Effect of operating parameters on molinate biodegradation

Patrícia Correiaa, Rui A.R. Boaventurab, Maria A.M. Reisc, Olga C. Nunesa,∗

aLEPÆ, Departamento de Engenharia Química, Faculdade de Engenharia, Universidade do Porto, R. Dr. Roberto Frias, 4200-465 Porto, Portugal
bLSRE, Departamento de Engenharia Química, Faculdade de Engenharia, Universidade do Porto, R. Dr. Roberto Frias, 4200-465 Porto, Portugal
cCQFB/REQUINTE, Department of Chemistry, FCT, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

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Abstract

The effect of operating conditions during molinate degradation by the defined mixed bacterial culture DC, previously described as able to mineralize molinate, was evaluated in a batch reactor. Parameters such as the rate of molinate degradation, the dissolved organic carbon (DOC) consumption and the accumulation of molinate degradation products were monitored along the culture growth. The effect of conditions such as temperature, pH, aeration rate, salinity, and presence of additional carbon and/or nitrogen sources, was tested independently. Degradation of molinate in river water was also evaluated. Culture DC was able to grow and to mineralize molinate at all the conditions assayed. Temperature was the factor with the strongest influence on bacterial growth and molinate mineralization. The lowest and the highest rate values of growth (0.010 and 0.110 h⁻¹) and of molinate degradation (0.027 and 0.180 g molinate g⁻¹ cell dry wt h⁻¹) were obtained at 15 and 35 °C, respectively. In cultures with approximately 187 mg l⁻¹ of molinate, 2-oxo-molinate was the major molinate degradation product accumulated in the medium, in concentrations below 0.133 mg l⁻¹.

Degradation of molinate was also evaluated in a continuous stirred tank reactor (CSTR). Operating the CSTR at a hydraulic retention time (HRT) of 83 h, fed with medium containing molinate concentrations ranging from 1 to 3 mM, culture DC degraded the herbicide with specific degradation rates similar to those obtained in the batch systems.

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1. Introduction

Several studies have described the environmental contamination with pesticides resultant from agricultural practices, accidental spillage or uncontrolled release of industrial effluents. The contamination of air, soils, surface and ground waters, and the trophic chain with pesticides has evident negative impacts on public health, and on biological diversity. Molinate (S-ethyl perhydroazepine-1-carbothioate), a herbicide used worldwide to protect rice crops, has been detected in natural waters in concentrations up to 100 µg l⁻¹ (Mabury et al., 1996). In the environment, molinate can be (photo-) chemically or biologically degraded into partially oxidized products such as molinate-acid, molinate-alcohol, hydroxy-molinate, oxo-molinate, molinate-sulfoxide, and molinate-sulfoxide (Soderquist et al., 1977; Golovleva et al., 1981; Imai and Kuwatsuka, 1986; Cochran et al., 1997; Konstantinou et al., 2001). Molinate and some of its degradation products, such as molinate-sulfoxide, have adverse effects on the health of humans and animals (Golovleva et al., 1981; Cochran et al., 1997; Jewell et al., 1999; California EPA, 2000). Several microorganisms have been described in the literature as able to co-metabolize molinate into the above-mentioned metabolites (Zyakun et al., 1983; Imai and Kuwatsuka, 1986).

∗Corresponding author. Tel.: +351 225081917; fax: +351 225081449.
E-mail address: opnunes@fe.up.pt (O.C. Nunes).
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and a few have been described as capable of their further mineralization (Imai and Kuwatsuka, 1986). A bacterial mixed culture (culture DC) was described as able to mineralize molinate when grown with molinate as the only carbon, nitrogen and energy source (Barreiros et al., 2003).

The development of strategies to cleanup molinate contaminated sites and to prevent future contaminations is important, because the deliberated application of the herbicide on rice fields promotes its dissemination through the surrounding environment (air, surface and underground water, soil and biotic community) (Soderquist et al., 1977; Albanis et al., 1998; Tsuda et al., 1998; Castro et al., 2005), in concentrations that may exceed the legal recommended highest values (Castro et al., 2005).

Despite the high efficacy and the low cost of bioremediation for the removal of toxic compounds from polluted sites, this method presents some constraints. The extent and rate of biodegradation depend, not only on the properties of the xenobiotic and the presence of microorganisms able to degrade the target compound, but also on environmental conditions such as temperature, pH, availability of nutrients, dissolved oxygen or other final electron acceptors (Strobel and Sullivan, 1999). The adequate settling of these conditions must be established before a bio-remediation technology is implemented. The effect of these conditions on molinate biodegradation was never reported.

