

Biodegradation of *n*-eicosane and VOCs: Cometabolism in a marine consortium

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Recibido 15 de enero de 2013, Aceptado 10 de febrero de 2013

Abstract

Several batch scale assays were performed in order to establish a correlation between the microbial removals of *n*-eicosane, in presence of different concentrations of volatile organic compounds (VOCs) which showed a partial removing. The selected VOCs were toluene and benzene. Results with benzene showed that the removal of this aromatic compound was decreased in presence of *n*-eicosane and the lowest removal was obtained when VOC concentration was higher. The removal of hydrocarbon was increased when VOC concentration was increased. In the assays with toluene, *n*-eicosane reached a higher removal when VOC concentration was increased, but the aromatic compound showed a decrease in its elimination dynamics. Control assays performed with VOCs at 28 mg/L without hydrocarbon showed higher removal dynamics for benzene than toluene. Also control assays of *n*-eicosane at two different concentrations but without any VOC showed that its removal dynamic decreased in the absence of the aromatic compounds for both assays. The kinetic adjustment obtained for toluene (Hill kinetic model) showed that the removal rate of this compound increased while its concentration at water-phase was higher. Although, when it was used a concentration of 50 mg/L or higher, the removal rate became almost constant. For benzene, the higher removal rate was reached with 38 mg/L. With higher concentration, the kinetic model showed inhibition, so the adjustment analyses fitted for a Monod kinetic model. *n*-Eicosane showed a better adjustment with the Haldane kinetic model with an initial concentration of 188 mg/L for the highest removal rate.

Keywords: VOC, Eicosane, Biodegradation, microcosm, seawater.

1. Introduction

Nowadays there is a great improvement of different technologies highly useful for control of oil spills in the sea and freshwater aquifers [1] (Hi-Point Industries, 1991). Biosorbents involve a physical process that has been considered economic, high effective and with an easily final disposal. These materials can be inoculated with microorganisms able to eliminate some heavy molecular weight oil fractions, but their efficiency is limited by factors like the toxicity of the non-polar substances, the bacterial time period for acclimation to the salinity or temperature conditions and the lack of an adequate source of nutrients that allows the cellular growth [2] (Wang *et al.*, 1996). Some microbial consortia isolated from the bottom of the sea had demonstrated a high hydrocarbon elimination capacity after a short-time period of specific acclimation in a microcosm system [3] (Ricalde, 2002) in presence of specific volatile organic compounds (VOCs), this activity improvement supposes a cometabolism process [4] (Vázquez, 2000). The principal aim of the present

work was to study the biological elimination of *n*-eicosane in presence of VOCs: toluene and benzene. Assays were performed with a marine microbial consortium that could be inoculated in biosorbents [5] (Lajud Nahum, 2003).

2. Experimental Section

2.1 Experimental Background

Assays consisted in the removal of initial *n*-eicosane concentrations in presence of different toluene and benzene volumes. The experimental procedure followed was settled down by Vazquez [4]. For acclimation of the microbial consortium to the recalcitrant compounds (hydrocarbon and VOCs), previous described microcosms system was applied [6] (Hernández *et al.*, 2002). Volatile solids suspended (VSS), dissolved oxygen (DO) and optical density (OD) determinations were performed according to the Standard Methods [7].

