Physiologic impact of 2-chlorophenol on denitrification process in mixture with different electron sources

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Abstract

The aim of this study was to evaluate the physiologic behavior of sludge in the absence and presence of 2-chlorophenol (2-CP) with different electron donors (phenol, glucose, and acetate) during denitrification process. In batch assays with phenol in the presence of 2-CP, a significant decrease of phenol consumption efficiencies (E_{phenol}) up to 99% was observed regarding the cultures without 2-CP. However, in most of the cases, nitrate consumption efficiencies ($E_{\text{NO}_3^-}$), and yields of nitrogen gas (Y_{N_2}) and bicarbonate ($Y_{\text{HCO}_3^-}$) were high, showing that the denitrifying respiratory process successfully occurred with phenol and 2-CP. The specific consumption rates of nitrate ($q_{\text{NO}_3^-}$) and phenol (q_{phenol}) decreased up to 6.0 and 32.3 times, respectively. In assays with glucose in the presence of 2-CP, the denitrifying performance was not significantly altered in terms of efficiencies and product yields; however, $q_{\text{NO}_3^-}$ was up to 1.6 times smaller than that obtained without 2-CP whereas q_{glucose} was increased up to 1.17 times. In assays with acetate plus 2-CP, the $E_{\text{NO}_3^-}$, E_{acetate} , and Y_{N_2} values remained high but 2-CP caused a decrease in $Y_{\text{HCO}_3^-}$. Moreover, $q_{\text{NO}_3^-}$ and q_{acetate} increased up to 1.4 and 2.0 times, respectively. These results show that the negative or positive effects of 2-CP on denitrification process depend on the type and concentration of electron source. The obtained physiologic and kinetic information might be useful to define strategies to maintain successful denitrification processes in wastewater treatment bioreactors fed with 2-CP.

Keywords 2-Chlorophenol · Denitrification · Electron donors · Physiologic impact

Introduction

Contamination of groundwater by nitrate has been reported and its treatment is recommended to obtain good quality drinking water (Mazari-Hiriart et al. 2005). Consumption of drinking water with high concentration of nitrate may result in methemoglobinemia in infants and possibly in cancer (Ward et al. 2010). Denitrification is a biological respiratory process that consists of the reduction of nitrate or nitrite to N_2 and implies energy generation through a series of reactions catalyzed by specific enzymes (Cuervo-López et al. 2009). Denitrification is an

important process used in wastewater treatment for nitrogen removal and better water quality (Lu et al. 2014). It is well documented that organotrophic denitrification can be successfully applied for the simultaneous removal of nitrate and aromatic recalcitrant molecules used as reducing sources for nitrate reduction into nitrogen gas (Texier et al. 2012; Cervantes et al. 2017; Chouhan et al. 2017). Previous studies clearly evidenced that depending on the type of electron donor used, the denitrifying respiratory process can be modified in a different manner, by changing the values of substrate consumption efficiencies, product yields and/or specific rates of substrate consumption and product formation (Calderer et al. 2010; Ge et al. 2012). For example, Baytshtok et al. (2009) observed that the exchange of methanol to ethanol as electron source caused a decrease in the specific rates of nitrate or nitrite removal. Peña-Calva et al. (2004) obtained different consumption efficiencies and product yields of the denitrifying process depending on the tested electron donors: acetate, toluene, *m*-xylene, and benzene. Physiologic and kinetic

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information is useful to better understand how to carry out denitrifying processes with high values of efficiencies, yields and velocities in wastewater treatment systems receiving effluents contaminated with recalcitrant aromatic compounds (Martínez et al. 2007; van der Waals et al. 2017).

