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ALTERNATIVE FOR PHENOL BIODEGRADATION IN OIL CONTAMINATED WASTEWATERS USING AN ADAPTED BACTERIAL BIOFILM LAYER

PRILAGOĐENI BAKTERIJSKI BIOFILM KAO ALTERNATIVA BIORAZGRADNJI FENOLA U OTPADNIM VODAMA U NAFTNOM RUDARSTVU

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Abstract

The project studied the biodegradation potential of phenols in an industrial wastewater from an oil field in the province of Santander, Colombia. An elevated potential was established, according to three important factors: the great abundance of microorganisms found in the wastewater and sludge samples collected, the bacterial adaptation to high phenol concentrations (10 mg/l) and the elevated elimination efficiencies (up to 86%) obtained in the laboratory tests. The laboratory scale treatment system, which consisted of fixed-bed bioreactors with adapted bacterial biofilm, was optimized using a 2^2 factorial experimental design. The selected variables, studied in their maximum and minimum level were: HRT (hydraulic retention time) and the presence or absence of GAC (granular activated carbon) layer. The response variable was phenol concentration. The optimum treatment conditions for low and high phenol concentrations (2.14 y 9.30 mg/l), were obtained with the presence of GAC and 18 hours of HRT. The best result for the intermediate phenol concentration (6.13 mg/l) was obtained with a 24 hour HRT and the presence of GAC. Nevertheless, the presence of the GAC layer was not significantly important in terms of phenol removal. Moreover, the increase of HRT from 18 to 24 hours, showed no significant improvement in phenol removal.

Sažetak

U okviru projekta proučavan je potencijal biorazgradivosti fenola u otpadnoj vodi iz naftne industrije u provinciji Santander u Kolumbiji. Utvrđeno je povećanje potencijala s obzirom na tri čimbenika: veliki broj mikroorganizama pronađenih u prikupljenim uzorcima otpadne vode i taloga, prilagodbu bakterija na velike koncentracije fenola (10 mg/l) i povećanje djelotvornosti uklanjanja (do 86%) dobiveno laboratorijskim ispitivanjima Laboratorijski sustav obrade koji se sastojao od bioreaktora sa fiksnim slojem sa prilagođenim tankim bakterijskim slojem, optimiziran je korištenjem faktorskog eksperimentalnog pristupa 2^2. Odabrane varijable , kod kojih se pratila maksimalna i minimalna razina, bile su: hidrauličko vrijeme zadržavanja (engl. hydraulic retention time – HRT) i prisustvo ili izostanak sloja zrnastog aktivnog ugljena. (engl. granular activated carbon – GAC). Varijabla koja se pratila bila je koncentracija fenola. Optimalni uvjeti obrade za male (2,14 mg/l) i velike koncentracije fenola (9,30 mg/l) dobivene su uz prisustvo sloja zrnastog aktivnog ugljena i hidrauličkog vremena zadržavanja u trajanju od 18 sati.. Najbolji rezultat za umjerenu koncentraciju fenola (6,13 mg/l) dobiven je uz hidrauličko vrijeme zadržavanja od 24 sata i uz prisustvo sloja aktivnog ugljena. Unatoč tome prisustvo sloja aktivnog ugljena nije bilo od značaja u smislu uklanjanja fenola. Štoviše, Povećanje hidrauličkog vremena zadržavanja sa 18 na 24 sata nije značajno poboljšalo uklanjanje fenola.

INTRODUCTION

Petroleum Industries causes significant water contamination in the stages of exploration, well cementation and oil extraction. In the extraction stage, water is continuously contaminated due to the direct discharge of these industrial wastewaters, which is a common activity in oil fields in Colombia. These wastewaters contain several pollutants such as phenols, which are commonly disposed of without a proper treatment. Phenol is highly toxic, corrosive, and mutagenic. It is also known as a carcinogenic and teratogenic agent, which affects both the environment and human beings. Phenol removal from the industrial wastewaters is necessary, prior to the wastewater discharge. Almost all petroleum industries have traditionally used physicochemical processes to treat

their contaminated waters. These wastewaters contain elevated organic loads, their treatment is quite expensive and they contain significant concentrations of specific pollutants. Given these characteristics, the biological treatment of these waters is an attractive alternative.

In the last decades several biological processes for the decontamination of petroleum wastewaters, such as the aerobic and anaerobic degradation, have been studied in Colombia (Delgado et al, 1993). Nevertheless, as mentioned before, specific chemical processes have been tested in petroleum wastewaters to accomplish phenol removal. There is a great abundance of phenol removal experiences in aerobic media. Even though aerobic media processes generally contribute to high elimination efficiencies, anaerobic processes do not generate great amounts of waste sludge, and their energy consumption is minimal, which contributes to low maintenance costs. Several studies have indicated the possibility of designing such kinds of biological processes (Field and Sierra, 1989). Phenol biodegradation has been accomplished with bacteria such as *Pseudomonas cepacia* (Folsom et al, 1990), *Pseudomonas putida-P8* and *Cryptococcus elinovii-H1* (Yucel, 1989), with phenol concentrations up to 3.2 g/l.

