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Effects of inorganic ions on autotrophic denitrification by *Thiobacillus denitrificans* and on heterotrophic denitrification by an enrichment culture

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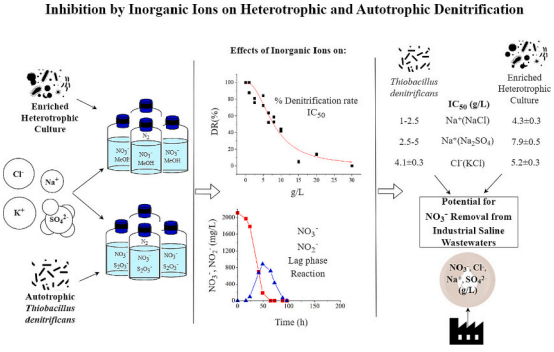
HIGHLIGHTS

- High content of ions in industrial wastewaters pose a challenge for denitrification.
- Batch assays were run with Na^+, Cl^-, K^+, SO_4^{2-} and NO_x-SO_2 scrubber wastewater.
- Single ions inhibited autotrophic and heterotrophic denitrification.
- Cl^- had the most important role in decreasing denitrification rate.
- Denitrification has potential for treatment of NO_x-SO_2 scrubber wastewater.

GRAPHICAL ABSTRACT



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ABSTRACT

Salinity of nitrate-laden wastewaters, such as those produced by metal industries, tanneries, and wet flue gas cleaning systems may affect their treatment by denitrification. Salt inhibition of denitrification has been reported, while impacts of individual ions remain poorly understood whilst being relevant for wastewaters where often the concentration of a single ion rather than the salts varies. The aim of this study was to determine the inhibition by inorganic ions (Na^+, Cl^-, SO_4^{2-} and K^+) commonly present in saline wastewaters on denitrification and reveal its potential for the treatment of such waste streams, like those produced by NO_x-SO_x removal scrubbers. The inhibitory effects were investigated for both heterotrophic (enrichment culture) and autotrophic (*T. denitrificans*) denitrification in batch assays, by using NaCl, Na_2SO_4, KCl and K_2SO_4 salts at increasing concentrations. The half inhibition concentrations (IC_{50}) of Na^+ (as NaCl), Na^+ (as Na_2SO_4) and Cl^- (as KCl) were: 4.3 ± 0.3, 7.9 ± 0.5 and 5.2 ± 0.3 g/L for heterotrophic, and 1–2.5, 2.5–5 and 4.1 ± 0.3 g/L for autotrophic denitrification, respectively. Heterotrophic denitrification was completely inhibited at 20 g/L Na^+ (as NaCl), 30 g/L Na^+ (as Na_2SO_4) and 30 g/L Cl^- (as KCl), while autotrophic at 8 g/L Na^+ (as NaCl), 10 g/L Na^+ (as Na_2SO_4) and 15 g/L Cl^- (as KCl). In both cases, Cl^- addition had the most important role in decreasing denitrification rate, while Na^+ at 1 g/L stimulated autotrophic denitrification but rapidly inhibited the rate at higher concentrations. Nitrite reduction was less inhibited by the ions than nitrate reduction and both the osmotic pressure and

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the toxicity of the single ions played key roles in the overall inhibition of denitrification. Eventually, both autotrophic and heterotrophic denitrification showed potential for the treatment of a saline wastewater from a NO_x-SO_2 removal scrubber from a pulp mill.

1. Introduction

Nitrate discharge has detrimental impacts on the environment (e.g., eutrophication) as well as on human health, and, hence, requires control. Biological nitrate removal is an effective treatment widely employed, for example, in wastewater treatment plants and in groundwater remediation (Chan-Pacheco et al., 2021; Rivett et al., 2008). Biological treatment represents a cost-efficient management of secondary pollution and, thus, has potential also for denitrification of industrial wastewaters (Park and Yoo, 2009). However, industrial wastewaters, like those produced in tanning and food industries (Lefebvre and Moletta, 2006), metal industries (Luo et al., 2021), as well as coal-fired power plants (Koenig and Liu, 2004), often present challenging conditions for denitrifying microorganisms, such as high concentrations of nitrate, metals, and high salinity. For instance, wet scrubbers simultaneously removing NO_x and SO_x from industrial flue gases transfer the pollutants from the gaseous to the liquid phase, thus generating nitrogen and sulphur laden liquid waste streams (Gholami et al., 2020) that need to be treated. Moreover, the chemicals used in the scrubbing process (for a review see Chen et al. (2021)) result in variable concentrations of chloride and sodium ions in the generated wastewater.

Both heterotrophic and autotrophic denitrification have been studied for industrial application, such as for wastewaters from agrifood industry (De Lucas et al., 2005), mining industry (Di Capua et al., 2017b), fishery industry (Mariángel et al., 2008) and flue gas desulphurization (FGD) scrubbers (Vredenbregt et al., 1997; Wei et al., 2017). On one hand, heterotrophic denitrification of industrial wastewaters often requires supplementation of organic electron and carbon sources, therefore increasing the costs of the treatment (Park and Yoo, 2009). However, many industries produce large quantities of organic waste streams that represent potential low-cost carbon and electron sources for heterotrophic denitrification. For instance, in kraft pulp mills, evaporator condensates contain methanol (MeOH) and other organic compounds (Meyer and Edwards, 2014) that can be utilised as carbon and electron sources. Methanol supports heterotrophic denitrification (C/N = 0.93 g/g) according to the following reaction (1) (Foglar and Briški, 2003):

$$1.09\ CH_3OH + NO_3^- + H^+ \rightarrow 0.074\ C_5H_7O_2N + 0.463\ N_2 + 0.72\ CO_2 + 2.421\ H_2O \quad (1)$$

On the other hand, autotrophic denitrification obtains electrons for nitrate reduction from reduced inorganic sulphurous compounds, and does not require addition of organic substrate (Di Capua et al., 2019). For instance, the chemolithoautotrophic *Thiobacillus denitrificans* reduces nitrate to nitrogen gas by oxidizing thiosulphate (S/N = 3.92 g/g) according to reaction (2) (Mora et al., 2014):

$$S_2O_3^{2-} + 1.16NO_3^- + 0.035CO_2 + 0.519HCO_3^- + 0.11NH_4^+ + 0.124H_2O \rightarrow 0.11C_5H_7O_2N + 0.578\ N_2 + 0.435H^+ + 2SO_4^{2-} \quad (2)$$

Salinity of industrial wastewaters, which can reach 150 g/L (Lin et al., 2021), poses a challenge for both autotrophic and heterotrophic denitrification. In a saline environment, Na^+ cations interfere with the enzyme activity (Lin et al., 2021; Wang et al., 2016) and at high concentrations they increase internal osmotic pressure of microbial cells, leading to the breakage of the cell membrane (Yang et al., 2013). In addition, specific toxicity of other ions and their interactions may affect the inhibitory effect (Lin et al., 2021; Macêdo et al., 2019).

Inhibition by salts has been reported for heterotrophic denitrification in batch (Macêdo et al., 2019; Mariángel et al., 2008; Wang et al., 2016; Zhao et al., 2013) and continuous reactor studies (Dinçer and Kargi, 1999; Glass and Silverstein, 1999; Luo et al., 2021; Panswad and Anan, 1999), as well as for autotrophic denitrification in some batch studies (Chen et al., 2022a; Claus and Kutzner, 1985a; Koenig and Liu, 2004). Most of these studies focus on the inhibition caused by NaCl and only few consider the impacts of other salts (e.g., Na_2SO_4, Na_3PO_4, $MgCl_2$, $CaCl_2$). Therefore, the effects of the single ions, such as Cl^- in absence of Na^+, have not been delineated for denitrification. Since in industrial processes other chemicals than NaCl are often present in variable ratios, Na^+ and Cl^- ions concentrations can vary independently from each other in the wastewater. For this reason, the focus on the inhibition by the single ion is of importance, in view, for example, of a biological nitrogen removal process to treat the wastewater from wet flue gas cleaning systems. Moreover, to the best of the authors knowledge, the potential of autotrophic and heterotrophic denitrification to treat high ions content wastewaters from NO_x-SO_2 removal scrubber has so far not been reported. This becomes relevant especially for industries producing nitrogenous waste streams, such as pulp mills, where both inorganic and organic electron donors for their denitrification would be available.

