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A Mathematical Model Of Bacteria Capable Of Complete Oxidation Of Ammonium Predicts Improved Nitrogen Removal And Reduced Production Of Nitrous Oxide

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Abstract*—The removal of excess nutrients from water ecosystems requires oxidation of toxic ammonium by two types of bacteria; one oxidizes ammonium to nitrite and the other oxidizes nitrite to nitrate. The oxidation of ammonium is often incomplete and nitrite accumulates. Nitrite is also toxic, and is converted by the ammonium-oxidizing bacteria to nitrous oxide, a powerful greenhouse gas. Here we use mathematical modeling to analyze a potential solution to the problems related to incomplete oxidation of ammonium. We propose that a single engineered nitrifying bacterium should be capable of complete oxidation of high concentrations of ammonium to nitrate. Our model is based on available data on ammonium- and nitrite-oxidizing bacteria. The model predicts that insertion of highly expressed genes of a nitrite oxidation system into the genome of an ammonia-oxidation bacterium should result in complete oxidation of ammonium to nitrate in nutrient-overloaded conditions. Due to its increased capacity to fully oxidize ammonium to nitrate, the proposed bacterium would display dramatically reduced production of nitrous oxide, and therefore might have great potential to reduce the greenhouse effect of nutrient-overloaded water systems

Keywords— mathematical model; ammonium oxidation; nitrous oxide

I. INTRODUCTION

Eutrophication is a global ecological problem related to excessive nutrient supply to water ecosystems, such as lakes and rivers, due to leakages of field fertilizers and wastewater. The increase in concentrations of limiting nutrients, mainly phosphorus and nitrogen compounds (ammonium, urea, nitrite), increases biomass production in water bodies. The increased level of dying organic material in turn depletes dissolved oxygen (O_2), which causes irreversible changes in ecosystems exacerbated by algae blooms and increased pH [1-3]. One efficient method for biological removal of nitrogen is harvesting of floating plants [2, 4-6]. However, plant growth is inhibited by high pH and high levels of toxic ammonium and nitrite in eutrophic systems [2, 3, 7].

Nitrifying bacteria can promote survival of plants, fish and other aquatic organisms by reducing toxicity of nitrogen compounds [5, 7, 8]. In freshwater ecosystems, the first step of nitrification (oxidation of ammonium to nitrate) is performed by ammonium-oxidation bacteria (AOB) (and by ammonium-oxidation archaea in marine and soil environments), which derive energy from oxidation of ammonium to nitrite. This process requires a final electron acceptor, most commonly O_2 [9]. This first step of nitrification reduces the amount of ammonium, which is very toxic for aquatic ecosystems when present in large amounts, but increases the amount of nitrite, which is still considerably toxic [7, 8]. However, AOB grow in symbiosis with nitrite-oxidizing bacteria (NOB), which perform the second step of nitrification by oxidizing nitrite to nitrate [10-12]. Although nitrate is still toxic for humans when it is present in ground waters, its presence in natural water systems such as lakes and rivers results in considerable reduction of toxicity towards plants, fish and other organisms compared to nitrite and ammonium [7]. Therefore NOB are essential participants in restoration of eutrophic water systems, which can also involve consumption of nitrogen compounds by harvested plants [2, 4-6]. AOB and NOB can tolerate the elevated pH (up to 9) of eutrophic systems [13, 14]. Moreover, they reduce pH by excreting protons during the oxidation reactions.

Although nitrification is beneficial for remediation, it requires the synchronized growth of two types of nitrifying cells. This happens at normal nitrogen levels, when AOB and NOB grow together in symbiotic biofilms [10]. However NOB appear to be more sensitive to various environmental conditions than AOB [12, 15, 16]. In particular, increased levels of nitrogen load cause inhibition of NOB growth, resulting in accumulation of toxic nitrite [15, 17]. Similarly, nitrite often accumulates in eutrophic waterbodies under reduced-O_2 conditions due to lower affinity of NOB for O_2 compared to AOB [18, 19].

Accumulation of nitrite under low-O_2 conditions leads to another ecological problem. When O_2 levels are low, AOB/NOB use other electron acceptors, in particular nitrite, to get energy for their survival [20,

21]. This results in the sequential reduction of nitrite to nitric oxide (NO) and then to nitrous oxide (N_2O) [21-24] through the so-called "nitrifier denitrification" pathway. AOB and NOB do not possess the last enzyme of the denitrification pathway, which reduces N_2O to dinitrogen [25, 26]. Therefore they produce the greenhouse gas N_2O under these conditions. As a greenhouse gas, N_2O has ~300-fold higher ability to trap heat in the atmosphere compared to CO_2 [27]. Additionally, N_2O reacts with ozone, and since N_2O is very stable this causes substantial depletion of the ozone layer of the planet [27]. The level of N_2O in the atmosphere is increasing by 0.31 % annually, and nitrifying bacteria are the main contributors to the emission of this dangerous greenhouse gas from waterbodies, including eutrophic lakes and wastewater treatment plants [18, 28-30].

