Bioenergetics analysis of ammonia-oxidizing bacteria and the estimation of their maximum growth yield

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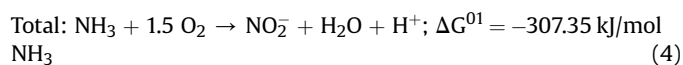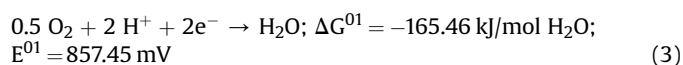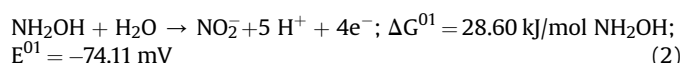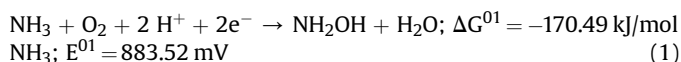
The currently accepted biochemistry and bioenergetics of ammonia-oxidizing bacteria (AOB) show an inefficient metabolism: only 53.8% of the energy released when a mole of ammonia is oxidised and less than two of the electrons liberated can be directed to the autotrophic anabolism. However, paradoxically, AOB seem to thrive in challenging conditions: growing readily in virtually most aerobic environment, yet limited AOB exist in pure culture. In this study, a comprehensive model of the biochemistry of the metabolism of AOB is presented. Using bioenergetics calculations and selecting the minimum estimation for the energy dissipated in each of the metabolic steps, the model predicts the highest possible true yield of 0.16 gBio/gN and a yield of 0.13 gBio/gN when cellular maintenance is considered. Observed yields should always be lower than these values but the range of experimental values in literature vary between 0.04 and 0.45 gBio/gN. In this work, we discuss if this variance of observed values for AOB growth yield could be understood if other non-considered alternative energy sources are present in the biochemistry of AOB. We analyse how the predicted maximum growth yield of AOB changes considering co-metabolism, the use of hydroxylamine as a substrate, the abiotic oxidation of NO, energy harvesting in the monooxygenase enzyme or the use of organic carbon sources.

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1. Introduction

Ammonia-oxidizing bacteria (AOB) have a fundamental role in the biogeochemical cycling of nitrogen and their presence both in natural and engineered industrial systems is generally desired as a mechanism to regulate the supply of loss of nitrogen from the environment (Prosser, 2005). For these reasons, AOB have been studied and their growth parameters have been extensively assessed (Kowalchuk and Stephen, 2001; Chain et al., 2003; Geets et al., 2006). They are able to oxidize NH_3 to NO_2^- to generate energy and reducing power for autotrophic growth (Sayavedra-Soto and Arp, 2011) following the catabolic steps showed in Equations (1)–(4)

$$NH_3 + O_2 + 2\,H^+ + 2e^- \rightarrow NH_2OH + H_2O;\ \Delta G^{01} = -170.49\ \text{kJ/mol}\ NH_3;\ E^{01} = 883.52\ \text{mV} \tag{1}$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5\,H^+ + 4e^-;\ \Delta G^{01} = 28.60\ \text{kJ/mol}\ NH_2OH;\ E^{01} = -74.11\ \text{mV} \tag{2}$$

$$0.5\,O_2 + 2\,H^+ + 2e^- \rightarrow H_2O;\ \Delta G^{01} = -165.46\ \text{kJ/mol}\ H_2O;\ E^{01} = 857.45\ \text{mV} \tag{3}$$

$$\text{Total:}\ NH_3 + 1.5\,O_2 \rightarrow NO_2^- + H_2O + H^+;\ \Delta G^{01} = -307.35\ \text{kJ/mol}\ NH_3 \tag{4}$$

The catabolism of AOB begins with the reaction catalysed by ammonia monooxygenase (AMO) in which NH_3 is oxidised to hydroxylamine with concomitant reduction of oxygen to water (Equation (1)). The reaction is highly exergonic. It was initially assumed that this energy is harvested by the cell (Drozd, 1976; Hollocher et al., 1982) but no experimental evidence has been provided (Sayavedra-Soto and Arp, 2011). Thus, the energy of this reaction is considered dissipated and therefore the only energy available for growth is a maximum of 136.86 kJ per mole of NH_3 recovered from the electron transport chain (Equation (3)) and corresponding to a 53.8% of the total energy of the catabolic process (307.35 kJ/mol NH_3, Equation (4)). Besides the two electrons

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released from the NH_3 oxidation to hydroxylamine, another two electrons are needed to turn over the AMO enzyme. These are coming from the subsequent hydroxylamine oxidation (Equation (2)), further reducing the number of electrons available for growth (Fig. 1). Overall, less than 53.8% of the energy released from catabolism and less than two of the electrons liberated from NH_3 oxidation are available for growth.

