THE EFFECT OF ARTIFICIAL SWEETENERS ON THE GROWTH AND METABOLIC ACTIVITY OF *Nitrosopumilus maritimus* STRAIN SCM1

Elisa Martinez Calado

BACHELOR THESIS

BACHELOR OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

Academic supervisor: Dr. Maria Teresa Blay Olivé (mteresa.blay@urv.cat)

Professional supervisors: Prof. Dr. Martin Könneke (martin.koenneke@uol.de), Dr. Bert Engelen (bert.engelen@uol.de)

In cooperation with: Benthic Microbiology Research Group, School V | Institute for Chemistry and Biology of the Marine environment (ICBM), Carl von Ossietzky Universität Oldenburg

Tarragona, September 2025

This work was conducted based on the results obtained during the external internship at the Institute for Chemistry and Biology of the Marine Environment (ICBM) at the Carl von Ossietzky Universität of Oldenburg, Germany.

Index

Abstract

Artificial sweeteners are increasingly detected in aquatic environments, yet their effect on marine microorganisms remains largely unknown. This study investigated the impact of these micropollutants on the growth and metabolic activity of *Nitrosopumilus maritimus*, a marine ammonia-oxidizing archaeon with a key role in the marine nitrogen cycle. Cultures were exposed to different concentrations of five artificial sweeteners (saccharin, sucralose, aspartame, acesulfame K and cyclamate), and nitrite accumulation was used as an indicator of growth and ammonia oxidation activity. Results showed that the culture exposed to 3000 ng/L of sucralose exhibited growth inhibition. A subsequent screening experiment was conducted with concentrations between 1000 ng/L and 3000 ng/L. No significant inhibition was observed under any of the experimental conditions, suggesting the initial result may have been due to experimental variability or an outlier. These findings indicate that *N. maritimus* can sustain normal activity and growth in presence of artificial sweeteners at environmentally relevant concentrations.

Keywords: Artificial sweeteners, ammonia-oxidizing archaeon, micropollutants, nitrogen cycle, nitrification.

Abbreviations

AOA: Ammonia-oxidizing archaea

AOB: Ammonia-oxidizing bacteria

AS: Artificial Sweetener

MGI: Marine-group I

SCM1: Synthetic *Crenarchaeota* Media 1

SAC: Saccharin

SUC: Sucralose

ASP: Aspartame

ACE: Acesulfame K

CYC: Cyclamate

AMO: Ammonia monooxygenase

HAO: Hydroxylamine oxidoreductase

MCO: Multicopper oxidases

NNS: Nonnutritive sweeteners

GDA: Glutaraldehyde

SD: Standard deviation

LMM: Linear mixed-effects model

1. Introduction

1.1. The Marine Nitrogen Cycle

Nitrogen-containing compounds are essential nutrients in the ocean. Due to the role of nitrogen as key macronutrient, its availability strongly influences primary production, marine food webs and global biogeochemical cycles [1,2]. A lack of this nutrient has the capacity to limit biological productivity in many marine ecosystems.

Nitrogen exists in more chemical forms than most other major elements, with a broad range of chemical transformations that are unique to this element. Nearly all of these transformations are done by marine organisms as part of their metabolism, either to synthetize structural components, or to gain energy for growth. The most abundant form of nitrogen on Earth is nitrogen gas (N_2), which is not bioavailable. All other bioavailable forms are known as “fixed nitrogen”, such as ammonium (NH_4^+), nitrite (NO_2^-) or nitrate (NO_3^-). The general unavailability of N_2 for marine organisms explains the importance of nitrogen fixation (conversion to organic *N*) and denitrification (conversion of nitrate to N_2) [3]. The balance between these two processes determines the amount of bioavailable nitrogen in the ocean, which in turn influences overall marine productivity (Figure 1).

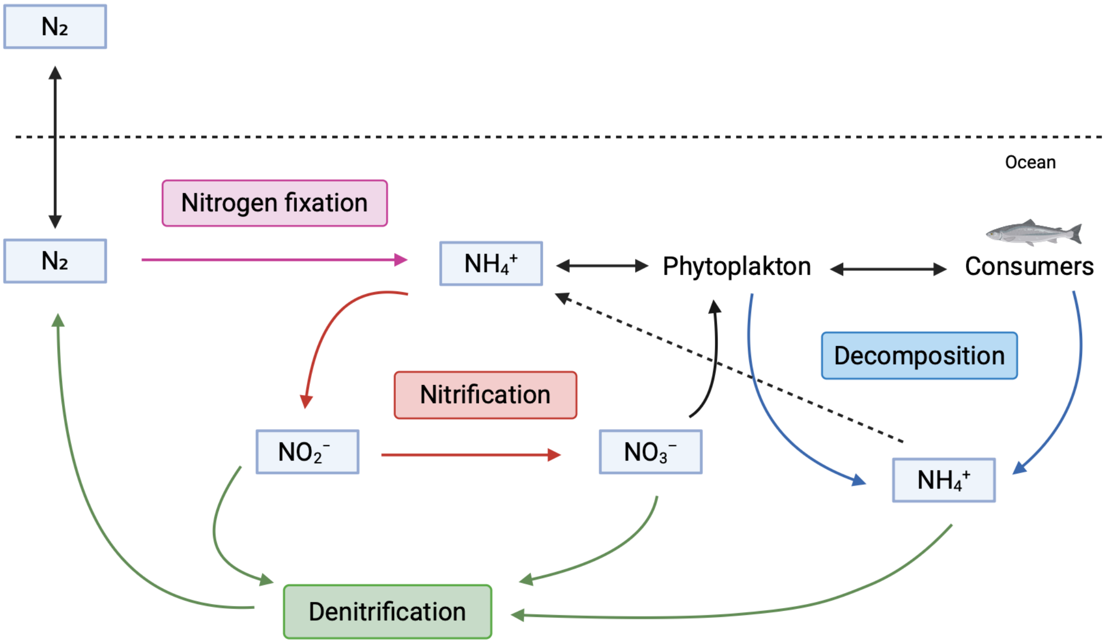

Figure 1: Simplified Marine Nitrogen cycle. The figure illustrates the key processes in the marine nitrogen cycle, highlighting the movement of different chemical forms of nitrogen: Ammonia (NH_4^+), Nitrite (NO_2^-), Nitrate (NO_3^-) and Nitrogen gas (N_2). The pink arrow represents nitrogen fixation, where N_2 is converted to bioavailable NH_4^+. Part of it is taken up by phytoplankton, while the majority enters the nitrification pathway represented by the red arrows. In nitrification, NH_4^+ is converted to NO_2^- and then to NO_3^-. Nitrate is the main nitrogenous compound used by primary producers (phytoplankton) in the ocean and it is a major nutrient required for photosynthesis in marine ecosystems. Nitrogen is assimilated by phytoplankton and moves through the marine food web; it is eventually returned to the environment through decomposition of organic matter and waste which is represented by blue arrows. Finally, green arrows represent the denitrification process by which NH_4^+, NO_2^- and NO_3^- are converted back into N_2. This gas may reenter the cycle through nitrogen fixation, or it can be exchanged with the atmosphere. Together these processes maintain the balance of nitrogen in the ocean and support marine productivity [4].

The temporal variations in the different forms of fixed nitrogen in the ocean are largely controlled by primary production. Primary production is the process by which autotrophic organisms, which are mainly phytoplankton in the marine environment convert inorganic carbon (CO_2) into organic matter. This transformation usually occurs through photosynthesis, using sunlight as energy source, or through chemosynthesis [5]. In marine ecosystems, phytoplankton are the dominant primary producers, forming the base of the marine food web.

Nitrogen plays an important role in the metabolism of phytoplankton as an essential nutrient. The lack of bioavailable nitrogen in the marine environment limits their growth and reproduction, which in turn constrains carbon fixation and reduces overall primary productivity. This impacts higher trophic levels and the efficiency of energy transfer through the food web [6]. So, when nitrogen is in short supply, phytoplankton growth is limited, which impacts carbon fixation and the entire marine food web.

At the global scale, biological nitrogen fixation is the largest source of nitrogen to the ocean, while anaerobic microbial processes are responsible for nitrogen losses [7,8]. These inputs and losses are connected by nitrification, a two-step aerobic process [1]:

1. Ammonia oxidation (step 1):

$$NH_4^+ + 1{,}5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$$

(This step is primarily carried out by ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB))

2. Nitrite oxidation (step 2):

$$NO_2^- + 0{,}5O_2 \rightarrow NO_3^-$$

(This step is typically carried out by nitrite oxidizing bacteria)

1.2. The Ecological Role of Ammonia Oxidizing Archaea

In the last few decades, scientific advances have significantly deepened our understanding of the global nitrogen cycle, especially through the discovery of new microbial processes and key microbial players. This includes the discovery of AOA which are now known to constitute a major fraction of the microbial biomass on Earth and play a vital role in the global biogeochemical cycling of nitrogen, particularly in oligotrophic (nutrient-poor) regions in the ocean [9].

Archaea were discovered in the marine plankton [10] and it is now well understood that they are not limited to extreme environments as was once thought, but are, in fact, among the most abundant microorganisms in the ocean [11]. One particularly important group is the Marine group I (MGI) archaea, which are significantly abundant in global oceans and were originally discovered through ribosomal RNA gene sequencing [12]. More recently, metagenomic, biogeochemical and microbiological studies established the ability of MGI archaea to oxidize ammonia, linking this group to a central process in the global nitrogen cycle [13,14,15].

Within the domain Archaea, members of the phylum *Thaumarchaeota* are among the most abundant prokaryotes on Earth and are widely distributed in marine, terrestrial, and geothermal environments. All studied *Thaumarchaeota* oxidize ammonia (even when it is present at very low concentrations) to generate energy. At the same time, they fix inorganic carbon (CO_2) into organic matter, meaning that they can function as primary producers. This combination of ammonia oxidation and carbon fixation makes them key players in both nitrogen and carbon cycles, especially in oligotrophic environments as the open ocean [16].

A key representative of this group is *Nitrosopumilus maritimus*, which is a marine AOA with a remarkably high affinity for ammonia, enabling them to thrive in the open ocean, even when ammonium is present at low nanomolar concentrations [14,17,18]. *N. maritimus* gains its energy from ammonia oxidation coupled to oxygen consumption and can even produce oxygen when depleted, which may explain their regular occurrence in anoxic waters [17]. Importantly, they have the capacity to oxidize ammonia in the absence of organic carbon sources, confirming its role as chemolithoautotroph and primary producer.

Phylogenetically, *N. maritimus* and related AOA were originally placed in the MGI *Crenarchaeota* based on 16S rRNA gene markers. However, comprehensive genomic analyses have demonstrated that these organisms form a distinct lineage characterized by unique core genes, different from *Crenarchaeota*. As a result, they have been classified into a different phylum, known as *Thaumarchaeota* [19].

For nearly a century, ammonia oxidation was thought to be carried out only by *Bacteria*, until isolation of mesophilic (moderate-temperature-loving) and thermophilic (heat-loving) AOA (including *N. maritimus*) revealed an archaeal role [12]. While researchers have made progress in cultivating enrichment cultures of AOA in the laboratory, achieving a fully isolated, uncontaminated culture has proven very challenging. To this date, only *N. maritimus* has been successfully isolated in pure culture and cultivated. This particular strain was obtained from a gravel sample taken from a tropical marine aquarium [14].

N. maritimus was the first reported chemolithoautotrophic nitrifier identified within the MGI-group in the domain Archaea and the first mesophilic isolate within the phylum *Crenarchaeota* [14,20]. Its metabolic versatility and ecological distribution show their importance in global carbon and nitrogen cycling, particularly in oligotrophic marine environments [12,14].

The cells of *N. maritimus* are known to be extremely small, they appear as straight rods with a diameter of 0.17 to 0.22 μm and lengths between 0.5 and 0.9 μm. In addition, their growth is correlated with a near-stoichiometric conversion of ammonia to nitrite [14]. This process can be described by the following overall reaction:

$$NH_3 + 1{,}5\ O_2 \rightarrow NO_2^- + H_2O + H^+\ (\Delta G^{0,} = -235\ kJ\ mol^{-1})$$

The cultures are grown aerobically in the dark at 28ºC in Synthetic *Crenarchaeota* Media (SCM1). Due to their small size, *N. maritimus* cells are not visible to the naked eye. Therefore, their growth is typically monitored by measuring nitrite accumulation in the medium. An increase in nitrite concentration in the medium corresponds to increased cellular activity and biomass production [14].