Chlorophenols are recalcitrant compounds that are widely distributed due to their different applications, among them the wood preservation (Persson et al. 2008). Chlorophenols and their derivatives can cause alterations as carcinogenicity in living beings (Igbinosa et al. 2013). According to Vlastos et al. (2016), the 2-chlorophenol (2-CP) is a compound that causes chronic toxicity in aquatic organisms. The LC_{50} value of 2-CP for juvenile *Carassius auratus* was of 30.72 mg L⁻¹ (Yang et al. 2009). The toxic or inhibitory effects of chlorophenols on aerobic biological processes have been previously described (Pérez-Alfaro et al. 2013; Wang et al. 2015). Under nitrifying conditions, Pérez-Alfaro et al. (2013) have observed that the main negative effect was at the level of the ammonium oxidation process, which was 100% inhibited in the presence of 2.5 mg 2-CP-C L⁻¹. Experiments with 2-CP and other chlorophenols under anaerobic conditions such as methanogenesis or denitrification have been mainly focused on the chlorinated compound consumption (Bae et al. 2002; Beristain-Montiel et al. 2012). According to Sanford and Tiedje (1997), the reductive dechlorination and denitrification processes can occur simultaneously at low nitrate concentrations, where organic compounds can serve as electron donors for denitrification and reductive dechlorination. Also, it has been suggested that the stoichiometric reduction of nitrate during removal of 2-CP is possible; however, the production of N₂ was not measured (Bae et al. 2002). On the other hand, Chang et al. (2003) concluded that nitrate competes with 2-CP as electron acceptor because in their study, the 2-CP removal efficiency decreased in the presence of nitrate. As the performance of the denitrifying process is intrinsically linked to the electron donor employed, it might be thought that the effect of 2-CP on the denitrifying process will depend on the electron donor employed. More detailed physiologic and kinetic information is required to understand better the impact of chlorophenols on denitrification when different reducing sources are used. This type of studies could provide information to improve the performance of denitrifying reactors in the presence of recalcitrant compounds such as chlorophenols.

The aim of this study was to contribute to new knowledge generation on the effects of 2-CP on the respiratory behavior of a physiologically stabilized denitrifying sludge when denitrification was conducted with several concentrations of different electron donors: phenol, glucose, and acetate. Response variables such as efficiencies and specific rates of

substrate consumption as well as yields and specific rates of product formation were used to evaluate the physiologic behavior of the denitrifying sludge.

Materials and methods

Inoculum source

The denitrifying sludge employed for batch cultures was obtained from a continuous upflow anaerobic sludge blanket (UASB) reactor. The reactor of 1.4 L was operated at 30 °C and a hydraulic retention time of 2 days. The reactor was fed with two solutions: one containing the nitrogen source (2.42 g NaNO₃ L⁻¹) and the second solution had the following composition (g L⁻¹): 0.8 acetate-C, 2.16 K₂HPO₄, 0.54 KH₂PO₄, and 0.03 MgSO₄. The concentrations of trace elements were the following (mg L⁻¹): 70 FeCl₃·6H₂O, 7.5 CuSO₄·5H₂O, 7 CaCl₂·2H₂O, 5 MnCl₂, and 1 Na₂Mo₄·H₂O. Under these conditions, carbon and nitrate consumption efficiencies were around 98%. The $Y_{HCO_3^-}$ was 0.96 ± 0.02 and Y_{N_2} was 0.79 ± 0.04 . Thus, from the respiratory point of view, the denitrifying sludge was physiologically stable. The sludge produced at these conditions was centrifuged at 4000g for 10 min and washed with a solution of NaCl (9 g L⁻¹) at least three times prior to be used as inoculum for batch cultures.

Batch assays

All assays were performed in 545-mL serological bottles with 400 mL of culture medium and 145 mL of headspace. The basal mineral medium used was (g L⁻¹) 0.72 K₂HPO₄, 0.27 KH₂PO₄, 0.026 NaHCO₃, and 0.03 MgSO₄·7H₂O. Trace elements were added as previously described in the continuous culture. The bottles were sealed with rubber caps and aluminum backs. To obtain anaerobic conditions, a stream of He was bubbled by 6 min.

