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1 **Stimulation of pyrene mineralization in freshwater sediments by bacterial**
2 **and plant bioaugmentation**

3

4 Yves Jouanneau^{1*}, John C. Willison¹, Christine Meyer¹, Serge Krivobok^{1,2}, Nathalie
5 Chevron³, Jean-Luc Besombes⁴ and Gérard Blake³

6

7 ¹Laboratoire de Biochimie et Biophysique des Systèmes Intégrés, Département de Réponse et
8 Dynamique Cellulaires, CNRS UMR 5092, CEA-Grenoble, F-38054 Grenoble Cedex 9,
9 France.

10 ²Laboratoire d'Écologie Alpine (LECA), UMR CNRS 5553, Université Joseph Fourier-
11 Grenoble 1, BP 53, F-38041 Grenoble Cedex 09, France.

12 ³Laboratoire d'Optimisation et Conception en Ingénierie de l'Environnement (LOCIE),
13 ESIGEC - Université de Savoie, 73376 Le Bourget du Lac, France.

14 ⁴Laboratoire de Chimie Moléculaire et Environnement (LCME), ESIGEC - Université de
15 Savoie, 73376 Le Bourget du Lac, France.

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18 *Corresponding author:

19 Yves Jouanneau

20 CEA-Grenoble, DRDC/BBSI,

21 F-38054 Grenoble Cedex 9, France.

22 Tel. : 33 (0)4.38.78.43.10 ; Fax : 33 (0)4.38.78.51.85

23 Email : yjouanneau@cea.fr

1 **Abstract**

2 As a means to study the fate of PAHs in freshwater sediments, pyrene mineralization was
3 examined in microcosms spiked with ¹⁴C-pyrene. Some microcosms were planted with reeds
4 (*Phragmites australis*) and/or inoculated with a pyrene-degrading strain, *Mycobacterium* sp.
5 6PY1. Mineralization rates recorded over a 61-d period showed that reeds promoted a
6 significant enhancement of pyrene degradation, which possibly resulted from a root-mediated
7 increase of oxygen diffusion into the sediment layer, as indicated by *in situ* redox
8 measurements. In inoculated microcosms, mineralization reached a higher level in the absence
9 (8.8%) than in the presence of plants (4.4%). Mineralization activity was accompanied by the
10 release of water-soluble pyrene oxidation products, the most abundant of which was identified
11 as 4,5-diphenanthroic acid. Pyrene was recovered from plant tissues, including stems and
12 leaves, at concentrations ranging between 40 and 240 μg/g dry weight. Plants also
13 accumulated labeled oxidation products likely derived from microbial degradation. Pyrene-
14 degrading strains were 35-70 fold more abundant in inoculated than in non-inoculated
15 microcosms. Most of the pyrene-degrading isolates selected from the indigenous microflora,
16 were identified as *M. austroafricanum* strains. Taken together, the results of this study show
17 that plants or PAH-degrading bacteria enhance pollutant removal, but their effects are not
18 necessarily cumulative.

1 **Introduction**

2 Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants which
3 form upon combustion of fossil fuels and organic compounds. Oil spills occurring during
4 transport, use or disposal operations are other main sources of PAH pollution. Oil spills were
5 shown to have long-term toxic effects on marine wild life, essentially because of the
6 persistence of PAHs in seashore sediments (1). The fate of these compounds in natural
7 ecosystems and their impact on living organisms is, however, poorly understood. Once in
8 soils or sediments, PAHs undergo various transformations including sequestration in organic
9 matter and microbial degradation. Sequestered PAHs represent a fraction which is not
10 extractable in solvent as a result of either a tight but non-covalent binding to the humic
11 material present in organic matter (2), or the formation of covalently-bound residues between
12 oxidized PAH metabolites and the humic material (3,4). Sequestration and the poor water-
13 solubility of PAHs are frequently invoked to explain the resistance of PAHs to microbial
14 degradation in soils and sediments (5).

15 In order to improve or accelerate removal of PAHs from contaminated soils, bioaugmentation
16 treatments involving selected microorganisms (6) or plants (7) have been proposed. Using
17 selected bacteria, successful enhancement of PAH degradation in soils (8), and sediment (9)
18 have been reported in laboratory studies. However, the success of inoculation experiments is
19 often limited as the survival of the selected strains and their *in situ* degradation activity
20 depends on whether soils offer favorable conditions, such as suitable pH and salinity (10).
21 Moreover, PAH biodegradation does not always correlate with reduced toxicity, as toxic
22 byproducts may accumulate in soils (11). Little information is available about the nature and
23 quantity of metabolites released in soils upon PAH degradation.

24 Phytoremediation, i.e. the use of plants to help in the removal of contaminants, is considered a
25 promising approach to eliminate organic pollutants such as PAHs from the soil (7).

1 Laboratory or greenhouse experiments show that various types of plants promote PAH
2 degradation in soils by stimulating microbial metabolism in the rhizosphere (12,13).
3 Dissipation of PAHs in the rhizosphere has been correlated with a decrease in toxicity (14).
4 However, it is still unclear how roots exert their positive effect. In addition, the effect of
5 byproducts resulting from the biodegradation of PAHs and their possible uptake by plants are
6 rarely studied in phytoremediation experiments, although one study has addressed the
7 distribution of pyrene and its metabolites in soil and plants following degradation in the
8 rhizosphere (15). Little is also known about the potential of aquatic plants such as reeds to
9 promote the removal of PAHs from contaminated sediments.

10 In this study, we investigated the biotransformation of pyrene in a natural fresh water
11 sediment which had not been previously exposed to PAH contamination. Sediments were
12 spiked with ¹⁴C-labeled pyrene, and the mineralization of this compound was studied in
13 microcosm systems. We investigated the effects of reeds and of a PAH-degrading bacterium
14 (16) on pyrene degradation, analyzed the metabolites released, and monitored the
15 accumulation of radiolabeled by-products by the plants. The possible mechanisms by which
16 the rhizosphere of reeds stimulates microbial PAH degradation in aquatic sediment, are
17 discussed.

1 **Experimental Section**

2

3 **Plant and bacterial organisms.**

4 *Mycobacterium* sp. 6PY1 was previously isolated from a PAH-contaminated soil and selected
5 for its ability to grow on pyrene as sole carbon source (16). This bacterium was grown on a
6 mineral salts medium [MSM, (17)] supplemented with benzoate (5 mM), as previously
7 described (16). Young *Phragmites australis* plants (4 leaves, 1-month old) as well as cuttings
8 were obtained from a laboratory culture. Plants showing the closest morphological aspects
9 were selected in order to obtain comparable planted microcosms. Plant growth was
10 monitored by the cumulated length of leaves and mass increase.