The aim of this work was to reveal the effects of inorganic ions (Na^+, Cl^-, SO_4^{2-} and K^+) on both heterotrophic (enrichment culture) and autotrophic denitrification (*T. denitrificans*), to better understand the limits and the potential of these biological processes for nitrogen removal from high ion content wastewaters. *T. denitrificans* was selected as the model microorganism for autotrophic denitrification as also used in earlier studies (Di Capua et al., 2017a, 2017b; Zhao et al., 2004; Zou et al., 2016). Pair of ions were added as model compounds (NaCl, Na_2SO_4, KCl and K_2SO_4) in batch tests and the inhibitory effects were evaluated on denitrification rate, nitrite accumulation and duration of lag phase. The order of importance of the ions in decreasing denitrification rate was identified with statistical analysis of the data. Moreover, denitrification potential was demonstrated with real NO_x-SO_2 removal scrubber wastewater from a pulp mill.

2. Materials and methods

2.1. Denitrifying microorganisms: source, cultivation, and growth media

A heterotrophic denitrifying culture was enriched from a denitrifying activated sludge of a municipal wastewater treatment plant (WWTP) located in Helsinki (Finland). The most abundant bacterial genera in the sludge were identified by Kruglova et al. (2020) as *Candidatus Microthrix, Trichococcus, Rhodobacter, Hyphomicrobium* and *Geothrix*. During the enrichment period of three months the culture was regularly transferred to fresh medium after NO_3^- and NO_2^- were removed. The final growth medium (Table S1) was modified from Zou et al. (2014) by addition of ammonia, iron, and yeast extract at pH 7, to overcome nutrient limitation (Fig. S1). Nitrate concentration was increased to 2 g/L to simulate a high-nitrate content wastewater. Methanol was supplemented in excess as electron donor (C/N = 1.3 g/g). During the first two months of the enrichment, the pH of the medium was initially buffered by Na_2HPO_4/NaH_2PO_4 buffer solution and $NaNO_3$ was used as nitrate source. Later, KNO_3 and K_2HPO_4/KH_2PO_4 were used to control the sodium concentration in the medium (Table S1).

Autotrophic denitrifying microorganism *Thiobacillus denitrificans* (DSM 12475) was acquired from Leibniz-Institute DSMZ (Germany). The pure culture was aseptically incubated for three weeks using the growth medium recommended by DSMZ (Leibniz-Institute DSMZ, n.d.), to obtain an active culture. Subsequently, the final medium was modified to minimize the concentration of sodium and increase nitrate

concentration to 2 g/L to simulate a high-nitrate content wastewater (Table S1). Thiosulphate and bicarbonate were added in excess to obtain a S/N ratio of 4.62 g/g and a C/N ratio of 0.86 g/g, and achieve sufficient denitrification and alkalinity, respectively. The pH of final medium was around 7.5. Nutrient solution was autoclaved at 121 °C for 20 min while the other solutions were sterilized by filtration through polyethersulfone (PES) membrane (pore size 0.2 μm). *T. denitrificans* culture was maintained by regular transfers to fresh media, once NO_3^- and NO_2^- were removed and to avoid $S_2O_3^{2-}$ and SO_4^{2-} accumulation. *T. denitrificans* was cultivated in the final medium for three months prior to the experiments.

Both cultures were incubated in 117 mL glass serum bottles with 60 mL liquid volume, the one with heterotrophs in non-aseptic and the *T. denitrificans* in aseptic conditions. After the addition of the respective growth medium, the bottles were sealed with butyl rubber stoppers and aluminium crimps and purged for 20 min with N_2 to create anoxic conditions. After the inoculation (10 % *v*/v), the heterotrophic denitrifying enrichment culture and *T. denitrificans* culture were incubated (150 rpm) at 35 °C and 30 °C, respectively. Incubation temperature for *T. denitrificans* was set to 30 °C as at 35 °C no growth was observed in three weeks of incubation (results not shown).

2.2. Denitrification inhibition by ions: batch assays with model compounds

The effect of chloride and sodium concentrations on both autotrophic and heterotrophic denitrification was studied in batch tests in serum bottles (total number of assays: 134) with addition of NaCl, Na_2SO_4 and KCl. Each salt was tested at final concentrations of Cl^- (as KCl) or Na^+ (as NaCl or Na_2SO_4) in the range 1–30 g/L, corresponding to molar concentrations of 0.02–1.3 mol/L and to salinity of 0.2–9.2 % (*w*/w). Growth medium and incubation were as reported in Table S1 and Section 2.1. If needed, pH was adjusted to around 7 with 10 M KOH. The final working volume of each bottle was 60 mL with 10 % (*v*/v) inoculum. Both tests with autotrophic and heterotrophic denitrification were started between 5 and 7 days after the last culture transfer.

Positive controls (no addition of salt solution) were used in both autotrophic and heterotrophic tests. All the incubations were performed in duplicates and sampled until no NO_3^- and NO_2^- were detected (<100 mg/L) or until 42 days (1000 h) if NO_3^- had not been removed. The sampling frequency was increased when the turbidity of the medium increased visually. From the start to the end of the incubation, the pH remained in the range 6.7–7.8 and 8.2–7.0 for heterotrophic and autotrophic denitrification, respectively.

Since high concentrations of K^+ and SO_4^{2-} were present in the Cl^- and Na^+ tests (Fig. 1), their possible inhibitory effects were studied in a complementary batch test with K_2SO_4 addition, following the same procedures as above. The concentrations tested for heterotrophic denitrification were 15 and 35 g/L K^+, while for autotrophic denitrification were 15 and 20 g/L K^+, taking into account the potassium already present in the media.