1.3. Ammonia Oxidation Pathways of Bacteria and Archaea

The stoichiometry of ammonia oxidation of *N. maritimus* is similar to the one characterized for aerobic, obligate chemolithoautotrophic AOB [20], but the biochemical pathway in *N. maritimus* is completely different and very unique to archaea. All AOB share a common pathway (Figure 2) where ammonia is first turned to hydroxylamine (NH_2OH) using an enzyme called ammonia monooxygenase (AMO). Then NH_2OH is converted to nitrite by the hydroxylamine oxidoreductase (HAO) complex. This process releases electrons which are passed through a standard electron transport chain involving cytochrome C, which are iron-containing proteins, and producing energy. In the case of *N. maritimus*, AMO is also used to start the reaction, but there is a lack of genes encoding for the HAO enzyme complex and for the cytochrome C proteins, this is an indication for an alternative archaeal pathway. It relies instead to a completely different system mainly based on copper-containing proteins which also suggest an alternative electron transfer mechanism. Several of these proteins are multicopper oxidases (MCOs), which are believed to replace he role of HAO by oxidizing hydroxylamine or a related intermediate, such as nitroxyl (HNO), to nitrite.

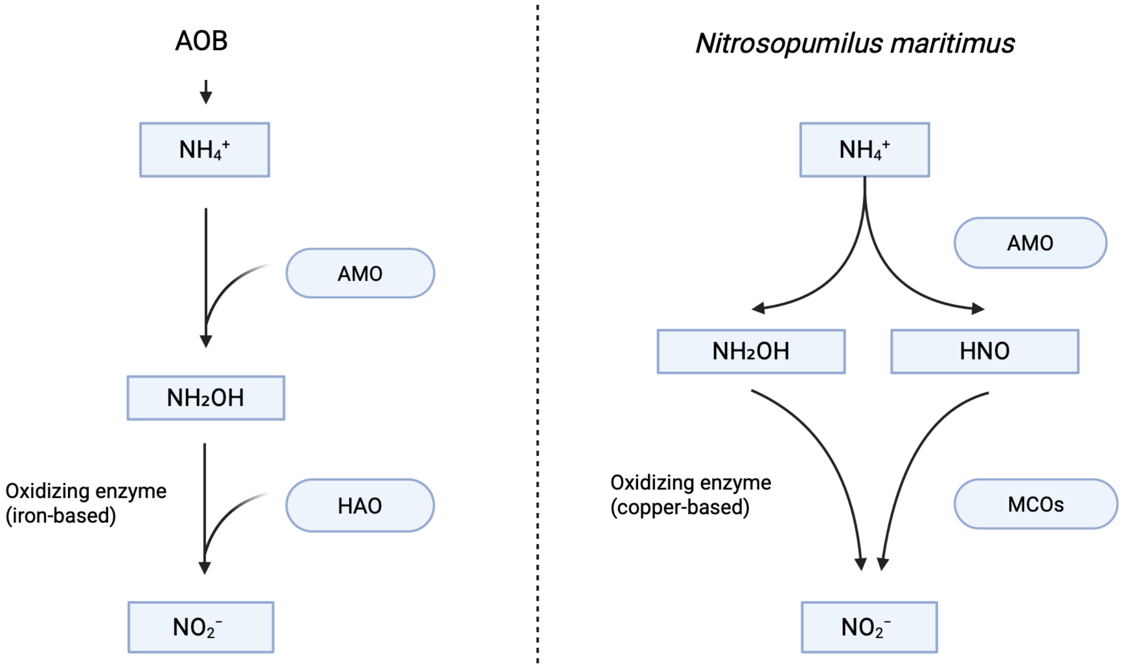

Figure 2: Comparison of ammonia oxidation pathways in AOB and *Nitrosopumilus maritimus*. The left side of the figure represents the common pathway all AOB share, where ammonia (NH_4^+) is first oxidized to hydroxylamine (NH_2OH) by the enzyme AMO. Hydroxylamine is then further oxidized to nitrite (NO_2^-) by the HAO complex, an iron-based enzyme. This process releases electrons that enter a cytochrome C mediated electron transport chain, contributing to energy production. The right side of the figure shows the proposed alternative pathway for *N. maritimus*. While AMO is also involved in the ammonia oxidation, *N. maritimus* lacks genes for HAO and cytochrome C. Two parallel pathways have been proposed: one where NH_2OH is produced and then oxidized to NO_2^- via copper-based MCOs and another where AMO produces directly HNO, which is then oxidized to NO_2^- by a distinct MCO. These copper-based enzymes suggest a unique archaeal electron transport mechanism different from the iron-based mechanism in AOB [12].

In addition, *N. maritimus* has three key genes (amoA, amoB and amoC) that code for the AMO enzyme, which starts the process of oxidizing ammonia. They are completely different to the ones encoding for AMO in bacteria, which also indicates that the archaeal version of AMO might work differently. The archaeal pathway is thought to be simpler and possibly more efficient than bacteria, ideal for a low-nutrient environment [12].

There are two possible pathways proposed for the oxidation process in *N. maritimus* (Figure 2, right). One in which hydroxylamine is oxidized by a copper-containing analog of HAO, and another where AMO produces HNO instead of hydroxylamine. In the latter case, HNO would be oxidized by a different multicopper enzyme than NH_2OH.

N. maritimus, like all known AOB, grows chemolithoautotrophically by using inorganic carbon as the sole carbon source [14]. However, whereas AOB use the Calvin-Bassham-Bensson cycle with the CO_2-fixing enzyme ribulose bisphosphate carboxylase/oxygenase (RubisCO) as the key enzyme, there is an absence of genes in *N. maritimus* coding for RubisCO and other enzymes for this cycle. This points to an alternative pathway of carbon fixation. The mechanism which is most likely used by these organisms is the 3-hydroxypropionate / 4-hydroxybutyrate (3HP / 4HB) pathway originally found in thermophilic *crenarchaeota* like *Metallosphaera sedula* [12,21]. Thus, while AOB and *N. maritimus* carry out the same process of ammonia oxidation, they use completely different biochemical pathways.

1.4. Artificial Sweeteners as Micropollutants

Given the widespread distribution and the ecological significance of *N. maritimus* in the ocean, understanding how emerging micropollutants affect its physiology is essential. Micropollutants, such as artificial sweeteners (ASs) or pharmaceutical compounds, are an emerging concern for the public health. They enter the marine environments via wastewater treatment plants, aquaculture runoff, hospital effluents and shipping activities. Their occurrence in marine environments is a public health concern due to their adverse effects on ecological resilience and human and animal health [22].

An AS is a synthetic substance employed as a sugar substitute because of its greater sweetness and lower caloric intake. The sweetness of these compounds is measured in relation to the reference sugar sucrose.

Nonnutritive sweeteners (NNSs) are defined as sweetening agents that have a higher sweetening intensity and a lower calorie content per gram compared with nutritive sweeteners such as sucrose or corn syrup. NNSs can be of synthetic or natural origin [23]. There are currently seven food sugar substitutes which are approved by the European Union, including: saccharin or E 954 (SAC), sucralose or E 955 (SUC), aspartame or E 951 (ASP), acesulfame K or E 950 (ACE), and cyclamate or E 952 (CYC) [24]. They are usually associated to the consumption of dietary products but ASs are also commercially used in other products such as chewing gum, frozen desserts, fruit juices and gelatines [25].

Studies have shown that after ingestion, ASs are excreted mostly unchanged from the human body, they are classified as metabolically inert. SUC, for example, is known to be highly stable and hydrophilic, this contributes to its poor absorption, rapid elimination, and also to a minimal metabolism of the absorbed fraction, and a lack of bioaccumulation potential in humans [26]. In the last decade, their presence and behavior in the environment has become object of study since they have been detected at high concentrations in influents and effluents from wastewater treatment plants, showing low or even negative removal percentages during wastewater treatment [25].

In a previous study regarding the occurrence and vertical distribution of micropollutants of emerging concern (pharmaceutical compounds, UV filters, and AS) in the sea surface microlayer and near-surface layer in the southern North Sea, three out seven ASs examined were found in 100% of all samples analyzed. These ASs were SAC, SUC and ACE [22]. SAC is one of the most popular ASs owing to its low price, and sweetness that is about 300 times greater than sugar. It is considered an emerging contaminant and widely detected in the environment; it is considered to have potential ecotoxicity risks to aqueous organisms and human beings [27].

Given the emerging concern over ASs as micropollutants in the marine environment and their potential threat to public health, as well as the widespread abundance and ecological importance of the archaeon *N. maritimus* in the ocean, this bachelor thesis aimed to investigate the effects of different concentrations of five AS (SAC, SUC, ASP, ACE, CYC) on the growth and metabolism of *Nitrosopumilus maritimus*.

2. Hypothesis and Objectives

ASs are increasingly recognized as emerging micropollutants of the marine environment due to their widespread use in daily life, poor metabolization, and low to negative removal in wastewater treatment plants. Given their potential ecological impact, the hypothesis of this thesis is that high concentrations of ASs interfere with the growth and ammonia-oxidizing activity of *Nitrosopumilus maritimus*, a key archaeon deeply involved in marine nitrogen cycling.

Therefore, the main objective of this project is determining the impact of different concentrations of five ASs (SAC, SUC, ASP, ACE and CYC) on the growth and metabolism of *N. maritimus* under controlled laboratory conditions and therefore evaluate their potential disruption to the marine nitrogen cycling process.

3. Materials and Methods

To determine the effect of ASs on the growth of *N. maritimus*, different culture media were prepared at low (10 ng/L), medium (500 ng/L) and high (1000 ng/L) concentrations of each compound. In addition, a culture was prepared with a medium that reflected the environmental concentration of each AS, based on values reported in a previous study about the vertical distribution of micropollutants in the southern North Sea coastal waters [22].

3.1. Media Preparation

To begin with the project, the SCM1 medium, was prepared (named after "Candidatus *Nitrosopumilus maritimus 1")*. Different solutions were made and later mixed to form the complete medium that supports the growth of *N. maritimus*. All liquid volumes were measured using graduated cylinders, and solutes were weighed using analytical balances (Quintix, Sartorius, Germany). All reagents used for the preparation of the medium were obtained from Carl Roth GmbH (Karlsruhe, Germany), unless stated otherwise and the solutions were stored in laboratory bottles unless noted differently.

Throughout the entire project and in all experiments involving *N. maritimus*, this same SCM1 medium was used to ensure proper cultivation conditions. The following solutions were prepared in the laboratory:

First, 1 L of $FeNaEDTA$ solution (7.5 mM) and 1 L of Modified Non-Chelated Trace Element Solution (Mod. Trace Element) were prepared. The second one is a mix of various solutes that enrich the medium for optimal *N. maritimus* growth. The solutes were: HCL (100 mM), H_3BO_3 (0.5 mM), $MnCl_2 * 4H_2O$ (0.5 mM), $CoCl_2 * 6H_2O$ (0.8 mM), $NiCl_2 * 6H_2O$ (0.1 mM), $CuCl_2 * 2H_2O$ (0.01 mM), $ZnSO_4 * 7H_2O$ (0.5 mM) and $Na_2MoO_4 * 6H_2O$ (0.15 mM).

For the preparation of the Mod. Trace Element solution, since only $Na_2MoO_4 \cdot 2H_2O$ was available in the lab (instead of $Na_2MoO_4 \cdot 6H_2O$), a conversion had to be calculated to ensure the correct molar concentration in the medium.

Additionally, 500 mL of $HEPES$ buffer (1 M $HEPES$, 0.6 M, pH 7.8) were prepared and the pH was measured to be 7.85, so no adjustment had to be done. Also, 200 mL of the $NaHCO_3$ (1 M) solution was made and stored in serum bottles with approximately one-third of the bottle left empty. The bottles were sealed with rubber stoppers to allow withdrawal using syringes and were flushed with CO_2 to improve handling. Furthermore, 200 mL of 1 M NH_4Cl solution and 1 L of 0.4 g/L KH_2PO_4 solution were prepared.

To start the preparation of the SCM1 medium, inorganic salts such as $NaCl$, $MgSO_4$, $MgCl_2$, $CaCl_2$, and KBr were dissolved in 1 L of distilled water, according to the laboratory protocol.

All solutions were autoclaved and cooled down to room temperature to proceed with the media preparation with sterile solutions. Once cooled, all six previously prepared solutions were added into the liter of SCM1 medium using glass pipettes, the volumes were added according to the lab protocol. The mixing process was done in the laminar flow hood to prevent from contamination. The final pH of the medium was 7.57 (target range: 7.55 – 7.6), so no adjustment was necessary. The surplus solutions were stored in the fridge at 4 °C. Once the medium was ready, two aliquots of 50 mL were prepared, and 1.5 mL of *N. maritimus* from a pre-existing culture (strain SCM1, originally isolated from a marine tropical aquarium at the Seattle Aquarium, Washington, USA; [14]) was inoculated into each. The aliquots were named Culture 1.1 and Culture 1.2 and were incubated at 28 °C for the rest of the experiment.

