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Pangenome-Scale Mathematical Modelling of ANAMMOX Bacteria Metabolism

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Abstract: Removal of fixed nitrogen compounds such as ammonium and nitrite from wastewater is of critical importance for balancing the nitrogen cycle and protecting aquatic environments from eutrophication. ANaerobic AMMonium OXidising (ANAMMOX) bacteria have recently been employed for fixed nitrogen removal purposes in wastewater treatment processes. These specialised bacteria convert ammonium and nitrite into nitrogen gas anaerobically, thereby reducing the amount of energy required for aeration in conventional wastewater treatment processes. However, slow growth rates of ANAMMOX remain a major obstacle towards their widespread use in industrial wastewater treatment processes. Thus, a pangenome-scale, constraint-based metabolic model, *i*RB399, of ANAMMOX bacteria has been developed to design strategies for accelerating their growth. The main metabolic limitation was identified in the energy metabolism of these bacteria, concerning the production of ATP. The extremely low efficiency of the electron transport chain combined with very high growth-associated maintenance energy is likely to be responsible for the slow growth of ANAMMOX. However, different ANAMMOX species were found to conserve energy using a variety of different redox couples, and the modelling simulations revealed their comparative advantages under different growth conditions. *i*RB399 also identified dispensable catabolic reactions that have demonstrably beneficial effects on enhancing the growth rates of ANAMMOX bacteria. Thus, the pangenome-scale model will not only help identify and overcome metabolic limitations of ANNAMOX bacteria, but also provide a valuable resource for designing efficient ANNAMOX-based wastewater treatment processes.

Keywords: ANAMMOX; constraint-based metabolic model; eutrophication; nitrogen cycle; pangenome; wastewater treatment processes

Citation: Bielski, R.G.; Islam, M.A. Pangenome-Scale Mathematical Modelling of ANAMMOX Bacteria Metabolism. *SynBio* **2024**, *2*, 70–84. https://doi.org/10.3390/synbio2010005

Academic Editor: Yasuo Yoshikuni

Received: 30 November 2023
Revised: 28 January 2024
Accepted: 2 February 2024
Published: 8 February 2024

1. Introduction

The oxidation of fixed nitrogen compounds to inert nitrogen gas is a key process in nature and in the wastewater treatment industry. The last century has seen a dramatic change in the anthropomorphic effects on the nitrogen cycle. Before the invention of the Haber-Bosch process, humans had a relatively small impact on the nitrogen cycle. The largest sources of fixed nitrogen compounds were mineral deposits of nitre and guano [1]. Deposits of these compounds were a scarce resource, and the only alternative was a single chemical plant using the Birkeland–Eyde nitrate production process [2]. Today, the situation has completely changed, and humans produce roughly half of the fixed nitrogen compounds found on earth [3]. Now, the human needs for fixed nitrogen compounds, one of the major sources of nutrients and constituents of cellular DNA and amino acids, are almost entirely met by the Haber–Bosch process, as it contributes to 80% of the nitrogen present in human tissue samples [4]. This fixed nitrogen mainly originates from their use as agricultural fertilisers. However, a large portion of these artificial fertilisers is not absorbed by plants and ends up in rivers and ponds via agricultural run-off, causing eutrophication, aquatic habitat destruction, and nutrient pollution problems. Thus, ANaerobic AMMonium

OXidising (ANAMMOX) bacteria are very important for mitigating nutrient or fixed nitrogen pollution since they are responsible for oxidising 50% of all fixed nitrogen compounds in the nitrogen cycle [5].

ANAMMOX bacteria were discovered in 1995 and gradually implemented into wastewater treatment processes; now, over 114 full-scale ANAMMOX bacteria employing wastewater treatment plants exist in the world [6]. An increasing number of species from active plants and from the natural environment have been identified and described, and their genomes have been sequenced [7–9], providing an opportunity to reconstruct their metabolic networks to develop genome-scale metabolic models (GEMs). While their central carbon metabolism has been explained and elucidated in reasonable detail [10], many other aspects of their metabolism remain largely unexplored. These strictly anaerobic bacteria have been observed to consume a wide variety of inorganic nitrogen compounds and organic substrates. The finer details and mechanistic insight of how these substrates affect the overall ANAMMOX metabolism have far-reaching implications due to their applications in industrial wastewater treatment processes and wider role in the nitrogen cycle.

ANAMMOX bacteria have been described as generalists, not specialists [11], and their growth on a variety of different organic substrates have been investigated [10,12,13]. The physiological potential of ANAMMOX bacteria has previously been revealed by studying their core genome only [14]. However, exclusively core genome-based analysis cannot describe the full potential of ANAMMOX metabolism due to the lack of not considering the genes in dispensable and unique genomes. Also, the specialised traits that are present or missing in different ANAMMOX species can be identified by examining their dispensable and unique genomes [15]. Thus, a pangenome-scale analysis would be beneficial since it considers the core, unique and dispensable genomes of an organism together [16]. Previous studies on other organisms have successfully shown the predictive power of pangenome-scale modelling analysis as well [17]. Such analysis not only helps organise large amounts of genomic data but also enables a coherent, consistent, and mechanistic understanding of an organism's metabolic characteristics.

The slow growth rate of ANAMMOX bacteria remains a major obstacle to their implementation in wastewater treatment processes. Their doubling times generally range from 1.8 to 14 days [7,8,18,19], leading to longer plant start-up times and increased risk of plant downtime if ANAMMOX sludge is lost. It has been reported that ANAMMOX bacteria can use a wide variety of substrates for respiration, yet prior work on their pangenome has primarily focused on the genes involved in using ammonium and nitrate only [14]. Notably, the uptake rates of ammonium and nitrite of ANAMMOX are comparable to aerobes [20] and therefore should provide these bacteria with similar amounts of energy for growth. However, based on the available thermodynamic data [21], there is a clear inconsistency between the amount of energy ANAMMOX use for growth and the theoretical amount of energy available from substrates. This discrepancy suggests that there can be reasons other than the lack of energy in substrates causing these bacteria to grow slowly.