2.2 Experimental Units

Batch assays were performed by duplicate with



Viton™ cap closed vials of 250 mL capacity filled with 200 mL of a prepared seawater solution with a salinity of 34 UPS [8] (de la Lanza *et al.*, 2001) and addition of Macro- and Micronutrient solutions as described elsewhere [9] (Varela-Nieto, 1998) the injected volumes were 5 mL and 25 μ L, respectively. A not-characterized marine microbial consortium obtained from the sea bottom near an immersed discharge pipeline of an oil refinery in Salina Cruz, Oaxaca. It was acclimated 10 days in a 1 L capacity microcosm system as described in previous communications [3, 6, 10] (Hernández *et al.*, 2002; Ricalde, 2002; Cárdenas *et al.*, 1999) with a mixed toluene / benzene (1:1 volume ratio) gas current stripped into the reactor while 1 mL of *n*-eicosane was daily injected. The amount of initial biomass was 0.5 g VSS/L corresponding to an OD (at 660 nm) between 0.6-0.65 *A* and an initial *n*-eicosane concentration of 32 to 47 mg/L was established for assays with different water-phase concentrations of benzene, calculated by Henry's law air-water distribution ratio equation [11] (Mendoza, 2002). Experimental benzene water-phase concentrations were: 13, 29, 55 and 79 mg/L. For toluene assays the initial *n*-eicosane concentration was higher than in benzene assays (155 - 188 mg/L) because previous experiments [4] (Vazquez, 2000) used low and intermediate hydrocarbon initial concentrations varying between 28 to 44 mg/L and 82 to 105 mg/L, respectively. Obtained results showed a high-rate complete removal of the hydrocarbon as also with the different toluene concentrations tested. The applied water-phase concentrations of toluene were: 10, 24, 48 and 88 mg/L. 4 to 5 mg DO/L was reached in the liquid medium of each vial, applying 5-10 minutes of air sparging before adding the biomass amount and the organic compounds. Sterile controls (140 °C, 20 minutes at 1.5 bar) were prepared with benzene: 24 mg/L and *n*-eicosane: 29 mg/L; while with toluene: 29 mg/L and *n*-eicosane: 188 mg/L. Not sterile control assays for single compound removal were performed with 28 mg/L for experiments with each VOC (low concentration) and for *n*-eicosane low and high concentrations were used: 36 mg/L and 220 mg/L, respectively.

2.3 Experimental procedures

All the assays were performed by duplicate. Experimental vials were shaken at 100 RPM and incubated at room temperature, the amount of *n*-eicosane and VOCs removed daily during 25 to 35 days were determined by GC-FID analysis. Every day 1 mL of liquid suspension was removed from all

the vials, each volume was transferred to a closed tube of 1 mL capacity, centrifuged 1 minute at 2500 rpm and 500 μ L of the liquid phase isolated in another close tube and quickly extracted twice with *n*-hexane, collecting the non-polar phase in a final volume of 1 mL (complete tube capacity) and submitting this to GC-FID quantitative determination. The solid pellet remaining after centrifugation was also extracted with *n*-hexane, centrifuged again and the non-polar phase (same 1 mL as final volume) analyzed by CG-FID in order to determine *n*-eicosane and VOCs solid-phase sorbed amount. Concentration control curves for separated and mixed compounds were carried out in water phase but extracted as described above. Quantified concentrations after extraction were correlated to the initial concentrations in the vials with saline water medium.

2.4 Analyses

Intermediate metabolites were not determined neither than CO₂ production, so the term **removal** was applied for disappearance of hydrocarbon and VOCs during daily GC-FID analysis of the batch reactors liquid phase. *n*-Eicosane, toluene and benzene were determined by Gas-Liquid-Chromatography in a Hewlett Packard gas chromatograph series 6890, equipped with a flame ionization detector with a 200' x 0.01'' I.D. capillary column coated with 6 ring-polyphenylether. Inlet Split used 100:1; temperature programmed 50 to 200° C at 8° C/min, column flow rate: 1.5 mL/min. He Retention times: *n*-eicosane: 33-34 min, toluene: 7.6-8 min and benzene: 5.8-6 min [12] (Gulyas and Reich, 1995). Total removal of each compound was quantified as follows:

$$\text{NPLC} + \text{SPSC} = \text{NRC} \quad [1]$$

$$\text{TIC} - \text{NRC} = \text{RC} \quad [2]$$

where NPLC: non-polar liquid concentration, SPSC: solid-phase sorbed concentration, NRC: not-removed concentration, TIC: total initial concentration, RC: removed concentration.

Removal Efficiency (%) was calculated as follows:

$$[(C_i - C_{t^*}) / C_i] \cdot 100 = \text{RE} \quad [3]$$

where *C_i*: initial concentration, *C_{t*}*: concentration at specific time, RE: removal efficiency.