Both problems – incomplete nitrification and accumulation of N_2O – are mainly caused by uncoupling between the two nitrification steps (performed by AOB and NOB), which leads to accumulation of nitrite under various conditions. Therefore a potential solution of both problems might be integration of both nitrification steps into a single cell, which would allow more efficient coupling. However, the existence of two types of bacteria instead of one in nature presumably has a biological reason [31, 32]. The free energy of nitrite oxidation (-74 kJ/mol) is lower than that of ammonium oxidation (-275 kJ/mol). It was suggested that the relatively low free energy of nitrite oxidation is a possible reason why most of AOB have not evolved a nitrite oxidation system [31]. However, a complete nitrifying bacterium of a *Nitrospira* lineage has been recently found in a hot water pipe of an underground oil exploration well [33]. This bacterium thrives only under low ammonia and nitrite concentrations and therefore it cannot be directly used for the restoration of eutrophic waters. However the existence of this complete nitrifying bacterium suggests that there is a potential for genetic engineering of a more efficient complete nitrifier. Importantly, the levels of nitrite are relatively low in natural ecosystems, which might explain why a complete nitrifier with high capacity to utilize nitrite has not appeared during evolution. However, human activity has resulted in nutrient overload, which has transformed natural environments and has led to elevated nitrite levels, and this new ecological situation is calling for new solutions. The modern methods of bioengineering allow us to easily manipulate cellular genomes. Here we use mathematical modeling to analyze the potential implications of combining two nitrification steps in a single bacterium on nitrification efficiency and production of N_2O.

We started the model construction from consideration of how interactions between AOB and NOB might affect the efficiency of nitrification and production of N_2O under various environmental conditions observed in eutrophic freshwater ecosystems. Nitrification and N_2O production were previously modelled as contributors to remediation processes of wastewaters [34-37]. These models also included other participant organisms (*e.g.*, heterotrophic bacteria) and processes (*e.g.*, phosphate removal), which we are not considering here. Since AOB and NOB live and function together in a closed community on biofilms, where they perform complete nitrification, it is possible to study nitrogen removal by AOB/NOB cultures in isolation from other components of the ecosystem, which simplified our modeling.

To analyze the potential limitations of AOB and NOB nitrification, we included all regulation which is known to be involved, such as inhibition of both nitrification steps (ammonium and nitrite oxidation) and N_2O production by nitrite [13, 15, 38], inhibition of both nitrification steps by ammonia [14, 15], differences of O_2 requirements between AOB and NOB [10, 18, 19], and accelerated production of N_2O under low-O_2 and sufficient nitrite conditions [20-22, 24]. Since no single existing model includes all these processes simultaneously, we developed a new model of nitrification and N_2O production by AOB and NOB, which was based on integration of existing models and data. The parameters of our model were derived from the published data on cell cultures of AOB and NOB. After analysis of the key mechanisms which limit nitrification in this natural two-bacterial system, we applied the model to simulate a complete nitrifier bacterium, which might be built by extension of an AOB genome with nitrite oxidation genes from a NOB. Our model predicts that the complete nitrifying bacteria should be a useful tool in remediation and reduction of N_2O emission

II. MODEL DESCRIPTION

Our nitrification model was initially built for a mixture of AOB and NOB bacteria and then applied for modelling of the engineered complete nitrifying bacterium (CNB). The scheme of the main processes included in the model is shown in Fig. 1.

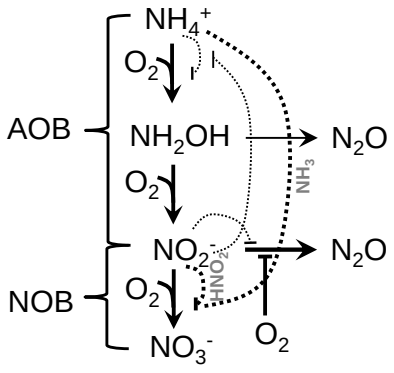

Fig. 1. Main steps of nitrification and N_2O production included in the model. Two stages of nitrification are: oxidation of ammonium to nitrite by AOB, via production of hydroxylamine (NH_2OH), and oxidation of nitrite to nitrate by NOB. Alternatively,

both stages can be performed by CNB. Nitrification requires oxygen (O_2). At low O_2 nitrite can be reduced to nitrous oxide (N_2O). N_2O can be also produced by NH_2OH oxidation, at low rates. The system is regulated through the inhibition of ammonium and nitrite oxidation and nitrite reduction by non-ionized forms of nitrite and ammonia (HNO_2 and NH_3). The main regulation, which is shown by thick dotted lines, strongly affects nitrification.

The first step of nitrification is the oxidation of ammonium to nitrite. Two AOB enzymes are involved in this step: firstly ammonium monooxygenase (AMO) oxidizes NH_4^+ to hydroxylamine (NH_2OH) [39] and secondly hydroxylamine oxidoreductase (HAO) oxidizes NH_2OH to nitrite (NO_2^-) [40]. The reactions of AMO and HAO are:

AMO: $NH_4^+ + O_2 + H^+ + 2e^- \rightarrow NH_2OH + H_2O$

HAO: $NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$

Like others [35] we assumed, based on the existing data, that $2e^-$ from HAO reaction return to AMO and another $2e^-$ are used to generate a proton gradient, with O_2 being a terminal e- acceptor ($2e^- + 0.5O_2 + 2H^+ \rightarrow H_2O$). Therefore the $2e^-$ required for oxidation of one molecule of ammonium by AMO are provided by the oxidation of one molecule of hydroxylamine by HAO. This limits the rate of AMO reaction, so it cannot be faster than the rate of HAO reaction (eq. 3 below). Also we assumed, similarly to other models [35, 37] that electron flux from HAO is tightly coupled to final electron transfer to O_2, so that O_2 - dependence of HAO is determined by the affinity of the terminal oxidase for O_2.