Extracellular electron transfer (EET) is the process by which some microorganisms exchange intracellular electrons with an extracellular electron donor/acceptor, including metal ions and artificial metal electrodes, across the cell membrane [22,23]. There are multiple mechanisms by which electrons can be transferred extracellularly, and in the case of ANAMMOX, the primary mechanism of EET is by Direct Electron Transfer (DET) with the anode [24]. DET is also used by methanogens and nitrifiers which have been used in anaerobic digestion (AD) [25]. In order to improve their applicability to AD, strategies have been developed to enhance DET in these organisms [26]. These strategies are likely to be applicable to ANAMMOX bacteria as well. Hence, pangenome-scale modelling analyses can be useful to quantify the effect of EET on ANAMMOX growth and nitrogen removal processes, providing key insights regarding the potential benefits of DET implementation for ANAMMOX-based wastewater treatment processes.

This study investigates how different carbon and energy sources in the growth medium affect the nitrogen removal rates and the growth rates of ANAMMOX bacteria through

constructing a pangenome-scale constraint-based metabolic model of ANAMMOX, *i*RB399. After developing the pangenome and examining the metabolic genes in the pangenome, we reconstructed the pangenome-scale metabolic network of ANAMMOX. The model incorporates core, dispensable, and unique metabolic genes to simulate the effects of substrates that are less commonly used to grow ANAMMOX bacteria. We also mapped the effect of these substrates on the magnitude and direction of metabolic fluxes through the central carbon metabolism. Finally, the efficiency of the ANAMMOX electron transport chain, and the effect of EET on ANAMMOX growth and nitrogen removal processes were simulated and explored using *i*RB399. These model-based analyses not only provide key insights into the potential benefits of DET implementation for ANAMMOX-based processes but also help identify metabolic limitations and generate experimentally testable hypothesis to accelerate the growth rate of ANAMMOX bacteria.

2. Results

2.1. Overview of the ANAMMOX Pangenome-Scale Model

In this study, we describe the construction of a pangenome-scale metabolic model of ANAMMOX bacteria, *i*RB399. The model was developed by using the pangenome-scale reconstructed metabolic network of ANAMMOX and named it according to the established naming conventions used in the BiGG database for metabolic models [27]. The reconstructed metabolic network contains 426 metabolites and demonstrates the metabolic activity of 344 genes (see Supplementary File S2) from *Candidatus Kuenenia stuttgartiensis* and 55 genes from other ANAMMOX bacteria genomes (Table 1). *i*RB399 further includes 408 reactions, 44 of which are exchange reactions, one biomass objective function reaction and one reaction for non-growth associated maintenance energy. Apart from cytoplasm and extracellular compartments, the model has an extra compartment, the anammoxosome, containing the reactions involved in the ANAMMOX nitrogen and energy metabolism, as well as the respiratory complexes.

Table 1. List of ANAMMOX genome sequences used in this study and downloaded from the NCBI public database [28].

Genbank ID	Species Name	Notes on NCBI
GCA_002009475.1	*Ca. Brocadia caroliensis*	Derived from metagenome
GCA_000987375.1	*Ca. Brocadia fulgida*	Derived from metagenome
GCA_017347445.1	*Ca. Brocadia pituitae*	Derived from environmental source
GCF_001753675.2	*Ca. Brocadia sapporiensis*	Included in RefSeq
GCA_000949635.1	*Ca. Brocadia sinica*	Included in RefSeq
GCF_000296795.1	*Ca. Jettenia caeni*	Missing strain identifier
GCA_005524015.1	*Ca. Jettenia ecosi*	Derived from metagenome
GCF_900232105.1	*Ca. Kuenenia stuttgartiensis*	Included in RefSeq
GCA_000786775.1	*Ca. Scalindua brodae*	Derived from metagenome
GCF_002443295.1	*Ca. Scalindua japonica*	Included in RefSeq
GCA_002632345.1	*Ca. Scalindua rubra*	Derived from metagenome
GCA_017368835.1	*Ca. Scalindua sediminis*	Derived from metagenome

At the species level, ANAMMOX bacteria are genetically very diverse. The unique, dispensable, and core genomes are comprised of 6453, 4954, and 925 genes, respectively. The unique genome was poorly annotated, with 74.8% being hypothetical genes as compared to 40.2% hypothetical genes in the dispensable genome and 10.2% hypothetical genes in the core genome. Overall, the unique hypothetical genes made up 39.1% of the total number of genes present in the pangenome (Figure 1). The dispensable genome contains genes, such as glutamate transporters, formate transporters and hydroxylamine oxidoreductase, enabling the catabolism of substrates other than ammonium and nitrite.

Analysis of the genes involved in the energy metabolism (Figure 2) revealed that the genes encoding the enzyme nitrite reductase were found to be missing in the genomes of *Ca. Brocadia pituitae*, *Ca. Brocadia sapporiensis* and *Ca. Brocadia sinica*. Hydroxylamine

reductase was also missing from the majority of the species examined. The rest of the genes related to nitrogen metabolism were detected in all the genomes analysed in this study.