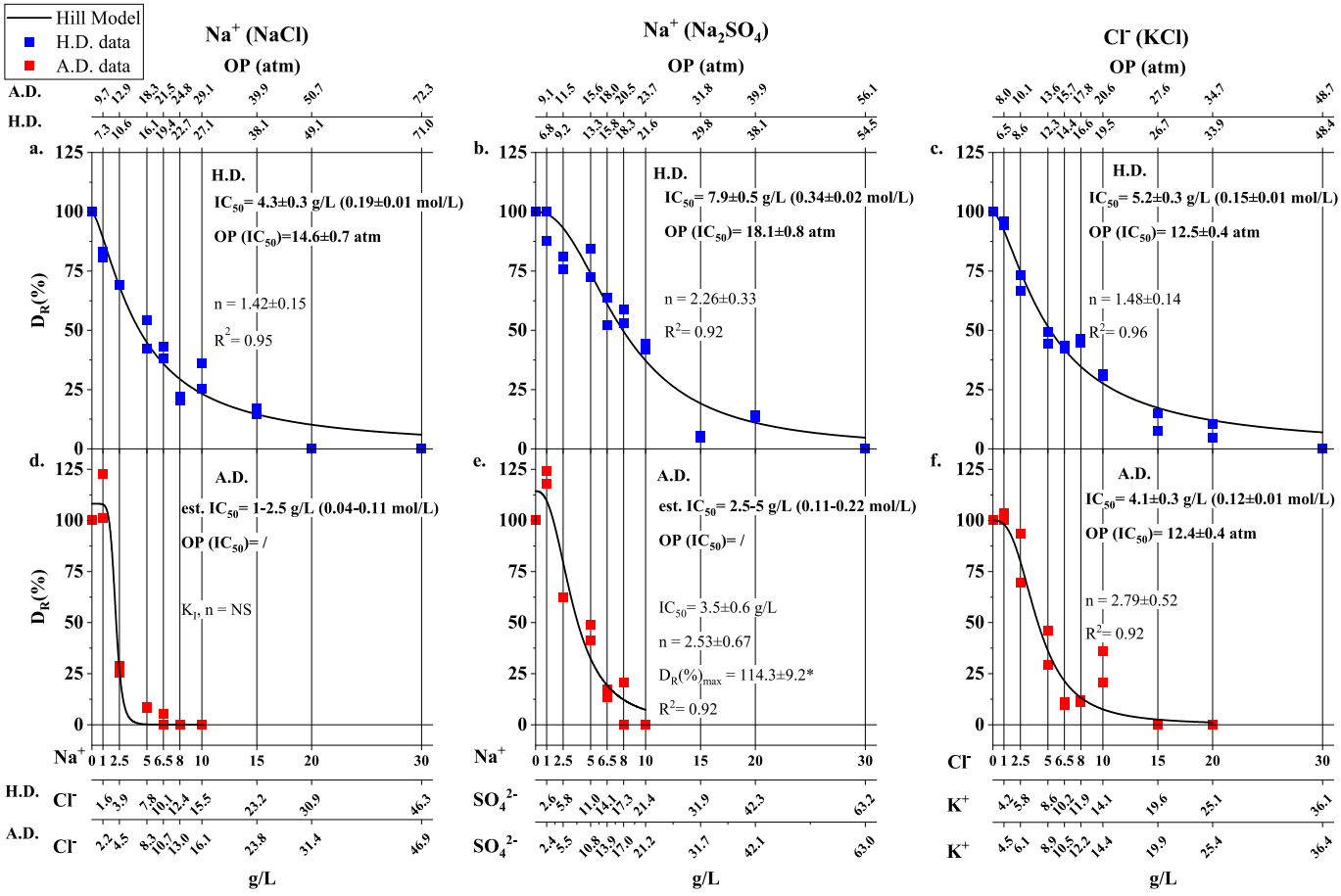

Fig. 1. Effects of ions concentrations on percentage denitrification rate (D_R (%)) for heterotrophic (H.D.: a, b, c) and autotrophic (A.D.: d, e, f) denitrification tests and fitting with Hill inhibition model. On the top x-axis the osmotic pressure (OP) is reported for H.D. (T = 35 °C) and A.D. (T = 30 °C). The second and third bottom x-axis in each plot refer to the total concentration of the second ion (in salt + growth medium) for heterotrophic (H.D.) and autotrophic (A.D.) denitrification, respectively. Two data points (duplicates) are shown for each ion's concentrations. NS: not significant. * Fitted value.

2.3. Source and composition of saline wastewater produced by a simultaneous NO_X-SO_2 removal scrubber system

Wastewater from a wet cleaning system removing NO_x-SO_2 from flue gases produced in two recovery boilers was obtained from a pulp mill. In the NO_x-SO_2 removal scrubber, NaOH is used as scrubbing agent, while ClO_2 and sulphurous compounds are added to increase NO_x solubility. Prior to the scrubber, an electrostatic precipitator is used at the plant to remove particles from the flue gases. A wastewater sample (2 L), collected from the first of the three stages of the scrubber, was stored in a plastic container (prewashed with 1 M nitric acid) for several months at 4 °C. During this period, analysis of samples taken at different frequencies showed that concentrations of anions did not change (results not shown).

The composition of the wastewater sample is presented in Table 1.

2.4. Denitrification batch assays on wastewater produced by a simultaneous NO_X-SO_2 removal scrubber system

Heterotrophic and autotrophic denitrification of real NO_x-SO_2 removal scrubber wastewater was studied in batch assays under non-aseptic conditions. Prior to the experiment, the pH of the wastewater was adjusted to 7 with 10 M KOH and solids were removed by settling. The wastewater supernatant was then added (88 % v/v) to serum bottles (60 mL working volume), together with addition (2 % v/v) of additive solution. The additive solution contained electron donor in excess (MeOH for heterotrophic denitrification (C/N = 1.3 g/g) and $S_2O_3^{2-}$ for autotrophic denitrification (S/N = 4.64 g/g)), and HCO_3^- (C/N = 0.86 g/g) in the test with *T. denitrificans*. The bottles with the wastewater and amendments were purged with N_2 for 20 min before inoculation (10 % v/v) with heterotrophic enrichment culture or *T. denitrificans*. Inoculated bottles with growth medium were incubated as positive controls. The experiment was run in duplicates. Denitrification was monitored by sampling every 24–72 h.

2.5. Analytical methods and calculations

2.5.1. Sampling and analyses

At every sampling time, 1 mL liquid sample was taken and filtered through 0.2 μm Chromafil Xtra syringe filters (Macherey-Nagel, Germany). Before the filtration, samples from the bottles with Cl^- concentration above 10 g/L were pre-treated with chloride elimination set (Hach LCW925) to reduce the Cl^- interference with the nitrite measurement (Glass and Silverstein, 1999). Samples from the heterotrophic denitrification test were stored at −20 °C, while the ones from the autotrophic denitrification test were stored at +4 °C, since at −20 °C excessive formation of precipitate was observed for samples from cultivation after being melted. An additional 0.5 mL sample was taken at the beginning and the end of the incubation for pH measurement with WTW pH-meter 330i. Concentrations of anions (Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, $S_2O_3^{2-}$) in all the filtered samples from the batch tests were analysed via ion chromatograph as reported by Hajdu-Rahkama and Puhakka (2022). Concentration of MeOH was measured in the samples from the heterotrophic denitrification tests via gas chromatograph equipped with a flame ionization detector according to Kokko et al. (2018).

Table 1
Characterization of the wastewater produced from a full-scale simultaneous NO_x-SO_2 removal scrubber system treating the flue gases produced in two recovery boilers of a pulp mill.

Analyte	Concentration (mg/L)	Analyte	Concentration (mg/L)
Cl^-	3 400	Al	1.14
NO_2^-	<100	As	<0.01
NO_3^-	1 100	B	24.70
SO_4^{2-}	13 500	Co	<0.01
$S_2O_3^{2-}$	<100	Cr	0.21
		Cu	0.01
		Fe	7.60
DOC[a]	13	K	22.16
DIC[b]	/	Mg	2.64
		Mn	0.08
pH	1.8	Mo	0.02
T (°C)	64.5	Na	5 200.00
		Ni	0.12
		P	0.29
		Pb	<0.01
		Se	0.02
		V	<0.01
		Zn	0.19

[a] Dissolved Organic Carbon.
[b] Dissolved Inorganic Carbon.

2.5.2. Calculations

Inhibitory effects of the ions on autotrophic and heterotrophic denitrification were monitored as denitrification rate, lag phase and nitrite accumulation. Denitrification rates (mg/L h^{-1}) were estimated by the linear regression tool in Origin 2019b software (100 interpolations with $R^2 > 90$, 4 with $72 < R^2 \leq 90$), considering the part with maximum slope in the plot of NO_3^- concentration vs. time. Percentage denitrification rates (D_R (%)) with respect to the average of positive controls were calculated. If D_R (%) > 0, the time (h) before the steepest part of the NO_3^- concentration curve, approximatively corresponding to the inflection point, was considered as the lag phase of denitrification. Stoichiometry of denitrification reactions was verified by comparing measured concentrations of consumed methanol and consumed/produced thiosulphate/sulphate with theoretical values obtained from Eqs. (1), (2).

The osmotic pressure (OP) and the ionic strength of the solution, due to the main ions in the growth media (Table S1) and the added inhibitory salts, were estimated as described by Fritzmann et al. (2007) and Belessiotis et al. (2016).

Modified non-competitive Hill model was adopted to describe the effect of sodium and chloride ions concentrations on the percentage denitrification rate (D_R (%)), as described by Lin et al. (2021) for Anammox process. The model was expressed with Eq. (3):

$$D_R(\%) = \frac{D_R(\%)_{max} \bullet K_I^n}{K_I^n + I^n} \quad (3)$$

where $D_R(\%)_{max}$ is the maximum percentage denitrification rate (100 %) related to the positive control, K_I (=IC_{50}) is the half-inhibition constant (g/L) corresponding to the ion concentration that results in 50 % inhibition, I is the independent variable representing the concentration of the ion (g/L) and n is the tuning parameter. The model was implemented and fitted to the experimental calculated percentage denitrification rates with the "Non-linear curve fit" tool in Origin 2019b software. R^2 and *p*-value for *t*-test (set at 0.05) were used to evaluate the goodness of the fit and the significance of the parameters (K_I, n). If the fitting of the data returned non-significant parameters, IC_{50} was expressed as a range based on the experimental data.