There appears to be a paradox, for AOB have metabolically speaking "one hand tied behind their back" and yet seem to thrive in challenging conditions. For example: i) AOB only consume free ammonia (NH_3) rather than the much more abundant ammonium ion (NH_4^+) (Suzuki et al., 1974) (at pH 7 only 0.6% is in the form of NH_3) and compete with microorganisms that have more affinity for it (Prosser, 2005); ii) AOB are found in conditions of low oxygen (Sliekers et al., 2005) or competing with microorganisms that have higher oxygen affinity (Geets et al., 2006); and iii) AMO exhibits high co-metabolic activity (Fig. 1). This enzyme oxidises many substrates other than ammonia, for no apparent energy gain and without further oxidation of the metabolite produced (Arp et al., 2002). Moreover, AOB grow better in complex natural or engineered systems but few are available in pure cultures (Kowalchuk and Stephen, 2001). Nearly all wastewater treatment plants can be induced to grow AOB, but few laboratories are able to sustain this group in pure culture. Finally, there appears to be a stable, and little understood, interdependence between AOB and heterotrophs (Khunjar et al., 2011; Keluskar et al., 2013).

In this work, we aim to describe in detail how AOB can harvest from the environment the energy and electrons necessary to grow on inorganic carbon. Known biochemistry and bioenergetics of the metabolic process are studied and a comprehensive model for AOB metabolism is proposed. We have used only thermodynamics analysis to calculate the maximum possible energy harvest per mole of NH_3 consumed. The minimum values for energy dissipation were selected through all the calculation process therefore, the yield calculated could be considered the maximum theoretically possible for AOB growth. Our results are compared against the theoretical and experimental values reported in literature. Other possible hypotheses about the biochemistry of AOB are also discussed and the growth yields associated to these are calculated. The bioenergetics analysis of the AOB metabolism aims to understand better its survival and therefore to be able to design more efficient processes for removal of nitrogen or other co-metabolites.

2. Materials and methods

2.1. Thermodynamic analysis of AOB metabolism and growth yield calculation

The biochemistry and bioenergetics of AOB metabolism are analysed in detail in the following sections. By means of this analysis, we approximate the energy harvested in each of the steps of the catabolic activity of the AOB per mole of nitrogen consumed ($\Delta G_{E+/N}$, Equation (5)). We do this by calculating the Gibbs energy of each chemical reaction of the catabolism and estimating the associated energy dissipation. At the same time, the energy required for each of the anabolic steps to form 1 mol of new biomass is approximated ($\Delta G_{E-/Bio}$, Equation (5)). For this, an average formula of biomass of $C_5H_7O_2N$ is considered with a molecular weight of 113 gBio/mol and a degree of reduction of 4 (McCarty, 2007). Further details on these calculations are presented in Section S1 and detailed in the Excel of Supplementary Information.

The energy balance of the AOB metabolism (Equation (5)), is calculated closing the carbon, nitrogen and electron balances of the catabolic and anabolic reactions. With this methodology, the maximum growth yield (Y_{XS}^{max}, in units of mole of biomass formed per mole of nitrogen consumed) together with the overall stoichiometry of the AOB metabolism can be assessed.

$$\frac{1}{Y_{XS}^{max}} = \frac{\sum_{j=1}^{j=S_{ana}} \Delta G_{E-/Bio}}{\sum_{i=1}^{i=S_{cat}} \Delta G_{E+/N}} + 1 \qquad (5)$$

In Equation (5), to calculate the energy balance between anabolism and catabolism, 1 mol of ammonia per mole of biomass is added in order to account for the nitrogen necessary to build new biomass ($C_5H_7O_2N$). S_{cat} and S_{ana} are the number of catabolic and anabolic steps respectively considered in the metabolism of AOB.

Based on the analysis presented, the growth yield of AOB is calculated and compared with the experimental values in the literature and those obtained applying two other widely used generic methods for yield calculation: The Thermodynamic Electron Equivalents revised Model (TEEM2) and The Energy Dissipation Method (see Section S2 and Excel of Supplementary Information for the description of these methods and the detailed calculation).

2.2. The biochemistry and bioenergetics of AOB catabolism

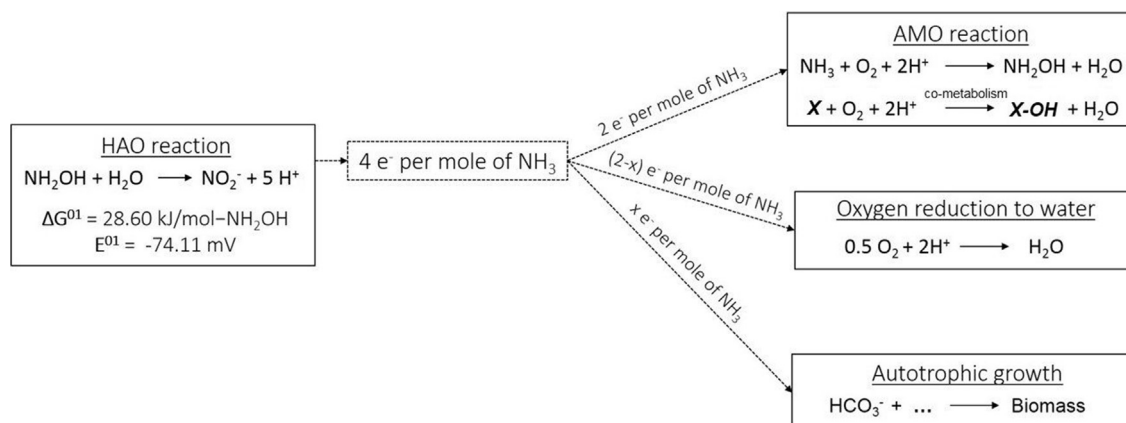The catabolic process of AOB can be divided into three main

Fig. 1. Hydroxylamine oxidation is the only source of electrons for the AOB metabolism. *X* refers to any co-metabolite.

steps, as presented in Equations (1)–(4) and further detailed in Fig. 2.