The aim of this study was to evaluate the effect of the operating conditions on molinate degradation by the defined mixed culture DC. This culture is composed by a mixture of five bacterial strains (ON1–ON5) purified from an enrichment culture of contaminated soil and water collected nearby a molinate producing industry (Barreiros et al., 2003). The optimal abiotic conditions for molinate degradation such as temperature, pH or salinity in a batch reactor were established. The capacity of culture DC to remove molinate from a contaminated water stream was also assessed in a continuous stirred tank reactor (CSTR) fed with different molinate concentrations. Accumulation of molinate degradation products was examined in the different conditions assayed.

### 2. Materials and methods

#### 2.1. Chemicals

Molinate (S-ethyl perhydroazepine-1-carbothioate) of 97% purity was obtained from Herbevex, Produtos Químicos (Estoril, Portugal). Some properties of molinate are given in Table 1. Molinate sulfoxide, molinate-sulfone, molinate-alcohol, and 2-oxo-molinate were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). All other reagents were analytical grade from commercial sources.

#### 2.2. Culture and growth media

Culture DC, previously isolated and stored as described by Barreiros et al. (2003) was grown in mineral medium B (Barreiros et al., 2003) containing molinate as the only source of energy, carbon and nitrogen, in concentrations from about 187 mg l\(^{-1}\) (1 mM), to 750 mg l\(^{-1}\) (4 mM). To prepare the inoculum [10% (v/v)], culture DC was grown in medium B with 4 mM molinate, in an Erlenmeyer flask, for 3 days at 30°C and 120 rpm. To study the effect of salinity on molinate degradation, medium B was supplemented with 10 g l\(^{-1}\) NaCl (179 mM). The effect of pH was assessed in cultures grown in mineral medium B buffered with phosphate buffer (27 mM) at pHs 6.3, 7.2, and 8.0. In order to study the effect of additional carbon and nitrogen sources on molinate depletion, medium B was supplemented with meat extract (1 g l\(^{-1}\)), yeast extract (1 g l\(^{-1}\)), or with meat extract (110 mg l\(^{-1}\)), tryptone (160 mg l\(^{-1}\)) and urea (30 mg l\(^{-1}\)) (OECD, 1995). All the media were autoclaved before their use.

#### 2.3. Experimental set-up

Culture DC was grown, under aseptic conditions, in a batch reactor with a volume of 500 ml, equipped with a cooling jacket, a sintered glass air diffuser, an exhaust, an inlet port for inoculation, and an outlet port for sampling. The reactor was operated with a 350 ml working volume, controlled temperature (refrigerated water bath, Huber), magnetic stirring (300 rpm), and pH monitoring. Aerobic conditions were maintained by using an air pump, that supplied sterile air through the diffuser.

The reactor used for the continuous assays was similar to that described for batch assays, except that in this case the reactor had a capacity of 11, and a working volume of 700 ml. Medium B with molinate concentrations ranging from 1 to 3 mM was fed to the reactor through a peristaltic pump with a flow rate of 8.4 ml h\(^{-1}\), corresponding to an hydraulic retention time (HRT) of 83 h.

#### 2.4. Operating conditions

The effect of operating conditions on molinate degradation by culture DC was assessed in cultures grown in mineral medium B [pH 7.2; NaCl 0.04%, w/v] with approximately 1 mM molinate. The conditions tested in the batch assays were (see Table 1): (a) temperature [15, 20, 35 and 35°C; 0.2 volume of air per volume of reactor per minute (vvm)]; (b) pH (6.3, 7.2 and 8.0; 30°C; 0.2vvm); (c) aeration rate (0.1 and 0.2 vvm; 30°C); (d) salinity [NaCl 0.04% and 1% (w/v); 30°C; 0.2vvm]; and (e) presence of additional nutrients [yeast extract and meat extract, and meat extract, tryptone and urea (as described above); 30°C; 0.2vvm]. The effect of each parameter was studied independently. To evaluate abiotic losses, a control was performed in the same medium, at 30°C, 0.2vvm, inoculated with a previously autoclaved culture.

#### 2.5. Degradation of molinate in river water

The ability of culture DC to remove molinate from real waters was studied using water from the river Pranto (central Portugal) collected at a site receiving tail waters from paddy rice fields situated on the banks of this river. The river water presented pH 8.0, conductivity of 1090 μS cm\(^{-1}\), and a DOC content of 1.3 mg l\(^{-1}\). The water was naturally contaminated with molinate at a concentration of 16 μg l\(^{-1}\) (Castro et al.,
and was spiked with molinate in order to obtain a final concentration of about 1 mM. No other nutrient was added. The experimental set-up and inoculation procedures were similar to those described above. The temperature of incubation was 30 °C. A non-inoculated control reactor was operated simultaneously to assess both abiotic losses and biodegradation of molinate by the autochthonous microbiota.

