Research Article

Predicting Accumulation of Intermediate Compounds in Nitrification and Autotrophic Denitrification Processes: A Chemical Approach

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Nitrification and sulfur-based autotrophic denitrification processes can be used to remove ammonia from wastewater in an economical way. However, under certain operational conditions, these processes accumulate intermediate compounds, such as elemental sulphur, nitrite, and nitrous oxide, that are noxious for the environment. In order to predict the generation of these compounds, an analysis based on the Gibbs free energy of the possible reactions and on the oxidative capacity of the bulk liquid was done on case study systems. Results indicate that the Gibbs free energy is not a useful parameter to predict the generation of intermediate products in nitrification and autotrophic denitrification processes. Nevertheless, we show that the specific productions of nitrous oxide during nitrification, and of elemental sulphur and nitrite during autotrophic denitrification, are well related to the oxidative capacity of the bulk liquid.

1. Introduction

Removal of reduced nitrogen species from wastewater is conventionally carried out by means of nitrification and denitrification biological processes, where ammonia nitrogen (NH₄⁺) is first converted to nitrate (NO₃⁻) and then to nitrogen gas (N₂) [1]. Wastewater nitrification occurs under aerobic conditions whereas denitrification is anaerobic. Nitrification is conducted in two consecutive steps by nitrifying microorganisms: ammonia conversion into nitrite by ammonia oxidizers (i.e., partial nitrification) and then nitrite conversion into nitrate by nitrite oxidizers. The most studied nitrifiers are ammonia-oxidizing bacteria (AOB), such as Nitrosomonas, and nitrite-oxidizing bacteria (NOB), where Nitrobacter is the most referenced genus. Nitrifiers are chemolithotrophic and their activity decreases as the pH is reduced below neutrality [2].

Typically, denitrification relies on the oxidation of organic carbon by heterotrophic bacteria, and readily biodegradable carbon sources such as methanol, ethanol, and acetate,
must be added externally to treatment plants [3]. Organic
electron donors are expensive and have high biomass yields,
leading to higher operational costs and sludge production [4].
Autotrophic denitriﬁcation is an alternative process for the
reduction of nitrate or nitrite (NO$_3^-$), which is accomplished
by oxidation of inorganic electron donors, including different
forms of sulfur such as sulphide [5]. Among denitriﬁying
bacteria that are able to use sulfur compounds as electron
donors is *Thiobacillus denitriﬁcans* which is the most studied.
This species of bacteria has its optimal growth conditions
at pH 7.5-8.0 [6]. Autotrophic denitriﬁcation supported by
sulfur possesses several advantages over common denitriﬁcation,
because sulfur compounds are more cost-effective and have
much lower sludge production [7]. Thus, the combination of
nitrification and sulfur-based denitriﬁcation is attracting
increasing interest in recent years. Particularly, these pro-
desses are gaining attention in order to remove nitrogen from
anaerobic reactor eﬄuents containing low organic matter
and high ammonia and sulphide (S$^-$) concentrations [7].
However, under certain operational conditions ammonia and
sulphide removal processes generate undesirable interme-
диates such as nitrous oxide (N$_2$O), nitrite, and elemental
sulphur (S$^0$). Nitrous oxide is a potent greenhouse gas, which
can be produced in the aerobic nitrification process by AOB
in presence of low dissolved oxygen (DO) concentrations
and nitrite accumulation [8]. Under aerobic conditions, AOB metabolism can generate N$_2$O through hydroxylamine
oxidation or nitrite oxidation [8] while NOB are not able
to produce this compound in presence of oxygen [9]. On
the other hand, the accumulation of nitrite and elemental
sulphur can occur during autotrophic denitriﬁcation [10].
The presence of these compounds should be avoided to
maintain the stability of the process due to the toxic effect of
nitrite on the sulphide oxidation rate [11] and the decrease of
biomass activity due to the formation of sulphur precipitates
[12]. The accumulation of both compounds could be related
to the $S/N$ ratio of the influent [13, 14]. S/N ratio may have
a major inﬂuence on the distribution of sulphide oxidation and
nitrate reduction end-products and on their simultaneous
removal [10, 15, 16].