To identify the ion with the highest effect on denitrification rate, a statistical analysis was performed on the experimental data, separately for heterotrophic and autotrophic denitrification. Concentrations (mol/L) of the ions (Na^+, Cl^-, SO_4^{2-} and K^+) were considered as the independent variables, while D_R(%) was the response variable (see supplementary materials). Partial Least Square Regression (PLSR), previously used for analysis of denitrification studies by Pan et al. (2023), and Random Forest Regression (RF) (Song et al., 2020) were implemented with Minitab software and Python "RandomForestRegressor" function, respectively, to analyse data characterized by strong collinearity. Eventually, standardized coefficients and variable importance values returned by PLSR and RF, respectively, were examined to determine which ion had the highest importance in decreasing denitrification rate.

3. Results

The effects of inorganic ions on percentage denitrification rates (D_R(%)), nitrite accumulation and lag phase for both heterotrophic and autotrophic denitrification are summarized in Figs. 1 and 2. Increasing concentration of the single ions inhibited autotrophic and heterotrophic denitrification to different extents. Despite the partial inhibition of denitrification, when D_R(%) > 0, over 95 % of the initial nitrate concentration was removed (Fig. S2-S7). Moreover, at almost all the studied ion concentrations, >95 % of the accumulated NO_2^- was eventually removed (Fig. S2-S7).

3.1. Effects of inorganic ions on heterotrophic denitrification by enrichment culture

For heterotrophic denitrification (Fig. 1a, b, c), the fitting of the experimental data with the modified Hill inhibition model, showed that 50 % rate inhibition (IC_{50}) with Na^+ (as NaCl), Na^+ (as Na_2SO_4) and Cl^- (as KCl) was obtained at 4.3 ± 0.3, 7.9 ± 0.5 and 5.2 ± 0.3 g/L, with the corresponding osmotic pressures ($OP(IC_{50})$) of 14.6 ± 0.7, 18.1 ± 0.8 and 12.5 ± 0.4 atm, respectively. At the same concentration of Cl^- (5.2 g/L), rate inhibition and cation concentration with NaCl and KCl were 42 % and 3.5 g/L Na^+, and 50 % and 8.8 g/L K^+ (also considering K^+ in the growth medium), respectively. Lower inhibition was observed by K^+ and SO_4^{2-} (as K_2SO_4) as the rate decreased by about 40 % at 15 g/L K^+ and 15 g/L SO_4^{2-}, while co-presence of 35 g/L K^+ and 40 g/L SO_4^{2-} (OP≈ 36 atm) resulted in partial inhibition only (Fig. 3). By expressing the IC_{50} in molar units (mol/L), in accordance with the results in g/L, Cl^- was more inhibitory than SO_4^{2-} when paired with Na^+ as 50 % inhibition was reached at 0.19 ± 0.01 mol/L Na^+ (as NaCl) and 0.34 ± 0.02 mol/L Na^+ (as Na_2SO_4), respectively. This was also seen by plotting OP vs D_R(%) (Fig. S11), since at similar OP, denitrification with addition of Na_2SO_4 had higher rates than with addition of NaCl, while with KCl it had the lowest rates. Also, Na^+ was more inhibitory than K^+ when paired with SO_4^{2-}, as about 40 % inhibition was reached at 0.28 mol/L Na^+ with 0.14 mol/L SO_4^{2-} (as Na_2SO_4) and 0.38 mol/L K^+ with 0.16 mol/L SO_4^{2-} (as K_2SO_4). On the other hand, IC_{50} of Cl^- as KCl (0.15 ± 0.01 mol/L) was lower than that of Na^+ as NaCl (0.19 ± 0.01 mol/L). Eventually, denitrification was completely inhibited at 20 g/L (0.87 mol/L) Na^+ as NaCl, 30 g/L (1.3 mol/L) Na^+ as Na_2SO_4 and 30 g/L (0.85 mol/L) Cl^- as KCl. Denitrification rate decreased with increasing OP and no denitrification activity was observed above OP of 40 atm (Fig. 1 and S11). When plotting D_R(%) with ionic strength (Fig. S12), large variations of the denitrification rates were observed at similar ionic strengths of the solution, further confirming that the type of the ions added had a key role in denitrification inhibition.

Inhibition by the ions was further characterized as increase in lag phase and variation in nitrite accumulation during denitrification (Fig. 2). For heterotrophic denitrification (Fig. 2c), lag phase was not clearly present at the lower Cl^- and Na^+ concentrations but started to increase from 8 g/L Na^+ and 15 g/L Cl^-. With K_2SO_4, the lag phase in the presence of 35 g/L K^+ was between 15 and 22 days. Longer lag phases was seen in the presence of Na^+ (as NaCl and Na_2SO_4) than of Cl^- (as KCl). For instance, at 20 g/L of the ion, lag phase was around 35 days for Na^+ (as Na_2SO_4) while around 10 days for Cl^- (as KCl).

In denitrification, nitrite (NO_2^-) can accumulate as an intermediate during nitrate reduction to nitrogen gas. In this study, in heterotrophic denitrification without salt addition, approximatively 1000 mg/L NO_2^- accumulated (Fig. 2a) and was followed by over 95 % removal within 120 h (Fig. S8). Starting from 5 g/L Na^+ (Fig. 2a), NO_2^- accumulation decreased, with the lowest accumulation for Na^+ (as NaCl). On the other hand, in the presence of Cl^- (as KCl), nitrite steadily accumulated up to 10 g/L Cl^-. No nitrite accumulated in the presence of and above 10 g/L Na^+ (as NaCl), 20 g/L Na^+ (as Na_2SO_4) and 20 g/L Cl^- (as KCl). With K_2SO_4, accumulation of NO_2^- decreased from around 1100 mg/L in the positive controls to around 800 mg/L at 15 g/L K^+ and was eventually removed by over 95 % (Fig. 3). Therefore, Cl^- had a higher effect than SO_4^{2-} while Na^+ higher than K^+ in decreasing nitrite accumulation. Moreover, NO_2^- accumulation was less affected than denitrification rate in the increase in OP and ionic strength (Fig. S12).

In summary, increasing Na^+ concentration resulted in longer lag phase than with increasing Cl^-, and nitrite accumulation (and

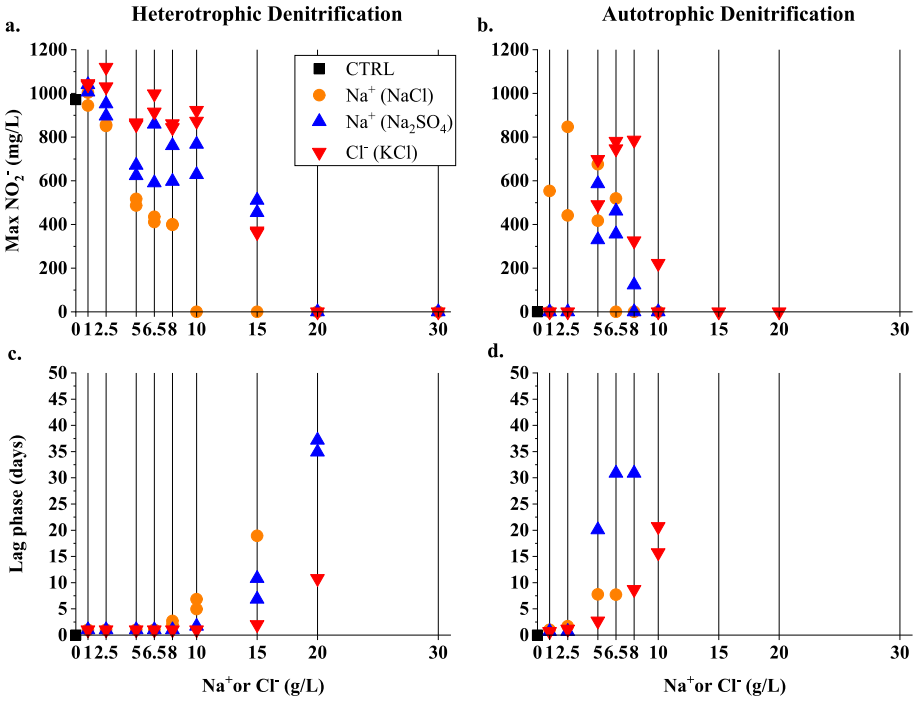

Fig. 2. Effects of ions' inhibition of heterotrophic (on the left) and autotrophic (on the right) denitrification on highest nitrite concentration accumulation during the incubation (a. and b.) and lag phase (c. and d.). Two data points (duplicates) are shown for each ion. Positive controls are indicated as "CTRL" and are represented by average values (n = 6) (plot c: standard error < 5 %).