To simulate the AMO and HAO reactions, we assume saturation kinetics with Michaelis-Menten terms for the substrates: total ammonium nitrogen, further called $[NH_4^+]_{tot}$, NH_2OH and O_2, similar to existing models [13, 35]. Ammonium oxidation is inhibited by non-ionized forms of ammonium and nitrite: ammonia (NH_3) and nitrous acid (HNO_2) [15]. The overall rate equations are:

$$V'_{AMO} = V_{mAMO} \cdot \frac{O_2}{O_2 + K_{1,O2}} \cdot \frac{[NH_4^+]_{tot}}{K_{NH4} + [NH_4^+]_{tot}} \cdot \frac{1}{1 + HNO_2 / K_{i,HNO2} + NH_3 / K_{i1,HN3}} \quad (1)$$

$$V_{HAO} = V_{mHAO} \cdot \frac{O_2}{O_2 + K_{2,O2}} \cdot \frac{NH_2OH}{NH_2OH + K_{NH2OH}} \quad (2)$$

Here O_2 stands for the concentration of the dissolved oxygen, which was varied in our simulations (Sections III.A and III.B), but kept constant during each timecourse. This corresponds to experiments with controlled aeration. The maximal O_2 concentration was assumed to be 0.2 mM [18]. The affinity of AMO for oxygen is $K_{1,O2}$ = 0.001 mM [35]. The dependence of hydroxylamine oxidation on oxygen is determined by the affinity of terminal oxidase for oxygen [35]; $K_{2,O2}$ = 0.016 mM. Since the electrons for AMO reaction come from the oxidation of NH_2OH, the rate of the AMO reaction is limited by the rate of the HAO reaction [37]:

$$V_{AMO} = \min(V'_{AMO}; V_{HAO}) \quad (3)$$

In all simulations the maximal rate constant of ammonium oxidation was chosen to be V_{mAMO} = 3.6 mM/h^{-1}, which corresponds to reaction rates typically observed in AOB cultures [24]. For example, for the initial concentrations of $[NH_4^+]_{tot}$ = 5 mM, O_2=0.2 mM and pH=7.5, this value of V_{mAMO} would give an ammonium oxidation rate of 3 mM/h [24]. The maximal rate constant of hydroxylamine oxidation was chosen to be equal to V_{mAMO}, similar to [35], V_{mHAO} = 3.6 mM/h^{-1}. The substrate affinities for AMO and HAO are K_{NH4}=0.2 mM and K_{NH2OH}=0.02 mM, respectively [35]. The inhibition constant by ammonia, $K_{i1,\ NH3}$, was estimated as 3 mM [15], while the inhibition constant for HNO_2 was estimated as $K_{i,\ HNO2}$ = 0.001 mM [13].

The rate of ammonium oxidation depends on pH [13, 15]. Like others [15, 34], we described the pH effect through the dependence of the equilibrium between ionized and non-ionized forms of ammonium and nitrite on pH [15, 34]. The concentrations of non-ionized inhibitors NH_3 and HNO_2 were expressed via total amount of ammonium ($[NH_4^+]_{tot}$) and total amount of nitrite (called $[NO_2^-]_{tot}$), pH and temperature (T) in ºC, as before [15, 34]:

$$[NH_4^+]_{tot} = NH_4^+ + NH_3; \quad NH_3 = \frac{NH_4^+ \cdot K_{eq,NH_4^+}}{H^+}, \quad \text{where}$$

$$K_{eq,NH_4^+} = e^{-6344/(T+273)}.$$

This leads to $NH_3 = \frac{[NH_4^+]_{tot}}{1 + 10^{-pH} \cdot e^{6344/(T+273)}}$.

Similarly, the expression for HNO_2 is [15, 34]:

$$HNO_2 = \frac{[NO_2^-]_{tot}}{1 + 10^{pH} \cdot e^{-2300/(T+273)}}$$

The pH levels were varied between different simulations, but were fixed during each timecourse, corresponding to experiments where nitrifiers are kept in appropriate buffers.

The second step of nitrification is the oxidation reaction of nitrite to nitrate (NO_3^-) by nitrite oxidoreductase (NXR, [41]):

$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$

Again, O_2 is the terminal e^- acceptor, leading to the O_2-dependence in the equation below. The NXR reaction was described similarly to AMO in agreement with existing data on nitrite oxidation, which is inhibited by free nitrous acid and ammonia and has Michaelis-Menten dependence on nitrite substrate [14, 15]:

$$V_{NXRox} = V_{mNXR} \cdot \frac{[NO_2^-]_{tot}}{[NO_2^-]_{tot} + K_{1,NO2}} \cdot \frac{O_2}{O_2 + K_{3,O2}} \cdot \frac{1}{1 + HNO_2 / K_{i,HNO2} + NH_3 / K_{i2,HN3}} \quad (4)$$

The maximal rate constant of NXR was chosen to be half of V_{mAMO}, which is typically observed in mixed cultures of AOB and NOB; V_{mAMO} = 1.8 mM/h^{-1} [36]. The affinity of terminal oxidase for oxygen is $K_{3,O2}$ = 0.062 mM [10, 42]; substrate affinity for nitrite is $K_{1,NO2}$ = 0.02 mM [14]. The inhibition constants for NH_3 and HNO_2 are estimated as $K_{i2,NH3}$= 0.03 mM [15] and $K_{i,HNO2}$ = 0.001 mM, respectively [14].

