no betão e sempre que haja um crack e água entra no sistema, as bactérias começam a precipitar o $CaCO_3$. O betão pode também ser encontrado em poços de petróleo. Devido às altas temperaturas e pressões, este sofre muitas tensões. Investigadores da Universidade de Aarhus decidiram aplicar o conceito de auto-regeneração do betão neste ambiente anaeróbico. Precisava-se de encontrar uma bactéria adequada para se adaptar a estas condições. *N. magadiense* foi a bactéria selecionada para o estudo. Este projeto é centrado na precipitação de $CaCO_3$ induzida por *N. magadiense*. Estudou-se a fisiologia de *N. magadiense* em diferentes condições. Investigou-se seu comportamento em termos de condições extremas, uma vez que é um poliextremófilo. Os testes do projeto envolveram uma redução do carbonato (HCO_3^-) dos meios ótimos, que entram em conflito com a precipitação microbiana ce CaCO3; o pH foi alterado para saber o comportamento de *N. magadiense*; uma substituição de fonte de carbono para medir a produção de $CO2/CO3^2-$ e condições extremas foram aplicadas e os resultados foram avaliados. Provou-se que gls N. magadiense não pode crescer sem carbonatos. Não foi encontrada uma boa fonte de carbono de substituição. [HCO_3^-] não pode ser muito reduzido, e [NaCl] não pode ser um substituto, pois [Cl^-] inibiu as células, e não é recomendado alterar o pH. O processo de redução de ferro é mais percetível a temperaturas mais baixas e o processo fermentativo é a temperaturas mais altas. Em conclusão, *N. magadiense* é um bom candidato para ser usado na indústria do petróleo, mas mais pesquisas são necessárias para passar à sua aplicação.

Palavras-chave: Betão, *cracking*, *N. magadiense*, auto-regeneração, processo, microbiano, precipitação, carbonato, cálcio, indústria, petrolífera

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Acronyms

AF_m Aluminate phases.

W/C water cement ratio.

N. magadiense Natranaerobaculum magadiense.

C-S-H calcium silicate hydrate.

HPLC High-performance liquid chromatography.

LC Liquid Chromatography.

NB Nutrient Broth.

OBD Overbalanced drilling.

PBS phosphate buffered saline.

PI Propidium iodide.

REV Representative Elementary of Volume.

S9 syto9.

UBD Underbalanced drilling.

Chapter 1

Introduction

1.1 Concrete, the most used material for constructions

Concrete is the most used man-made material, with over 12 billion tonnes manufactured annually, it is known worldwide because of its characteristics being similar to some natural rocks. [1–4] This consideration is based on its durability, strength and low cost. [1, 2]

Concrete's composition is mainly cement and water. It has been used for many years since it has a high-compressive strength, but, as a disadvantage,it has low tensile forces. This can be compensated by adding reinforcement steel to concrete which will take over this kind of forces. [1]

Many types of construction materials have been used from stone, bricks and tiles to, since Roman times onwards, concrete for its durability. [4] Nowadays, a hydraulic cement is used, the Portland cement. [1] In contrast with non-hydraulic cement, Portland cement, when reacted with water, turns into a much harder and insoluble material and can be used under water. Consequently, it will not deteriorate in wet and acidic environments at a high rate. [1] Sources of calcium, silicon, aluminium and iron will be blended to create this cement. [1] These sources are limestone, clay, some bauxite and iron ore and all are burned in the kiln at temperatures that can reach 1500°C. [1] The cement clinker made is most of all composed by alite ($3CaO \cdot SiO_2$), belite, ($2CaO \cdot SiO_2$), aluminate ($3CaOAl_2O_3$) and ferrite ($4CaO \cdot Al_2O_3 \cdot Fe_2O_3$). [1] Their respective composition percentage on Portland Cement, 50%, 24%, 11% and 8%. [1] After reacting with water, they give particular hydration products with diverse characteristics. Reaction rate and final strength product addition are important aspects on these compounds. [1]

1.1.1 Fly ash, blast furnace slag and silica fume, examples to improve concrete

Another aspect that has to be mentioned is the fact that, if researchers want to improve concrete, a variety of additives can be added. [1, 5] These additives will work on concrete's workability, pumpability, setting properties, mechanical performance and durability. [5] They can, as well, enable concrete's manufacture and construction, producing special products as high fluidity, high strength, underwater and sprayed concrete. [5] These added materials can come from industrial waste or recycled materials and can be polymer base, mineral or supplementary cementitious materials. [1, 5] Polymers can modify the concrete using synthetic rubber latexes and thermoplastic emulsions which are known for being chemically resistant concrete materials [6] but, due to environmental conditions, they cannot be used anymore, making the mineral and supplementary cementitious materials the most common used. [3] The cement production is a high energy consuming process, since raw materials are burnt at 1500°C, and releases to the atmosphere a large amount of CO_2 [1], 6-8% when producing Portland's cement. [3] Thus, has a negative impact economically- due to high amount of energy needed and spent - and environmentally. [1, 5]

To minimize these factors there are some materials that are utilized to enhance concrete's sustainability and improvement of its properties. [1]

Fly ash, silica fume, blast furnace slag are some examples to be studied. [1, 5] Fly ash, a coal-burning power plant waste product, has the ability of replacing 35-75% of cement in the concrete mix, since it is a source of reactive silica. [1] This material can improve strength by decreasing the water cement ratio (W/C) as well as resistance against chemical attacks, reducing the matrix permeability. [1]

Silica fume comes from silicon industry [1] and is a highly favoured material for its superior concrete durability properties. [2, 7–9] Blast furnace slag, from steel industry, and silica can be a good substitute of cement on concrete mix, as both have reactive silica and blast furnace slag has calcium as well. [1] Air-entraining agents, are other common additives used, since they enhance the freeze/thaw resistance, as well as setting or retarding agent and plasticizers, which permit a lower W/C and rises concrete strength. [1]

These admixtures, when added to concrete, link with calcium hydroxide loose while the hydration of cement in concrete is occurring, they form another cementitious compound, calcium silicate hydrate (C-S-H). [5]

Even if concrete is modified for a greater good, environmentally and economically speaking, it can perform negatively of what it was meant to do. These causes can be from poor design, poor construction, bad material selection, a rigid environment to work with or a combination of all these factors. [3]

As mentioned before, being one of the improvement aspects in concrete, its durability, has several processes that will influence in a negative way causing unwanted deterioration of concrete structures. [1] The main cause of this consequence is the process of cracking. [1] This affects concrete's permeability and can initiate various mechanisms, like corrosion. [1, 2] These cracks will accelerate corrosion allowing the access of corrosion agents to the steel surface. [3]

This is a major problem for civil engineers when they are working on maintaining ageing infrastructures. [2] Corrosion is the reaction of a refined metal with a non-metallic environment inducing the formation of oxides, sulphides, sulphates, chlorides and so on, changing physically and chemically metals properties. [2] Since it is a slow process and easy to detect, this problem only brings economic consequences, where it is spent billions of dollars per year to maintain, repair and replace the damage caused. [2] Freeze/thaw and alkali-silica reactivity are also other problems that can affect concrete and have to be taken on consideration. [2, 10]

1.1.2 The problem happens because of the pore transportation

Cracking results in various problems in concrete and these problems occur on the pores due to their transportation. [3] There are two types of concrete transport: ionic and moisture. [3]

1.1.2.1 Ionic Transport: an ion diffusion in saturated concrete

Ionic transport refers the diffusion of an ion in saturated concrete is studied by applying dedicated mathematical models. [3] These models can be simple, where it is tested only the complexity of the ionic transportation, and the multi-ionic, which relates the ionic transport but also another transport mechanism, like water movement when there are humidity gradients. [3] These ions can be transported by two phenomena: an electrochemical potential gradient and the changes caused by aqueous solution. [3, 11, 12]

Since the ions travel through the pores, their transport modelling should be at pore scale but that does not happen because it is an impossible task. [3] To turn the problem around, it was made a general mathematic model, the homogenization model, where it is averaged the pore sizes. [13–16] This technique counts with equations that are integrated of Representative Elementary of Volume (REV), e.g., a concept where it is measured the smallest volume in a sample that, when measuring the yield, will be insignificant to the whole sample [17], which leads to equation at material scale. [3]

Continuous hydration will affect significantly the concrete's transport properties when supplementary cementitious materials are added to the mix, like fly ash and granulated burning slag. [3]

The concrete's pore structure can be changed by chemical reactions, as well as its transport properties. [3] What happens is that it will start creating new phases and this leads to poor transport properties, by reducing the material porosity. [3] The contrary can open more pore space and, as a consequence, enhance its diffusion. [3] To conclude the ionic transport, in order to comprehend it, electrochemical potential fields, temperature and water content has to be evaluated. [3]

The electrochemical potential is normally mentioned in ionic transport and is applied using two different methods. [3] The first one is based on the null current density hypothesis, which means that there is no electrochemical potential in the transport. [3] The second one says that electrochemical potential can be counted relating it with the ionic concentration present in the solution. [3]

There is also another parameter that has to be noticed, the temperature field. Different approaches were proposed to state a prediction about the temperature distribution on porous materials. [3] The most comprehensive involves the resolution of the energy balance on each phase in the porous medium. [3, 18] Turns out that this parameter is hard to evaluate and, for that reason, is often neglected. [3]

1.1.2.2 The moisture transport

The other type of transport is the moisture transport. [3] This one has two approaches when working with it. [3] The first one involves a summary of all phases in the process: aqueous solution, water, vapor and dry air. [3] Here, various mass conservation equations are applied to result on the description of the global moisture fields. [3]

Some tests were made where it was studied the multiphase approach under isothermal conditions. [19] The results confirm that it could reproduce, in a proper way, in isothermal drying tests. [19] This is useful, but in a marginal way, when analysing concrete's durability because of the parameters that need to be resolved. [3, 19]

The second method is a simplified version of the first one. [3] It studies the water content in cement-based materials with the assumption that the gas pressure is always the same and corresponds to the atmospheric pressure. [3]

1.1.3 Cement is composed with isolated and interconnected pores

The cement paste structure is composed by pores, that can be isolated and interconnected pores. [1, 10] These last pores mentioned have different sizes and are distributed throughout the cement micro- and macro structure. [10] Three categories describe these interconnected pores, macropores, capillary pores and gel pores. [10]

The gel pores are present in the interlayer spacing within C-S-H and have dimensions from a few fractions to several nm. [10] These pores do not affect neither the concrete's durability nor its reinforcement protection, since they are too small to be transferring aggressive species. [10] The capillary pores are the empty spaces that are not filled by solid products, due to hydration of hardened cement paste. [10] They can reach diameters from 10 to 50 nm if the cement paste is nicely hydrated and made with low W/C but it can achieve values of 3-5 µm if the W/C is high or if it is not properly hydrated. [10] Largest pores, with a few mm size, are created from air entrapped while mixing and, by compaction of fresh concrete, they remain in the cement paste. [10] There are also air bubbles, with sizes from 0.05-0.2 mm, that can be present in cement paste as an air-entraining admixture agent, so there is a resistance to freeze/thaw cycles. [10]

This study is more centred on the microstructure of cement paste and, for that reason, is relevant to talk about the isolated and interconnect pores that exist in it. The capillary pores (connected pores) are one of the important parameters to enhance concretes durability. [10] They determine cement's permeability which permits the entry of chemicals and water on the concrete matrix. [1] This is relevant also on the cracking process, since cracking relate to both types of concrete pores (isolated and interconnected), this will considerably enhance permeability. [1]

Deterioration is a big deal in concrete and starts in concrete's permeability. Chloride attacks, carbonation, decalcification and sulphate attack are the most usual chemical attacks on concrete. [1, 4]

1.1.4 Chloride attack

Chloride attacks came from many different sources being present into the concrete or may diffuse within it from the outside. [4] Chloride ions happens to cause destabilization on the steel reinforcements passive film, accelerating corrosion. [1, 4] These ions are found in the matrix by piercing through the connected pore system. [1] These kinds of attacks start with an ingress and then the corrosion. [4] Chloride ingress is often compared with Fick's law of diffusion. [3] The first step consists on a suction, with most importance when the surface is dry. [3] This happens due to capillary action. [3] Salt water is easily absorbed by dry concrete. After this, the diffusion enters because there is some capillary movement of the salt water through the pores. [3]

On hydrated cement systems, the chloride can have advantages. [4] The cement-chloride interaction and because it links penetrating ions, can influence positively the durability of concrete since it slowly interrupts the ingress of these and other aggressive ions towards the steel. [4]

Some analyses were made on this kind of cement and it was concluded that chloride react with Aluminate phases (AF_m) [20–24], proving that is stable over a significant range of chloride concentrations [22, 25] (up to 3 mol/L). It can also be found but in a chloro-sulphate AF_m phase. [26] Some tests have been made, but recently there has been more interest in the role of Fe and the formation of chloride-bearing minerals. [27] The final conclusion of these tests was that ferrite links with the chloride-bearing minerals. [4] This was recorded in solid phase where cement paste was exposed to a solution of 10% NaCl. [28] At the pore solution/paste interface, chloride can also interact physically with the materials. [4]

To summarize this, the chloride ion reaches the passive layer damaging it, but it does not have any pH drop. Chloride works as a catalyst to corrosion. It is not consumed in the process, although it helps to break down the oxide passive layer on the steel allowing at high rate to proceed with the corrosion process. [4]

1.1.5 Carbonation

The carbonation process consists on the penetration of gaseous carbon dioxide (CO_2) in concrete partially saturated, starting a series of reactions with both ions mixed in the pore solution and the hydrated cement paste. [3, 4] The gas will dissolve in water and creates an acid, like it happens to other gases. [3] To summarize it, there is a list of different steps. The CO_2 penetrates the material. It will mix itself in the pore solution as $HCO_3{}^-$ and $CO_3{}^{2-}$. The $CO_3{}^{2-}$ molecules then induce a reaction with the dissolved calcium present forming a precipitation of, not only, calcite ($CaCO_3$), but also, other CO_2-based solid phases. [4] These reactions will induce a pH drop and, consequently, the dissolution of portlandite. [4] Basically it does not attack the cement paste, per se, it just neutralizes the alkalis in the pore water to form calcite carbonate which lines the pores. [3]

However, carbonation does not have a negative affect when acting by itself. [4] It can derive to a reduction of the material porosity which favours the development of a protective layer over the concretes surface. [4] Although the pH drop connected to the process will possibly derive to a detrimental effect on reinforced concrete structures since it destroys the passive layer around the rebars. [3, 4] Other mechanisms induce the formation of calcite by reducing the amount of calcium and hydroxide ions in the pore solution, which, therefore, triggers portlandite dissolution. [4] The formation of calcite instead of portlandite will cause a reduction on the porosity of the material, because calcite has a higher molar volume. [29, 30]

Carbonation damages more quickly when the concrete, that covers the reinforced steel, is fairly low. [3] It can be also caused when the cover is high, but the pore structure is open, meaning that the pores are connected in a way that allows CO_2 to enter at high rate and this happens when alkaline reserves in the pores are low. [3]

Usually present in old, badly build structures (with special attention to buildings) and reconstituted stone elements containing reinforcements which is often for having a small content of cement and for being widely porous. [3]

An advantage of carbonation is that is easy to notice and measure it, with a simple pH indicator, the usual is a solution of water and alcohol with phenolphthalein. [3]

Many models were made to predict the depth of carbonation. [31–34] Short story of these models is that none counts with the pH drop caused by carbonation because OH^- concentrations are ignored, which is prejudicial when evaluating the corrosion risk. [3] The cementitious materials, which is a remarkably alkaline pore solution, is also neglected. [3] Chemically speaking, if there is a significant Na^+ and K^+ concentration, this is likely to impact the carbonation process. [3]

As recent studies, carbonation and chloride binding are connected. [35]

1.1.6 Decalcification

Decalcification is, normally, known by the dissolution of portlandite with C-S-H in hydrated cement systems when exposed to water (pure and seawater). [3] The dissolution of these hydrates is the consequence of the leaching of ions, the majority are calcium and hydroxide ions, from the pore solution to the external environment. [3] This only affects structures that have been dealing with pure and acidic waters for long periods of time, like dams, water pipes and radioactive waste disposal facilities. [3] The ionic leaching is prejudicial to concrete since it rises the porosity and permeability as well as loss of mechanical strength. [3]

This process drives to the dissolution of calcium hydroxide and the decalcification of C-S-H. [36–38]

Calcium hydroxide is the most critical phase when exposed to water. [4] Its depletion will increase with the contact period. [36],[39–42]

The amount of leached calcium is directly correlated with the W/C. If there is a high W/C value, it will induce a high porosity, high permeability and high pore volume, as consequences, and a high initial value of portlandite content. [36, 43]

Bulk density and pore structure of hydrated cement paste are changed by decalcification. [4, 36]

1.1.7 Concrete exposed to sulphate-bearing solutions can show signs of deterioration

There are two ways that cement based materials can be in contact with sulphate: by external sulphate attack, where the sulphate bearing solutions can be natural or polluted ground waters, or by internal sulphate attack, where sulphate can, in fact, exist in the original mix. [44, 45]

