Influence of Biopreparations on the Bacterial Community of Oily Waste

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Abstract. Oil pollution is reported to be one the most serious environmental problems nowadays. Therefore, methods of remediation of oily polluted soils and oily wastes are of great importance. Bioremediation being a perspective method of sanitation of oil pollutions, includes biostimulation of the polluted sites’ indigenous microflora, and in some cases additional introduction of active strains able to decompose hydrocarbon. The efficacy of introducing such biopreparations depends on the interactions between the introduced microbes and the indigenous ones. In this study, the influence of bacterial consortium (Rhodococcus jialingiae, Stenotrophomonas rhizophila and Pseudomonas gessardii) introduction on the bioremediation of an oily waste sampled from a refinery situated in the Mari El region (Russia) was estimated. Single and multiple inoculations of the consortium in addition to moistening and aeration were compared with a control sample, which included only aeration and moistening of the waste. It was shown, that two of the three introduced strains (Rh. jialingiae and Ps.gessardii) gene copy numbers were higher in the inoculated variants than in the control sample and with their initial counts, which meant that these strains survived and included into the bacterial community of the wastes. At the same time, bacterial counts were significantly lower, and the physiological profile of waste microflora slightly altered in the inoculated remediation variants as compared with the control sample. Interestingly, no difference in the degradation rates of hydrocarbons was revealed in the inoculated remediation variants and the control sample.

1. Introduction
Oily wastes generated during the processes of oil extraction, transportation and refinery present a serious problem and are characterized by a complex multicomponent composition including oil products, mechanical impurities and water [1–4]. Disposal of oily wastes in the environment can lead to different toxic effects induced by hydrocarbons, radioactive elements and heavy metals [5–9].

Among many methods of oil waste disposal biological ones attract more and more attention. One of biological methods of oil waste disposal is bioaugmentation, which involves the introduction of microorganisms into the polluted object for bioremediation [10–12]. Single strains with high ability to decompose hydrocarbons as well as microbial consortia may be used for bioaugmentation. Usually for bioaugmentation used hydrocarbon oxidizing microorganisms of the following taxonomic groups: Mycobacterium, Sphingomonas, Pseudomonas, Acinetobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Brevibacterium, Corynbacterium, Flavobacterium, Nocardia, Pseudomonas, Dietzia, Methylobacterium, Rhodococcus, Vibrio for bacteria, and Chrysosporium, Bjerkander, Irpex, Agrocyebe, Lentinus, Trametes, Pleurotus, Botryosphaeria for fungi [13–18]. Wu et al. (2016) notice that the introduction of the strain Acinetobacter SZ-1 allowed to 34% reduce of total hydrocarbon content in contaminated soils [13]. Hassanshahian et al. (2014) achieved a 95% oil reduction in polluted water, using the strain Alcanivorax borkumensis SK2T [14]. Covino et al. (2016) used for bioaugmentation the following
species of fungi - *Pleurotus ostreatus* u Botryosphaeria rhodina* [15]. However, only the introduction of *P. ostreatus*, which reduced the total hydrocarbon content in the soil on 86.8%, was effective.

Some authors suppose that, the introduction of a consortium is more effective, since each strain of microorganisms will consume every component of oil [12,16,19,20]. Cerqueira et al. (2014) used the for bioremediation group of strains (*Stenotrophomonas acidaminiphila, Bacillus megaterium, Bacillus cibi, Pseudomonas aeruginosa, Bacillus cereus*) and their consortium [16]. Authors came to the conclusion that the most effective was the use of the consortium.

Pacwa-Plociniczak et al. (2016) described that a consortium of *Bacillus subtilis T0-1* and *Pseudomonas sp. P-1* is three times more effective than their individual application for bioremediation of contaminated soils, and Dellagnezze et al. (2016) found that the efficiency of using a consortium of bacteria reaches 99% for oil-polluted seawater [17,21].

The efficacy of bioaugmentation process directly depends on the influence of indigenous community on the survivability of the introduced strains [22,23]. The analysis of changes in taxonomic and functional diversity of the microbial community can help to estimate the response of the microbial community to the introduction of microorganisms, and thus to help to control bioaugmentation process [24,25].

The objective of our study is to estimate the changes of a man-made formation microbial community in response to the introduction of a bacterial consortium. To estimate the influence of the introduction oily waste inoculation process was carried out with the help of hydrocarbon-oxidizing strains. Bioaugmentation process was carried out one and three times, a sample with no inoculation was used as a control sample. In the course of the experiment the following parameters were estimated: oil product content in the samples, survivability of the introduced strains and the changes in the total number of bacteria using qPCR method, as well as the changes in the functional diversity of the communities using BIOLOG® EcoPlates.

2. Materials and methods

2.1 Experimental Design

Oily waste containing mechanical impurities and oil products was chosen as the research target. The oily waste used contained 177.8 g kg-1 of total petroleum hydrocarbons (TPH). The oily waste was collected from a refinery, Mari El, Russia (latitude: 54°50′26″ N, longitude: 52°27′08″ E).

Three variants of the experiment were carried out: a sample with no introduction of microorganisms (I), a sample with a single introduction of microorganisms (II), and a sample with triple introduction of microorganisms (III). The introduction of the bacterial consortium was carried out on day 1 (II), and on days 1, 28 and 56 (III) of the experiment in the amount necessary to achieve a final concentration in the sample equal 10^8 CFU g^-1. The experiment was carried out under the same conditions: constant room temperature, moistening and aeration.