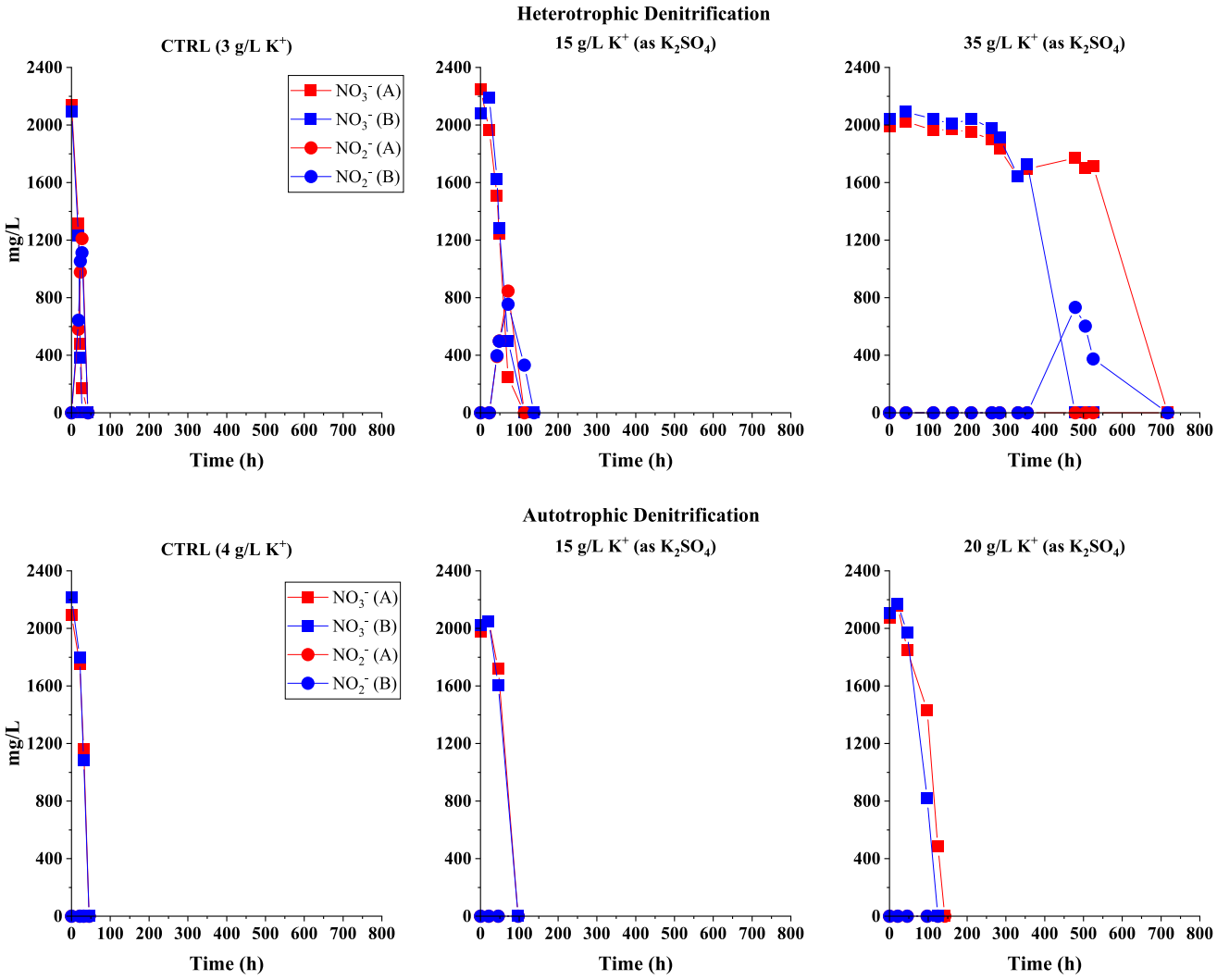

Fig. 3. Nitrate and nitrite concentrations plotted against time at different concentrations of K^+ (as K_2SO_4) for heterotrophic denitrification (first row) and autotrophic denitrification (second row). Two data points (duplicates) are shown for each concentration. CTRL plot refers to the positive control with the only inoculated growth medium in absence of salt addition.

subsequent removal) indicated that nitrite removal was less inhibited than nitrate removal by Cl^- and Na^+ and was less affected by K^+ and SO_4^{2-} than Na^+ and Cl^-.

At the end of the incubations, MeOH removal in heterotrophic denitrification (Fig. S10a) was in line with the stoichiometry and no clear correlation between electron donor consumption and increasing concentration of the ions was apparent. Eventually, after extending the incubation time by additional 30 days, nitrate was not removed at the highest concentrations of Cl^- and Na^+.

Eventually, since all the experiments were conducted with an anion paired with a cation, a statistical analysis of the data was conducted to better discern the effect of the single ions on denitrification rate. Based on the analysis of standardized coefficients for PLSR and variables importance for RF, Cl^- resulted to have the most important role in decreasing denitrification rate (Fig. S13), supporting the observations made. Further considerations on the order of inhibition of the other studied ions could not be made from the statistical analysis, as their ability in predicting the decrease in denitrification rate varied with the two methods.

3.2. *Effects of inorganic ions on autotrophic denitrification by T. denitrificans*

For autotrophic denitrification by *T. denitrificans*, D_R(%) increased at 1 g/L Na^+ (as NaCl and Na_2SO_4) (Fig. 1d, e, c), showing that low concentrations of sodium stimulated denitrification in comparison to the conditions in the positive controls. However, starting from 2.5 g/L Na^+ as NaCl and Na_2SO_4, denitrification rate started to decrease rapidly. Fitting of the experimental data with the Hill inhibition model, returned significant ($p < 0.05$) parameters for Cl^- (as KCl): $IC_{50} = 4.1 \pm 0.3$ g/L and calculated $OP(IC_{50}) = 12.4 \pm 0.4$ atm. Hill inhibition equation (Eq. (3)) did not adequately model Na^+ inhibition, thus, IC_{50} were taken as ranges from the experimental data for Na^+ (as NaCl) and Na^+ (as Na_2SO_4) and were 1–2.5 and 2.5–5 g/L, respectively. In molar units, the values of IC_{50} for Na^+ (as NaCl), Na^+ (as Na_2SO_4) and Cl^- (as KCl) were 0.04–0.11, 0.11–0.22 and 0.12 ± 0.01 mol/L, respectively. Moreover, since duplicates for autotrophic denitrification presented noticeable variation at certain concentrations of Cl^- and Na^+, these assays were rerun. The results of the rerun (Fig. S5, S6, S7), not included in Fig. 1, differed to some extent from the previous tests, which was attributed to sensitivity of *T. denitrificans* under close to fully inhibitory salt conditions. K^+ and SO_4^{2-} had lower inhibitory effects than Cl^- and Na^+ also on autotrophic denitrification rate as it decreased by around 60 % in the co-

presence of 20 g/L (0.51 mol/L) K^+ and 20 g/L (0.21 mol/L) SO_4^{2-} (Fig. 3). Also, at similar OP, denitrification with K_2SO_4 had higher D_R(%) than with the other salts (Fig. S11). Complete inhibition was observed at 8 g/L (0.35 mol/L) Na^+ as NaCl, at 10 g/L (0.44 mol/L) Na^+ as Na_2SO_4 and at 15 g/L (0.43 mol/L) Cl^- as KCl. Total inhibition was reached at similar molar concentrations of both Cl^- and Na^+.