2.6. Sampling, cell dry weight, molinate and DOC measurements

Culture samples were collected along regular intervals of time and further analysed for biomass, molinate and dissolved organic carbon (DOC) contents. Cell growth was monitored spectrometrically (OD610 nm). Cells dry weight was obtained through a calibration curve of optical density versus cells dry weight. Molinate concentration and DOC content in culture supernatants were analysed, respectively, by high performance liquid chromatography (HPLC) and a TOC apparatus. Culture samples were collected along regular intervals of time and further analysed for biomass, molinate and dissolved organic carbon (DOC) contents. Cell growth was monitored spectrometrically (OD610 nm). Cells dry weight was obtained through a calibration curve of optical density versus cells dry weight. Molinate concentration and DOC content in culture supernatants were analysed, respectively, by high performance liquid chromatography (HPLC) and a TOC apparatus. Culture samples were collected along regular intervals of time and further analysed for biomass, molinate and dissolved organic carbon (DOC) contents.

2.7. Analysis of molinate degradation products

The presence of molinate-alcohol, molinate-sulfoxide, molinate-sulfone and 2-oxo-molinate in culture supernatants at the end of the growth was assessed. Each sample (40 ml) was acidified (pH 3) with concentrated HCl and extracted twice with the same volume of n-hexane. The resulting 80 ml of organic extract was passed through a water phase membrane separator containing anhydrous sodium sulfate in order to remove any remaining aqueous phase. The organic extracts were dried under a nitrogen flow, and the residual extract was re-suspended in 1 ml of n-hexane, and analysed by HPLC as described before (Barreiros et al., 2003). The detection limit of the method for each component was of 0.025 mg l−1.

2.8. Determination of specific cell growth and molinate degradation rates

Specific cell growth (µ), molinate degradation (rs) and DOC degradation (rs) rates were calculated during the exponential growth phase, using the equations below:

\[ X = X_0 e^{\mu t - t_0} \]
\[ S = S_0 - \frac{r_s X_0}{\mu} (e^{\mu t - t_0} - 1) \]

where \( X_0 \) and \( S_0 \) are the biomass and substrate concentrations at the initial time (t0) and X and S are the biomass and substrate concentrations along the reactor operation time (t). It was assumed that the specific molinate and DOC degradation rates follows a zero-order kinetics relatively to molinate and to DOC content, respectively (given the high molinate and DOC concentration in the exponential growth phase).

The cell growth yield on molinate (Y) was calculated as

\[ Y = \frac{\mu}{r_s} \]

The above parameters were determined by fitting the experimental data of biomass, molinate and DOC concentration along the time. The commercial software Fig.P, from BioSoft®, was used to calculate these parameters and the associated 95% confidence interval. The specific molinate degradation rate (rs), in the CSTR was determined through the equation:

\[ r_s = \frac{Q}{V X} (S_i - S) \]

where V is the reactor volume, Q is the influent flow rate, and \( S_i \) and S are the molinate concentrations in the influent and effluent, respectively.

3. Results and discussion

The effect of the operating conditions on molinate mineralization by culture DC was studied in batch cultures grown under aseptic conditions with controlled temperature, aeration and stirring rates, and pH (culture medium buffering capacity). For each condition assayed it was determined the specific cell growth (µ) and molinate degradation (rs) rates, the cell growth yield (Y), the percentage of DOC consumed, and the accumulation of molinate degradation products.

3.1. Biodegradation of molinate as the only source of carbon, energy and nitrogen

3.1.1. Effect of temperature

The effect of temperature on molinate degradation by culture DC was studied in batch cultures grown in medium B aerated with an air flow rate of 0.2 vvm. The range of tested temperatures (15–35 °C) was chosen according to the average temperature of natural waters in central Portugal (approximately 15 °C in winter and 25 °C in summer), and to the operating temperatures of the biological wastewater treatment plants (around 15–20 °C). In order to evaluate the effect of temperature shifts, culture DC was also grown at 30 and 35 °C.