11

12 **Sediments**

13 The sediment samples were taken from a natural fresh water lake located in the Savoie region
14 of the French Alps (Lac d'Aiguebelette). The sediment was removed from an area of the
15 shore devoid of reeds and other plants. It consisted of light grey, calcareous particles, which
16 were sieved to below 2 mm and stored in 2 L bottles at 7°C until needed. A detailed physico-
17 chemical analysis (available upon request) revealed that the sediment had a pH of 7.7, a low
18 content of organic carbon (TOC = 1.4%), and low levels of heavy metals. The overall content
19 of the 16 priority PAHs was 30 ppb, including 2.9 ppb of pyrene.

20

21 **Set up of microcosms**

22 Microcosm experiments were carried out in 2.5 L polycarbonate jars designed for anaerobic
23 incubation of Petri dishes (GasPak, Fisher Labosi, France). A 5-mm hole was made through
24 the lid and was sealed with a rubber stopper. This modification allowed the insertion of a
25 250-mm needle (gauge 17) and a 50 mm needle (gauge 19) as air inlet and outlet,

1 respectively. In each empty jar, a mixture of [4,5,9,10-¹⁴C]-pyrene (2.6 μ Ci, specific activity
2 55 mCi/mmol; Amersham Pharmacia Biotech, Orsay, France) and 15 mg unlabeled pyrene
3 dissolved in cyclohexane was introduced, and the solvent was allowed to evaporate. Then,
4 300 g of wet sediment and 50 ml water were placed in the jars, which were subsequently
5 shaken on a rotary shaker at 120 rpm for 3.5 h. Five sets of microcosms were prepared which
6 differed by the following additions : A, no addition; B, planted with *P. australis*; C, planted
7 with *P. australis* and inoculated with strain 6PY1; D, inoculated with strain 6PY1; E, treated
8 with 0.33% w/w sodium azide (control). Duplicates of microcosms A, D and E; and triplicates
9 B and C were prepared. Strain 6PY1 was added as benzoate-grown cells so as to obtain an
10 initial population of 1.4×10^7 bacteria per g of wet sediment. The bacterial inoculum (3 ml) was
11 from an early stationary phase culture. Upon dilution in the microcosms, the residual benzoate
12 concentration was calculated to be lower than 0.6 μ M, which was judged negligible.
13 Inoculated as well as control microcosms were further shaken 30 min at 120 rpm. When
14 applicable, seven plants of *P. australis* including four young shoots and three cuttings, were
15 introduced per microcosm. Finally, the microcosms were supplemented with 250 ml sterile
16 water, closed, and incubated at room temperature ($23 \pm 2^\circ\text{C}$) in a fume cupboard. Microcosms
17 containing plants were illuminated 16 h per day with two daylight fluorescent lamps (Osram
18 Fluora, 36W/77). Each microcosm was gassed with humidified, sterile air at a roughly
19 constant flow rate (1-1.5 l/h) via a manifold connected to a compressed air cylinder.

20

21 **Measurements of ¹⁴CO₂ evolution**

22 The gas outlet of each microcosm was connected via silicon rubber tubing to a cartridge
23 containing 3 g of hydrophobic resin (Amberlite XAD-2, Supelco) to trap pyrene and other
24 organic molecules, followed by two consecutive CO₂ traps. Each CO₂ trap consisted of a
25 sealed glass tube containing a 15-ml solution of 1 N NaOH, with a 1-ml plunging pipette as a

1 gas-inlet and a 19-gauge needle as an outlet. The trapping solution was replaced every week.
2 The trapped radioactivity was estimated on 1-ml samples mixed with 7 ml of Ready Value
3 cocktail (Beckman) in scintillation vials. The mixtures were left overnight then counted using
4 a Beckman LS6500 scintillation counter.

5

6 **Redox potential measurements in the sediment layer**

7 Redox potentials were measured with small-size platinum electrodes (Orion Ionizer 407A or
8 Ingold XM100) at defined depths in the sediment layer. The electrodes were calibrated with
9 two different pH buffers (pH 4.01 and 7.00) saturated with quinhydrone. Redox potentials
10 were normalized versus the standard hydrogen electrode. For these measurements, separate
11 experiments were carried out in 12-L glass carboys containing a 10-cm deep sediment layer
12 planted with reeds. Redox determinations were made in the rhizosphere region at various
13 distances of the roots and in dark zones of the sediment as indicated in Fig.2B.

14

15 **Processing of the microcosms at the end of the incubation**

16 Plants were carefully removed, rinsed with water, and subjected to growth measurements.
17 Plants from triplicate microcosms were then pooled, and analyzed for radioactivity and
18 pyrene content as described below. The decanted water phase of each microcosm was filtered
19 through a 75 mm-diameter, 0.2 μm Nalgene filter, analyzed by scintillation counting, and
20 stored at 4°C in the dark in sterile screw-capped flasks until needed. Sediment samples (50 g
21 wet mass) from the microcosms were mixed with 100 ml of 0.1M Tris-HCl, pH 7.5, in 250-ml
22 closed flasks and incubated for 16 h at 20°C on a shaker at 200 rpm. After decantation for 24
23 h at 4°C, the supernatant aqueous phase was filtered through a glass fiber filter (Whatman
24 GF/B), subjected to radioactivity counting, and stored in closed bottles at 4°C. The washed
25 sediments were then subjected to organic solvent extraction.

1 **Extraction of pyrene and ¹⁴C-labeled molecules from water, sediments and plants**

2 Aqueous samples (25 to 40 ml) were acidified by adding trifluoroacetic acid to 0.1% final
3 concentration, then applied to solid phase extraction cartridges (Upticlean C18U, 500/6,
4 Interchim). After washing with 10 ml of 0.1% trifluoroacetic acid, the cartridges were eluted
5 successively with 50% and 100% methanol, 5 ml each. Since 80-90% of the bound
6 radioactivity was eluted with 50% methanol, only the latter extracts were further analyzed by
7 thin-layer chromatography. Sediment samples (10 g wet weight) were solvent-extracted by a
8 standard Soxhlet procedure, using either methanol or dichloromethane. Briefly, samples were
9 mixed with 30 g of anhydrous sodium sulfate, then loaded into cartridges for a 5h-extraction
10 using a Buchi apparatus. The extract was taken to dryness under a stream of nitrogen and
11 dissolved in 4 ml methanol before HPLC analysis or radioactivity counting.