Anaerobic phenol biodegradation has also been studied via an obliged bacterial consortium where an intimate interdependence was established among the bacteria (Knoll and Winter, 1989). Other research has been focused in mixed and pure culture phenomena. This criterion is considered just as important as the type of media or process (aerobic or anaerobic). Certain mechanisms for phenol transformation have been documented (Young and Häggblom,). Generally, these mechanisms imply diverse interaction between different bacterial species such as photosynthetic, denitrifying, sulphate-reducing and methanogenic bacteria.

In Colombia, ECOPETROL (Colombian Petroleum Company) and ICP (Colombian Petroleum Research Institute) have explored various treatment alternatives, which range from chemical oxidation to combined processes with biotechnology. Most of the biotechnology used in oil fields has been tested in aerobic media (Restrepo et al, 2007). An example of these combined processes is the use of air and photolysis with the addition of adapted bacterial broth to remove phenol. These kinds of technologies have been tested in ECOPETROL's fields since 2005.

Other authors have studied the possibility to combine the biotechnologies with the adsorption in activated carbon. The use of activated carbon is substantially interesting because the effective removal of phenol can be guaranteed when biological processes are not totally effective. Several studies have been undertaken. One of these is the biodegradation of phenol and aromatic amines with adapted bacteria in a fluidized bed of sand and activated carbon (Kock et al, 1991). Other researchers have concluded that anaerobic degradation of phenol with methane and carbon dioxide production occurs at the same time as phenol adsorption in GAC. One particular study demonstrated the bacterial bioconversion of the previously adsorbed phenol into carbon dioxide and methane in absence of another carbon source. This indicated the possible bio-regeneration of the activated carbon in anaerobic conditions (Craveiro, 1991).

The petroleum wastewater studied, generated in an oil field in Santander – Colombia, consists of the sum of the produced formation waters obtained during oil extraction and the water applied during the same processes. This wastewater is physically separated from the petroleum and is conducted to a series of oil traps, and finally it is temporarily stored in two facultative lagoons. From these lagoons, approximately 3 to 4 liters per second (48 to 63 gal/ min) are discharged to the creek. At that point, the wastewater phenol concentration ranges from 4 to 8 mg/l, which widely surpasses the maximum phenol concentration for wastewater discharge accepted under Colombian regulations (0.2 mg/l).

Considering the numerous disadvantages of aerobic processes, and taking into account that there have not been enough reported studies in biodegradation of phenol in fixed-bed anaerobic reactors, the project evaluated phenol biodegradation in up-flow fixed-bed anaerobic reactors with a bacterial biofilm layer previously adapted to phenol. The laboratory-scale process was optimized applying a 2^2 experimental design (two levels and two variables). HRT and absence or presence of a GAC layer, were the variables studied. The response variable selected was phenol concentration in the effluent of the reactors. The research was conceived with the purpose of demonstrating a significant decrease of phenol concentration in the effluent of the bioreactors, which could be attributed to bacterial biodegradation. The presence or absence of the GAC layer was introduced in the experiments with the intention of demonstrating its positive contribution to phenol removal when the adapted microorganisms are exposed to relatively high (toxic) phenol concentrations.

MATERIALS AND METHODS

Experimental procedure

The first stage in the project evaluated the phenol biodegradation potential through the study of the native microbial flora present in the wastewater. A physicochemical and microbiological characterization was performed in order to identify potential microorganisms, adequate for phenol biodegradation. Some of these microorganisms were selected for the adaptation and bio-augmentation processes.

Wastewater and sludge samples were collected in different wastewater deposits prior to its discharge.

The sampling procedures were conducted according to the EPA Standard Methods, method 1060. A unique compound sample was collected for the wastewater parameters analysis: BOD (Biological Oxygen Demand), COD (Chemical Oxygen Demand), dissolved oxygen, oil and grease, total suspended solids, total phenols, and TPH (Total Petroleum Hydrocarbons). Eight microbiological samples were collected, half of them corresponding to wastewater and the other half to sludge. These samples were treated according to the standard microbiological procedures and then sowed in the following modified culture media (agar): nutrient, brain-heart, yeast extract and rose bengal. A total of 128 plates were sowed, 64 for wastewater samples and 64 for sludge samples. The culture media was modified with the petroleum wastewater, each medium prepared contained 50% wastewater and 50% distilled water in volume. Once microorganisms had grown in the plates, several laminas were prepared for microscopic observation. Among the microorganisms found, bacteria were prevalent over fungi. Different bacteria were selected for the adaptation process taking into account presence of the following characteristics: special bacterial morphology, mucilaginous bacteria, endospores, and isolated colonies. Furthermore, these bacteria were grouped as gram positive, gram negative or belonging to the gender *Pseudomonas*.

All previously selected microorganisms were adapted sequentially to increasing phenol concentrations (0.5, 1, 2, 4, 6, 8 y 10 mg/l). The adaptation process was conducted by consecutive inoculations in modified aqueous brain heart broth (50% distilled water + 50% wastewater effluent) with the corresponding phenol concentration. The process was held under anaerobic conditions, after inoculation the containers were completely sealed. 25 liters of modified liquid growth media were prepared for the bioaugmentation process. The liquid media was prepared with a 0.3% and 0.1% content of molasses (for nutrition) and dibasic potassium phosphate (phosphorus supplement) respectively. The phenol concentration was adjusted to 10 mg/l and the container was sealed to guarantee anaerobic conditions.