Kinetic assays were performed with basal mineral medium, denitrifying sludge [0.5 g volatile suspended solids (VSS) L⁻¹], phenol, glucose or acetate at different initial concentrations (80, 130, and 180 mg C L⁻¹) and the respective nitrate concentrations (25, 38, and 50 mg N L⁻¹) for establishing in all cases a C/N ratio of 3.4 ± 0.2 . Other assays under similar experimental conditions but adding 2-chlorophenol (22.4 mg C L⁻¹) were also performed in all cases at a C/N ratio of 4.0 ± 0.1 .

Recovery assays

To determine the exposure effect to 2-CP on the denitrifying process, two different assays were also carried out. One

assay was made with denitrifying sludge previously exposed for 72 days to 2-CP (22 mg C L^{-1}) in the presence of nitrate and acetate. After this time, the sludge was twice washed with an isotonic solution of NaCl and incubated in mineral medium containing nitrate (83 mg N L^{-1}) plus acetate (100 mg C L^{-1}). Under the same culture conditions, a second assay was carried out with denitrifying sludge without prior exposition to 2-CP.

All batch assays were performed in duplicate. Liquid and headspace samples were taken periodically for determining the concentration of substrates and products. Up to fourteen bottles were used to allow the duplicate and independent sampling at different times. The liquid samples were filtered using a $0.45\text{-}\mu\text{m}$ membrane before being analyzed. Values obtained in all assays are represented as the mean $\pm$ standard deviation.

Response variables used for evaluating the denitrifying process were as follows: specific rates of substrate consumption and product formation (q , $\text{mg of N or C g}^{-1}$ of VSS h^{-1}), efficiency [E ($\text{mg of substrate consumed mg}^{-1}$ of substrate fed) $\times 100$] and yield (Y , $\text{mg of product mg}^{-1}$ of substrate consumed).

Statistical analysis

For determining differences among the results obtained with the different electron donors in the presence and absence of 2-CP on the denitrifying behavior process, individual consumption efficiencies and specific consumption rate values obtained in each assay were subjected to ANOVA analysis. Multiple comparison tests (Duncan, $\alpha = 0.05$) were also made. The computer package utilized was Number Cruncher Statistical System (NCSS).

Analytical methods

Organic and inorganic carbon concentrations were determined using a total organic carbon analyzer (TOC) (Shimadzu, TOC-VCSN, Japan). The phenolic compounds were measured by HPLC (Perkin Elmer series 200, USA) using a C-18 column (Phenomenex, USA) and UV detector at 274 nm, where the mobile phase was acetonitrile–water (60:40 v/v) flushed at 1.5 mL min^{-1} . Nitrate and nitrite were measured by capillary electrophoresis (Beckman Coulter, ProteomeLabTM, PA 800, USA) at 214 nm, with a melted silica capillary of 60 cm length and $75 \mu\text{m}$ internal diameter; the electrolyte used was a mixture of 5 mL of Na_2SO_4 (0.1 M), 5 mL of NaCl (10 mM) and 5 mL of commercial solution CIA Pak OFM anion-BT (Waters) and 35 mL of deionized water. The gas composition in the headspace (CO_2 , CH_4 , N_2 , and N_2O) was determined by gas chromatography (Varian 3350, USA) with a thermal conductivity detector and a steel column packed with Poropak Q 80-100

Mesh using helium as carrier gas (16 mL min^{-1}). The temperatures of the column, injector, and detector were 50, 100, and 100°C , respectively. Glucose concentration was measured using a YSI 2700 biochemical analyzer (YSI Inc.) and a YSI membrane 2365. Acetate concentration was determined using a gas chromatograph equipped with a flame ionization detector (Hewlett-Packard 5890, USA) as reported by Martínez-Gutiérrez et al. (2014). VSS were quantified according to standard methods (APHA 1998).