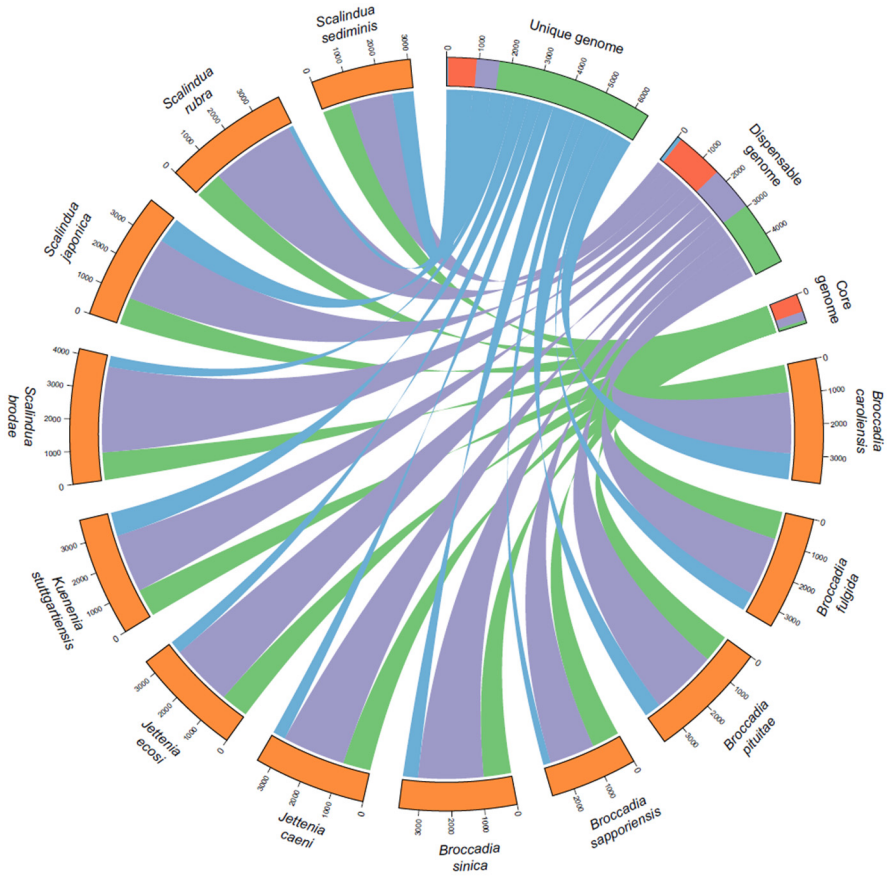

Figure 1. The gene distribution in the ANAMMOX pangenome. The Figure was generated using CIRCOS [29]. The functional distribution of genes is shown in the core, dispensable and unique genomes as blue for transporters, red for metabolic genes, purple for non-metabolic genes, and green for hypothetical genes. The width of the ribbons represents the number of genes belonging to the unique, dispensable or core genome.

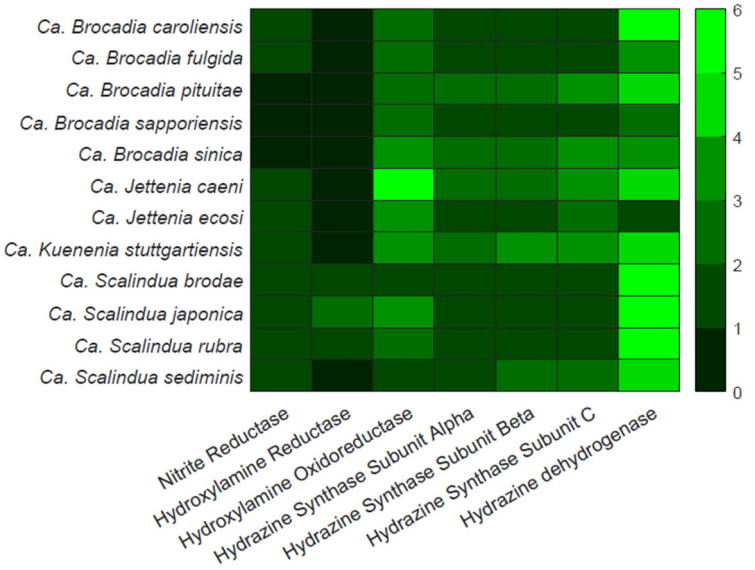

Figure 2. Prevalence of genes in the ANAMMOX pangenome related to energy metabolism. The number of genes found in each genome is shown by differing shade of green.

2.2. Redox Activities of ANAMMOX Bacteria

Figure 3 shows the distribution of genes found in the ANAMMOX pangenome, enabling different pathways of energy metabolism in *i*RB399 for generating energy and supporting ANAMMOX cell growth. The figure is also showing how the simulation results generated using *i*RB399 are compared to previously reported in vivo cell growth in the literature [10,13,24,30–34]. Although propionate was reported to support ANAMMOX growth, model simulations revealed that propionate cannot support ANAMMOX growth as the sole electron donor but can be assimilated when another donor is present. Notably, it was the only electron donor identified to be unable to support growth as the sole electron donor, as all the other donors are reported to support ANAMMOX growth as the sole electron donor in the growth medium. Simulations with *i*RB399 were conducted with alternative electron acceptors, and it was found that glucose, glutamate and formate supported ANAMMOX growth as the sole electron donor with nitrite, nitric oxide and nitrate as electron acceptors.