Sulphate ions tend to react with ionic species of the pore solution to precipitate gypsum ($CaSO_4 \cdot 2H_2O$), ettringite ($[Ca_3Al(OH)_6 \cdot 12H_2O]_2 \cdot (SO_4)_3 \cdot 2H_2O$) or thaumasite ($Ca_3[Si(OH)_6 \cdot 12H_2O] \cdot (CO_3) \cdot SO_4$) [44] or a combination of all these phases. The precipitation of this compounds can induce to expansion, strength loss, spalling and severe degradation of concrete. [3] A consequence of the formation of gypsum and ettringite could be the expansion and ultimately cracking. Ettringite is often identified as the predominant since it provokes instability on hydrated cement systems, when in contact with sulphate solutions. [45, 46] The formation of gypsum gave the information that causes expansion of C3S hydrated pastes and can contribute, as a possibility, to degrading concrete in rich sulphate environments. [47]

In isolation, this attack does not happen. [3] The reason is that there has to be a cation balance in order to occur reactions with sulphate, since it is not ignored. [3]

There is a lot of reasons that drive to degradation of concrete as sulphate being the reason. [48] An example is the W/C which has a reasonable effect on sulphate ion penetration and consequent expansion. [48–50] This is why there is a W/C maximum limit on standard concrete structures when exposed to sulphate. [3] Additives like silica fume, fly ash and blast furnace slag, demonstrate that there is a better resistance to sulphate attack once it is reduced the permeability. [49–52] This consists on a reduction of the specimen expansion suffering a sulphate attack. [3] Nevertheless, their ability to limit damage is significantly less relevant when there is $MgSO_2$ present [51], because magnesium, when reacted with sulphate, can change the reaction course and end up forming insoluble phases. [3]

Another product that can be originated by a sulphate attack is thaumasite. This formation only happens if ${SO_4}^{2-}$, C-S-H, ${CO_3}^{2-}$ and water are present. [44, 53, 54] This can come from ettringite by replacing Al^{3+} for Si^{4+} with ${CO_3}^{2-}$ present [53, 55–57] or it could come from the direct interaction with C-S-H, sulphates and carbonates. [55, 56] Supplementary cementing materials can be used to delay the thaumasite formation, but their influence depends on their source. It was discovered that blast furnace slag can improve the behaviour of limestone cements. This does not happen with fly ash because cement will remain vulnerable to thaumasite sulphate attack [58], it just retards the problem. [59]

In conclusion, supplementary cementing materials have a good and effective aspect on concretes resistance if they act quickly. [59]

Most of these attacks will drive to corrosion of the reinforced steel. [3]

Concrete, as mentioned before, contains pores that have a high concentration of soluble calcium, sodium, potassium oxides. [3] These components will create hydroxides, which will create an environment alkaline dense with pH 12-13. [3] These conditions will result in a passive layer formed in the steel surface. [3] This layer, with a probability of being part metal oxide/hydroxide and part mineral from the cement, is a dense, impenetrable film, which, if it is stable and maintained, will prevent further corrosion by keeping chemicals, like sulphate and chloride, and water far from steel. [3] However, a true passive layer is very concentrated, thin layer of oxide that can lead to corrosion at a very slow pace. [3]

This passive layer can only be untargeted by these attacks if the cracking process is minimized and, for that reason, it should be available a mechanism that could heal by sealing or plugging in new cracks, so it is reduced the concretes matrix permeability. [1] An ideal concept should be an active self-healing mechanism as it does not need to be labour-intensive manual checking and repair, resulting in a significant increase of money saved. [1]

1.2 Self-Healing Concrete, a concept to prevent concrete's cracking

The Self-Healing Concrete has been known for a long time. [60] Concrete has been showing that when there are some cracks in old structures, this tend to be lined with white crystalline material, meaning that concrete has the ability of self-seal cracks with chemical products and with the help of rainwater and carbon dioxide. [60] It was studied then, more recently [61, 62], and in water flow research, that cracked concrete under a hydraulic gradient tends to reduce, overtime, the permeability, in a gradual way. [60] This turns the suggestion that concrete could self-seal by itself, slowing the water flow rate. [60] Calcium carbonate, the product of reacting non-hydrated cement with carbon dioxide dissolved in water, is the main cause of self-sealing. [61] To sum it, under certain conditions, this phenomenon is well established. [60] This is important to watertight structures and to increase the duration of infrastructures service life. [60] Recently the term self-healing has been more and more mentioned, a mechanism that can, in a proper way, recover concrete materials creates an astonishing interest beyond the science community. Some demonstrations were made over a resonance frequency of an ultra-high-performance concrete damaged by freeze-thaw actions [60] and the stiffness of pre-cracked specimens [60] showed that these two problems can be recovered by water immersion. [60] Another research proved that the recovery of flexural strength was verified in pre-cracked concrete beams when exposed to compressive loading at the beginning. [60] It is noticed that self-healing was related to continuous cement hydration within the cracks. [60] As it was previously studied, as a permeability matter, that the width of the concrete cracks, which was discovered that can be critical for self-healing to happen, was artificially restricted using a feedback-controlled equipment and/or by compression to close the preformed attack. [60] These experiments settled that self-healing concept can be used in concrete materials. [60]

The engineering behind the self-healing concept was simulated on a polymeric material using encapsulated chemicals. [63, 64] There is been a lot of experiments that involve encapsulation, sensing and actuation to discharge the encased chemicals into concrete cracks. [65–67] One of the approaches that was a demonstration of air-curing polymers that were released to a crack with the vision that it could induce to recover the composite elastic modulus. [60] What happened was that the formation of cracks in concrete were the responsible term for chemicals being released. This originates the breaking of the embedded brittle hollow glass fibres that had the polymer. Some other approaches were used. [60] One consisted in a repair agent encapsulated, at the cracked location, in a film pipe that melts under heating. A heating device is, also, embedded to be a heat resource to the film pipe, when it is supplied an electric current, other one was based on a speculation of an injection of microorganisms to provoke the precipitation of calcium in the concrete crack. [68–70] These represents creative ways to induce the self-healing concept artificially in concrete materials. [60]

Concrete is a unique material for following reason: it has micro-reservoirs of non-hydrated cement particles widely spread and available for self-healing. [60] These particles are known to last a long period of time and, especially in concrete with low W/C, correspond to a quantity of non-hydrated cement around 25% or higher. [60] This is another path called autogenous self-healing which turn to be an economical way comparing with the other ways, to get the self-healing concept, mentioned before. [60] This autogenous phenomenon was shown to be effective in transport and mechanical recovery traits. Although, the reliability of this process is unknown, since it is not regularly studied the quality of this concept, but it should be noticed because mechanical strength recovery is a condition of weak calcite. [60]

As mentioned before concrete is the most used material on the construction sector and it is a strong, durable and cheap. [71] For that reason, there are six important parameters that need to be considered in order to create an effective autogenous self-healing functionality. This self-healing needs to be ready to be activated when and where is needed. It needs to last the same time as the service life of a structure which could be decades. It has to be economically manageable for highly cost-sensitive constructions industry, where large volumes of materials are used on a daily basis. Consistency in a wide range of typical concrete structure environments. This process has, also, to be able to repair multiple cracking damages. So, to summarize, pervasiveness, stability, economics, reliability, quality and repeatability are the six traits for a good autogenous self-healing functionality. [1, 60]

There are various types of mechanisms or agents for autonomous self-healing concrete repair. [1] As a viable mechanism, a secondary mineral formation can be involved since it matches with the material matrix, meaning that is does not affect in a negative way but enhances concrete durability by closing new cracks formed and, consequently, reduce the matrix permeability. [1]

Applying chemical agents such as the inclusion of still nonreacted cement particles in the concrete matrix is a viable way as it is related to some listed self-healing properties. [1] Other agents can also work in an equal way and, at the same time contribute to the self-healing property of concrete, like bacterial agents. [1] The more used ones are chemical agents [72–74], although, recently have been used bacterial agents [72, 75–77]. [71] The concrete matrix can be a very dry and extremely alkaline environment, which may cause, as a first impression, inhospitable for life, comparing with natural systems where occurs bacteria thrive. [1] These bacteria that can be used in the concrete matrix are desiccation-and/or alkali-resistant. The formation of spores is typical in this kind of bacteria and is known to manage high mechanical and chemical induced stresses. [78] Some of these species, since it produces spores, have a low-metabolic activity and extreme long lifetimes, being able to be viable for up to 200 years. [1]

Since bacteria can control the precipitation of dense calcium carbonate layers, crack sealing and lower the permeability of concrete surfaces, bacterial treatment on the durability improvement of concrete structures, ornamental stones and degrades limestone studies have been a point to focus. [1] In these studies bacteria as well as compounds that are required for mineral precipitation had contact in the structure's surface after crack setting or formation occurred, so it was not present, at the beginning, as a healing agent in the material's matrix. [1] The bacterial mechanism that induced calcite precipitation was, firstly, based on the enzymatic hydrolysis of urea. [1] This process consisted on the reaction of urea ($CO(NH_2)_2$) and water, producing CO_2 and ammonia (NH_3). A high pK value of the $NH3/NH4^+$ system, about 9.2, is caused and the reaction results on an enhance of the pH and, simultaneously, a shift in the carbonate equilibrium with the conversion of CO_2 in $HCO_3{}^-$ and $CO_3{}^{2-}$. This will provoke calcium carbonate ($CaCO_3$) precipitation when there are enough calcium ions (Ca^{2+}) in the system. [1] The disadvantage of the urease-based system is the fact that, environmentally speaking, there is an exorbitant production of ammonium to carbonate ions. [1, 71, 76]

For a bacterial self-healing mechanism, both bacteria and the biomineral precursor components, which is environmentally friendly, should be incorporated in the material. [1] The bacteria will be immobilized in the material and it is predicted that the precipitation of minerals and crack sealing should start in the moment that there is a crack and, consequently, the entrance of water in the system. [1] Figure 1.1 illustrates the described process.

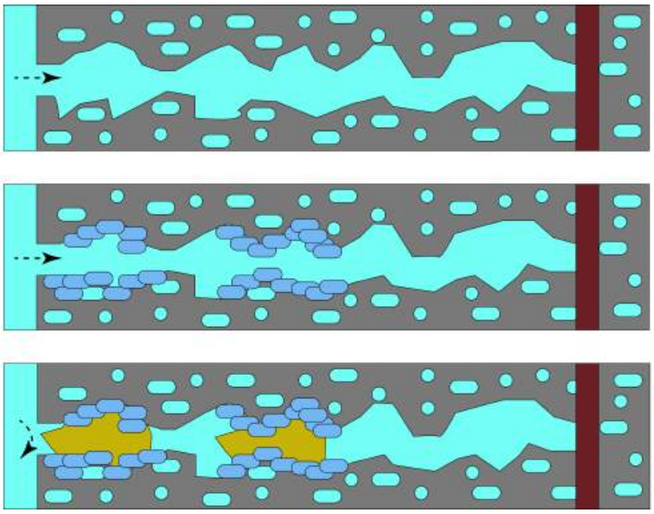

Figure 1.1: Microbial crack healing scenario: the bacteria provokes $CaCO_3$ precipitation while it is immobilized on the concrete adapted from [72]

To test the potential of calcite precipitation on concrete for durability improvement purposes and since the bacteria and mineral precursor compounds were added externally, because it was not taking part of concrete at the beginning, the healing mechanism was not considered as a truly self-healing. [1] A series of experiments were executed with the consideration that it should be selected, first of all, a selection of potential bacterial species that could be suitable. [1] Four species of alkali-tolerant (alkaliphilic) bacteria were capable of forming spores and were of the genus Bacillus. These species were cultivated and immobilized to test the potential bacterial mineral production and compatibility with concrete. [1]

Sporosarcina pasteurii were the culture selected. It was washed twice in tap water and, before being added to the concrete mix make up water, it was quantified the number of cells on the cell suspension using a microscopic counting. [1]

Yeast extract and peptone were added as carbon sources on the medium for the incubation of cement stone pieces with this culture. It was revealed, on the surface of the bacteria-embedded specimen, that a significant formation of calcite-like crystals. [1] This showed that alkali-resistant spore-forming bacteria can form minerals if the appropriate carbon source is accessible, when embedded in concrete's cement paste. [1]

These types of experiments shown that immobilized bacteria interfere in the precipitation of minerals. Additionally, some classes of needed carbon sources and bacteria don't affect, in a negative way, concrete strength characteristics. Concluding that bacterially controlled crack-healing in concrete by mineral precipitation is a viable process. [1] But it needs some arrangements in some areas as the effectiveness seal cracking of the bacterial mineral precipitation to make sure if it decreases significantly the cracked concrete's permeability. Thus, the embedded reinforcements can be protected from corrosion and, consequently enhance the material's durability. [1]

Moreover, when bacterial species take part of the concrete matrix, a selection is made, so they remain viable to last the predicted lifetime of the construction. [1] If this is possible, then it is compared with abiotic self-healing mechanisms so that all the listed self-healing characteristics for the most ideal agent prove to be bacterial. [1] The bacterial mineral production mechanism is metabolic, since bacteria metabolize the available carbon sources, in this case yeast extract and peptone, and under alkaline conditions. Occurs the production of carbonate ions which precipitate and react with the calcium ions present in the concrete matrix. [1] Carbonate ions can reach high concentrations where the bacteria are active and calcium carbonate, or calcite, crystals are formed. [1] But, for an autonomous self-healing, all compounds that are required for the healing reaction should be already incorporated in the concrete mix. [1]

1.2.1 The potential of calcite precipitation is the most significant factor that influences self-healing of concrete

As it was mentioned on the chapter 1.1, cracks are more likely to induce a reduction of the durability of concrete structures and micro cracks are cases that does not occur so regularly on ordinary concrete but if it happens, it will lead, substantially, to concrete's permeability. By that, it reduces the concrete's resistance against aggressive substances that pass through. [71, 73] But these microcracks are originated in harmful or unstable cracks. [71] The phenomenon that is studied in this chapter, the self-healing of concrete, can be based on chemical, physical and mechanical processes as its primary causes. [61, 71, 73] This concept has as its main influencing factor the precipitation of calcium carbonate. [61, 71, 79]

Recent studies of bacterial potential for calcium carbonate precipitation on the surface of concrete or limestone had, as a base, enzymatic hydrolysis of urea, as mentioned before. [69–71, 75, 77, 80–82]

In recent years, there has been a development of a two component self-healing system. [71] The first component corresponds to bacterial spores which after germination, will process organic compounds, the second component, catalysing their metabolic conversion to originate calcium carbonate. [1, 71, 76] These compounds are integrated on concrete, since they are mixed in fresh cement paste. [71] It was proved then that bacteria and calcium lactate, as an organic calcium salt, which behaves as a calcium carbonate precursor, acts positively on concrete's compressive strength. [71] It was also noticed that the mineral production due to bacterial functionality of incorporated, yet unprotected, two component self-healing agent was restricted to young concrete specimens, i.e. 1-7 years old. [71] Hypothetically, high alkalinity may have been the cause of a major crushing and inactivity of the incorporated bacterial spores, making a loss of viability and a reduction of the mineral-forming capacity on older specimens. [71] An alternative to resolve the latter problem, was the immobilization in porous expanded clay particles of the two-component biochemical healing agent principle as a protection on the concrete. [71] These expanded clay particles work as an internal reservoir and as a part of both structural element of concrete as well as a protective matrix for self-healing agent. [71] This suggests a possibility of viability increase of the biochemical self-healing agent functionality. [71]

This experience, as mentioned before, consisted on a mixture of calcium lactate and bacterial spores that were isolated from an alkaline lake soil, on Wadi An Natrun, Egypt. [71] Two cracked mortar specimens were used, one as a control and other as a bacteria-based. These mortars had a wide number of cracks with different widths and they were under tap water. [71]

This makes sure that the mortars were kept open to the atmosphere during incubation, allowing free diffusion of oxygen and carbon dioxide over the water-air surface. [71] These specimens were analysed on a stereomicroscopic as well as photographic imaging for crack healing measurements. [71]

Sampling on this experience based on the removal of the top part a massive columnar precipitate that was formed on the bacteria-based specimen on various crack surface locations. [71]

Aerobically, oxygen is consumed during metabolic bacterial calcium lactate conversion. [71] To measure the oxygen consumption, optical oxygen microsensors are used on these two specimens. [71] The Fick's first law of diffusion (eq. 1.1) is used to calculate this uptake by studying the changes caused on oxygen concentration in the linear part of the gradient in the diffusive boundary layer. [71, 83]

$$J = -D_{O_2} \frac{dC(z)}{dz} \tag{1.1}$$

D_{O_2} is diffusion coefficient of O_2 in water, C(z) the O_2 concentration at depth z. [71]

At the end of this experiment applying the two-component biochemical self-healing agent promote and upgrade the capability of concrete self-heal. [71] Also, it was concluded that, with the oxygen measurements, bacterial spores embedded in clay particles proved to enhance the bacterial activity and functional for several months after concrete casting, turning this process viable. [71]

The ability of microbes on increasing the crack healing properties of concrete was assumed to be due to a combination of direct and indirect calcium carbonate formation where the direct way is the precipitation of $CaCO_3$ is metabolically converted through calcium lactate and the indirect way is a reaction of CO_2 molecules, metabolically formed before, with $Ca(OH)_2$ minerals, that is present in the concrete matrix. [71]

Since the bacteria, that is active on the concrete, consume oxygen, the healing agent will have an important role since it can act as an oxygen diffusion barrier which will protect, in the end, the steel reinforcements against corrosion. [71] However, the possibility of oxygen being removed from the concrete matrix by bacteria in order to inhibit reinforcement corrosion as never been studied, although it could, potentially be a beneficial process. [71]

Some advantages of bacteria-based concrete could, presumable, be on maintenance reduction, repair costs and service life extension of concrete constructions, but characteristics as long-term durability and cost efficiency of this recent type of concrete are needed to be resolved and studied before applying it. [71]

1.3 Self-Healing Cement applied on the Oil Industry

This study as mentioned on the previous chapter, showed that there is a possibility to occur a self-healing mechanism on concrete using bacteria as healing agents. This causes a major development on engineering. However, this test was only tested with aerobic bacteria.

