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Biofiltration onto pine bark for the treatment of water contaminated with atrazine: influence of sorbent on *Pseudomonas* sp. strain adp

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Abstract – A combination of adsorption and biodegradation processes was studied as a potential for the treatment of water contaminated with atrazine. Pine bark was used as a cheap and readily available sorbent, and *Pseudomonas* sp. strain ADP as atrazine degrader. *Pseudomonas* sp. strain ADP was found to be able to grow in the aqueous bark extract and to use the soluble bark organics as carbon sources. However, a lag period of about 25 hours was observed in cultures in sterile bark extract for the growth and atrazine biodegradation as compared with the cultures grown in a mineral medium. In pine bark aqueous extract, *Pseudomonas* sp. strain ADP was shown to mineralize 75% of atrazine in 330 hours (initial concentration of $44.2 \mu\text{g}\cdot\text{L}^{-1}$). The presence of sterile pine bark particles (liquid to solid ratio of 20) in mineral medium reduced the yield down to 50% due to atrazine sorption onto bark.

atrazine / mineralization / adsorption / pine bark / *Pseudomonas* ADP sp. / water

1. INTRODUCTION

The herbicide atrazine (6-chloro-N 2 -ethyl-N 4 -isopropyl-1,3,5-triazine-2,4-diamine) is used extensively in many parts of the world to control weeds, primarily in the production of corn. Because of its high persistence (DT50 from 11 to 248 days in different soils) (Stolpe and Shea, 1995) and its solubility ($30 \text{ mg}\cdot\text{L}^{-1}$), and in spite of the prohibition of its use since June 2003 in France, many practices have been developed to minimize the pollution of surface and groundwater resources.

Bioremediation has been suggested for water and soil decontamination. Numerous microorganisms have been isolated from atrazine-contaminated soils and have shown that atrazine could be used as a nitrogen source (Behki et al., 1993; Cook and Hutter, 1981; Erickson and Lee, 1989; Mandelbaum et al., 1995; Radosevich et al., 1995; Yanze-Kontchou and Gschwind, 1994; Migrain et al., 1993). However, a majority of the organisms described did not mineralize the atrazine (Migrain et al., 1993). Only a few bacterial isolates have been described as atrazine mineralizers (Mandelbaum et al., 1995; Radosevich et al., 1995; Yanze-Kontchou and Gschwind, 1994; Migrain et al., 1993).

Mandelbaum et al. (1995) isolated *Pseudomonas* sp. strain ADP and showed that it could use atrazine as a sole nitrogen source but metabolized much more than required for its nitro-

gen assimilation, due to the presence of enzymes in the cytoplasm or the periplasm of the cells. Thus, *Pseudomonas* sp. strain ADP in a studied media containing $1 \text{ mg}\cdot\text{L}^{-1}$ of atrazine (corresponding to $0.7 \text{ mg}\cdot\text{L}^{-1}$ of nitrogen) was able to mineralize 80% of the herbicide in carbon dioxide.

Adsorption processes have also been tested to remove pollutant from contaminated waters, using activated carbon (Feakin et al. 1995a), natural materials or waste as sorbents: notably, a wide variety of cheap sorbents have already been tested for water treatment such as beetroots (Rima et al., 2004), onion skin (Kumar et Dara, 1980), bagasse (Pernel, 1999) and pine bark specifically for pesticide adsorption (Bras et al., 1999).

The efficiency of biological processes is limited by the low concentration of atrazine in contaminated waters, and adsorption processes must include relatively complex recovery or disposal processes, including the elimination of the pollutant after the treatment. The combination of adsorption and biological techniques is a promising alternative. Such combined processes have already been developed with activated carbon (Feakin et al., 1995a; Jones et al., 1998; Venus et al., 2000) to concentrate atrazine onto the sorbent and thereby improve the efficiency of biological degradation in a “bioregeneration” stage (Feakin et al., 1995b).

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In our work, pine bark was selected as a cheap sorbent readily available in Portugal and in the south of France.

