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BIODEGRADATION OF HYDROCARBONS IN POLLUTED SOILS USING β-CYCLODEXTRIN AS A COADIUVANT

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The biodegradation of pollutants depends on their bioavailability for soil microorganisms. Hydrocarbons are hydrophobic compounds which are poorly soluble in water and thus poorly bioavailable.

Cyclodextrins interact with hydrocarbons to produce inclusion complexes that are more water soluble, so they can be more easily degradated. Cyclodextrins, moreover, are cheap, natural and biocompatible compounds widely used for medical and food purposes.

In this paper we studied the use of cyclodextrin to enhance the degradation rate of dodecane by natural microbial strains isolated from a petroleum-polluted soil. We used three types of soil texture: sand, loamy sand and clay, in order to understand their influence on the biodegradation rate.

Probably due to the oxygen diffusion required for microbial metabolism, the biodegradation kinetic in clay is slower (half-reaction time = 199.3 h) than in loamy sand (half-reaction time = 101.7 h) and in sand (half-reaction time = 76.9 h). In all tests cyclodextrins increased the biodegradation time gain (24.2-40.9%).

It was checked the level of sorption of hydrocarbon on soil and the eluviation through a soil profile analized. Cyclodextrin did not show any effect on eluviation of hydrocarbon, that was anyway absent.

Биодеградация загрязнителей зависит от их доступности для почвенных микроорганизмов. Углеводороды – гидрофобные соединения, которые плохо растворимы в воде и таким образом слабо пригодны для микробов.

Циклодекстрины реагируют с углеводородами, образуя инклюзионные комплексы, которые более растворимы в воде, и таким образом легко разрушаемы. Циклодекстрины – недорогие, природные и биосовместимые соединения, широко используемые для медицинских и пищевых целей.

Изучено использование циклодекстринов для усиления интенсивности деградации додекана природными микробными штаммами, выделенными из почвы, загрязненной нефтью. Для выяснения степени разрушения додекана использовались 3 типа почв разной текстуры: песок, глинистый песок и глина. Вероятно, благодаря требуемой диффузии кислорода для микробного метаболизма, кинетика биодеградации в глине протекает медленно (время полуреакции = 199,3час), чем в глинистом песке (время полуреакции = 101,7час) и в песке (время полуреакции = 76,9). Во всех опытах циклодекстрины повышали период биодеградации на 24,2 – 40,9%. Проверялись уровень сорбции углеводорода в почве и элувиация в почвенном профиле. Циклодекстрин не оказал какого – либо эффекта на элувиацию углеводорода во всех испытанных случаях.

Աղտոտիչների կենսաքայքայումը կախված է նրանց մատչելիությունից հողային մանրէների համար: Ածխաջրածինները հիդրոֆոբ միացություններ են, որոնք վատ են լուծվում ջրում և այդպիսով քիչ պիտանի են մանրէների համար:

Ցիկլոդեքստրինները ռեակցիայի մեջ են մտնում ածխաջրածինների հետ, առաջացնելով ինկլյուզիոն համալիրներ, որոնք առավել լուծելի են ջրում, և այդպիսով հեշտ քայքայվող: Բացի դրանից, ցիկլոդեքստրինները էժան, բնական և կենսահամատեղելի միացություններ են, լայնորեն օգտագործվում են բժշկական և սննդային նպատակներով:

Ուսումնասիրվել է դոդեկանի քայքայման արագության մեծացման համար ցիկլոդեքստրինների օգտագործումը նավթով աղտոտված հողից անջատված բնական մանրէային շտամներով: Դոդեկանի քայքայման մակարդակի որոշման համար օգտագործվել են 3 հողային տիպեր ավազ, կավային ավազ և կավ: Հավանաբար, մանրէային մետաբոլիզմի համար պահանջվող թթվածնի դիֆուզիայի շնորհիվ կենսաքայքայման կինետիկան ընթանում է ավելի դանդաղ կավ (կես ռեակցիայի ժամանակը =199.3 ժամ), քան կավային ավազի (կես ռեակցիայի ժամանակը =101.7 ժամ) և ավազի (կես ռեակցիայի ժամանակը =76.9 ժամ) մեջ: Բոլոր դեպքերում ցիկլոդեքստրինները բարձրացնում են կենսաքայքայման ժամանակը 24.2-40.9% ով: Ստուգվել է ածխաջրածինների սորբցիայի մակարդակը հողում և հետազոտվել է էլուվիացիան հողային կտրվածքով: Բոլոր փորձարկված դեպքերում ցիկլոդեքստրինը ածխաջրածինների էլուվիացիայի վրա ազդեցություն չի թողել:

Introduction

Xenobiotic chemicals enter a specific biogeochemical cycle when discharged in an environmental compartment. Their transport and distribution not only depend upon diffusion and transport patterns within each compartment, but also on partition processes between the various compartments: air, water or land [1].

The remediation of polluted soil can be achieved by chemical, physical or biological treatment. In recent few years, bioremediation processes have been preferred because of their low cost and capability of fully degrading pollutants. In particular, *in situ* bioremediation aims at cleaning up contaminated soils, reducing health hazards and/or the ecotoxicological risks of spreading contamination, without moving the soil itself [2,3,4,5,6].

One main factor that influences the extent of pollutant biodegradation is their bioavailability; this is a priority research objective in the bioremediation field. [6,7]. Hydrocarbon bioavailability is very poor; in fact, they are hydrophobic, so they pass very slowly from a non-aqueous to the aqueous phase liquid in which they are metabolised by microorganisms [7, 8]. Moreover, in the soil they are adsorbed to clay or humus fractions [7, 9]. The addiction of surfactants can increase their bioavailability due to increased water solubility [10,11,12,13]; on the other hand, eluviation through the soil profile can also be enhanced and, as a consequence, the risk of the pollution of groundwater increased. Moreover, surfactants are frequently toxic and poorly biocompatible; sometimes they are considered as pollutant compounds.

α, β and γ cyclodextrins are cyclic oligosaccharides formed by 6, 7 or 8 α-1,4-linked glucose units respectively [14]. Since they have toroidal hydrophobic cavities with a hydrophilic shell, they are water-soluble and form inclusion complexes with hydrophobic molecules of a size compatible with their hydrophobic core. In this way, the aqueous solubility of several compounds is increased by cyclodextrins through their dynamic equilibrium exchange with guest molecules, which then dissociate from the cyclodextrin complex and become available for catabolism. Cyclodextrins are natural, non toxic compounds and harmless to microorganisms and free enzymes [15]. Due to their properties, cyclodextrins can be proposed as a surfactant to be used in soil and water bioremediation from hydrophobic xenobiotics. Schwarz and Bar (1995) found that cyclodextrins enhanced degradation of toluene and p-toluic acid [16]. Wang et al. (1998) demonstrated a better biodegradation of phenanthrene in the presence of cyclodextrins [17]. In our laboratory, hydrocarbon degradation has been investigated in the aqueous phase using microbial colonies isolated from a petroleum-polluted soil. Aliphatic (dodecane, tetracosane) and poliaromatic (naphthalene, anthracene) hydrocarbons were degraded and their degradation

kinetics were accelerated by β-cyclodextrin. Hence, β-cyclodextrin enhanced the water solubility of the hydrocarbons and consequently increased their bioavailability for microorganisms [18].

In this work, our aim was to investigate the application of β-cyclodextrin as a surfactant for *in situ* hydrocarbon polluted soil bioremediation. First, we checked the risk of increased groundwater pollution due to the increased water solubility of the hydrocarbons. We checked, in the presence and in absence of β-cyclodextrin, the sorption and the eluviation of dodecane, an aliphatic, low-molecular weight hydrocarbon, in three soils differing in texture and, consequently, in permeability and drainage properties: a sand, a loamy sand and a clay. Then we checked the degradation kinetics of dodecane in the same three soils, inoculated with a hydrocarbon degrading selected microbial population, simulating a farming system.