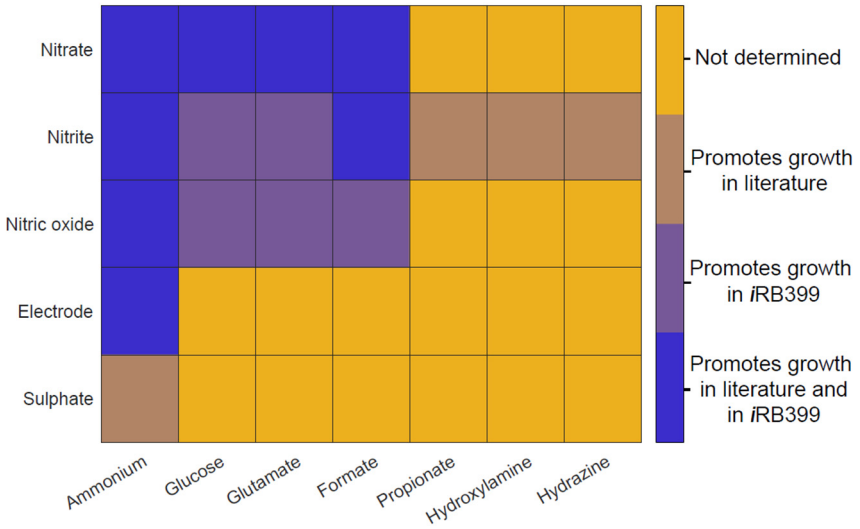

Figure 3. Distribution of genes enabling energy metabolism pathways in the ANAMMOX pangenome involving 7 different electron donors (along x-axis) coupled to 5 different electron sinks/acceptors (along y-axis). Colouration is used to show whether the redox couple has been observed promoting growth in the literature or in *i*RB399 or both.

Figure 4 shows the simulated growth rates of ANAMMOX bacteria using *i*RB399. Since propionate was unable to support growth under any conditions as the sole electron donor, it is not shown in the figure. Propionate uptake was only observed in model simulations when ammonium was also available in the in silico growth medium. Glucose was identified to be the best electron donor overall and the electron acceptors followed the same pattern through the range of electron donors with the exception of formate. Formate resulted in the slowest growth rate as an electron donor in *i*RB399. For most simulations, there was a close relationship between ATP production fluxes and growth rates, with the exception being nitric oxide as the electron acceptor and formate or glucose as the electron donor. In both cases, the model was able to produce more ATP with nitric oxide than with nitrite or nitrate; however, the observed growth rates were still similar to the results obtained from the use of other electron acceptors.

Growth simulations using the ratio of consumption of electron donor to electron sink (Figure 5) shows that the ratio increased with the reducing potential of the donor and decreased with increasing oxidation state of the acceptor. The exceptions were where the final product was not nitrogen gas. For example, for nitric oxide oxidation of glucose, *i*RB399 predicted that nitric oxide would be reduced to ammonium instead of being oxidised to nitrogen gas.

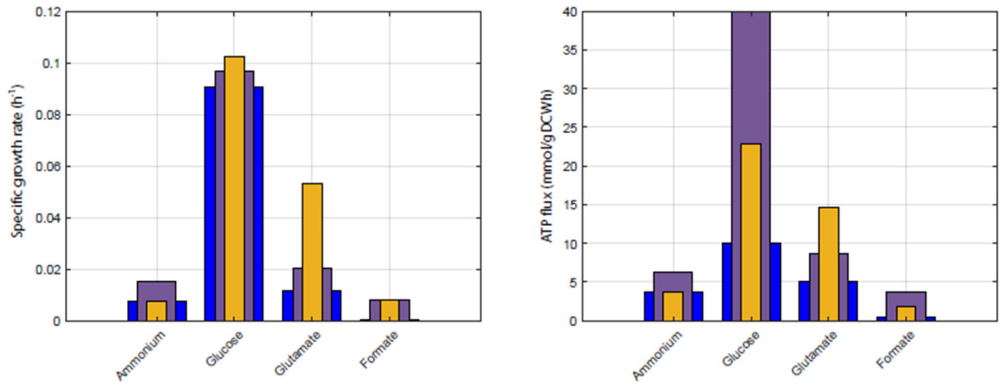

Figure 4. In silico ANAMMOX growth simulation with *i*RB399. ANAMMOX growth rates (**left**) and ATP production fluxes (**right**) were simulated using *i*RB399 and the COBRA toolbox [35], and constrained by the availability of electron donors (5 mmol/gDCW.h of donor in each experiment) with an excess of nitric oxide (purple), nitrate (yellow) and nitrite (blue) as electron acceptors.

Glucose, glutamate, and formate can all be oxidized by ANAMMOX bacteria, but not directly. The organics are oxidized in order to reduce nitrate or nitrite to ammonium, so that it can then be converted to hydrazine as an intermediate to produce nitrogen. While this mechanism allows for generalization, it also severely limits the amount of energy that can be used for cellular respiration since the final step is very inefficient. Simulations with *i*RB399 show that extracellular electron transfer (EET) via an electrochemical cell or nitric oxide can provide an alternative electron sink for ANAMMOX bacteria. EET is mutually exclusive to nitrite-based respiration, so it cannot be used to supplement the availability of electron sinks [24].