For autotrophic denitrification (Fig. 2d), lag phase increased starting from 5 g/L Na^+ and Cl^-, in the range 3–20 days, and showed longer lag phases in the presence of Na^+ (as NaCl and Na_2SO_4) than of Cl^- (as KCl). With K_2SO_4, the lag phase was between 2 and 4 days at 20 g/L K^+ (Fig. 3).

In the autotrophic denitrification test, no nitrite accumulated in the positive controls (Fig. 2b). With increasing concentrations of Na^+ and Cl^-, NO_2^- accumulation initially increased but then, for the highest concentrations of the ions tested, decreased to below 100 mg/L. Nitrite accumulation started at 1 g/L Na^+ (as NaCl), while it accumulated at 5 g/L Na^+ (as Na_2SO_4) and Cl^- (as KCl). No nitrate accumulated during autotrophic denitrification at any of K^+ (as K_2SO_4) concentrations tested (Fig. 3). Cl^- and Na^+ affected nitrite accumulation more than SO_4^{2-} and K^+, and nitrite removal was less inhibited than nitrate removal, similarly to what observed for heterotrophic denitrification.

For autotrophic denitrification, thiosulphate consumption and sulphate production decreased with increasing concentrations of Na^+ (Fig. S10b and S10c) with respect to the stoichiometry, while Cl^- (as KCl) had a lower effect.

Also, for autotrophic denitrification by *T. denitrificans*, both PLSR and RF returned that Cl^- had the most important role in decreasing denitrification rate (Fig. S13), similarly to what seen for heterotrophic denitrification.

3.3. Denitrification of real wastewater from simultaneous NO_x-SO_2 removal scrubber

The potential of heterotrophic and autotrophic denitrification of real scrubber wastewater was studied in batch tests. In all the tests with inoculated wastewater and electron donor/carbon supplementation, nitrate removal was slower than in positive controls (<50 h for 95 % removal) but it was eventually over 95 % (Fig. 4). Initial nitrate (around 800 mg/L) was removed over 95 % in <80 h by autotrophic denitrification (Fig. 4b) and within 150 h by heterotrophic denitrification (Fig. 4a), at Cl^-, Na^+ and SO_4^{2-} concentrations in the wastewater (OP around 10 atm) of 3 g/L, 4.5 g/L and 12 g/L, respectively (considering dilution caused by culture conditions). Electron donors and products were in line with the stoichiometries of nitrate removal (Fig. 4d and e). Nitrite did not accumulate in batch tests.

In summary, both heterotrophic and autotrophic denitrification removed nitrate from the scrubber wastewater.

4. Discussion

This study revealed the inhibitory effects of chloride, sodium, potassium, and sulphate ions on autotrophic and heterotrophic denitrification as determined by nitrate removal rate, nitrite accumulation and lag phase of the batch incubations. Also, nitrate was successfully removed from real NO_x-SO_2 scrubber wastewater.

4.1. Effects of the ions on heterotrophic and autotrophic denitrification

The effects of single ions on denitrification have not been delineated in detail in previous studies, which have focused mainly on the salts (Table 2). For instance, Cl^- inhibition has been reported for heterotrophic denitrification as NaCl (Holm Kristensen and Jepsen, 1991; Panswad and Anan, 1999) but not as KCl, thus including also Na^+ inhibition. Also, denitrification inhibition by K^+ (as K_2SO_4) has not been described by others, and shows, when compared with other salts in Table 2, the lowest effect in almost all the cases. The results for Na^+ (both Na_2SO_4 and NaCl) inhibition on heterotrophic denitrification rate were similar to those reported by Wang et al. (2016) and Zhao et al. (2013), showing

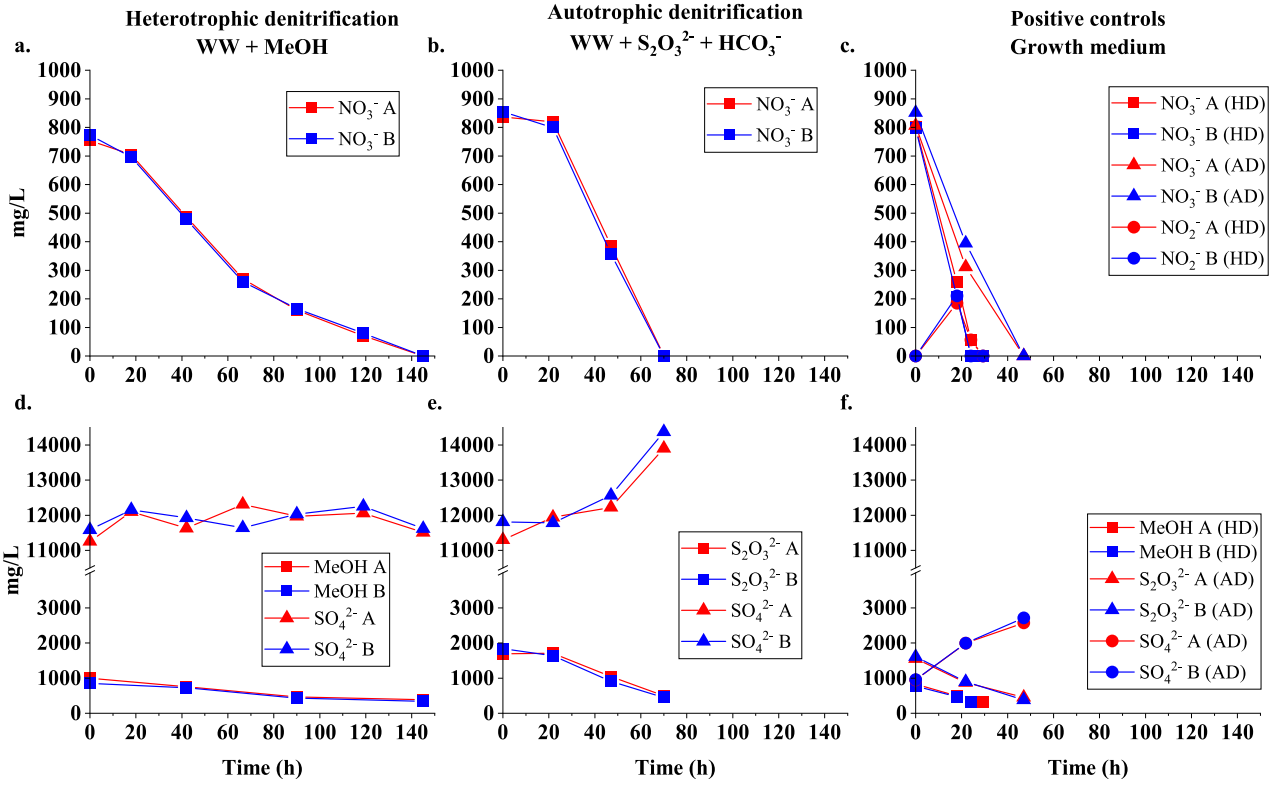

Fig. 4. Nitrate and nitrite concentrations (a, b, c) and MeOH, thiosulphate and sulphate concentrations (d, e, f) vs. time for heterotrophic and autotrophic denitrification of simultaneous NO_x-SO_2 removal scrubber wastewater (WW) with electron/carbon source supplementation. Duplicates (A and B) are shown in each plot.

Table 2
Comparison of studies investigating salinity inhibition on heterotrophic and autotrophic denitrification. All the results refer to batch studies conducted at temperatures ranging from 22 to 37 °C. The concentrations of the added salt reported in the references have been converted into concentration of the added ion for the comparison with this study.