12 Before extraction, plants from triplicate microcosms were pooled (only plants grown from
13 seedlings were considered). Plants were cut out into five lots: roots, lower stems, upper stems,
14 lower leaves, upper leaves. Each lot was air-dried, weighted, and solvent-extracted as above.
15 Extracts were concentrated to a volume of 2 ml, and subjected to scintillation counting, HPLC
16 and TLC analysis.

17

18 **Chromatographic analyses**

19 HPLC analysis was carried out on a Waters system equipped with a photodiode array detector
20 (model 996), piloted by a Millennium software. Separation were performed on a VYDAC C18
21 column (25 cm by 4.6 mm i.d., Interchim), operated isocratically at 22°C, at a flow rate of 1
22 ml/min with methanol as mobile phase. The sample injection volume was 10 µl. For
23 calibration, standard solutions of pyrene in methanol were used, at concentrations ranging
24 between 5 and 50 µg.ml⁻¹ (5 to 50 ppm). Calculations of concentrations in samples were made
25 on the basis of peak area.

1 TLC was performed on silica plates (20× 20 cm; Merck) to which 5- μ l samples were applied.
2 Plates were developed first with hexane/acetone (80/20), and then with
3 benzene/acetone/acetic acid (85/10/5). Radioactive spots were localized after exposure of the
4 plates to X-ray films (Kodak Biomax) for at least one week at 20°C. Spots of interest were
5 isolated by scraping the silica plate and transferring the material into Pasteur pipettes. Labeled
6 compounds were eluted with 1.2 ml methanol. After evaporating the solvent under vacuum,
7 the dry samples were derivatized with BSTFA/TCMS (99:1) (Supelco) and subjected to GC-
8 MS analysis as previously described (16,18).

9

10 **Microbiological analyses**

11 Total bacteria present in sediment samples were counted by epifluorescence microscopy after
12 extraction and staining of the bacteria with a kit allowing to distinguish between live and dead
13 cells (*BacLight* viability kit, Molecular probes, Oregon, USA). Bacteria were extracted from
14 1.0 to 1.5 g of wet sediment by 3 cycles of a treatment consisting of Vortex shaking for 10 s
15 in 3 volumes of sterile phosphate buffer, followed by a 1-min incubation in a ultrasonic bath
16 (Fisher, model FB11011). After letting the suspension decant for 5 min, 100- μ l samples were
17 mixed with the same volume of *BacLight* stain and incubated for 15 min. Aliquots (5 μ l) were
18 then observed under a Zeiss Axioskop microscope, and the bacteria present in 10 fields were
19 counted. Bacterial counts per g of sediment were then calculated. Enumeration of bacteria
20 able to degrade pyrene was performed by plating aliquots of suspension on solid MSM
21 medium coated with a layer of solid pyrene, using a method adapted from (19). The presence
22 of pyrene-degrading micro-organisms was indicated by the appearance of circular clearing
23 zones in the opaque pyrene layer after 7-10 d incubation at 25°C.

24

1 **Isolation and identification of pyrene-degrading strains**

2 Pyrene-degrading strains were isolated from the center of clearing zones on pyrene-coated
3 plates and identified by PCR amplification and sequencing of the 16S rRNA gene, as
4 described previously (20). The PCR products from various isolates were sequenced partially
5 and the complete sequence was then determined for representative strains (C-6, C-7, C-8).
6 The sequence of C-8 was found to be identical to the previously determined sequence of
7 *Mycobacterium* sp. 6PY1, which is closely related to the species *M. gilvum* (16). The
8 sequences of strains C-7 (*Stenotrophomonas* sp.) and C-6 (*M. austroafricanum*) have been
9 deposited in the EMBL Nucleotide Sequence Database under the accession numbers
10 AJ871633 and AJ871634, respectively.

11

12 **Results**

13 **Effects of *P. australis* and *Mycobacterium* 6PY1 on pyrene mineralization in freshwater** 14 **sediment**

15 The sediment used in this study was from a natural lake in the French Alps. It consisted
16 mainly of limestone, with a low content of organic matter (TOC of 13 g/kg dry matter), and a
17 low background level of PAHs (approx. 30 μ g/kg dry matter).

18 Twelve microcosms containing identical amounts of sediment spiked with [¹⁴C]-pyrene were
19 prepared as described under Materials and Methods, and incubated for 61 days in closed jars.

20 The microcosms were divided into five sets, some of which were planted with *P. australis*
21 (B), some were inoculated with *Mycobacterium* sp. 6PY1 (D), some received both types of
22 organisms (C) while non-inoculated (A) or biocide-treated (E) microcosms served as controls.

23 Strain 6PY1 was previously described as a pyrene-degrading bacterium isolated from a
24 polluted soil (16). Benzoate-grown cells were used to inoculate the microcosms since
25 benzoate was found to induce the pyrene catabolic enzymes (16). Quantitative monitoring of

1 the $^{14}\text{CO}_2$ evolved by the microcosms showed that pyrene mineralization became significant
2 after 30 days in non-inoculated microcosms confirming that the natural sediment contained
3 microorganisms able to mineralize pyrene (Fig. 1). *P. australis* appeared to stimulate pyrene
4 biodegradation, as the mineralization rates observed in B microcosms reached higher levels
5 than in A microcosms devoid of plants (Fig. 1A). This stimulation was probably due to the
6 effect of rhizosphere on microbial metabolism, as will be discussed later. Microcosms
7 inoculated with strain 6PY1 showed an immediate and high rate of pyrene mineralization,
8 which slowly declined over 9 weeks of incubation (Fig. 1B). This result suggested that the
9 inoculated bacterial strain not only enhanced pyrene degradation but also survived in the
10 microcosms over a long period of time, an assumption that is supported by the
11 microbiological analyses presented below. In C microcosms which had received both plants
12 and strain 6PY1, the mineralization rate was found to be lower than in D microcosms which
13 only received strain 6PY1. Hence, plants and selected bacteria which separately enhanced
14 pyrene biodegradation appeared to have antagonistic effects when used in combination.