Overall, the maximum energy available is 307.35 kJ per mole of NH_3 (Equation (4)). However, not all this energy can be used effectively by the cell as there are biochemical limitations. The biochemistry and bioenergetics of each of the catabolic steps are evaluated in detail in the subsequent sections.

2.3. First step: ammonia oxidation to hydroxylamine

The oxidation of ammonia to hydroxylamine by AMO occurs with the concomitant reduction of one molecule of oxygen to water and releasing energy. One molecule of ammonia releases two electrons when oxidised to hydroxylamine while four electrons are required to reduce one molecule of oxygen. Therefore, two electrons have to come from some other electron donor. Since hydroxylamine oxidation is the only known source of electrons in the metabolism of AOB, hydroxylamine has to be the source of these two electrons (Figs. 2 and 3). How the electrons are transported to the AMO enzyme is still not elucidated. Ubiquinol has been proposed as electron donor but without experimental evidence (Simon and Klotz, 2013).

Although this is an exergonic reaction, there is no known mechanism for capturing the energy released, which has led to the
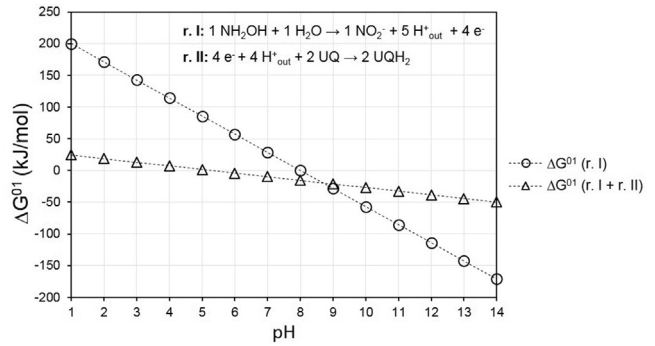

Fig. 3. Gibbs energy of the hydroxylamine oxidation reaction: Without considering the reduction of ubiquinone (r.I, dotted line with circles) and considering the reduction of ubiquinone coupled (r.I + r.II, dotted line with triangles).

assumption that this energy is fully dissipated as heat (Yuan and VanBriesen, 2002). This is the case of a wasteful metabolism: the energy released in AMO reaction (170.49 kJ/mol NH_3) represents 55.47% of the total amount of energy available in the AOB catabolism (307.35 kJ/mol NH_3) and it is enough to translocate up to 10.2 protons from the negative to the positive side of the cell membrane (calculated estimating the proton motive force according Equation
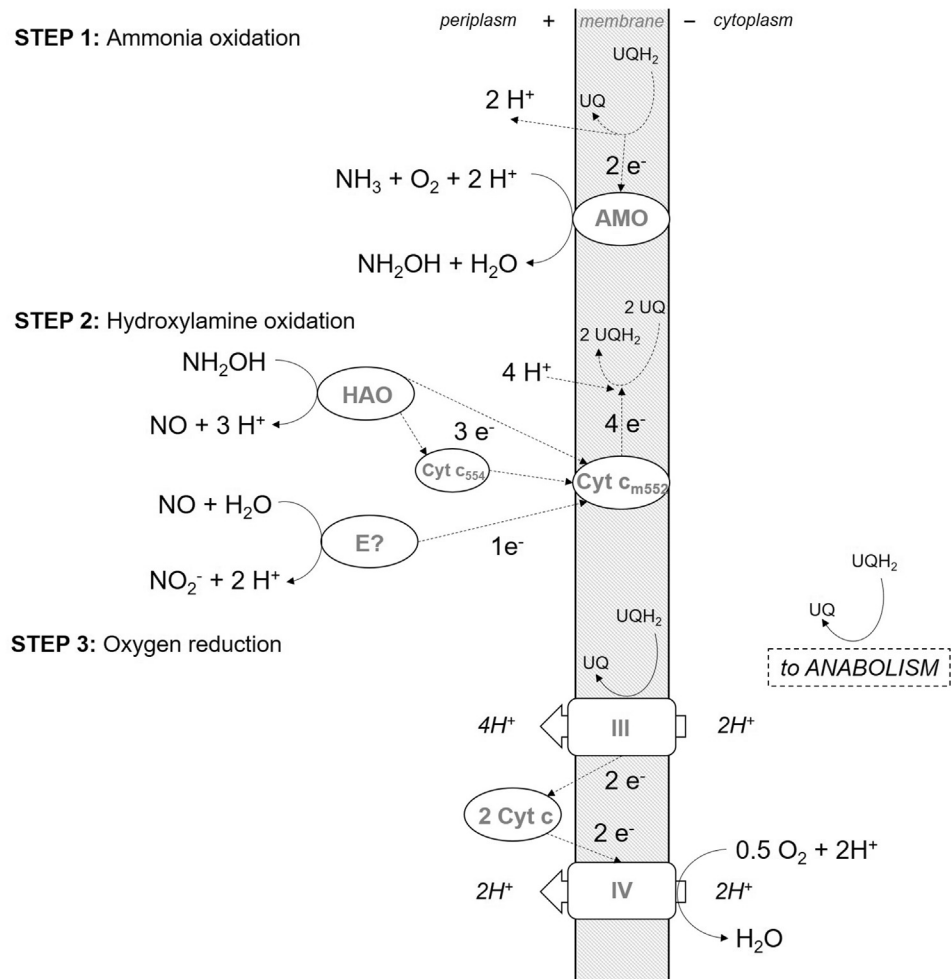