Optimization

A 2^2 factorial experimental design was used to optimize the biodegradation process. Two variables were studied: HRT in a maximum (+, 24 hours) and minimum (-, 18 hours) level and the presence (+) or absence (-) of a GAC layer. Phenol concentration was the response variable. Table 1 shows the complete set of experiments conducted. These experiments were conducted by triplicate on three different phenol concentrations, low, medium and high, corresponding to 2, 6 and 10 mg/l. The experiments were performed in laboratory scale bioreactors. The table shows the characteristics of each bioreactor according to the HRT and GAC absence or presence.

Table 1 Conducted experiments
Tablica 1. *Provedena eksperimenta*

Reactor	2 [mg/l]		6 [mg/l]		10 [mg/l]	
	HRT	GAC	RT	GAC	RT	GAC
1	-	-	-	-	-	-
2	+	-	+	-	+	-
3	-	+	-	+	-	+
4	+	+	+	+	+	+

In addition to the experiments described in table 1, several controls were established. Table 2 shows the 28 control experiments proposed.

Table 2 Control experiments
Tablica 2. *Kontrolna eksperimenta*

Absence of Hydraulic Retention Time					
2 [mg/l]		6 [mg/l]		10 [mg/l]	
HRT	GAC	HRT	GAC	HRT	GAC
0	-	0	-	0	-
0	+	0	+	0	+
Light effect without a microbial layer			Light effect with a microbial layer		
HRT	GAC	Light	HRT	GAC	Light
-	+	+	-	+	+
-	-	-	-	-	-
-	-	+	-	-	+
-	+	-	-	+	-
+	+	+	+	+	+
+	-	-	+	-	-
+	-	+	+	-	+
+	+	-	+	+	-

Bioreactor Design and preparation

The bioreactor was designed taking into account the characteristics of a regular up-flow fixed-bed anaerobic reactor, with a rectangular base and a perforated plate for water distribution. Glass was the material selected for construction. The plate was elaborated in acrylic. The designed model is detailed in figure 1. The reactor had a maximum capacity of 5 liters. Along with the support media (50% porosity), the volume decreased to 2.5 liters, approximately. The reactors were batch operated. Stratified crushed stone was used as the support media. Its characteristics are included in table 3. The GAC layer was distributed on top the crushed stone.

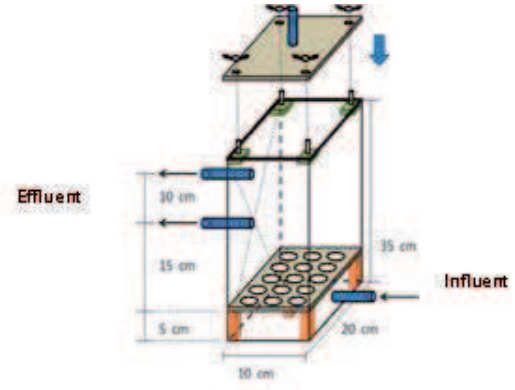

Figure 1 Standard bioreactor model.
Slika 1. *Standardni model bioreaktora*

Table 3 Support media characteristics. Each bioreactor was constructed using the same specifications.
Tablica 3. *Karakteristike potpornog medija. Svaki bioreaktor konstruiran je uz primjenu istih specifikacija.*

Characteristics		Value
Total Bed height (cm)		20
Approximated Bed porosity (%)		45
Head loss (cm)		< 1
Standard comercial GAC (kg)		0.25
Approximated height of the bed layers (cm)		
1rst Layer	Crushed stone diameter (5 – 7 cm)	10
2nd Layer	Crushed stone diameter (3 – 5 cm)	5
3rd Layer	Crushed stone diameter (1 – 3 cm)	5
4th Layer	GAC	2

The tests were conducted using chlorine free water and the corresponding amount of phenol, obtaining the concentrations described previously (2, 6 and 10 mg/l). Mariotte Bottles were used to control both the water level in the reactor and the up-flow current, as shown in figure 2.

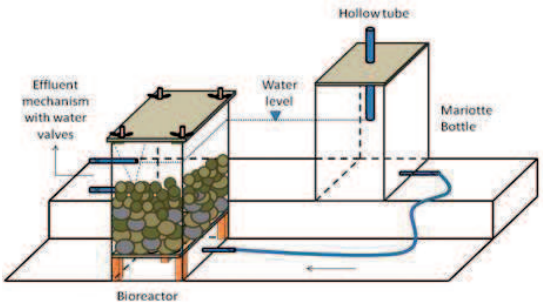

Figure 2 Water distribution, a mariotte bottle was connected to the bioreactor in order to maintain both the water level and the up-flow current.
Slika 2. *Distribucija vod.e Mariottova boca je spojena na bioreactor s ciljem održavanja razine vode uz željeni protok*

Prior to the laboratory experiments, the packed reactors were exposed to a preliminary treatment. This preliminary treatment included the addition of both the dissolution of Arabic gum (adherent agent) and the augmented microbial broth. The arabic gum dissolution remained 2 days inside the bioreactor. The augmented microbial broth had a one week retention time. Both the Arabic gum and the microbial broth were taken out of the reactors when the retention time was completed. This treatment was conceived in order to enhance the adhesion of the microbial broth to the packed layer, contributing to the formation of the corresponding microbial layer or biofilm. In order to confirm the biofilm formation, turbidity was monitored during a complete week. In addition to turbidity, increases in mass were also determined as an indication of biofilm formation.