15

16 **Carbon balance of pyrene transfer and degradation**

17 At the end of the incubation, the distribution of pyrene and labeled metabolites in the
18 aqueous, gaseous and solid phases of the microcosms was determined. The volatile organic
19 fraction was trapped on hydrophobic resin cartridges placed at the microcosm outlets, and
20 analyzed. It consisted essentially of pyrene and accounted for less than 0.06% of the initial
21 amount based on HPLC quantification. The amount of radioactivity recovered from the
22 overlying water of the microcosms varied widely, reaching levels that correlated with the
23 pyrene mineralization rates (Table 1). This result indicated that pyrene biodegradation was
24 associated with the release of water-soluble metabolites, the accumulation of which suggested
25 that they were not further degraded by microorganisms. The nature of these metabolites was

1 investigated below. In control microcosms where no pyrene mineralization occurred, the
2 presence of the PAH in the overlying water was undetectable by HPLC analysis. It could not
3 be detected either upon TLC analysis of the radioactive material extracted from the water
4 phase (see below and Fig. 3). Analysis of sediment washings also showed a correlation
5 between the amount of released radioactivity and the extent of pyrene mineralization. The
6 amount found in washings was even greater than in the overlying water, indicating that a large
7 proportion of the metabolites were present in interstitial water and sequestered in the pores of
8 the sediment.

9 The residual concentration of pyrene in the sediment was estimated either by ^{14}C -counting or
10 by HPLC determination, each method involving a separate extraction procedure. The two sets
11 of data, presented in Table 1, gave consistent results, showing that the most extensive removal
12 of pyrene occurred in microcosms inoculated with strain 6PY1, in which mineralization was
13 the highest. Significant pyrene removal was also observed in microcosms harboring plants,
14 while no difference in pyrene content was noticeable between microcosms containing
15 sediment only and the control microcosms containing biocide. These data are qualitatively in
16 accord with those presented above, in that the extent of pyrene removal was correlated with
17 the levels of pyrene biodegradation in relevant microcosms. However, the recovery of pyrene
18 in control microcosms, or the recovery of labeled carbon in other microcosms (calculated as
19 the sum of the radioactivity found in the gaseous, liquid and solid phases of the microcosms)
20 ranged between 43% and 65% (Table 1). Possible explanations for such low recoveries will
21 be discussed later.

22

23 **Bioaccumulation of pyrene and metabolites in *P. australis***

24 During the 61-day incubation period in microcosms, plants showed good growth as judged
25 from the increase in the length of stems and leaves, and from the overall gain of plant mass.

1 The incorporation of pyrene and labeled metabolites in plants was estimated in solvent
2 extracts of the plant tissues by HPLC quantification and radioactivity counts (Table 2). Pyrene
3 was detectable in all tissues of *P. australis* grown on contaminated sediments, the highest
4 levels being found in roots. Substantial amounts of pyrene were also measured in stems and
5 leaves, with higher accumulations in the lower part of these tissues. Interestingly, plants
6 grown on sediments which had been inoculated with strain 6PY1 accumulated much less
7 pyrene than plants grown on non-inoculated sediment (Table 2), suggesting that bacterial
8 biodegradation significantly reduced the bioavailability of the PAH for plants. Nevertheless,
9 these plants incorporated a similar amount (roots) or twice as much (stems and leaves) of
10 labeled compounds as plants grown in non-inoculated microcosms, suggesting that they
11 accumulated water-soluble metabolites resulting from bacterial catabolism. Calculations
12 based on the specific activity of ^{14}C -pyrene indicated that pyrene accounted for about 8% of
13 the radio-labeled compounds found in roots and around 1% or less in stems and leaves. Even
14 in plants from non-inoculated microcosms where PAH biodegradation was limited,
15 metabolites rather than pyrene appeared to be the major labeled compounds found in plant
16 tissues (Table 2).

17

18 **Evidence for root-mediated oxygen diffusion into the sediment layer**

19 In the course of the microcosm incubation, the sediment layer, initially light grey in color,
20 turned dark, presumably because the anaerobic conditions which prevailed below the surface
21 favored the precipitation of metallic sulfides. Looking through the transparent bottom of the
22 jars, we noticed that plant roots were bordered with clear zones contrasting with the dark
23 background (see Fig. 2A). This observation suggested that plant roots released oxygen into
24 the sediment, thereby promoting the oxidation of sulfides in the vicinity of the roots. In order
25 to test this hypothesis, redox measurements were carried out through the sediment layer of

1 planted microcosms using microelectrodes. As illustrated in Fig.2B, positive redox potentials
2 were found in the clear zones surrounding the roots whereas negative values were recorded in
3 the dark parts of the sediment. Hence, it is inferred that the plant-mediated delivery of oxygen
4 to the rhizosphere might be the primary explanation for the stimulation of bacterial pyrene
5 mineralization by *P. australis*.

6 .

7 **Identification of ^{14}C - labeled metabolites**

8 The radioactive compounds found in the overlying water of each microcosm were extracted
9 and analyzed by thin layer chromatography. The autoradiography revealed only three resolved
10 spots, the intensity of which was correlated with the extent of pyrene mineralization in the
11 microcosms (Fig. 3). None of these spots was detectable in the control microcosms containing
12 biocide. The major spot, with an R_f of 0.38, was identified as 4,5-diphenanthroic acid based
13 on the following criteria: identical R_f by TLC, identical retention time by GC and same mass
14 spectrum as the authentic compound, which has been previously characterized (J.C. Willison,
15 unpublished data). The compound that migrated with a R_f of 0.58 was identified as 4-
16 phenanthroic acid based on the same criteria, whereas the third spot of lowest intensity (R_f of
17 0.45) was too scarce to give a significant signal by GC-MS. As discussed below, the two
18 identifiable compounds are known to be metabolites produced by pyrene-degrading bacteria
19 such as *Mycobacterium* 6PY1. Curiously, in one of the replicate microcosms (C2), the spot of
20 4,5-diphenanthroic acid was undetectable, suggesting that it might have been converted to
21 more hydrophilic byproducts which were not isolated by the extraction method used. Such a
22 conversion might have been carried out by bacterial strains able to metabolize 4,5-
23 diphenanthroic acid, which would have grown to a higher density in this particular
24 microcosm.

1 It should be noted that pyrene was undetectable in the overlying water of the microcosms,
2 even in controls in which biodegradation was inhibited. On the other hand, analysis of
3 sediment extracts by TLC showed that the ^{14}C -label was almost entirely associated with
4 pyrene, while trace amounts of the above-cited metabolites were detected in those
5 microcosms where biodegradation was highest (data not shown).