The objective of our study was to estimate the capacity of *Pseudomonas* sp. strain ADP to grow and degrade atrazine as the sole nitrogen source in the presence of the bark soluble compounds (using an aqueous bark extract), and in the presence of bark particles. For the first purpose (in bark extract), atrazine primary degradation was studied at relatively high initial concentration (about 50 mg·L⁻¹) to allow a significant growth of the biomass. Atrazine mineralization was also quantified using a lower concentration (about 40 µg·L⁻¹) corresponding to a pollution of natural waters. Then, mineralization experiments were conducted in pine bark aqueous extract and in the presence of pine bark particles using ¹⁴C-labeled atrazine.

2. MATERIALS AND METHODS

2.1. Chemical treatments

Atrazine PESTANAL in powder of 98% chemical purity and [U-ring-¹⁴C]-atrazine (chemical purity 98.6%, 9.1 × 10¹¹ Bq·mmol⁻¹) were purchased from Riedel de Haën and Sigma-Aldrich, respectively. Stock solution of 4 mg·L⁻¹ of pure [U-ring-¹⁴C]-atrazine (specific activity of 17 MBq·L⁻¹) was prepared in methanol and stored at 4 °C.

2.2. Pine bark preparation

Experiments were performed with *Pinus pinaster* bark remaining from non-treated trees. Barks were collected from a sawmill located in the north of Portugal. The bark was composed of 99% of organic matter, with lignin as the major constituent (more than 50% of organic matter). About 15% of the bark organic matter was easily extractable with water.

The bark was ground, sieved to a particle size < 200 µm, dried at 100°C until constant weight and stored at room temperature in closed flasks. Dried pine bark (particle size < 200 µm) was sterilized by 2 successive autoclavings separated by 20 hours, at room temperature.

Aqueous extract was obtained by suspending bark particles (bark particles size < 200 µm) in distilled water for 24 hours on a rotary shaker (150 rpm) with a liquid/solid ratio of 20 mL·g⁻¹. The solution was filtered at 0.45 µm with an acetate cellulose membrane (Whatman) and then sterilized by filtration at 0.2 µm acetate cellulose membrane (Roth). Total organic carbon and nitrogen concentrations in the solution obtained were, respectively, 100 mg·L⁻¹ and 4 mg·L⁻¹.

2.3. Bacterial inoculum

Atrazine-degrading bacteria *Pseudomonas* sp. strain ADP was supplied by DSMZ (No. 11735). It was isolated and described as an atrazine-degrading bacteria by Mandelbaum et al. (1995). The atrazine-mineralizing capacity of *Pseudomonas* sp. strain ADP was verified in our laboratory.

2.4. Analytical methods

Cell growth was followed by monitoring the absorbance at 600 nm (OD₆₀₀) using a Milton Roy Spectronic 401 spectrophotometer (see cell growth solutions Sect. 2.5).

Atrazine primary biodegradation was monitored by HPLC analyses performed on a WATERS module I Plus equipped with a RP-18 Supelcosil column (particle size 5 µm; length × ID 25 cm × 4.6 mm) at room temperature. The isocratic mobile phase used was acetonitrile:water (50:50, vol:vol) at 1 mL·min⁻¹. A UV detector set at 220 nm was used. The volume of injection was 20 µL. The retention time of atrazine was 7 minutes. The limit of detection was 0.03 mg·L⁻¹.

Aerobic bacterial activity was also evaluated by monitoring the Biochemical Demand in Oxygen (BDO) in 500 mL flasks equipped with Oxitop[®] heads (commercialized by WTW). The Oxitop[®] heads measured the pressure in the flask. A trap filled with NaOH pastilles captured CO₂ resulting from the bacterial respiration.

Atrazine mineralization was followed by monitoring ¹⁴CO₂ which was captured in a NaOH trap placed at the bottom of the cap of the flasks. The trap was filled with 0.5 mL of 1N NaOH, replaced periodically. ¹⁴CO₂ was titrated by adding 7 mL of liquid scintillation cocktail (Ultima Gold) and counting on a TRI-CARB 2100TR Packard Liquid Scintillation Analyzer. Assays were triplicated. The averaged results were written in the text; error bars indicate the maximal and minimal value of the triplicates.