Analytical methods

Phenol was determined using standard photometric techniques compatible with Merck's Spectroquant Nova 60. The procedure conducted, which involved the use of the appropriate phenol photometric kit, is analogue to EPA 420.1 and US Standard Methods 5530. To ensure reliability, several phenol calibration curves (concentration vs. absorbance) were tested for both low and high concentration levels at different wave lengths (450, 500 and 525 nm). The wavelengths selected correspond to the recommended range for red colored samples (color obtained in the samples when using the selected phenol photometric kit). The low range calibration curve was elaborated with the following concentrations: blank, 0.05 mg/l, 0.1 mg/l, 0.2 mg/l, 0,5 mg/l and 1 mg/l. The high range calibration curve was obtained with the following concentrations: blank, 1 mg/l, 2 mg/l, 4 mg/l, 6 mg/l, 8 mg/l, 10 mg/l and 12 mg/l. Each calibration concentration was obtained by successive dilutions from a 1000 mg/l initial solution.

Experimental design analysis

The data obtained from the experiments were processed as shown in table 4, y_1 to y_4 correspond to the mean of the experiments conducted by triplicate. As shown in table 4, several data and responses were obtained:

The mean: indicates the central value, around which the others are distributed.

Individual response or principal effects: (Response to C^+ or HRT) indicates the variation in the response when a variable changes. It was calculated as expressed in table 4.

Table 4 Experimental design responses
Tablica 4. *Reakcije na izvedbu eksperimenta*

HRT (h)	GAC		Mean	Response to C+
	C -	C +		
18	Y_1	Y_3	$(Y_1 + Y_3)/2$	$Y_3 - Y_1$
24	Y_2	Y_4	$(Y_2 + Y_4)/2$	$Y_4 - Y_2$
Mean	X_1	X_2	$(X_1 + X_2)/2$	$[(Y_3 - Y_1) + (Y_4 - Y_2)]/2$
Response to HRT 24 h - 18 h	$Y_2 - Y_1$	$Y_4 - Y_3$	$[(Y_2 - Y_1) + (Y_4 - Y_3)]/2$	

RESULTS AND DISCUSSION

The results of the physicochemical characterization are included in table 5. Phenol concentration detected in the water sample taken (May, 2007), was 8.02 mg/l. This reading was extremely high considering that 0.2 mg/l is the maximum concentration permitted in wastewaters effluents. In other samples taken in the same location, phenol concentration ranged from 4.6 mg/l (March, 2007) to 0 mg/l (October, 2007). This variability could be attributed to the climatic behavior in the area, as well as the oil production demand in the oil field. This variation also exposed the need to study bacterial behavior in different phenol concentrations.

Table 5 Physicochemical analyses results (May, 2007). The method column corresponds to the US Standard method applied.
Tablica 5. *Rezultati fizikalno-kemijske analize (svibanj, 2007). Metoda odgovara US standardnoj primijenjenoj metodi.*

ANALYSIS	RESULT	METHOD
COD [mg O_2/L]	1199.4	5220-B
BOD [mg O_2/L]	384.0	5210-B
Total suspended solids [mg SS/L]	40.0	2540-D
Oil and grease [mg 0 & G/L]	32.0	5520-D
Dissolved oxygen [mg O_2/L[	1.202	4500-O-C
Phenol [mg/L of phenol]	**8.02**	5530-C
Barium [mg/L of Ba]	N.D	3500-BA
TPH [mg/L]	7.4	SM 5520 F

Microorganism growth was found in all the plates. Bacteria were found in all the 64 plates conceived for its growth. Some bacteria even grew in fungi (yeast-extract) plates. Fungal growth was also observed. Different bacterial morphology was evident, with predominance of gram negative bacillus and cocci bacillus. There was also a significant quantity of gram positive bacteria, especially cocci and bacillus. Five different species from the gender *Pseudomonas* were identified. A noteworthy number of mucilaginous bacteria were detected, as well as isolated bacteria, 14 colonies in total. Mucilaginous bacteria represent an important finding in fixed-bed treatments because they can contribute to the biofilm formation. They were also an indication of abundant bacterial population. Fungi were classified in the following gender: *Cladosporium, Penicillium, Fusarium, Aspergillus* and *Phoma.*

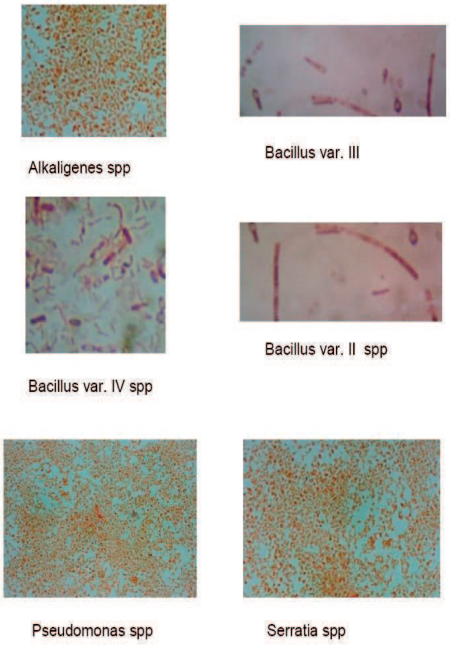

Figure 3 Bacteria identified from biofilm samples
Slika 3. *Bakterije identificirane sa uzoraka biofilmova*