Results and discussion

Assays with phenol in the absence and presence of 2-chlorophenol

When phenol was used as electron donor in the absence of 2-CP, it was observed that the main fraction of nitrate was consumed by the denitrifying sludge before 100 h, independently of the phenol concentration employed (Fig. 1). However, phenol consumption was not complete within the same period. In fact, once nitrate was mainly consumed, phenol consumption decreased, suggesting that phenol consumption was linked to nitrate depletion. These results can be explained considering that the stoichiometric value of C/N ratio for complete oxidation of phenol by denitrification is close to 1; however, the experimental C/N ratio used was 3.4 ± 0.2 . Thus, phenol was present in excess as electron donor with respect to the available nitrate as terminal electron acceptor. These conditions were established in these reference assays to favor the reductive dechlorination of 2-CP when this would be added into the cultures (Martínez-Gutiérrez et al. 2014). In all reference assays, after 336 h and irrespective of the phenol concentration supplemented, nitrate was totally consumed and reduced to N_2 , obtaining Y_{N_2} values close to 1 without a significant difference among them (Table 1). Likewise, the phenol consumed was mainly mineralized through the denitrifying respiratory process, as $Y_{\text{HCO}_3^-}$ values were higher than 0.60. The oxidation of phenol linked to denitrification has been previously reported in the literature (Beristain-Cardoso et al. 2009; Sahariah and Chakraborty 2012). In the present work, it was observed that the denitrifying metabolic pathway was not influenced in spite of using an excess of phenol as reducing source, as Y_{N_2} values remained close to 1. Nevertheless, the increasing phenol concentration caused a significant decrease in the efficiency (E_{phenol}) ($\alpha = 0.05$) (Table 1) and the specific rate (q_{phenol}) ($\alpha = 0.05$) (Fig. 2) of phenol consumption. This might be related to alterations in cellular membrane such as changes in fatty acid composition as previously reported for phenolic compounds (Fischer et al. 2010).

Fig. 1 Phenol (**a**) and nitrate (**b**) consumption by the denitrifying sludge in the absence (full figure and continuous line) and presence (infill figure and discontinuous line) of 2-CP at different initial concentrations of phenol

Table 1 Response variables of the denitrifying process with different concentrations of phenol as electron donor in the absence and presence of 2-CP

Compound concentrations (mg C or N L ⁻¹)	E_{phenol} (%)	$E_{\text{NO}_3^-}$ (%)	$Y_{\text{HCO}_3^-}$ (mg C mg ⁻¹ C consumed)	Y_{N_2} (mg N mg ⁻¹ NO ₃ ⁻ -N consumed)
Phenol (80) + NO ₃ ⁻ (25)	80.9 ± 13.1	100.0 ± 0.0	0.90 ± 0.21	0.90 ± 0.01
Phenol (80) + NO ₃ ⁻ (25) + 2-CP (22.4)	1.1 ± 0.8	98.0 ± 2.8	0.88 ± 0.11	0.97 ± 0.02
Phenol (130) + NO ₃ ⁻ (38)	30.5 ± 1.9	98.3 ± 0.5	0.74 ± 0.02	1.08 ± 0.12
Phenol (130) + NO ₃ ⁻ (38) + 2-CP (22.4)	16.9 ± 0.3	90.0 ± 14.1	0.80 ± 0.06	0.91 ± 0.12
Phenol (180) + NO ₃ ⁻ (50)	30.2 ± 6.3	98.8 ± 0.5	0.60 ± 0.03	0.87 ± 0.01
Phenol (180) + NO ₃ ⁻ (50) + 2-CP (22.4)	15.8 ± 15.7	75.9 ± 14.1	0.82 ± 0.11	1.07 ± 0.08

Efficiency (E) and yield (Y) values were determined at 336 h of culture

Addition of 2-CP to the culture medium resulted in the decrease of phenol consumption efficiencies (E_{phenol}) up to 99%. Surprisingly, $E_{\text{NO}_3^-}$ remained higher than 90% in the assays amended up to 130 mg phenol-C L⁻¹ and it only decreased by 23% with 180 mg phenol-C L⁻¹ (Table 1). In all cases, the process remained mainly catabolic as the $Y_{\text{HCO}_3^-}$ and Y_{N_2} values were higher than 0.80 and 0.91, respectively. These results showed that the denitrifying respiratory pathway was still occurring in spite of the presence of 2-CP. As shown in Fig. 1 in the presence of 2-CP, after 96 h of culture, the phenol concentrations remained almost constant whereas nitrate was still consumed. In

these assays, 2-CP was partially consumed, obtaining $E_{2\text{-CP}}$ values ranging between 15.5 and 31.6% after 336 h. These results suggest that nitrate reduction to N₂ by denitrification might be linked to the oxidation of the phenol produced by the reductive dechlorination of 2-CP (Sanford and Tiedje 1997). This could also explain why E_{phenol} values remained low because part of the detected phenol would be product of the reductive dechlorination of 2-CP.