Materials and methods

Sorption and eluviation assays. Three mineral soils with different textures were examined: a sand (sand 97.6%, silt 1.6%, clay 0.8%), a loamy sand (sand 80.4%, silt 17.8%, clay 1.8%) and a clay (sand 3.9%, silt 60.3%, clay 35.8%). Organic matter was absent. Soils were dried in an oven at 120°C for 7 h. The amount of dodecane retained by matrix potential, due to sorption or capillary forces, was determined. 80 ml of water with 67 µl of dodecane were added to 100 g of soil and mixed for 4 hours, then the gravitational water was drained and the amount of dodecane present in it analysed. The dodecane retained by the soil was calculated as the difference between the total amount added and the amount detected in gravitational water. The same assay was carried out in each of the three soils, without cyclodextrin or with 1% (w/w) β-cyclodextrin added to the mixture.

Eluviation tests were performed in two glass columns, 3 cm diameter and 30 cm high, filled with 280 g sand. 188 µl of dodecane were added on the surface of the two columns, and 1% (w/w) of water-melted β–cyclodextrin was added to one column. Then 850 ml of water were applied at the surface of each column and left to elute by gravity. The drained water was collected in 20 ml fractions and each fraction was analysed for its dodecane content.

Biodegradation kinetics. 700 g of each soil, previously dried in an oven at 120°C for 7 h, were posed to a depth of 10 cm depth in an impermeable, open box. 470 µl dodecane and 1% (w/w) water-melted β-cyclodextrin were added, then 100 ml of microbial culture were spread as inoculum and 200 ml of mineral growth medium (LMM) were added. Daily, water was balanced and soil homogeneity and aeration were maintained by vigorous and accurate mixing. The temperature was kept at 20°C. The tests were performed by monitoring the dodecane decrease, in the presence and absence of β-cyclodextrin and with the three different soil textures (sand, loamy sand and clay).

Microbial inoculum. Microbial colonies were isolated from a petroleum-polluted soil by dispersing a sterile water suspension of soil in MMA (Mineral Medium Agar: 0.8 g/l K_2HPO_4, 0.2 g/l KH_2PO_4, 0.05 g/l $CaSO_4 2H_2O$, 0.5 g/l $MgSO_4 7H_2O$, 0.09 g/l $FeSO_4 7H_2O$, 1 g/l $(NH_4)_2SO_4$, 15 g/l agar) with 4% v/v dodecane as the sole carbon source in Petri dishes, and incubating them at 28°C for 5 days. A random pool of 54 colonies was transferred to 200 ml of LMM (Liquid Mineral Medium: as MMA, without agar) with 4% v/v dodecane in a conical flask and incubated on an oscillatory shaker at 140 rpm for 5 days at 28°C. This culture was used as inoculum for the degradation kinetic assays.

Dodecane analysis. For the biodegradation kinetics assays, 15 g of soil sample were mixed with 6 ml of water and extracted by shaking with three successive aliquots of a total volume of 30 ml of toluene. The mixture was centrifuged at 4000 rpm for 5 min and the organic phase was transferred to a fresh tube. 100 µl of a standard solution of 1% w/v naphthalene in toluene were added to 1 ml of extract. The samples were analysed by a gas chromatograph GC HP 5890 series II equipped with a flame ionization detector on a HP1 Cross-Linked Methyl Silicone capillary column, 15 m long and 0.32 mm i.d., film thickness 1.0 mµ. The operating conditions were: temperature from 100 to 250°C at

20°C/min; pressure 13 psi; injection volume 4 µl. For the sorption and eluviation assays, dodecane was extracted and analysed as previously described [18].

