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1 # Anaerobic biotransformation of nitroanilines enhanced by the
2 presence of low amounts of carbon materials

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9

10 ## Abstract

11 Three microporous activated carbons -original (AC_0), chemical oxidized with HNO_3
12 (AC_{HNO3}) and thermal treated (AC_{H2})-, and three mesoporous carbons - xerogels (CXA
13 and CXB) and nanotubes (CNT)-, were tested on the biological reduction of *o*-, *m*- and
14 *p*-nitroaniline (NoA) at a concentration above the half maximal inhibitory concentration
15 (IC_{50}) for a methanogenic consortium degrading a mixture of volatile fatty acids (VFA)
16 containing acetate, propionate and butyrate. NoAs were only partially reduced in the
17 absence of carbon materials (CM). Rates were dependent on the nitro group position,
18 increasing in the order *metha*>*para*>*ortho*. CM lead to NoAs almost total reduction and
19 at higher rates. With AC_0 and AC_{H2}, rates increased 3-fold, 4-fold and 8 fold for *o*-, *m*-
20 and *p*-NoA, respectively.

21

22 *Keywords*: anaerobic bioreduction; activated carbon; carbon nanotubes, carbon xerogel,
23 nitroanilines.

24 # Introduction

25 NoAs are commonly used in the industrial production of pharmaceuticals and synthetic
26 dyes originating contaminated wastewaters (Harter, 1985). They have also been
27 reported as products of anaerobic reduction of azo dyes (Donlon et al., 1997; Garrigós et
28 al., 2002) and explosives (Spain, 1995). In soils, herbicide microbial degradation also
29 originates nitroanilines. They are categorized as toxic and mutagenic substances and
30 concern on their removal is logic (Malca-Mor and Stark, 1982; Chung et al., 1997).
31 Some published results on biological degradation of NoAs under anaerobic conditions
32 have shown their transformation via reduction of the nitro group, forming nitroso and
33 hydroxylamino intermediates to the corresponding amines, through a six-electron
34 transfer mechanism donated by co-substrates (Spain, 1995; Razo-Flores et al., 1997a).
35 However, NoAs biological reduction has been described as proceeding at very low rates
36 and/or need acclimatized biomass (Saupe, 1999; Khalid et al., 2009). Redox mediators,
37 compounds that can be reversibly oxidized and reduced, shuttling the electrons from a
38 co-substrate to the organic compound to be degraded, can help as electron carriers,
39 increasing the rates of biotransformation of contaminants (Van der Zee and Cervantes,
40 2009). This is very important for the efficient operation of advanced biological reactors
41 with granular anaerobic sludge, such as the upflow anaerobic sludge bed (UASB), on
42 organic compounds removal, as the electron transfer limitations can lead to poor
43 performance (need of long hydraulic retention times to reach a satisfactory extent) or
44 even collapse of anaerobic reactors (Cervantes et al., 2001). Insoluble CM have been
45 shown as a feasible redox mediators for the microbial reduction of azo dyes presenting
46 advantages in comparison with soluble quinones, such as their easier removal from the
47 medium and the no need of continuous addition (Van der Zee et al., 2003; Pereira et al.,
48 2010; Pereira et al., 2014). Besides, CM can be modified in order to gain advantage of
49 their unique specific proprieties (Figueiredo el al., 1999; Pereira et al., 2010;
50 Amezquita-Garcia et al., 2013).
51 In the present study, different CM, including microporous (AC_0, AC_{HNO3} and AC_{H2})
52 and mesoporous CM (CX, CNT) were explored for the first time as redox mediators on
53 the anaerobic biological reduction of nitroanilines. Three NoAs differing only in the
54 position of nitro group, *ortho*, *metha* and *para* (*o*-, *m*- and *p*-NoA) were tested. The
55 potential toxic effect of NoA and final degradation products was evaluated for a
56 methanogenic consortium degrading VFA.
57

58 # Experimental

59

60 ## Chemicals

61 *o*-NoA (98%), *m*-NoA (98%), *p*-NoA (>99%), *m*-phenylenediamine (*m*-Phe, 98%), *p*-
62 phenylenediamine (*p*-Phe, 98%) were purchase from Sigma. Acetonitrile (ACN) was
63 purchased from Panreac at HPLC analytic grade.
64

