Annuaire de l'Université de Sofia "St. Kliment Ohridski"
Faculte de Biologie
2015, volume 100, livre 4, pp. 332-343
First National Conference of Biotechnology, Sofia 2014

BACTERIAL ABUNDANCE AND ACTIVITY IN OIL POLLUTED AND RESTORED ARTIFICIAL WETLANDS

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Keywords: oil pollution, wetland, heterotrophic and oil-degrading bacteria

Abstract: The oil industry has a great environmental risk throughout ship accidents and the impact of waste generated during the oil refining or production of petrochemicals and their derivatives. The waste waters of petrochemical industry commonly contain gross amounts of oil and suspended solids. After the purification processes, in some cases the wastewaters are discharged into artificial wetlands for sedimentation and biodegradation of refractory petrochemicals and organic matter.

This paper studied the dynamics of heterotrophic and oil-degrading bacteria in a polluted and a restored artificial wetland of petrochemical plant concerning the local specificity of wetlands and the seasonal changes. The oil polluted wetland was characterized both with higher water and lower sediment abundances of heterotrophs comparing to that of restored wetland. The number of oil-degrading bacteria was relatively similar in the two environments, except that in the sediments of restored wetland, which exceeded up to several times the number of oil-degrading bacteria elsewhere. The share of oil-degrading bacteria in the community of heterotrophs was higher (0.63% vs. 0.04%) in the water column and in the sediments (3.01% vs. 0.009%) of the restored wetland compared to the polluted one. The total activity of water and sediment heterotrophic bacteria was not significantly different between the wetlands with average value ranging from 4.5±2.0 μg O_2 l^{-1} to 5.3±1.3 μg O_2 l^{-1}. In a contrast, the relative bacterial activity, calculated per cell of heterotrophs, differed significantly between the water of polluted vs. restored wetland (81.8±182 x 10^{-4} μg O_2 l^{-1} vs. 30.3±34.4 x 10^{-4} μg O_2 l^{-1}), and between their sediments (0.07±0.1 x 10^{-4} μg O_2 l^{-1} vs. 0.04±0.05 x 10^{-4} μg O_2 l^{-1}). The ANOVA analysis indicated a significant contribution of water temperature in the variance of heterotrophs abundance

and activity, and water temperature and wetland local conditions, both contributing in the variance of oil-degrading bacteria abundance.

INTRODUCTION

Petroleum-based products are the major source of energy for industry and daily life and their importance for contemporary human society forces the petroleum industry all over the world. The petroleum industry is of great environmental risk throughout ship accidents and the impact of waste generated during the oil manufacturing. The amount of natural crude oil seepage was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year (Kvenvolden and Cooper, 2003). Release of hydrocarbons into the environment, whether accidentally or due to human activities is a main cause of water and soil pollution (Holliger et al., 1997). Biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment (Atlas, 1992; Amund and Nwokoye, 1993; Lal and Khanna, 1996, Ulrici, 2000), and it is cheaper than other remediation technologies (Leahy and Colwell, 1990). Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and on the amount of the hydrocarbons present. Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi. The reported efficiency of biodegradation ranged from 6% (Jones et al., 1970) to 82% (Pinholt et al., 1979) for fungi, 0.13% (Jones et al., 1970) to 50% (Pinholt et al., 1979) for soil bacteria, and 0.003% (Hollaway et al., 1980) to 100% (Mulkins Phillips and Stewart, 1974) for marine bacteria. Many scientists reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil (Bartha and Bossert, 1984), fresh water (Cooney, 1984), and marine environments (Atlas, 1985; Floodgate, 1984). Bacteria are the most active agents in petroleum degradation, and they work as primary degraders in oil polluted environments (Rahman et al., 2003; Brooijmans et al. 2009). In earlier days, the extent to which bacteria, yeast, and filamentous fungi participate in the biodegradation of petroleum hydrocarbons was the subject of limited study, but appeared to be a function of the ecosystem and local environmental conditions (Leahy and Colwell, 1990). Different factors influencing hydrocarbon degradation have been reported by Cooney et al. (1985). One of the important factors that limit biodegradation of oil pollutants in the environment is their limited availability to microorganisms (Barathi and Vasudevan, 2001) and environmental temperature (Atlas, 1981).

The aim of the paper was to study the seasonal site specific dynamics of bacterial number (heterotrophic and oil-degrading bacteria) and activity

(heterotrophic bacteria) in polluted and restored artificial wetlands of a factory that produces petrochemicals.