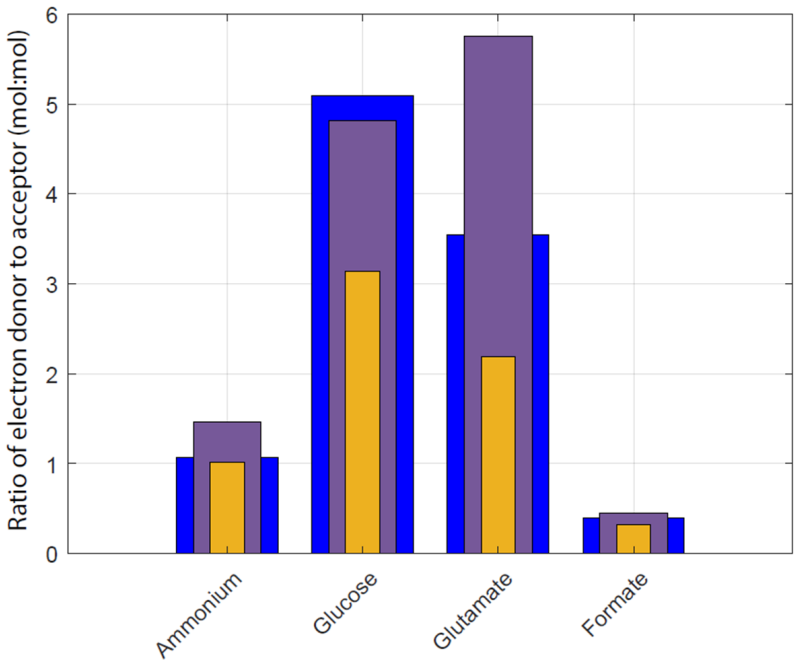

Figure 5. Ratio of electron donor to electron acceptor consumption in growth simulations of ANAMMOX using *i*RB399 and the COBRA toolbox [35]. Electron acceptors used are nitric oxide (purple), nitrate (yellow) and nitrite (blue). In each case, the uptake flux of the electron donor was constrained to 5 mmol/gDCW.h.

2.3. Flux Determination of the Central Carbon Metabolism

The magnitude and direction of fluxes through reactions in the central carbon metabolism of ANAMMOX bacteria (Figure 6) vary depending on the nature of the substrates being consumed. Generalisations cannot be made about the direction of cycle being dependant on whether the cell is engaged in lithotrophy or heterotrophy. In simulation A of Figure 6, the oxidative TCA cycle is being used to provide the cell with energy and reduce nitrite to ammonium. The ammonium is then transported to the anammoxosome to be consumed in the ANAMMOX pathway to produce nitrogen and carbon dioxide as final products. Simulation B shows the central carbon metabolism during chemolithoautotrophy and largely follows the experimentally determined fluxes reported previously [10]. The TCA cycle runs in the oxidative direction and carbon is fixed through the Wood-Ljungdahl pathway as in simulation A. Simulation C demonstrates why generalisation for the directions of fluxes do not apply to the trophic mode of ANAMMOX bacteria. The TCA cycle runs in the reductive direction in order to assimilate carbon in C. In all simulations, the TCA cycle does not produce malate, and aspartate takes its place as an intermediate.

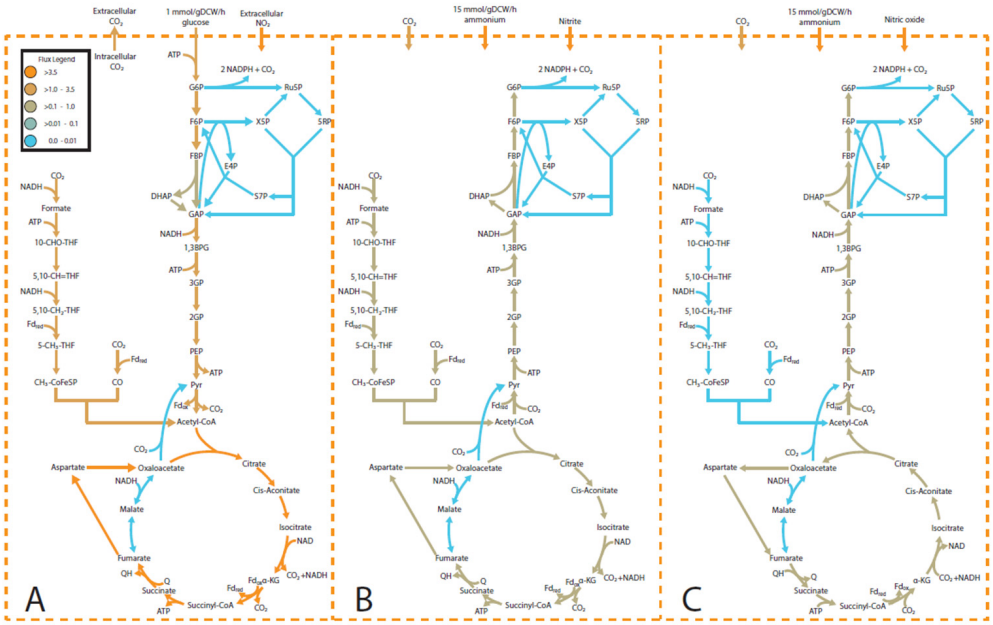

Figure 6. Simulation of metabolic fluxes in the central carbon metabolism under different substrate constraints. (**A**) shows the effect of glucose supplementation with nitrite as the electron sink, (**B**) shows the effect of ammonium and nitrite, and (**C**) shows the effect of ammonium and nitric oxide. Abbreviations: 1,3BPG, 1,3-Bisphosphoglyceric acid; 10-CHO-THF, 10-Formyltetrahydrofolate; 2GP, 2-Phosphoglycerate; 3GP, 3-Phosphoglycerate; 5,10-CHO=THF, 5,10-Methenyltetrahydrofolate; 5-10-CH2-THF, 5,10-Methylenetetrahydrofolate; 5-CH3-THF, 5-Formyltetrahydrofolate; CH3-CoFeSP, Methylcorrinoid protein; DHAP, Dihydroxyacetone phosphate; E4P, Erythrose 4-phosphate; F6P, Fructose 6-phosphate; FBP, Fructose bisphosphate; G6P, Glucose 6-phosphate; GAP, Glyceraldehyde 3-phosphate; PEP, Phosphoenolpyruvate; Pyr, Pyruvate; R5P, ribose-5-phosphate; Ru5P, Ribulose 5-phosphate; S7P, Sedoheptulose 7-phosphate; X5P, D-Xylulose 5-phosphate; α-KG, α-ketoglutarate.