65 ## Preparation and Characterization of Carbon Materials

66 Microporous CM comprise the commercial NoritROX0.8 AC_0 and two samples with
67 different chemical composition on the surface, maintaining the original textural
68 properties, prepared from chemical ($AC_{HNO3)}$ and thermal (AC_{H2}) treatment of AC_0. As
69 mesoporous CM, two CX synthesized by the sol–gel process at pH 6.25 (CXA) and
70 5.45 (CXB) to obtain materials with different textural properties and a commercial CNT
71 (Nanocyl 3100, 95%, diameter of 9.5 nm, an average length of 1.5 μm) were used.
72 Preparation and characterisation of tested CM are already described in Pereira et al.
73 (2010, 2014).

74
75 **Biological assays**
76 Biological reduction of nitroanilines was conducted in 70 mL serum bottles, sealed with
77 a butyl rubber stopper, containing 25 mL of medium. The primary electron donating
78 substrate of the medium was composed of 2 g L^{-1} chemical oxygen demand (COD) of a
79 NaOH-neutralised volatile fatty acids (VFA) mixture, containing acetate, propionate
80 and butyrate in a COD based ratio of 1:10:10. Basal nutrients were also added: NH_4Cl
81 (2.8 g L^{-1}), $CaCl_2$ (0.06 g L^{-1}), KH_2PO_4 (2.5 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (1 g L^{-1}). Medium was
82 buffered at a pH of 7.3 ± 0.2 with $NaHCO_3$ (2.5 g L^{-1}). Anaerobic granular sludge
83 collected from an anaerobic Internal Circulation reactor of a brewery wastewater
84 treatment plant was the inoculum at a concentration of 2.5 ± 0.5 g L^{-1} volatile suspended
85 solids (VSS). Nitroanilines were added at the final concentration of 1 mM. The effect of
86 the different CM on biological reduction was tested at a concentration of 0.1 g L^{-1}. This
87 concentration is in accordance with a previous published work (Pereira et al., 2010) in
88 which AC concentrations from 0.1 g L^{-1} to 0.6 g L^{-1} were tested and lead to an increase
89 of the dye adsorption (from less than 10 % to 65 %), without accelerating the dye
90 reduction rates, beyond the concentration of 0.1 g L^{-1}. These results are important once
91 activated carbon is costly and therefore the use of low amounts is an advantage for
92 biological processes application. Furthermore, as a redox mediator, AC is recycled from
93 its oxidized and reduced states and thus should be effective at low concentrations.
94 Sludge was incubated overnight at 37 ºC in a rotary shaker at 120 rpm. After the pre-
95 incubation period, NoAs and VFA´s (2 gCOD L^{-1}) were added with a syringe from the
96 stock solution to the desired concentration. Controls without CM and without biomass
97 were also conducted. All experiments were prepared in triplicate.
98
99 **Effect of nitroamines and final products on a methanogenic consortium**
100 Serum bottles of 25 mL, containing 12.5 mL of buffer solution with 3.05 g L^{-1} sodium
101 bicarbonate and 1 g L^{-1} of Resazurin, were supplemented with 0.4 g anaerobic granular
102 sludge which corresponds to 2.1 ± 0.2 g of volatile suspended solids (VSS) per litre, and
103 the headspace was flushed with a mixture of N_2/CO_2 (80/20 vol/vol). The final pH was
104 7.2 ± 0.2. Following the addition of 0.125 mol L^{-1} Na_2S, under strict anaerobic
105 conditions, the flasks were incubated overnight at 37 ºC and 120 rpm. After that period,
106 the mixture of VFA 1:10:10 (acetate, propionate and butyrate as mass of COD) at the
107 final concentration of 2 gCOD L^{-1}, and the solutions to be tested, were added and the
108 Flasks were maintained at 37 ºC and 120 rpm during the entire assay. The pressure was
109 measured every 60 min by using a hand-held pressure transducer able of measuring a
110 pressure variation of ± 202.6 kPa (0 to 202.6 kPa) with a minimum detectable variation
111 of 0.5 kPa, corresponding to 0.05 mL of biogas in a 10 mL headspace. The assay was
112 finished when the pressure remained stable. 500 µL of sample volume were collected
113 every day using a gas-tight syringe and methane content of the biogas was measured by
114 gas chromatography using a Chrompack Haysep Q (80–100 mesh) column (Chrompack,
115 Les Ulis, France), with N_2 as carrier gas at 30 mL min^{-1} and a flame-ionization detector.
116 Temperatures of the injection port, column, and flame-ionization detector were 110, 35
117 and 220 ºC, respectively. The values of methane production were corrected for the
118 standard temperature and pressure conditions (STP). In the biodegradability
119 experiments the methane production was expressed as mg COD-CH_4 g_{VSS}^{-1} day^{-1}. In
120 order to determine the activities, the values of pressure (calibrated as an analogical
121 signal in mV) were plotted as a function of time and the initial slopes of the methane
122 were calculated. SMA values were determined dividing the initial slope by the VSS
123 content of each vial at the end of the experiment and were expressed in mL CH_4 g_{VSS}^{-1}