N_2O production by nitrifying bacteria is largely related to reduction of nitrite by AOB under O_2 – limited conditions [20-24]. Similarly to [35], we assumed that NH_2OH is used as an electron donor for N_2O production, which in our model was described as one step (integrating the reduction of nitrite to NO by nitrite reductase (NIR) and the reduction of NO to N_2O by nitric oxide reductase (NOR) for simplicity. The stoichiometry of nitrite and NH_2OH during the reduction of nitrite to N_2O was estimated as 1:1 based on the balance of electrons required for NO_2^- reduction (NIR + NOR) and provided by NH_2OH oxidation (HAO):

$$NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O \text{ (NIR)}$$
$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \text{ (NOR)}$$
$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \text{ (HAO)}$$

The summary equation for N_2O production from NO_2^- and NH_2OH is:

$$NH_2OH + NO_2^- + H^+ \rightarrow N_2O + 2H_2O$$

(HAO, NIR and NOR: $NH_2OH + H_2O + NO_2^- + H^+ \rightarrow 2NO_2^- + 6H^+ + 4e^- \rightarrow 2NO + 2H_2O + 2H^+ + 2e^- \rightarrow N_2O + 3H_2O$)

The rate of N_2O production during the reduction of nitrite at low O_2 concentration is described by Michaelis-Menten dependence on nitrite and hydroxylamine substrates [35]. We also included the observed inhibition of N_2O production by HNO_2 and O_2 [35, 38]:

$$V_{N2O\_NO2} = V_{mN2O\_NO2} \cdot \frac{[NO_2^-]_{tot}}{[NO_2^-]_{tot} + K_{2,NO2}} \cdot \frac{NH_2OH}{NH_2OH + K_{NH2OH}} \cdot \frac{1}{1 + HNO_2 / K_{i,HNO2} + O_2 / K_{i,O2}} \quad (5)$$

The inhibition of N_2O production by nitrous acid was included based on existing data, with the inhibition constant $K_{i,\ HNO2}$ = 0.001 mM [38]. The inhibition constant for O_2 was taken from [35] as $K_{i,O2}$ =0.003 mM. The substrate affinity for nitrite was estimated as $K_{2,NO2}$ =0.4 mM [43].

In addition to major production of N_2O via denitrification at low O_2 concentrations, a minor flux to N_2O via oxidation of NH_2OH by HAO contributes to N_2O accumulation under normal O_2 conditions [38, 44, 45]:

$$V_{N2O\_NH2OH} = V_{mN2O\_NH2OH} \cdot \frac{NH_2OH}{NH_2OH + K_{NH2OH}} \quad (6)$$

Based on the observed rates of N_2O production under low and high O_2 [46], we estimated the corresponding maximal rates as: V_{mN2O_NO2} = 0.09 mM/h^{-1}; V_{mN2O_NH2OH} = 0.0018 mM/h^{-1} (for the chosen maximal rates of AMO, HAO).

The kinetics of nitrification and accumulation of N_2O are described by the following system of ordinary differential equations (ODEs):

$$\frac{d[NH_4^+]_{tot}}{dt} = -V_{AMO} \quad (7)$$

$$\frac{dNH_2OH}{dt} = V_{AMO} - V_{HAO} - V_{N2O\_NO2} - V_{N2O\_NH2OH} \quad (8)$$

$$\frac{d[NO_2^-]_{tot}}{dt} = V_{HAO} - V_{NXRox} - V_{N2O\_NO2} \quad (9)$$

$$\frac{dNO_3^-}{dt} = V_{NXRox} \quad (10)$$

$$\frac{dN_2O}{dt} = V_{N2O\_NO2} + 0.5 \cdot V_{N2O\_NH2OH} \quad (11)$$

The simulations of the engineered complete nitrifying bacterium (CNB) were done assuming that parameters of nitrite oxidation, such as the affinity for O_2 and inhibition constants, are the same as for ammonium oxidation, due to the same electron transport chain (see Section III.C for more details). Therefore in simulations of CNB, the parameters $K_{3,O2}$ and $K_{i2,HN3}$ in equation (4) were replaced by $K_{2,O2}$ and $K_{i1,HN3}$:

$$V_{NXRox} = V_{mNXR} \cdot \frac{[NO_2^-]_{tot}}{[NO_2^-]_{tot} + K_{1,NO2}} \cdot \frac{O_2}{O_2 + K_{2,O2}} \cdot \frac{1}{1 + HNO_2 / K_{i,HNO2} + NH_3 / K_{i1,HN3}} \quad (4')$$

We assumed, similarly to other models [34, 35] that all reactions are far from equilibrium and therefore irreversible. The efficiency of nitrification presented in Results was calculated at varying initial concentrations of ammonium and pH. The oxidation of ammonium and nitrite by AMO and NXR enzymes was considered to be inhibited by ammonia or nitric acid when the corresponding rates were < 5% of maximal rates, at saturated substrate levels. The system of ODEs was solved using MATLAB, integrated with the stiff solver ode15s (The MathWorks UK, Cambridge). The MATLAB code of the model is available from the authors upon request.