Fig. 2. The catabolic process of AOB. For clarity, only electron transport is detailed and not the energetic necessities of each step. Cyt refers to the cytochromes involved in the metabolic process. Complex III and IV of the respiratory-transport-chain are the sites where electron transport is coupled to proton translocation. Complex III is the ubiquinol-cytochrome c reductase and complex IV is the cytochrome c oxidase. Figure adapted from Simon and Klotz (2013).

S(1) of Supplementary Information).

2.4. Second step: hydroxylamine oxidation to nitrite

The second step is the oxidation of hydroxylamine to nitrite and occurs in the periplasm mediated by a bundle of enzymes in the "*Hydroxylamine Ubiquinone Redox Module*". Recently, the discovery of NO as the product of hydroxylamine oxidoreductase enzyme (HAO), added a new and currently unknown enzyme to the diagram, that is responsible of carrying the oxidation of NO to NO_2^- (Caranto and Lancaster, 2017). In this bundle of enzymes, the flow is assumed to be sequential and electroneutral. Sequential because the electrons flow from hydroxylamine to cytochrome *c554*, then to cytochrome *cm552*, and then to reduce *ubiquinone* to *ubiquinol* (UQ/UQH$_2$) (Fig. 2). Electroneutral because protons released in the reaction are consumed by the reduction of UQ/UQH$_2$ (Simon and Klotz, 2013).

In standard conditions, hydroxylamine oxidation is endergonic (28.60 kJ/mol NH$_2$OH) and none of these enzymes have been described coupled with energy sources. Nevertheless, four positive charges are generated in this reaction as the cytochromes only accept the four electrons (Fig. 2). Because the reaction is confined to the periplasm, this increases the membrane potential of the cell but also makes the Gibbs energy of the reaction highly dependent on the pH of the environment. The Gibbs energy calculations for this reaction predict that it would only be feasible at pH > 8 (Fig. 3).

The reaction of hydroxylamine oxidation is only possible if it couples with the reduction of two mole of ubiquinone using the protons released to the periplasm. Then, the overall reaction becomes exergonic for a larger range of pH (pH > 5) (Fig. 3).

The two moles of ubiquinol produced are the only source of reductive power generated in the catabolic process. The ubiquinol produced feeds with electrons the AMO enzyme, the oxygen reduction and the anabolism of AOB (Fig. 1).

2.5. Third step: oxygen reduction to water

The reduction of oxygen to water occurs at the end of the electron transport chain. The electrons flow from the oxidation of hydroxylamine and enter in the respiratory chain at the potential level of ubiquionone (Chain et al., 2003; Simon and Klotz, 2013). This is the only electron transport in the catabolic process of AOB proven to be coupled with proton translocations that lead to ATP production (Chain et al., 2003).

In Fig. 2, the complex III (ubiquinol-cytochrome c reductase) and complex IV (cytochrome *c* oxidase) are both coupling sites (i.e. the electron flow is coupled with proton translocations across the membrane). The consensus is that four protons are translocated for every two electrons traveling through complex III and two in complex IV which implies a total of six protons are translocated (White, 2007).

If no energy was dissipated, the Gibbs energy calculated is enough for the translocation of 8.8 protons. If six protons are assumed translocated, then the process has an efficiency of 68.56% in energy transfer. This is a conservative assumption: lower energy dissipation is assumed than by other methods proposed (McCarty, 2007).

2.6. The energetics demand of the AOB anabolism

Formation of biomass requires energy. In the literature, there are several theoretical and experimental approaches presented for estimating this necessary amount. Earlier reports showed that the energetic requirement may depend on the carbon source used, the type of microbial cell, and the conditions of the experiment (Farmer

and Jones, 1976; Stouthamer and Bettenhaussen, 1977). Nevertheless, some authors have proposed a fixed energy requirement to simplify the estimation of growth yield for different functional groups. For example, Stouthamer and Bettenhaussen (1973) suggested an average maximum value of 25 g dry-weight-biomass/mol ATP for the formation of any microbial cell, although lower yields were measured when using glucose as carbon source in aerobic conditions (Farmer and Jones, 1976).