2.5. Atrazine primary degradation

Pseudomonas sp. strain ADP was cultivated in a 250-mL Erlenmeyer flask containing 50 mL of mineral medium (MM) or aqueous pine bark extract, which was supplemented or not with additional sources of carbon (glucose 1 g·L⁻¹) and nitrogen (ammonium sulphate 1 g·L⁻¹).

The mineral medium used contains phosphate buffer (27 mM, pH 7.2), CaCl₂·2H₂O (0.2 mM), NaCl (7.56 mM), MgCl₂·6H₂O (0.81 mM), FeCl₂·4H₂O (5.19 µM), HCl (1.3 µL, 25%), ZnCl₂ (0.07 mg·L⁻¹), MnCl₂·4H₂O (0.1 mg·L⁻¹), H₃BO₃ (0.062 mg·L⁻¹), CoCl₂·6H₂O (0.19 mg·L⁻¹), CuCl₂·2H₂O (0.017 mg·L⁻¹), NiCl₂·6H₂O (0.024 mg·L⁻¹), and NaMoO₄·2H₂O (0.036 mg·L⁻¹).

Glucose (1 g·L⁻¹) was supplied as the carbon source in the mineral medium (MM). Ammonium sulphate (1 g·L⁻¹) or atrazine (50 mg·L⁻¹) were supplied as a nitrogen source.

Pseudomonas sp. strain ADP was inoculated at about 10⁶ cell·mL⁻¹ (initial OD₆₀₀ = 0.06). Cultures of *Pseudomonas* sp. strain ADP containing 50 mL of medium were incubated for 60 hours at 30 °C and stirred on an orbital stirrer (150 rpm). All assays were triplicated.

2.6. Atrazine mineralization

[U-ring-¹⁴C]-atrazine was introduced at a concentration of 44.2 µg·L⁻¹ (9.2 kBq/flask) as the sole nitrogen source. Cultures of *Pseudomonas* sp. strain ADP were gently stirred with a magnetic stirrer at 20 ± 2 °C in a biometer. Glucose was supplied as the sole carbon source at 0.25 g·L⁻¹ (i.e. 100 mg TOC·L⁻¹)

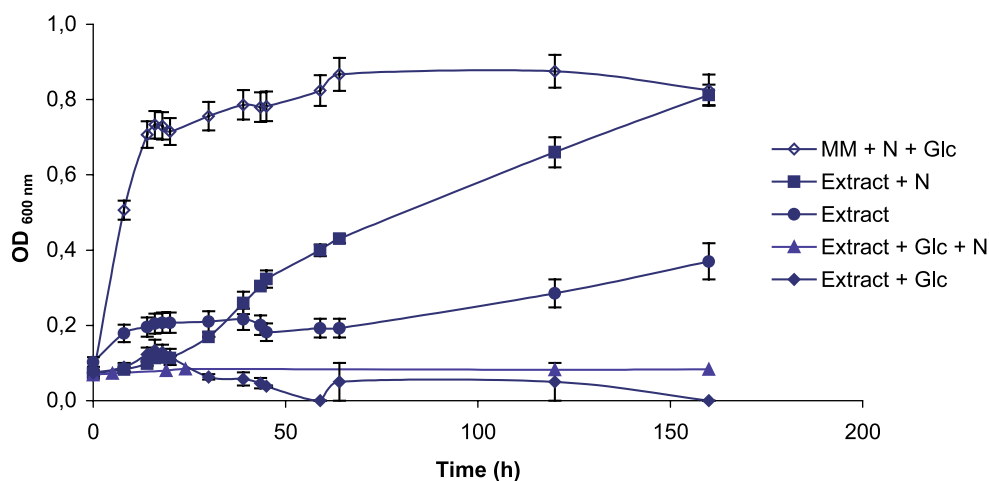


Figure 1. Growth of *Pseudomonas* sp. strain ADP in sterile bark extract (Extract) or in sterile mineral medium (MM) supplemented or not with additional carbon (glucose $1 \text{ g}\cdot\text{L}^{-1}$, Glc) and nitrogen (ammonium sulphate $1 \text{ g}\cdot\text{L}^{-1}$, N) sources, at 30°C , 150 rpm. Mean of triplicated assays; error bars present the maximal and minimal values of the triplicates.

when mineral medium (MM) was used. When bark extract was used as the cultivation medium or when pine bark particles were suspended in MM (at $\text{L/S} = 20$), the bark soluble organic compounds were the sole carbon sources ($\text{TOC} = 100 \text{ mg}\cdot\text{L}^{-1}$). All assays were triplicated. The averaged results were written in the text; error bars indicate the maximal and minimal value of the triplicates.