In the presence of 2-CP, a longer time (3.5 times) was required for the consumption of the main fraction of nitrate compared with the assays without 2-CP (Fig. 1). Similarly, $q_{\text{NO}_3^-}$ and q_{phenol} decreased up to 6.0 and 32.3 times, respec-

Fig. 2 Specific consumption rates in the absence and presence of 2-CP at different concentrations of the electron sources (phenol, glucose or acetate). **a** Consumption of nitrate and **b** consumption of carbonaceous compounds

tively (Fig. 2). The lowest $q_{\text{NO}_3^-}$ and q_{phenol} values were determined in the assays amended with 2-CP ($\alpha=0.05$), showing that 2-CP had inhibitory properties on denitrification process. Thus, results indicate that phenol consumption rate was the most affected by the presence of 2-CP as the lowest q_{phenol} values were obtained when 2-CP was added to the denitrifying culture ($\alpha=0.05$).

The results showed that although nitrate reduction into N_2 was not significantly affected by the presence of 2-CP (the Y_{N_2} values were close to 1), phenol consumption efficiencies as well as specific consumption rates of both phenol and nitrate were significantly decreased ($p \leq 0.001$) by the addition of $22.4 \text{ mg } 2\text{-CP-C L}^{-1}$ (Table 1).

Assays with glucose in the absence and presence of 2-chlorophenol

When glucose was used as reducing source in the absence of 2-CP, nitrate and glucose were concomitantly consumed within 24 h (Fig. 3). No significant effect of glucose concentration was observed on $E_{\text{NO}_3^-}$ and E_{glucose} as their values were near 100% in all cases (Table 2). Glucose was mainly mineralized whereas nitrate was reduced to N_2 . The $Y_{\text{HCO}_3^-}$ values varied from 0.64 to 0.82 and Y_{N_2} values from 0.65 to 0.77. These results showed that the denitrifying process was successfully carried out using glucose as the electron donor.

When 2-CP was added to the culture, the denitrifying performance was similar to that observed without 2-CP (Fig. 3) and most of the E_{glucose} , $E_{\text{NO}_3^-}$, Y_{N_2} , and $Y_{\text{HCO}_3^-}$ values did not significantly change (Table 2). Under these conditions, the consumption efficiency of 2-CP was negligible in all cases. Thus, in spite of the presence of 2-CP, the results showed that the sludge kept an effective denitrifying respiratory process. Nevertheless, the presence of 2-CP provoked a significant effect on specific consumption rate of nitrate ($p=0.0001$) as the highest $q_{\text{NO}_3^-}$ values were obtained in the absence of 2-CP whereas the lowest $q_{\text{NO}_3^-}$ values were obtained when 2-CP was present ($\alpha=0.05$) (Fig. 2). In fact, the presence of 2-CP resulted in an average decrease of 1.6 ± 0.3 times in $q_{\text{NO}_3^-}$. On the other hand, it must be noted that increases in specific consumption rate of glucose (q_{glucose}) were observed due to the increase in the initial glucose concentration in the absence as in the presence of 2-CP (Fig. 2). In fact, statistical difference in q_{glucose} was observed when the different concentrations of glucose were assayed ($p=0.001$). Thus, the lowest q_{glucose} values were obtained at $80 \text{ mg glucose-C L}^{-1}$ whereas the highest q_{glucose} values were achieved at $180 \text{ mg glucose-C L}^{-1}$, regardless of the presence of 2-CP ($\alpha=0.05$). At the highest concentrations of glucose (130 and 180 mg C L^{-1}), the 2-CP addition seemed to favor the glucose consumption with higher values for q_{glucose} up to 1.39 times.