<table>
<thead>
<tr>
<th>Table 1 – Physical properties of molinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical structure</td>
</tr>
<tr>
<td>Molecular weight</td>
</tr>
<tr>
<td>Water solubility</td>
</tr>
<tr>
<td>Henry’s law constant</td>
</tr>
<tr>
<td>Organic coefficient</td>
</tr>
</tbody>
</table>

Data from Mabury et al. (1996).
Culture DC was able to grow and to degrade molinate under all temperatures tested. The occurrence of cell growth at expenses of molinate was evidenced by the fact that the exponential growth phase was coincident with the molinate consumption, and growth ceased after molinate had been consumed (Fig. 1A–D). Furthermore, there was a direct association between the DOC decrease and molinate depletion, suggesting that molinate was mineralized (Fig. 1). The high values of molinate and DOC contents in the abiotic control, when compared with those of cultures, indicate that abiotic losses are negligible (Fig. 1E). The cell growth yield increased from 15 to 35°C, with values in the range of those usually found for the degradation of xenobiotic compounds (Grady et al., 1999). In the range of temperatures assayed, specific cell growth ($\mu$) and molinate degradation ($r_s$) rates increased with temperature from 0.010 to 0.110 h$^{-1}$, and from 0.027 to 0.180 g molinate g$^{-1}$ cell dry wt h$^{-1}$, respectively (Table 2). This variation follows the Arrhenius equation.
Table 2 – Specific growth ($\mu$) and molinate degradation ($r_s$) rates, percent of DOC consumption and cell growth yield ($Y$) of culture DC grown in medium B with about 1 mM of molinate under different batch operating conditions.

<table>
<thead>
<tr>
<th>Operating condition</th>
<th>$\mu$ (h$^{-1}$)</th>
<th>$r_s$ (g molinate g$^{-1}$ cell dry wt h$^{-1}$)</th>
<th>DOC reduction (%)</th>
<th>$Y$ (g cell dry wt g$^{-1}$ molinate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.010 ± 0.001</td>
<td>0.027 ± 0.001</td>
<td>55</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>20</td>
<td>0.022 ± 0.001</td>
<td>0.046 ± 0.003</td>
<td>73</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>30</td>
<td>0.058 ± 0.002</td>
<td>0.110 ± 0.010</td>
<td>77</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td>35</td>
<td>0.110 ± 0.020</td>
<td>0.180 ± 0.030</td>
<td>84</td>
<td>0.60 ± 0.10</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.3</td>
<td>0.057 ± 0.003</td>
<td>0.130 ± 0.020</td>
<td>66</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>7.2</td>
<td>0.058 ± 0.002</td>
<td>0.110 ± 0.010</td>
<td>77</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td>8.0</td>
<td>0.045 ± 0.002</td>
<td>0.140 ± 0.020</td>
<td>74</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>Aeration (vvm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 (3 mg O$_2$ l$^{-1}$)</td>
<td>0.064 ± 0.001</td>
<td>0.090 ± 0.010</td>
<td>85</td>
<td>0.70 ± 0.10</td>
</tr>
<tr>
<td>0.2 (5 mg O$_2$ l$^{-1}$)</td>
<td>0.058 ± 0.002</td>
<td>0.110 ± 0.010</td>
<td>77</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td>Salinity (% NaCl, w/v)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.04</td>
<td>0.058 ± 0.002</td>
<td>0.110 ± 0.010</td>
<td>77</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td>1</td>
<td>0.038 ± 0.002</td>
<td>0.100 ± 0.010</td>
<td>62</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>River water</td>
<td>0.042 ± 0.001</td>
<td>0.096 ± 0.005</td>
<td>70</td>
<td>0.44 ± 0.03</td>
</tr>
</tbody>
</table>

At 15 °C, culture DC was able to remove only 55% of the initial DOC concentration, whereas at 30 and 35 °C, these values reached 77% and 84%, respectively (Table 2). In order to evaluate if the remaining organic compounds were residual molinate or degradation products of molinate, extracts of culture supernatants from the end of the incubation period were analysed (Table 3). The highest residual molinate concentration (0.766 mg l$^{-1}$) was detected in the organic extract of culture supernatant grown at 35 °C, while for the other temperatures, molinate was not detected (<0.025 mg l$^{-1}$) or was present in concentrations that did not exceed 0.055 mg l$^{-1}$ (Table 3). Previous studies showed that molinate undergoes oxidative metabolism forming either non-toxic ring-hydroxylated metabolites or toxic metabolites derived from sulfoxidation of molinate (Golovleva et al., 1981; Imai and Kuwatsuka, 1986; Jewell et al., 1999). Thus, the accumulation of molinate-sulfoxide in the treated water should be avoided since it has been reported as being more persistent and more toxic to animals than molinate (Golovleva et al., 1981; Jewell et al., 1999). In the present study, molinate-sulfoxide, molinate-sulfone and alcohol-molinate were not detected (detection limit of 0.025 mg l$^{-1}$) in any organic extract of the cultures grown at different temperatures, whereas 2-oxo-molinate was detected in the organic extracts of cultures grown at 15, 30 and 35 °C, in concentrations below 0.050 mg l$^{-1}$.