Under a thermodynamic point of view, the formation of
redox intermediates could be predicted considering the Gibbs
free energy ($\Delta G$) of the potential reactions, normalized to
the number of moles of electrons (e$^-$) transferred in such reac-
tions, since the most energetically favorable reactions would
be preferentially used by microorganisms [18]. Alternatively,
to predict the reactions that can take place in a speciﬁc aque-
ous system, Scott and Morgan [17] proposed the use of a
conservative parameter called oxidative capacity (OXC)
which represents the total number of transferable electrons in
a given system. This parameter is deﬁned as the equivalent
sum of all oxidants that can be reduced with a strong reduct-
ant to an equivalence point. At every equivalence point a
diﬀerent electron condition deﬁnes a reference level of elec-
trons. By using the OXC concept, the information about the
chemical composition of the bulk liquid is condensed into a
single descriptive parameter which can be easily calculated as

$$\text{OXC (equivalents/L)} = \sum n_i \cdot [\text{Ox}_i] - \sum n_i \cdot [\text{Red}_i]$$

where [Ox]$_i$ and [Red]$_i$ represent the concentration (molar)
of the individual oxidants and reductants of the system and
$n_i$ is the number of equivalent electrons that are transferred.

The objective of this research is to determine if the Gibbs
free energy or the oxidative capacity are useful parameters
to predict the possible accumulation of intermediate com-
ponents during nitrification and autotrophic denitriﬁcation
processes.

2. Materials and Methods

2.1. Experimental Data. The production of intermediate
compounds in nitrification and autotrophic denitriﬁcation
processes was analyzed using data from experimental bench-
scale reactors. Measurements of N$_2$O emissions in nitrifi-
cation were those obtained by Campos et al. [9], during
the operation of a nitrifying bioﬁlm airlift suspended (BAS)
reactor under different dissolved oxygen concentrations. The
nitrifying BAS reactor was of 2.6 L, continuously fed with
a synthetic medium containing 500 mg NH$_4^+$-N per liter,
operated at 23°C and a hydraulic retention time of 8 h
under three diﬀerent DO concentration conditions: 1, 2,
and 5 mg O$_2$ per liter. After 1 week of operation, once a
constant composition of the liquid phase was achieved for
each condition, quick changes (3-5 min in length) in the DO
concentration were carried out (Table 1). DO concentrations
tested in these quick-change assays were 0.5, 1, 2, and 5 mg O$_2$
per liter. Thus, a total of 12 conditions were evaluated for the
production of N$_2$O (Table 1). Periodical samples of the outlet
gas were taken until verifying that a constant concentration
of N$_2$O was reached for each DO concentration tested.

The accumulation of elemental sulfur and nitrite during
autotrophic denitriﬁcation was studied using data from
Fajardo et al. [12], for a denitriﬁying sequencing batch reactor
(SBR) simultaneously removing nitrate and sulphide oper-
dated during 220 days. The SBR had a working volume of 1 L
and was fed with a synthetic medium containing nitrate and
sulphide (500 mg NO$_3^-$-N/L and 100-450 mg S$^{2-}$/L) using
different loading rates in 9 stages (Table 1). The reactor was
operated at 30°C and at a ﬁxed hydraulic retention time of
1 d.

2.2. Calculations. The reactions involved during the analyzed
processes of ammonia oxidation and of autotrophic denitriﬁ-
cation with sulphur compounds and related to the production
of NO$_3^-$, N$_2$O, and S$^0$ as intermediate compounds, are listed
in Table 2. The Gibbs free energy value was calculated taking
into account the concentrations of the diﬀerent compounds
for each operational condition, normalized to the number of
moles of e$^-$ transferred in the reaction ($\Delta G/e^-$).

To determine the oxidative capacity for the diﬀerent oper-
ating conditions, ﬁrst, in order to make a “redox ladder” [17],
the redox potential of each half reaction involved in ammonia
oxidation and autotrophic denitriﬁcation was calculated as a
function of pH (Table 3), using the experimental data sum-
marized in Table 1. Table 4 shows that, for both nitrification
and denitriﬁcation, the related redox potential values were
similar for all the operational stages. A “redox ladder” was
set using the calculated potentials (Figure 1). In the case
Table 1: Summary of performed experiments of nitrification and autotrophic denitrification processes [9, 12] for the evaluation of the production of N$_2$O, S$^0$ and NO$_3^-$ as intermediate compounds (subscript "i" indicates "influent" and subscript "e" indicates "effluent").