Researchers from Aarhus University, AU, decided to study the possibility of having a self-healing property on concrete that is used underground, in oil well perforations.

The cement is used on oil wells to restrict fluid movements between the formations and bonding and supporting the casing. [84] Cementing is slurry mixing process which includes cement and water, being pumped downwards through the casing to points that are critical in the annulus, around the casing or in the open hole below the casing sting. [84]

There are two types of cementing operations: Primary and Remedial. [84] Primary provides a zonal isolation, which is not related, in a direct way, with the production but it is a dependent step for a correct performance, allowing either the production or the simulation operations to be conducted. [84] The process consists on pumping slurry down the casing until it reaches the annulus. [84] However, there are modified techniques that are used for special situations, as it is shown on Table 1.1. [84]

Table 1.1: Modified techniques for Primary cementing

Cementing through pipe and casing	Normal displacement technique
Stage cementing	For wells with critical fracture gradients
Inner-string cementing tubing	For larger diameter pipes
Outside or annulus cementing tubing	For surface pipe and large casing
Reverse-circulation cementing	For critical formations
Delayed-set cementing	For critical formations and placement improvement
Multiple-string cementing	For small diameter tubing

Remedial is used essentially to reinforce primary cementing, in case there are some problems associated with it. [84] It is also a very costly process so, for economical and most successful way, is better to prevent this from happening by planning, designing and execute correctly all drilling, primary cementing and completion operations. [84] Cement has an aid role as well when isolating oil-production zones. [84] Cement protects casing from corrosion. [84] Prevents blowouts, forming rapidly a seal. [84] Preserves the casing from shock loads in deeper drilling. [84] Also, covers zones of lost circulation or thief zones. [84] A correct cement placement requires , at the beginning, the examination of the well parameters, that defines the well needs and designs placement techniques and fluids to meet the needs for the life of the well, where its design is influenced by fluid properties, mechanics and chemistry followed by calculating the fluid composition and executing laboratory tests to make sure that the fluid (slurry) design matches all the well needs. [84] The next step involves a proper hardware to implement the design made at the beginning, calculate the volume of fluids (slurry) that need to be pumped and blend, mix and pump fluids into the annulus. [84] After this, the treatment is controlled in real time, comparing with the design made and making the necessary changes. Finally, it is appraised the results comparing it with the projected design and applying the necessary changes for future jobs. [84] Well parameters are essential to know the wellbore and its characteristics when designing the cement job. [84] Depth, influencing not only the quantity of wellbore fluids involved, wellbore fluid volume, the friction pressures, hydrostatic pressures, temperature and the cement slurry design but also controls the hole and casing sizes. [84] Because of high temperatures, high pressures and corrosive fluids are characteristics of extremely deep wells and it has to have a different type of design. [84]

The amount of cement required for the well is determined by the wellbore geometry. Alongside with the casing dimensions, wellbore geometry ascertains the annular volume and the necessary fluid. [84]

The temperature is divided by three categories: bottom hole circulation temperature (BHCT), bottom hole static temperature (BHST) and temperature differential. [84] BHCT corresponds to the temperature that cement is exposed while circulating past the bottom of the casing. It conducts the cement setting time (thickening time) and can be measured by temperature probes which flow in the drilling fluid. [84] BHST have in consideration a static condition where there is no fluids flowing the wellbore and not cooling the wellbore. [84] It plays an important role in the strength development of the cured cement. [84] The temperature differential is, essentially, the temperature between the top and bottom of the cement placement and it becomes significant when cement is placed on deep wells, since there is a considerable temperature difference. [84] As a consequence, it is common to design two distinct types of cement slurries to better accommodate the temperature difference. [84] To maintain the wellbore integrity, the hydrostatic pressure that is exerted by, for example, the cement and drilling fluid cannot be higher that the fracture pressure of the weakest formation. [84] The fracture pressure stands for the upper safe pressure limitations of the formations which is before its break down, the pressure needed to extend formation's fractures. [84] The fluids present in the wellbore, the hydrostatic pressure, along with the friction pressures, created by fluid movement, cannot, as well, surpass the fracture pressure which can conduct to formation break down. [84] This cannot happen because if it does happen, formation is not controlled and, consequently, it results on a lost circulation. [84] Lost circulation, or fluid loss, needs to be controlled for successful primary cementing. [84]

Pressure experienced in the wellbore, as well as BHST, influences the strength development of the cement. [84]

The formation composition can cause some compatibility problems, since shale formations are sensitive to fresh water and, if there is a need to take precaution measures, such as the presence of swelling clays and high-pH fluids, it should be taken into consideration that formations could slough off. [84] Complex features as flowing fluids, high pressure fluids, corrosive gases that some formations may also have require some special attention. [84]

Applying the self-healing concept on this concrete could improve a lot the overall process as a manufacturing and economic point of view. But, for that reason, it is needed to know the well conditions and if there are bacteria that could grow on those conditions. It is known that high pressures, high temperatures and high alkalinity are factors that needs to be considered for a bacteria growth. These characteristics will lead to a selection of a special kind of bacteria, called the polyextremophilic bacteria.

1.4 Polyextremophilic selection

There are a variety of bacteria that can grow under extreme conditions, like high sodium salt concentrations ([Na^+]), alkaline pH and exalted temperatures, and thus conditions with anaerobic halophilic polyextremophiles. [85]

Sodium carbonates, as well as supplemented NaCl, are the responsible agents for the [Na^+]. [85]

Archaea are the most well-known extreme halophiles, but there are also some extreme halophilic bacteria. [85] These bacteria were isolated from different extreme environments such as solar thalassohaline salterns, which is originated from marine waters, athalassohaline and ancient thalassohaline salt lakes, located in Wadi An Natrum, Egypt and Great salt lake, UT, USA respectively, marine environments and fermented fish sauces. [85]

In terms of aerobic and anaerobic bacterial species, they are an equal distribution on the extreme halophilic class, with four facultative anaerobes as an addition, such as *Halomonas sinaiensis* and *Thiohalorhabdus denitrificans*. [85] Bacterial extreme halophiles display various physiological and nutritional properties, belonging to different phylogenetic groups. (Table 1.2)

Table 1.2: Phylogenetic groups of extrema halophiles, adapted from [85]

Order	Phylum
Actinomycetales	Actinobacteria
Sphingobacteriales	Bacteroidetes
Bacillales *Halanaerobiales* *Natranaeriobiales*	Firmicutes
Rhizobiales *Rhodospirillales*	Proteobacteria
Chromatiales *Oceanospirillales* *Pseudomonadales*	Proteobacteria

Most extreme halophiles are mesophilic, an organism that grows at moderate temperature, or neutrophilic, an organism that thrives at neutral pH, slightly thermophilic extreme halophiles, with abundant alkaphilic extreme halophiles too. [85]

A little percentage of extreme halophiles have the possibility to grow optimally under alkaline conditions as well as at elevated temperatures. [85] But if all these three conditions (high pH, high temperature and extreme halophile) are needed for optimal growth, these microorganisms are called polyextremophiles. [85]

There was a selection between the polyextremophilic conditions and only the anaerobic microorganisms *Natranaerobius themophilus*, *Natranaerobius "jonesii"*, *Natranaerobius truepiri* and *Natronovirga wadinatrunensis* demonstrate their optimum values at extreme conditions. [85] So, basically, this selection points to the *Natranaerobiacecae* family.

1.5 *N. magadiense* is the bacteria to study

Natranaerobiacecae is a bacterial family affiliated with the class *Clostridia* in phylum Firmicutes. [86] Currently there are three types of genus in this family, the *Natranaerobium* which as two species included, *Natronovirga*, with one specie, and *Natranaerobaculum*, with one as well. (Table 1.2) [86] All the member of this family are fermentative polyextremophiles, which, to grow, they need large quantities of salt and a high pH, can tolerate high temperatures, meaning that they are thermotolerant and they are, also, Gram positive species. [86] These types of bacteria where found in the anaerobic sediments of soda lakes with a high saline concentration of the Wadi En Natrun, Egypt and Lake Magadi, Kenya. [86]

Table 1.3: Types of genus in the *Natranaerobiacecae* family, adapted from [86]

Genus	Number of Species	Type of Species	General Properties
Natranaerobius	2	*Natranaerobius thermophilus*	Anaerobic obligate, Gram positive rods, endospores not noticed. Alkaphilic and thermophilic, halotolerant chemoorganothrophs. Cell wall have a significant lack of murien and meso-diaminopimelic acid.
Natronovirga	1	*Natronovirga wadinatrunensis*	Anaerobic obligate, Gram positive rods, endospores not noticed. Alkaphilic and thermophilic, halotolerant chemoorganothrophs.
Natranaerobaculum	1	*Natranaerobaculum magadiense*	Anaerobic obligate, Gram positive rods, endospore- forming. Alkaphilic and thermophilic, halotolerant chemoorganothrophs.

Analysing these species more carefully for the objective of this project, it is known that one of these bacteria must confirm some aspects to be a good candidate to be applied on concrete to act as a self-healing agent. These aspects are: Polyextremophile, Spore-forming, Anaerobic and a metabolism of proton consumption, which will maintain the pH and tolerate high pressures.

Table 1.4: *Natranaerobiacecae* family species specifications, adapted from [86]

Genus	***Natranaerobius***		***Natronovirga***	***Natranaerobaculum***
Species	***Natranaerobius thermophilus***	***Natranaerobius trueperi***	***Natronovirga wadinatrunensis***	***Natranaerobaculum magadiense***
Cell size (μm)	0.2-0.4 x 3-5	0.6 x 2-3	0.3-0.4 x 4.5	0.2-0.5 x 3-7
Endospore formation	-	-	-	+
Motility		-	-	+(slow)
pH range for growth and optimum	8.3-10.6 (9.5)	8.0-10.8 (9.5)	8.5-11.5 (9.9)	7.5-10.7 (9.25-9.5)
Temperature range for growth and optimum (ºC)	35-56 (53-55)	26-55 (52)	26-55 (51)	20-57 (40-50)
Total Na^+ range for growth and optimum (M)	3.0-5.0 (3.3-3.9)	3.5-4.5 (3.7)	3.1-5.3 (3.9)	0.5-2.7 (0.9)
Utilization of carbohydrates	+	+	+	-

By the evaluation on Table 1.3, it is shown that *N. magadiense* (Figure 1.2) is, in fact, the only bacteria, from this family, that could work on the Self- Healing Cement Project. [87]

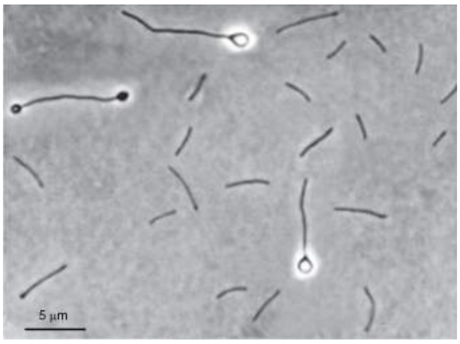

Figure 1.2: Microscopic view of *N. magadiense*, provided by [87]

To summarize everything, *N. magadiense* even though it is a polyextremophile and spore-forming, metabolic proton consumer is a probability, since it uses, as electron donor, peptides from yeast extract. Having an electron donor and a proton consuming metabolism, the pH will rise and, chemically, the following equation will occur

$$CO_2 \rightarrow CO_3^{2-} \tag{1.2}$$

And with Ca^{2+} present in the cement could create,

$$CO_3^{2-} + Ca^{2+} \rightarrow CaCO_3 \tag{1.3}$$

Which is essential to self-heal a crack, being the main component of concrete. In this study,the main goal of this project was to check if *N. magadiense* could induce the precipitation of $CaCO_3$, and so *N. magadiense* is tested, not only, at different pressures, temperatures and pH, but also, the bacteria's carbon source is changed.

Chapter 2

Materials and Methods

Materials and Experimental methods will be referred in this chapter. It is going to be mentioned, in a general way, how the researcher did the experimental medium (standard medium), filled the sample vials, prepared the inoculum, analyzed the amount of proteins present in the medium and determined the amount of cation Iron (II), Fe^{2+}, and total Fe in the vials, at the beginning and end of the experiment.

2.1 The Standard medium and microorganism

- A medium meant for species from the same family as *N. magadiense*. All the ingredients for this medium were provided by Sigma-Aldrich.

Table 2.1: Standard medium components

Distilled Water (Milli Q), 1L	
Potassium fosfate (KH_2PO_4), 0.2g	Magnesium chloride hexahydrate ($MgCl_2 \cdot H_2O$), 0.1g
Ammonium chloride (NH_4Cl), 1.0g	Potassium chloride (KCl), 0.2g
Sodium chloride (NaCl), 16.0g	Sodium carbonate (Na_2CO_3), 68.0g
Sodium bicarbonate ($NaHCO_3$), 38.0g	Yeast Extract, 2.0g
Nutrient Broth (NB), 8.0g	Vitamin Solution, 10 mL

Since the beginning of every experiment made is the production of the standard medium, it was added 60% of the MilliQ water required, all the components shown on table 2.7 were added.

The vitamin solution was an exception due to the fact that, at temperatures higher than 60°C, provokes vitamin degradation.

Na_2CO_3 and $NaHCO_3$ are slowly added to the solution that must be, at the same time, mixed and heated because it is used a large quantity of these two components to make this medium.

The 40% of MilliQ water left is added when all components are dissolved, letting it be agitated for 5 min more, without heating it.

After the medium being mixed and cooled to room temperature, the pH value is measured. The pH value for all experiments need be around 9 to 10, since *N. magadiense* growth is higher at this range.

The solution is then transferred to a glass bottle, with a rubber lid and metal ring lid attached, and sterilized on the autoclave. This process will force all O_2 particles to be moved into the gas phase, making the liquid phase anaerobic.

From there, the bottle is quickly sealed to prevent any contact with the surround environment.

Flushing the medium with N_2 is required in order to have an anaerobic environment. 50 min to one hour is the duration of this step to make sure that the medium is completely anoxic.

Along with the previous step, the temperature will decrease and then it will be possible to add the vitamin solution. This is done with a syringe, 22 µm diameter filter and a 5 µm diameter needle attached to it, to prevent the entrance of more O_2 in the system apart from the one that already exists in the vitamin solution. Having O_2 in it will induce to a continuous N_2 flushing for 10-15 min more.

After this period of N_2 washing, it is moved to the vial filling process.

2.1.1 Inoculum

- Corresponds to *N. magadiense* and it was provided by Aarhus University.

Table 2.2: Inoculum's Preparation

Standard medium	Inoculum (1 week old)

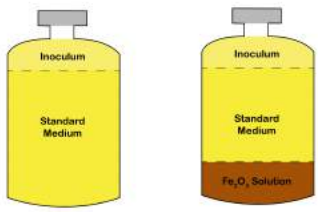

Figure 2.1: Inoculum preparation scheme

For bacterial fermentative purposes, it was added 10% of inoculum – *N. magadiense* growth media – equivalent to the overall flask volume and filled with standard medium. To assess the bacterial growth effect with the presence of Fe, another option was, to the two components mentioned above, adding a solution of Fe (Fe_2O_3).

2.1.2 Sampling vials

The vials are sterilized long side with the medium and flushed for about a minimum of 2-4 min.

These tubes can be incubated at atmospheric pressure or to vary this parameter. The capacity will change with the goal of the experiment. Ferric Oxide (Fe_2O_3) is facultative since in depends on what is going to be analysed, as well as the inoculum's amount used.

Processing the vials filling, and depending on what is the desired volume, the medium can vary between 82-99% (V/V), Fe_2O_3 from 0 to 28% (V/V) since their concentration can be changed and the inoculum can be the equivalent of 1% as well as 10% (V/V). At the end, it should reach 100%. Some samples were analysed as abiotic controls, which will be explained in more detail on Chapter 3.