These results suggest that the organic compounds that most contributed to the residual DOC values obtained by the end of growth phase (around 35–40 mg l$^{-1}$) were those released by cell lysis, although it could not be excluded the hypothesis of being products of direct molinate degradation not detected by the methodology used (HPLC/UV).

In a previous study (Barreiros et al., 2003), it was shown that in culture DC (mixture of five isolates, ON1–ON5), isolate ON4 is the first organism attacking molinate, with the release of metabolites such as ethanethiol and possibly N-carboxy hexamethyleneimine. Ethanethiol is not further metabolised by strain ON4, being consumed by other members of culture DC, while the last compound supports the growth of several members, including of isolate ON4. Isolate ON4, named as Gulosibacter molinativorax (Manaia et al., 2004), grows in nutritive medium between 10 and 41 °C, with an optimal growth temperature of 35–37 °C while the other isolates, belonging to genera Pseudomonas, Stenotrophomonas, and Achromobacter have lower optimal growth temperatures.

![Fig. 2 – Arrhenius fitting of specific cell growth rate ($\mu$) and molinate degradation rate ($r_s$) with temperature inverse (T$^{-1}$). Symbols: $\bullet$, specific cell growth rate; $\square$, molinate degradation rate; $-$, specific cell growth rate Arrhenius fitting; $-$-$-$, molinate degradation rate Arrhenius fitting.](image-url)

(Fig. 2), leading to activation energies of 84387 and 67481 J mol$^{-1}$ for cell growth and molinate degradation, respectively.

At 15 °C, culture DC was able to remove only 55% of the initial DOC concentration, whereas at 30 and 35 °C, these values reached 77% and 84%, respectively (Table 2). In order to evaluate if the remaining organic compounds were residual molinate or degradation products of molinate, extracts of culture supernatants from the end of the incubation period were analysed (Table 3). The highest residual molinate concentration (0.766 mg l$^{-1}$) was detected in the organic extract of culture supernatant grown at 35 °C, while for the
presumably around 28–30°C (Palleroni, 1984; Tsuji et al., 1982). Since molinate mineralization is achieved only when all culture DC members are present, it can be anticipated that temperatures of incubation above 35°C would reduce or even halt molinate mineralization.

3.1.2. Effect of pH

At 30°C, the pH range assayed did not affect the specific molinate degradation rate. The values obtained for cultures grown at pH 6.3 and 8.0 were similar to that found at pH 7.2 (0.110 g molinate g\(^{-1}\) cell dry wt h\(^{-1}\)) (Table 2). The DOC removal was slightly lower in the culture grown at pH 6.3 (66%) than in those grown at higher pH (74% and 77%) (Table 2). The analysis of the organic extracts of cultures supernatants did not reveal the accumulation of significant amounts of residual molinate (maximum of 0.037 mg l\(^{-1}\)) or molinate degradation products. Only 2-oxo-molinate was detected, at a low concentration (0.088 mg l\(^{-1}\)), in the organic extract of the culture grown at pH 6.3 (Table 3).

3.1.3. Effect of aeration rate

The influence of aeration on molinate degradation was studied in cultures grown with air flow rates of 0.1 and 0.2vvm, corresponding to approximately 3 and 5 mg l\(^{-1}\) of dissolved oxygen, respectively. The specific cell growth and molinate degradation rates obtained in both experiments were not statistically different for the two oxygen concentrations (Table 2). The DOC removal by culture DC (between 77% and 85%) and the low amounts of residual molinate (0.064 mg l\(^{-1}\)) and 2-oxo-molinate (0.038 mg l\(^{-1}\)) accumulated at the end of the growth (Table 3), indicate that molinate was efficiently mineralized with both dissolved oxygen concentrations, showing that neither of these concentrations were limiting for the molinate degradation.

The culture DC was not able to grow in medium B in the absence of oxygen, with molinate as nutrient and nitrate as final electron acceptor (results not shown). However, under these conditions, molinate was partially degraded (90%), but not mineralised, since only 28% of the DOC content was removed after 80 h of incubation. The present results are in accordance with those previously reported for molinate degradation in soils under flooded and non-flooded conditions (Thomas and Holt, 1980; Imai and Kuwatsuka, 1982), suggesting that molinate mineralization only occurs in aerobiosis.