Results and Discussion

Sorption and eluviation assays. The amount of dodecane retained by the matrix potential of soil was 97.81% in sand, 99.42% in loamy sand and 99.80 % in clay (figure 1). Therefore, the amount of dodecane that remains in drained gravitational water is very low after just 4 hours of contact with the soil. The dodecane retained by matric potential rises from sandy to clay texture, but the difference between sand, loamy sand and clay is very low.

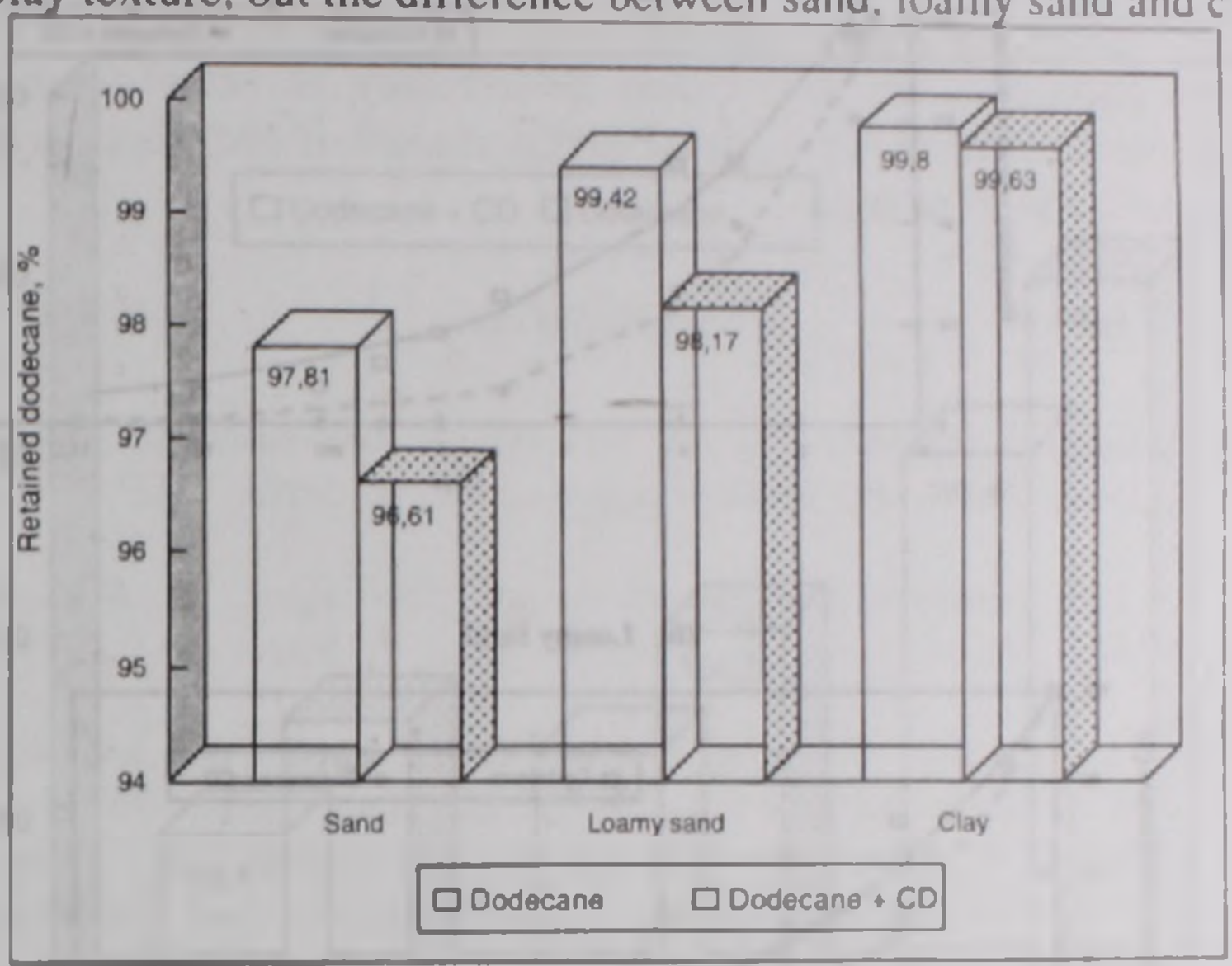

Figure 1. Retained amount of dodecane by sand, loamy sand and clay, due to sorption or capillary forces.