<table>
<thead>
<tr>
<th>Nitrification</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>NH$_4^+$-N$_i$ (mg/L)</th>
<th>NH$_4^+$-N$_e$ (mg/L)</th>
<th>NO$_2^-$-N$_i$ (mg/L)</th>
<th>NO$_2^-$-N$_e$ (mg/L)</th>
<th>DO tested (mg/L)</th>
<th>N$_2$O-N production (gN$_2$O-N/g VSS⋅d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>7.5</td>
<td>23</td>
<td>500</td>
<td>124</td>
<td>170</td>
<td>170</td>
<td>0.5</td>
<td>10.6</td>
</tr>
<tr>
<td>Stage 2</td>
<td>7.5</td>
<td>23</td>
<td>500</td>
<td>124</td>
<td>170</td>
<td>170</td>
<td>1.0</td>
<td>18.7</td>
</tr>
<tr>
<td>Stage 3</td>
<td>7.5</td>
<td>23</td>
<td>500</td>
<td>124</td>
<td>170</td>
<td>170</td>
<td>2.0</td>
<td>13.8</td>
</tr>
<tr>
<td>Stage 4</td>
<td>7.5</td>
<td>23</td>
<td>500</td>
<td>124</td>
<td>170</td>
<td>170</td>
<td>5.0</td>
<td>12.4</td>
</tr>
<tr>
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<td>23</td>
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<td>124</td>
<td>170</td>
<td>170</td>
<td>0.5</td>
<td>2.3</td>
</tr>
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<td>500</td>
<td>124</td>
<td>170</td>
<td>170</td>
<td>1.0</td>
<td>9.8</td>
</tr>
<tr>
<td>Stage 7</td>
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<td>23</td>
<td>500</td>
<td>124</td>
<td>170</td>
<td>170</td>
<td>2.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Stage 8</td>
<td>7.5</td>
<td>23</td>
<td>500</td>
<td>124</td>
<td>170</td>
<td>170</td>
<td>5.0</td>
<td>6.6</td>
</tr>
<tr>
<td>Stage 9</td>
<td>7.5</td>
<td>23</td>
<td>500</td>
<td>9</td>
<td>3</td>
<td>451</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Stage 10</td>
<td>7.5</td>
<td>23</td>
<td>500</td>
<td>9</td>
<td>3</td>
<td>451</td>
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<td>Stage 11</td>
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<td>23</td>
<td>500</td>
<td>9</td>
<td>3</td>
<td>451</td>
<td>2.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Stage 12</td>
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<td>500</td>
<td>9</td>
<td>3</td>
<td>451</td>
<td>5.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Autotrophic denitrification</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>NO$_3^-$-N$_i$ (mg/L)</th>
<th>S$^2$-S$_i$ (mg/L)</th>
<th>NO$_3^-$-N$_e$ (mg/L)</th>
<th>S$^2$-S$_e$ (mg/L)</th>
<th>NO$_3^-$-N$_e$ (mg/L)</th>
<th>SO$_4^{2-}$-S$_e$ (mg/L)</th>
<th>S$^2$ specific production (mg S$^2$/g VSS⋅d)</th>
<th>NO$_3^-$ specific production (mg NO$_3^-$-N/g VSS⋅d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1*</td>
<td>7.8</td>
<td>30</td>
<td>450</td>
<td>200</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Stage 2</td>
<td>8.0</td>
<td>30</td>
<td>450</td>
<td>250</td>
<td>0.9</td>
<td>110</td>
<td>294</td>
<td>291</td>
<td>125</td>
<td>6.5</td>
</tr>
<tr>
<td>Stage 3</td>
<td>8.6</td>
<td>30</td>
<td>450</td>
<td>300</td>
<td>0.7</td>
<td>12</td>
<td>291</td>
<td>128</td>
<td>30.8</td>
<td>18.3</td>
</tr>
<tr>
<td>Stage 4</td>
<td>9.3</td>
<td>30</td>
<td>450</td>
<td>200</td>
<td>1.0</td>
<td>22</td>
<td>362</td>
<td>24</td>
<td>32.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Stage 5*</td>
<td>7.5</td>
<td>30</td>
<td>450</td>
<td>100</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Stage 6</td>
<td>8.2</td>
<td>30</td>
<td>450</td>
<td>150</td>
<td>0.9</td>
<td>94</td>
<td>306</td>
<td>266</td>
<td>-13.0</td>
<td>15.7</td>
</tr>
<tr>
<td>Stage 7</td>
<td>8.4</td>
<td>30</td>
<td>450</td>
<td>300</td>
<td>1.0</td>
<td>13</td>
<td>309</td>
<td>160</td>
<td>24.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Stage 8</td>
<td>7.7</td>
<td>30</td>
<td>450</td>
<td>350</td>
<td>0.9</td>
<td>77</td>
<td>239</td>
<td>375</td>
<td>-3.2</td>
<td>12.9</td>
</tr>
<tr>
<td>Stage 9</td>
<td>7.7</td>
<td>30</td>
<td>450</td>
<td>450</td>
<td>0.9</td>
<td>79</td>
<td>154</td>
<td>474</td>
<td>-3.3</td>
<td>13.2</td>
</tr>
</tbody>
</table>