3.1.4. Effect of salinity

In rice crop fields, the surrounding natural waters may contain relatively high concentrations of NaCl (Duarte et al., 2003), since some paddy fields are in river banks at 5–10 km from the river mouth, where the effect of tides may be of some significance. Furthermore, effluents from molinate manufactures may contain high concentrations of salts. At high concentrations, salts such as NaCl may be inhibitory for non-halotolerant microorganisms or, by opposite, may favour the growth of halotolerant/halophilic microorganisms. Although isolate ON4 is able to grow in complex media with NaCl 7% (w/v) (Manaia et al., 2004), the effect of this salt on molinate mineralization by culture DC was not evaluated before. Indeed, it was known neither the halotolerance of the other members of culture DC, nor the effect of the salt on molinate metabolism.

The presence of NaCl 1% (w/v) in culture medium did not affect the specific molinate degradation rate (about

<table>
<thead>
<tr>
<th>Operating conditions</th>
<th>Degradation products concentration (mg l(^{-1}))</th>
<th>Molinate</th>
<th>2-oxo molinate</th>
<th>Molinate-sulfoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature (°C)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.055</td>
<td>0.042</td>
<td>&lt; 0.025</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.055</td>
<td>&lt; 0.025</td>
<td>0.027</td>
<td>&lt; 0.025</td>
</tr>
<tr>
<td>30</td>
<td>&lt; 0.025</td>
<td>0.027</td>
<td>&lt; 0.025</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>0.766</td>
<td>0.050</td>
<td>&lt; 0.025</td>
<td></td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.3</td>
<td>0.029</td>
<td>0.088</td>
<td>&lt; 0.025</td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>0.037</td>
<td>&lt; 0.025</td>
<td>0.027</td>
<td>&lt; 0.025</td>
</tr>
<tr>
<td><strong>Aeration (vvm)</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.1 (0.3 mg O(_2) l(^{-1}))</td>
<td>0.064</td>
<td>0.038</td>
<td>&lt; 0.025</td>
<td></td>
</tr>
<tr>
<td><strong>Salinity (% NaCl, w/v)</strong></td>
<td>10.002</td>
<td>0.061</td>
<td>&lt; 0.025</td>
<td></td>
</tr>
<tr>
<td><strong>Additional nutrients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molinate, meat extract, tryptone and ureaa</td>
<td>&lt; 0.025</td>
<td>&lt; 0.025</td>
<td>&lt; 0.025</td>
<td></td>
</tr>
<tr>
<td>Molinate, yeast and meat extractsb</td>
<td>0.194</td>
<td>0.027</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td><strong>River water</strong></td>
<td>1.676</td>
<td>0.133</td>
<td>&lt; 0.025</td>
<td></td>
</tr>
</tbody>
</table>

\(\text{a} \) Molinate 1 mM, 110 mg l\(^{-1}\) of meat extract, 160 mg l\(^{-1}\) of tryptone and 30 mg l\(^{-1}\) of urea.

\(\text{b} \) Molinate 1 mM and 1 g l\(^{-1}\) of yeast extract and of meat extract each.
0.1 g molinate g\(^{-1}\) cell dry wt h\(^{-1}\)). However, under these conditions, the specific cell growth rate was lower (0.038 h\(^{-1}\)) than that obtained in medium B without NaCl supplement (0.058 h\(^{-1}\)). These results may suggest that in the presence of NaCl more molinate is being used for cell maintenance in detriment of growth. Despite of being one of the lowest values among those obtained for other operating conditions, the DOC removal by culture DC in the presence of NaCl 1% (w/v) (62%), seems to indicate that molinate was also mineralized under these conditions. In agreement with these results, the organic extract of the culture supernatant presented the highest amount of residual molinate (10 mg l\(^{-1}\)), which corresponded to 4% of the initial molinate content. Nevertheless, among the searched metabolites, only 2-oxo-molinate was detected at a low concentration (0.061 mg l\(^{-1}\)).

Altogether, the results obtained suggest that molinate degradation by culture DC is not hampered by the presence of salt, although it is expected that the increase of NaCl concentration in culture medium may reduce the herbicide mineralization.

### 3.2. Effect of alternative nutrients on molinate biodegradation

Natural waters contain complex mixtures of organic compounds, which, eventually, may be used by microorganisms as energy, carbon and/or nitrogen sources. In the presence of alternative and energetically favourable organic compounds, microorganisms may not degrade xenobiotic compounds.