For air-water distribution ratio was applied

Henry's law equation as described in Mendoza, 2002.

$$K_{aw} = \frac{x_a/V_a}{x_w/V_w} = \frac{x_a V_w}{x_w V_a} \Rightarrow x_w \left(K_{aw} \cdot \frac{V_a}{V_w} \right) = x_a \quad [4]$$

where K_{aw} : Henry's dimensionless air-water partition coefficient, x_a : air molar fraction, x_w : liquid mole amount, V_a : specific gas phase volume, V_w : specific liquid phase volume.

3. Results and Discussion

3.1 Removal dynamics

For the assays performed with single benzene and *n*-eicosane obtained results are showed in Table 1 as C1 and C2. For pure benzene (28 mg/L), 90% of removal was reached at day 5 and until day 35 the removal only could be enhanced to 95 %. *n*-Eicosane alone (36.6 mg/L) reached a 90 % removal in 2.6 days but until day 10 only 95 % was removed and the same percentage was quantified at day 25. Comparison between these results and those observed for the assays carried out with different mixture ratios of the recalcitrant compounds (1, 2, 3 and 4), showed that the same removal percentage (95 %) was reached for the experiments with a VOC concentration range varying between 13 to 55 mg/L, while in the assay with a higher aromatic concentration (79 mg/L) only a 28 % was reached at day 35. For the assays performed in presence of hydrocarbon with low benzene concentrations: 13 and 29 mg/L, the 90 % of removal was reached at days 5.1 and 4.5, respectively. The highest removal for both assays until day 25 was 95 % but this percentage was reached around days 11 and 12. These results are similar to the dynamics observed for disappearance of pure benzene but 95 % of VOC removal in this assay was reached between days 35 and 36. The assay with 79 mg/L of benzene in presence of the hydrocarbon showed the lowest removal dynamic: only 28 % removed until day 35.

n-Eicosane in the mixed assays presented a similar initial removal dynamic for each experiment: 90 % of removal reached between 1.8 and 2.2 days. Highest removal percentages (96 – 97 %), without improvement until day 25, were reached in a longer time period (12 - 15 days) than for the control assay (C2) performed without aromatic compound, described above.

Sterile Controls were performed with Benzene: 25 mg/L plus *n*-eicosane: 30 mg/L. Obtained results showed that the highest benzene removal was reached at day 35: 8.89 % and for *n*-eicosane: 36 %.

Not sterile assays performed only with benzene, reached the 90 % of removal in a shorter time period compared to the assays performed in presence of *n*-

Table 1. Removal of single (C1 and C2) and mixed benzene and *n*-eicosane (1, 2, 3, 4) in batch assays performed with a marine microbial consortium as biocatalyst, suspended in a lab prepared seawater solution.

Assay	Initial Concentration (mg / L)	Removal over than 80 % and time to reach (% : d)	90% Removal at time: (d)	Highest Removal (%) and time to reach it (% : d)
Benzene (C1)	28	83: 3	5	95: 34.6***
Benzene + HC (1)	12.7	dnp*	5.1	95: 11.61
Benzene + HC (2)	28.9	86: 3.2	4.5	95: 12.61
Benzene + HC (3)	54.8	84: 6.2	7.8	95: 14.89
Benzene + HC (4)	78.6	Not reached	Not reached	28: 34.6***
<i>n</i> -Eicosane (C2)	36.6	84: 1.2	2.6	95: 9.86
<i>n</i> -Eicosane + VOC (1)	47.3	83: 1.15	1.8	97: 11.61
<i>n</i> -Eicosane + VOC (2)	42.7	83: 1.15	2.2	96: 12p.61
<i>n</i> -Eicosane + VOC (3)	41.2	82: 0.57	1.8	96: 14.89
<i>n</i> -Eicosane + VOC (4)	32.84	88: 1.3	dnd**	97: 13.2***

Notes: * datum not presented. ** datum not determined. *** These assays were analyzed during 35 days for the rest the time experiment lasted 25 days.

eicosane but its highest removal percentage was reached lately. The assay with hydrocarbon alone, reached a 90 % of removal in a longer time than in assays performed in presence of benzene and it was not reached a 97 % as highest removal like in the last mentioned. However, for the assays with both compounds, reaching the highest removal percentage occurred in longer time periods in comparison with the assay without VOC (single *n*-eicosane).