*Stable values were not achieved
of ammonia oxidation, NH$_4^+$ was selected as the electron reference level from the redox ladder while bisulphide (HS$^-$) was chosen for autotrophic denitrification, since these are the main species at the analyzed pH conditions. Then, the OXC was calculated according to (2) and (3) for ammonia oxidation and denitrification processes, respectively:

OXC (equivalents/L) = 4 · [DO] / 16000 + 6 · [NO$_2^-$-N] / 14000

OXC (equivalents/L) = 5 · [NO$_3^-$-N] / 14000 + 3 · [NO$_2^-$-N] / 14000 + 8 · [SO$_4^{2-}$-S] / 32000

where [DO] is the dissolved oxygen concentration (mg O$_2$/L), [NO$_3^-$-N] is the nitrogen concentration as nitrate (mg NO$_3^-$-N/L), [NO$_2^-$-N] is the nitrogen concentration as nitrite (mg NO$_2^-$-N/L), and [SO$_4^{2-}$-S] is the sulfur concentration as sulphate (mg SO$_4^{2-}$-S/L).

Table 2: Reactions involved during ammonia oxidation and autotrophic denitrification processes, number of e$^-$ transferred and ΔG (kJ) for each reaction.

<table>
<thead>
<tr>
<th>Process</th>
<th>Reaction</th>
<th>e$^-$</th>
<th>ΔG (kJ)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial nitrification</td>
<td>(i) NH$_4^+$ + 3/2 O$_2$ → NO$_2^-$ + 2 H$^+$ + H$_2$O</td>
<td>6</td>
<td>−273.0 + RT ln [NO$_2^-$]$^<em>$ [H$^+$]$^{3/2}$ / [NH$_4^+$]$^</em>$ [O$_2$]$^{3/2}$</td>
</tr>
<tr>
<td></td>
<td>(ii) 2 NH$_4^+$ + O$_2$ → N$_2$O + 2 H$^+$ + 3 H$_2$O</td>
<td>8</td>
<td>−526.6 + RT ln [N$_2$O]$^<em>$ [H$^+$]$^{3/2}$ / [NH$_4^+$]$^</em>$ [O$_2$]$^{3/2}$</td>
</tr>
<tr>
<td>Autotrophic denitrification</td>
<td>(iii) 5 HS$^-$ + 2 NO$_3^-$ + 7 H$^+$ → S + N$_2$ + 6 H$_2$O</td>
<td>10</td>
<td>−990.1 + RT ln [N$_2$]$^<em>$ [HS$^-$]$^</em>$ [NO$_3^-$]$^<em>$ [H$^+$]$^</em>$</td>
</tr>
<tr>
<td></td>
<td>(iv) 5 HS$^-$ + 8 NO$_3^-$ + 3 H$^+$ → 5 SO$_4^{2-}$ + 4 N$_2$ + 4 H$_2$O</td>
<td>40</td>
<td>−3726.9 + RT ln [SO$_4^{2-}$]$^<em>$ [N$_2$]$^</em>$ [HS$^-$]$^<em>$ [NO$_3^-$]$^</em>$ [H$^+$]$^*$</td>
</tr>
<tr>
<td></td>
<td>(v) HS$^-$ + NO$_3^-$ + H$^+$ → S + NO$_2^-$ + H$_2$O</td>
<td>2</td>
<td>−136.6 + RT ln [HS$^-$]$^<em>$ [NO$_3^-$]$^</em>$ [H$^+$]$^*$</td>
</tr>
<tr>
<td></td>
<td>(vi) HS$^-$ + 4 NO$_3^-$ → SO$_4^{2-}$ + H$^+$ + 4 NO$_2^-$</td>
<td>8</td>
<td>−499.7 + RT ln [HS$^-$]$^<em>$ [NO$_3^-$]$^</em>$ [SO$_4^{2-}$]$^<em>$ [H$^+$]$^</em>$</td>
</tr>
<tr>
<td></td>
<td>(vii) 5 S + 6 NO$_3^-$ + 2 H$_2$O → 5 SO$_4^{2-}$ + 4 H$^+$ + 3 N$_2$</td>
<td>30</td>
<td>−2736.8 + RT ln [SO$_4^{2-}$]$^<em>$ [H$^+$]$^</em>$ [N$_2$]$^*$</td>
</tr>
<tr>
<td></td>
<td>(viii) S + 3 NO$_3^-$ + H$_2$O → SO$_4^{2-}$ + 2 H$^+$ + 3 NO$_2^-$</td>
<td>6</td>
<td>−414.9 + RT ln [SO$_4^{2-}$]$^<em>$ [H$^+$]$^</em>$ [N$_2$]$^*$</td>
</tr>
</tbody>
</table>