3.2. Nitrite Quantification

The following solutions were made to measure nitrite accumulation in the media: 250 mL of Sulfanilamide / NED reagent solution were prepared using orthophosphoric acid, sulfanilamide, and N-(1-naphthyl) ethylenediamine dihydrochloride according to the lab protocol. The solution was stored in a lab bottle the dark at 4 °C.

To determine the concentration of nitrite in each media bottle, a standard curve was created. A 100 mM sodium nitrite stock solution was first prepared, from which serial dilutions were made to obtain standards of 10 mM, 1 mM, 0.5 mM, 0.25 mM, 0.1 mM, and 0.05 mM.

After preparing the dilutions for the standard curve, glass tubes were set up for the colorimetric assay. According to the lab protocol, the sulfanilamide reagent was added to each tube with distilled water and the corresponding sample. The mixture was incubated for 10 minutes at room temperature. Following incubation, the samples were vortexed and measured in triplicate using a UV-Vis spectrophotometer (UV-1280, Shimadzu, Japan) at 545 nm and distilled water was used as the blank reference. The average values were used to construct the calibration curve and to obtain the calibration curve equation for the determination of the nitrite concentrations (Table 10, Figure 12). The calibration curve equation was $y = 1.1888x + 0.0408$ (R^2 = 0.99) where y represents the absorbance and x the nitrite concentration in mM.

This colorimetric assay allowed nitrite quantification, correlating pink coloration with nitrite accumulation. The darker the pink color gets, the more nitrite there is in the media, and higher absorbances are obtained, as a result of the metabolic activity of *N. maritimus*. First, the growth of Culture 1.1 and Culture 1.2 (without AS) was monitored to establish a reference for the normal growth pattern of *N. maritimus* under standard conditions (Table 6). The absorbances were obtained at 545 nm with the UV-Vis spectrophotometer (UV-1280, Shimadzu, Japan) over a period of two weeks.

With this data, the nitrite concentration in the medium was calculated with the help of the nitrite calibration curve equation (Figure 12). Absorbance values were substituted into the calibration curve equation to calculate the corresponding nitrite concentration.

3.3. Growth Monitoring

The growth of *N. maritimus* is reflected by the nitrite accumulation in the media, as nitrite is a direct byproduct of its ammonia-oxidizing activity. To monitor this growth, samples were taken every 2 – 3 days from the previously prepared aliquots of 50 mL (Culture 1.1 and Culture 1.2) over a two-week period. All sampling procedures were carried out under sterile conditions in a laminar flow hood to avoid contamination. From each culture, 1.5 mL samples were taken and stored in Eppendorf tubes for subsequent nitrite analysis, performed following the procedure described above. Nitrite concentrations were determined using the previously established nitrite calibration curve equation ($y = 1.1888x + 0.0408$) (Figure 12), with values calculated by isolating x (nitrite concentration). This exact procedure was repeated for both cultures over two weeks to follow the exponential growth of *N. maritimus* under optimal conditions.

3.4. Artificial Sweeteners and *N. maritimus*

To determine the potential effect of five different ASs (SAC, SUC, ASP, ACE and CYC) on the growth of *N. maritimus,* twenty-one Erlenmeyer flasks, each with a final volume of 50 mL, were prepared. Twenty flasks represented different sweetener concentrations of each AS (low, medium, high, and environmental), and one was a control without sweeteners. The low (10 ng/L), medium (500 ng/L) and high (1000 ng/L) concentrations were standardized across all five AS. In contrast, the environmental concentration for each AS was based on data from a previous study investigating the vertical distribution and risk assessment of pharmaceuticals and other micropollutants in the southern North Sea coastal waters [22]. For exact concentrations of ASs used in each culture, please refer to Table 7 in the appendix.

The initial concentration of the stock solutions for each AS was 5 mg/mL. To prepare each media with the desired concentration of AS and facilitate accurate volume handling, serial dilutions were made, first to 500 µg/L, and then to the final working concentration of 5 µg/L. These resulting AS solutions were sterilized using 0.2 µm cellulose acetate syringe filters and stored for later use in the fridge at 4 °C.

The required volumes of each AS to add to the flasks were calculated using the standard dilution formula ($Ci * Vi = Cf * Vf$). For example, to reach a final concentration of 10 ng/L in a 50 mL flask, the calculated addition was 100 μL of the 5 μg/L working solution.

To complete the experimental setup, SCM1 medium and calculated volumes of sweeteners were added to sterile Erlenmeyer flasks to the final volume of 50 mL (Table 8). Then, 1.5 mL of *N. maritimus* from Culture 1.1 was inoculated into each. All steps were carried out under sterile conditions in the laminar flow hood.

The Erlenmeyer flasks were sealed with paper stoppers and covered also with aluminum foil to avoid possible contaminations during incubation. The flasks were incubated at 28 °C, and nitrite concentration was measured every 2 – 3 days as previously described. Also, standards were measured at each time point to ensure data accuracy.

3.5. Sucralose Screening

Based on the results obtained from the initial growth monitoring, which showed that *N. maritimus* growth was inhibited almost completely at the SUC concentration of 3000 ng/L, a follow-up screening was conducted to determine the specific concentration (between 1000 ng/L and 3000 ng/L) at which growth inhibition occurred. This range was selected because 1000 ng/L corresponds to the highest concentration condition in the initial experimental setup, while 3000 ng/L was the environmentally relevant concentration used as the medium-level condition. Since growth was observed at 1000 ng/L, but was inhibited at 3000 ng/L, it was assumed that the inhibition must occur somewhere between these two concentrations.

For this screening, previously autoclaved Erlenmeyer flasks were prepared with SUC concentrations of 1000 ng/L, 1500 ng/L, 2000 ng/L, 2500 ng/L, and 3000 ng/L, each in triplicate. Additionally, three control flasks without AS were included, resulting in a total of 18 cultures. The dilutions were made with the previously prepared 500 µg/L sterile stock solution of SUC which was sterilized via 0.2 µm syringe filter and stored in the fridge at 4 ºC. The required volumes of stock solution were calculated using the standard dilution formula ($Ci * Vi = Cf\ Vf$). For example, to reach 1000 ng/L in a 50 mL flask, an appropriate volume of 0.1 mL of SUC [500 µg/L] was added to the culture medium.

To simplify pipetting, 1 L of SCM1 medium was prepared and inoculated with 30 mL of *N. maritimus* from the control culture of the previous experiment. This ensured that each 50 mL flask received the equivalent concentration of *N. maritimus* as if 1.5 mL had been added individually, consistent with the inoculation volume used in all previous growth monitoring experiments. Then, the calculated AS volumes were added to each flask (Table 9).

Samples were taken from each flask at the beginning and end of the experiment in Eppendorf tubes for ammonium and cell count determinations. Glutaraldehyde (GDA) 25% was added for cell fixation in the cell counting samples, and the culture samples for the ammonium measurement were filtered through 0.1 µm syringe filters. All samples were stored in the fridge at 4 °C.

Growth monitoring was done every 2 – 3 days, just as in the previous experiments. However, results were inconsistent: the control samples showed no growth, while 3000 ng/L cultures showed excessive growth. This was most likely due to labeling errors during sample preparation.

Therefore, the screening experiment was repeated using Culture 1.2. New ammonium and cell count samples were collected and stored under the same conditions for further analysis. The Erlenmeyer flasks were autoclaved and properly cleaned again to avoid contaminations, and the process was done the same way as before, preparing triplicates of each concentration for more accurate results.

Statistical analyses were performed in R (version 4.5.1; R Core Team, 2025) [28] using the packages *lme4 [29], lmerTest [30], emmeans [31], ggplot2* [32] *and tidyverse [33]*. First, nitrite concentrations from triplicates were summarized as mean ± standard deviation (SD) to provide an overview and for graphical representation. Then, to test the effect of AS concentration and incubation time, a linear mixed-effects model (LMM) was applied, with concentration and time as fixed factors and replicate as a random factor. The results of this model were reported as F and p values based on a Type III ANOVA table with Satterthwaite's approximation for degrees of freedom. Finally, to further evaluate potential differences between treatments, post-hoc pairwise comparisons between treatments were conducted using estimated marginal means (*emmeans*) with Holm adjustment for multiple testing.

3.6. Ammonia Quantification

Ammonia measurements were made at the beginning (T_0) and at the end (T_f) of the project to monitor the metabolic activity of *N. maritimus*, which oxidizes ammonia to nitrite as its energy source. These determinations provide insight into the nitrification process of the cultures and complements the nitrite measurements to confirm active ammonia consumption.

For this measurement the Salicylate nitroprussidine method was used. First, 100 mL of the salicylate reagent was made in the lab and stored in a bottle, sodium salicylate, (tri)sodium citrate, disodium tartrate dihydrate and sodium nitroprusside were the reagents used for the elaboration of this solution. Then 100 mL of the $NaOH$ solution (1.5 M) and 60 mL of the hypochlorite reagent were made according to the lab protocol.

The ammonia measurement was done with the samples taken from the SUC screening at the beginning and at the end of the incubation period. These samples were previously filtered through 0.1 µm syringe filters to remove *N. maritimus* cells and stored in the fridge at 4 ºC. This filtration setup ensured that only the ammonia present in the culture medium was measured, excluding any potential interference from cellular material.

A standard curve was prepared for ammonia quantification, following the same procedure used for nitrite determinations. A stock solution of ammonium chloride (NH_4Cl, 55.43 mM) was diluted to obtain 1 mM working solution, which served as the basis for the serial dilutions. The concentrations used for the standard curve were: 0.5 mM, 0.25 mM, 0.1 mM and 0.05 mM (Table 11, Figure 13). The calibration curve equation obtained was: $y = 3.2041x + 0.0255$ (R^2 = 0.99), where y represents the absorbance and x the ammonia concentration in mM.

A total of forty-two tubes were used in this procedure, including thirty-six for the samples taken at the beginning (T_0) and at the end (T_f) of the screening, five for the standard curve and one for the blank which was done with distilled water.

All solutions were mixed in glass tubes; they were vortexed and incubated in the dark at room temperature for 45 minutes. The absorbances were read at 650 nm with the UV-Vis spectrophotometer (UV-1280, Shimadzu, Japan) and there was a color development of the samples to different shades of green / blue according to the amount of ammonia in the media.

This colorimetric assay allowed quantification of ammonia in the media by measuring absorbances with the UV-Vis spectrophotometer. Lower ammonia concentrations resulted in more intense coloration and therefore higher absorbance readings.

3.7. Cell Counting

Cell counting was done to track and confirm the growth of *N. maritimus*, but also to check the media for possible contaminations during the screening. Because these cells are extremely small and don't show up well with typical optical density methods, counting the actual number of cells gives us a more accurate picture of their growth.

To preserve the cells for later analysis, samples taken at the beginning (T_0) and end (T_f) of the experiment were fixed using 25% GDA. Specifically, 190 µL of 25% GDA was added to 1 mL of each sample.

The equipment used was a filtration system (filter flask) and an adequate tube for installing a vacuum, a 0.1 µm polycarbonate filter (∅ 25mm), the TAE buffer, and SYBR Green as staining solution. For this procedure, 0.1 mL of sample was mixed with 5 mL of TAE buffer and filtered through the vacuum filtration system using a 0.1 µm polycarbonate filter (∅ 25mm). Prior to use, the filtration setup was rinsed with ethanol and distilled water. After filtering the sample and buffer mixture, the vacuum was released, and the setup was washed with 10 mL of TAE. This washing step was repeated twice, with the vacuum reapplied each time.

Following filtration, the membrane filter was placed on a microscope slide with SYBR Green stain, covered with a cover slip, and observed under the light microscope (Axiolab 5, Zeiss, Germany) equipped with a 100x oil immersion objective and a filter set suitable for SYBR Green. The samples analyzed were $T_{0.1}$- control (one of the three control triplicates at the beginning of the experiment) and the $T_{f.1}$- control (the same sample but after a period of twenty-four days), both without AS in the medium.