Removal dynamics obtained in assays performed with a high concentration of *n*-eicosane and different volumes of toluene are showed by separate in Table 2 and 3, respectively. The data from the experiments with single and mixed organic compounds showed notorious differences in the removal dynamics for

these assays. In Table 2 can be observed that *n*-eicosane alone presented a lower removal dynamic when compared against the assays with toluene addition. Furthermore, data indicate that as VOC concentration was increased, less time was needed for removal of similar hydrocarbon high concentrations.

The biocatalyst did not present the same capacity to remove toluene as benzene, indicated by the comparison done between assay C4 in Table 3 and assay C1 in Table 1, respectively. Even more, close similar concentrations were used for both VOCs in all the assays performed in presence of the hydrocarbon.

Table 2. Removal data for assays performed with *n*-eicosane high initial concentration and different toluene volumes.

Assay (key)	HC Initial concentration (mg/L)	Removal percentage after one day (%)	Time to reach 80% of removal* (d)
(C3) <i>n</i> -Eicosane	188.086	44	1.9
(A1) <i>n</i> -Eicosane plus VOC	174.812	75	1.63 with highest removal: 79%
(A2) <i>n</i> -Eicosane plus VOC	154.783	70	1.5
(A3) <i>n</i> -Eicosane plus VOC	154.783	65	1.6
(A4) <i>n</i> -Eicosane plus VOC	155.995	80	1

Notes: * all the assays lasted 26 days.

The highest removal percentage for toluene was 55 % in presence of *n*-eicosane and 45 % without the hydrocarbon (Table 3). These results are related to those described by Vázquez [4]. The increasing of toluene concentration resulted in a shorter time period needed to reach a 30 % of removal, so while VOC concentration was increased a high removal of the same aromatic compound was enhanced. However, after 52 mg/L of initial toluene concentration the removal dynamics kept almost constant (Table 3).

In the sterile controls performed with toluene (29 mg/L) and *n*-eicosane (188 mg/L) the highest removal for toluene was 4 – 5 % while a 30 % was reached for the hydrocarbon after 25 days as time assay.

3.2 Graphic description

Curves describing the removal of the saturated hydrocarbon and benzene in some experiments are

Table 3. Removal data for assays performed with different toluene concentrations and an established percentage (v/v) of *n*-eicosane (varying between 154 - 188 mg / L).

Assay (key) VOC Initial concentration (mg/L) plus HC	Removal after			Highest removal and time to reach it* (%:d)	Time to reach a 30 % of removal (d)
	Day 1 (%)	Day 2 (%)	Day 15 (%)		
(C4) Toluene: 27.307	15	20-23	37.5	44.8: 20.94	7.7
(A1) Toluene: 9.979 plus HC	12.5	15	30.5	31: 15.7	14.8
(A2) Toluene: 24.36 plus HC	13	dnd**	31	39: 20.9	14.3
(A3) Toluene: 51.75 plus HC	21	20	40	45.7: 22.7	3.3
(A4) Toluene: 88.41 plus HC	7	20	53	54.6: 26.7	3.4

Notes: * all the assays lasted 26 days. ** datum not determined.

showed in Figures 1a and 1b. Dynamics of *n*-eicosane and toluene disappearance can be observed in Figure 2a and 2b. For each set of graphics, two assays with mixed compounds, one with a low aromatic concentration and the other with the highest, are showed as also are the assays performed with separated compounds. Standard deviation bars were not represented because in all the assays it was obtained a deviation lower than (\pm) 5 %.