Summing everything, the vials are filled with:

$$Medium\ (l) + Fe_2O_3\,(l) + Inoculum\ (l) \tag{2.1}$$

Posterior to the vial filling, they are incubated for 1 week and, subsequently, some samples are taken so the researchers can evaluate how did *N. magadiense* behaved to the conditions that were given.

The incubation can be either in an incubator at 1 atm and 50°C or in a reactor that supports pressures to 600 bar. For that, is used the pressure vials (Figure 2.2). The procedure of incubating in these reactors is illustrated in figure 2.3.

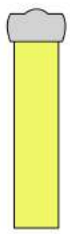

Figure 2.2: Pressure vial

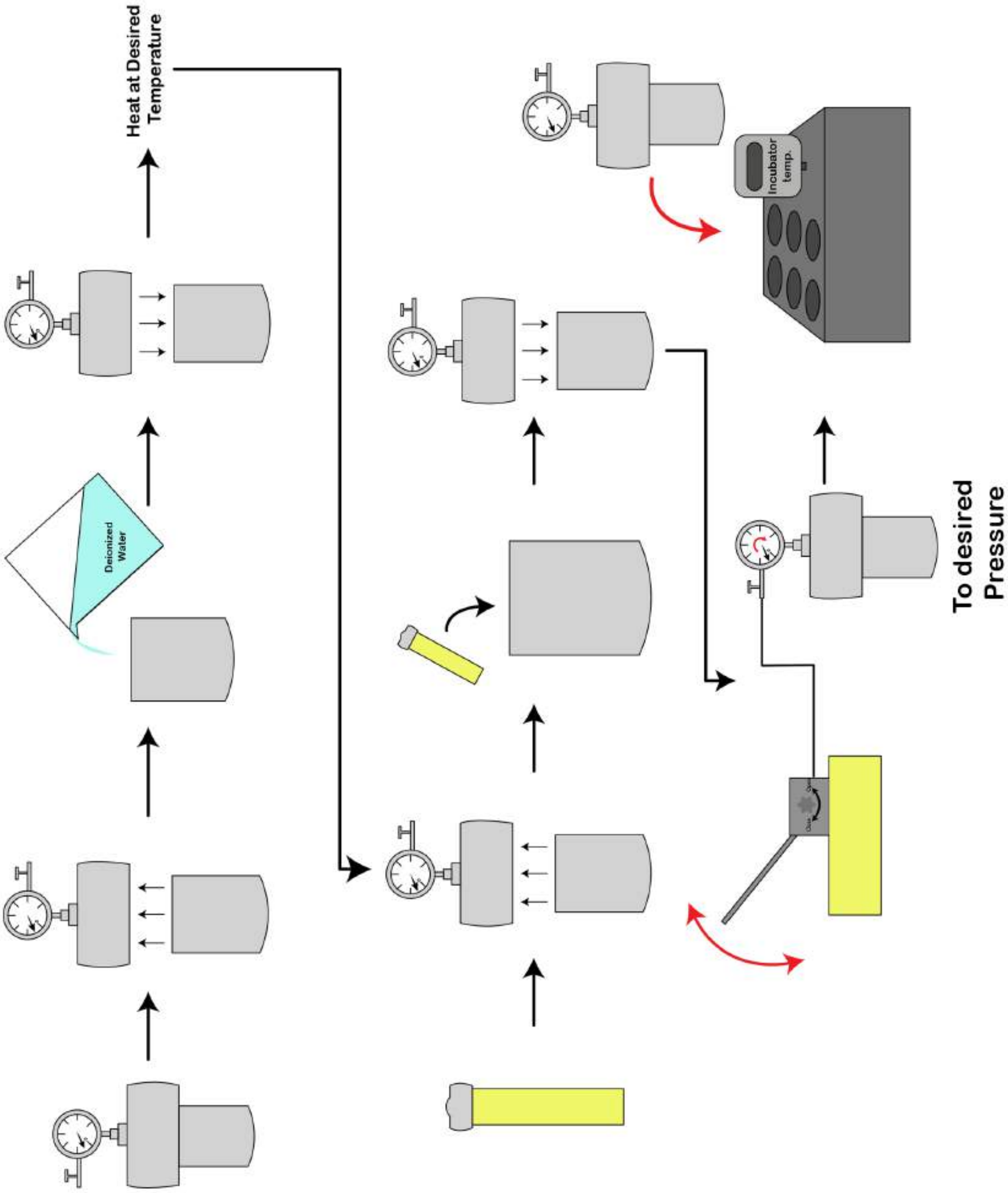

Figure 2.3: Process of incubation of a pressure vial using reactors

These tests can be Fe extraction, protein assay, cell counting.

The cell counting can be measured using the microscope or by the Flow Cytometer. Cell counting, by microscope, counts with a chambered microscope slide (hemocytometer) and a counter. The cell number that is got over this reading is the average of five of sixteen grids, implemented in the hemocytometer. (Figure 2.4)

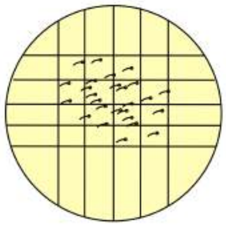

Figure 2.4: Microscopic view of bacteria in the hemocytometer

The number of cells that was read, is then calculated through that average times 4 million and the dilution factor, resulting the real cell number.

$$Average\ (Cell\ counted) \times 4000000 \times\ DF = Real\ Number\ of\ Cells \tag{2.2}$$

Using this technology, the flow cytometer, a range of measurements are applied on cells, cell organelles, and other objects suspended in a liquid and in a stream at rates equivalent of various thousand per second through a flow chamber. [88] The Figure 2.5 represents how the cell counting is analysed. [88] The process consists is a light beam that reads the number of cells and some other impurities on the sample. [88]

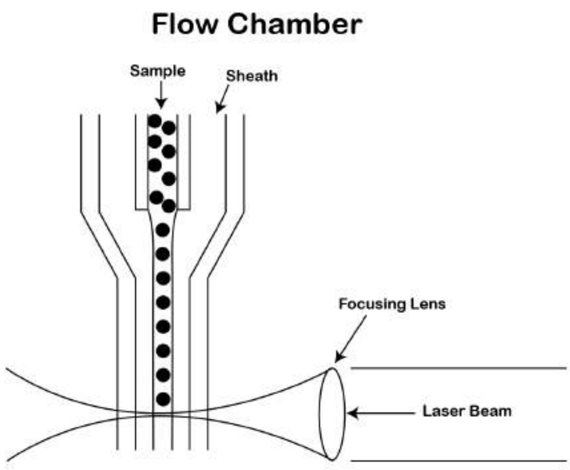

Figure 2.5: Scheme of how does Flow cytometer work, adapted from [88]

The samples are treated before, to make sure that the reading is effective. Samples that have Fe particles must be filtered using a 11 µm pore diameter so these particles are retained in it and only the cells pass through. To assure that cells are in suspension, phosphate buffered saline (PBS) is used. This solution is commonly used in biomolecule research. [89] Consists on a solution of 4% PBS in water, being kept at -20°C. PBS prevents cell activity, immobilizing them.

For fermentation purposes, it was sampled 100 µL from the sampling vials to an Eppendorf with 5 µL of PBS. From there, 10 µL of the first sample were taken to 990 µL of PBS making a 100x dilution. With that, in a microwell plate, 40 µL of the previous sample were taken to be mixed 20 µL, with 179 µL of PBS and 1 µL of syto9 (S9) [90], and 20 µL with 178 µL of PBS, 1 µL of S9 and 1 µL of Propidium iodide (PI) [91]. 10 µL of the samples with Fe were added to a 10 mL of PBS and filtered. From that, 200 µL were taken to a microwell plate, adding S9 as well.

2.2 *N. magadiense*'s growth

For this experiment, it was used the standard medium, since the main goal was to check the *N. magadiense* growth.

Since the inoculum did not have iron in the mixture, it was possible to count the cells.

Sampling was the only difference between vials.

On a 10 mL volume, which was added to the vial, a percentage of inoculum was added.

Two tests were made, where one had 1% (0.1 mL) of inoculum and the other 10% (1.0 mL). On both cases, the remaining added volume was standard medium.

The conditions for this experiment were 1 bar, as pressure, 50 ºC, as optimum temperature, and pH 10. It was incubated for 2 weeks.

2.3 Replacing a complex carbon source with a simple and unique one

In this experiment, two carbon screenings were made to see the possibility of a replacement between a complex carbon source and a simple and unique one.

Since the objective was to test different carbon sources, the standard medium was made without the default carbon source (Yeast Extract and NB).

On the first carbon screening, 20 carbon sources were studied and had, as concentration, 2 g/L. (Table 2.3)

Table 2.3: Carbon sources analysed on the first carbon screening

Acetate	Pyruvate	Citrate	Glysine	Formate
Cysteine	Oxalic Acid	Taurine	Lysine	Butyrate
Yeast Extract	Creatinine	Lactate	Glycerol	Succinic Acid
Propionate	Betaine	Glutanime	Malic Acid	Aspartic Acid

The carbon sources were introduced in the sample vial. The sample volume used were 10 mL. This sample had 1% of inoculum (0.1 mL), 0.4 mL of iron, as Ferric Oxide, the carbon source (0.200 g) and the standard medium previously made without the default carbon source.

The second carbon screening had the same procedure, apart of the concentration of carbon source used which, in this case, was 8 g/L. (Table 2.4)

Table 2.4: Carbon sources analysed on the second carbon screening

Acetate	Pyruvate	Citrate
Cysteine	Oxalic Acid	Taurine
Nutrient Broth	Creatinine	Lactate

Lactate and Glycerol were only available in liquid phase, so the corresponding volume for 2 g of Lactate and Glycerol was determined. Same applied to the second carbon screening.

2.4 Effect of carbonates on *N. magadiense*'s growth

On this experiment, it was tested the effect of carbonates in *N. magadiense* growth.

For that, it was reduced the amount of HCO_3^- present in the medium. Since the objective was to reduce the amount of HCO_3^-, maintaining the concentration of Na^+, seven conditions were studied, where in three of them, it was replaced the anion HCO_3^- with Cl^-. (Table 2.5)

Table 2.5: Sample's specifications

A/D	100% HCO_3^-
B	10% HCO_3^-
C	1% HCO_3^-
E	10% HCO_3^-+ Added NaCl
F	1% HCO_3^-+ Added NaCl
G	0% HCO_3^-+ Added NaCl
H	0% HCO_3^-

In order to do this experiment, seven media were made (100 mL each), taking into consideration the specifications present in table 2.5.

It was calculated the amount of Na^+ that needs to be added in order to have the same concentration as in the standard medium.

The amount of carbon source, Yeast Extract and NB, used was 1 g/L and 4 g/L, respectively.

The conditions for this experiment were 50 ºC, as temperature, pH 10 and, as pressure, 1 bar.

Hence, with a reduction of HCO_3^-, the pH drops drastically and, in order to maintain the pH at 10, it was used a solution of NaOH. The incubation was for 1 week.

To know the effect of carbonates, iron cannot be added on this test, so it would be easier the cell counting.

2.5 Cl^-, SO_4^{2-} and PO_4^{3-} with *N. magadiense*

The main goal of this experiment was to check the different *N. magadiense* growth behaviours under different pH values.

It was made the same procedure as in the section 2.4.

Three different media were made, with yeast extract and NB as carbon sources. The concentrations were the exact same as in section 2.4.

At the end it was tested the number of cells present on each sample using a protein assay instead of cell counting.

2.6 The polyextremophilicity of *N. magadiense*

The last test made in this project was the influence of temperature and pressure on *N. magadiense*.

Since this project was to be applied in oil wells, the pressure values used correspond to the top, middle and bottom. The values used were 1, 150 and 300 bar.

The temperature varied in a interval of -10ºC and +10ºC of 50ºC, the optimum temperature for growth. The pH of this experiment was the optimum, 10.

It was used the standard medium and it was sampled to pressure vials, due to a variation of pressures. (Figure 2.2)

These pressure vials have a volume of 4 mL. Then they were introduced on a reactor, as represented on figure 2.3. This experiment had an incubation of 1 week.

Since the goal was to test the growth and the activity of *N. magadiense* at this conditions, both fermentative and Fe reduction processes were tested, using a protein assay (section 2.8) and an iron assay (section 2.7). On the mixture there was 10% of inoculum, 90 mmol of ferrous oxide (Fe_2O_3) and the remaining volume filled with standard medium. Hence, with the data got at the end of this experiment it was made a design of experiments (DOE)

2.6.1 Design of Experiments (DOE)

DOE is a type of design that compares the behaviour of more than two factors, or parameters. Hence, it provides enough data to observe changes that might have occurred in one or more characteristics of an experimental unit, the entity that is subjected to certain measurements of one or more characteristics, and to identify the possible causes. The involving factors can be controlled or not, where a controlled factor is a variable that researchers change its values with the intuit of figuring out the effect of one or more responses. Each factor value is denominated as a level, or treatment, and can be qualitative or quantitative. [92] The effect of a factor is the average change observed on the response when a factor changes its level. Response stands for the results gotten in an experiment, meaning, it is the characteristic for what researchers are interested in optimizing. [92] Before implement a DOE, it requires a systematic approach so that its application would lead to positive results. This approach, generally, needs to have the following details:

- Constitution of an investigation team;
- A clear definition of the experiment objectives, as a conception of a new product or quality improvement of products that already exist, etc...
- Relevant information taken previously like experiments that were made;
- Responses selection, i.e. it is chosen the quality characteristics, as its respective measurement methods;
- Selection of the controllable factors to test as well as its levels and, if it can be executed properly, select the noise factors and respective levels;
- Brief analysis of the possible interactions between factors, knowing that there is at least an interaction between two factors when the effect of one factor in the response depends on the level of the other factor;
- An identification of which factors will remain constant during the experiment;
- Identifying the experiment restrictions;
- Design the number of experiments that are meant to be made and, based on this decision, the number of factors and levels, plan the experiment using the most appropriate matrix, that is, define the experiment layout;
- Plan how many replicates it is needed to be done;
- Realize all experiments, or trials, randomly.

On DOE, there are two types of analysis to be taken in to consideration. These are the analysis of variance (ANOVA) and the residual analysis. [92] ANOVA is a statistical technique that allows the comparison between parameters of two or more populations.

This analysis has the possibility to distribute the total variability of the experimental data with all the diverse components that cause that effect, to determinate the components that are statistically significant. [92] The residual analysis is, no more than, estimates of the experimental errors, which confirms the validation of the mathematical model and ANOVA analysis of the design proposed. This analysis needs to have the following aspects:

1. The Normality verification – a practical form, yet subjective, of verifying if the residuals have a normal distribution which comes with the elaboration of a Normal Plot. If the residuals are arranged in an approximately straight line, it can be concluded the normality assumption is reasonable satisfied; [92]

2. Residuals and experimental order – a simple graph that relates residuals in function of random order that experiments were made and, without a doubt, is the easiest method to evaluate the independence of the design. If a hypothesis of the independence is reasonably satisfied, residuals should be distributed randomly on a graph, without showing any special trend; [92]

3. Knowing the residuals by predicted and estimate values – Simple method to verify if the variance is constant. Consists on a graph that relates residuals with predicted or estimate values. If the assumption of variance is constant is not strongly violated, the graph will not present any special structure. In practical cases, variance usually is increasing or decreasing with the values that were observed (or their average), which affects the graph as putting the residuals on a funnel form. [92]

Hence, to strengthen the DOE's study, it should be used a data transformation that determines the more adapt transformation parameter (λ), in order to, maintain the variance homogeneity, i.e. the results got by a graph that relates residuals with predicted values, and data normality. Box and Cox transformation method is the data transformation method, since it is very precise. [92]

2.7 Iron assay

- Iron extraction method to count all the Fe^{2+} reduced in a sample.

Table 2.6: Iron Assay material compounds

Ferrozine reagent	Hydrochloric acid (HCl), 0.5M	Iron (II) stock
solution, 10 Mm	Hydroxylamine-hydrochloride HAHCl, 1M ($NH_2OH \cdot HCl$)	

The components of every reagent were available at Aarhus University.

For this assay, the samples have to be in an environment with HCl, which as the ability of stopping the reduction process. 20 µL are mixed with 80 µL of HCl in an Eppendorf and is mixed until there is no particle precipitation. To help the particle suspension, mixing it time to time is suggested as well as diluting it to 5-10 times. When there is no precipitation, it is ready to move to the standard curve. These 9 concentrations are prepared using a Fe^{2+} stock solution, 10 mM, dissolving 0.392 g of iron ammonium sulphate ($NH_4Fe(SO_4)_2 \cdot 6\,H_2O$) in 0.5 M HCl (42 mL of 37% HCl in 500 mL of water and diluted to 1 litre) to 100 mL. This stock solution is stored cool. Form this solution, 2.5 mL are transferred to be diluted with 0.5 M HCl to 10 mL in a volumetric Flask. The Fe concentrations are 0/25/50/100/200/400/800/1000/1500 µM. The samples are diluted again with a dilution factor of 5-10. Then, the researchers mixed 20 µL of sample and 80 µL HCl in a microwell plate (figure 2.6), folded it with aluminium foil and assuring the complete absence of light, left it resting for 25 min in a drawer.