The adaptation process was successful for all selected groups. Changes in color and turbidity in the aqueous culture media were an indication of continuous growth. After conducting the optimization tests, several samples from the bioreactors were collected to perform an initial assessment of potential degradation bacteria. These bacteria corresponded to the different microorganisms in the mixed adapted pool consortia that were able to degrade phenol in the packed stratum. The bacteria identified were: Bacillus var I, Bacillus var.II, Bacillus var.III, Bacillus var.IV, Bacillus var.V, Proteus spp, Enterobacter spp., Lactobacillus spp., Diplococcus spp., Fusobacterium spp., Streptococcus spp., Enterobacter spp., Alkaligenes spp., Flavobacterium spp., Klebsiella spp., Serratia spp., Pseudomonas spp., and Corynebacterium spp. (See Figure 3).

During the optimization process, phenol concentration was determined indirectly using the calibration curves (concentration vs. absorbance). Among the three studied wavelengths, the 525 nm wavelength was selected for the analyses because it showed an adequate linearity (correlation coefficient close to 1), both for low and high range concentrations. Figures 4 and 5 show the calibration curves obtained.

Figure 6 shows the evolution of turbidity in the preliminary treatment with microbial broth. Each one of the series relates to a particular bioreactor. Turbidity was variable in time. On day 1, turbidity was low because of the addition of aqueous growth media to the microbial broth (dilution). Bioreactors 10 and 12 showed a different behavior, which could be attributed to normal starting fluctuations. On the following days turbidity increased, decreased on day 3 and progressively increased during days 5 and 6. An exact tendency was observed in each reactor. The elevated turbidity demonstrated significant increases of bacteria number in the augmented broth. The fluctuations observed may be an indication of the bacterial layer formation (biofilm) in the crushed stone strata. Also, the McFarland method was used to confirm turbidity results in terms of colony-forming units (CFU/ml), as shown in figure 7.

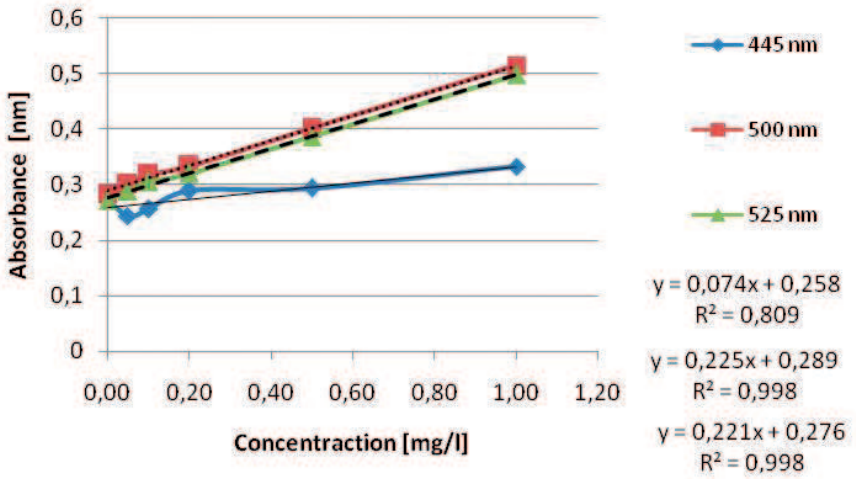

Figure 4 Calibration curve for low range. The linearization curves included in the right correspond to 445, 500 and 525 nm respectively
Slika 4. *Kalibracijske krivulje za male raspone. Krivulje lineariziranja prikazane desno odgovaraju veličinama 445, 500 i 525 nm*

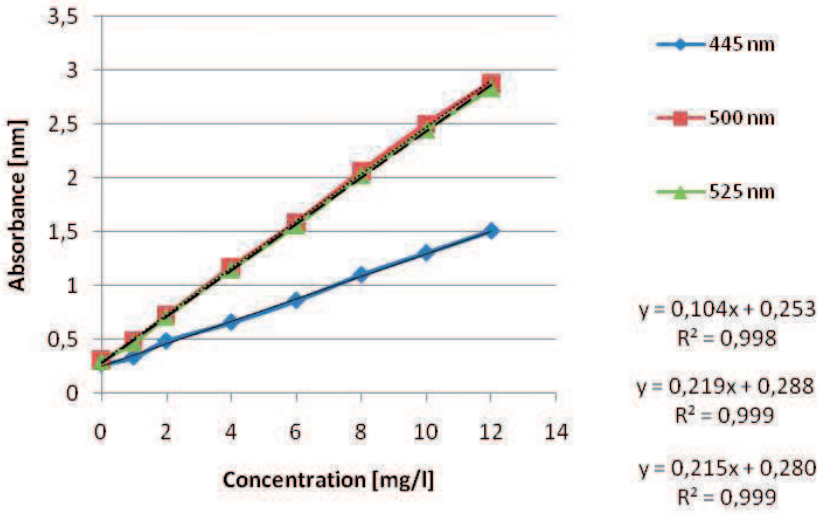

Figure 5 Calibration curve for high range. The linearization curves included in the right correspond to 445, 500 and 525 nm respectively
Slika 5. *Kalibracijske krivulje za velike raspone. Krivulje lineariziranja prikazane desno odgovaraju veličinama 445, 500 i 525 nm*