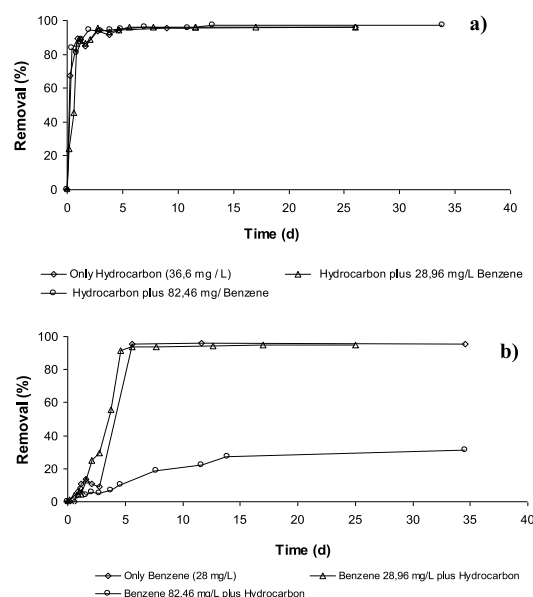


Figure 1. a) Dynamics of *n*-eicosane disappearance in batch scale reactors. Hydrocarbon concentration range varying between 33 - 47 mg/L (Table 1, assays C1, 2 and 4). b) Removal dynamics for different benzene concentrations (Table 1 assays C2, 2 and 4) in presence or absence of *n*-eicosane.

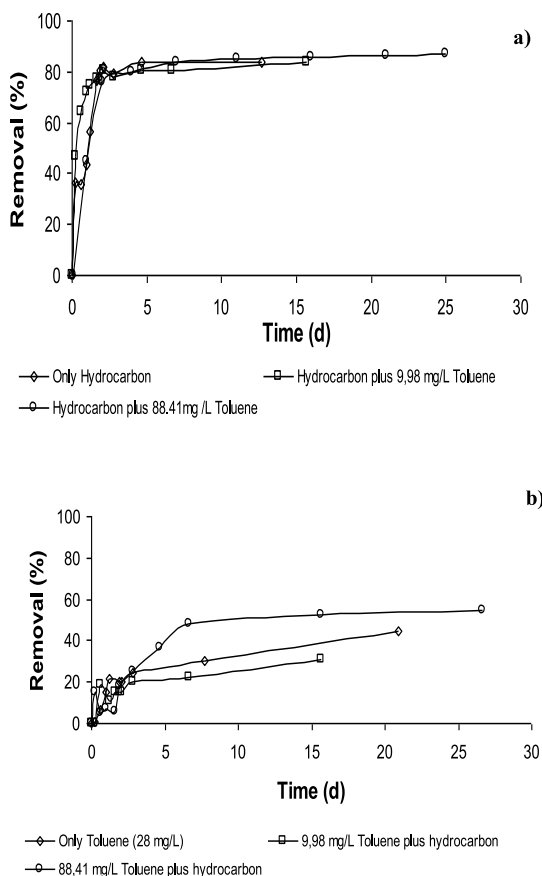


Figure 2. a) Dynamics of *n*-eicosane disappearance in batch scale reactors. Hydrocarbon concentration range was between 154 -188 mg/L (Table 2, assays C3, A1 and A4). b) Removal dynamics for different toluene concentrations (Table 3 assays C4, A1 and A4) in presence or absence of *n*-eicosane.

3.3 Kinetic fitting

Different mathematical fitting models were applied in order to describe the removal kinetics of VOCs in presence of *n*-eicosane. The models that better adjustment presented were Haldane kinetic model for *n*-eicosane, Hill kinetic model for toluene and for benzene a Monod kinetic curve showing a lower activation with those VOC concentrations upper than 38 mg/L.

The kinetic curves showed that when increasing the toluene concentration, the removal rate of the HC was higher, until reaching a constant disappearance rate with an initial concentration of 49 - 50 mg/L of toluene (See Figure 3). The maximum removal rate with benzene was reached at an initial concentration of 37 - 38 mg/L. For benzene, the kinetic model that showed the better adjustment was similar to a Monod curve but lower activity was observed when assays were performed

with higher concentrations than the initial one at which highest removal rate was reached (See Figure 4). For *n*-eicosane, the experimental data presented a high adjustment with the Haldane kinetic model (See Figure 5). The kinetic curves for the hydrocarbon established 188 mg/L as the highest removal rate initial concentration, exceeding this concentration also low removal activity was observed.