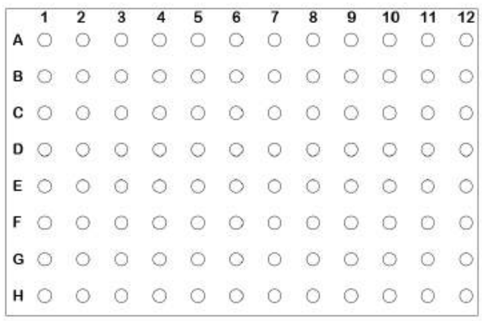

Figure 2.6: Scheme of a microwell plate

After 25 min, it was added 100 µL of Ferrozine reagent that will react with the Fe particles, changing colour instantaneously (Figure 2.7).

Figure 2.7: The results after an Iron extraction test

Ferrozine, reacting with Fe^{2+}, will create a stable magenta complex that is highly soluble in water and can be used to measure iron in water. [93] Known for being a recommended chelator of Fe^{2+} and applied in biological samples. [94–96] This solution is made diluting 0.1 g of Ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonsyre)-1,2,4-triazine) in 100 mL of 50 mM HEPES-buffer. Is also buffered to pH 7 with 2M NaOH. The HEPES-buffer is a dilution of 1.2 g of HEPES-buffer, N-2-hydroxyethylpiperazin, with 100 mL of water. Dissolving 8 g of NaOH in 100 mL of water with consistent stirring, it is obtained NaOH (2M) solution.

In this assay, not only it was measured the amount of Fe^{2+} in the samples, but also, the amount of Fe^{3+} and, consequently, the total amount of Fe. The total amount of Fe was determined using the same method as the determination of Fe^{2+}, until it was transferred to the microwell plate. Here, it was used Hydroxylamine-hydrochloride, 10% (wt/vol) of $NH_2OH \cdot HCl$ with HCl 1M , to reduce it and, from there, Ferrozine was used to verify the existing amount. [97] The Fe^{3+} that exists in the samples was determined by subtracting the total Fe with Fe^{2+}. HCl and $NH_2OH \cdot HCl$ are two components that prevent oxidation of Fe^{2+} and reduction of Fe^{3+}, respectively. [97]

After preparing every posterior step, it was analysed the absorbance in the FLUOstar Omega. A technology used, especially, for microwell plate format assay. This equipment is also very practical, precise and cheap for all the applications needed.

page

2.8 Protein assay

- Technique to measure proteins.

Table 2.7: Reagent A Compounds, for 1L)

Potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6$), 2g	Sodium Carbonate (Na_2CO_3), 100g	Sodium hidroxide, (NaOH), 500mL (1N)	Water, H_2O

Table 2.8: Reagent B Compounds

Potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6$), 2g	Copper (II) Sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$), 1g	Sodium hidroxide (NaOH), 1N 10 mL (1N)	Water (H_2O), 90 mL

Table 2.9: Reagent C Compounds

Folin-Ciocalteau Phenol reagent	Water, H_2O

The components of every reagent were available at Aarhus University and provided by Sigma-Aldrich.

For this assay, the samples were stored in the freezer at -20°C and, to start this process, it was centrifuged and washed with water to be able to determine the amount of proteins that exists in a cell. The centrifugation can be from 5-10 min and, simultaneously, the sample is washed with water by introducing the same volume as the initial before centrifugation. Then a second centrifugation is made and the sample is washed a second time but the volume is a bit less than the first one, so it is easier to detect the amount of proteins present in the samples. It was then considered that the sample volume should be higher that the considered, at first, to use in the assay, so it can be analysed again if the test results are dubious. Quantifying proteins is an essential metric to be determined when researchers have a protein purification test, need to determine yields or mass balance, or even verify the activity/potency of the proteins. [98] Protein assays are spectrophotometric assays and can be either UV absorbance methods or dye-binding assays, colorimetric and fluorescent-based detection. [98] From these assays this report will only be covering a dye binding assay, the Lowry protein assay. This assay has as base a two-step procedure. [98] Starting with a reaction in alkaline solutions caused by proteins, leading to a reduction of Copper (Cu^{2+} to Cu^{+}), with an enhancement stage, Folin Ciocalteu reagent is reduced creating a blue colour capable of reading the absorbance at 750 nm as maximum wavelength. [98] This assay is also very sensitive to interfering agents and, since it has been applied, there has been some changes in order to decrease this sensitivity, boost the dynamic range and the speed and consequent stability of the colour formation. [99] Its sensitivity is applied by the Folin Ciocalteu reagent and can lead to a response not linear. [100] However, this reagent is also recommended for its simplicity. [100] Three reagents are used A, B and C, as referenced on Materials section. This assay is based in Lowry's assay, which consists on discovering, by absorbance, the amount of proteins in a sample. To start, there is the preparation of the reagents. The reagents A and B can be kept for 2 to 3 months. Reagent C is made considering that 1 volume of Folin Ciocalteu reagent is diluted to a volume of H_2O which is 15 times more the Folin Ciocalteu's volume.

Having all reagents prepared, the assay starts with a standard curve where it is used 2000 mg/L of Bovine serum albumin in the same buffer containing the unknown sample. In this case it was water. To have a standard curve, a sequence of dilutions has to be done. The concentrations vary from 0 g/L to 1000 g/L.

Adding 1.0 mL of standard/sample to 90 mL of Reagent A, mixing as follow. This is then incubated for 10 min at 50°C.

The solution was cooled to room temperature for 10-15 min after the 10 min incubation.

Then 0.1 mL of Reagent B is added, mixed and incubated at room temperature for 10 min.

Immediately after 10 min incubation, 3 mL of Reagent C is added, mix and incubated at 50°C for 10 min.

The sample was then cooled at room temperature, having a final volume of 5 mL.

Then, the results can be measured in a spectrophotometer with 1 cm cuvettes or in the FLUOstar Omega, using a microwell plate.

This assay was essentially to measure the number of proteins in samples that had Fe in its composition. For that reason, to be analysed in a spectrophotometer or FLUOstar Omega, the samples had to be centrifuged. From there, with the cell counting and protein assay of the inoculum, because it is very difficult to count cells in samples with Fe, it is possible to, approximately, estimate the amount of cells that exists in the samples.

Chapter 3

Results and Discussion

3.1 *N. magadiense*'s growth

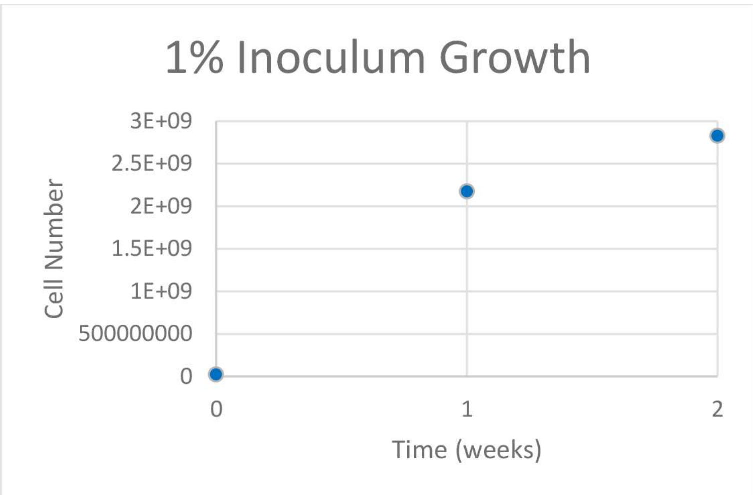

Figure 3.1: Impact of the 1% Inoculum in cell growth

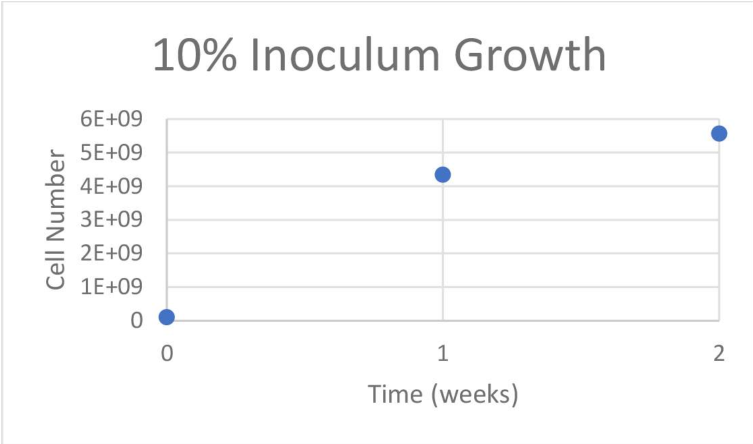

Figure 3.2: Impact of the 10% Inoculum in cell growth

This test involved the impact of *N. magadiense*'s growth, evaluating its speed by changing the amount added to the medium. To measure this impact, it was added to the standard medium 1% inoculum (Figure 3.1) and 10% inoculum (Figure 3.2), where this percentage corresponds to the volume that is going to be used in the sample (10 mL). The method used to count the cells in this trial was the one described previously. It is observed that, with 10% inoculum, the growth rate is substantially higher than in the test with 1% inoculum test. Looking at the growth equations, after 1 week, of each sample, it is noticed that the growth of the 10% inoculum trial corresponds to the double the growth of the 1% inoculum trial. The inoculum impact is only noticed at the beginning of the cell growth, because after a period of time, the cell number, on each sample, will be the same. It was made an approximate prediction on the day that the samples would have the same number of cells (Figure 3.3).

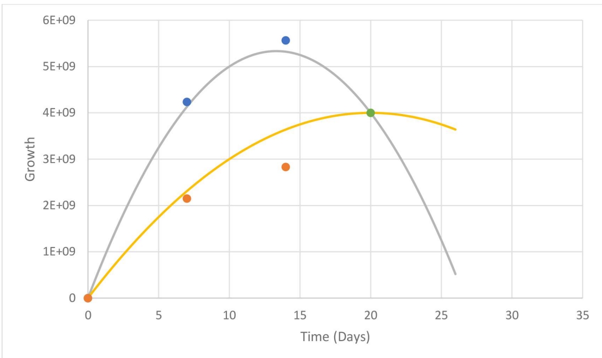

Figure 3.3: Approximate behaviour of how the cells grow depending on the inoculum rate

This is not the correct way of doing the growth measurement, since cell growth would behave through an exponential equation. The correct way would imply more data during the experimental time period instead of having just an idea of how *N. magadiense* grows in these conditions. The correct way would be making an experimental analysis to evaluate the cell growth in shorter periods of time. The values would give an exponential equation, which demonstrate the theoretical behaviour of *N. magadiense*, since calculating the cell number using a microscope can have some reading mistakes. To get the point when the number of cells is equal, one would have to use both mathematical equation to find the common value. However, with this module, it is possible to predict, approximately, the time that the two samples will have the same number of cell. This figure shows that on the day 20 the growth would be about 4000 million cells, which corresponds to the dead state of the sample with 10% inoculum and the highest cell number in the 1% inoculum trial.

It is roughly concluded is that *N. magadiense* would grow between 2-3 weeks exponentially, depending on the amount of inoculum used. Higher inoculum induces a faster growth.

3.2 The possible candidates to replace a complex carbon source

At the beginning of this study, it was made a carbon screening, in order to, discover a simple and unique carbon source to replace yeast extract and nutrient broth, which are very complex, in order to make it easier to measure the amount of CO_2 produced. This test is made using the Iron (Fe) assay shown on the materials and methods section. Twenty different carbon sources were analysed (Table 2.3)

Yeast Extract was the Positive control. The negative control had none carbon source, since it was tested that *N. magadiense* do not need carbohydrates to grow. [87]

Since the medium is rich of Na_2CO_3 and $NaHCO_3$, it is impossible to measure the activity of the cells by fermentative process. So, to evaluate that, and since *N. magadiense* can reduce Fe^{3+} into Fe^{2+}, a solution of Fe was added to the mixture (inoculum and medium). The test was controlled by colour changing, meaning that, at the beginning of the experiment, samples had all an orange colour and, at the end, it should appear as a brown/black colour, being influenced by the Fe reduction.

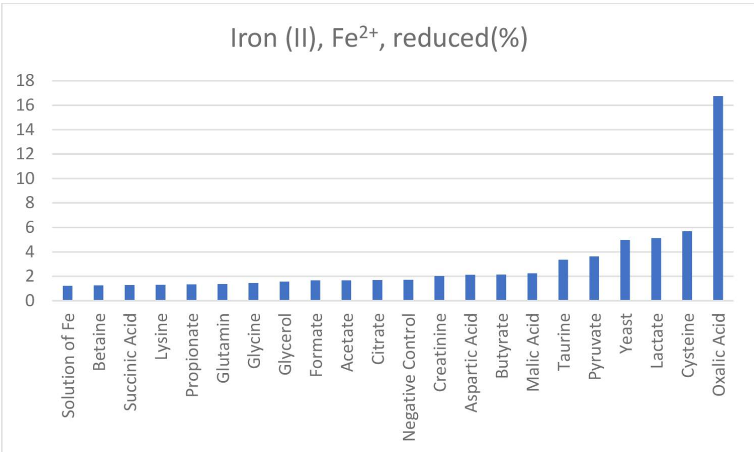

Figure 3.4: The iron reduction caused in samples with a different simple carbon source

With the results shown on Figure 3.4, it was concluded that carbon sources that, after 1 week, had values of Fe reduction lower than the negative control were unfavourable for *N. magadiense*'s growth. This left the researchers with Creatinine, Aspartic Acid, Butyrate, Malic Acid, Taurine, Lactate, Cysteine, Oxalic Acid.

After this screening, another one was made using fewer carbon sources. In this screening it was made biotic and abiotic tests to make sure if the carbon sources were reacting chemically or enzymatically with Fe, reducing it.

The selection of carbon sources had into consideration the ones that showed significant values, since the previous screening (Table 2.4). The concentration of the carbon sources raised to 8 g/L, to evaluate, as well, the impact on the Fe reduction.

Compounds like Acetate and Lactate are proved to reduce Fe^{3+} into Fe^{2+} biotically. [101]

It was concluded that these results were dubious. However, with the latter information, there's a probability that Acetate, Citrate, Butyrate, Pyruvate, Lactate, Formate and Propionate could be good candidates to replace Yeast and Nutrient Broth in the medium. Cysteine and Oxalic Acid, with the second carbon screening, proved to have a chemical reaction with ferrous oxide (Fe_2O_3). So, compounds with the same characteristics as these two chemical species, will react on an equal way, but the result could be less noticeable. Cell counting, using a microscope or a flow cytometer, is a test that could support this test because then it is possible to know the behaviour of *N. magadiense* with each carbon source.

3.3 Carbonates are essential for *N. magadiense*'s growth

Given the fact the cement self healing is based on the precipitation of $CaCO_3$ whenever there are large amounts of carbonates in the system, this process being chemical and biological, is in this cases, mainly chemical making it difficult to detect the bacterial activity. To be able to understand the bacterial activity, in this study, the amount of carbonates was reduced and replaced by a source of chloride provided by sodium chloride, since this compound is present in ocean waters. Yeast extract and NB are the carbon sources used with quantities of 1 g/L and 4 g/L, respectively. In this trial, seven different conditions were studied where it was changed the $HCO_3{}^-$ quantity on each sample, with the possibility of replacing the amount taken with NaCl. All the samples had a pH value between 9-10, since it is the range of optimum cell growth. [86, 102] NaOH was used to raise the pH of samples that had values lower than this range. This happens because of the reduction of $HCO_3{}^-$, which is a buffer compound, that maintains pH at the pretended range. (Table 2.5)

The samples A/D, B, C and H tested the impact of $HCO_3{}^-$, by reducing its amount. It helped to understand *N. magadiense* behaviour with the absence of carbonates. The absence of carbonate will target the cell growth of *N. magadiense*, since it is an essential compound. [87] The other ones were to test if the amount of $HCO_3{}^-$ could be replaced with a presence of a larger concentrations of sodium ion [Na^+]. The specifications for all samples are the following equations.

$$100\%\ HCO_3^- \rightarrow 68\ g\ of\ Na_2CO_3\ and\ 38\ g\ of\ NaHCO_3 \tag{3.1}$$

$$10\%\ HCO_3^- \rightarrow 6.8\ g\ of\ Na_2CO_3\ and\ 3.8\ g\ of\ NaHCO_3 \tag{3.2}$$

$$1\%\ HCO_3^- \rightarrow 0.68\ g\ of\ Na_2CO_3\ and\ 0.38\ g\ of\ NaHCO_3 \tag{3.3}$$

$$0\%\ HCO_3^- \rightarrow 0\ g\ of\ Na_2CO_3\ and\ 0\ g\ of\ NaHCO_3 \tag{3.4}$$

$$MW(Na_2CO_3) = 2 \times 22.99 + 12.01 + 3 \times 16.00 = 105.99\ gmol^{-1} \tag{3.5}$$

$$MW(NaHCO_3) = 22.99 + 12.01 + 1.01 + 3 \times 16.00 = 84.01\ gmol^{-1} \tag{3.6}$$

$$MW(NaCl) = 22.99 + 35.45 = 58.44\ gmol^{-1} \tag{3.7}$$

The sample A/D correspond to the normal medium. It is shown on Figure 3.5 that *N. magadiense* increased its population at a very high rate, since the experiment had a duration of 1 week. The concentrations present in the medium of $HCO_3{}^-$ and Na^+ are, respectively, 1.082 M and 2.01 M.