In this case, to analyse the complex anabolic process, we divide it in several steps: reversed electron transport, fixation of inorganic carbon and biomass formation from glucose (Table 1). AOB uses inorganic carbon as main carbon source, therefore the first step is to fix the CO_2 in the Calvin cycle (Chain et al., 2003). In this process, 3 mol of CO_2 are reduced to 1 mol of phosphoglceraldehyde (PGAL) consuming 9 mol of ATP (White, 2007). PGAL is used as building block to produce the necessary proteins, phosphates, lipids and amino acids that generate new cells (Chain et al., 2003). The reduction of CO_2 in the Calvin cycle requires 20 electrons per mole of biomass. Because the electron donor is NAD(P)H. This in turn requires the reduction of NAD(P)$^+$ which further increases the energy demand of autotrophic growth and reduces the growth yield. The reduction of NAD(P)$^+$ is known to be energetically costly as it requires an increase in membrane potential which is thought to promote higher rates of energy dissipation (Sarewicz and Osyczka, 2015) and therefore significantly decrease the maximum growth yield (Liu et al., 2007; Heijnen and Kleerebezem, 2010; Heijnen and Van Dijken, 1992). Five times more energy is needed to transport electrons backwards in the electron transport chain than the energy harvested when the electrons flow goes forward (Kim and Geoffrey Michael, 2008).

The electrons used to reduce NAD(P)$^+$ in AOB metabolism can only come from the oxidation of hydroxylamine (Fig. 1) (Chain et al., 2003). The work of Aleem (1966) gives the stoichiometry of cytochrome *c* oxidised by NADP$^+$ versus ATP consumed. As cytochrome *c* is previously reduced by hydroxylamine, and by neglecting the energy difference that could exist between NADH and NADPH in physiological conditions (Spaans et al., 2015), we can calculate the amount of energy consumed by reverse electron transport and add it to the overall energy demand (see Section S1 in Supplementary Information).

To simplify the complex formation of new cells, we assume the formation of glucose from PGAL (which releases 2 mol of ATP) and we estimate the energy demand for the further formation of biomass from glucose using the value proposed by McCarty (McCarty 1971, 2007; Rittmann and McCarty, 2001) of 10.5 g dry-weight-biomass/mol ATP when glucose is the carbon source_EN-REF_8. We consider this value conservative as Kleerebezem and van Loosdrecht (2010) have shown that it underestimates the energy needed for cell formation.

2.7. Summary: the bioenergetics of AOB metabolism

The bioenergetics calculations for the overall AOB metabolism are presented in Table 1. The detailed balance of electrons, carbon and nitrogen used to calculate the maximum growth yield is presented in the Excel of the Supplementary Information.

Together with the energy needed for the formation of new biomass, some energy is required for maintenance of the actual cells and to compare the experimental yield with the one obtained theoretically this energy requirement also needs to be evaluated. This is not trivial as it is not clear if maintenance is a constant because it could decrease in periods of starvation (Geets et al., 2006). The calculation of the energy requirement for maintenance is discussed in Section S2 and detailed in the Excel of the Supplementary Information.

Table 1
Bioenergetics calculations for overall AOB metabolism. When the electron carriers are involved in the transfer of electrons or their physiological potential are unknown, only electrons with zero potential are considered. The ΔG^{01} values are calculated based on the Gibbs free energies of formation presented in Table S1 of Supplementary Information.

Catabolism

1. Ammonia oxidation:
   a) $UQH_2 \rightarrow UQ + 2H^+_{out} + 2e^-$; $\Delta G^{01}_{2,c} = -19.30$ kJ/molNH$_2$OH; $E^{01} = -100.00$ mV
   b) $NH_3 + O_2 + 2H^+_{out} + 2e^- \rightarrow NH_2OH + H_2O$; $\Delta G^{01}_1 = -170.50$ kJ/molNH$_3$; $E^{01} = 883.52$ mV
   TOTAL: $\Delta G^{01}_1 = -151.20$ kJ/molNH$_3$
   Energy harvested: $\Delta G_{E+/N,\,1} = 0$ kJ/molNH$_3$
2. Hydroxylamine oxidation:
   a) $NH_2OH + H_2O \rightarrow NO_2^- + 5\,H^+_{out} + 4e^-$; $\Delta G^{01}_{2,a} = 28.60$ kJ/molNH$_2$OH; $E^{01} = -74.11$ mV
   b) $2UQ + 4H^+_{out} + 4e^- \rightarrow 2UQH_2$; $\Delta G^{01}_{2,b} = -38.59$ kJ/molNH$_2$OH; $E^{01} = 100.00$ mV
   TOTAL: $\Delta G^{01}_2 = -9.99$ kJ/molNH$_2$OH;
   Energy harvested: $\Delta G_{E+/N,\,2} = 0$ kJ/molNH$_2$OH
3. Oxygen reduction:
   $0.5\ O_2 + UQH_2 \rightarrow H_2O + UQ$; $\Delta G^{01}_3 = -146.16$ kJ/molH$_2$O;
   Energy harvested: $\Delta G_{E+/N,\,3} = 6 \cdot \Delta\mu_{H^+} = 100.15$ kJ/molH$_2$O (31.48% energy dissipation)