To study the effect of the presence of readily metabolisable carbon/nitrogen sources on molinate degradation, culture DC was grown in medium B containing molinate (1 mM) supplemented with low concentrations of meat extract, tryptone and urea, according to the OECD method 303A (1995), that establish the standard conditions that should be used to assess the biodegradability of an organic compound. A second assay was performed supplementing medium B, with molinate 1 mM, with high concentrations of yeast and meat extracts (1 g l\(^{-1}\) each). Comparing molinate degradation profiles obtained for these two cultures with that obtained with molinate as the single source of energy, carbon and nitrogen, it can be observed that, even in the presence of additional nutrients, molinate was biologically removed (cf. Fig. 3A and B with Fig. 1C). By the end of the incubation periods, a residual concentration of 0.2 mg l\(^{-1}\) of molinate was only detected in the organic extract of the culture grown with higher content of nutrients (Table 3). In order to compare the performance of culture DC grown only with molinate and when additional nutrients were present, parameters depicted in Table 4 \((r_s', DOC\) reduction and \(Y\) \) were calculated taking into consideration the total DOC present in the medium (including molinate and all the other carbon sources). No significant discrepancies were observed for the percentage of DOC reductions and for the cell growth yields obtained in any of these assays. However, the presence of additional nutrients seems to increase the kinetic parameters \(\mu\) and \(r_s'\) (Table 4).

The only degradation products detected in the organic extracts of culture supernatants by the end of these incubations were 2-oxo-molinate and molinate-sulfoxide (around 0.03 mg l\(^{-1}\) each), in the culture grown with high content of nutrients (yeast and meat extracts, 1 g l\(^{-1}\) each). These results show that the degree of molinate degradation by culture DC is not prejudiced by the presence of additional nutrients. Moreover, as expected, the presence of readily metabolized carbon and nitrogen sources stimulate the specific cell growth rate as well as the specific DOC depletion rate. The growth factors present in yeast and meat extracts may also have contributed for the increase of the kinetic parameters. Paca et al. (2002) also observed an increase of specific degradation rate of phenol by *Candida tropicalis* when grown in the presence of additional essential nutrients.

### 3.3. Aerobic biodegradation of molinate in river water

Previous studies showed that culture DC was able to degrade molinate from naturally contaminated waters from rice paddies, reducing herbicide concentrations ranging from 10.5 to 1550 \(\mu\)g l\(^{-1}\) to values below 0.48 and 2.2 \(\mu\)g l\(^{-1}\), respectively (Castro et al., 2005). These results suggested that...
culture DC could be used in the treatment of molinate polluted waters. However, due to the low initial molinate concentrations used in that study, it was not possible to compare the performance of culture DC grown in naturally contaminated waters and synthetic media. In this way, in the present study culture DC was grown in river water spiked with 1 mM of molinate. The cell dry weight, molinate and DOC concentrations along time of incubation of culture DC grown in river water are presented in Fig. 4. Along 120 h of incubation, the molinate concentration in the uninoculated control presented a mean value of 178.6 ± 9.8 mg l⁻¹ (Fig. 4), showing that losses by volatilization and biodegradation by natural microbiota of river water were negligible. Comparing the kinetic parameters obtained when culture DC was grown in river water (µ = 0.042 h⁻¹ and rₘ = 0.096 g molinate g⁻¹ cell dry wt h⁻¹) with those obtained for the same culture in medium B at pH = 8.0 (µ = 0.045 h⁻¹ and rₘ = 0.140 g molinate g⁻¹ cell dry wt h⁻¹), it can be observed that culture DC grows and degrades molinate with similar rates in both media. The values of DOC removal were also similar in both cultures: 70% and 74%, respectively. By the end of the growth in river water, only residual molinate (1.676 mg l⁻¹) and 2-oxomolinate (0.133 mg l⁻¹) were detected. The present results, together with those reported by Castro et al. (2005), indicate that culture DC shows potential to be used to decontaminate real polluted waters.

3.4. Aerobic biodegradation of molinate in a continuous reactor

A CSTR was operated at different feeding molinate concentrations. The temperature, aeration rate and pH were set to 30 °C, 0.2 vvm, and 7.2, respectively. Culture DC was allowed to grow in medium B with molinate 1 mM (197 mg l⁻¹) at batch conditions during 10 h, and then switched to continuous operation at a hydraulic residence time (HTR) of 83 h. The steady state was attained after approximately 95 h of operation. The molinate concentration and molinate degradation rate (rₘ) stabilized at 6 mg l⁻¹ and 0.07 g molinate g⁻¹ cell dry wt h⁻¹, respectively (Fig. 5). At day 5 (after 130 h of operation), the molinate concentration in the feeding medium was raised to 2 mM (330 mg l⁻¹), allowing for an increase of the biomass content from 40 to 80 mg l⁻¹. After about 200 h of operation, the mean values of molinate concentration and of molinate degradation rate (rₘ) stabilized at 10 mg l⁻¹ and 0.05 g molinate g⁻¹ cell dry wt h⁻¹, respectively. At day 11 (after 261 h of operation), the influent molinate concentration was raised again to 3 mM (520 mg l⁻¹). The mean values of biomass and molinate concentration stabilized at about 90 and 3 mg l⁻¹, respectively, while the molinate degradation rate (rₘ) stabilized at 0.07 g molinate g⁻¹ cell dry wt h⁻¹ (Fig. 5). The oscillations of molinate concentration in the effluent throughout the assay (3–10 mg l⁻¹) may be due to small variations in the feed flow rate. The analysis of the organic extracts of the effluent after 551 h of operation revealed that,