124 day^{-1}. Background methane production due to the residual substrate was subtracted. Test
125 included series containing increasing NoAs in the range of 0.25 to 1 mM, to evaluate
126 their effect on the methanogenic consortium activity. The final products of biological
127 reduction were also tested. Two controls were made in the same conditions, one
128 containing only VFAs and the other without any substrate (blank assay). All batch
129 experiments were performed in triplicate. The effect of tested compounds was evaluated
130 by comparing with the control containing only VFAs.
131
132 **Analytical techniques**
133 Reactions were monitored spectrophotometricaly in a 96-well plate reader (ELISA BIO-
134 TEK, Izasa) and by HPLC. NoAs show a yellow colour with maximum wavelengths at
135 410 for *o*-NoA, 350 for *m*-NoA and 380 nm for *p*-NoA. At select intervals, samples
136 were withdrawn (300 µL), centrifuged at 5000 rpm for 10 min to remove the biomass
137 and/or CM and diluted to obtain less than one absorbance unit. The UV-vis spectra
138 (200–800 nm) were recorded and nitroaniline concentration calculated at λ_{max}. Molar
139 extinction coefficients were calculated at λ_{max}: $\varepsilon_{410\ nm}$= 1.345mM^{-1} cm^{-1} for *o*-NoA; ε_{350}
140 $_{nm}$= 0.582 mM^{-1} cm^{-1} for *m*-NoA and $\varepsilon_{380\ nm}$= 3.104 mM^{-1} cm^{-1} for *p*-NoA. Reduction
141 extent (RE) was calculated according to equation RE (%) = $[(A_0-A_t)/A_0]*100$, where
142 A_0, is the absorbance at λ_{max} at the beginning of incubation and A_t, the absorbance at
143 λ_{max} at a selected time (t). First-order reduction rate constants were calculated in
144 OriginPro 6.1 software, applying the equation $Ct = C_0 + Ci\ e^{-kt}$, where C_t is the
145 concentration at time t; C_0, the offset; C_i, the concentration at time initial time; k, the
146 first-order rate constant (h^{-1}) and t, is the accumulated time of the experiment.
147 HPLC analyses were performed in a HPLC (JASCOAS-2057 Plus) equipped with a
148 Diode Array Detector detector. A C18 reverse phase Nucleodur MNC18 column
149 (250x9x4.0 mm, 5 µM particle size and pore of 100 Å from Machenerey-Nagel,
150 Switzerland) was used. Mobile phase was composed of the solvents: A (ultrapure water)
151 and B (Acetonitrile). Compounds were eluted at a flow rate of 0.5 mL min^{-1} and at room
152 temperature, with isocratic condition containing 50 % of A and 50% of B, during 20
153 min. Compounds elution was monitored at λ_{max} of compounds (410, 350 and 380 nm)
154 and at 230 nm for reduction products (5-ASA and phenylenediamines).
155
156 ## Results and Discussion
157
158 **Carbon materials as redox mediators on NoA biological reduction**
159 Biological reduction of structurally related NoAs by granular anaerobic biomass and the
160 effect of different CM as redox mediators was studied and compared. During the
161 reaction, the yellow colour decreased and, in the presence of CM, the solution turned
162 colourless. As monitored by spectrophotometry, a decrease of the visible spectra was
163 observed (fig. 1A). In addition, the reactions were followed by HPLC, by which NoA
164 and products are separated and can be analyzed individually. Figures 1B show the
165 HPLC chromatograms for 3-NoA reduction in the presence of AC_{H2}. A decrease of the
166 NoA peak was observed at the maximum wavelength of the NoA. At 230 nm, both NoA
167 removal and product formation could be monitored, confirming the reduction of the
168 NoA. As compared with standards, the products of nitroanilines reduction were
169 identified as the expected products, the correspondent phenylenediamines, which is in
170 agreement with literature (Razo-Flores et al., 1997b; Razo-Flores et al., 1999; Saupe,
171 1999; Bhushan et al., 2006). According to previous literature, nitroreductases convert
172 nitro groups either to nitroso derivatives, hydroxylamines or amines through six
173 electron successive addition from cosubstrates to nitrocompounds. The high reactivity