2.4. The ANAMMOX Electron Transport Chain

In order to improve the growth rate of ANAMMOX bacteria, it is important to consider the efficiency of the electron transport chain (ETC) and the availability of ammonium in the growth medium. Based on the maximum theoretical thermodynamic efficiency estimation of the ETC of ANAMMOX bacteria (see Supplementary File S1 for details), it was theorised that one proton is translocated per electron transferred from the donor to the acceptor through the ETC. This observation suggests that the operating efficiency of the ANAMMOX ETC is 16.7% (Supplementary File S1), which further implies that the production of ATP,

and by extension, growth is substantially limited due to the inefficiency of the ETC or the energy metabolism. This observation is further supported by model simulations (Figure 7), demonstrating that the growth rate can be increased significantly if the ETC efficiency can be increased as compared to increasing the availability of electron donor (ammonium) in the medium. The respiratory complexes involved in the ETC were all placed in the anammoxosome compartment in the model since this is the place where these reactions are reported to take place in vivo [36].

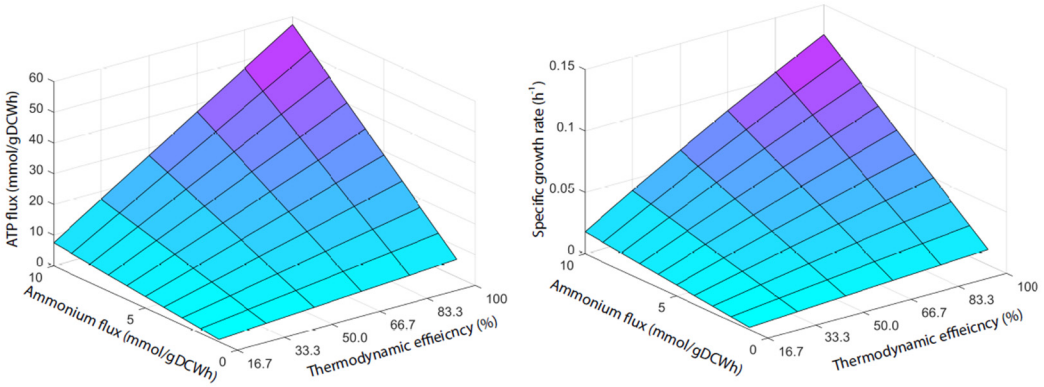

Figure 7. Analysis of the thermodynamic efficiency of the ANAMMOX electron transport chain (ETC) expressed in % efficiency. Thermodynamic efficiency was measured by the number of protons translocated per electron transferred through the ETC. This is compared with the ammonium uptake flux to demonstrate the sensitivity of the system to both inputs. The sensitivity of ATP flux (**left**) and the growth rate (**right**) with ammonium uptake flux and ETC efficiency is compared to further demonstrate the similar pattern observed between them.

3. Discussion

Growth simulations using *i*RB399 demonstrate that the exceptionally slow growth rates of ANAMMOX bacteria can be explained by the low efficiency of the ETC and the difficulty in producing ATP for the growth-associated maintenance energy (GAM). The efficiency of the ETC determines the amount of energy in terms of ATP that is produced by the oxidation of the electron donor. This low thermodynamic efficiency of the ETC is supported by the observation that when ANAMMOX bacteria utilise alternative redox couples, those should provide significantly more energy than ammonium and nitrite. However, they still grow very slowly and are outcompeted by other organisms. This low efficiency therefore seems to apply to all redox couples.

Since neither the GAM nor the ETC efficiency can be influenced by external factors, it is impossible to ameliorate this deficiency of ANAMMOX growth rate by changing the growth medium. The growth rate is also very sensitive to the ETC efficiency. This is true for all substrates since in order to produce ATP from oxidative phosphorylation, all electrons pass through the ETC and only translocate one proton each. In *i*RB399, organic electron donors are also subject to this mechanism since they are oxidised to reduce nitrite, nitrate and nitric oxide to ammonium in order to produce hydrazine and then nitrogen as the final product. Since the final product in these cases is still nitrogen, the low electron transport efficiency applies.

ANAMMOX bacteria are mainly known for their ability to oxidize ammonium and nitrite into nitrogen gas anaerobically using the ANAMMOX pathway. In spite of this specialised function, these bacteria have been known to be generalists rather than specialists. This observation is actually supported by the construction of the ANAMMOX pangenome, which shows that ANAMMOX bacteria have a large number of genes enabling a diverse range of metabolic pathways and are capable of consuming a variety of different substrates to produce energy. Their metabolic reconstruction shows that they are able to pair a large variety of electron donors and acceptors to produce ATP. There are also transporters which could not be confidently annotated due to lack of evidence but could allow ANAMMOX

bacteria to catabolise a larger variety of electron donors not shown in this study. Notably, there were no electron donors that could only be consumed by one species. Each pathway involved in utilising the redox couples identified was present in more than one species. Overall, it was shown that there is a wide variety of substrates that can be exploited for growing ANAMMOX bacteria, and they are likely removing all of these substrates from the wastewater they treat as well.

The propensity to use electron donors to always produce ammonium followed by hydrazine is unique to ANAMMOX bacteria and distinguishes them from other nitrogen-oxidizing bacteria. While they may not be specialized in their substrate use, they are extremely specialised in the final steps of the energy conservation process, which appears to severely limit their growth, as has been demonstrated by growth simulations using *i*RB399. While this specialised energy metabolism not only limits their growth but also restricts them to almost always convert fixed nitrogen compounds in any oxidation state to nitrogen gas regardless of what the electron donor is. This capability is very useful in wastewater treatment applications because it reduces the strain on process controllers to make sure that a specific electron acceptor needs to be always present in the system.