Denitrification	Microorganisms and source	Initial NO_3^- (g/L)	Energy source	Denit. activity inhibition %	Ion concentration (g/L)		Reference
Heterotrophic	Enrichment culture from a denitrifying compartment of a WWTP	2	Methanol	50 % 50 % 50 % ~40 %	4.3 ± 0.3 7.9 ± 0.5 5.2 ± 0.3 15	Na^+ (as NaCl) Na^+ (as Na_2SO_4) Cl^- (as KCl) K^+ (as K_2SO_4)	This study
	Granular sludge from a lab-scale denitrifying reactor	0.44	Acetate	50 % 50 % 50 %	4.54 7.03 3.14	Na^+ (as NaCl) Na^+ (as Na_2SO_4) Na^+ (as Na_3PO_4)	Wang et al. (2016)
	Biomass adapted at 24 g/L NaCl for a year in a batch reactor	0.44	Fish blood water	50 %	18	Na^+ (as NaCl)	Mariángel et al. (2008)
	Inoculum from lab-scale partial denitrification system	0.26	Acetate	71 %	~4	Na^+ (as NaCl)	Ji et al. (2018)
	Seed from lab-scale sequencing batch reactor	0.18	Methanol	31 % ~60 %	~4 ~8	Na^+ (as NaCl) Na^+ (as NaCl)	Zhao et al. (2013)
Autotrophic	*T. denitrificans* (DSM 12475)	2	Thiosulphate	50 % 50 % 50 % ~60 %	1–2.5 2.5–5 4.1 ± 0.3 20	Na^+ (as NaCl) Na^+ (as Na_2SO_4) Cl^- (as KCl) K^+ (as K_2SO_4)	This study
	T. denitrificans enriched from soil	0.92	Thiosulphate	~35 % ~60 %	12 ~5	Na^+ (as NaCl) Na^+ (as Na_2SO_4)	Claus and Kutzner (1985a)
	Enrichment culture from tidal flats	0.44	Elemental sulphur	10 %	8 12	Na^+ (as NaCl) Na^+ (as Na_2SO_4)	Koenig and Liu (2004)
	Enrichment culture from fish cannery wastewater treatment	0.44	Thiosulphate	10 %	4	Na^+ (as NaCl)	Campos et al. (2008)
	Enrichment culture from an anoxic tank of a WWTP	0.11	Sulphide	50 %	26	Na^+ (as NaCl)	Chen et al. (2022a)

higher inhibition by the co-presence of Na^+ and Cl^- rather than Na^+ and SO_4^{2-}. On the other hand, higher tolerance to Na^+ paired with Cl^- was observed by Mariángel et al. (2008) by using denitrifying biomass after a long-term enrichment under saline conditions.

Inhibitory effects on denitrification were also observed in nitrite accumulation and in the increase in lag phase duration. Nitrite accumulation during denitrification depends on many factors, such as nitrate and phosphate concentrations, pH, dissolved oxygen, temperature (Philips et al., 2002). In our study, without ions addition, heterotrophic denitrification accumulated around 1000 mg/L NO_2^- (70 % of the initial nitrogen) and reduced it further (>95 %) with no clear inhibition. In comparison, Glass et al. (1997) reported that heterotrophic denitrification at pH 8 removed 6500 mg/L NO_2^- and was not completely inhibited. With increasing concentrations of the ions, especially Na^+, nitrite accumulation decreased, similarly to what reported by Zhao et al. (2013).

Increasing concentrations of the inorganic ions rapidly decreased autotrophic denitrification rates by *T. denitrificans*. Furthermore, low concentrations of sodium (up to 1 g/L in this study), enhanced the rates, indicating that *T. denitrificans* requires Na^+ for optimal growth. Therefore, Hill inhibition model as written in Eq. (3) did not consider the initial increase in denitrification rate and, thus, did not comprehensively model the sodium effects on autotrophic denitrification. For this reason, IC_{50} were estimated as a range from the experimental data. In our study, Na^+ (as NaCl) had the lowest inhibitory concentration reported for autotrophic denitrification if compared to other reports (Table 2). Moreover, the co-presence of Na^+ and Cl^- resulted more inhibitory than Na^+ and SO_4^{2-}, as also reported by Koenig and Liu (2004). Inhibition by Na^+ as Na_2SO_4 was close to that reported by Claus and Kutzner (1985a) (Table 2), who attributed the inhibitory role to SO_4^{2-}. Contrarily, 20 g/L SO_4^{2-} at the beginning of incubation did not completely inhibit autotrophic denitrification in our study, similarly to the observations of Chung et al. (2014). The different tolerances to ions were likely due to the origin of autotrophic as well as heterotrophic denitrifying microorganisms (different in all the studies as reported in Table 2). As for Cl^- inhibition in absence of Na^+ of autotrophic denitrification, which has not been previously reported, our study showed IC_{50} at 4.1 ± 0.3 g/L Cl^- (as KCl).

For autotrophic denitrification, no nitrite was detected in the positive controls. Hence, salinity also inhibited nitrite reduction and NO_2^- accumulated with increasing concentration of the ions. Claus and Kutzner (1985a) reported 70 % denitrification inhibition on *T. denitrificans* at 1000 mg/L NO_2^-. In our study, the highest NO_2^- accumulation for autotrophic denitrification was around 850 mg/L, and, hence, nitrite inhibition on *T. denitrificans* could not be completely excluded. However, in almost all the cases, nitrite was removed by >95 % and its accumulation decreased with the increase in the ions' concentrations once the nitrate removal become slower. Therefore, despite an initial inhibition of nitrite reduction for autotrophic denitrification, denitritation seemed to be less inhibited by the inorganic ions than

denitratation, as also reported by others for heterotrophs (Mariángel et al., 2008; Zhao et al., 2013) and autotrophs (Chen et al., 2022a).

Eventually, autotrophic denitrification reaction showed to be more affected by Na^+ than the other ions. Consumption of $S_2O_3^{2-}$-S by *T. denitrificans* in the presence of 2.5 g/L Na^+ and above was lower than the stoichiometry (Fig. S10b), indicating that NO_3^- and NO_2^- were not completely reduced to N_2 and other intermediates (not measured in this study) could have possibly accumulated. For instance, Chen et al. (2023) reported accumulation of N_2O with autotrophic NO_2^- removal at increasing NaCl concentrations, indicating that nitrous oxide reduction was the limiting step. Moreover, SO_4^{2-}-S production was lower than $S_2O_3^{2-}$-S consumption (Fig. S10c), suggesting that inhibitory concentrations of Na^+ could also affect the sulphur end products of denitrification reaction.

4.2. Sensitivity of denitrification activity to the single ions

As shown above, the studied ions affected denitrification activity to different extents. Na^+ is toxic at high intracellular levels and can disrupt the cell membrane because of unbalanced osmotic pressure (Yang et al., 2013). Moreover, also anions can affect the activity and stability of many enzymes (for a review, see Zhao (2005)), such as Cl^- and SO_4^{2-} (Lin et al., 2021). Different explanations for the mechanisms of ions and salts inhibition on denitrification have been reported in the literature. For instance, Zhao et al. (2013) reported that osmotic stress played a primary role in the inhibition of denitrification by NaCl and that Na^+ could affect the activity of denitrification enzymes. Moreover, Koenig and Liu (2004) reported for an autotrophic culture enriched from tidal flats that the decrease in denitrification rate was dependent on OP but independent of the type of the salt when studying the effect of a single salt (NaCl or Na_2SO_4) on denitrification. They showed that inhibition started above 20 atm, while in our study at that OP autotrophic denitrification rates were already below IC_{50} for cultures not originally enriched from a saline environment. On the other hand, with multi-ions solution (NaCl, $MgCl_2$ and $CaCl_2$), Macêdo et al. (2019) reported for a heterotrophic culture not cultivated in saline environment that the specific toxicity and the interactions between the salts were the key factors, regardless of the osmotic pressure applied. In the range 11–13 atm, their denitrification rates were inhibited over 70 % against the 50 % observed with KCl in our study. Considering that in our study at the same OP the pairs of ions decreased denitrification rate at different extents, the inhibition by ions was likely due to both the osmotic stress and the toxicity of the single ions. However, the exact mechanisms of ions' inhibition in this study cannot be further disclosed.