MATERIALS AND METHODS

Sites and sampling

Two artificial wetlands (polluted - PWL and restored - RWL) of petrochemical plant "Nova Plama" AD were studied to assess the seasonal and site specific effects on bacterial number and activity. The sampling wetlands outstand from one another at about 200 m and are linked to each other by a system of channels and siphons and small ponds.

Water and sediment samples were collected at three sampling sites for each wetland from December 2002 to August 2003 using a Limnos type water sampler (1.0 L) and a rod operated sediment core sampler, respectively. The samples for microbiological analysis were stored in 4ºC and transported to the laboratory within 4 hours.

Environmental variables

During the sampling, water temperature (T) and dissolved oxygen (DO) were measured by OXI-196 WTW, and water pH and conductivity by a HI 98129 pH-meter. The concentrations of inorganic nutrients (NO_3-N, NH_4-N and PO_4-P) were determined spectrophotometrically according to APHA (1989). The concentrations of nitrites (NO_2-N), sulphates (SO_4), chlorides (Cl), and calcium ions (Ca^{2+}) and water alkalinity were measured according to Clesceri et al. (1989) and Golterman et al. (1978), respectively. Chemical oxygen demand (COD) was measured according to the guide of the Bulgarian State Standards (BSS 17.1.4.02-77, 1977), and the sediment organic matter was determined using the Turin method (Kaurichev 1978).

Microbiological analysis

The amount of water and sediment heterotrophic and oil-degrading bacteria were counted using the technique of Most Probable Number (Colwell 1979) and cultivating the samples on nutrient broth (heterotrophic bacteria) and salt solution amended with hexadecane (1%) as a carbon source (oil-degrading bacteria). The bacterial sediment inoculums were prepared by dissolving 1 g sample in 50 ml NaCl (0.9%) and shaking on a rotary shaker (160 rpm) for 30 min. The samples were cultivated at dark for 5 days (heterotrophic bacteria) and 12 days (oil-degrading bacteria). Results were obtained using the Mac-Kredy's table for bacterial number calculation.

The metabolic activity of heterotrophic bacteria was measured using the method of Biological Oxygen Demand (BSS 17.1.4.07-78, 1978) for 5 days cultivation of water samples and samples of sediment inoculums.

Statistical analysis

The results are processed using software SPSS (ver. 6) for Windows. The significance ($p<0.05$) of the influence of the independent variables (wetland conditions, season) on the microbiological parameters (dependent variables) was determined using dispersion analysis (Shafe 1980). Correlation coefficients are calculated and statistical significance is proved at $p<0.05$ and $p<0.01$.

RESULTS AND DISCUSSION

Environmental characteristics

There was no significant difference between the environments of the RWL and PWL (Table 1).

Table 1. Environmental characteristics of the wetlands, expressed by means and standard deviations (SD) (n=9).

Parameter	Dimension	Restored wetland			Polluted wetland		
		Range	Mean	SD	Range	Mean	SD
T	°C	0.0-28.0	12.6	11.4	0.5-29.0	12.8	11.8
pH	-	7.1-8.7	8.1	0.6	7.2-8.7	8.2	0.5
DO	mg l^{-1}	1.1-14.1	7.8	4.2	2.0-15.7	8.0	3.6
PO_4-P	mg l^{-1}	0.1-0.3	0.2	0.1	0.1-0.4	0.3	0.1
NH_4-N	mg l^{-1}	0.3-1.5	0.9	0.7	0.4-1.9	0.9	0.6
NO_3-N	mg l^{-1}	0.0-0.2	0.1	0.1	0.1-0.7	04	0.2
NO_2-N	mg l^{-1}	0.0-0.4	0.1	0.1	0.0-0.2	0.1	0.1
SO_4	mg l^{-1}	39.4-93.9	58.0	15.8	33.3-130.3	84.4	34.4
Cl	mg l^{-1}	224-540	382.3	99.3	188-660	406.3	153.7
Ca	mg l^{-1}	30.0-67.0	47.0	12.8	28.0-64.0	48.0	10.2
Total alkalinity	meq l^{-1}	5.0-10.0	8.0	1.9	5.0-10.5	8.4	2.0
COD	mg O_2 l^{-1}	12.0-91.0	53.7	24.3	18.0-98.0	63.4	33.3
Organic matter in sediments	%	3.6-16.8	8.2	4.2	5.9-18.5	13.9	6.0

In both wetlands temperature increases steadily during the sampling period, except in January 2003 when its minimum values of 0°C and 0.5°C were registered in the restored and polluted wetland, respectively. The time of measure was differentiated into two seasons – cold with water temperatures below 10°C (December 2002 – March 2003) and warm with temperatures above 10°C (April 2003 - August 2003). During the cold period after a minimum of DO

concentration in January and February 2003 (ice cover), its values increased with increasing the temperature. During the warm season peaks were registered in the DO concentration in June 2003 for the RWL and in July 2003 for the PWL.