Anabolism

4. Reversed electron transport:
   $10NAD(P)^+ + 10\ UQH_2 \rightarrow 10\ NAD(P)H + 10\ UQ$; $\Delta G^{01} = 1210.01$ kJ/molBio;
   Energy demanded: $\Delta G_{E-/Bio,\,4} = 10 \cdot 5 \cdot \Delta G_{ATP} = 2612.50$ kJ/molBio (53.68% energy dissipation)
5. Calvin cycle:
   $5HCO_3^- + 10NAD(P)H + 15H^+ + 40/3ATP \rightarrow 5/6Glucose + 10H_2O + 10NAD(P)^+ + 40/3ADP + 40/3Pi$
   $\Delta G^{01} = 430.27$ kJ/molBio (*without ATP*);
   Energy demanded: $\Delta G_{E-/Bio,\,5} = 40/3 \cdot \Delta G_{ATP} = 696.67$ kJ/molBio (38.24% energy dissipation)
6. Biomass formation from glucose:
   $5/6Glucose \rightarrow 1$ Biomass ($C_5H_7O_2N$)
   Energy demanded: $\Delta G_{E-/Bio,\,6} = 11.96 \cdot \Delta G_{ATP} = 624.79$ kJ/molBio (Approximation)

3. Results and discussion

3.1. Yield calculation: comparison with other methods

Based on the methods and assumptions described above, we have estimated the maximum growth yield (Y_{XS}^{max}) for AOB to be 0.16 gBio/gN-NH3. This value drops to 0.13 gBio/gN-NH$_3$ when maintenance was considered (see Section S2 and Excel of Supplementary Information). The calculation is conservative as low values of energy dissipation were considered: no losses of energy in proton translocations or in the ATP formation are assumed, and the conservative value of McCarty (2007), together with the energy estimated to fix CO$_2$, was used to calculate the energy requirements in the formation of new cells (Kleerebezem and van Loosdrecht, 2010). Therefore, the resulting yield can be considered a maximum thermodynamic threshold for AOB. In Table 2, we compared our results with the maximum growth yield given by The Energy Dissipation Method (Heijnen and Van Dijken, 1992) and the TEEM2 (McCarty, 2007) (see Section S2 and Excel of Supplementary Information).

The calculation approximated by applying the TEEM2 method directly gives a value of 0.59 gBio/gN-NH3, far larger than the one calculated here and the one estimated by The Energy Dissipation Method. In McCarty (2007) a modification of the method is introduced to correct the growth yield overestimation when an oxygenase reaction is involved in the catabolic process. The energy of this reaction is considered fully dissipated, so it must be removed from the overall energy of the catabolic process (Yuan and VanBriesen, 2002). If the correction is applied to the AOB metabolism, the TEEM2 method returns a value of 0.43 (Table 2), still far larger than the value calculated by the calculations presented in this manuscript.

The growth yield obtained with The Energy Dissipation Method (Heijnen and Van Dijken, 1992) is similar to the one presented in this study. However, when we directly apply this method, we close the energy balance considering the full energy released in the oxidation of NH$_3$ (307.35 kJ/mol NH$_3$) and therefore we implicitly assume that the energy of the monooxygenase reaction is harvested. Kleerebezem and van Loosdrecht (2010) also claim that a TEEM2 type correction is necessary for the monooxygenase reaction. This correction means subtracting the energy of the monooxygenase reaction from the overall ΔG^{01} value of catabolism. However, when the correction is applied, the calculated yield reduces to 0.06 gBio/gN-NH$_3$: a very low value for the AOB growth yield if we compare it with the experimental values presented in Table 3 (see Excel of Supplementary Information for detailed calculations).

3.2. Yield calculation: comparison with experimental values

The experimental growth yield values measured for AOB encompass the full range between 0.04 and 0.45 gBio/gN-NH$_3$ (Table 3). This makes the comparison between experimental and theoretical calculations rather difficult. Considering that the

Table 2
Comparison between theoretical and experimental yields obtained with different methods and the current approach in this study.

Theoretical growth Yield (Y_{XS}^{max})		gBio/gN-NH$_3$
According to this study		0.16
According to The Energy Dissipation method	No correction	0.13
	Monooxygenase correction	0.06
According to TEEM2 method ($\varepsilon = 0.3$)	No correction	0.59
	Monooxygenase correction	0.43

Table 3
Experimental growth yields values for AOB found in literature.

Experiment	Growth Yield (gBio/gN-NH$_3$)	Reference
Mixed culture	0.040–0.13	Sharma and Ahlert (1977)
N. europea	0.063–0.17	Keen and Prosser (1987)
N. europea	0.063	Engel and Alexander (1958)
N. europea	0.098–0.155	Wezernak and Gannon (1967)
N. europea	0.102	de Bruijn et al. (1995)
Mixed culture	0.105	Ahn et al. (2008)
Mixed culture	0.127	Jubany et al. (2008)
Mixed culture	0.140	Blackburne et al. (2007)
Mixed culture	0.147	Ciudad et al. (2006)
Mixed culture	0.148	Fang et al. (2009)
Mixed culture	0.180	Liu and Wang (2012)
Nitrifiers	0.1–0.3	Tchobanoglous and Burton (1991)
Mixed culture	0.203	Chandran and Smets (2000)
Mixed culture	0.33–0.340	Rittmann (1987), Rittmann et al. (1999), Rittmann and McCarty (2001),
Mixed culture	0.45	Bellucci et al. (2011)