Fig. 3 Glucose (a) and nitrate (b) consumption by the denitrifying sludge in the absence (full figure and continuous line) and presence (infill figure and discontinuous line) of 2-CP at different initial concentrations of glucose

Table 2 Response variables of the denitrifying process with different concentrations of glucose as electron donor in the absence and presence of 2-CP

Compound concentrations (mg C or N L ⁻¹)	E_{glucose} (%)	$E_{\text{NO}_3^-}$ (%)	$Y_{\text{HCO}_3^-}$ (mg C mg ⁻¹ C consumed)	Y_{N_2} (mg N mg ⁻¹ NO ₃ ⁻ -N consumed)
Glucose (80) + NO ₃ ⁻ (25)	86.9 ± 7.2	98.9 ± 0.9	0.82 ± 0.02	0.65 ± 0.10
Glucose (80) + NO ₃ ⁻ (25) + 2-CP (22.4)	97.7 ± 0.9	99.6 ± 0.5	0.48 ± 0.06	0.77 ± 0.10
Glucose (130) + NO ₃ ⁻ (38)	100.0 ± 0.0	92.1 ± 1.9	0.68 ± 0.01	0.77 ± 0.07
Glucose (130) + NO ₃ ⁻ (38) + 2-CP (22.4)	92.9 ± 4.4	77.6 ± 2.9	0.70 ± 0.09	0.73 ± 0.01
Glucose (180) + NO ₃ ⁻ (50)	100.0 ± 0.0	92.8 ± 0.9	0.64 ± 0.04	0.72 ± 0.02
Glucose (180) + NO ₃ ⁻ (50) + 2-CP (22.4)	97.9 ± 1.1	87.5 ± 13.7	0.70 ± 0.05	0.83 ± 0.10

Efficiency (E) and yield (Y) values were determined at 24 h of culture

The results showed that although denitrification pathway was not significantly altered by the presence of 2-CP in terms of efficiencies and yields, the process was kinetically affected when 2-CP was present, provoking an inhibitory effect on the nitrate reduction while a beneficial effect seemed to be observed for the glucose oxidation process.

Assays with acetate in the absence and presence of 2-chlorophenol

Figure 4 shows the consumption profiles of acetate and nitrate with and without 2-CP. In the absence of 2-CP, both compounds were mainly consumed within 44 h. At this time, $E_{\text{NO}_3^-}$ and E_{acetate} were higher than 95 and 76%, respectively.

Acetate was mineralized to HCO_3^- and nitrate was converted into N_2 , as both $Y_{\text{HCO}_3^-}$ and Y_{N_2} were higher than 0.87 (Table 3). These yield values are similar to those found in other work where acetate was used for denitrification (Martínez et al. 2007). The $q_{\text{NO}_3^-}$ and q_{acetate} increased while the concentration of acetate and nitrate also increased (Fig. 2), similar to that observed in the presence of glucose. In these assays, higher specific rates were achieved as the electron donor concentration was increased like a first-order reaction. In fact, a clear denitrifying profile was observed similar to the kinetic profile obtained by Peña-Calva et al. (2004).

When the denitrifying sludge was in contact with 2-CP, the consumption profiles were similar to those observed

Fig. 4 Acetate (a) and nitrate (b) consumption by the denitrifying sludge in the absence (full figure and continuous line) and presence (infill figure and discontinuous line) of 2-CP at different initial concentrations of acetate

Table 3 Response variables of the denitrifying process with different concentrations of acetate as electron donor in the absence and presence of 2-CP