The water pH was neutral to alkaline as values varied in ranges 7.1-8.7 (RWL) and 7.2-8.7 (PWL). In both wetlands the water PO_4-P and NO_3-N concentrations were low and slightly varying during the period of measurement. More dynamic in time was the content of water NH_4-N. Its peak was during the winter, especially in February 2003 (RWL) and February and March 2003 (PWL). The NO_2-N content varied widely in both wetlands having the highest values in August 2003 (on average for both wetlands 0.4±0.01 μg l^{-1}). As a whole, the SO_4 content in the restored wetland was lower than in the polluted with maximum concentration in May 2003 and minimum in June 2003. In the polluted wetland the maximum and minimum concentrations were in April 2003 and August 2003. Ca and Cl concentrations varied in a different range in both wetlands and there was no a certain tendency in their trends during the sampling period. Alkalinity varied in a very narrow range in both wetlands with higher values during the warm season and lower during the cold one. COD varied widely in both wetlands with minimum in June 2003 (RWL and PWL) and maximum in April (RWL) and August (PWL) 2003.

The organic matter content in the sediments was higher in the PWL has a similar seasonal pattern with that of RWL – relatively high values during the cold season and lower values during the warm one.

Number of water and sediment bacteria

The number of RWL and PWL water and sediment bacteria (heterotrophic and oil-degrading) was counted during the year in order to analyse their seasonal dynamics (Fig. 1).

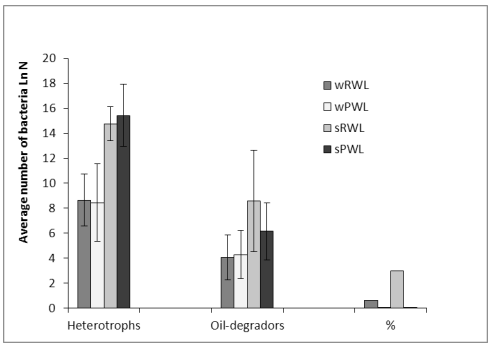

Figure 1. Number (Ln N) of water (wRWL and wPWL) and sediment (sRWL and sPWL) bacteria (heterotrophs and oil-degradors) inhabiting restored (RWL) and polluted (PWL) wetlands. Oil-degradors are represented as a percent (%) of heterotrophic bacteria.

The abundance of the heterotrophic bacteria was high both in the RWL ($5.5\pm7.3x10^6$ cfu g^{-1}) and in PWL ($54.9 \pm124.7x10^6$ cfu g^{-1}) sediments, and PWL ($83.7\pm49.8x10^6$ cfu g^{-1}) water column. There was a significant difference ($p<0.05$) between the average amount of water and sediment heterotrophic bacteria for each wetland. Insignificant differences ($p>0.05$) were recorded between wetlands both in their numbers of water and sediment heterotrophic bacteria.

The seasonal patterns of water heterotrophic bacterial dynamics were similar in the two wetlands having a maximum number in January 2003 (RWL – $14.2x10^4$ cfu ml^{-1} and PWL – $75.0 x10^5$ cfu ml^{-1}), and a minimum for RWL in March 2003 ($4.7x10^2$ cfu ml^{-1}) and PWL in May 2003 ($1.0x10^2$ cfu ml^{-1}). The highest numbers of sediment heterotrophic bacteria in both wetlands were determined in March 2003 (RWL – $2.3x10^7$ cfu g^{-1} and PWL – $38.2x10^7$ cfu g^{-1}), while the lowest was determined for RWL in August 2003 ($3.3x10^5$ cfu g^{-1}) and PWL in June 2003 ($1.8x10^5$ cfu g^{-1}). In the PWL sediments a wider fluctuation of bacterial abundance was registered compared to that in the RWL.

Oil-degrading bacteria comprised a small part of the heterotrophic bacteria 0.63% (RWL water) and 3.01% (RWL sediments), and 0.04 % (PWL water) and 0.01% (PWL sediments). The average number of water oil-degrading bacteria was higher in the PWL ($3.5\pm7.4x10^2$ cfu ml^{-1}) than in the RWL ($1.8\pm2.6x10^2$ cfu ml^{-1}) in the opposite to the sediment oil-degradors which were more abundant in RWL ($16.5\pm28.6x10^4$ cfu g^{-1}) then in PWL ($52.3\pm14.2x10^3$ cfu g^{-1}).