Table 3: Half reactions involved during ammonia oxidation and autotrophic denitrification written as reduction processes ($E_{H^+}$ was calculated considering [Ox]/[Red]=1 [15]).

<table>
<thead>
<tr>
<th>Process</th>
<th>Half reaction</th>
<th>$E_{H^+}$ (V)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial nitrification</td>
<td>1/4 O$_2$ + H$^+$ + e$^-$ → 1/2 H$_2$O</td>
<td>1.23 − 2.303 · $RT / F$ · pH</td>
</tr>
<tr>
<td></td>
<td>1/8 N$_2$O + 5/4 H$^+$ + e$^-$ → 1/8 H$_2$O + 1/4 NH$_4^+$</td>
<td>2.04 − 2.303 · $RT / F$ · 5 · pH</td>
</tr>
<tr>
<td></td>
<td>1/6 NO$_2^-$ + 4/3 H$^+$ + e$^-→ 1/6$ NH$_4^+$ + 1/3 H$_2$O</td>
<td>0.89 − 2.303 · $RT / F$ · 4 · pH</td>
</tr>
<tr>
<td>Autotrophic denitrification</td>
<td>1/5 NO$_3^-$ + 6/5 H$^+$ + e$^-$ → 1/10 N$_2$ + 3/5 H$_2$O</td>
<td>1.25 − 2.303 · $RT / F$ · 6 · pH</td>
</tr>
<tr>
<td></td>
<td>1/3 NO$_2^-$ + 4/3 H$^+$ + e$^-→ 1/6$ N$_2$ + 2/3 H$_2$O</td>
<td>0.42 − 2.303 · $RT / F$ · 4 · pH</td>
</tr>
<tr>
<td></td>
<td>1/8 SO$_4^{2-}$ + 9/8 H$^+$ + e$^-→ 1/8$ HS$^- + 1/2$ H$_2$O</td>
<td>0.25 − 2.303 · $RT / F$ · 8 · pH</td>
</tr>
<tr>
<td></td>
<td>1/2 S + 1/2 H$^+$ + e$^-→ 1/2$ HS$^-</td>
<td>−0.11 − 2.303 · $RT / F$ · 1 · pH</td>
</tr>
</tbody>
</table>

* $R$ is the ideal gas constant (8.31·10$^{-5}$ kJ/mol·K); $T$ is the operational temperature (K); concentrations are given as molar (M).

* $F$ is the Faraday constant (96,500 C/mol)
Table 4: E° calculation of the redox half-reactions involved during ammonia oxidation and autotrophic denitrification processes under the operating conditions of each studied stage.