The pangenome shows the different reactions and pathways associated with each ANAMMOX species. However, this analysis is not complete without considering substrate affinity compared to other ANAMMOX species. For example, *Ca. Brocadia* sp. 40 generally outcompetes other ANAMMOX bacteria in wastewater treatment systems because of its higher affinity for nitrite at higher concentrations than most other ANAMMOX species [20]. This observation also applies to EET, where different species have very different reaction kinetics while performing EET with different responses to voltage, current density and substrate concentration [24]. It is important to note here that the kinetics of ammonium affinity while performing EET are not yet established in the literature. On a mol-per-mole basis, EET is a comparable electron acceptor to nitrite in terms of ATP synthesis in the cell. However, the kinetics of ammonium absorption by the cell might be different when performing EET leading to different growth rates at the same concentration of ammonium when compared to nitrite in vivo.

The most promising finding of this study is that ANAMMOX bacteria should be capable of utilising glucose as an electron donor while using nitric oxide as an electron acceptor. This reaction produces carbon dioxide and ammonium while providing these bacteria with more energy than the ammonium to nitrite pathway. This is due to the additional energy provided by glycolysis. The reason nitric oxide and not nitrite or nitrate are suggested as electron acceptor is that other denitrifiers are not as tolerant of nitric oxide as ANAMMOX. Therefore, by growing the seed sludge for a wastewater treatment reactor using glucose and nitric oxide as substrates, a relatively pure culture of ANAMMOX bacteria could in theory be grown quicker than is possible with ammonium and nitrite. Using nitrite and nitrate with glucose would not be useful since it would result in other organisms proliferating. This redox couple is possibly not feasible with all ANAMMOX species, as shown in Figure 2, since it requires the nitrite reductase activity as well. It should also be noted that nitric oxide may also inhibit growth in practice [37].

There is a large amount of genetic diversity among ANAMMOX bacteria, more than is generally observed between physically similar organisms. For example, between 44% and 57% of *Escherichia coli* genes were core genes in a study of 17 genomes [38], as compared to only 26% being core genes in the ANAMMOX pangenome. This small pool of core genes is puzzling because generally these organisms look nearly identical and have only been identified as different species by genetic analysis. Furthermore, half of the annotated dispensable genes appear to have non-metabolic functions. This makes it very difficult to determine what effect they are having on the cell's physiology and metabolism. Supplementary File S2 provides further details of how metabolic and non-metabolic genes are distributed in the pangenome. There are no pathways known to be unique to one species of ANAMMOX bacteria. This is likely due to the poor annotation of the pangenome, making it impossible to identify enzymes involved in other pathways. With improved genome annotations or

the identification of new species, more genes encoding enzymes in other pathways can be identified. Improved gene function identification can alter how sludge is generated in future as species might be pre-selected for beneficial traits, allowing a wider selection of substrates to be removed from wastewater.

There is a large number of Heme-C cytochromes that have very specific roles in ANAMMOX metabolism [39,40]. Some thermodynamically unfavourable reactions have been suggested to be integral to ANAMMOX metabolism, yet there is no information as to where the energy comes from to make them feasible. Nitrite oxidation to nitrate coupled to carbon dioxide reduction has been long accepted as the means by which ANAMMOX bacteria fix carbon. However, this is thermodynamically unfeasible without energy input or electron bifurcation. Furthermore, experiments have been performed where carbon could be fixed without producing nitrate [30]. In the ANAMMOX genome there are several genes encoding reactions that could potentially reduce nitrate with low energy redox carriers. This poses a problem since it presented the model with the possibility of carrying out thermodynamically unfeasible reactions. To prevent creating futile cycles, these reactions were made irreversible. The fluxes were checked across different growth scenarios for these thermodynamically unfeasible loops, and it is expected that a different mechanism is responsible for production of nitrate from nitrite which is unrelated to carbon fixation.

This study has shown that EET likely provides the cell with as much or slightly less energy than the conventional ANAMMOX reaction per mole of nitrogen in the feed. However, it should also be considered that this configuration brings alternative limiting factors such as that the bacterium must make contact with the electrode. It is therefore likely that this technology will require a rethinking in its approach before it is considered for use in wastewater treatment plants since it is demonstrably inferior in its current form. Accelerating the process by applying a voltage to the electrode is also likely to bring new engineering challenges, as high voltage applied to the electrode can cause the formation of reactive oxygen species (ROS) such as hydrogen peroxide, damaging the bacterial cell membrane and other cellular components [41]. These challenges may reduce the efficiency of EET and be detrimental to the overall performance of the system. Nonetheless, removal of nitrogen in an anaerobic bioelectrochemical system (BES) has been performed with multiple species in the same reactor which was shown to allow not only ammonium but also nitrate, nitrite and hydroxylamine to be oxidised on the anode [42]. This result demonstrates that EET via BES has potential as an alternative to the nitrite pathway in ANAMMOX bacteria.

Our study implies that future ANAMMOX sludge production might be possible at much higher rates if feedstocks contain glucose and nitric oxide. We also suggest that more research needs to be conducted to identify which organic substrates might be useful for ANAMMOX bacteria as there are transporters which could not be identified during gene annotation. Although we analysed the limitations in ANAMMOX energy metabolism and the ETC using *i*RB399, more research needs to be conducted to overcome these limitations. If the inefficiency in the ETC can be remedied, there is potential to greatly improve ANAMMOX growth rates. The ETC efficiency remains the greatest obstacle for effective utilisation of energy by the cell and by extension, to growth.