The amount of Na^+, in weight percentage (wt%), of each compound (Na_2CO_3, $NaHCO_3$ and NaCl) corresponds,

$$\%Na_{(Na_2CO_3)} = \frac{2 \times 22.99}{105.99} \times 100 = 43.4\% \tag{3.8}$$

$$\%Na_{(NaHCO_3)} = \frac{22.99}{84.01} \times 100 = 27.4\% \tag{3.9}$$

$$\%Na_{(NaCl)} = \frac{22.99}{58.44} = 0.394 \times 100 = 39.3\% \tag{3.10}$$

$$m(Na^{+}) = 68.0 \times 0.434 = 29.51\ g \tag{3.11}$$

$$m(HCO_3^{-}) = 68.0 - 29.51 = 38.49\ g \tag{3.12}$$

$$m(Na^{+}) = 38.0 \times 0.274 = 10.412\ g \tag{3.13}$$

$$m(HCO_3^{-}) = 38.0 - 10.412 = 27.59\ g \tag{3.14}$$

$$m(Na^{+}) = 16.0 \times 0.393 = 6.288\ g \tag{3.15}$$

$$m(Cl^{-}) = 16.0 - 6.288 = 9.712\ g \tag{3.16}$$

$$m(Na^{+}) = 29.51 + 10.412 + 6.288 = 46.21\ g \tag{3.17}$$

$$m(HCO_3^{-}) = 38.49 + 27.59 = 66.08\ g \tag{3.18}$$

$$m(Cl^{-}) = 9.712\ g \tag{3.19}$$

$$[Na^{+}] = \frac{46.21}{22.99} = 2.01\ M \tag{3.20}$$

$$[HCO_3^{-}] = \frac{66.08}{61.02} = 1.082\ M \tag{3.21}$$

$$[Cl^{-}] = \frac{9.696}{35.45} = 0.274\ M \tag{3.22}$$

For all samples with 10% HCO_3^{-} (B and E) the amount that of Na^{+} that was necessary to replace Na_2CO_3 and $NaHCO_3$ with NaCl was calculated by the following way,

$$61.2 \times 0.434 = 26.56\ g\ Na \tag{3.23}$$

$$34.2 \times 0.274 = 9.37\ g\ Na \tag{3.24}$$

$$26.56 + 9.37 = 35.92\ g\ Na \tag{3.25}$$

The same process was made for the samples with 1% (C and F) and 0% (G and H) HCO_3^{-},

$$67.32 \times 0.434 = 29.22\ g\ Na \tag{3.26}$$

$$37.62 \times 0.274 = 10.31\ g\ Na \tag{3.27}$$

$$29.22 + 10.31 = 39.53\ g\ Na \tag{3.28}$$

$$68 \times 0.434 = 29.51\ g\ Na \tag{3.29}$$

$$38 \times 0.274 = 10.41\ g\ Na \tag{3.30}$$

$$29.51 + 10.41 = 39.92\ g\ Na \tag{3.31}$$

Resulting on the following quantities of NaCl, required to maintain the concentration of Na^{+} in the system,

$$m(NaCl):\ \frac{39.3}{100} = \frac{35.91}{x} \leftrightarrow\ x = 91.1\ g \tag{3.32}$$

$$m(NaCl):\ \frac{39.3}{100} = \frac{39.53}{x} \leftrightarrow\ x = 100.33\ g \tag{3.33}$$

$$m(NaCl):\ \frac{39.3}{100} = \frac{39.92}{x} \leftrightarrow\ x = 101.32\ g \tag{3.34}$$

On B, C and H samples, [$HCO_3{}^-$] and [Na^+] were reduced a lot, presenting the following values,

$$m(Na_2CO_3) = 6.8\ g \tag{3.35}$$

$$m(NaHCO_3) = 3.8\ g \tag{3.36}$$

$$m(Na_2CO_3) = 0.68\ g \tag{3.37}$$

$$m(NaHCO_3) = 0.38\ g \tag{3.38}$$

$$m(Na^+) = 6.8 \times 0.434\ + 3.8 \times 0.274 + 6.304 = 10.30\ g \tag{3.39}$$

$$m(HCO_3^-) = 6.8 \times (1 - 0.434) + 3.8 \times (1 - 0.274) = 6.61\ g \tag{3.40}$$

$$m(Na^+) = 0.68 \times 0.434\ + 0.38 \times 0.274 + 6.304 = 6.703\ g \tag{3.41}$$

$$m(HCO_3^-) = 0.68 \times (1 - 0.434) + 0.38 \times (1 - 0.274) = 0.661\ g \tag{3.42}$$

$$[Na^+] = \frac{10.30}{22.99} = 0.448\ M \tag{3.43}$$

$$[HCO_3^-] = \frac{6.61}{61.02} = 0.108\ M \tag{3.44}$$

$$[Na^+] = \frac{6.703}{22.99} = 0.291\ M \tag{3.45}$$

$$[HCO_3^-] = \frac{0.661}{61.02} = 0.0108\ M \tag{3.46}$$

The E, F and G trials had an excess of [Cl^-].

$$\%Cl_{(NaCl)} = \frac{35.45}{58.44} = 0.607 \times 100 = 60.7\% \tag{3.47}$$

$$m(Cl^-) = \frac{60.7}{100} = \frac{x}{91.1} = 55.30\ g \tag{3.48}$$

$$m(Cl^-) = \frac{60.7}{100} = \frac{x}{100.59} = 61.06\ g \tag{3.49}$$

$$m(Cl^-) = \frac{60.7}{100} = \frac{x}{101.58} = 61.66\ g \tag{3.50}$$

$$[Cl^-] = \frac{55.30}{35.45} = 1.560\ M \tag{3.51}$$

$$[Cl^-] = \frac{61.06}{35.45} = 1.722\ M \tag{3.52}$$

$$[Cl^-] = \frac{61.66}{35.45} = 1.739\ M \tag{3.53}$$

N. magadiense is a halotolerant, but the range of tolerance is between 0-1.55 M with an optimum at 1.2-1.4 M. [87] The chloride ion concentration [Cl^-] is higher than the values of tolerance, which inhibit the cells and, consequently there is no significant growth. In addiction, large quantities of chloride could originate an attack on the passive film of the reinforcement steel inside the concrete, since chloride attacks can be caused inside the concrete, accelerating corrosion. [1, 4] If the objective is to reduce HCO_3^- due to the "infinite" amount present on concrete, to through eventual mass transactions between the mineral precursor, where *N. magadiense* is located, with the exterior of it, the recommended measures to improve this test should be to maintain the amount of Cl^- in the system, increase [Na^+] with a component that does not have any element that could influence corrosion or other problem on concrete, such as sulphur (S) or chloride (Cl^-). However, this situation would need further investigation.

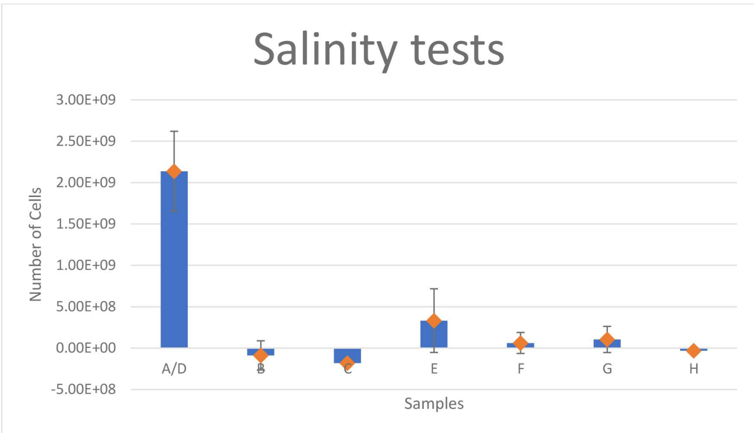

Figure 3.5: Carbonate is essential for *N. magadiense*

The results on Figure 3.5 were taken by counting cells using a microscope. The counting consisted on the procedure wrote on the material and methods chapter. The reading is done on a specific area of 4x4 nm through two specific process. On Figure 3.6, there are two possibilities to count the cells. The red lines represent the boundaries where the cells can be counted, i.e. when a cell is stepped in one of each line, the cell is valid to be counted. However, the count can be made using method A or method B, never both.

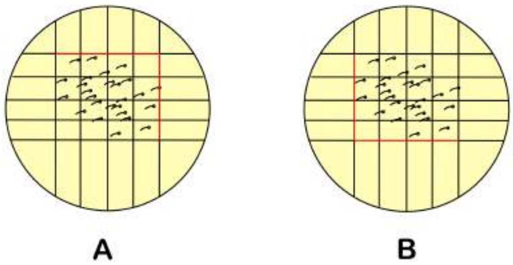

Figure 3.6: Two methods to count the existing cell in the sample

After repeating this measurement five times, it was made an average of the number of cells that were counted. Then, to the value gotten, it is multiplied 4 million, since it corresponds to the volume correction of the grid which each square measured has a volume of 1 nL, to get to mL, the volume of the sample.

3.4 Chloride inhibits *N. magadiense* and pH cannot be changed

This trial involved the change of pH. This can only be made if the [HCO_3^-] is reduced, so it was used 10% of the normal amount added to the medium. This experiment was similar to the salinity test, where HCO_3^- is switched with other ions, which in this case was Cl^-, SO_4^{2-} and PO_4^{3-}. It was required to know which amount of Na that was going to be replaced. Since this calculation was made precisely, the desired amount was 35.91 g of Na, by replacing $NaHCO_3$ and Na_2CO_3 with to 91.1 g of NaCl, 110.83 g of Na_2SO_4 and 85.30 g of Na_3PO_4.

$$MW\left(SO_4^{2-}\right) = 96.02\ gmol^{-1} \tag{3.54}$$

$$MW\left(PO_4^{3-}\right) = 94.93\ gmol^{-1} \tag{3.55}$$

$$MW\left(Na^{+}\right) = 22.99\ gmol^{-1} \tag{3.56}$$

$$MW\left(Cl^{-}\right) = 35.45\ gmol^{-1} \tag{3.57}$$

$$MW\left(NaCl\right) = 22.99 + 35.45 = 58.44\ gmol^{-1} \tag{3.58}$$

$$MW\left(Na_2SO_4\right) = 2 \times 22.99 + 96.02 = 142\ gmol^{-1} \tag{3.59}$$

$$MW\left(Na_3PO_4\right) = 3 \times 22.99 + 94.93 = 163.90\ gmol^{-1} \tag{3.60}$$

$$\%Na_{(Na_2SO_4)} = \frac{2 \times 22.99}{142} = 0.324 \times 100 = 32.4\% \tag{3.61}$$

$$\%Na_{(Na_3PO_4)} = \frac{3 \times 22.99}{163.90} = 0.421 \times 100 = 42.1\% \tag{3.62}$$

$$\%Na_{(NaCl)} = \frac{22.99}{58.44} = 0.394 \times 100 = 39.3\% \tag{3.63}$$

Necessary amount of [Na^+] = 35.91 g

$$m\left(Na_2SO_4\right) : \frac{32.4}{100} = \frac{35.91}{x} \leftrightarrow\ x = 110.83\ g \tag{3.64}$$

$$m\left(Na_3PO_4\right) : \frac{42.1}{100} = \frac{35.91}{x} \leftrightarrow\ x = 85.30\ g \tag{3.65}$$

$$m\left(PO_4^{3-}\right) = 85.30 - 35.91 = 49.39\ g \tag{3.66}$$

$$m\left(SO_4^{2-}\right) = 110.83 - 35.91 = 74.92\ g \tag{3.67}$$

$$m\left(Cl^{-}\right) = 55.29\ g \tag{3.68}$$

$$\left[PO_4^{3-}\right] = \frac{m\left(PO_4^{3-}\right)}{MW\left(PO_4^{3-}\right)} = \frac{49.39}{94.93} = 0.520\ M \tag{3.69}$$

$$\left[SO_4^{2-}\right] = \frac{m\left(SO_4^{2-}\right)}{MW\left(SO_4^{2-}\right)} = \frac{74.92}{96.02} = 0.780\ M \tag{3.70}$$

$$\left[Cl^{-}\right] = \frac{m\left(Cl^{-}\right)}{MW\left(Cl^{-}\right)} = \frac{55.36}{35.45} = 1.562\ M \tag{3.71}$$

$$[Na^+] = \frac{m(Na^+)}{MW(Na^+)} = \frac{35.91}{22.99} = 1.562\ M \tag{3.72}$$

The [Na$^+$] is the same on each compound, changing only the anions concentration. Na_3PO_4 is known to be a buffer that raises pH and stabilises it between 11 and 12. A protein assay was used to measure the data on this trial, the Lowry protein assay. [98] The procedure was the one that is explained in the materials and methods chapter. This method was used in order to find a way of counting the number of cells in samples with a compound that would prejudice cell counting. An example of a particle that is harmful for cell counting is Fe. This process consisted on knowing the amount of protein in mg/mL. With this value it is calculated the number of cells present in 1 mL. Then, with the information given in the literature [87], it is discovered the cell volume and, consequently the volume that an average cell occupy 1 mL of Sample. A ratio is made and with that and the amount of proteins present in 1 mL of sample, it is able to make an assumption on the possible number of cells present in the sample. (Table 3.4) To simplify,

$$Number\ of\ cells\ (Sample) = \frac{Proteins(Sample)}{\frac{Proteins}{Cell}} = \frac{Cell}{Proteins} \times Proteins(Sample) \tag{3.73}$$

Table 3.1: Number of cells on each sample

NaCl	1.47E+05
Na_2SO_4	1.25E+06
Na_3PO_4	3.20E+05

In this case, the inoculum used was always the same where the base of the inoculum has the same conditions as a normal media, with 2 g/L of yeast and 8 g/L of NB. This inoculum was used due to its conditions had an insignificant influence on the experiment development. Hence, to measure the amount of protein that exist in the media, as it was told in the methods, an absorbance test is made with a standard curve at the beginning of the test. Albumin bovine serum is the compound for the standard curve. This curve is to relate the amount of protein that is used (mg/mL) with the absorbance itself. From this curve, an equation is obtained which was, then, used to determine the amount of proteins that existed on each sample. The absorbance of the samples is measured using either a 1 mL cuvette in a spectrophotometer or on the FLUOstar Omega. Of these two techniques the latter is faster and can analyze more than one sample, simultaneously.