Compound concentrations (mg C or N L ⁻¹)	E_{acetate} (%)	$E_{\text{NO}_3^-}$ (%)	$Y_{\text{HCO}_3^-}$ (mg C mg ⁻¹ C consumed)	Y_{N_2} (mg N mg ⁻¹ NO ₃ ⁻ -N consumed)
Acetate (80) + NO ₃ ⁻ (25)	85.8 ± 4.7	100.0 ± 0.0	0.94 ± 0.01	0.87 ± 0.01
Acetate (80) + NO ₃ ⁻ (25) + 2-CP (22.4)	88.8 ± 0.3	100.0 ± 0.0	0.67 ± 0.01	1.0 ± 0.04
Acetate (130) + NO ₃ ⁻ (38)	76.3 ± 4.9	95.4 ± 6.5	0.90 ± 0.04	0.95 ± 0.01
Acetate (130) + NO ₃ ⁻ (38) + 2-CP (22.4)	83.4 ± 0.2	99.8 ± 0.3	0.45 ± 0.01	0.97 ± 0.04
Acetate (180) + NO ₃ ⁻ (50)	91.6 ± 0.3	96.4 ± 4.6	0.95 ± 0.01	0.98 ± 0.21
Acetate (180) + NO ₃ ⁻ (50) + 2-CP (22.4)	94.9 ± 0.3	100.0 ± 0.0	0.51 ± 0.07	0.95 ± 0.04

Efficiency (E) and yield (Y) values were determined at 44 h of culture

without 2-CP (Fig. 4) and E_{acetate} and $E_{\text{NO}_3^-}$ were higher than 83%. Nevertheless, in all cases, the consumption efficiency of 2-CP was negligible during 44 h. In all cases, Y_{N_2} values were higher than 0.95 but $Y_{\text{HCO}_3^-}$ values decreased between 29 and 50%. At same acetate concentration used, no significant differences were found between E_{acetate} , $E_{\text{NO}_3^-}$ and Y_{N_2} values observed in the presence of 2-CP and those obtained in its absence (Table 3), but significant effect of 2-CP ($p=0.001$) was determined on $Y_{\text{HCO}_3^-}$. These results indicated that in spite of the presence of 2-CP, acetate and nitrate were mainly consumed by denitrification but the oxidative pathway of acetate was altered by the addition of 2-CP, diminishing its mineralization. Finally, results

also showed that $q_{\text{NO}_3^-}$ and q_{acetate} increased while the concentrations of nitrate and acetate were increased as observed in the cultures without 2-CP (Fig. 2). In fact, q_{acetate} and $q_{\text{NO}_3^-}$ were significantly increased about 2-fold and 1.4-fold, respectively, in the assays where 2-CP was added to the culture ($\alpha=0.05$). This improved denitrification due to the presence of 2-CP might be related to changes in the cell membrane and mechanisms by which these compounds are transported.

Results obtained in the present work evidenced that the metabolic and kinetic differences in the behavior of the denitrifying sludge due to the presence of 2-CP depended on the electron source employed. A summary of the global

kinetic results indicates that the significant effect of 2-CP on the denitrifying kinetic performance depended on both concentration ($p=0.0001$) and type of the electron donor ($p=0.0001$). In this sense, the highest carbon consumption rate was achieved in the assays with 2-CP and 180 mg glucose-C L⁻¹ whereas the lowest carbon consumption rate was determined in the presence of 2-CP and phenol as electron source. The highest specific rate obtained with glucose might be related to the involvement of oxidative glycolytic pathway which is well known to be faster than the oxidation of acetate under anaerobic conditions (Chidthaisong and Conrad 2000). In fact, when 2-CP was present, q_{glucose} was up to twofold higher than q_{acetate} ($\alpha=0.05$). The highest $q_{\text{NO}_3^-}$ (29-fold higher) was achieved in the assays with 180 mg acetate-C L⁻¹ amended with 2-CP while the lowest $q_{\text{NO}_3^-}$ was determined in the presence of 2-CP and phenol. Likewise, in the presence of 2-CP, twofold higher values of $q_{\text{NO}_3^-}$ were achieved when acetate was used in comparison when glucose was the electron donor ($\alpha=0.05$).