3. RESULTS AND DISCUSSION

3.1. Growth of *Pseudomonas* sp. strain ADP and atrazine primary degradation in sterile pine bark extract

Figure 1 shows the capacity of *Pseudomonas* sp. strain ADP to grow in the presence of bark soluble organics contained in the extract, in different nutritive conditions (addition of carbon and/or nitrogen source) and compared with the growth obtained in classical nutritive conditions (in mineral medium supplemented with carbon and nitrogen).

Cultures of *Pseudomonas* sp. strain ADP in bark extract spiked with $(\text{NH}_4)_2\text{SO}_4$ revealed a lag phase of about 25 hours compared with the cultures grown in the mineral medium (MM) spiked with $(\text{NH}_4)_2\text{SO}_4$ and glucose $1 \text{ g}\cdot\text{L}^{-1}$ (Fig. 1). However, a similar OD was obtained in both cultures after 150 hours. This result reveals the capacity of *Pseudomonas* sp. strain ADP to use the organic compounds from the bark as carbon sources after a phase of adaptation. When the extract was not supplemented with $(\text{NH}_4)_2\text{SO}_4$, Figure 1 shows that nitrogen concentration was too low (about $4 \text{ mg}\cdot\text{L}^{-1}$, against $210 \text{ mg}\cdot\text{L}^{-1}$ in mineral medium) or in a form not easily available to support bacterial growth correctly. The addition of an additional nitrogen source was therefore shown to stimulate considerably the growth of *Pseudomonas* sp. strain ADP in bark extract. Surprisingly, the addition of glucose to bark extract was found to induce an inhibition of the growth of the strain, in the

presence or not of an additional nitrogen source (respectively, $\text{C/N} = 100/200$ with $(\text{NH}_4)_2\text{SO}_4$ $1 \text{ g}\cdot\text{L}^{-1}$, and $\text{C/N} = 100/0.8$ without $(\text{NH}_4)_2\text{SO}_4$). The formation of aggregates was observed in these cultures which could be defensive cell forms, suggesting a possible phenomenon of toxicity of glucose as reported by other authors (Heinze et al., 1978).

Other assays conducted in pine bark extract (Fig. 2) at different concentrations of glucose (0, 0.25, 0.5 and $1 \text{ g}\cdot\text{L}^{-1}$) confirmed this hypothesis; respiratory activity of *Pseudomonas* sp. strain ADP in pine bark extract (measure of BDO) was optimum when glucose was not added (the C/N rate in extract was then 100/4 and corresponded to bacterial nutritive needs). When glucose was added to bark extract, bacterial activity decreased with addition of glucose.

When atrazine was present as the sole nitrogen source (Fig. 3a), the growth of *Pseudomonas* sp. strain ADP both in mineral medium (MM, glucose $1 \text{ g}\cdot\text{L}^{-1}$) and in sterile bark extract was extremely limited as compared with the growth observed with $(\text{NH}_4)_2\text{SO}_4$ (Fig. 3b). However, Figure 3 shows that more than 95% of atrazine was degraded in 24 hours in both cases, suggesting that growth and atrazine degradation were not strictly correlated.

After 55 hours of incubation, the biodegradation rates observed in Figure 3 are quite similar in mineral medium and in sterile pine bark extract. However, a lag period of 24 hours was observed in bark extract. This phenomenon may be explained by the adaptation of inoculated cells to the bark extract. Soluble organic compounds of bark present in the extract may not be degraded as easily as glucose by the microorganisms. *Pseudomonas* sp. strain ADP was able to degrade atrazine in non-favorable conditions.