Fluorescent cells appeared as bright green spots and were counted manually in ten different fields of view. The average number of cells per field was calculated and used to estimate the total number of cells on the filter for both samples according to the following equation:

$$Total\ cells\ on\ the\ filter = \frac{Total\ cell\ count}{Square\ area} * Effective\ Filter\ area$$

The cell concentration in the culture was then calculated from the total number of cells on the filter using the protocol formula:

$$\frac{Cells}{mL} = (Total\ cells\ on\ the\ filter) * (Dilution\ factor) * \frac{Culture\ volume}{Volume\ added}$$

All counts and calculations were performed in triplicates whenever possible, ensuring reliability and reproducibility of the obtained cell concentration values.

3.8. Additional Methodological Information

The ChatGPT language model (OpenAI, May 2025 version) was employed to enhance the linguistic clarity and coherence of the text and to provide guidance on the use of R for statistical analysis and data visualization. All scientific content and interpretations remain the original work of the author.

4. Results

4.1. Growth of *N. maritimus* with Artificial Sweeteners

To assess the impact of five different ASs (SAC, SUC, ASP, ACE and CYC) on the growth of *N. maritimus*, cultures were set-up in media containing different concentrations of each AS. The cultures prepared were at low (10 ng/L), medium (500 ng/L) and high concentration (1000 ng/L), as well as the environmental concentration of each AS found in a previous study [22].

The growth was monitored indirectly by measuring nitrite accumulation in the media with a colorimetric assay. This assay quantifies nitrite by correlating the intensity of pink coloration with nitrite concentration: the darker the pink, the higher the nitrite level produced by the metabolic activity of *N. maritimus*.

As a first step, the growth of two control cultures without AS (Culture 1.1 and Culture 1.2) was monitored over a period of two weeks to establish a baseline growth pattern under optimal conditions. The calculated nitrite concentrations for these control cultures were subsequently plotted to facilitate visualization and comparison:

Table 1: Concentration of nitrite in Culture 1.1 and Culture 1.2 over a two-week period. The values were calculated with the nitrite calibration curve equation (Figure 12) and the absorbance values of the nitrite determinations (Table 6).

$[NO_2^-]$ mM in Culture 1.1	$[NO_2^-]$ mM in Culture 1.2
0.00185061	0.00185061
0.02203903	0.02288022
0.15746972	0.16588156
0.36355989	0.41318977
0.76648721	0.87415882
1.10548452	1.10043742

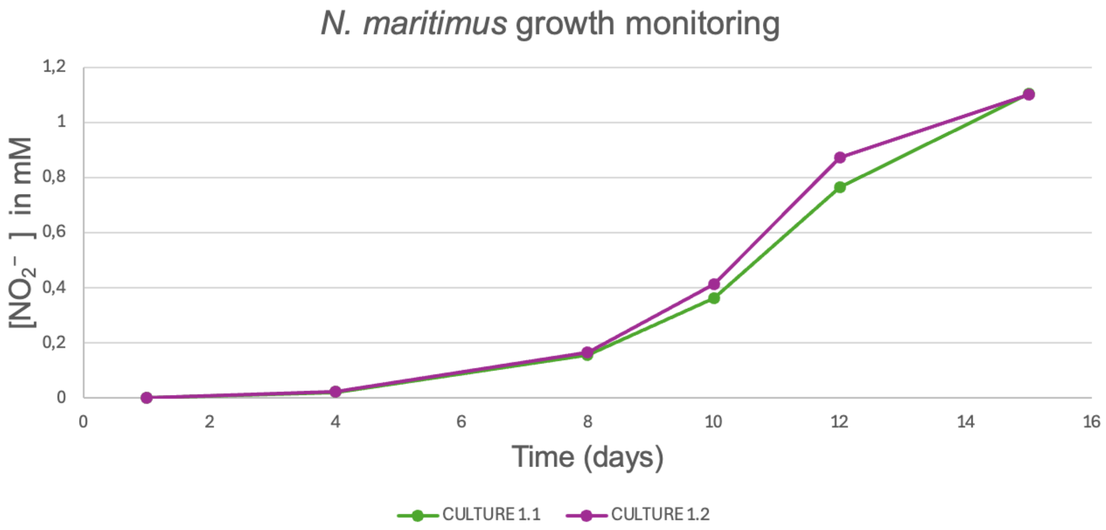

Figure 3: Nitrite concentrations from Culture 1.1 and Culture 1.2 over a two-week period in *N. maritimus* cultures. The figure presents a graphical representation of nitrite concentration values from Table 1. The nitrite accumulation in the culture is used as an indicator of the activity of *N. maritimus*, as it directly correlates with its growth. The graph shows exponential increase in nitrite concentrations, reflecting the exponential growth phase of the cultures in optimal conditions.

As for the determination of the potential effect of ASs in the growth of *N. maritimus*, growth was monitored in 21 Erlenmeyer flasks, each with a volume of 50 mL. Twenty contained different concentrations of the five ASs, while one flask served as a control culture without sweetener. For this experiment, the Culture 1.1 was used as the active culture to establish all new ones.

Samples were taken over a two-week period of time for nitrite determinations. As in the previous experiment, the absorbance values were used to calculate nitrite concentrations using the calibration curve equation from Figure 12. The results of the nitrite concentrations in each culture can be found in the appendix (Table 12). In addition, a graphical representation was plotted for each AS for a better visualization of the growth of *N. maritimus* over time:

Figure 4: Nitrite concentration values obtained from the control culture during the ten-day growth experiment with *N. maritimus*.

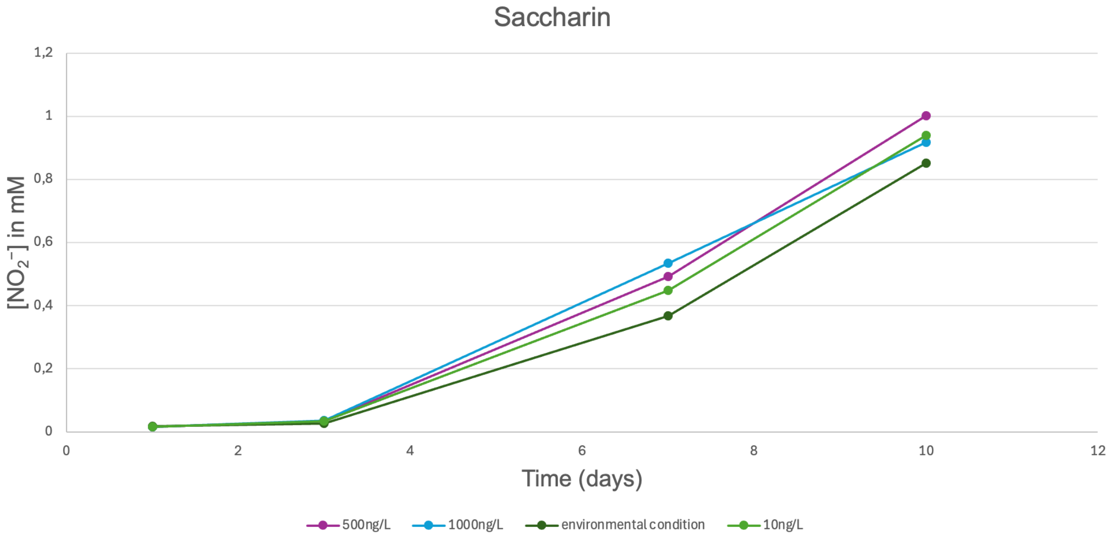

Figure 5: Nitrite concentration values obtained from the cultures with SAC during the ten-day growth experiment with *N. maritimus*.

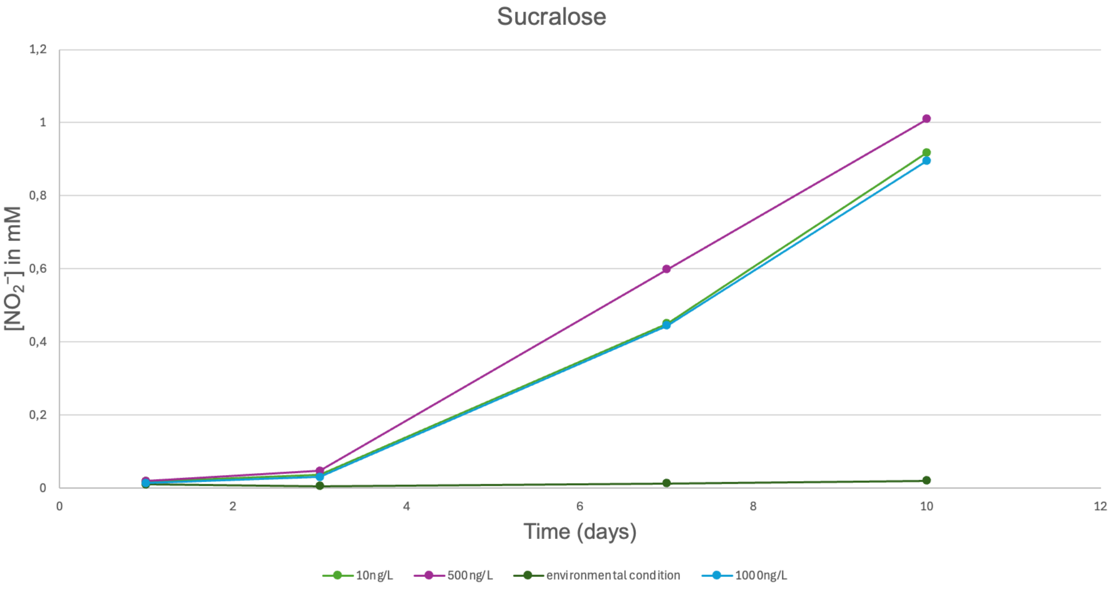

Figure 6: Nitrite concentration values obtained from the cultures with SUC during the ten-day growth experiment with *N. maritimus*. Cultures containing concentrations of 10 ng/L, 500 ng/L and 100 ng/L of SUC, showed nitrite accumulation in the media, indicating active growth of *N. maritimus*. The culture with the medium containing a concentration of 3000 ng/L of SUC showed no significant nitrite accumulation, suggesting an absence of growth under this condition.

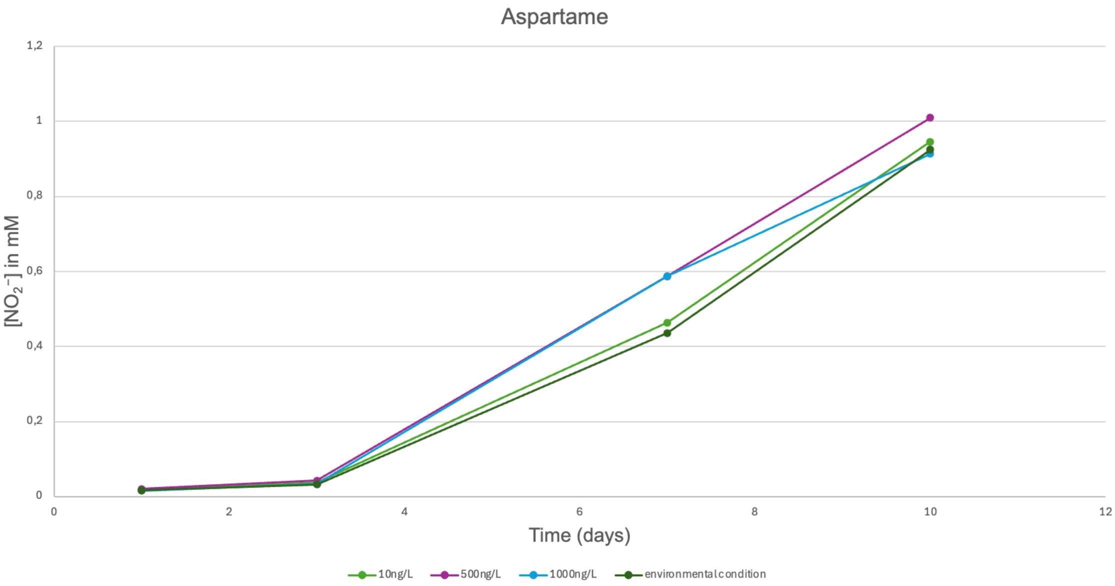

Figure 7: Nitrite concentration values obtained from the cultures with ASP during the ten-day growth experiment with *N. maritimus*.