Average Pure Culture: 0.11 σ = ± 0.04.
Average Mixed Culture: 0.21σ = ± 0.12.
Total Average: 0.17 σ = ± 0.10.

calculations conducted in this study are conservative with the estimations of the energy losses in the metabolic process, the values observed experimentally are expected to be in all cases lower than the calculated ones. This is true for the values reported in pure culture. However, the values reported when measured in experiments with mixed cultures are in average 65% higher than the observed yield predicted through the metabolic analysis. Nevertheless, the results observed by Ciudad et al. (2006), Blackburne et al. (2007), Jubany et al. (2008) and Fang et al. (2009) agree with the theoretical value estimated in our calculations.

3.3. Yield calculation: NO$_2^-$ as a nonenzymatic product of NO oxidation

The possibility that NO is oxidised abiotically is discussed in Caranto and Lancaster (2017). In the presence of O$_2$, nonenzymatic NO oxidation to NO$_2^-$ occurs and could compete with the activity of the unknown enzyme that carries this last step in AOB metabolism. Caranto and Lancaster 2017 show that the Gibbs energy of the oxidation of hydroxylamine is higher if NO instead NO$_2^-$ is the product. We also observe this, but as the oxidation of hydroxylamine is not coupled with energy harvesting and its oxidation to NO only releases three electrons instead of the four released when it is oxidised to NO$_2^-$, the maximum yield calculated for AOB when NO is considered the final product, drops to 0.10 gBio/gN-NH$_3$ (0.08 gBio/gN-NH$_3$ including maintenance, see Excel of Supplementary Information). Comparing these values with the experimental ones presented in Table 3, they can be considered too low and therefore the abiotic NO oxidation not representative.

3.4. Is the energy of the ammonia oxidation fully wasted?

The AMO reaction has not been shown to be coupled with any known energy harvesting mechanism (Simon and Klotz, 2013) and therefore it is considered as a necessary first metabolic step but without energetic benefit (Chain et al., 2003; Whittaker et al., 2000; Vajrala et al., 2013). However, how the electrons released in the hydroxylamine oxidation are provided to the AMO enzyme is also not solved and this electron transport could be associated with some energy harvesting process like proton translocation.

If no energy harvesting is coupled with AMO activity, then the ammonia-oxidizing bacteria will only harvest the energy of two electrons released from the oxidation of the hydroxylamine. The same number of electrons that are entering the electron transport chain for the nitrite-oxidizing bacteria (NOB). This can sound contradictory as the growth yield of NOB is lower than half of the value of the growth yield of AOB (Blackburne et al., 2007; Jubany et al., 2008). The explanation comes from the energy balance of the metabolism of NOB. The electron transfer for NOB is not fully elucidated (Simon and Klotz, 2013), however, the oxidation of HNO$_2$ into HNO$_3$ has a reduction potential around of 328 mV, which implies that ubiquinone cannot be considered the electron carrier of the reaction (UQ/UQH$_2$ 100 mV). Therefore, because electrons cannot enter at the level of the ubiquinone, in the electron transport chain the number of translocated protons per mole of nitrate consumed must be lower than six. An analysis of the energy balance of NOB is presented in the Excel of Supplementary Information. Assuming the same process for the anabolism of NOB as for the AOB, a maximum growth yield of 0.10 gBio/gN-NO$_2$ is calculated. This value perfectly agrees with the values presented by Jubany et al. (2008) and Blackburne et al. (2007).

The idea that the energy of the AMO reaction is lost is also supported because AOB fed hydroxylamine have shown a higher yield than AOB fed with NH$_3$ (Vajrala et al., 2013; de Bruijn et al., 1995). This observation agrees with our model. If we calculate the growth yield considering hydroxylamine as substrate, it reaches a value of 0.31 gBio/gN-NH$_2$OH (0.27 gBio/gN-NH$_2$OH including maintenance, see Excel of Supplementary Information).

3.5. Is the low selectivity of AMO enzyme of any benefit for AOB growth?

AOB are known to co-oxidize a broad spectrum of different substrates (carbon monoxide, methane, hydrocarbons, aromatic compounds, etc.) (Sayavedra-Soto and Arp, 2011). This secondary activity is considered as a fortuitous degradation and attributed to the low selectivity of the AMO. It is classified as co-metabolism, occurring concomitantly with NH$_3$ oxidation, and it is considered a futile microbial activity that is not yet fully biochemically described or understood (Wackett, 1996).

Co-oxidation of substrates other than NH$_3$ will lead to the futile use of electrons, electrons that will then not be available for microbial growth. In general, AOB cannot oxidize the product of these co-metabolisms or use them as carbon source. Therefore, the co-oxidation of substrates other than NH$_3$ will inevitably reduce the growth yield of AOB.