4. Materials and Methods

4.1. Selection of ANAMMOX Genomes

A total of 12 genomes (Table 1) were selected from the NCBI [28] public database based on completeness and certainty that the genomes belonged to ANAMMOX bacteria. Most of the available genomes were rejected at this stage for not containing genes related to nitrogen metabolism such as hydrazine dehydrogenase or because they scored poorly in the genome completeness and quality checking process. Genome quality checking was conducted using CheckM—v1.0.18 [43] in the Kbase environment [44].

4.2. Pangenome Generation

The ANAMMOX pangenome was generated using OrthoMCL—v2.0 [45] in Kbase [44] by analysing the 12 genomes shown in Table 1. The genes were then manually sorted by function and presence in the core, dispensable and unique genomes to identify the genes in each category as shown in Figure 1. The figure was generated using CIRCOS [29].

4.3. Formulation of the Biomass Objective Function of iRB399

The biomass objective function was developed based on the biomass composition of *Ca. K. stuttgartiensis* since it is the most widely studied species of ANAMMOX bacteria. The detailed biomass composition, including the amount of all cellular macromolecules, such as amino acids, DNA, RNA, fatty acids, and lipids, of *Ca. K. stuttgartiensis* was generated from its genome sequence and the published literature describing its cellular compositions and physiology (see Tables S3–S10 in Supplementary File S1). The amount of amino acids in the cell has been previously reported [46] and the DNA, RNA and amino acid composition was estimated from the genome sequence. The amount of lipids has been estimated from the literature [47]. The cell wall composition was determined partially from the literature [48]. All compositions were calculated using the basis of one gram dry cell weight.

4.4. Calculation of Growth-Associated Maintenance

The GAM accounts for the energy (in the form of ATP) necessary to replicate a cell, including for macromolecular synthesis (e.g., proteins, DNA, and RNA). The GAM is best determined in chemostat growth experiments [49]. The growth-associated maintenance of *K. stuttgartiensis* was calculated using the published correlation of ammonium consumption and growth rate [21]. This calculation is explained in detail in Supplementary File S1.

4.5. Metabolic Network Reconstruction

Using one of the publicly available genome sequences of *Ca. K. stuttgartiensis* (GenBank ID: GCF_900232105.1, RefSeq ID: GCF_900232105.1), the draft metabolic model was automatically generated using RASTtk—v1.073 [50] and Model SEED [51] apps in the Kbase [44] environment. The resultant draft genome was then manually curated to remove reactions which were unnecessary, unbalanced or contained incorrect metabolite identifiers. Gaps in the metabolic network were filled with reactions generated with FastGapfill v.2.0.0 [52] and manually added to the model. Genes and reactions involved in the central carbon metabolism was completed with results from published ^{13}C and ^{2}H isotope tracing experiments [10]. The reactions in the metabolic network were split into two compartments, the cytosol and the anammoxosome. The anammoxosome contains the reactions involving nitrogen and energy metabolism, as well as the ANAMMOX reaction [36]. Therefore, the anammoxosome compartment in the model contains the respiratory complexes and the ANAMMOX pathway and there is no transportation of redox carriers in or out of the anammoxosome compartment.

4.6. Model Validation

The model was checked using MEMOTE [53] for consistency and scored 52% overall. However, when stoichiometric consistency (0%) was excluded, this score rose to 90.3%. Further details about the model and the MEMOTE report are available in Table S2, Supplementary File S1.

4.7. In Silico Analysis of ANAMMOX Metabolism

The FBA was used for the in silico analysis and simulation of the ANAMMOX growth on ammonium, glucose, glutamate, and formate as electron donors and nitrite, nitric oxide, nitrate and extracellular electrode as electron acceptors. The COBRA toolbox [35] was used for FBA implementation, and all simulations were conducted in MATLAB (the MathWorks Inc., Natick, MA, USA) using the GUROBI optimization solver (Gurobi Optimization, LLC, Beaverton, OR, USA). The model reactions and genes, as well as the details of constraints used to simulate ANAMMOX growth with *i*RB399, are provided in Tables S1 and S2 in Supplementary File S2.

5. Conclusions

In conclusion, ANAMMOX growth is limited by bottlenecks in the energy metabolism, and there are no alternative electron acceptors that offer substantial advantages over nitrite for growing pure ANAMMOX cultures. When ANAMMOX performed EET using an electrode as electron acceptor, it was shown in model simulations to be an inferior electron acceptor to nitrite. It may still be possible to increase the overall growth rate by optimizing the EET conditions. This study identified that a very large portion of the energy produced from the anaerobic oxidation of ammonia appears to be lost either due to inefficiency in the ANMMOX ETC or by another unknown process. Using *i*RB399, we predict that a medium containing nitric oxide and glucose should provide ANAMMOX bacteria with the most energy while still being theoretically toxic to most other organisms. Thus, the reconstructed metabolic network and the developed model can be used as ANAMMOX knowledgebase to design novel experiments regarding ANAMMOX gene functions, physiology, and metabolism, as well as to apply and analyse the efficacy of different ANAMMOX species in wastewater treatment processes. Our detailed pangenome-scale metabolic model also provides the opportunity for researchers to test hypothesis in silico before performing experiments in vivo.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/synbio2010005/s1, Supplementary File S1: Details of ANAMMOX biomass composition and the ETC thermodynamic efficiency estimation; Supplementary File S2: Details of ANAMMOX pangenome, and genes, reactions and metabolites of *i*RB399.

Author Contributions: R.G.B.—visualization, data curation, methodology, software, validation, formal analysis, investigation, writing—original draft preparation; M.A.I.—conceptualization, resources, supervision, project administration, funding acquisition, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Loughborough University Doctoral Scholarship to RGB and a Royal Society Research Grant (RGS\R1\221313) awarded to MAI.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Materials.

Acknowledgments: We thank anonymous reviewers for providing excellent feedback to help improve the quality of the published version of the paper.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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