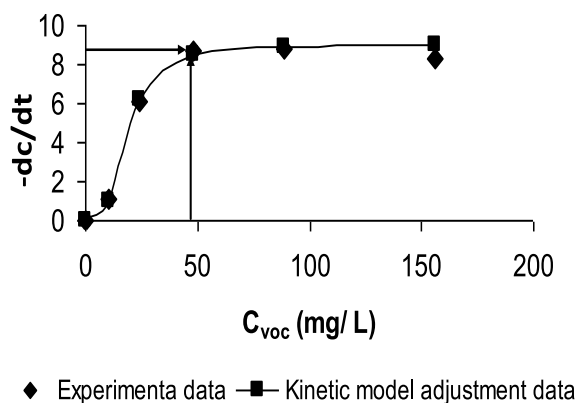


Figure 3. Toluene removal kinetic (Hill model).

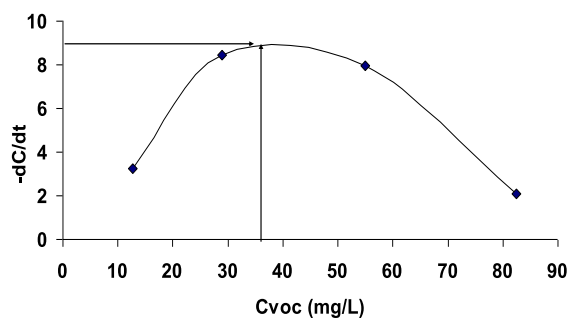


Figure 4. Benzene removal kinetic: Monod curve showing lower activation at aromatic concentrations higher than 37 - 38 mg/L.

4. Conclusions

When only the aromatic compound was added (benzene: 28 mg/L and toluene: 27.307 mg/L), benzene reached higher disappearance percentage than toluene. It was observed that *n*-eicosane was more easily removed (until 188 mg/L) than the aromatic compounds, which is in agreement with findings of Vázquez [4]. The disappearance of *n*-eicosane was improved by the presence of benzene or toluene, reaching a maximum value around 90 and 80 % removal, respectively. A higher

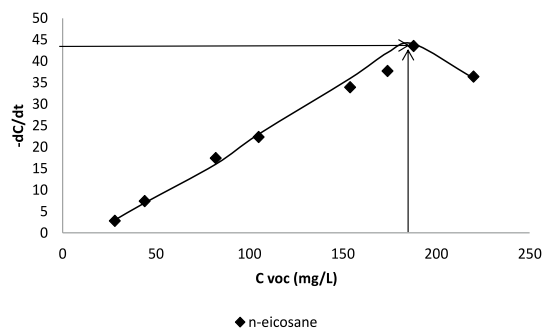


Figure 5. *n*-eicosane removal kinetic: Haldane adjustment with lower activation at concentrations upper than 188 mg/L.

disappearance rate for VOCs was observed when the concentration of both aromatic compounds was increased until a plateau is reached. This behavior is consistent with that reported by Wang *et al* [2] for a characteristic microbial co-metabolism. The kinetic fitting for toluene showed that its removal rate increased as its concentration at water-phase increase up to 50 mg/L, where it became almost constant. For benzene, the higher removal rate was achieved with an initial water-phase concentration between 37 - 38 mg/L; at higher concentrations no further activation is observed. For *n*-eicosane, its higher degradation rate was with an initial concentration of 188 mg/L. With this concentration, the highest removal rate was accomplished; at higher concentration a lower removal rate was observed. Microorganisms which were acclimated within the microcosms system enhanced their hydrocarbon removal capacity as has been previously described by Hernández *et al* [6]. Biosorbents could be inoculated with those bacterial consortia whose biodegradation capacity was enhanced with a continuous acclimation by using the microcosm technique.

5. Acknowledgements

Funding for this research was obtained through projects CGPI-IPN 2002072, CONACYT 27583B and 123047. The authors express a sincerely grateful acknowledgement to Professor Ana Vázquez Roca from University of Coruña, Spain, who established the initial techniques applied in this work and performed the first experimental results with low and intermediate *n*-eicosane initial concentration in presence of toluene.

6. References and Notes

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