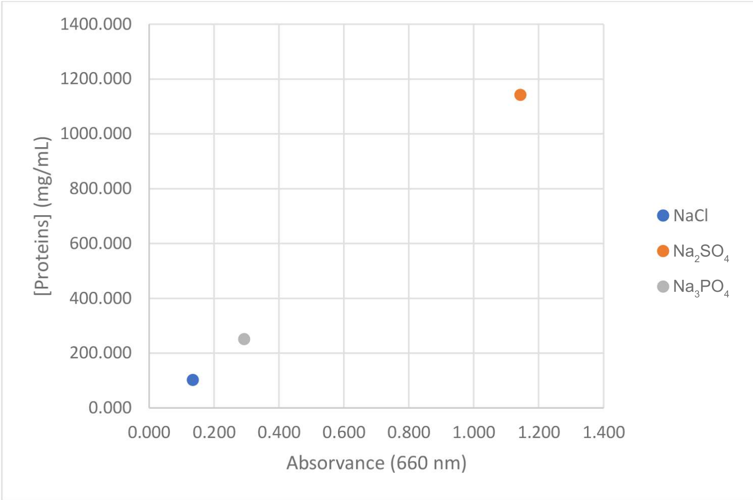

Figure 3.7: pH changes cell growth and an excess of chloride, inhibits the cells

The results show that the compound that reacts better with *N. magadiense* is, in fact, Na_2SO_4. However, the anion ${SO_4}^{2-}$ has a negative effect on concrete, since enhances the probability of a sulphate attack. [44, 45] The $[Cl^-]$ is higher that the range that *N. magadiense* can tolerate, inhibiting the cells from growing, and, for that reason, it represents the lowest value. The pH value is in the pretended range for optimum growth, only the $[Cl^-]$ does not match neither the optimum (1.2-1.4 M) nor range (0-1,55 M). [87]

3.5 *N. magadiense*, a cell that can tolerate extreme conditions

Table 3.2: Extreme conditions applied to *N. magadiense*

RUN	TEMPERATURE (ºC)	PRESSURE (bar)	IRON REDUCTION (%)	CELLS (cell/mL)
1	40	1	45.431	5.349E8
2	50	1	38.835	4.810E8
3	60	1	9.548	8.669E8
4	40	150	41.774	4.029E8
5	50	150	38.919	3.906E8
6	60	150	8.294	7.896E8
7	40	300	38.731	4.494E8
8	50	300	44.435	4.303E8
9	60	300	12.552	7.426E8

This analysis of *N. magadiense* consisted on a relation of cell number with extreme conditions, being high temperature and high pressure. (Table 3.5) It was measured the cell growth using the method presented on Material and Methods Chapter. It was used reactors that could handle pressures up 400 bar, since the pressure in an oil well is about 5000 psi (≈344 bar). [84] Wells can be drilled OBD or UBD. [84] The conventional drilling is the OBD, i.e. the pressure on the bottom of the well is higher than the formation pressure. [84] In this type of wells, a primary well-control mechanism is provided by a column of fluid of a specific density, in the holes. [84]

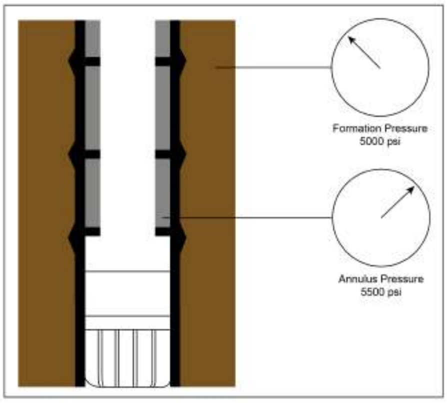

Figure 3.8: Schematic view of OBD, adapted from [84]

On UBD, a lighter fluid substitutes the column fluid used on OBD, and, intentionally, the well is designed to have a pressure, at the bottom, lower than the pressure of the formation. [84]

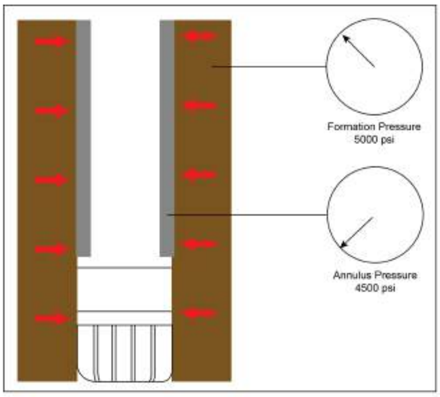

Figure 3.9: Schematic view of UBD, adapted from [84]

For that reason, similar pressures were needed to be analyzed in order to see *N. magadiense*'s growth and, consequently, cell activity under these kind of pressures. The temperature is also an important term as it is changing between 40°C and 60°C. This step was taken because it was meant to see how *N. magadiense* reacts out of the optimum temperature, 50 ºC. [87], and 1 bar. The parameters in oil wells are dependent on the depth of the well, i.e. for each 60 ft (≈18.3 m), the temperature is raised 1°F (0.556°C). [103] The experiment had, as an activity indicator, iron incorporated in the medium. 90 mM of Fe (III) was the maximum concentration used before. [87] It was made also an inoculum with this concentration in order to see how well *N. magadiense* behaves with the presence of Fe at normal conditions, 1 bar and 50°C. [87] The medium used had the addition of 90 mmol of Fe so the dozing to pressure vials would be easier and quicker. To the cell number results in this experiment, a design of experiment (DOE) was made in order to have an accurate study about *N. magadiense*.

3.5.1 DOE applied to the conditions presented in this experiment

The tool that was used for this evaluation was the Design Expert 10.0.2. A mathematical model was applied based on the experiment results, which will serve as an idea of how the experiment worked analyzing, as well, other different scenarios to enhance this study. This develops a model that is reinforced with an ANOVA analysis, were it is evaluated if the model suggested for the experiment is significant. The best model for this trial is, in fact, the quadratic model. (Table 3.6)

Table 3.3: ANOVA´s analysis of the Fe reduction process model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob>F	
Model	1983.66	5	396.73	56.44	0.0036	significant
A-Temperature	1520.86	1	1520.86	216.37	0.0007	
B-Pressure	0.60	1	0.60	0.086	0.7885	
AB	23.57	1	23.57	3.35	0.1645	
A^2	430.69	1	430.69	61.27	0.0043	
B^2	7.41	1	7.41	1.05	0.3800	
Residual	21.09	3	7.03			
Total	2004.75	8				

It was taken, as an example, the Fe reduction process. The ANOVA confirmed that the quadratic model is the model to be used as it demonstrates that the chance on having a noise disturbance in the model is very low (0.37%). This low value is called the p-value, which determines the probability of the F value occur. The F value is the Mean Square of each model term in relation with the residual Mean Square. Since the p-value is too low, it shows that there is a low chance of this large F value occur due to noise. Thus, to be significant, the values of p-value must be under 0.0500. Hence, it shows also which parameters are more significant on the model, and, consequently, in the experiment. Hence, values greater than 0.1000 indicates that the model terms are not significant for the experiment. Therefore, it is verified that A and A^2 are the most significant parameters of the model. Thus, the ANOVA analysis of this process, indicates that the model terms used are significant, making the model suitable to be applied to this situation. If, in any case, this does not happen, it is required to reduce the number of the models parameters. Thus, reducing the model may induce its improvement.

The residual analysis was made based on the experiment results after the ANOVA's following the aspects mentioned before.

Taking the residual analysis of the Fe reduction process, the first analysis is the normal distribution of the residuals, which conclude that the design assumption of the normality is reasonable satisfied, since residuals are arranged in a way that represents, approximately a straight line. (Figure 3.10)

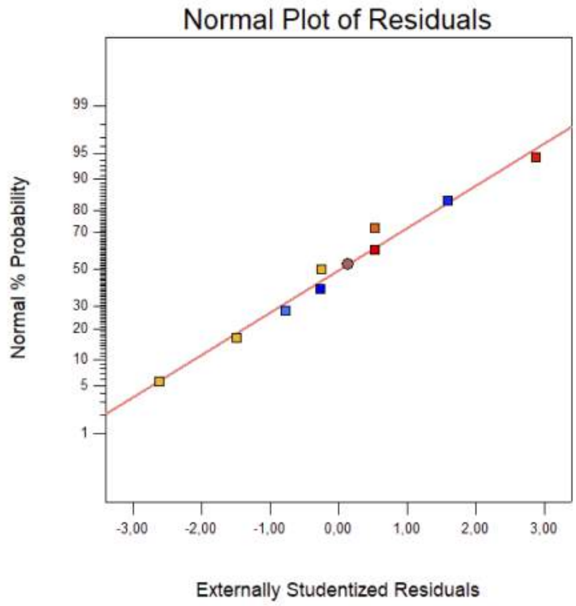

Figure 3.10: Design's normality

The second analysis is to see the independence of the design, between residuals and the order of experimental execution. Looking at figure 3.11, it can be concluded that, apparently, the experiment does not violate the independence hypothesis.

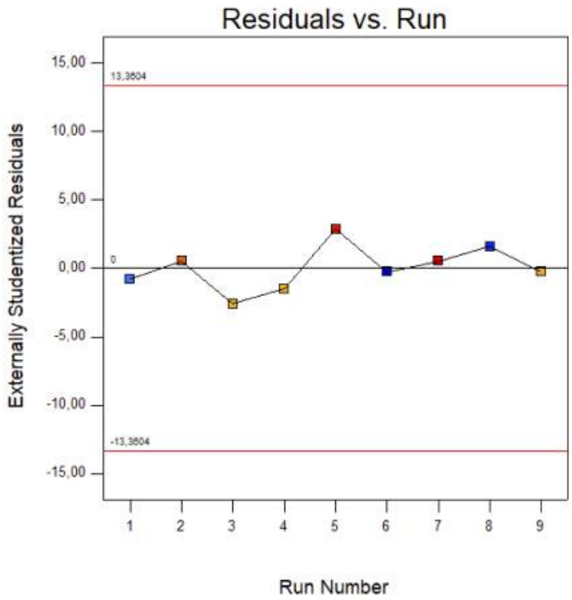

Figure 3.11: Design's Independence

The third residual analysis is the homogeneity and by evaluating the graph, that was made based on an assumption, it does not seem to strongly breach the variance homogeneity presumption. (Figure 3.12)

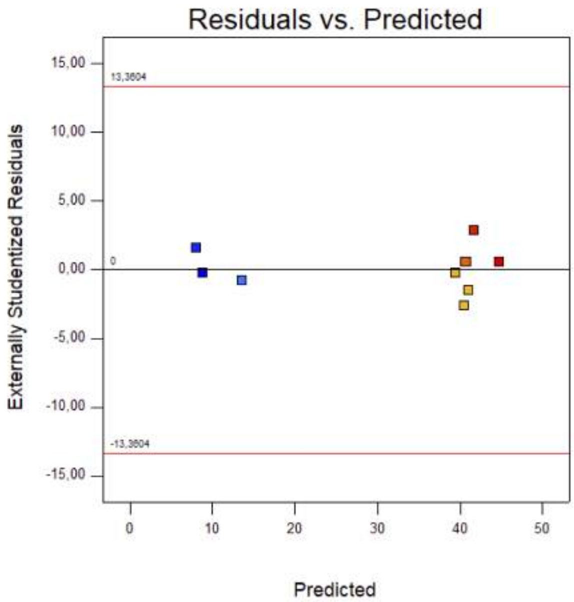

Figure 3.12: Design's homogeneity

In this platform, when it is created a mathematical model, it will relate the results obtained during the experiment with the model's predicted values. Residuals turns to be the difference of both. These residuals are assumed to have a normal distribution (Gauss Curve). Since there are no results outside the boundaries (red lines), one can conclude that none of the values observed have critical errors.

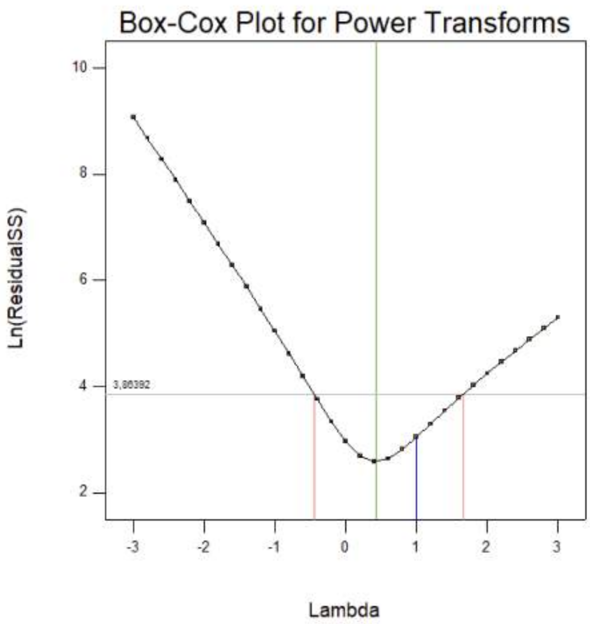

Figure 3.13: Box-Cox plot to verify if the model is adapted to the experiment

To make sure that the model requires any transformation because if the hypothesis of variance is constant is strongly violated, it is necessary to find the most adequate λ. [92] Fortunately, this is not the case, but it is verified by a Box-Cox plot to be sure that this is the best transformation. The objective of this transformation is to find the best estimate of λ for a minimal residual variation ($SS_{Residual}$), which is determined by a plot with the residual variation and the transformation parameter relation. To calculate the confidence interval, it is necessary to determine critical value of the residual variation ($SS_{Critical}$). This term is defined by,

$$\text{SS}_{critical} = \text{SS}_{minimal\ residual}\left[1 + \frac{t^2_{\frac{\alpha}{2};\nu}}{\nu}\right]$$

Where $SS_{Critical}$ is the critical variation value, $SS_{MinimalResidual}$ the minimal residual value, ν the number of degrees of freedom associated to the minimal residual variation and a t-Student distribution value for a significance level α and ν degrees of freedom, $t^2_{\frac{\alpha}{2};\nu}$. If λ=1 is inside the confidence interval, there is no need to make any transformation. This criterion is verified on figure 3.13.

Followed by all the analysis made, the model graphs were contour and 3D Surface, where it can analyze the behaviour of, in this case, *N. magadiense* on the studied conditions. Being a design of two factors (temperature and pressure) with three levels each, as shown on table 3.5.

Two responses were studied, the reduction of iron and the cell number, representing, respectively, the iron reduction process and the fermentative process.

The results of this experiment are shown on figures 3.14 and 3.15. It is shown that the model adapts to the experimental data. Three temperatures were studied (40°C, 50°C and 60°C), as well as three different pressures (1 bar, 150 bar and 300 bar).

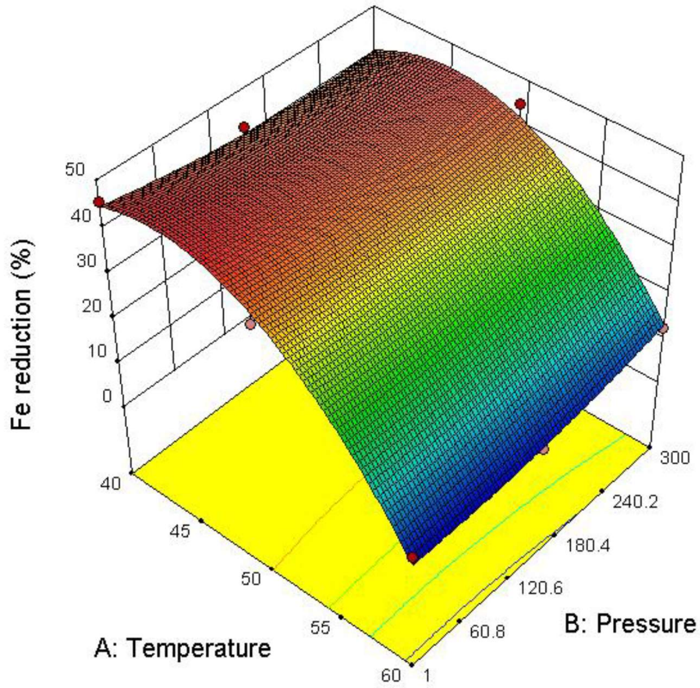

Figure 3.14: 3D surface plot that relates iron (Fe) reduction, temperature and pressure

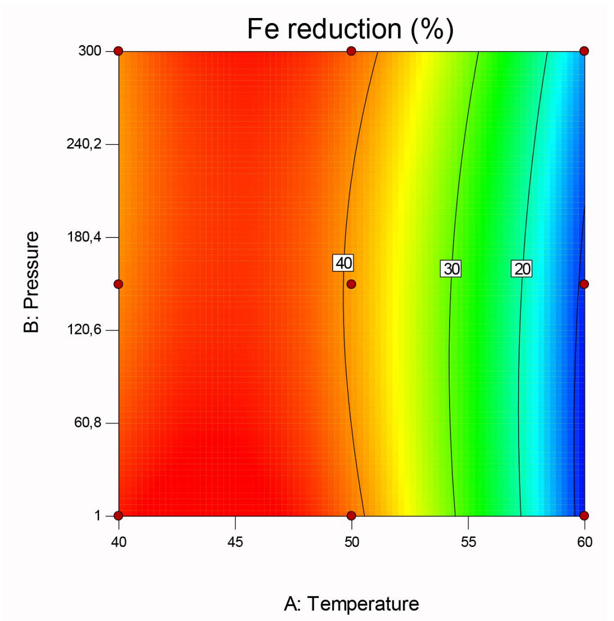

Figure 3.15: Contour plot that relates iron (Fe) reduction, temperature and pressure

Figure 3.15 shows that the reduction of iron is more significant between 40°C to 50°C and at 1 bar, then, along with the pressure increase, it tends to be more salient around 45°C. There are two types of processes: fermentative and the Fe reduction. The fermentative is between the cells and the carbon source that exists in the media. The Fe reduction process is when there is electron transfer between ferrihydrite and the carbon source (Yeast and NB); this is caused by *N. magadiense*'s metabolism, since it has a proton consumer metabolism. Hence, while it is occurring the Fe reduction, it will happen the oxidation of carbon dioxide (CO_2). After 50°C, *N. magadiense* tends to discard Fe and start to resort only on fermentative process. So, the Fe that is reduced is mainly at 40°C and 1 bar as it shown the figure 3.14, a 3D Surface plot that relates all the conditions that are being studied (Fe reduction, temperature and pressure). This plot represents parabolic function where the point where there is maximum reduction is around 45°C and 1 bar. Overall, the pressure does not change to much the behaviour of *N. magadiense* and the main impact agent is temperature, as it is possible to analyze in the ANOVA table.

The contrary happens in the number of cells, figure 3.16 and 3.17. The number of cells was determined by the amount of proteins present in the samples, since counting cells is a very difficult task due to the amount of Fe particles in the sample. The calculation was made as previously, using the equation 3.66.