When β-cyclodextrin is added, the fraction of dodecane that remains in gravitational water is slightly higher: the amount retained by the soil becomes 96.61% in sand, 98.71% in loamy sand and 99.63% in clay (figure 1). It can be observed that the decrease is very small in clay (0.17%) and slightly higher in sand (1.2%). In any case, β-cyclodextrin induces a very slight increase in the solubility of the hydrocarbon in gravitational water; this solubility proved to be very low in all the chosen soils, although they were devoid of organic matter.

The eluviation assays, performed by eluting the hydrocarbon with water through the different soils in glass columns, confirmed that β-cyclodextrin does not increase the eluviation of dodecane. In these assays a water volume corresponding to the average annual rainfall in Italy was used. No traces of dodecane were found in drained water.

Hence, the risk of groundwater pollution due to β-cyclodextrin because of the increased solubility of the hydrocarbon can be excluded.

*Biodegradation kinetics.*Dodecane was fully degraded in sand in 329 hours, while this time was reduced to just 192 hours when β-cyclodextrin was added (figure 2a). The half-reaction time (T1/2), that is the time at which the xenobiotic concentration becomes half its initial value, was 76.9 h without β-cyclodextrin and 45.4 h with β-cyclodextrin (figure 3). Similar results were obtained in loamy sand: dodecane full degradation was reached in 401 h without β-cyclodextrin, while 278 h were sufficient when β-cyclodextrin was added

(figure 2b); the half-reaction time varied from 101.7 h to 67.2 h, respectively (figure 3). It is evident that in sand and loamy sand the addition of β-cyclodextrin causes a significant acceleration of biodegradation kinetics, due to the increased bioavailability of hydrocarbon that passes into the aqueous phase in the form of inclusion complexes. The degradation time gain due to β-cyclodextrin was 40.9% in sand and 33.9% in loamy sand (table 1).