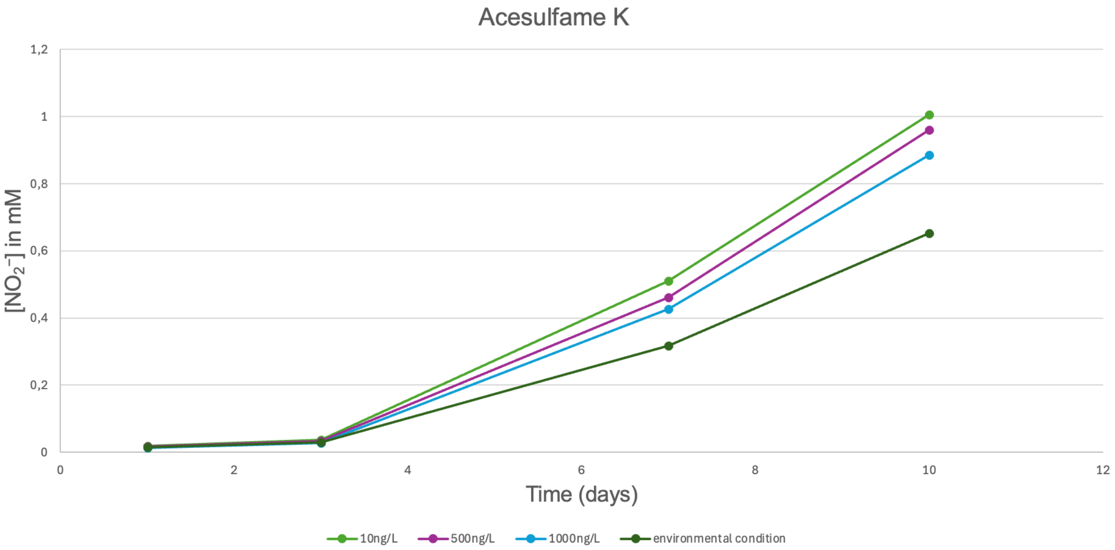

Figure 8: Nitrite concentration values obtained from the cultures with ACE during the ten-day growth experiment with *N. maritimus*.

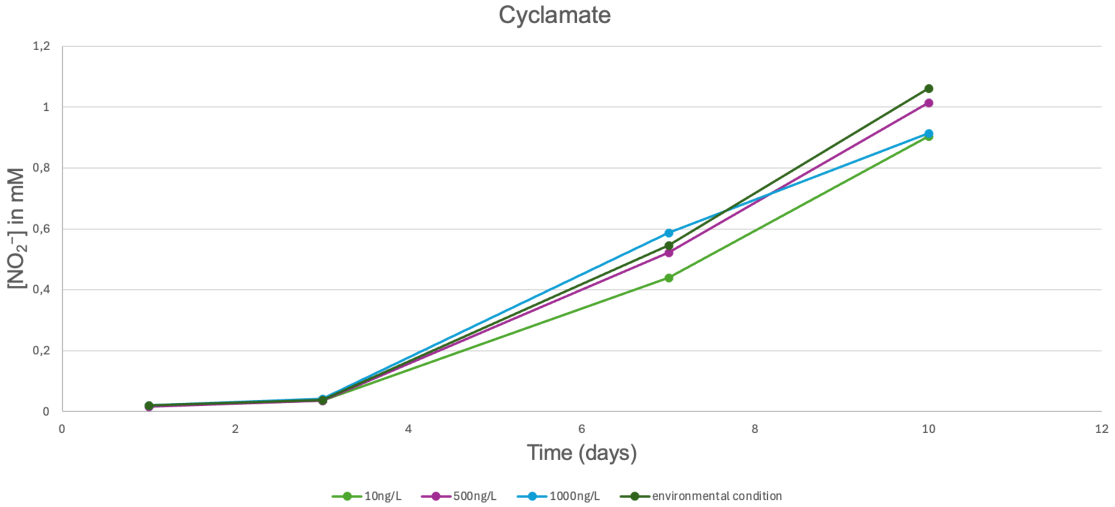

Figure 9: Nitrite concentrations obtained from the cultures with CYC during the ten-day growth experiment with *N. maritimus*.

There was a growth inhibition observed in the culture with the media containing a concentration of 3000 ng/L of SUC (Figure 6), which was the environmental concentration found in the southern North Sea coastal waters [22].

4.2. Sucralose Screening

Following the result obtained in the previous experiment, a screening experiment was conducted to determine the exact concentration at which SUC inhibits the growth of *N. maritimus*.

A total of eighteen Erlenmeyer flasks were prepared, including triplicates for the control condition and for each of the following SUC concentrations: 1000 ng/L, 1500 ng/L, 2000 ng/L, 2500 ng/L and 3000 ng/L. The use of triplicates ensured more accurate and reliable results for the screening.

The SUC screening was repeated twice because of the appearance of inconsistent results. While the control triplicates did not grow, the 3000 ng/L cultures grew excessively:

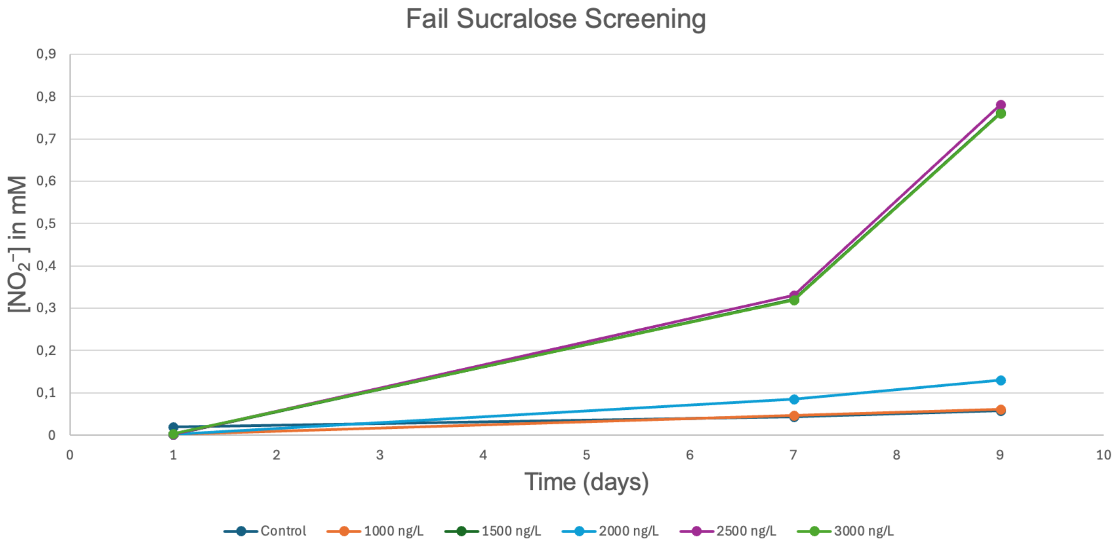

Figure 10: Nitrite concentration data obtained during the first SUC screening experiment. The figure shows nitrite accumulation in *N. maritimus* cultures over a nine-day period. This screening was repeated due to incoherent results: cultures exposed to 1000 ng/L and 2500 ng/L of SUC, as well as the control condition without AS, showed no growth, while cultures containing concentrations of 1500 ng/L, 2000 ng/L and 3000 ng/L of SUC grew exponentially.

This first screening was carried out using the control culture of the previous experiment as the inoculum (former Culture 1.1). For the second screening however, Culture 1.2 was used. This culture had not been stored in the incubator prior to inoculation and was therefore inactive at the time of use. Since reactivating the cells is a long process, the experiment was carried out over an extended period of nearly a month. Absorbance measurements were used to calculate nitrite concentrations using the nitrite calibration curve equation from Figure 12, and the resulting values can be found in the appendix in Table 13.

As the nitrite production in *N. maritimus* cultures was quantified in triplicate, the resulting data were summarized as mean values and SDs. The mean provides a measure of the central tendency of nitrite accumulation for each condition, whereas the SD indicates the variability among replicates, reflecting the reproducibility of the experiment. The calculated results were as follows:

Table 2: Mean nitrite concentrations and corresponding SDs from triplicate cultures in the SUC screening experiment. Values were calculated from the individual measurements reported in Table 13.

Days		**Control (mM)**	**1000 ng/L (mM)**	**1500 ng/L (mM)**	**2000 ng/L (mM)**	**2500 ng/L (mM)**	**3000 ng/L (mM)**
Day 1	Mean	0.00128982	0.00128982	0.00100942	0.00128982	0.00128982	0.00185061
	SD	0.00097132	0.00048566	0.00084118	0.00048566	0.00097132	0.00084118
Day 3	Mean	0.0024114	0.00269179	0.00269179	0.0024114	0.00269179	0.00269179
	SD	0.00048566	0.00084118	0	0.00048566	0	0
Day 8	Mean	0.00381337	0.00409376	0.00353297	0.00381337	0.00381337	0.00437416
	SD	0.00097132	0.00048566	5.3115E-19	0.00048566	0.00048566	0.00145697
Day 10	Mean	0.00437416	0.00381337	0.00409376	0.00437416	0.00437416	0.00437416
	SD	0	0.00097132	0.00097132	0.00084118	0.00084118	0.00084118
Day 15	Mean	0.02792732	0.01194482	0.01587035	0.01026245	0.01026245	0.01054284
	SD	0.01680264	0.00222556	0.00971316	0.00145697	0.00084118	0.00097132
Day 20	Mean	0.21607223	0.22756842	0.22083894	0.21719381	0.22027815	0.22504486
	SD	0.01293164	0.00725242	0.01517244	0.02623902	0.02026422	0.00128493
Day 22	Mean	0.53880664	0.54834006	0.54357335	0.51637506	0.55478914	0.54525572
	SD	0.04419488	0.01583424	0.02039764	0.05403478	0.02963708	0.00656986
Day 24	Mean	0.96584791	0.97622252	0.99164424	0.93388291	0.99500897	0.96865186
	SD	0.07042376	0.02800441	0.01120176	0.03360529	0.00596787	0.00850942

Across all conditions, SD values for each day were low, demonstrating consistent results between the replicates of the second sucralose screening experiment. To visualize the overall growth dynamics of *N. maritimus* with different concentrations of AS, a line graph was generated in R using the package *ggplot2*. The figure shows mean nitrite concentrations for each condition across the 24-day incubation period, with error bars which represent the SD of the triplicates. This provides an overview of the central tendency and the variability among replicates:

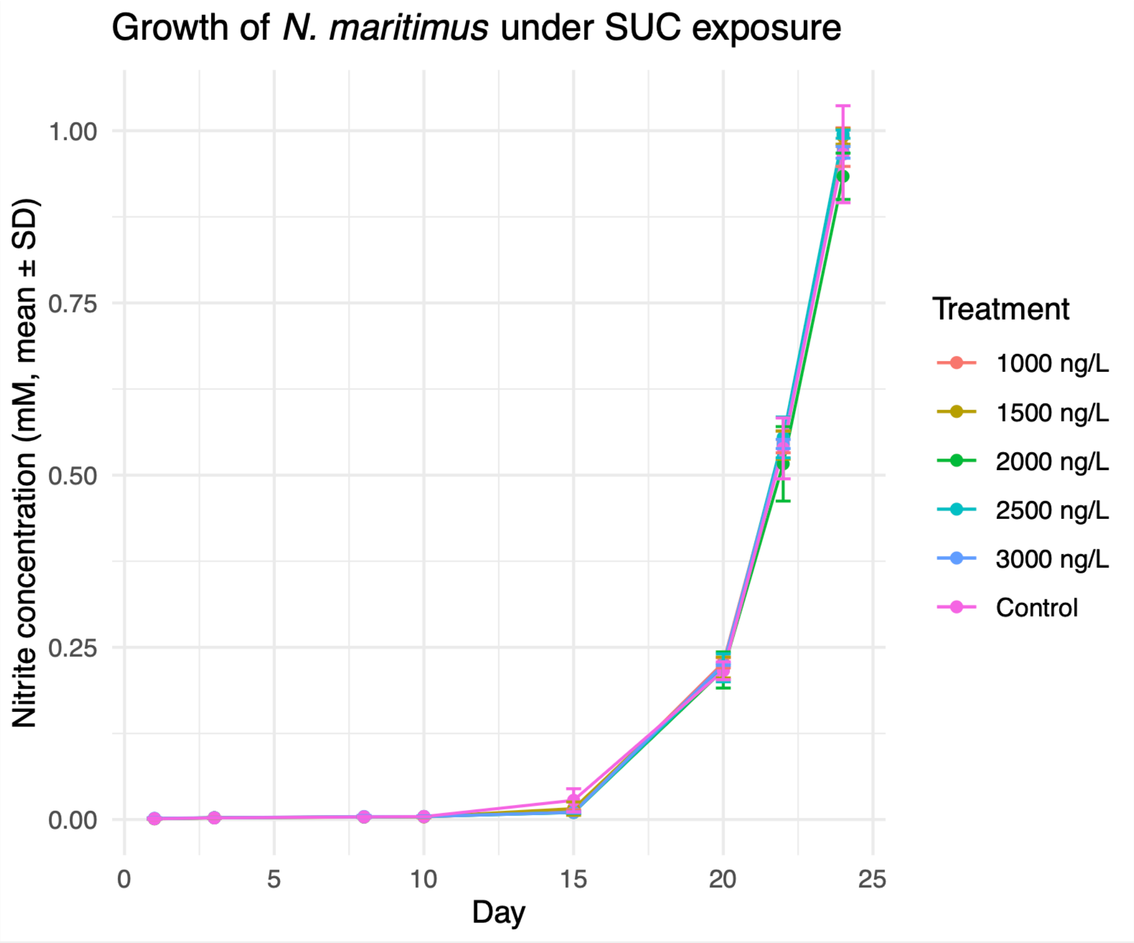

Figure 11: Growth of *N. maritimus* cultures over 24 days under different concentrations of SUC. The data represents mean nitrite concentrations (mM) ± SD of triplicate cultures (n=3). The curves show a significant increase in nitrite accumulation over time (LMM, p<0.001), while no significant differences were detected between concentrations.