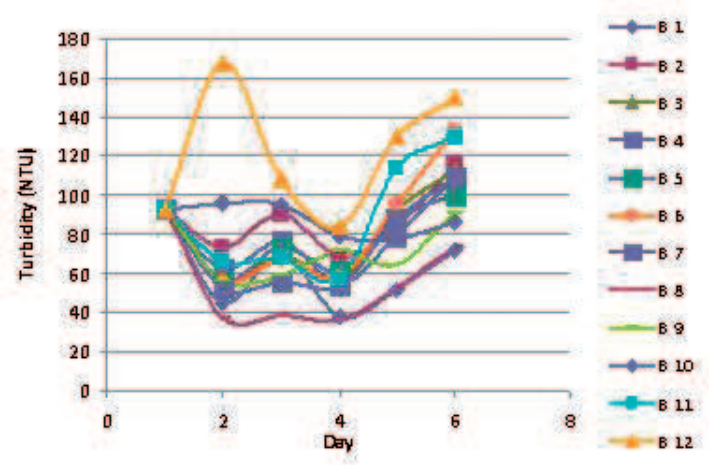

Figure 6 Turbidity data collected during the preliminary treatment
Slika 6. *Podaci o zamućenosti sakupljeni tijekom primarne obrade*

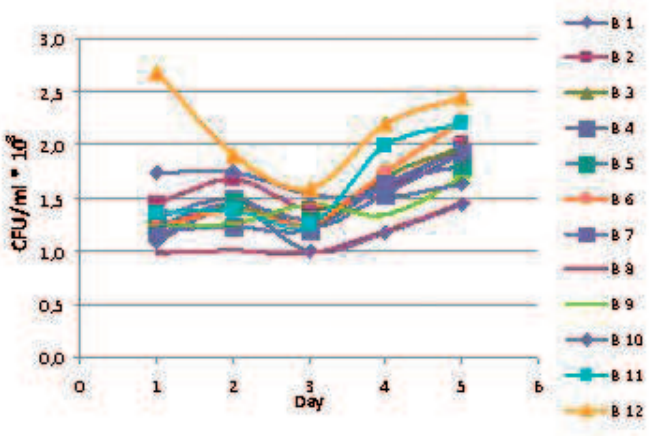

Figure 7 Bacterial concentration in each bioreactor.
Slika 7. *Koncentracija bakterija u bioreaktorima*

Table 6 Mass readings of selected crushed stone pieces.
Tablica 6. *Očitavanje masa odabranih drobljenih uzoraka*

Reactor	Mass Day 2 (g)	Mass Day 5 (g)	Difference (mg)
1	8,3	8,33	**30**
2	9,87	9,93	**60**
3	7,2	7,27	**70**
4	11,66	11,71	**50**
5	2,83	2,86	**30**
6	5,26	5,27	**10**
7	5,29	5,31	**20**
8	9,96	10	**40**
9	8,59	8,6	**10**
10	4,94	4,95	**10**
11	12,12	12,15	**30**
12	13,18	13,19	**10**

Bacterial layer formation was confirmed by comparing mass of a selected stone in the packed bed of each bioreactor. As revealed in table 6, this difference was found in each reactor with values ranging between 10 and 70 mg.

During the optimization process, high efficiencies were reported for all the concentrations studied under anaerobic conditions. These efficiencies ranged from 72 to 86% (phenol removal efficiency). This initial assessment suggests a high phenol biodegradability potential using directly selected and adapted bacteria obtained from the petroleum wastewater. According to table 7, the most favorable conditions for low and high phenol concentration (≅ 2.14 and 9.30 mg/l) were obtained with a HRT of 18 hours with the presence of the GAC layer (83 and 86%, respectively). For the intermediate phenol concentration, a 24 hour HRT with GAC presence was more efficient (81%). Figure 8 shows a general diagram of the results of the experiments. The efficiency was calculated referred to the bioreactor's initial concentration (Ci).

Table 7 Global Optimization results
Tablica 7. *Optimiranje rezultata*

Ci = 2.14 [mg/l]			Ci = 6.13 [mg/l]			Ci = 9.30 [mg/l]		
HRT	**GAC**	**%**	**HRT**	**GAC**	**%**	**HRT**	**GAC**	**%**
-	**-**	**77**	**-**	**-**	**72**	**-**	**-**	**78**
+	**-**	**79**	+	**-**	**63**	+	**-**	**85**
-	+	**83**	**-**	+	**75**	**-**	+	**86**
+	+	**81**	+	+	**81**	+	+	**81**

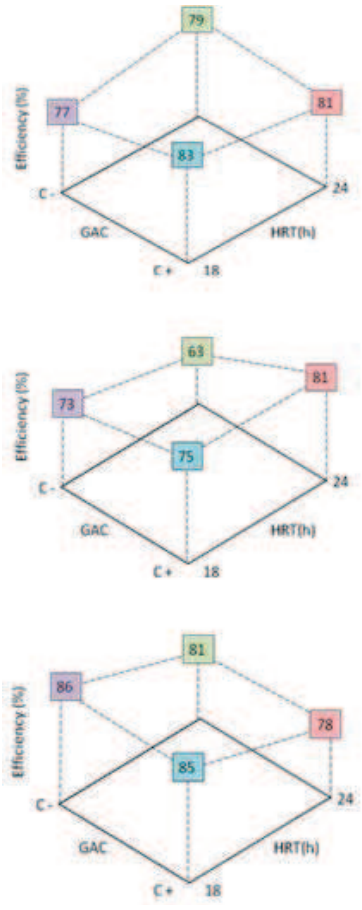

Figure 8. Phenol removal efficiences for 2, 6 and 10 mg/l respectively
Slika 8. *Efikasnost uklanjanja fenola za 2, 6, i 10 mg/l*

Tables 8 to 10 include the experimental design responses for each studied concentration.