III. Results and Discussion

The modelled system includes a mixed culture of AOB and NOB or a monoculture of the proposed engineered bacterium (complete nitrifying bacterium, CNB). The model was focused on analysis of two experimentally observed phenomena: incomplete nitrification under high nitrogen loads [15] and production of N_2O under reduced O_2 conditions.

Therefore the model integrates various aspects of existing data and models, which are important in restriction of nitrification under various environmental conditions (pH, O_2; [13-15, 34] and production of N_2O [35, 36, 38]). The main processes included in the model are schematically shown in Fig. 1. Thus, the model describes O_2-accelerated nitrification and O_2-inhibited production of N_2O and their inhibition by non-ionized forms of ammonium (ammonia, NH_3) and nitrite (nitrous acid, HNO_2) [15, 38]. The inhibition of the second nitrification step - nitrite oxidation – by NH_3 and HNO_2 has a particularly strong effect on nitrification as described below. The mechanism of this inhibition seems to be largely related to inhibition of the electron transport chain [17].

During model construction, we made a couple of simplifying assumptions, as described in Section 2 in more detail. Briefly, we assumed Michaelis-Menten substrate dependence for all enzymes, which agrees with available data [13, 14, 35, 36]. We also assumed that pH and O_2 levels are maintained during each timecourse. Next, similar to existing models, we made a simplifying assumption that the transport of all compounds in and out of the cells is not rate-limiting, and therefore this factor was not included. Besides, we did not consider the dilution of nitrite due to its diffusion from AOB to NOB, because AOB and NOB cells live in densely packed mixed colonies forming biofilms, where nitrite, the product of an AOB cell, quickly enters the nearest NOB cell [10, 47]. Next, our model was not intended to provide quantitative description of bacterial growth, but rather to analyse the changes in enzymatic activities under different conditions. To model a mixed culture, we simply assumed a certain ratio of AOB and NOB enzyme activities, which is typically observed in a balanced culture of AOB and NOB, competent for complete nitrification.

After considering a mixed culture of AOB and NOB, we applied the model to CNB. We assumed that the CNB is based on an AOB bacterium, whose genome is extended by genes of the nitrite-oxidizing system. Therefore, the CNB uses the electron transport chain (ETC) from the AOB host, which is expected to dramatically change the nitrification efficiency. Indeed, the dependence of the whole process of nitrite oxidation on O_2 concentration is largely determined by the affinity of the terminal oxidase (a final element of ETC) for O_2 [34, 42]. Similarly, the inhibition of nitrite oxidation by NH_3 and HNO_2 seems to be largely related to the poisoning of the ETC by these compounds [15, 17, 48]. Therefore, in our model we assumed that the change of ETC components of CNB compared to NOB results in changes of the affinity of the nitrite oxidizing system of CNB for O_2 and changes in the NH_3 and HNO_2 toxicity. In particular, we assumed that these key characteristics of nitrite oxidation are identical for ammonium and nitrite oxidation (see Section 2). This avoids uncoupling between the AOB and NOB, as further discussed below. In the following we compared the behaviour of a mixture of AOB and NOB with the proposed CNB uni-cellular systems with respect to their capacity for complete nitrification of ammonium to nitrate, and for production of N_2O.

A. Working range of AOB and NOB nitrification at normal oxygen levels

The kinetics of nitrification in our model depend on the concentration of applied ammonium, in agreement with existing data [15]. For moderate concentrations of ammonium, it is completely converted to NO_3^- (at constant oxygen and pH, Fig. 2A). However, higher ammonium concentrations lead to inhibition of nitrification by non-ionized ammonium and nitrite (Fig. 2B; Fig. 1).