To further evaluate potential differences between treatments, data were analyzed using a LMM in R (packages *lme4* and *lmerTest*), with concentration and time as fixed factors and replicates as random factors. The statistical analysis confirmed that nitrite production increased significantly over time, demonstrating that the cultures were actively growing (F(1,132) = 217.4, $p < 0.001$). In contrast, no significant effect of concentration was detected (F(5,132) = 0.003, p = 1.000), and the concentration × time interaction was also not significant (F(5,132) = 0.017, p = 0.999). Post-hoc pairwise comparisons between treatments (Holm-adjusted) further confirmed that none of the concentrations differed significantly from the control.

Table 3: Results of the LMM testing the effects of concentration, time, and their interaction on nitrite production in *N. maritimus*.

Effect	Df (num, den)	F-value	p-value
Concentration	(5, 132)	0.003	1.000
Time (Day)	(1,132)	217.4	<0.001***
Concentration x Day	(5,132)	0.017	0.999

Significance codes: *** p < 0.001

Together, these results demonstrate that the tested concentrations of SUC did not significantly alter nitrite production by *N. maritimus*.

4.3. Ammonia Quantification

Ammonia measurements were conducted at the beginning and at the end of the SUC screening to assess the metabolic activity of *N. maritimus* and to further confirm the proper growth of the archaeon in the medium. The results of the concentration of ammonia in the media were as follows:

Table 4: Ammonia concentrations on day one of the SUC screening experiment. This table shows the initial ammonia concentrations on day one for each experimental condition of the second SUC screening experiment. All conditions were conducted in triplicate to ensure data accuracy and reproducibility.

Sample	Initial $[NH_4^+]$ (mM)		
Control	0.78633626	0.76511345	0.77229175
1000ng/L	0.77541275	0.77073125	0.76917075
1500ng/L	0.77759745	0.77354015	0.76729815
2000ng/L	0.76230455	0.76230455	0.76511345
2500ng/L	0.76136825	0.76511345	0.75949565
3000ng/L	0.77197965	0.76386505	0.76105615

Table 5: Ammonia concentrations on day twenty-four of the SUC screening experiment. The table shows the final ammonia concentrations found in each experimental condition of the *N. maritimus* cultures after twenty-four days of growth.

Sample	Final $[NH_4^+]$ (mM)		
Control	0.06070347	0.10689429	0.19397023
1000ng/L	0.1200025	0.10751849	0.14309791
1500ng/L	0.11157579	0.27917356	0.1346712
2000ng/L	0.08848038	0.16619331	0.34159358
2500ng/L	0.08067788	0.11282419	0.10783059
3000ng/L	0.1387285	0.11407259	0.09409819

4.4. Cell Counting

Cell counting was performed both to confirm the proper growth of *N. maritimus* and to assess the cultures for any possible contaminations during the screening experiment. The samples analyzed were the $T_{0.1}$- control sample and the $T_{f.1}$- control sample (the same sample but after twenty-four days of growth) both without AS.

No visible contamination was observed under the microscope. Cell concentrations were calculated using the formulas described in section 3.7. For example, for the $T_{0.1}$- control sample at the beginning of the screening, the total number of cells on the filter was determined as follows:

$$Total\ cells\ on\ the\ filter = \frac{19{,}2}{96100\ \mu m^2} * 314160000\ \mu m^2 = 62766.618\ cells$$

Using this result, the cell concentration in the culture was then calculated as:

$$(62766.618 \text{ cells on the filter}) * (1) * \frac{50\ mL}{0.1\ mL} = 31383309 \frac{cells}{mL} = 3.14 * 10^7\ \frac{cells}{mL}.$$

Thus, for the $T_{0.1}$- control sample of the sucralose screening, the calculated cell concentration in the culture was $3.14 * 10^7\ \frac{cells}{mL}$. After twenty-four days of growth, the corresponding $T_{f.1}$- control sample showed a concentration of $5.9 * 10^7\ \frac{cells}{mL}$.

5. Discussion

5.1. Artificial Sweeteners as Emerging Micro-Contaminants

ASs are widely consumed in considerable quantities nowadays as sugar substitutes because of their high intensity sweetness [34,35]. Their worldwide production and consumption is on the rise, for example, they have grown by more than 5% in recent years in the United States [36]. In addition, ASs are excreted mostly unchanged from the human body after ingestion, as they are considered metabolically inert [26] and end up in wastewaters. These and other food ingredients have been detected at high concentrations in influents and effluents from wastewater treatment plants, showing low or even negative removal percentages during wastewater treatment [25].

This persistence, along with their widespread presence in aquatic environments, has led to the recognition of ASs as emerging micro-contaminants [27]. Therefore, the main objective of this study is to evaluate the possible effect of the presence of ASs in the marine environment on the growth and metabolism of *N. maritimus*, an AOA that belongs to the MGI *Thaumarchaeota*, one of the most widely distributed and abundant groups of microorganisms that play a significant role in the global nitrogen cycle [37].

5.2. Baseline Growth Pattern of *N. maritimus*

To start working with *N. maritimus* in the laboratory, two initial cultures were prepared to establish a reference for the normal growth pattern. The resulting growth curve (Figure 3) displayed the characteristic phases observed in microbial cultures: lag phase, exponential phase and stationary phase [38]. For optimal growth in the subsequent experiments, *N. maritimus* was inoculated during the exponential phase, as this is when the cells are most metabolically active and dividing at their maximum rate. For successful inoculation of *N. maritimus* in a new culture, the absorbance (at 545 nm) from nitrite determinations should ideally range between 0.7 and 1.0. Values outside this range (either too low or too high) can delay the onset of exponential growth of the archaeon and may reduce its overall viability in the new medium.

N. maritimus is known for its slow growth and high sensitivity to changes in environmental conditions. This has been demonstrated, for instance, by extended lag phases and reduced growth rates following the removal of α-ketoglutarate from the medium. Additionally, compounds like pyruvate and oxalacetate have been shown to enhance growth, indicating a response even to low concentrations of organic molecules [39]. These findings support the hypothesis that the presence of AS in the culture medium may influence its growth or metabolic activity.

More recent studies have classified *N. maritimus* as an “aeonophile”, a type of ultra-slow-growing extremophile that maintains metabolic activity even in energy-limited environments, often without frequent cell division [40]. This also supports the idea that small environmental disturbances, such as chemical stressors or suboptimal growth conditions, may delay or suppress growth, even if metabolic processes like ammonia oxidation continue at low levels. Such organisms may need extended recovery periods following a disruption, and the growth dynamics may also change significantly.

5.3. The Effect of Artificial Sweeteners on *N. maritimus*

In the first experiment conducted in the laboratory, the growth of *N. maritimus* was analyzed in the presence of various concentrations (low, medium, high and environmental) of five different ASs (SAC, SUC, ASP, ACE and CYC).

The initial results showed no significant differences in growth between the different cultures, except for one. The culture exposed to the environmental concentration of SUC (3000 ng/L) exhibited no growth throughout the ten-day duration of the experiment (Figure 6). This growth inhibition can be observed indirectly through the concentration of nitrite contained in the media which was determined through sampling procedures every 2 - 3 days.

N. maritimus plays a key role in the marine nitrogen cycle by catalyzing the first and rate-limiting step of nitrification, the oxidation of ammonia to nitrite [14,41].

Therefore, the accumulation of nitrite in the media is directly linked to its metabolic activity and serves as an indicator of its growth. While all other cultures of *N. maritimus* presented exponential growth after the third day, the culture with the media containing 3000 ng/L of SUC showed no nitrite accumulation and therefore no growth in the ten-day duration of the experiment.

If the presence of SUC in the medium can inhibit the metabolic activity of *N. maritimus*, this could disrupt nitrification in marine ecosystems by slowing ammonia oxidation, leading to ammonia accumulation and reduced nitrite availability. Such disturbances may affect the overall nitrogen cycle, with downstream impacts on primary production and the ecosystem nutrient balance.

5.4. Sucralose Screening and Inconsistent Results

Following this result, a screening was conducted to identify the exact concentration of SUC where the growth of *N. maritimus* is inhibited. Concentrations ranging from 1000 ng/L to 3000 ng/L were tested, based on previous observations where the archaeon exhibited normal growth at 1000 ng/L but showed no growth at 3000 ng/L of SUC.

The screening was repeated because of the observation of inconsistent results. (Figure 10). In this initial attempt, the control cultures and those containing 1000 ng/L and 2000 ng/L of SUC showed no growth, while cultures with SUC concentrations of 1500 ng/L, 2500 ng/L and 3000 ng/L exhibited exponential growth. These results were inconsistent with the findings of the previous experiment, in which growth was inhibited at 3000 ng/L of SUC. Moreover, the control condition should have shown exponential growth as was observed in all previous cultures without ASs containing in the media. Due to these discrepancies, the screening experiment had to be repeated.

A labelling error during culture preparation is the most likely explanation of the inconsistent results obtained during the first SUC screening experiment, however other potential sources of variability include accidental media contamination or pipetting errors during the experimental setup. Considering the slow growth and high susceptibility of *N. maritimus* strain SCM1 to environmental changes, even minor deviations in experimental setup could have impacted its metabolic activity and growth.

5.5. Second Sucralose Screening Experiment

The second SUC screening was prepared following the same protocol as the first one, however, this time another inoculum (Culture 1.2) was used. This culture had been stored in a drawer at room temperature for a few weeks, since the preparation of the first experiment, and was no longer active at the time of inoculation. *N. maritimus* is a highly sensitive archaeon that requires incubation in SCM1 medium at 28ºC in the dark for optimal growth [14]. Storing this culture outside of these conditions led to the inactivation of the cells. Reactivating *N. maritimus* is a slow process, which is why the growth in this second SUC screening experiment was monitored for almost a month, in contrast to the typical two-week period of growth when an actively growing culture is inoculated into a freshly prepared medium. This also supports the classification of *N. maritimus* as an “aenophile”, a proposed class of extremophile, as was mentioned before [40].

In this second screening attempt (Figure 11), the exact activation point of the cells can clearly be observed around day 15 of growth monitoring and the exponential growth is shown in the graph from day 20 to day 24. No notable difference can be observed between the cultures with different concentrations of SUC and the control triplicates in the experiment. The resulting data were summarized as mean values, representing the central tendency of nitrite accumulation for each condition, and SDs, which reflect the variability among replicates and indicate the reproducibility of the experiment.

To further support these findings, statistical analysis using a LMM in R confirmed that nitrite production increased significantly over time, but no significant differences were detected between concentrations or in the concentration × time interaction. Post-hoc pairwise comparisons (Holm-adjusted) likewise showed no significant contrasts between treatments. These results indicate that the previously observed inhibition at 3000 ng/L SUC was likely an experimental anomaly rather than a reproducible effect of sucralose.

Based on all of this, it can be concluded that the previously observed growth inhibition of the *N. maritimus* culture with the medium containing 3000 ng/L of SUC was likely a false result. This discrepancy may have been caused by medium contamination during culture preparation, pipetting inaccuracies, or errors during sample extraction for growth monitoring. Other possible sources of variation include poor mixing of the AS in the culture medium or even inconsistent incubation conditions.

5.6. Additional Confirmation of Growth

Additional experiments were conducted to confirm the proper growth of *N. maritimus*. Since ammonia is consumed as part of its metabolic activity, ammonia concentrations were measured at both the beginning and the end of the SUC screening experiment. The results of the ammonia measurements (Table 4 and Table 5) with the Salicylate nitroprussidine method show a significant decrease in ammonia concentrations in each of the triplicates with concentrations of SUC and in the control cultures. These results are as expected, *N. maritimus* oxidizes ammonia into nitrite, therefore, while nitrite concentrations increase in the medium, ammonia concentrations decrease as a consequence of their growth and metabolic activity.