and instability of nitroso derivatives difficults their detection. The aromatic amines formed are usually difficult to be further degraded under the anaerobic conditions, however have the possibility, in some cases, to be degraded by following aerobic processes (Van der Zee and Villaverde, 2005).

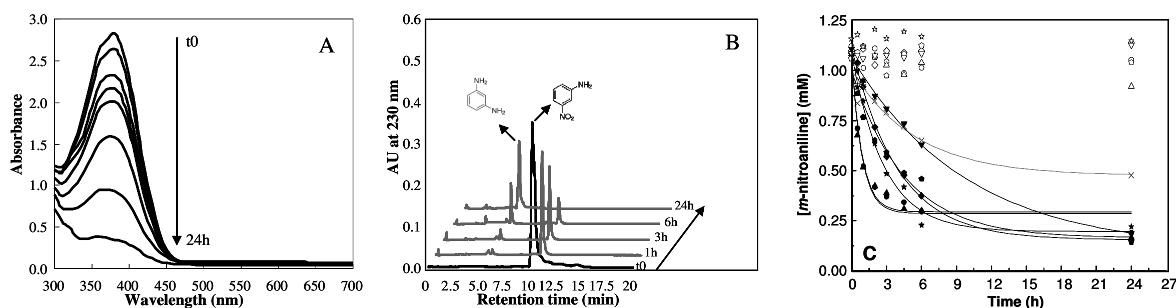

Figure 1. Biological reduction of *m*-NoA with AC_0 as monitored by UV-vis spectroscopy (**A**) and HPLC (**B**). First-order rate curves of *m*-NoA biological reduction (**C**). **x**, no CM; ●, AC_0; ▲, AC_{H2}; ♦, AC_{HNO3}; ⬟, CXA; ★, CXB and ▼, CNT. Black symbols - biotic and white symbols - abiotic assays.

As observed in figure 1C, NoAs reduction followed first-order kinetics and higher rate was obtained for the *m*-NoA which was 2x higher than the obtained for *p*-NoA and 4x higher than the obtained for *o*-NoA, revealing the effect of the position of the nitro substituents in the molecule. In the absence of CM, the extent of biological reduction in the equilibrium (~24h) were 32±1, 56±4 and 52±2, for *o*-NoA, *m*-NoA and *p*-NoA, respectively (Table 1).

Table 1. Extent (%) and rates (d^{-1}) of nitroaniline biological reduction (1 mM), effect of different carbon materials (0.1 g L^{-1}). Controls without biomass reveal that any adsorption to carbon materials occurs (data not shown).

	***o*-NoA**		***m*-NoA**		***p*-NoA**	
Condition	**(%)**	**(h^{-1})**	**(%)**	**(h^{-1})**	**(%)**	**(h^{-1})**
Control	32 ± 1	0.07 ± 0.01	56 ± 4	0.26 ± 0.11	52 ± 2	0.14 ± 0.02
AC_0	97 ± 2	0.15 ± 0.02	98 ± 1	1.14 ± 0.04	89 ± 1	1.05 ± 0.01
AC_{H2}	97 ± 3	0.22 ± 0.03	97 ± 1	1.12 ± 0.01	92 ± 1	0.96 ± 0.04
AC_{HNO3}	94 ± 1	0.10 ± 0.03	95 ± 1	0.23 ± 0.01	94 ± 1	0.18 ± 0.01
XA	93 ± 2	0.10 ± 0.01	94 ± 1	0.22 ± 0.03	93 ± 1	0.14 ± 0.01
XB	91 ± 1	0.09 ± 0.01	92 ± 1	0.36 ± 0.01	91 ± 1	0.15 ± 0.01
CNT	94 ± 6	0.10 ± 0.01	91 ± 1	0.10 ± 0.01	93 ± 2	0.07 ± 0.01