In some laboratory experiments, the ratio of co-oxidation of other molecules to ammonia has surprisingly high values (Hyman

et al., 1985; Hyman and Wood, 1984). If a ratio of 2:1 NH_3 to co-metabolite oxidised is considered, then the estimated true yield is just of 0.09 gBio/gN-NH_3 (see Excel of Supplementary Information). The growth yield of AOB with intense co-metabolic activity has not been extensively documented. Taher and Chandran (2013) reported a biomass production unaffected by high co-metabolic activity and other authors claim to observe AOB cultures maintained only by degrading co-substrates (Forrez et al., 2009; De Gusseme et al., 2009). Clearly, if we assume that the current biochemical model for AOB metabolism is correct, this cannot be possible: concomitant consumption of NH_3 is needed to observe co-metabolic activity.

The energy released in the AMO reaction allows for the maximum translocation of 10 protons. If, for example, only the translocation of 2 protons are considered coupled to the AMO reaction (which implies an 80.42% of energy dissipation), the maximum possible growth yield calculated would increase to 0.21 gBio/gN-NH_3 (0.18 gBio/gN-NH_3 including maintenance, see Excel of Supplementary Information). This could be a plausible value if we consider the range presented in Table 3.

However, to date we were not able to find any strong reason that supports energy harvesting in the AMO enzyme activity. This implies that AOB do not benefit from co-metabolism and indeed this can only be detrimental for its growth.

3.6. Is the presence of heterotrophic bacteria beneficial to AOB growth?

Heterotrophs and AOB are competitors for ammonia and especially oxygen. Nevertheless, in conditions of low organic matter concentrations, commensalism between AOB and heterotrophs has been proven, with AOB releasing organic compounds which are substrates for heterotrophs (Rittmann et al., 1994; Dolinšek et al., 2013).

Moreover, heterotrophs can degrade the products of the co-oxidation reactions catalysed by the AMO enzyme. This reduces the product inhibition observed in AOB and favours the complete degradation of complex molecules (Khunjar et al., 2011; Taher and Chandran, 2013). At the same time, heterotrophs are known to degrade organic matter to easily degradable substrates like pyruvate, lactate, acetate: known carbon sources for AOB in specific conditions (Chain et al., 2003; Schmidt, 2009). The use of an organic carbon for growth not only reduces the energetic cost of new biomass formation but also the electrons needed for the process, all together increasing AOB capacity to survive in unfavourable conditions. It has been observed that the growth yield of AOB increases 130% when using organic substrates (Prosser, 2005). In our study, if pyruvate is assumed to be the only carbon source for AOB, a maximum yield of 0.61 gBio/gN-NH_3 (0.35 gBio/gN-NH_3 including maintenance, see Excel of Supplementary Information) is obtained. However, AOB heterotrophic activity does not occur easily. For unidentified reasons, the growth of AOB on organic sources is inhibited at higher concentrations of potential carbon sources (Prosser, 2005) or by CO_2 presence (Schmidt, 2009).

The metabolism of AOB could be more nuanced than previously thought. It is possible that ammonia oxidizers harvest small amounts of energy from the co-metabolism of a variety of substrates or by partial use of organic carbon sources to grow. This could explain why observed yields of AOB are often higher than conventional theory would predict, especially when AOB grows in mixed microbial communities. There may exist a mutual benefit between AOB and heterotrophs (Sedlacek et al., 2016) but it might be difficult to observe, because it may rely on secondary substrates for both bacterial populations. However, in unfavourable conditions, this commensalism could be a source of small quantities of energy harvested per mole of electron donor or carbon source and

co-metabolism in AOB, could not have a direct energy benefit but could help promote the presence of heterotrophs around AOB colonies.

4. Conclusions

An in-depth analysis of the bioenergetics of the catabolism of AOB could not find strong reasons to conclude a direct benefit from ammonia oxidation other than the production of hydroxylamine as a source of electrons for the reductive catabolic and anabolic processes needed for AOB survival. This implies that co-metabolic activity would be always detrimental for AOB as it is a futile loss of highly necessary electrons. Nevertheless, even assuming a high co-metabolic activity of 2:1 NH_3:co-metabolite, the growth yield calculated would allow AOB to survive (0.08 gBio/gN-NH_3).

When comparing our predicted growth yield with the one obtained by generic methods for the calculation of microbial growth yields, the latter were found wanting. The application of the TEEM2 method resulted in higher growth yields than observed, and the Dissipation Method gives a value smaller than most of the experimental values (Table 3).

A maximum true yield (Y_{XS}^{max}) of 0.16 gBio/gN-NH3 (0.13 gBio/gN-NH3 when maintenance was considered) is predicted in our analysis. These values are in concordance with the experimental ones observed by Blackburne et al. (2007), Jubany et al. (2008), and others. Although in the literature there is a large range of experimental values and the average of these are above the one theoretically presented in this study.

The higher yields observed versus the ones theoretically predicted could only be explained with non-considered energy sources in the biochemistry of the AOB. Possibilities for AOB to harvest more energy per mole of NH_3 oxidised could be by either the aforementioned hypothetical energy coupling in the AMO enzyme reaction or the feasible consumption of organic carbon sources that might occur in specific conditions. Both could be the explanation of a more robust growth of AOB within mixed cultures with heterotrophic activity.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.01.054.

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