Cell counting was also performed to further confirm proper growth of *N. maritimus* and to check the cultures for any potential contaminations during the screening experiment. One of the control triplicates of the screening was used for this analysis, with samples taken at the beginning and at the end of the experiment. For the initial control sample ($T_{0.1}$- control) a total of $3.14 * 10^{7} \frac{cells}{mL}$ was calculated. After a period of 24 days for the same sample ($T_{f.1}$- control) a total of $5.9 * 10^{7} \frac{cells}{mL}$ were observed. The number had increased indicating sustained growth throughout the experiment as expected. Additionally, no signs of contamination were observed during microscopy, confirming that the experiment proceeded successfully and that no external interference affected the growth of the archaeon.

6. Conclusion

The results obtained during this research project indicate that the presence of AS (specifically SAC, SUC, ASP, ACE and CYC) at low, medium and high concentration as well as environmentally relevant concentrations, do not significantly affect the growth or metabolic activity of *Nitrosopumilus maritimus*.

Initial experiments suggested a possible inhibitory effect of SUC at the environmentally relevant concentration of 3000 ng/L. However, subsequent screening experiments and control validations, including ammonia measurements and cell counting procedures, demonstrated consistent growth along all tested conditions (concentrations between 1000 ng/L and 3000 ng/L).

These findings confirm that *N. maritimus* is capable of sustaining normal cell growth and metabolic activity in the presence of AS, which are recognized as emerging micropollutants of the marine environment. Consequently, its vital contribution to the global nitrogen cycle is not likely to be compromised by the persistence of these compounds, even considering their low or even negative removal percentages during wastewater treatment processes.

Nevertheless, the widespread and long-term use of AS, their frequent detection in aquatic ecosystems and the growing evidence of their potential chronic ecotoxicity [27] raise serious concerns for the environmental and public health. Therefore, the development of successful removal processes for AS, along with other micropollutants of the marine environment, is crucial to avoid further damage to the marine ecosystems. More research studies may be required to investigate their long-term impact on marine life.

7. Bibliography

[1] Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB. Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proc Natl Acad Sci U S A. 2005;102(41):14683–8.

https://doi.org/10.1073/pnas.0506625102.

[2] Hutchins DA, Capone DG. The marine nitrogen cycle: new developments and global change. Nat Rev Microbiol. 2022;20:401–14.

https://doi.org/10.1038/s41579-022-00687-z.

[3] Gruber N. The Marine Nitrogen Cycle: Overview and Challenges. In: Nitrogen in the Marine Environment. 2nd ed. Academic Press; 2008. p. 1–50.

[4] Webb P. Introduction to Oceanography. Roger Williams University; 2019. Available from: https://rwu.pressbooks.pub/webboceanography/.

[5] Falkowski PG. Evolution of the nitrogen cycle and its influence on the biological sequestration of CO2 in the ocean. Nature 1997;387:272–5.

https://doi.org/10.1038/387272a0.

[6] Falkowski PG, Raven JA. Aquatic photosynthesis. Malden, MA: Blackwell Science; 1997.

[7] Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, et al. Nitrogen cycles: past, present, and future. Biogeochemistry. 2004;70:153–226.

https://doi.org/10.1007/s10533-004-0370-0.

[8] Gruber N, Sarmiento JL. Global patterns of marine nitrogen fixation and denitrification. Global Biogeochem Cycles. 1997;11:235–66.

https://doi.org/10.1029/97GB00077.

[9] Wright CL, Lehtovirta-Morley LE. Nitrification and beyond: metabolic versatility of ammonia oxidising archaea. ISME Journal 2023;17:1358–68.

https://doi.org/10.1038/s41396-023-01467-0.

[10] Fuhrman JA, McCallum K, Davis AA. Novel major archaebacterial group from marine plankton. Nature. 1992;356:148–9.

https://doi.org/10.1038/356148a0.

[11] Karner MB, DeLong EF, Karl DM. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature 2001;409:507–10.

https://doi.org/10.1038/35054057.

[12] Walker CB, De La Torre JR, Klotz MG, Urakawa H, Pinel N, Arp DJ, et al. Nitrosopumilus maritimus genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. Proc Natl Acad Sci U S A 2010;107:8818–23.

https://doi.org/10.1073/pnas.0913533107.

[13] Wuchter C, Abbas B, Coolen MJL, Herfort L, Van Bleijswijk J, Strous M, et al. Archaeal nitrification in the ocean. Proc Natl Acad Sci U S A. 2006;103(33):12317–22.

https://doi.org/10.1073/pnas.0600756103.

[14] Könneke M, Bernhard AE, De La Torre JR, Walker CB, Waterbury JB, Stahl DA. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 2005;437:543–6.

https://doi.org/10.1038/nature03911.

[15] Leininger S, Urich T, Schloter M, Schwark L, Qi J, Nicol GW, et al. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 2006;442:806–9.

https://doi.org/10.1038/nature04983.

[16] Könneke M, Schubert DM, Brown PC, Hügler M, Standfest S, Schwander T, et al. Ammonia-oxidizing archaea use the most energy-efficient aerobic pathway for CO2 fixation. Proc Natl Acad Sci U S A 2014;111:8239–44.

https://doi.org/10.1073/pnas.1402028111.

[17] Kraft B, Canfield DE. Microbe Profile: Nitrosopumilus maritimus. Microbiology (United Kingdom) 2022;168. https://doi.org/10.1099/mic.0.001207.

[18] Schouten S, Hopmans EC, Baas M, Boumann H, Standfest S, Könneke M, et al. Intact membrane lipids of "Candidatus Nitrosopumilus maritimus," a cultivated representative of the cosmopolitan mesophilic group I crenarchaeota. Appl Environ Microbiol 2008;74:2433–40.

https://doi.org/10.1128/AEM.01709-07.

[19] Spang A, Hatzenpichler R, Brochier-Armanet C, Rattei T, Tischler P, Spieck E, et al. Distinct gene set in two different lineages of ammonia-oxidizing archaea supports the phylum Thaumarchaeota. Trends Microbiol 2010;18:331–40.

https://doi.org/10.1016/j.tim.2010.06.003.

[20] Martens-Habbena W, Berube PM, Urakawa H, De La Torre JR, Stahl DA. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature 2009;461:976–9.

https://doi.org/10.1038/nature08465.

[21] Chapman RD, Heidemann M, Albert TK, Mailhammer R, Flatley A, Meisterernst M, et al. Transcribing RNA polymerase II is phosphorylated at CTD residue serine-7. Science. 2007;318:1780–2.

https://doi.org/10.1126/science.1145977.

[22] Adenaya A, Quintero RR, Brinkhoff T, Lara-Martín PA, Wurl O, Ribas-Ribas M. Vertical distribution and risk assessment of pharmaceuticals and other micropollutants in southern North Sea coastal waters. Mar Pollut Bull 2024;200.

https://doi.org/10.1016/j.marpolbul.2024.116099.

[23] Ruiz-Ojeda FJ, Plaza-Díaz J, Sáez-Lara MJ, Gil A. Effects of Sweeteners on the Gut Microbiota: A Review of Experimental Studies and Clinical Trials. Advances in Nutrition, vol. 10, Oxford University Press; 2019, p. S31–48.

https://doi.org/10.1093/advances/nmy037.

[24] European Commission. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of

analytical methods and the interpretation of results. Off J Eur Communities. 2002;L221:8–36.

Available from:

https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32002D0657.

[25] Baena-Nogueras RM, Traverso-Soto JM, Biel-Maeso M, Villar-Navarro E, Lara-Martín PA. Sources and trends of artificial sweeteners in coastal waters in the bay of Cadiz (NE Atlantic). Mar Pollut Bull 2018;135:607–16.

https://doi.org/10.1016/j.marpolbul.2018.07.069.

[26] Roberts A, Renwick AG, Sims J, Snodin DJ. Sucralose Metabolism and Pharmacokinetics in Man. Food Chem Toxicol 2000;38 Suppl 2:S31–41.

https://doi.org/10.1016/s0278-6915(00)00026-0.

[27] Pang L, Borthwick AGL, Chatzisymeon E. Determination, occurrence, and treatment of saccharin in water: A review. J Clean Prod 2020;270.

https://doi.org/10.1016/j.jclepro.2020.122337.

[28] R Core Team. R: A Language and Environment for Statistical Computing 2025. Vienna, Austria: R Foundation for Statistical Computing; 2025.

Available from: https://www.R-project.org/

[29] Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw 2015;67:1–48.

https://doi.org/10.18637/jss.v067.i01.

[30] Kuznetsova A, Brockhoff PB, Christensen RHB. lmerTest Package: Tests in Linear Mixed Effects Models. J Stat Softw 2017;82:1–26.

https://doi.org/10.18637/jss.v082.i13.

[31] Searle SR, Speed FM, Milliken GA. Population marginal means in the linear model: An alternative to least squares means. American Statistician 1980;34:216–21.

https://doi.org/10.1080/00031305.1980.10483031.

[32] Wickham H. ggplot2: Elegant Graphics for Data Analysis. 2nd ed. Springer-Verlag New York; 2016.

https://doi.org/10.1007/978-3-319-24277-4.

[33] Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, et al. Welcome to the tidyverse. J Open Source Softw 2019;4:1686.

https://doi.org/10.21105/joss.01686.

[34] Kroger M, Meister K, Kava R. Low-calorie sweeteners and other sugar substitutes: A review of the safety issues. Compr Rev Food Sci Food Saf 2006;5:35–47.

https://doi.org/10.1111/j.1541-4337.2006.tb00081.x.

[35] Subedi B, Kannan K. Fate of artificial sweeteners in wastewater treatment plants in New York State, U.S.A. Environ Sci Technol 2014;48:13668–74. https://doi.org/10.1021/es504769c.

[36] Sylvetsky AC, Rother KI. Trends in the consumption of low-calorie sweeteners. Physiol Behav 2016;164:446–50.

https://doi.org/10.1016/j.physbeh.2016.03.030.

[37] Könneke M, Lipp JS, Hinrichs KU. Carbon isotope fractionation by the marine ammonia-oxidizing archaeon Nitrosopumilus maritimus. Org Geochem 2012;48:21–4.

https://doi.org/10.1016/j.orggeochem.2012.04.007.

[38] Prescott LM, Harley JP, Klein DA. Microbiology. 5th Edition. Boston: McGraw-Hill; 2002.

[39] Qin W, Heal KR, Ramdasi R, Kobelt JN, Martens-Habbena W, Bertagnolli AD, et al. Nitrosopumilus maritimus gen. nov., sp. nov., Nitrosopumilus cobalaminigenes sp. nov., Nitrosopumilus oxyclinae sp. nov., and Nitrosopumilus ureiphilus sp. nov., four marine ammonia-oxidizing archaea of the phylum Thaumarchaeota. Int J Syst Evol Microbiol 2017;67:5067–79.

https://doi.org/10.1099/ijsem.0.002416.

[40] Lloyd KG, Steen AD. Defining ultra-slow-growing extremophilic microorganisms as aeonophiles. Nat Microbiol 2025.

https://doi.org/10.1038/s41564-025-02048-x.

[41] Martens-Habbena W, Berube PM, Urakawa H, De La Torre JR, Stahl DA. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature 2009;461:976–9.

https://doi.org/10.1038/nature08465.

8. Appendix

8.1. Nitrite Determinations Culture 1.1 and Culture 1.2.

Table 6: Absorbance values at 545 nm for Culture 1.1 and Culture 1.2. Results from the nitrite measurements of the *N. maritimus* cultures without AS in the medium over a two-week period. They reflect nitrite accumulation and were used to establish a baseline growth pattern under optimal culture conditions.

	Culture 1.1	Culture 1.2
Day 1 (31.03)	0.043	0.043
Day 2 (03.04)	0.067	0.068
Day 3 (07.04)	0.228	0.238
Day 4 (09.04)	0.473	0.532
Day 5 (11.04)	0.952	1.08
Day 6 (14.04)	1.355	1.349

8.2. Artificial Sweeteners in *N. maritimus* Growth Experiments

Table 7: Concentrations of ASs in each culture in the growth experiments with *N. maritimus*. The table presents five different ASs which were used for the growth experiment with *N. maritimus* at low, medium, high and environmental concentrations. The environmental concentration of each AS was determined in a previous study [22].