6

7 **Enumeration and identification of pyrene-degrading strains**

8 The bacterial population of the sediment after 61 days was found to be higher in microcosms
9 which harbored plants than in those which did not, and was higher in those which received a
10 bacterial amendment than in non-inoculated microcosms (Fig. 4). The proportion of living
11 bacteria (green cells with the *BacLight* stain) was higher than 90% in all microcosms. The
12 number of pyrene-degrading bacteria was 35- to 70-fold higher in sediments inoculated with
13 strain 6PY1 than in non-inoculated controls.

14 Two different strains able to degrade pyrene were isolated from the sediment of non-
15 inoculated microcosms which harbored plants. Based on the analysis of 16S rRNA gene
16 sequences, one of these strains, C-6, was identified as a strain of *Mycobacterium*
17 *austroafricanum* (100% sequence identity in a 1274 nt overlap with *M. austroafricanum*
18 ATCC 33464; accession no. X93182) and the other strain, C-7, was identified as a member of
19 the genus *Stenotrophomonas*, with close affiliation to *S. maltophilia* (99.1% sequence identity
20 in a 1302 nt overlap with *S. maltophilia* LMG 10857; accession no. AJ131117). Members of
21 both species have been previously shown to degrade various PAHs, including pyrene. Three
22 out of 4 strains isolated from inoculated microcosms containing plants were found to have
23 identical 16S rRNA sequences to strain 6PY1, with the other strain being identified as *M.*
24 *austroafricanum*. Lastly, all five isolates recovered from the sediment of unamended
25 microcosms were identified as *M. austroafricanum*. Hence, it seems likely that the latter

1 species was present in the indigenous microflora of the sediment, and that this bacterium
2 played a major role in the observed pyrene mineralization in non inoculated microcosms.

3

4 **Discussion**

5 **Fate of pyrene in the sediment and *in situ* biodegradation**

6 In this study, we examined the fate and biotransformation of pyrene in the sediment from a
7 natural aquatic ecosystem. The distribution of pyrene in the microcosms, as monitored by
8 radioactivity counting and chromatographic measurements, showed that it was exclusively
9 found in the sediment layer and virtually absent from the overlying water. However,
10 calculations based on radioactivity counting indicated that only around 60% of the labeled
11 carbon initially introduced in the microcosms was recovered at the end of the 2-month
12 incubation. A plausible explanation is that part of the pyrene was tightly bound to sediment
13 particles or sequestered in micropores, and could not be extracted with solvent. Previous
14 studies have shown that PAHs and pyrene in particular become partly non-extractable due to
15 sequestration in soil or sediment, and that the proportion of non-extractable PAH increases
16 with aging (2-4). For instance, Guthrie et al (2) found that the solvent recovery of pyrene from
17 a spiked sediment gradually decreased by 53 to 77% upon aging for 60 days. In the same
18 study, the authors showed that pyrene was sequestered as a complex with humic acids. The
19 formation of non-extractable PAH residues in soil was shown, in other studies, to increase
20 with the molecular mass and the Kow of the PAH (21).

21 The chosen sediment was characterized by a low background level of PAHs, and a low
22 organic content allowing limited microbial development, as judged from the relatively low
23 bacterial density ($< 10^8$ cells/g sediment). Nevertheless, indigenous bacteria endowed with
24 pyrene-mineralizing activity were readily detected in this habitat, consistent with the
25 observation that PAH-degrading bacteria are well-adapted to oligotrophic conditions (5).

1 Pyrene mineralization was observed after a delay of at least 20 days, during which pyrene-
2 degrading bacteria probably multiply at the expense of the supplied PAH used as carbon
3 source. This delay might also be partly due to the time of induction of pyrene catabolic
4 enzymes, at least at the beginning of the incubation, since in most bacterial species studied so
5 far, the synthesis of such enzymes appeared to be inducible (16,22,23).

6 Pyrene degradation led to the accumulation in the microcosms of a few water soluble
7 metabolites. Two of these metabolites, identified as 4,5-diphenanthroic acid and 4-
8 phenanthroic acid, were previously found in axenic cultures of several *Mycobacterium* strains
9 grown on pyrene (24-26). Interestingly, the same metabolites were detected in inoculated and
10 non-inoculated microcosms, suggesting that the pyrene degradation pathway of indigenous
11 bacteria was similar to that proposed for strain 6PY1 (16). Consistent with this proposal, we
12 observed the most abundant pyrene-degrading bacterial species isolated from the sediment
13 was a *M. austroafricanum* strain. The accumulation of the two metabolites also indicated that
14 they were poorly degraded by the microbial community of the sediment. Hence, 4,5-
15 diphenanthroic and 4-phenanthroic acids might be considered easily detectable and relevant
16 biomarkers for pyrene biodegradation activity in contaminated ecosystems.

17 **Plant-mediated stimulation of pyrene degradation**

18 *P. australis* plants were found to stimulate pyrene biodegradation in non-inoculated
19 microcosms,. Previous studies describing the stimulating effect of plants on the removal of
20 organic pollutants from contaminated soils have led to the general idea that plants may favor
21 bacterial metabolism through root exudates, not only by providing carbon sources, but also by
22 secreting substances which could enhance bacterial degradation of organic pollutants (27). In
23 the present study, the beneficial effect of *P. australis* on pyrene degradation may result
24 primarily from the ability of plant roots to bring oxygen into the sediment. Redox
25 measurements inside the sediment layer, strongly suggested that oxygen diffused from the

1 roots into the sediment layer, thereby stimulating bacterial metabolism. Considering the high
2 oxygen demand of PAH catabolism, the delivery of oxygen by plant roots might be expected
3 to particularly favor pyrene mineralization. Furthermore, pyrene-degrading bacteria were
4 found to be twice as abundant in microcosms harboring plants as in control microcosms.
5 In microcosms pre-inoculated with strain 6PY1, pyrene mineralization was found to be
6 diminished about two-fold due to the presence of plants. This observation, which apparently
7 contrasts with the stimulating effect discussed above, might have one of the following
8 explanations : (i) recycling of mineralized carbon through CO₂ fixation, (ii) enhanced
9 competition against strain 6PY1 in the rhizosphere (iii) inhibition of PAH catabolism by
10 compounds released in root exudates. The first hypothesis supposed that plants would have
11 incorporated labeled carbon equivalent to the difference in the amount of released ¹⁴C-plant incorporation could not account for more
12 between microcosms with and without plants. Calculations based on the radioactivity
13 recovered from plant tissues indicated that ¹⁴C-plant incorporation could not account for more
14 than 2% of this difference, thus ruling out a significant recycling of the labeled carbon
15 through CO₂ fixation. On the other hand, the plant rhizosphere did not create conditions
16 disadvantaging strain 6PY1 or other PAH-degrading bacteria, since these bacteria were found
17 to be as abundant in planted and non-planted sediments. As last hypothesis, it is possible that
18 root exudates delivered by plants either provide carbon sources to bacteria that are more
19 readily assimilated than pyrene, or generate compounds which could lower induction of
20 catabolic enzymes in pyrene-degrading bacteria. Accordingly, it was recently observed that
21 root exudates inhibited the expression of naphthalene catabolic genes in a PAH-degrading
22 *Pseudomonas* strain (28).