Recovery assays

To assess the effect of previous exposure to 2-CP on the denitrifying process, an assay with denitrifying sludge previously exposed to 2-CP (22 mg C L⁻¹) for 72 days in the presence of nitrate and acetate was carried out and compared to another assay with denitrifying sludge without prior exposure to 2-CP. In cultures without previous exposure to 2-CP, acetate and nitrate were completely consumed within 15 h, whereas in the cultures with sludge previously exposed to 2-CP, 31 h, were required for the whole acetate and nitrate consumption. In the cultures without previous exposure to 2-CP, the values of q_{acetate} and $q_{\text{NO}_3^-}$ were 168.2 ± 1.4 mg acetate-C g⁻¹ VSS day⁻¹ and 140.6 ± 0.2 mg NO₃⁻-N g⁻¹ VSS day⁻¹, respectively; while in the assays with sludge previously exposed to 2-CP, the values obtained for q_{acetate} and $q_{\text{NO}_3^-}$ were 43.9 ± 1.4 mg acetate-C g⁻¹ VSS day⁻¹ and 35.5 ± 5.8 mg NO₃⁻-N g⁻¹ VSS day⁻¹, respectively, representing a decrease of 74% for both specific rates. These results show that 2-CP caused a negative impact on the denitrifying sludge activity that persisted even after 2-CP was removed. Both reductive and oxidative pathways in the denitrifying process were influenced. These results might be related to the longtime of exposure to 2-CP, as previously suggested by Pérez-Alfaro et al. (2013). The previous exposure to 2-CP also provoked that Y_{N_2} and $Y_{\text{HCO}_3^-}$ decreased by 48 and 17%, respectively, resulting in values of 0.27 ± 0.01 mg N mg⁻¹ NO₃⁻-N consumed and 0.59 ± 0.01 mg C mg⁻¹ acetate-C consumed, respectively. The Y_{N_2} diminishment might be explained by nitrite

accumulation, which resulted in a higher nitrite yield ($Y_{\text{NO}_2^-} = 0.64 \pm 0.01$ mg N mg⁻¹ NO₃⁻-N consumed). Since nitrite was accumulated, it is possible to assume that the denitrifying pathway was affected at the level of nitrite reductase enzyme as previously observed in a continuous denitrifying reactor fed with phenolic compounds (Meza-Escalante et al. 2008). Thus, the results obtained in the present work indicated that sludge with long contact to chlorophenols would require special attention when being used for wastewater treatment to prevent nitrogenous and carbonaceous intermediates from the denitrifying pathway.

Conclusions

Results obtained in the present work evidenced that the impact of 2-CP on denitrification can occur at metabolic and kinetic levels and depends on both type and concentration of the electron donor used for nitrate reduction. In the presence of phenol as electron source and 2-CP, high nitrate consumption efficiencies were obtained while N₂ and bicarbonate were the main products, showing that the denitrifying respiratory process was carried out. However, the specific rate of nitrate reduction was inhibited by 2-CP. The high decreases obtained in the efficiency and specific rate of phenol consumption might be related to the formation of phenol by reductive dechlorination of 2-CP, masking the real capacity of the sludge for consuming phenol under these conditions. When glucose was used as electron donor, the presence of 2-CP did not change the metabolic behavior of the sludge as similar values for efficiencies and yields were observed. However, the results indicated that the presence of 2-CP provoked modifications at the kinetic level, being slower the nitrate reduction step and faster the glucose oxidation. When acetate was added as the reducing source, 2-CP only caused a decrease in the mineralization process while denitrification was successfully performed with an increase in its velocity.

To our knowledge, this is the first time that physiologic and kinetic data are determined to describe the distinct effects of 2-CP on denitrification process depending on type and concentration of diverse electron donors. The heterogeneous effects observed point out the importance to consider consumption efficiencies, product yields and respiratory rates as essential parameters for understanding better the global impact of chlorophenols on denitrification. These results might also represent a practical application in wastewater treatment, as efficient removal and high consumption rates of easily consumable organic matter such as acetate or glucose can be achieved even in the presence of recalcitrant pollutant such as 2-CP by a denitrifying process.

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Compliance with ethical standards

Conflict of interest The authors of this manuscript declare that there is no conflict of interest.

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