Seasonal patterns of water oil-degrading bacteria were similar to that of heterotrophic bacteria with maximum in January 2003 (RWL – $8.0x10^2$ cfu ml^{-1} and PWL – $23.0x10^2$ cfu ml^{-1}) and minimum in February 2003 (5.0 cfu ml^{-1} RWL and PWL). Sediment oil-degradors reached the maximum amount in January 2003 (PWL – $4.3x10^4$ cfu g^{-1}) and July 2003 (RWL - $74.7x10^4$ cfu g^{-1}).

There was no significant difference ($p>0.05$) between the number of oil-degrading bacteria inhabiting both the different substrata in each wetland and between the same substrata of different wetlands.

Metabolic activity of bacteria

Metabolic activity of heterotrophic bacteria was measured as a total for the whole community, as well as per cell (Fig. 2). There was no significant difference ($p>0.05$) in the metabolic activity between water and sediment heterotrophic bacteria in each wetland, and between the bacteria from one and the same substrata of different wetlands.

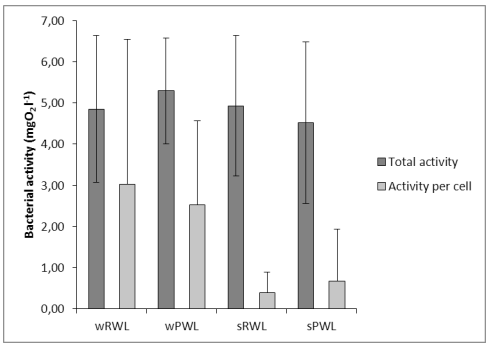

Figure 2. Metabolic activity of heterotrophic bacteria, expreced as total activity (mg O_2/l) and activity per cell (^10^{-3} mg O_2 l^{-1} water activity and ^10^{-5} mg O_2 l^{-1} sediment activity), inhabiting water column (wRWL and wPWL) and sediments (sRWL and sPWL) of recreated (RWL) and unrecreated (PWL) wetlands.

During the cold part of the sampling period there was a small difference between the activity of water heterotrophic bacteria of the two wetlands as in the PWL it was 15.2% higher than in the RWL. During the warmer months, the bacterial activity increased in both wetlands. In general, the activity of water heterotrophic bacteria was higher in the PWL (5.3±1.3 mg O_2 l^{-1}) than in the RWL (4.9±1.8 mg O_2 l^{-1}), but the difference was not significant ($p>0.05$). Water bacterial activity fluctuated in a wider range in the RWL (from 2.2 mg O_2 l^{-1} in January 2003 to 7.7 mg O_2 l^{-1} in August 2003) than in PWL (from 2.7 mg O_2 l^{-1} in December 2002 to 6.5 mg O_2 l^{-1} in August 2003).

The seasonal profiles of metabolic activity of sediment heterotrophic bacteria in the two wetlands have similar trends during the sampling period - after the winter minimum (RWL -2.3 – 4.0 mg O_2 l^{-1} and PWL - 1.1 – 2.0 mg O_2 l^{-1}), the activity increased rapidly staying relatively constant until June 2003 with maximal value of 6.7 mg O_2 l^{-1} (RWL) and 6.8 mg O_2 l^{-1} (PWL).

Environmental factors controlling bacterial number and activity

Bacterial number and activity variances were analysed concerning the season (cold and warm season) and local wetland environments (Table 2).

Table 2. Analysis of bacterial variance.

Dependent variable	Habitat	Source of variation	Analysis of variance			
			dF	SS	F	p
Heterotrophic bacteria	water	wetland	1	0.19	0.17	0.68
		season	1	7.06	6.50	**0.02***
		wetland and season	1	0.35	0.32	0.58
	sediment	wetland	1	1.79	2.58	0.13
		season	1	5.19	7.50	**0.02***
		wetland and season	1	1.02	1.48	0.24
Oil-degrading bacteria	water	wetland	1	0.04	0.06	0.81
		season	1	0.39	0.54	0.48
		wetland and season	1	0.03	0.04	0.84
	sediment	wetland	1	6.16	3.64	0.08
		season	1	2.03	1.20	0.29
		wetland and season	1	10.08	5.96	**0.03***
Bacterial activity	water	wetland	1	0.71	0.40	0.54
		season	1	13.22	7.44	**0.02***
		wetland and season	1	0.53	0.30	0.59
	sediment	wetland	1	0.78	0.28	0.60
		season	1	13.21	4.74	**0.05***
		wetland and season	1	1.94	0.70	0.42
dF-degrees of freedom; SS-sums of squares; F-F value; p-significance of F *-significance level<0.05						

The analysis of variance showed that the number and metabolic activity of water and sediment heterotrophic bacteria were determined by the season but not by the local wetland environment. In the opposite, oil-degrading bacteria were under the control of both season and local wetland environment.