23 **Pyrene bioaccumulation by plants**

24 Pyrene analysis in plant tissues revealed that it accumulated at concentrations varying along a
25 decreasing gradient from roots to leaf tips. This distribution suggested that pyrene entered the

1 plant through the roots and diffused to the upper parts through the vascular system. The
2 amounts of pyrene found in roots (250 $\mu\text{g/g}$), stems and leaves (5 times less on average) were
3 much greater than the limit of solubility of pyrene in water (0.16 mg/L) indicating that a
4 significant bioaccumulation of the PAH occurred in the plants. In microcosms inoculated with
5 strain 6PY1, plants accumulated 10- to 20-fold less pyrene but instead, incorporated
6 radiolabeled oxidation products. In this case, bacterial metabolism probably diminished the
7 availability of pyrene to plant roots, and also caused a release of hydrosoluble metabolites
8 which probably penetrate more easily into the plants. The exact nature of the labeled products
9 found in the plants remains to be determined, as well as the mechanism by which they
10 become sequestered.

11

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- 6

TABLE 1: Distribution of pyrene and labeled compounds released in microcosms after 61 d ($\%$)^a.

Microcosm	Organisms	Mineralized	Soluble ¹⁴ C-label released in	Residual pyrene	Total ¹⁴ C		
Series ^b	introduced	pyrene ^c	overlying water	Sedim. washings	¹⁴ C counts	HPLC	recovery
A(2)	None	0.18 \pm 0.07	0.55 \pm 0.02	1.04 \pm 0.06	58.9 \pm 8.9	65.3 \pm 5.5	60.7 \pm 9.0
B(3)	<i>P. australis</i>	0.46 \pm 0.12	0.82 \pm 0.01	1.08 \pm 0.14	51.8 \pm 8.2	48.7 \pm 5.3	54.2 \pm 8.5
C(3)	<i>P. australis</i> + 6Y1	4.4 \pm 0.7	1.69 \pm 0.09	4.61 \pm 0.22	35.2 \pm 6.2	35.1 \pm 2.6	45.9 \pm 7.2
D(2)	strain 6PY1	8.85 \pm 0.3	1.88 \pm 0.3	5.83 \pm 0.19	26.2 \pm 2.1	31.8 \pm 0.0	42.8 \pm 2.9
E(2)	none (+ azide)	0.062 \pm 0.005	0.63 \pm 0.004	1.00 \pm 0.05	62.8 \pm 13.8	59.1 \pm 2.5	64.5 \pm 13.9

^aPercents were calculated on the basis of the initial amount of ¹⁴C-pyrene applied (\pm standard error)

^bReplicate numbers for each microcosm series are indicated in parentheses

^cNumbers are taken from Fig. 1 and refer to the endpoints at 61 d.

1
2 **TABLE 2 : Pyrene and ^{14}C radioactivity recovered from the tissues of *P. australis* grown**
3 **on ^{14}C -pyrene-spiked sediments**

Tissues	Plant extracts from non-inoculated microcosms (B)			Plant extracts from microcosms inoculated with strain 6PY1(C)		
	$^{14}\text{C}^{\text{a}}$	Pyrene ^a	Pyrene ^b	$^{14}\text{C}^{\text{a}}$	Pyrene ^a	Pyrene ^b
	(10^3 cpm/g)	($\mu\text{g/g}$)	(% of ^{14}C)	(10^3 cpm/g)	($\mu\text{g/g}$)	(% of ^{14}C)
Roots	186	242	50	160	32.2	7.8
Upper leaves	94.5	46.7	19	170	2.23	0.5
Lower leaves	96.3	282	112	157	4.04	1.0
Upper stems	76.3	40.3	20	144	4.88	1.3
Lower stems	78.7	58.2	28	157	2.92	0.7

4 ^aDetermined from scintillation counts or HPLC assays. Data are normalized to gram
5 of tissue dry mass

6 ^bFraction of the radioactivity in the tissues that corresponds to undegraded ^{14}C -pyrene,
7 as calculated from the pyrene content of tissues (HPLC assay) and the specific activity
8 of ^{14}C -pyrene ($0.173 \mu\text{Ci/mg}$ as determined experimentally from the solution used to
9 spike the sediment).

1 **Figure legends**

2

3 **FIGURE 1 : Effects of *P. australis* or strain 6PY1 on pyrene mineralization in**
4 **microcosms.**

5 Microcosms contained 300 g of sediments (wet mass) spiked with 15 mg of pyrene, including
6 2.6 μCi ^{14}C -pyrene. Some microcosms harbored *P. australis* plants (\blacktriangle), others contained
7 sediments only (\bullet); Sediments were either non inoculated (Part A) or inoculated with strain
8 6PY1 (Part B). Biocide-treated microcosms served as controls (\circ). Extents of mineralization
9 are averages of data from duplicate or triplicate microcosms and bars indicate standard errors.

10

11

12 **FIGURE 2 : Redox changes in the sediment layer in the vicinity of plant roots.**

13 Pannel A : Picture view taken from the bottom of a microcosm showing clear zones of the
14 sediment along the roots, against a dark background due to metal sulfide precipitation.

15 Pannel B : Redox potential measurements across a section of the sediment layer in the vicinity
16 of the rhizosphere of a young *P. australis* plant.

17

18 **FIGURE 3 :TLC analysis of the ^{14}C -labeled metabolites released in the overlying water**
19 **of the microcosms**

20 Extracts from the water phase of microcosms were subjected to TLC analysis, followed by
21 autoradiography. Microcosms contained reeds (B), reeds and strain 6PY1(C), strain 6PY1
22 alone (D). Series A and E were controls with no addition and biocide added, respectively.

23 Samples of the following authentic chemicals were run in a separate lane and detected under
24 UV light: 1, Phthalic acid; 2, 4,5-Diphenanthroic acid; 5, Pyrene. Metabolites 2 and 4 were

1 identified by GC-MS as 4,5-diphenanthroic acid and 4-phenanthroic acid, respectively (see
2 text).