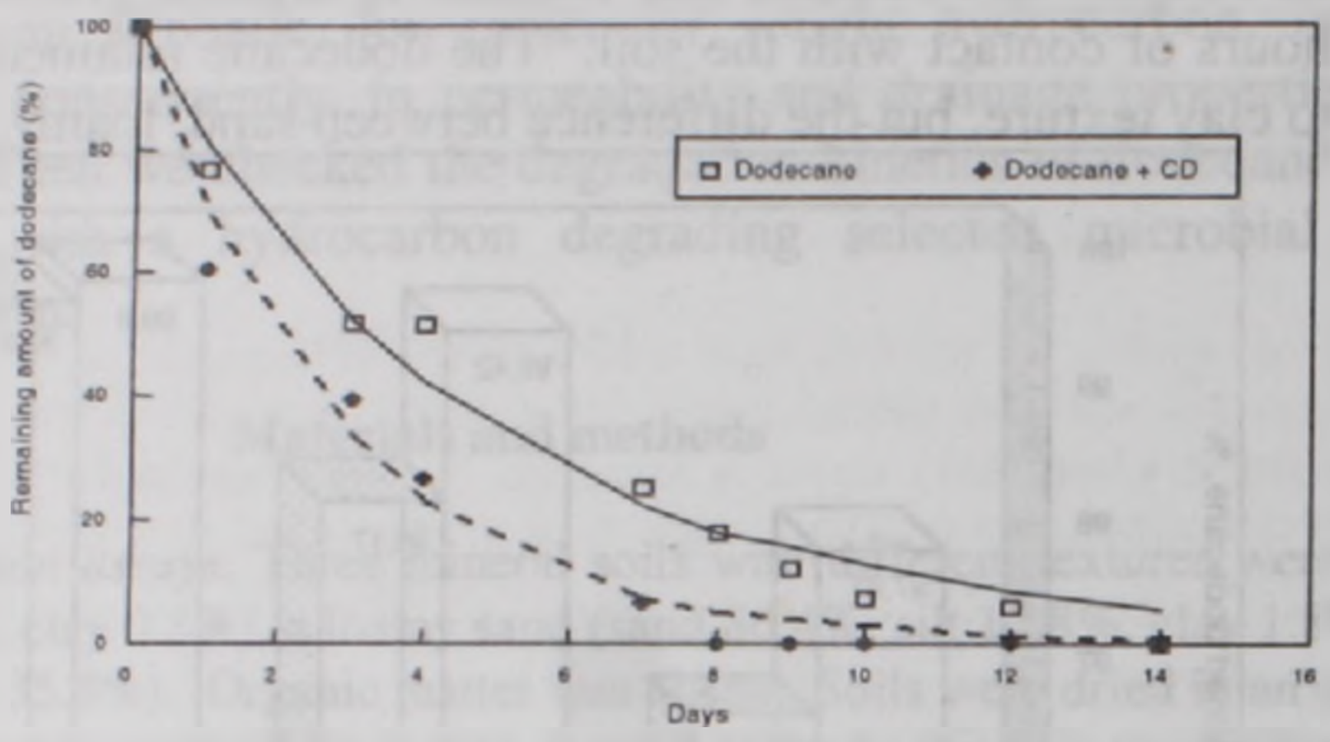

Figure 2. Percentage of remaining dodecane during the microbial degradation in sand (2a), loamy sand (2b) and clay (2c). Data are reported as media of two replicate determinations.

Table 1. Degradation time on sand, loamy sand and clay (hours)

	Sand	Loamy sand	Clay
Full degradation time without β-cyclodextrin	76.9	101.7	199.3
Degradation time with β-cyclodextrin	45.4	67.2	151.4
Degradation time gain (due to cyclodextrin)	40.9%	33.9%	24.2%
Required time	1	1.48	3.33