As for the order of inhibition of anions and cations, based on the results of our study in mol/L, Cl^- was more inhibitory than SO_4^{2-}, while Na^+ more than K^+. However, Cl^- as KCl had lower IC_{50} than as NaCl. This could indicate that K^+ was more inhibitory than Na^+ when paired with Cl^-, but, as shown in the test with K_2SO_4 in terms of denitrification rate and nitrite accumulation, and as reported by others (Chen et al., 2022b; Fang et al., 2011), bacterial cells seem to be less affected by potassium than sodium ions. Hence, despite more investigations on possible combined inhibition by K^+ and Cl^- are required, in this study, the major inhibitory anion and cation were attributed to Cl^- and Na^+. Furthermore, to better understand the effects of the single ions, the available data were analysed with two different statistical methods both returning Cl^- as the ion with the most important effect on denitrification rate. However, by using salts to study ions' inhibition, only pair of ions can be tested and not all the combinations are possible without adding a third ion (e.g., Na^+-K^+ and Cl^--SO_4^{2-}), which can affect the outcomes of the statistical analysis. Eventually, considering also that the OP associated to the salt at IC_{50} was lower for KCl and NaCl than Na_2SO_4, it can be concluded that Cl^- had very likely the highest toxic effect on both autotrophic and heterotrophic denitrification.

In general, heterotrophic enrichment culture showed higher tolerance to the ions than *T. denitrificans*. Even though composition of microbial community was not characterized, it was very likely that species tolerant to salinity became selectively enriched in the heterotrophic culture. Such selective enrichment has been reported as microbial community change with increasing salinity (Ji et al., 2018; Macêdo et al., 2019; Wang et al., 2018). On the other hand, *T. denitrificans* was the sole denitrifying species in the autotrophic tests, and more sensitive to the ions. The microorganism-based tolerance for ions in nitrogen removal processes, can also be observed for freshwater Anammox bacteria (Lin et al., 2021) and *Acinetobacter* sp. (heterotrophic nitrification and aerobic denitrification) (Chen et al., 2022b), as the former was more sensitive to K^+ rather than Na^+, while the latter was more inhibited by Na^+ than K^+, but for both SO_4^{2-} had the main inhibitory role. Therefore, the source of microorganisms needs to be properly selected for biological nitrogen removal from saline industrial wastewaters based not only on the substrate and the salinity, but also on the inorganic ions present.

4.3. Potential of biological denitrification for the treatment of high-ion-content industrial wastewaters: example of NO_x-SO_2 removal scrubber

Wet scrubbing is a common technology used to treat flue gases emitted by industries as well as vessels (Córdoba, 2015; Deng et al., 2021), which generates a liquid waste. The characteristics of this wastewater depend mainly on the flue gas composition and the type of scrubbing process. In the case of NO_x-SO_x removal scrubbers, ClO_2 and sodium based sulphurous compounds are used to enhance NO_x solubility (Schmid et al., 2022), while NaOH is used as a scrubbing agent (Chen et al., 2021), resulting in high and variable concentrations in the wastewater of not only nitrogenous and sulphurous compounds, but also chloride and sodium, as reported in Table 1 and Table S3 for a system treating flue gases from a pulp mill. To complete the NO_x removal and reduce the overall impact of emissions on water and air, denitrification can represent an effective solution to be implemented along with air emission control. Therefore, in this study, a first investigation on the potential of denitrification to treat wastewater from NO_x-SO_x removal scrubbers was made, starting from the possible inhibitory effects given by the main ions present.

According to the results of the tests with the model compounds, heterotrophic denitrification removed 2 g/L NO_3^- at Cl^-, Na^+ and SO_4^{2-} concentrations higher than those present in the real wastewater samples (Table 1 and Table S3), while *T. denitrificans* (DSM 12475) was more inhibited. Nitrite accumulated and was removed in both autotrophic and heterotrophic denitrification in the presence of the ions, showing promising results to avoid harmful discharge of NO_2^-. However, accumulation of other intermediates, such as N_2O, needs to be carefully considered for the environment. On the other hand, in the test with real wastewater, autotrophic denitrification was faster than heterotrophic denitrification in removing 800 mg/L NO_3^- and no NO_2^- accumulated at 3 g/L Cl^-, 4.5 g/L Na^+ and 12 g/L SO_4^{2-}, showing lower inhibition even in the presence of multiple ions. This could be due, for instance, to the presence of several metals (Table 1), to which autotrophic denitrification seem to have higher tolerance (Claus and Kutzner, 1985b); to other unidentified inhibitors for heterotrophic denitrification and boosters for autotrophic denitrification; or to lack of nutrients as NH_4^+ that has been reported as not essential for *T. denitrificans* (Koenig and Liu, 2001). Thus, the most likely inhibitory factor in the NO_x-SO_2 removal scrubber wastewater for *T. denitrificans* was salinity, similarly to what reported for flue gas desulphurization scrubber wastewater by Koenig and Liu (2004). Nevertheless, salinity tolerance seem to be enhanced in continuous flow reactors, where heterotrophic denitrification has been reported at 71.2 g/L NaCl (Glass and Silverstein, 1999) and 45 g/L Cl^- (Vredenbregt et al., 1997), and autotrophic denitrification at 33 g/L NaCl (Zhao et al., 2004), combined with biofilm systems, which present higher tolerance to toxicity (Liu et al., 2022).

The application of autotrophic denitrification to wastewaters from NO_x-SO_2 removal scrubber represents an interesting solution, as the electron donors could come from the scrubbing process, where large

amounts of $S_2O_3^{2-}$ and SO_3^{2-} are employed (Schmid et al., 2022). Moreover, autotrophic denitrification is reported to have lower N_2O gas production than heterotrophic (Cui et al., 2019), which tends to increase with increasing salinity (Zhao et al., 2013). However, one of the disadvantages of autotrophic denitrification is the large production of SO_4^{2-} (Di Capua et al., 2019), which is already present at high concentrations in the scrubber wastewaters (Table 1). A partial solution to this problem could be the application of mixotrophic denitrification that decreases sulphate production (Sahinkaya et al., 2011). Furthermore, also the variability in composition of NO_x-SO_2 removal scrubber wastewaters (Table 1 and S3) needs to be considered for the development of biological treatments.

Overall, the presence of organic waste streams (Meyer and Edwards, 2014) and the use of $S_2O_3^{2-}$ and SO_3^{2-} in the scrubbing process of flue gases highlight the attractiveness of pulp industry for the application of both autotrophic and heterotrophic denitrification. Therefore, denitrification could represent a potential solution for nitrate removal from NO_x-SO_2 removal scrubber wastewaters, as well as other saline industrial wastewaters, requiring further investigations in larger scale bioreactors.

5. Conclusions

In this study, inhibition by inorganic ions commonly present in saline wastewaters was investigated in batch assays on heterotrophic (enrichment culture) and autotrophic (*T. denitrificans*) denitrification. Moreover, real saline industrial wastewater from a NO_x-SO_2 removal scrubber was used to test denitrification potential. The main conclusions of this study are:

- Cl^- shows to be more inhibitory than Na^+, K^+ and SO_4^{2-} for heterotrophic and autotrophic denitrification rates. Autotrophic denitrification rate by *T. denitrificans* is increased by low Na^+ but at higher concentrations is rapidly inhibited.
- In both autotrophic and heterotrophic denitrification, nitrate removal is more inhibited than nitrite removal by the increasing concentrations of the ions.
- *T. denitrificans* (DSM 12475) shows lower IC_{50} than the heterotrophic enrichment culture and requires further considerations in view of a possible industrial application.
- Both autotrophic and heterotrophic denitrification efficiency is over 95 % for real NO_x-SO_2 removal scrubber wastewater, with autotrophic being faster than heterotrophic, showing the potential of autotrophic denitrification for this saline wastewater.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Niko Kalinainen reports a relationship with Valmet Technologies Oy that includes employment.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.165940.

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