<table>
<thead>
<tr>
<th>Nitrification</th>
<th>N₂O reduction to NH₄⁺ (V)</th>
<th>O₂ reduction to H₂O (V)</th>
<th>NO₂⁻ reduction to NH₄⁺ (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>1.49</td>
<td>0.79</td>
<td>0.30</td>
</tr>
<tr>
<td>Stage 2</td>
<td>1.49</td>
<td>0.79</td>
<td>0.30</td>
</tr>
<tr>
<td>Stage 3</td>
<td>1.49</td>
<td>0.79</td>
<td>0.30</td>
</tr>
<tr>
<td>Stage 4</td>
<td>1.49</td>
<td>0.79</td>
<td>0.30</td>
</tr>
<tr>
<td>Stage 5</td>
<td>1.49</td>
<td>0.79</td>
<td>0.30</td>
</tr>
<tr>
<td>Stage 6</td>
<td>1.49</td>
<td>0.79</td>
<td>0.30</td>
</tr>
<tr>
<td>Stage 7</td>
<td>1.49</td>
<td>0.79</td>
<td>0.30</td>
</tr>
<tr>
<td>Stage 8</td>
<td>1.49</td>
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<td>0.30</td>
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<td>Stage 9</td>
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<td>0.30</td>
</tr>
<tr>
<td>Stage 10</td>
<td>1.49</td>
<td>0.79</td>
<td>0.30</td>
</tr>
<tr>
<td>Stage 11</td>
<td>1.49</td>
<td>0.79</td>
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<th>NO₃⁻ reduction to N₂ (V)</th>
<th>NO₂⁻ reduction to N₂ (V)</th>
<th>SO₄²⁻ reduction to HS⁻ (V)</th>
<th>S reduction to HS⁻ (V)</th>
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</thead>
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<td>Stage 8</td>
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<td>Stage 9</td>
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<td>-0.34</td>
</tr>
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</table>

Figure 1: “Redox ladder” for the ammonia oxidation and autotrophic denitrification processes (adapted from Scott and Morgan [17]).
individual value to fall into 95% of the time. Determined, being the range in which one can expect any was considered significant. A 95% prediction interval was generated of N

free energy does not explain nitrous oxide generation. The

characteristic (p-values and R²) and standardized residuals were studied. The confidence and prediction intervals were calculated by using the F distribution and analysis of variance (ANOVA) test. P-value ≤ 0.05 was considered significant. A 95% prediction interval was determined, being the range in which one can expect any individual value to fall into 95% of the time.

3. Results and Discussion

3.1. Gibbs Free Energy. The calculation of the Gibbs free energy shows that, for all of the operational conditions tested in the nitrification experiments, oxidation of ammonia to nitrous oxide provides higher energy per mol of e⁻ transferred to microorganisms than its oxidation to nitrite (Figure 2(a)). This fact could justify that nitrous oxide production was always detected although the predominant product was always nitrite (Table 1). The literature reports that N₂O could be also generated during heterotrophic denitrification [9]. However, in this process, the energy available in the NO₃⁻/NO₂⁻ reduction to N₂ was higher compared with the reduction to N₂O [18] and, therefore, the Gibbs free energy does not explain nitrous oxide generation. The generation of N₂O during heterotrophic denitrification is generally associated to the effect of environmental conditions on the different nitrogen oxide reductases involved in the four reductive steps of complete denitrification. Denitrification enzymes receive their electrons from a common source (i.e., the ubiquinone/ubiquinol pool of the respiratory electron transport chain [19]), and limitations in the electron supply rate or in substrate availability can result in electron competition among these enzymes and accumulation of nitrogen oxide intermediates [20]. Also, it has been shown that pH affects the electron donor oxidation rate supplying electrons and the nitrogen reductases activity [21]. In the case of ammonia oxidation (such as the observed in the studies experiments), it has been shown that the production of N₂O can be related to an imbalanced metabolic activity and enzymatic regulation of AOB, especially under cyclic transitions in DO concentrations, or to chemical decomposition and oxidation of intermediate compounds [8]. Therefore, the key factors controlling intermediate compounds formation such as N₂O during nitrification may not only be energy availability but also environmental conditions.

For the autotrophic denitrification experiments, according to the calculated Gibbs free energy (Figure 2(b)), the most thermodynamically favorable reactions are those where elemental sulfur is consumed (reactions (vii) and (viii) of Table 2), which would explain the consumption of this compound observed during the operational stages 6, 8, and 9 (Table 1). Nevertheless, according to the free energy calculations the least favorable reactions are those where elemental sulfur is the end product (reaction (iii) and (v) of Table 2). This fact that does not agree with the experimental results found during stages 2, 3, 4, and 7 where S⁰ accumulation was observed. On the other hand, despite the fact that the reduction of nitrate into nitrogen gas provides more energy than its reduction to nitrite (Figure 2(b)), NO₃⁻ was detected during almost all operational stages of autotrophic denitrification process (Table 1). In sulfur-based autotrophic denitrification systems the formation of intermediate compounds has been related to the feeding S/N ratio and the operational conditions [9, 16], which are not evaluated using free energy analyses.
According to Seto and Iwasa [22], the behavior of chemotrophic microorganisms under anaerobic conditions is affected by the low level of energy available from redox reactions and, therefore, it would be expected that Gibbs free energy per electron mol transferred \( (\Delta G/e) \) was an appropriate parameter to predict the accumulation of intermediate products at least for autotrophic denitrification. Nevertheless, the discrepancies between experimental data and those obtained by the theoretical calculations indicate the opposite.