Table 8 Principal effects in terms of phenol removal efficiency for 2 mg/l
Tablica 8. *Glavni efekti s obzirom na djelotvornost odstranjivanja fenola za 2 mg/l*

HRT (h)	GAC		Mean	Response to C+
	C -	C +		
18	77.1	83.0	80.0	6.0
24	78.6	80.9	79.7	2.3
Mean	77.8	81.9	79.9	4.1
Response to HRT 24 h - 18 h	1.5	-2.2	0.3	

Table 9 Principal effects in terms of phenol removal efficiency for 6 mg/l
Tablica 9. *Glavni efekti s obzirom na djelotvornost odstranjivanja fenola za 6 mg/l*

HRT (h)	GAC		Mean	Response to C+
	C -	C +		
18	72.5	74.8	73.6	2.3
24	63.4	81.5	72.4	18.1
Mean	67.9	78.1	73.0	10.2
Response to HRT 24 h - 18 h	-9.1	6.7	-1.2	

Table 10 Principal effects in terms of phenol removal efficiency for 10 mg/l
Tablica 10. *Glavni efekti s obzirom na djelotvornost odstranjivanja fenola za 10 mg/l*

HRT (h)	GAC		Mean	Response to C+
	C -	C +		
18	77.9	85.6	81.7	7.7
24	84.6	81.4	83.0	-3.2
Mean	81.2	83.5	82.4	2.2
Response to HRT 24 h - 18 h	6.8	-4.1	1.3	

Experimental results for ≅ 2 mg/l of phenol

In presence of GAC, phenol removal efficiency increased by 6 and 2.3% for HRTs of 18 and 24 hours, respectively. GAC represented an average 4.1% increase of efficiency. This positive effect could be attributed to the adsorption of phenol, which had not been yet consumed by bacteria, on the surface of the GAC layer. Nevertheless, this contribution is not highly significant given that GAC adsorption is more common for high phenol concentrations. Considering the effect of HRT in absence of the GAC layer, an increase from 18 to 24 hours showed a 1.5% increase of phenol removal efficiency. Increasing the HRT for 6 more hours (18 to 24) was also not significant in these experiments. This is consistent with the biodegradation kinetics, where the degradation of chemical substances or pollutants (in this case, phenol) occurs mostly at the beginning of the process. GAC presence in the 24 hour experiment decreased the efficiency by 2.2%. This effect might have occurred due to phenol desorption from the GAC layer, which could have possibly made phenol less available for bacteria, decreasing its population. Even so, only little variations were identified in removal efficiencies, when considering the variables GAC and HRT, demonstrating both that phenol was indeed biodegraded in the first 18 hours and that the adsorption mechanism was unimportant in these experiments.

Experimental results for ≅ 6 mg/l of phenol

The presence of GAC increased phenol removal efficiency by 2.3 and 18.1% for HRTs of 18 and 24 hours, respectively. In these experiments, GAC represented an average 10.2% efficiency increase, which was more significant. Considering the effect of HRT in absence of the GAC layer, an increase from 18 to 24 hours reported a 9.1% decrease in phenol removal efficiency. In its presence, the efficiency increased by 6.7%. Efficiency might have decreased in the 24 hours experiments (without GAC) because of the stress bacteria suffered when exposed to concentrations three times higher than the previous ones. This could have caused massive bacterial dead on the surface of the biofilm. These non-living cellular structures may have absorbed phenol during the process which was then desorbed and eliminated in the effluent, causing the efficiency decrease. In this way, the GAC layer was important in retaining previously desorbed phenol which was then adsorbed onto GAC increasing the efficiency by 18.1%, as stated before. This phenomenon was not relevant for the 18 hour HRT because phenol that was absorbed on the cellular structures was probably unavailable in the circulating water.

Experimental results for ≅ 10 mg/l of phenol

The presence of GAC increased phenol removal efficiency by 7.7% for a HRT of 18 hours. In contrast, for a HRT of 24 hours, the efficiency decreased by 3.2%. GAC represented an average 2.2% efficiency increase. For the 18 hours HRT, GAC had a positive effect. As stated before, adsorption on GAC is more relevant for high concentrations, consistent with the 7.7% increase in efficiency. Nonetheless, the efficiency decreased in the 24-hour experiment, revealing phenol non-availability due to prior absorption on GAC. Considering the effect of HRT, in absence of the GAC layer, an increase from 18 to 24 hours demonstrated a 6,8% increase in phenol removal efficiency, while in presence of GAC, the efficiency decreased in 4,1%. At this point, bacteria were presumed to be highly adapted to increasing phenol concentrations which is indeed related to the existence of a stronger biofilm layer. This fact was demonstrated for a 24-hour HRT, both by the increase in phenol removal efficiency without the GAC layer, and the efficiency decrease with the GAC layer. Phenol absorption on GAC diminishes its availability for bacterial consumption, confirming the fact previously described for the 24-hour response on GAC.