3

4 **FIGURE 4 : Enumeration of total and pyrene-degrading bacteria in microcosms**

5 Bacteria were extracted from sediment samples taken from the microcosms after 61 d of
6 incubation. The total population was estimated by epifluorescence microscopy, while pyrene-
7 degrading bacteria were counted after plating on selective solid medium. Bars indicate
8 standard errors.

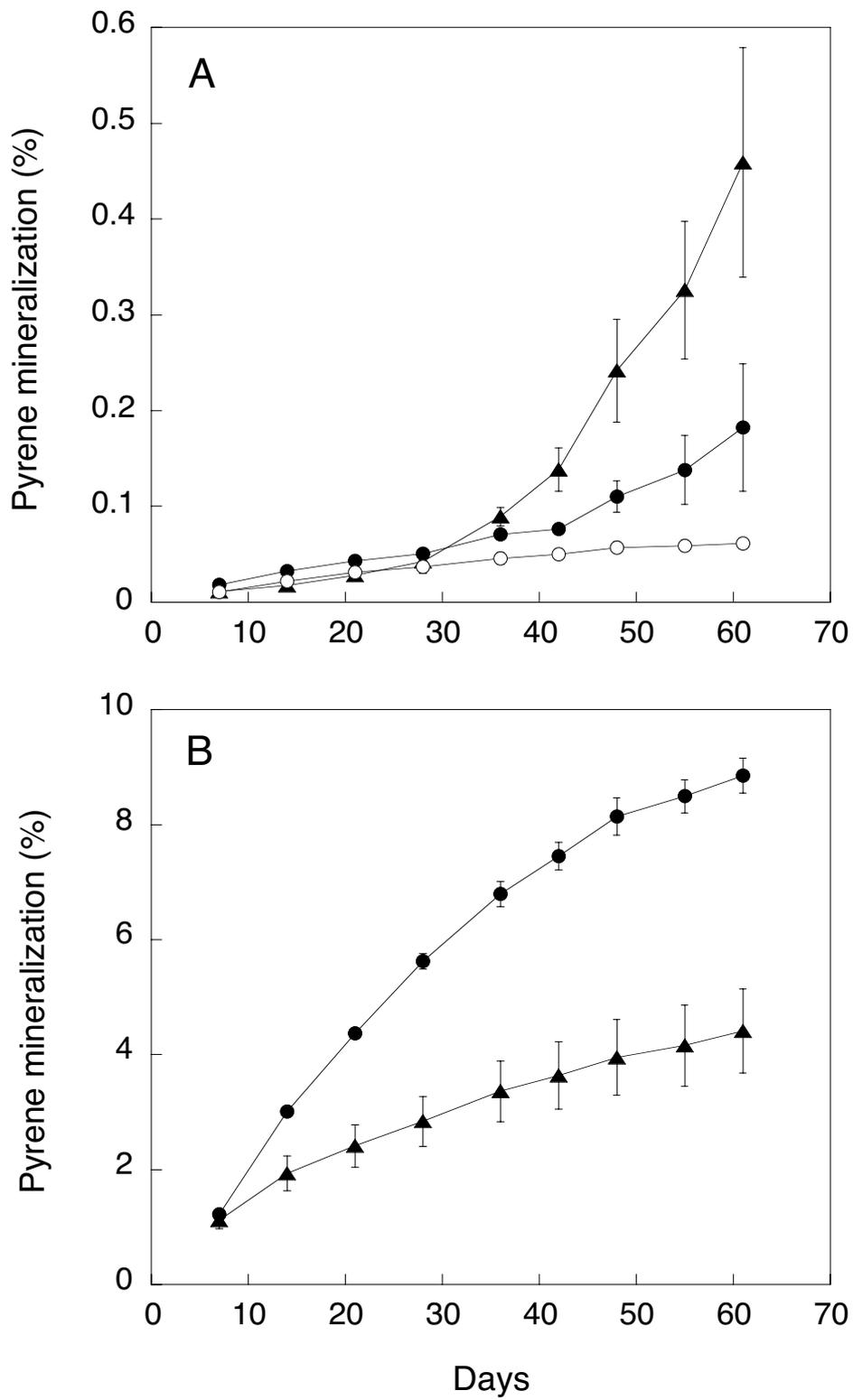
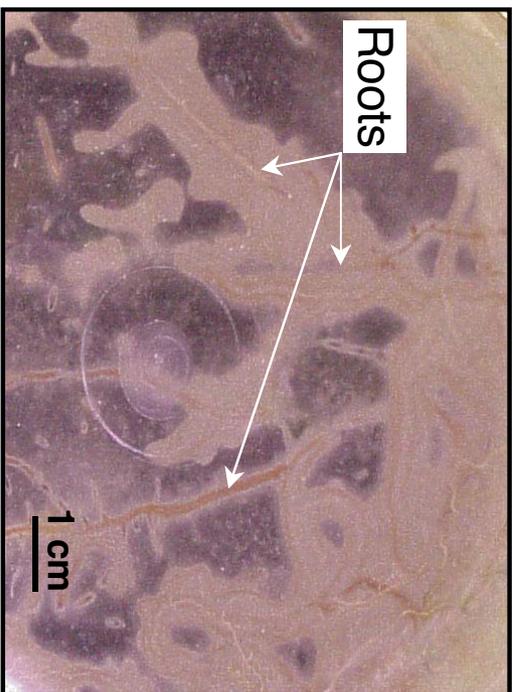


Fig. 1, Jouanneau et al.