	Saccharin [ng/L]	Sucralose [ng/L]	Aspartame [ng/L]	Acesulfame K [ng/L]	Cyclamate [ng/L]
Low conc.	10	10	10	10	10
Medium conc.	500	500	500	500	500
High conc.	1000	1000	1000	1000	1000
Environmental conc.	50	3000	1,3	30	200

Table 8: Volumes of ASs added into each experimental condition for *N. maritimus* growth monitoring. A total of 21 Erlenmeyer flaks with a final volume of 50 mL were used for the growth experiment (20 with varying concentrations of ASs and one control flask without AS, used as growth reference).

	Saccharin (µL)	Sucralose (µL)	Aspartame (µL)	Acesulfame K (µL)	Cyclamate (µL)	Control (µL)
10 ng/L	100	100	100	100	100	-
500 ng/L	5000	5000	5000	5000	5000	-
1000 ng/L	10000	10000	10000	10000	10000	-
Environmental condition	500	30000	13	300	2000	-

8.3. Sucralose Volumes in the Screening Experiment

Table 9: Volumes of SUC [500 ng/L] and SCM1 media with *N. maritimus* added in each Erlenmeyer flask. The table presents the volumes of SUC and SCM1 medium (pre-inoculated with *N. maritimus*) added to each experimental condition in the SUC screening experiment. A total of 18 Erlenmeyer flasks were used: 15 flasks with varying concentrations of SUC and 3 control flasks without AS.

	1000 ng/L	1500 ng/L	2000 ng/L	2500 ng/L	3000 ng/L
Sucralose [500 ng/L] (mL)	0.1	0.15	0.2	0.25	0.3
SCM1 + *N. maritimus* (mL)	49.9	49.85	49.8	49.75	49.7

8.4. Calibration Curves

8.4.1. Nitrite Calibration Curve

Table 10: Absorbance values obtained at 545 nm from the nitrite standard solutions. Five different nitrite standard solutions were prepared with concentrations ranging from 0.05 mM to 1 mM. The table presents the corresponding absorbance values measured for each standard.

[ ] in mM	Average ABS
0.05	0.073
0.1	0.132
0.25	0.36
0.5	0.70366667
1	1.194

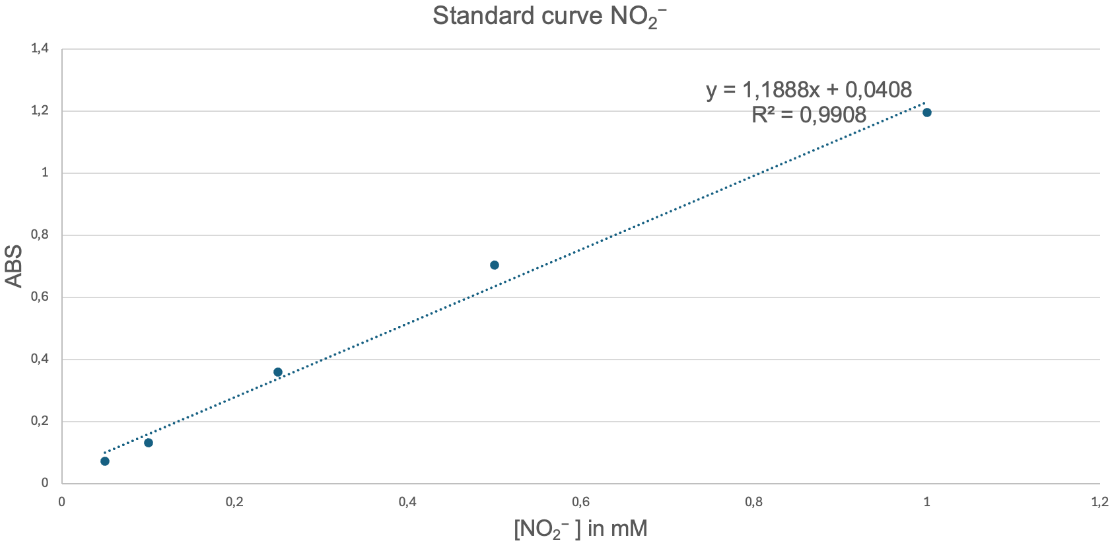

Figure 12: Standard curve for nitrite quantification based on absorbance values of the standard solutions at 545nm. This figure shows the standard curve generated from the data in Table 10, with nitrite concentrations (x-axis) plotted against absorbance (y-axis). The resulting linear equation ($y = 1.1888x + 0.0408$) allows the calculation of the nitrite concentration in subsequent experimental samples.

8.4.2. Ammonia Calibration Curve

Table 11: Absorbance values obtained at 650 nm from the ammonia standard solutions. Four different standard solutions were prepared with concentrations ranging from 0.05 mM to 0.5 mM. The table shows the corresponding absorbance values measured for each standard solution.

$[NH_4^+]$ in mM	**ABS 650 nm**
0	0
0.05	0.18
0.1	0.36
0.25	0.865
0.5	1.606

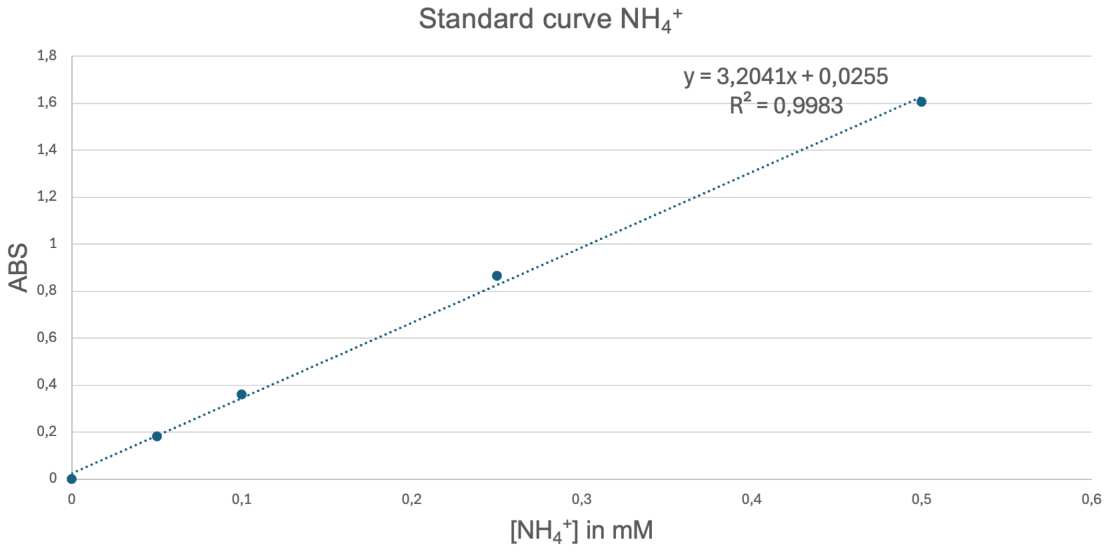

Figure 13: Standard curve for ammonia quantification based on absorbance values of the standard solutions at 650 nm. This figure shows the standard curve generated from the data in Table 11, with ammonia concentrations (x-axis) plotted against absorbance (y-axis). The resulting linear equation ($y = 3.2041x + 0.0255$) allows the calculation of the ammonia concentration of the samples from the screening experiment.

8.5. Nitrite Concentrations During the First Growth Experiment

Table 12: Nitrite concentrations (mM) during the ten-day duration of the growth experiment with *N. maritimus*. The cultures had different concentrations five ASs: SAC, SUC, ASP, ACE, CYC. A control culture without AS was also included as a growth reference for the other experimental conditions.

Control		
	Day 1	0.01362719
	Day 3	0.02708614
	Day 7	0.3845895
	Day 10	0.89518843

		10ng/L	**500ng/L**	**1000ng/L**	**Environmental condition**
SAC	Day 1	0.01615074	0.01615074	0.01615074	0.01699192
	Day 3	0.0346568	0.03381561	0.03549798	0.02708614
	Day 7	0.44851952	0.4922611	0.53432032	0.36776581
	Day 10	0.9397712	1.00201884	0.91705922	0.85144684

		10ng/L	**500ng/L**	**1000ng/L**	**Environmental condition**
SUC	Day 1	0.01783311	0.01867429	0.01362719	0.00942127
	Day 3	0.03549798	0.04643338	0.02960969	0.00521534
	Day 7	0.45020188	0.59825034	0.44431359	0.01194482
	Day 10	0.9179004	1.0095895	0.89602961	0.01951548

		10ng/L	**500ng/L**	**1000ng/L**	**Environmental condition**
ASP	Day 1	0.01783311	0.02035666	0.01615074	0.01699192
	Day 3	0.03718035	0.04222746	0.03297443	0.03213324
	Day 7	0.46366083	0.58731494	0.58731494	0.43590175
	Day 10	0.94565949	1.0095895	0.91369448	0.92462988

		10ng/L	**500ng/L**	**1000ng/L**	**Environmental condition**
ACE	Day 1	0.01951548	0.01615074	0.01362719	0.01446837
	Day 3	0.03633917	0.03297443	0.02792732	0.02876851
	Day 7	0.50992598	0.46197847	0.42748991	0.31729475
	Day 10	1.00538358	0.96080081	0.8859354	0.65208614

		10ng/L	**500ng/L**	**1000ng/L**	**Environmental condition**
CYC	Day 1	0.01867429	0.01615074	0.02035666	0.02035666
	Day 3	0.03633917	0.03549798	0.04054509	0.03718035
	Day 7	0.44010767	0.52170256	0.58815612	0.5460969
	Day 10	0.90528264	1.01547779	0.91453567	1.06258412

8.6. Nitrite Concentrations in the Second Sucralose Screening

Table 13: Nitrite concentrations obtained during the second SUC screening experiment. The table presents the nitrite concentration in each Erlenmeyer flask measured in the second SUC screening experiment. Six different experimental conditions were analyzed (control, 1000 ng/L, 1500 ng/L, 2000 ng/L, 2500 ng/L and 3000 ng/L) over a period of twenty-four days. Each condition was performed in triplicate, resulting in a total of 18 Erlenmeyer flasks (50 mL each) to ensure reproducibility and improve data reliability.

Days	Control (mM)	1000ng/L (mM)	1500ng/L (mM)	2000ng/L (mM)	2500ng/L (mM)	3000ng/L (mM)
Day 1	0.00016824	0.00185061	0.00100942	0.00100942	0.00016824	0.00269179
	0.00185061	0.00100942	0.00185061	0.00185061	0.00185061	0.00185061
	0.00185061	0.00100942	0.00016824	0.00100942	0.00185061	0.00100942
Day 3	0.00269179	0.00353297	0.00269179	0.00185061	0.00269179	0.00269179
	0.00185061	0.00269179	0.00269179	0.00269179	0.00269179	0.00269179
	0.00269179	0.00185061	0.00269179	0.00269179	0.00269179	0.00269179
Day 8	0.00437416	0.00437416	0.00353297	0.00437416	0.00437416	0.00605653
	0.00269179	0.00353297	0.00353297	0.00353297	0.00353297	0.00353297
	0.00437416	0.00437416	0.00353297	0.00353297	0.00353297	0.00353297
Day 10	0.00437416	0.00269179	0.00353297	0.00353297	0.00353297	0.00353297
	0.00437416	0.00437416	0.00353297	0.00437416	0.00437416	0.00437416
	0.00437416	0.00437416	0.00521534	0.00521534	0.00521534	0.00521534
Day 15	0.0237214	0.01110363	0.01026245	0.01110363	0.01026245	0.01110363
	0.01362719	0.01026245	0.01026245	0.01110363	0.00942127	0.01110363
	0.04643338	0.01446837	0.02708614	0.00858008	0.01110363	0.00942127
Day 20	0.22224092	0.2340175	0.20625841	0.23317631	0.22981157	0.22392328
	0.22476447	0.22897039	0.23654105	0.23149394	0.19700538	0.22644684
	0.20121131	0.21971736	0.21971736	0.18691117	0.2340175	0.22476447
Day 22	0.5789031	0.55871467	0.52506729	0.55366756	0.5789031	0.54189098
	0.5460969	0.55619112	0.56544415	0.5410498	0.52170256	0.5410498
	0.49141992	0.5301144	0.54020861	0.45440781	0.56376178	0.55282638
Day 24	1.01884253	0.96921265	1.0045424	0.97089502	0.99613055	0.96332436
	0.99276581	1.00706595	0.98603634	0.92547106	0.98855989	0.96416555
	0.8859354	0.95238896	0.98435397	0.90528264	1.00033647	0.97846568