Global optimization analysis

Taking into account the results obtained, the presence of a GAC layer in the bioreactor did not constitute an important advantage for the biodegradation process, especially when phenol concentrations were high. The efficiencies increases were generally less than 10%. Similarly, the experiments showed that prolonged HRTs were not an indication of efficiency increases. For low and high phenol concentrations (2 and 10 mg/l), the bacterial layer was able to biodegrade phenol in the first 18 hours of HRT. This shows that raising the HRT to 24 hours was also not significant. For the tests conducted with 6 mg/l of phenol, the 24 hours HRT tests showed a phenol removal efficiency increase of 2%, confirming that most of phenol

degradation occurred within the first 18 hours. According to this, both, the use of GAC, and the prolongation of the HRT do not reflect major efficiency increases.

Control experiments

Table 11 summarizes the data obtained in the control experiments. In the absence of HRT (fast filtration), the GAC layer was essential, being the only mechanism contributing to phenol removal. The control experiments conducted with biofilm layer reported elevated efficiencies (up to 97%). The bioreactors exposed to direct light showed higher efficiencies, especially when the three factors were combined (light, GAC and HRT). This phenomenon proved how light participates in phenol biodegradation. The absence of any of the factors showed lower efficiencies. These experiments (controls) illustrated higher efficiencies compared to the previous ones (optimization) thanks to the microbial layer progressive adaptation and size increase.

Table 11 Control tests results in terms of phenol removal efficiency
Tablica 11. *Kontrolni rezultati ispitivanja s obzirom na efikasnost odstranjivanja fenola*

Absence of Hydraulic Retention Time								
Ci = 2,14 mg/l			Ci = 6,13 mg/l			Ci = 9,30 mg/l		
HRT	GAC	%	HRT	GAC	%	HRT	GAC	%
0	-	-13	0	-	14	0	-	17
0	+	2	0	+	19	0	+	34

Light effect without microbial layer (biofilm)				Light effect with microbial layer (biofilm)			
HRT	GAC	Light	%	HRT	GAC	Light	%
-	+	+	59	-	+	+	96
-	-	-	1	-	-	-	85
-	-	+	4	-	-	+	76
-	+	-	20	-	+	-	81
+	+	+	45	+	+	+	97
+	-	-	-6	+	-	-	81
+	-	+	4	+	-	+	86
+	+	-	-47	+	+	-	81

The bioreactors used for the control experiments in absence of a microbial layer were washed and disinfected with chlorine after being previously used in the optimization. An efficiency equivalent to 4% was obtained in absence of GAC and HRT due to the contribution of light (possible photo-degradation). The negative efficiencies reported in table 10 might be attributed to phenol desorption from both the GAC layer and other dead cellular structures (-47 and -6%, respectively). The experiments with GAC and light presence showed significant efficiencies, 59% and 45%, for 18 and 24 hours, respectively, demonstrating the significance of the GAC adsorption as well as the photo-degradation mechanism when microorganisms are absent.

In absence of the three factors (biofilm, GAC layer and HRT), the efficiencies obtained were quite low (1% and 4%). This evidence confirmed phenol biodegradation by means of the adapted biofilm attached to the crushed stone media inside the bioreactors.

Pre-pilot testing

After conducting the laboratory and control tests, some bioreactors were tested with oil wastewater as affluent. An 18-hour HRT was selected for the tests given the diverse findings concerning the experimental design analyses and the global analysis discussed previously. Table 12 shows the results obtained, the parameter Ci refers to affluent concentration, while Cf to effluent concentration. Results show that GAC presence is not favorable for phenol biodegradation in these bioreactors. This could happen because phenol is adsorbed in the GAC layer becoming less available for bacterial degradation, phenol concentration lowers and by the same mean the biodegradation rate.

Table 12 Pre-pilot testing results
Tablica 12. *Rezultati prethodnih ispitivanja*

GAC	Ci [mg/l]	Cf [mg/l]	Phenol removal efficiency %
+	9,69	6,49	33,02
+	8,54	6,31	26,11
-	8,46	2,16	74,47
-	9,77	4,72	51,69

CONCLUDING REMARKS

Three important findings indicated the elevated phenol biodegradation potential of the studied petroleum wastewater: the great abundance of isolated microorganisms from wastewater and sludge samples collected in the effluent of the oil field, the bacterial adaptation to high phenol concentrations and the elevated phenol removal efficiencies obtained in the optimization experiments. The optimum biodegradation conditions for low and high phenol concentrations (2,14 y 9,30 mg/l) were obtained with the presence of GAC and 18 hours of HRT. The best result for the intermediate phenol concentration (6,13 mg/l) was obtained with 24 hours of HRT and the presence of GAC. When phenol concentration was elevated, the use of GAC had a positive effect with a HRT of 24 hours. On the contrary, for low phenol concentrations, most of the biodegradation occurred in the first 18 hours and the GAC

layer increased the efficiency by 4%. Phenol adsorption on the surface of the GAC layer became evident when there were no other mechanisms available for phenol elimination, especially when high concentrations were studied. Phenol elimination is more successful when all the analyzed factors are combined: bacterial layer biodegradation, light and GAC presence. The pre-pilot testing confirmed that phenol biodegradation is successful when oil wastewater is applied directly to bioreactors without the GAC layer.

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