Table 4 – Specific growth (µ) and dissolved organic carbon degradation (rₘ) rates, percent of DOC consumption and cell growth yield (Y) of culture DC grown in medium B with about 1 mM molinate and in the same medium supplemented with different nutrients (T = 30 °C, pH = 7.2 and aeration rate = 0.2 vvm)

<table>
<thead>
<tr>
<th>Medium B carbon/nitrogen sources</th>
<th>µ (h⁻¹)</th>
<th>rₘ (g DOC g⁻¹ cell dry wt h⁻¹)</th>
<th>DOC reduction (%)</th>
<th>Y (g cell dry wt g⁻¹ DOC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molinatea</td>
<td>0.058 ± 0.003</td>
<td>0.060 ± 0.020</td>
<td>77</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Molinate, mea extract, tryptone and ureaabb</td>
<td>0.220 ± 0.020</td>
<td>0.180 ± 0.060</td>
<td>79</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Molinate, yeast and mea extractsc</td>
<td>0.240 ± 0.020</td>
<td>0.230 ± 0.040</td>
<td>80</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

a Molinate 1 mM.
b Molinate 1 mM, 110 mg l⁻¹ of yeast extract, 160 mg l⁻¹ of tryptone and 30 mg l⁻¹ of urea.
c Molinate 1 mM and 1 g l⁻¹ of yeast extract and of meat extract each.

Fig. 4 – Cell growth, molinate degradation and DOC consumption by culture DC grown in water from the river Pranto spiked with 1 mM molinate (T = 30 °C and aeration rate = 0.2 vvm). Symbols: - - - - - - - - , biomass concentration; - - - - - - - - , molinate concentration; - - - - - - - - , DOC concentration; □, molinate concentration in uninoculated control; ______, fitting of Eq. (1) for biomass and of Eq. (2) for molinate.
beside molinate, only 2-oxo-molinate was detected (0.110 mg l\(^{-1}\)).

The results obtained show that culture DC is capable of molinate degradation in continuous systems, presenting herbicide degradation rates similar to those obtained in the batch reactor (0.110 g molinate g\(^{-1}\) cell dry wt h\(^{-1}\)). Carrasco et al. (1992) showed that a mixed microbial culture was able to remove 97% of molinate, when using the herbicide as the only source of carbon, in a CSTR at a HRT of 23 days, fed with mineral medium with 50 mg l\(^{-1}\) of molinate, and ammonia as nitrogen source. Comparatively, culture DC was considerably more efficient, showing capacity to use higher amounts of molinate as the only source of carbon and nitrogen, and to remove 97–99% of molinate in a CSTR with a residence time of about 3.5 days.

4. Conclusions

The main conclusions of this work can be summarized as following:

1. Under aerobic conditions, culture DC is able to mineralize molinate when the herbicide is the only source of energy, carbon and nitrogen, or in the presence of additional readily metabolised nutrients. Among the operating conditions tested (temperature, pH, aeration rate and salinity), temperature is the environmental factor with more evident influence on molinate degradation rate; the lowest value was obtained at 15 ºC and the highest at 35 ºC.

2. In cultures with approximately 187 mg l\(^{-1}\) of herbicide, among molinate degradation products, 2-oxo-molinate and, more rarely, molinate sulfoxide were detected, but in concentrations below 0.133 and 0.029 mg l\(^{-1}\), respectively, suggesting that these compounds are not major metabolic degradation products.

3. Culture DC is able to mineralise the herbicide in river water spiked with approximately 187 mg l\(^{-1}\) of molinate with similar efficiency as in synthetic medium.

4. Culture DC is able to degrade molinate in continuous systems. In a CSTR, operating with a residence time of 3.5 days, culture DC reduced molinate concentrations from 520 to 3 mg l\(^{-1}\), at a degradation mean rate of 0.07 g molinate g\(^{-1}\) cell dry wt h\(^{-1}\).

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