Similar results were obtained for the bioreduction of *o*-, *m*- and *p*-nitroaniline reduction in samples of the river Elbe (Börnick et al., 2001). The effect of CM on extent and rates of NoAs reduction is also set in table 1. All the tested CM improved the extent and rate of NoAs reduction, demonstrating their effect as redox mediators. Almost total reduction was obtained in the presence of CM. Comparing the different carbon

201 materials, higher reduction rates were obtained with the microporous samples AC_0 and
202 AC_{H2}, leading to an improvement of 3-fold, 4-fold and 8-fold higher for *o*rtho, *m*etha,
203 and *p*ara NoA, respectively, as compared with the reaction in the absence of CM. In
204 previous results with azo dyes, better performance was achieved with the mesoporous
205 carbon materials, explained by the easier access of the larger molecules of the dye to the
206 internal surface of the catalyst. NoAs are smaller molecules and, the better results with
207 the microporous materials might be related with higher surface area of these materials
208 instead of the size of the porous. Similarly to the known redox mediator anthraquinone-
209 2,6-disulfonate (AQDS), the effect as redox mediator of activated carbon has been
210 attributed to the quinone groups on its surface (Van der Zee et al., 2003). In this study,
211 comparing between the three samples of microporous activated carbon, better results
212 were obtained with AC_0 and AC_{H2} than with the AC_{HNO3} sample. In fact, in spite of the
213 higher amount of quinone groups in AC_{HNO3} compared to the other samples, its effect is
214 surpassed by the large amount of carboxylic acids and anhydrides also present in this
215 sample, which are electron withdrawing groups. In a previous work, thermal
216 modification of AC surface chemistry improved its capacity as redox mediator for azo
217 dye reduction, which was related with the a high content of electron rich sites on their
218 basal planes (π electrons), known to be active sites, and by a low concentration of
219 electron withdrawing groups (Pereira et al., 2010). Sample AC_{H2} has the advantage of
220 keeping some of the quinone groups without the presence of the oxygen-containing
221 acidic groups (removed during the thermal treatment). Other characteristic of the
222 activated carbon materials involved is their pH_{pzc}. Due to activated carbon amphoteric
223 character, when in solutions at pH below their pH_{pzc} it became positively charged and at
224 pH above the pH_{pzc}, negatively charged. Therefore, at pH 7 AC_0 and AC_{H2} are
225 positively charged and AC_{HNO3} negatively charged. NoAs are ionisable organic
226 compounds, they can exist either as nondissociated or dissociated species in aqueous
227 phase, depending on the solution pH in relation to their dissociated constants (pKa).
228 Once the pKa of *o*-, *m*- and *p*-NoA are -0.28, 2.45 and 0.98, respectively (Yang et al.,
229 2008), in solution at pH 7, deprotonation will occur generating the NoA correspondent
230 anions. The electrostatic attraction forces between the positively charged carbons and
231 the negatively charged NoA will be favourable to the electron shuttling.
232 Contrarily to our results, Amesquita-Garcia el al. (2013) investigating the redox
233 mediator effect of activated carbon fibres, original, chemical oxidized and thermal
234 treated, on 4-nitrophenol and 3-chloronitrobenzene chemical (Na_2S) reduction, have
235 concluded that activated carbon fibres chemically oxidized are better redox mediators
236 due to the increased number of quinone groups. Liu et al. (2012), have discussed about
237 the mechanism of methanogenesis stimulation by activated carbon in metanogenic
238 digesters, the possibility of favouring the direct interspecies electron transfer (DIET)
239 under anaerobic conditions between bacteria and methanogens and the role of AC
240 surface quinone groups. Authors have demonstrated that activated carbon could
241 accelerate the DIET between *Geobacter metallireducens* and *Geobacter sulfurreducens*
242 or *Geobacter metallireducens* and *Methanosarcina barkeri*. Studies using AQDS
243 instead of AC put aside the potential responsibility of quinone groups and lead authors
244 to consider, instead, the possible contribution of AC high conductivity enabling
245 electrical connections between microorganisms. Consequently, the investment of the
246 cells on metabolic energy in producing conductive pili and the additional cytochromes
247 that are required for the DIET in the absence of AC is reduced.
248
249 **Effect of nitroanilines and final reduction products on methanogenic consortium**
250