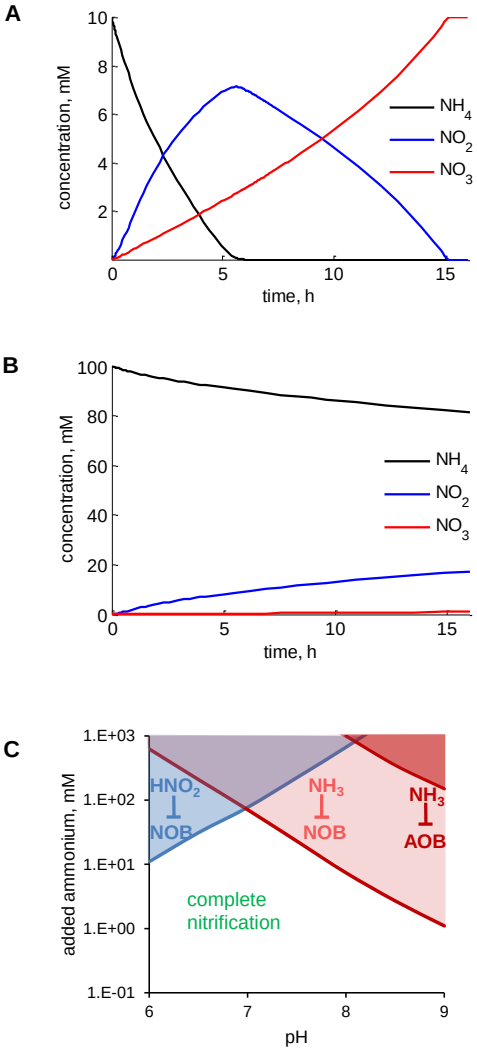

Fig. 2. Nitrification by a mixture of AOB and NOB at normal oxygen level (0.2 mM). A,B. Nitrification kinetics under normal (10 mM, A) and high (100 mM, B) levels of initial ammonium concentrations; pH=7. Black, blue and red lines correspond to the total concentrations of ammonium, nitrite and nitrate respectively. C. Dependence of nitrification efficiency on the initial concentration of added ammonium and pH. Ammonium oxidation by AOB or nitrite oxidation by NOB was considered to be not complete (inhibited by NH_3 or HNO_2) when the corresponding rate was less than 5% of the maximum rate.

Since the equilibrium between non-ionized and ionized forms of ammonium and nitrite is pH-dependent, the extent of the inhibition depends on pH [15]. The amount of HNO_2 increases at low pH and the amount of NH_3 increases at high pH; therefore different mechanisms of inhibition dominate at low and high pH [15]. The model demonstrates that complete nitrification happens only within a limited pH range and range of initial ammonium concentrations (Fig. 2C), in agreement with experimental observations [15]. The inhibition of the second stage of nitrification (nitrite oxidation) by NH_3 and HNO_2 has the most pronounced effect (Fig. 2C, Fig. 1). This results in accumulation of nitrite. In natural environments, nitrifiers live in biofilms, which might further increase the local concentrations of nitrite and thus cause more severe restrictions of the working range of nitrification.

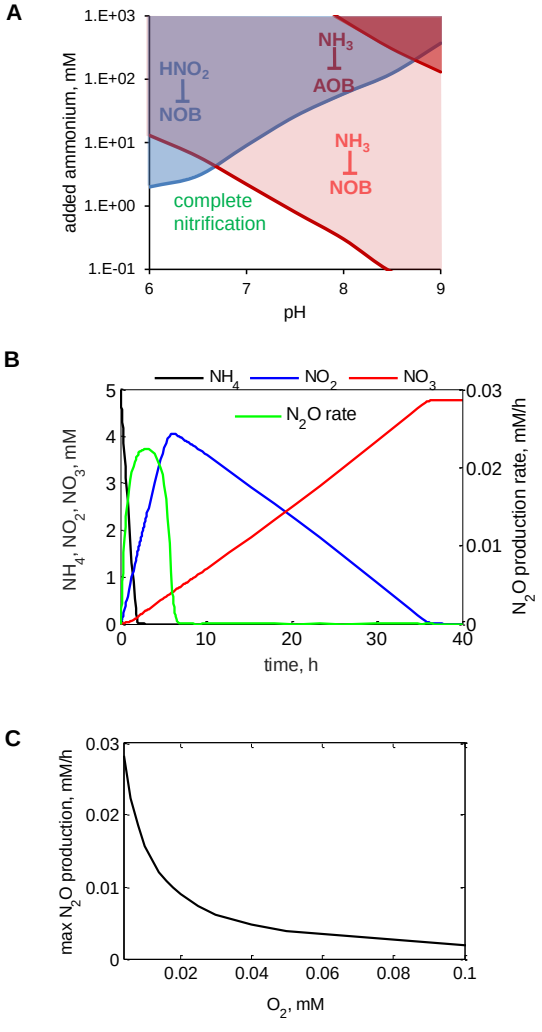

Fig. 3. Nitrification by AOB and NOB under reduced oxygen levels. A,B. The simulations were done for 0.006 mM O_2. A. Dependence of nitrification efficiency on the initial concentration of added ammonium and pH, calculated similarly to Fig. 2C. B. The kinetics of nitrification and production of N_2O. Black, blue and red lines correspond to the total ammonium, nitrite and nitrate concentrations respectively. Green line shows the rate of N_2O production. C. Dependence of the maximal rate of N_2O production on O_2 concentration. B,C. pH=7.5, initial ammonium 5 mM.

B. Working range of AOB and NOB nitrification and production of N_2O at reduced O_2 concentration

Under reduced-O_2 conditions, which often occur in eutrophic systems, nitrite oxidation might be additionally impaired due to lower affinity of NOB for O_2 compared to AOB [19]. Our model demonstrates that the range of pH and ammonium concentrations for complete nitrification is much narrower at reduced O_2 concentrations than at normal O_2 concentrations (Fig. 3A, Fig. 2C). In addition to the decreased efficiency of nitrification, reduced O_2 stimulates the reduction of nitrite to the greenhouse gas N_2O, mainly by AOB ([23, 24]; Fig. 1). Fig. 3B shows typical kinetics of AOB and NOB nitrification and accumulation of N_2O. Since the production of N_2O requires the presence of both electron acceptor (nitrite) and electron donor (hydroxylamine in our model; [35]), the production of N_2O ceases after the depletion of hydroxylamine (as in Fig. 3B) or ammonium. The maximal rate of N_2O production increases with decrease of O_2 (Fig. 3C), in agreement with experimental data [46]. This explains the observed high emission of N_2O by AOB from the low-O_2 regions of euthrophic lakes [18].