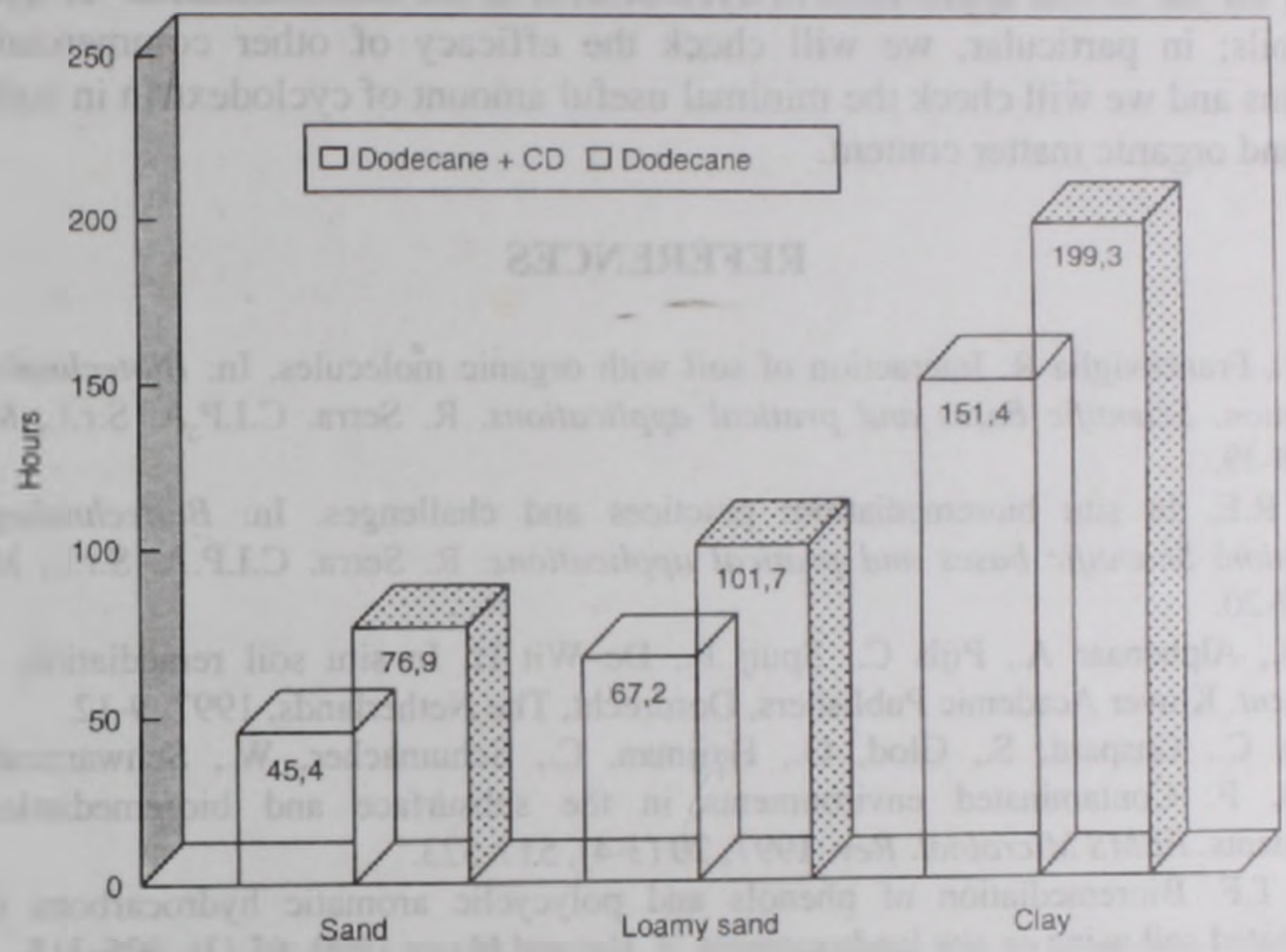

Figure 3. Dodecane half-reaction time in sand, loamy sand and clay calculated from data reported in Fig. 2

The positive effect of β-cyclodextrin was slightly less evident in clay. In fact, full dodecane biodegradation required 521 h without β-cyclodextrin and 497 h with β-cyclodextrin (figure 2c) with a 24.2% degradation time gain (table 1). Half-reaction time was reduced from 199.3 h to 151.4 h (figure 3). As expected, biodegradation was slower in clay, where the fine texture makes the diffusion of the oxygen required for microbial metabolism more difficult. Moreover, fine-textured soils have more total pore space than sandy soils, but these spaces are mostly micropores, that restrict the water flow. What may happen is that an intrinsically biodegradable compound is slowly diffused into soil micro- and nano-pores, which are so small that the compound within them is beyond microbial attack. That could be an explanation of the minor effect of β–cyclodextrin in clay, although its solubilization effect does not change. In any case, the addition of β–cyclodextrin resulted in an improvement in the biodegradation of dodecane in soil.

Conclusions

β–cyclodextrin used as a surfactant in bioremediation of hydrocarbon polluted soils enhances the bioavailability of hydrocarbons to the microorganisms; consequently, degradation kinetics are accelerated. The addition of β–cyclodextrin does not increase the risk of groundwater pollution even in mineral sandy soils. Therefore, the effectiveness of β–cyclodextrin in the improvement of biodegradation of hydrocarbons in soil has been demonstrated; its positive effect is influenced by the soil texture, but in any case the degradation time gain is always significant. Our next work will be the optimisation of several parameters for the *in situ* application of cyclodextrin in the bioremediation of hydrocarbon-polluted soils; in particular, we will check the efficacy of other commercial forms of cyclodextrins and we will check the minimal useful amount of cyclodextrin in soils differing in texture and organic matter content.

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