A



B

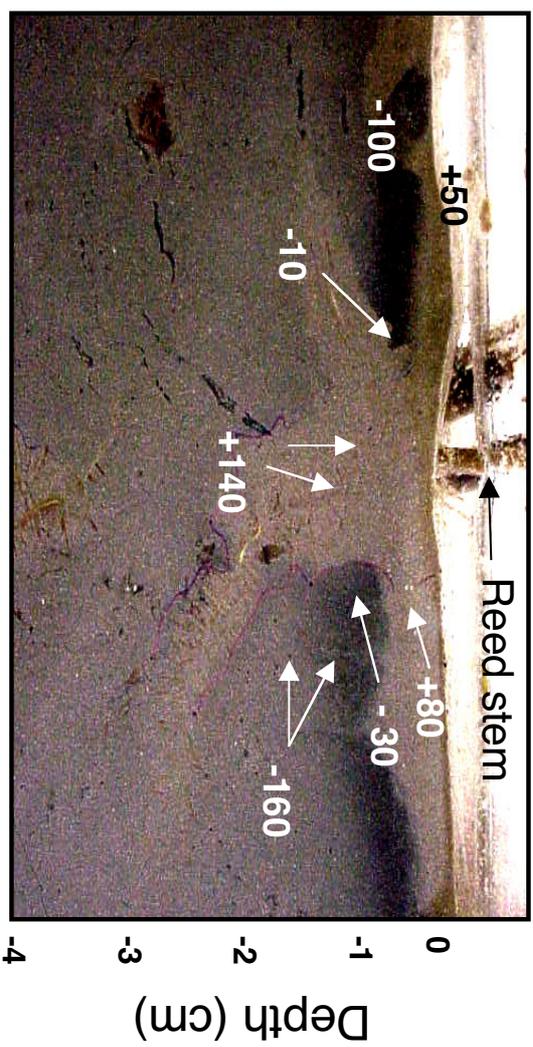


Fig. 2 : Jouanneau et al.

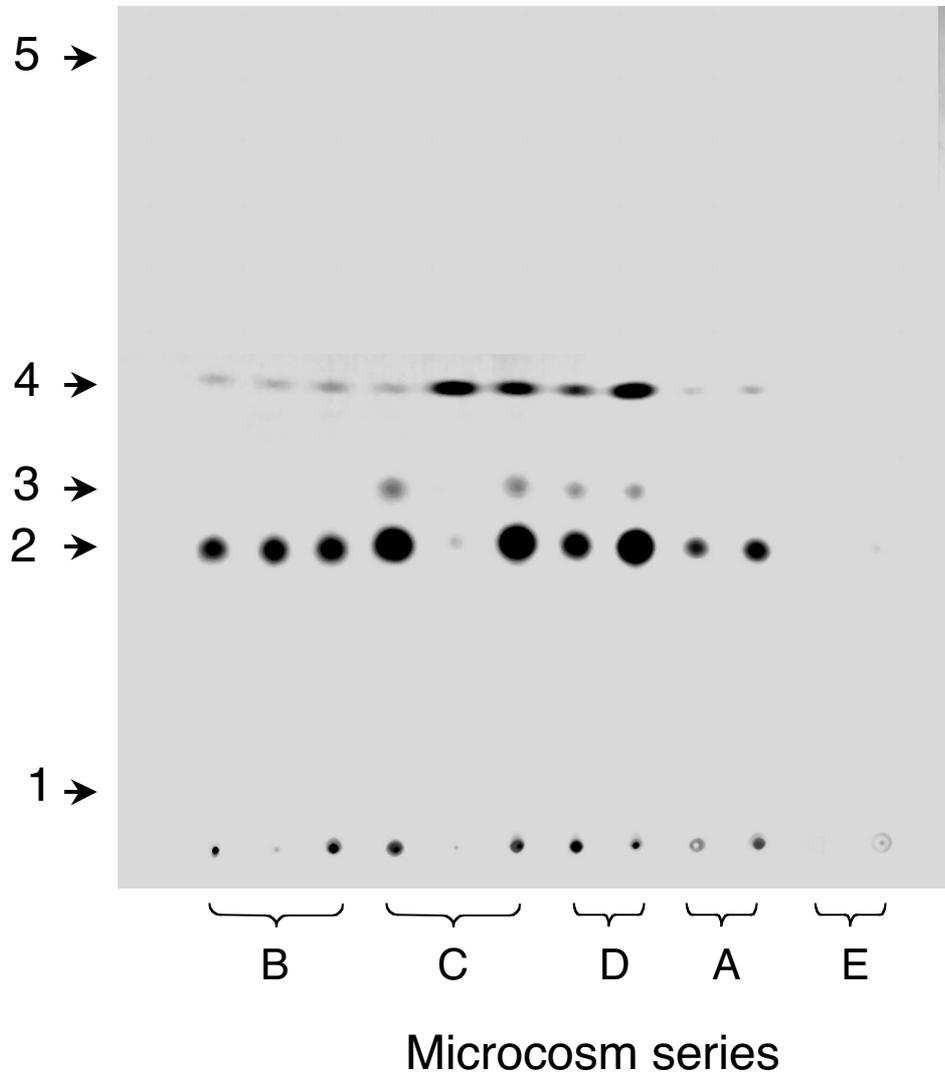


Fig. 3, Jouanneau et al.

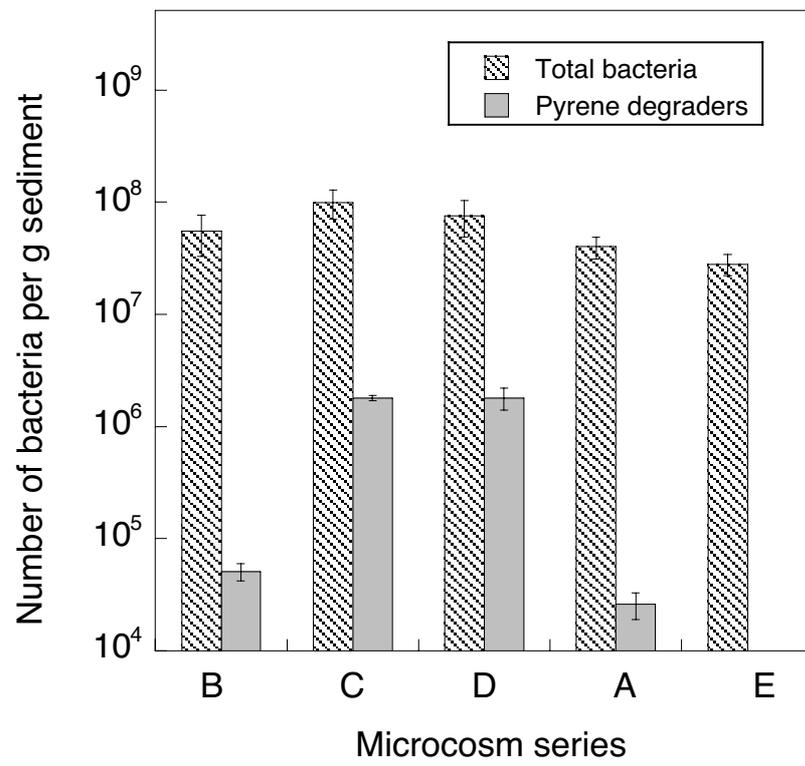


Fig.4 : Jouanneau et al