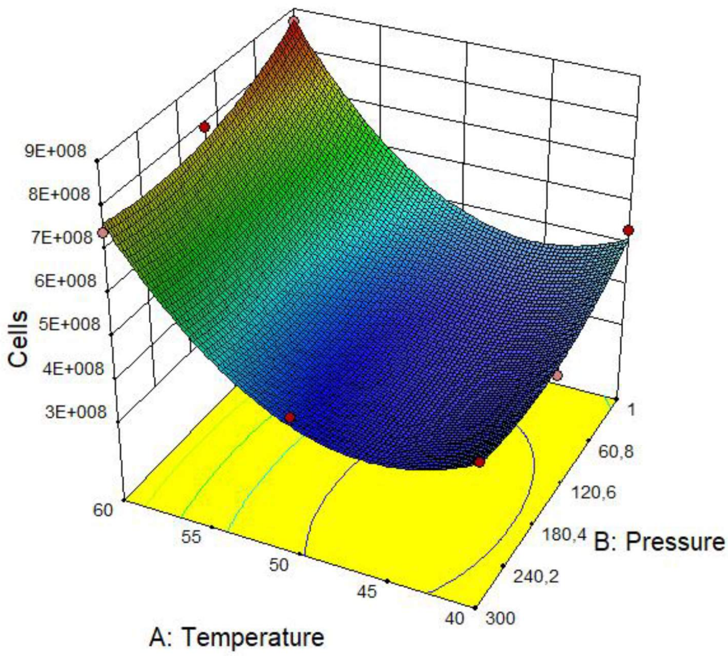

Figure 3.16: The number of cells is related with temperature and pressure

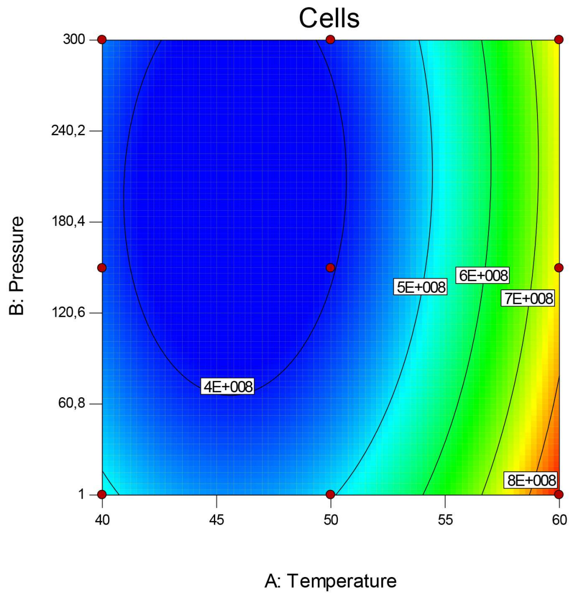

Figure 3.17: Contour plot that relates number of cells, temperature and pressure

It is concluded, through figures, that *N. magadiense* does not grow so well between 40°C and 50°C at high pressures, having a larger interval at around 150 bar. Thus, sum that the Fe reduction is more active in that same zone. It is more evident that *N. magadiense* grows better at 1 bar, since its highest cell number is at 1 bar and 60°C. It was concluded that *N. magadiense* behaves differently, depending on the conditions that they are in. For each response, a mathematical model was used to obtain their figures. These models can be used in order to study the best/worst conditions to work with *N. magadiense*. The models obtained were:

$$Fe\,reduction = -233.966 + 12.838\times T - 0.105\times P + 1.624E{-}3\times TP - 0.147\times T^2 + 8.614E{-}5\times P^2$$

$$Number\ of\ cells = 4.532 - 1.792E8\times T - 7.274E5\times P - 6519.4\times TP + 1.971\times T^2 + 2534.7\times P^2$$

Where T represents Temperature and P represents Pressure. These predictions depend on the importance of the responses, i.e. if it is very important to know what are the best conditions to have a Fe reduction, this platform (Design Expert 10.0.2) can optimize the model, based on the results. In case of maximizing Fe reduction and neglect cell growth, the results of that optimization would be the following,

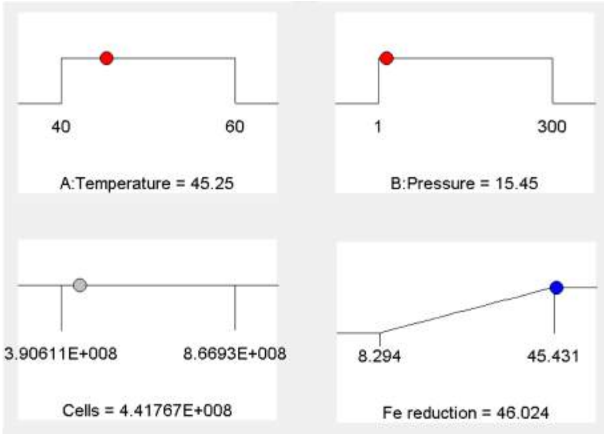

Figure 3.18: Optimization result if it was pretended to maximize Fe reduction

Summarizing it, the best conditions to maximize Fe reduction would be at 45.3°C and 15.5 bar, where the predicted value of Fe reduction would be 46.02%, with a number of cells of 4.418×10^8. If it was pretended the absolute opposite,

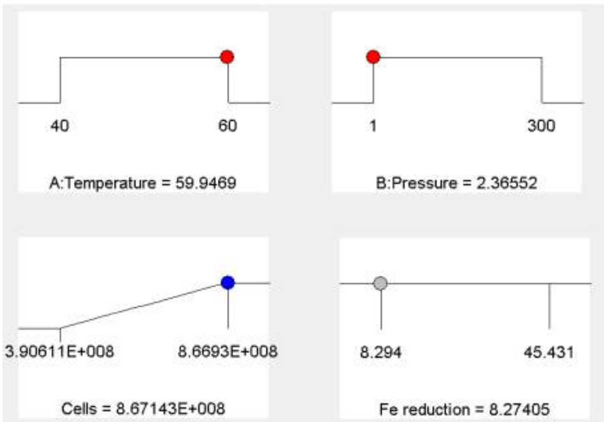

Figure 3.19: Optimization result if it was pretended to maximize the amount of cell in the system

The best conditions to maximize cell number would be at 59.9°C and 2.36 bar, where the predicted value of Fe reduction would be 8.27% and it would have in the sample 8.671×10^8 cells. Now, for knowledge purposes, since it is not pretended, the best conditions to minimize Fe reduction and number of cells, separately and respectively, would be,

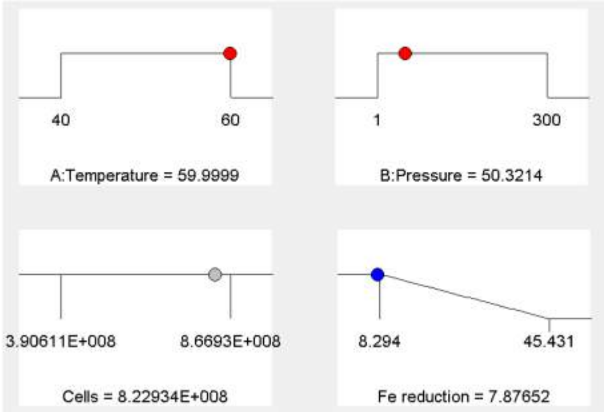

Figure 3.20: Optimization result if it was pretended to minimize Fe reduction

The worst conditions to Fe reduction would be at 60°C and 50.32 bar, where the predicted value of Fe reduction would be 7.88% and it would have in the sample around 8.229×10^8 cells.

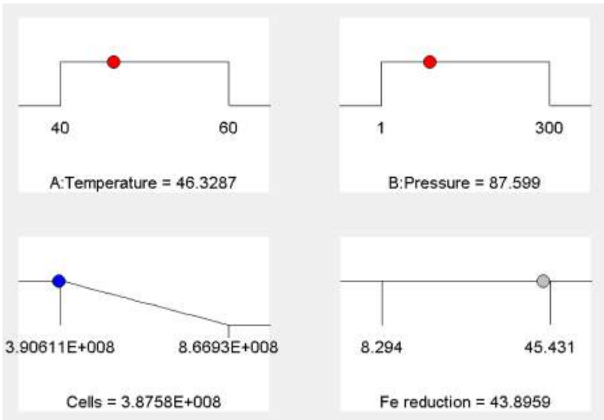

Figure 3.21: Conditions to minimize number of cells

To minimize cell number, it would be at 46.3°C and 87.6 bar, where the predicted value of Fe reduction would be 43.9% and there would be 3.876×10^8 cells in the sample. If it is given more importance to Fe reduction instead of number of cells, or vice-versa, but the pretended is to maximize each response the conditions would be,

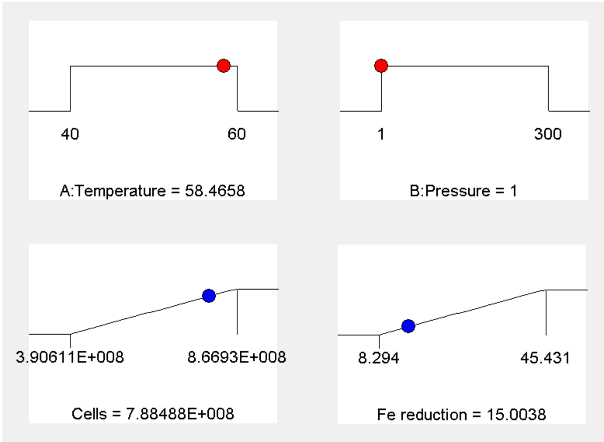

Figure 3.22: Conditions to give more importance to Fe reduction instead of number of cells

Giving more importance to Fe reduction maximization, the conditions would be at 40°C and 1 bar, where the predicted value of Fe reduction would be 44.72%, in a sample with 5.149×10^8 cells.

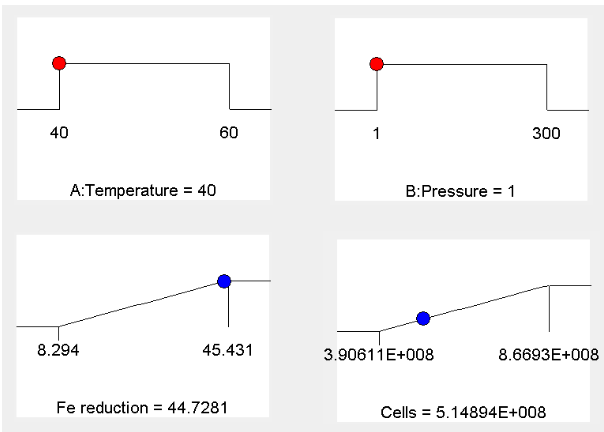

Figure 3.23: Conditions to give more importance to the Number of Cells instead of Fe reduction

Giving more importance to number of cells maximization, the conditions would be at 58.5°C and 1 bar, where the predicted a sample with 15% of Fe reduced and 7.885×10^8 cells. It is just missing the possibility that each response be equally important to maximize and minimize,

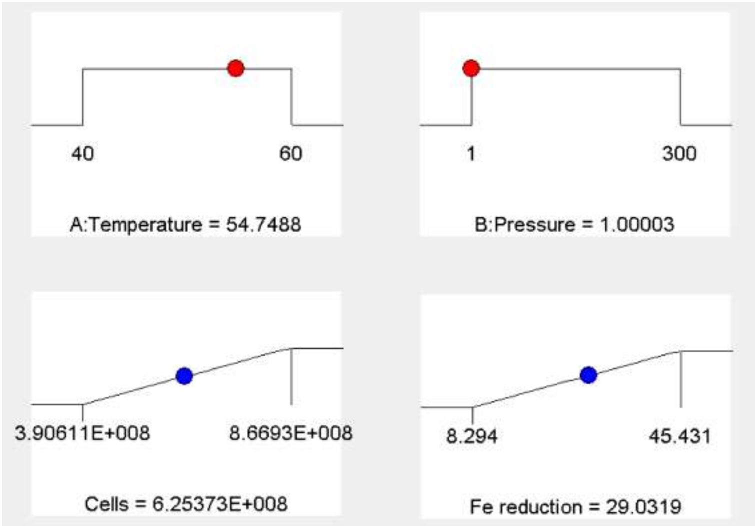

Figure 3.24: Conditions influenced by the equal importance of Fe reduction and number of cells on maximizing the results

Equal importance to maximize experimental responses, will get to conditions as presented in figure 3.24, 54.8°C and 1 bar, with values of Fe reduction and number of cells of 29.03% and 6.254×10^8, respectively.

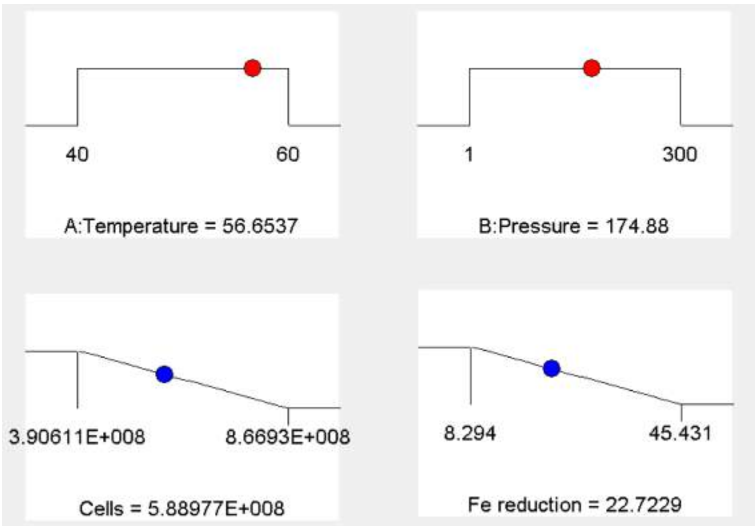

Figure 3.25: Conditions influenced by the equal importance of Fe reduction and number of cells on minimizing the results

Equal importance to minimize experimental responses, will be needed to be working at conditions as presented in figure 3.25, 56.7°C and 174.88 bar, with values of Fe reduction and number of cells of 22.72% and 5.889×10^8, respectively.

Chapter 4

Conclusion

To sum every aspect of this thesis, cracking as been a major problem in all kinds of concrete structures, being necessary to be reduced to prevent costly repairings. Self-Healing concept is the method that will help prevent this kind of concrete behaviour, being an economical, environmental, reliable and stable. However, this method tends to use microbial $CaCO_3$ precipitation as a concrete sealer. For this reason, during this research months, a possible bacteria, *N. magadiense*, was studied in order to accomplish all the requisites for $CaCO_3$ precipitation.

N. magadiense cannot grow without carbonates [87] and, if there is no cell growth, there is no activity. The activity can be through fermentative process or Fe redox cycle, when reducing Fe^{3+} into Fe^{2+}. Hence, it has a proton consumer metabolism, which means that it will make the environment pH rise and, consequently, it will occur a chemical reaction that converts CO_2 into ${CO_3}^{2-}$. This conversion is very useful and being, as well, an endospore forming bacteria, meaning that, it can remain in an environment for longer periods of time. *N. magadiense* a good candidate to be tested on cement with the purpose of developing the self-healing concept.

Since the purpose of study these bacteria were to be, possibly, used on oil well concrete, its physiology is an important step to consider in this project, initiated in AU University. On an oil well, *N. magadiense* would be subjected to high temperatures, high pressures (up to 344 bar, generically) and, also, high alkalinity, since it will be used in concrete. These conditions were evaluated, and it was confirmed that *N. magadiense* can growth and survive under these conditions.

As future considerations, it is recommended to find a simple and unique carbon source, to replace the complex one that is yeast and NB with an Fe extraction test, so it would be easier to measure *N. magadiense* cell activity and a cell counting procedure or protein assay, although, due to many external parameters that can influence the results, it may have consistency. [98] Customize the standard medium with the optimum conditions mentioned on the literature [87] and test its growth and activity. It would be recommended to evaluate *N. magadiense*'s behaviour when its limits are reached, i.e. test pressures higher that 300 bar, since if *N. magadiense* reacts well with the pressure change and consequent temperature variation, it can be used in all types of oil well, even those that can have pressures higher than 300 bar. [84]

Since $[Cl^-]$ was evaluated to concentrations that inhibit *N. magadiense*, it would be recommended to study its behaviour in its concentration range (0-1.55M). [87]

Hence, to apply this bacteria on concrete it was tested a reduction of HCO_3^- in the media. *N. magadiense* did not react well with the extreme reduction. In theoretical terms, when *N. magadiense* is present in the concrete, it should occur mass transfer changes between the exterior and interior of the environment where *N. magadiense* was meant to be situated. So, it would be advised, instead of this extreme conditions, to reduce to 50% the amount of HCO_3^- since it would be, in theory, the level of equilibrium between the system's interior and exterior. To replace the amount of HCO_3^- reduced, a compound that changes only the $[Na^+]$ would be the best choice, taking into consideration that the system may have components that could harm concrete, as sulphate, chloride and even excess of carbon dioxide. [3]

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