C. Improved nitrification and reduced production of N_2O in CNB

The negative effects of uncoupled ammonium and nitrite oxidation can be reduced in the CNB, which combines ammonium and nitrite oxidation in the same cell. The CNB might be developed with methods of genetic engineering, using AOB as a base, because AOB have higher tolerance to ammonium, higher affinity for O_2 and better growth compared to NOB [12, 15, 16]. As well as AOB genes, the CNB would have genes involved in nitrite oxidation and its regulation, including genes coding for the periplasmic NXR protein complex and membrane proteins that pass electrons from NXR to the ETC [11]. Potential candidate bacteria for the CNB are *Nitrosospira* (AOB) and *Nitrospira* (NOB) – the widespread freshwater AOB and NOB [39-43].

The uncoupling of ammonium and nitrite oxidation in mixed cultures is related to different sensitivity of AOB and NOB to NH_3 / HNO_2 inhibition and different affinities of AOB and NOB for O_2. The different affinities of AOB and NOB for O_2 are related to the different Km's of terminal oxidase components of AOB and NOB ETCs [35, 36, 42]. The NH_3 / HNO_2 toxicity also largely depends on the ETC properties, due to the poisoning of ETCs of AOB and NOB by ammonia, nitrite and the nitrite derivative NO [15, 17, 48]. Since in CNB ammonium and nitrite oxidation are coupled to the same ETC, in our model we assumed that the affinities of ammonium and nitrite oxidation to O_2, NH_3 and HNO_2 are the same. Our simulations demonstrate that this improves coupling between ammonium and nitrite oxidation, which reduces nitrite levels and

broadens the range of complete nitrification in CNB compared to a mixture of AOB and NOB (Fig. 4A,B, Fig. 3A,B). Additionally, the model predicts that the reduced accumulation of nitrite should decrease the production of N_2O by CNB compared to AOB (Fig. 4B, Fig. 3B). The production of N_2O is reduced in a broad range of possible O_2 concentrations (Fig. 4C) and the rate of N_2O production depends inversely on the activity of the nitrite-oxidizing enzyme NXR (Fig. 4D).

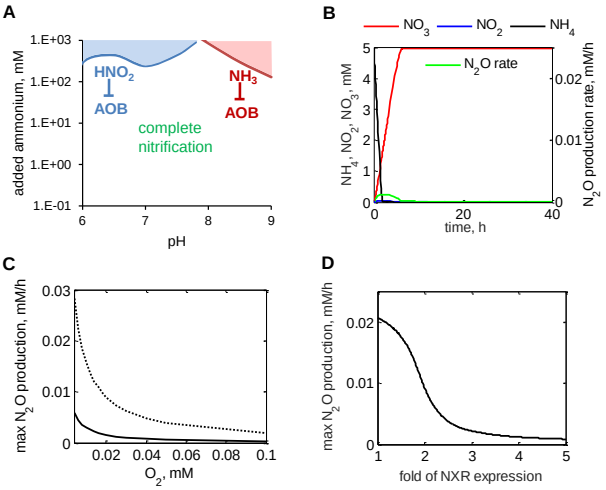

Fig. 4. Nitrification in CNB under reduced oxygen levels. A,B,D. The simulations were done for 0.006 mM O_2. A. Dependence of nitrification efficiency on the initial concentration of added ammonium and pH, calculated similarly to Fig. 2C. B. The kinetics of nitrification and N_2O production. Black, blue and red lines correspond to the total concentrations of ammonium, nitrite and nitrate respectively. The green line shows the rate of N_2O production. C. Dependence of the maximal rate of N_2O production on O_2 concentration. Solid line – CNB; dotted line – AOB, replotted from Fig. 3C. D. Dependence of the peak rate of N_2O production by CNB on NXR activity (fold expression relative to NOB). The simulations on A-C were done for 3-fold increased NXR expression relative to NOB. B-D. pH=7.5, initial ammonium 5 mM.

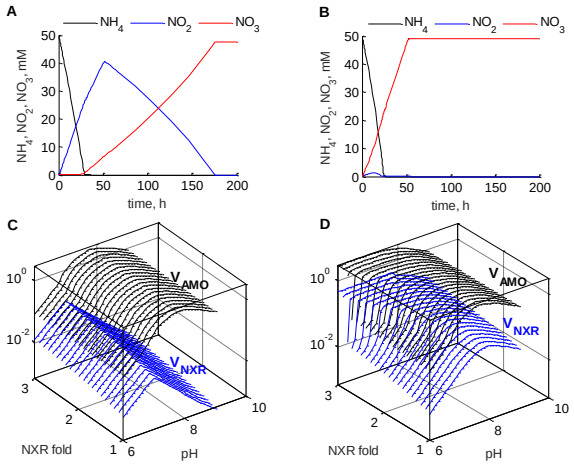

Fig. 5. Acceleration of the nitrification in CNB compared to two-bacterial system. A,B. Nitrification by a mixture of AOB and NOB (A) or by CNB (B) at 50 mM of initial ammonium, pH=8 and 3-fold increase of NXR level (VmNXR = 5.4 mM/h-1). Black, blue and red lines correspond to the total concentrations of ammonium, nitrite and nitrate respectively. C,D. Dependence of the minimal rates of AMO and NXR (for saturated substrate concentrations > 3Km) on pH and fold-change in NXR. Oxygen level is 0.006 mM.