251 The inhibitory effects of the three NoAs and their reduction products on the activity of
252 acetoclastic methanogenic bacteria were evaluated (table 2). The results revealed that
253 the concentrations of compounds tested in biological assays were above the IC_{50}, which
254 may also explain the low extent of reduction in the absence of CM. Among the NoAs,
255 similarly to the biological reduction results, the position of the nitro group had an effect
256 on methanogenic activity and a notorious higher toxic effect was observed for *o*-NoA.
257 The IC_{50} for *ortho* substituted NoA was 0.23 mM and for *metha* and *para* substitutions
258 was 0.67 mM and 0.51 mM, respectively. The lower reduction obtained for *o*-NoA
259 among the NoA tested, in all the tested conditions, may also be due to its higher toxic
260 effect on methanogenic consortium. Reduction products of NoA biotransformation in
261 the presence of AC_{H2} was also evaluated and up to 77 % of detoxification was obtained.
262 The results obtained are in accordance with literature reporting that aromatic nitro-
263 substituents are responsible for severe methanogenic toxicity, while correspondent
264 aromatic amines present lower toxic effects (Donlon et al., 1997; Razo-Flores et al.,
265 1997a).
266
267
268 **Table 2.** Potential toxic effect on acetoclastic methanogenic bacteria degrading VFA.

Chemical	Concentration (mM)	Activity (mLCH$_4$@PTN gVSS^{-1} d^{-1})	IC_{50} (mM)
***o*-NoA**	0.00 0.25 0.50 1.00	161.5 ± 10.1 73.6 ± 0.6 26.1 ± 1.3 * 0	0.23
Products of *o*-NoA bioreduction		124.7 ± 6.0	N.a.
***m*- NoA**	0.00 0.25 0.50 1.00	157.0± 9.31 123.7 ± 8.0 108.8 ± 10.5 35.9 ± 1.72 *	0.67
Products of *m*- NoA bioreduction		107.7 ± 3.2	N.a.
***p*-NoA**	0.00 0.25 0.50 1.00	129.2 ± 3.0 84.3 ± 4.6 48.3 ± 1.5 18.4 ± 2.5	0.51
Products of *p*-NoA bioreduction		98.0 ± 0.2	N.a.
MY1	control 0.125 0.25 0.50 1.00	199.1 ± 10.1 162.9 ± 15.2 129.9 ± 9.9 69.9 ± 4.6 0	0.44
Products of MY1 bioreduction		190.3 ± 5.3	N.a.
5-ASA	Control 0.20 0.40 0.80 1.00 2.00 4.00	178.8 ± 14.5 157.4 ± 4.2 167.1 ± 7.9 147.3 ± 6.9 118.3 ± 1.3 47.1 ± 1.4 0	2.0

269 N.a. - not applicable; * *, Metanogenic activity calculated after 1 day lag phase.

Conclusions

The efficiency of microporous (AC_0, and AC_{HNO3}, AC_{H2}) and mesoporous carbon materials (CXA, CXB and CNT) as redox mediators on isomeric NoAs reduction was compared. Rates were dependent on the nitro group position, increasing in the order *metha>para>ortho*. The presence of CM increases both the extent and the rates of compounds bioreduction. The surface area of carbon materials had greater responsibility than the pore sizes, with better results obtained for AC_0 and AC_{H2}. The pH_{pzc} of the materials is also an important factor on reduction reactions, and at pH 7 the electrostatic attraction between the positively charged carbons AC_0 and AC_{H2}, and the NoA anions favors the electron transfer. The high extent of compounds reduction in the presence of CM even when present at toxic levels to the methanogenic consortium, and the detoxification obtained with the mediated treatment, demonstrates the effectiveness of the process and their promising application in continuous high rate bioreactors

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