### 3.2. Oxidative Capacity

Analyses by linear regression showed significance and a strong to very strong relationship between the OXC of the bulk liquid and the production of intermediate compounds, in nitrification and autotrophic denitrification processes (Figure 3). In the case of nitrification, \( \text{N}_2\text{O} \) specific production increases with the increase of the OXC of the bulk liquid (Figure 3(a), \( R^2 = 0.704 \), \( p < 0.05 \)). That is, \( \text{N}_2\text{O} \) formation is promoted by both high DO and nitrite concentration. This agrees with the results of He et al. [23] who observed that \( \text{N}_2\text{O} \) production was favored by high redox potential inside nitrifying biofilms. However, some studies reported that \( \text{N}_2\text{O} \) formation decreased when the system was operated at high DO levels [24]. This cannot be attributed to the own effect of the dissolved oxygen but to the decrease of nitrite accumulation during the operation at high DO concentrations. In fact, Castro-Barros et al. [25] found an increase in the production of nitrous oxide at higher DO concentrations when nitrite was added to a nitrifying system. Moreover, high oxidative capacity and redox potential values are already reported in the literature as the most important parameters responsible for \( \text{N}_2\text{O} \) production during nitrification in soils [4].

In the case of autotrophic denitrification, sulphur generation seems to be promoted by less oxidative environments while the opposite trend is observed for nitrite (Figures 3(b) and 3(c), \( R^2 = 0.791 \) and \( 0.929 \), \( p < 0.05 \)). Generally, the formation of both intermediate compounds is related to the feeding S/N ratio: an excess of electron donor causes the accumulation of elemental sulphur while an excess of electron acceptor leads to the generation of nitrite [13, 14]. Nevertheless, this is not a valid criterion to predict the formation of intermediates since some studies showed that \( \text{S}^0 \) can be accumulated even under sulphide limitation conditions [10, 12] and, on the other hand, nitrite production was observed in the systems operated with an excess of sulphide [26]. Accumulation of \( \text{S}^0 \) during autotrophic denitrification is also related to high pH values (\( > 8.5 \)) [12]. This could be attributed to the effect of the pH on the redox state, probably due to a
shift in the $\text{H}_2\text{S}/\text{HS}^-/\text{S}^2-$ equilibrium, since higher pH values lead to lower redox potentials (lower oxidative capacity). This agrees with the results obtained in sulfide-oxidizing bioreactors showing that the formation of elemental sulfur is optimal at low redox potentials [27]. A similar tendency has also been observed during the aerobic oxidation of sulfide: the yield of $\text{S}^0$ increases as the DO concentration decreases due to the decrease of the redox potential [28].

The redox potential corresponds to the activity of the electrons present in the bulk liquid that influences the NAD$^+$/NADH ratio within cells. This ratio controls gene expression and enzyme synthesis for the overall cell metabolic activities [29]. Therefore, it is reasonable to think that the redox potential value inside bioreactors can affect the metabolite generation and, therefore, the spectrum of products obtained depending on the extracellular redox conditions. In fact, electrofermentation is a novel technique that is being used to change the overall performance in mixed-culture fermentations, by altering both microbial community structure and metabolic patterns [30]. Also, electrochemical control of the redox potential in mixed culture bioreactors has been shown to regulate microbial metabolites production [31].

In summary, the results show that there is good agreement between calculated OXC values and the specific rate of production of nitrous oxide, elemental sulfur, and nitrite, as intermediate compounds in the analyzed nitrification and autotrophic denitrification systems. This suggests that the OXC calculation can be used to assess and predict the generation of these intermediate compounds.

4. Conclusions

The value of the Gibbs free energy calculated for the evaluated operational conditions cannot be used in order to predict the formation of nitrous oxide, elemental sulphur, and nitrite during nitrification and autotrophic denitrification processes. Nevertheless, the oxidative capacity of the bulk liquid appears as a useful tool to predict the accumulation of these intermediates. The oxidative capacity is a parameter simple to calculate and may provide a valuable starting point for the evaluation of the accumulation of undesirable intermediate compounds in wastewater treatment systems.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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References