Therefore, upregulation of NXR expression in CNB, for example by increasing the strength of the ribosome-binding site or the promoter, should reduce N_2O even further. Thus, a 3-fold increase of the NXR maximal rate in CNB compared to NOB causes a 9-fold reduction of N_2O production (Fig. 4B, Fig. 3B) and substantial extension of the nitrification range (Fig. 4A, Fig. 3A). The rates of ammonium and nitrite oxidation by CNB are higher than in two-bacterial system (Fig. 5A,B). This acceleration of the nitrification is observed in a broad range of pH and NXR activities (Fig. 5C,D).

An important consideration for the potential engineering of the proposed CNB is the metabolic cost of the production of additional NXR enzymes. Although the oxidation of nitrite is energetically favourable (ΔG ~ -74 kJ/mol), it provides less energy than ammonium oxidation (ΔG ~ -275 kJ/mol). Therefore we envisage that in order to be competitive with a two-bacterial system, the genome of the CNB should be further modified to remove some of the genes which are not necessary any more. In particular, the expression of genes dealing with toxic nitrite and its intermediate NO might be reduced, because nitrite levels are predicted to be low due to the high expression of NXR genes in the CNB. In particular, the expression of denitrification genes that eliminate nitrite in AOB might be reduced. There are two main denitrification enzymes in nitrifying bacteria: NIR and NOR. Interestingly, NOR, which reduces NO to N_2O is a highly abundant protein in nitrifying bacteria, which can comprise 10-30 % of the total protein content [12]. Therefore, a reduction of NOR level in the CNB might be an especially promising approach. As well as economizing cellular resources, the reduction of NOR level should result in substantial (up to 70 %; [45]) decrease of N_2O production. To make the CNB even more competitive with the two-bacterial system, the use of CNB for the restoration of eutrophic systems might be restricted to oxygenated zones. In practice, this could be achieved by an immobilization of biofilms with CNB colonies at shallow places of eutrophic lakes. In this case, the expression of other genes required for cell survival in absence of oxygen might also be reduced, which would increase the efficiency of CNB in oxygenated environments. Additionally, inclusion of transcriptional and posttranscriptional regulators of NXR expression from NOB into the CNB genome might be a potentially useful approach. For example, NXR is known to be co-expressed with NIR, which is in turn induced by nitrite or its intermediate NO in NOB [48]. This suggests that NXR expression might be feedback-regulated by the

actual nitrite/NO levels, which might help a CNB to reduce the cost of NXR expression. However, the molecular mechanisms of the regulation of NXR expression in NOB are not completely understood [11, 54]. Therefore it might be necessary to selectively and reversibly insert nitrogen-regulatory genes from NOB to CNB and test the nitrification efficiency. The reversible genomic transformations might be done using serine integrases and their recombination directionality factors (RDFs), which allow insertion and removal (if required) of DNA fragments with genes, promoters and ribosome-binding sites [55, 56]. The existence of multiple efficient serine integrases [57] allows individual insertion/removal of multiple genes or gene clusters, such as NOR-related genes and transcriptional regulators of NXR expression. Another important consideration for the practical implementation of CNB is the choice of a host cell. For example, the recently discovered complete nitrifier *Ca.Nitrospira inopinata* [33] might be a potential host. The *Ca.Nitrospira inopinata* belongs to the NOB group, but it possesses ammonium-oxidizing genes, which allow it to perform complete nitrification of ammonium to nitrate [33]. The possible disadvantage of using *Ca.Nitrospira inopinata* as a host for the CNB is its relatively low efficiency of nitrification. Thus, it was shown that it can oxidize up to 1 mM of ammonium, but NXR activity is relatively low, which results in initial accumulation of nitrite. Also, this bacterium has limited capacity to oxidize external nitrite. Therefore, additional genomic transformations would be required to apply this bacterium to eutrophic environments, which have high ammonium (>10 mM) and nitrite concentrations [18]. Therefore we think that using another nitrifying bacterium as a host for CNB would be a better solution. In particular we suggest that using of an AOB instead of a NOB would be beneficial, due to higher tolerance of AOB to ammonium and higher affinity for oxygen. Finally, we would like to mention that using a CNB in remediation of natural ecosystems should be done in a controlled way, possibly by using the ability of nitrifiers to form biofilms.

IV. Conclusions

Two separate types of bacteria are required for the complete oxidation of ammonium to nitrate (nitrification). High concentration of ammonium in eutrophic ecosystems often leads to uncoupling between the two bacteria, resulting in incomplete nitrification and accumulation of nitrite. This delays restoration of eutrophic lakes and rivers and elevates the emission of nitrous oxide – a powerful greenhouse gas. To resolve these ecological problems, we propose using a complete nitrifying bacterium (CNB) created by inserting nitrite-oxidizing genes into the genome of an ammonium-oxidizing bacterium. Our mathematical model demonstrates that this type of bacterium has the potential to improve the nitrification efficiency and reduce the emission of nitrous oxide from nutrient-overloaded water systems.

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