3.2. Mineralization at low concentrations of atrazine

The mineralization of atrazine (i.e. conversion into CO_2) was studied at low concentrations ($44.2 \text{ }\mu\text{g}\cdot\text{L}^{-1}$) using ^{14}C molecules. Assays were conducted in mineral medium, in sterile

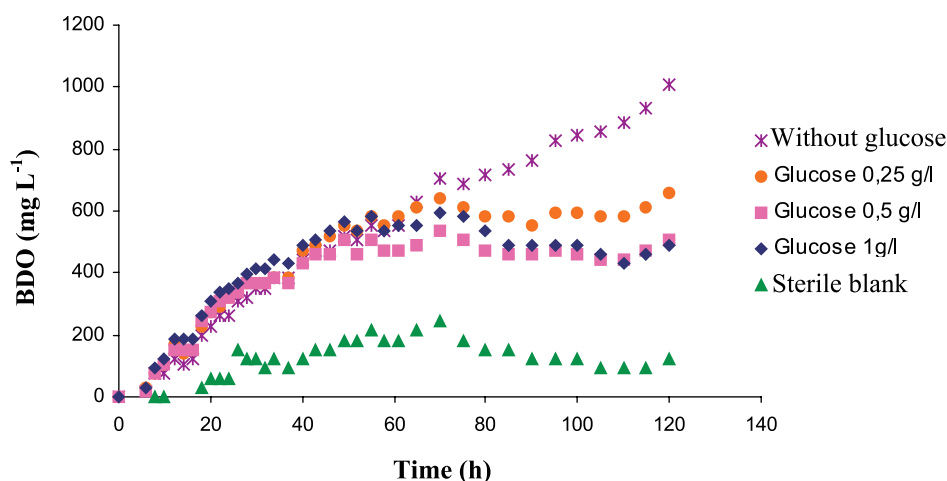


Figure 2. Biochemical demand in oxygen (BDO) of *Pseudomonas* sp. strain ADP in sterile bark extract (Extract) supplemented or not with different concentrations of glucose (0, 0.25, 0.5 or 1 g·L⁻¹, Glc) and nitrogen (ammonium sulphate 1 g·L⁻¹), at 30 °C, 150 rpm. The sterile blank contained glucose at 1 g·L⁻¹. Mean of triplicated assays.

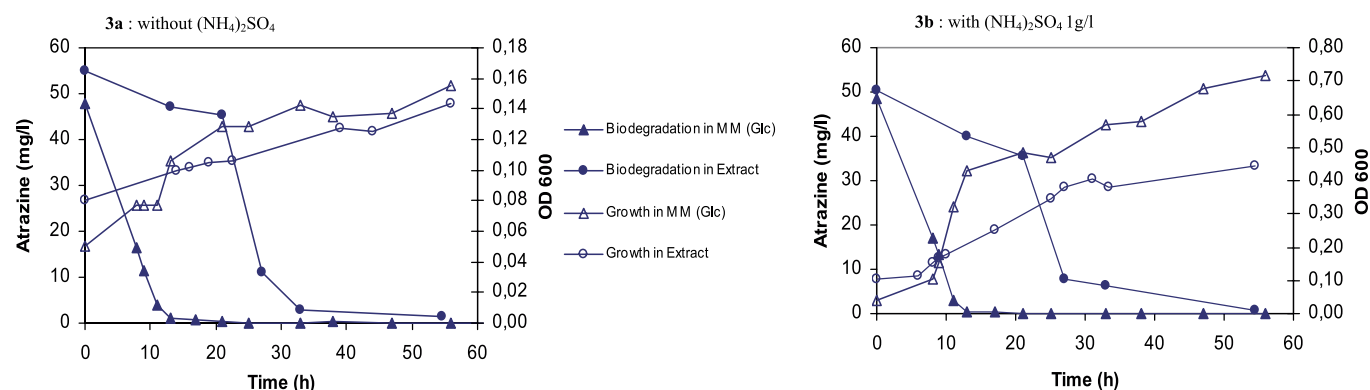


Figure 3. Growth of *Pseudomonas* sp. strain ADP (open dots) and atrazine degradation (full dots) in sterile mineral medium (triangles) or bark extract (circles) spiked with atrazine (50 mg/L) and supplemented (2b) or not (2a) with ammonium sulphate 1 g/L as nitrogen source, 30 °C, 150 rpm agitation. Mean of triplicated assays.

bark extract and with the presence of pine bark particles (L/S ratio of 20) in mineral medium. Atrazine was the sole nitrogen source in these assays since atrazine primary biodegradation was found to be rapid under these conditions (Fig. 3) and mineralization extremely reduced when atrazine was not the sole nitrogen source (Gendrault, 2004).