Partial correlation analysis was performed between microbiological and abiotic environmental parameters (Table 3). There was no correlation between microbial parameters and abiotic factors like DO, alkalinity, SO_4, Cl, Ca, NO_3-N. Heterotrophic bacterial number (concerning the season) and bacterial activity (concerning the local wetland conditions) correlated positively and significantly ($p<0.05$) with pH. The number of oil-degrading bacteria correlated

(season) significantly ($p<0.05$) and positively with pH, NO_2-N and PO_4-P, and negatively with NH_4-N. There were significant ($p<0.05$) positive correlations between the numbers of heterotrophic and oil-degrading bacteria concerning wetland conditions (0.51) and season (0.49). A significant negative correlation (-0.52; $p<0.05$) between sediment number of heterotrophic bacteria and sediment organic matter was calculated when the season and wetland interaction was used as a controlling factor.

Table 3. Correlation analysis between bacterial and environmental parameters of the wetland water column.

Parameters	**Controlling for:**	**pH**	**NH_4-N**	**NO_2-N**	**PO_4-P**
Heterotrophic bacteria	Wetland	-0.22	0.25	0.06	-0.26
Oil-degrading bacteria		0.15	-0.39	0.44	0.28
Bacterial activity		**0.49***	-0.17	0.36	0.36
Heterotrophic bacteria	Season	**0.50***	-0.21	0.23	0.06
Oil-degrading bacteria		**0.55***	**-0.72****	**0.51****	**0.48***
Bacterial activity		0.03	0.39	0.28	0.04
*$p<0.05$; **$p<0.01$					

Artificial wetlands are often used as a wastewater treatment facility in petrochemicals production. This system promotes sustainable use of local resources, which is more environmental friendly. There were no significant differences between the investigated wetlands concerning the abiotic and the bacterial parameters. The sediment organic matter was higher in PWL than in the RWL, although the difference in its content was not significant ($p>0.05$). The environmental and microbiological parameters varied in a very similar range in both wetlands - their profiles had a seasonal pattern depending on the water temperature (cold and warm season).

The higher abundance of bacteria during the cold season, especially in January 2003 (water heterotrophs) and in March 2003 (sediment heterotrophs) is considered to be a stress reaction which promotes a shift in microbial communities stimulating the proliferation of psychrophilic bacteria. During the cold season remarkable differences were recorded between the heterotrophic bacteria of the two wetlands when the water and sediment bacteria of PWL are 35 and 12 times higher than that of RWL, respectively. These differences are dramatically

reduced during the warm season and the number of heterotrophic bacteria got more similar. An increasing number of the heterotrophic bacteria during the cold season has been reported also by other authors (Tuomi et al. 1997).

The number of oil-degrading bacteria in the water column of RWL and PWL and sediments of PWL had a similar trend as that of the heterotrophic bacteria. The opposite trend was recorded for the RWL sediment oil-degrading bacteria which were more abundant in the warm season, exceeding the number of bacteria in PWL by 730 times.

In general, the metabolic activity of water and sediment heterotrophic bacteria was higher during the warm season than the cold one, and the differences between the two wetlands were not significant referring to each of the seasons.

Taking into account that the bacterial metabolic activity varied seasonally in a relatively narrow range in a contrast to the high fluctuating bacterial number, the bacterial activity per cell was calculated. This activity was higher during the warm season for both wetlands as in sediments it was 8 and 44 times higher for RWL and PWL, respectively. Such strong differences were not measured in the water column.

CONCLUSION

The analysis of variance showed that the number and metabolic activity of water and sediment heterotrophic bacteria were determined by the season but not by the local wetland environment. The similar heterotrophic bacterial number in wetland sediments despite the different organic matter contents were, probably, due to the presence of more persistent or non-degradable organic matter in PLW. Oil-degrading bacteria were under the control of both season and wetland environment as their number correlated significantly with pH, NO_2-N and PO_4-P (positively), and NH_4-N (negatively).

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