Figure 4 shows that 80% of atrazine was mineralized in the mineral medium in about 100 to 150 hours. With a similar initial carbon concentration (100 mg·L⁻¹), the mineralization was delayed in the sterile bark extract as compared with atrazine mineralization in mineral medium, corresponding to the adaptation phase of the cells; however, 75% of the atrazine initially present in the extract was mineralized in 330 hours.

The presence of bark particles in mineral medium reduced the mineralization down to 50%, indicating that atrazine bioavailability was reduced by its adsorption onto pine bark. Actually, at the same initial concentration, other studies showed that

only 5% of atrazine adsorbed onto pine bark particles after 330 hours of incubation was extractable, and 95% irreversibly linked to the particles and not available for microorganisms (results not shown) (Gendrault, 2004).

4. CONCLUSION

Pseudomonas sp. strain ADP was able to grow and degrade atrazine in the presence of bark organic soluble compounds and use them as a carbon source, suggesting that bark is not toxic for *Pseudomonas* sp. strain ADP and could be used as a sorbent phase in biofiltration for the treatment of water contaminated with atrazine.

Growth of *Pseudomonas* sp. strain ADP in bark extract was greatly increased by (NH₄)₂SO₄, but atrazine primary biodegradation was not significantly affected: primary biodegradation

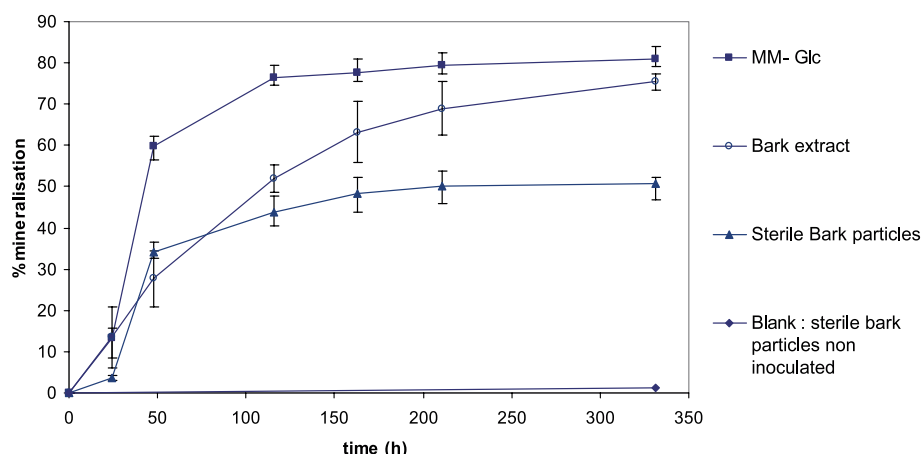


Figure 4. [U-ring- ^{14}C]-atrazine mineralization by *Pseudomonas* sp. strain ADP in mineral medium (MM - Glc), in sterile bark extract and in MM containing sterile pine bark (L/S of 20), at 20 °C. Atrazine was the sole source of nitrogen. Mean of triplicated assays; error bars presented the maximal and minimal values of the triplicates.

and growth are not directly correlated. The biofiltration may be applied without adding a nitrogen source, privileging the atrazine consumption by the microorganisms implied in the treatment. As *Pseudomonas* sp. strain ADP was able to degrade and consume the bark soluble organic compounds for its development, the addition of a carbon source was not necessary during the treatment. Moreover, the addition of glucose in assays in bark aqueous extract was found to induce an inhibition of the bacterial growth and activity.

Pseudomonas sp. strain ADP mineralized about 80% of atrazine in liquid development cultures (MM) with glucose as a carbon source. With bark soluble organics as carbon sources, a lag period is observed, but the overall yield of mineralization is not affected. When particles of bark were present in the medium, an adsorption phenomenon lowered the atrazine availability for the *Pseudomonas* sp. strain ADP, and reduced the mineralization down to 50%.

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