Three hydrocarbon-oxidizing strains belonging to the species of *Rhodococcus jialingiae, Stenotrophomonas rhizophila* and *Pseudomonas gessardii* previously extracted from the oily waste under analysis were chosen for the introduction.

2.2 Chemical and biological analyses

Hydrocarbon content was assessed using IR-spectrometry with an AN-2 analyser (LLC Neftehimavtomatika-SPb, Russia).

Community level physiological profiles (CLPP) were assessed by the BIOLOG® ECOplates (BIOLOG, Hayward, CA). Each 96-well plate consists of three replicates of 31 sole carbon substrates and a water blank. The plates were incubated at 25 °C for 168 h, and the color development in each well was recorded as optical density (OD) at 590 nm with a plate reader (Thermo Scientific Multiskan MK3, Shanghai, China) at regular 24 h intervals.

Microbial activity in each microplate, expressed as average well-color development (AWCD) was determined as follows: AWCD = ΣOD/31, where OD is the optical density value from each well. The Shannon–Weaver index (H) was calculated using an was calculated using Eq. H = - ΣpiLn pi, where pi is the ratio of the activity on each substrate (ODi) to the sum of activities on all substrates (ΣODi).
2.3 Molecular analysis
Total genomic DNA was extracted from the samples using the FastDNA® SPIN Kit for Soil (Bio101, Qbiogene, Heidelberg, Germany) according to the manufacturer’s instructions. The DNA samples were stored at -20 °C or analyzed immediately.

Quantitative polymerase chain reaction (qPCR) was conducted using 16S 984f /1378r primers for bacteria and species-specific primers listed in Table 1 [26–28]. Species-specific primers were designed based on the available draft genomes using Primer—BLAST tool from NCBI and assessed by Oligo 6 software. PCR reactions were conducted with a 0.1 U µl-1 SynTaq Polymerase, 1x Buffer with SYBR Green, 2.5 mM MgCl₂, 200 µM dNTPs each, 0.2 µM primer each and 1 µl of DNA template in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Munich, Germany). The qPCR program for total bacteria consisted of initial denaturation at 95 °C for 5 min followed by 39 three-step cycles of 62–60 °C for 45 s, 95 °C for 15 s, and 72 °C for 30 s. PCR amplification for introduced species was conducted using the following protocol: 15 min at 95 °C, followed by 39 cycles at 95 °C for 30 s, 30 s at Tm shown in Table 1, and 30 s at 72 °C. The standard curves were generated using serial DNA dilutions of DNA of Bacillus pumilus, Rh. jialingiae, S. rhizophila, Ps. gessardii. The concentration of DNA was measured on a Qubit 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) using the Picogreen ds DNA reagent (Invitrogen Ltd., Paisley, UK). Three replicates for each sample were used for qPCR analyzes. All of qPCR assays performed with efficiency of more than 94%, R² values greater than 0.99.

| Primers | Sequence (5’-3’) | Tm (°C) | Phylogenetic affiliation          |
|---------|------------------|--------|-----------------------------------|
| JiF     | TAATACGTAGGGTGCAAGCGTT | 60     | Rhodococcus spp.                 |
| JiR     | ATTTCACCGCTACACCAGGAAT |        |                                   |
| PGeF    | ATACAGAGGGTGCAAGCGTT  | 60     | Pseudomonas spp.                 |
| PGeR    | GCATTTCACCGCTACACAGG  |        |                                   |
| StF     | CGTGCGTAGGTGTTGGTTTGAAG | 60     | Stenotrophomonas spp.          |
| StR     | ATTTCACTGCTACACCGGAAT |        |                                   |

2.4 Statistical analysis
Sampling and chemical analyses were carried out in triplicate and biological analyses in quintuplicate, and all results were expressed on an air-dried sample basis. Statistical analyses were performed using Origin 8.0 (OriginLab, Northampton, USA) and R Statistical Software (R 3.0.0, R Foundation for Statistical Computing Version, Vienna, Austria) [29] packages. The confidence of data generated in the present investigations has been analyzed by standard statistical methods to determine the mean values and standard errors (SEs). The means were compared using Fisher’s least significant difference at α=0.05. The values in figures were expressed as mean ±SE of the corresponding replicates.

3. Results and discussion
The efficacy of bioaugmentation is evaluated first of all according to the rate of the contaminant decomposition. In the two inoculated samples as well as in the control sample, similar hydrocarbon content dynamics was observed – it decreased from 60 g kg⁻¹ to 25-27 g kg⁻¹ during 63 days, and further remained stable. Single and triple introduction of the hydrocarbon-oxidizing consortium did not influence significantly on the consumption of oil products in the samples. It can be explained with poor survivability of the species introduced or with competitive suppression by the indigenous community [23]. However, using qPCR analysis it was revealed that two of the three introduced strains did survive and even grew in the remediated waste (Figure 1 a-c).
Figure 1. Specific Gene Copy Numbers of the Introduced Strains in the Process of Bioremediation
(I, II, III – Variants of Bioremediation Methods; a – *Rh. jialingia*; b – *Ps. gessardii*; c – *S. rhizophila*).
Thus, the number of microorganisms belonging to the *Rh. jialingia* group after the first inoculation in sample II grew considerably from $1.94 \times 10^6$ gene copy number g$^{-1}$ on the first day and reaching the maximum on day 56 – $2.47 \times 10^7$ gene copy number g$^{-1}$. The second and the third inoculations (sample III) did not help to increase the number of the species. After 70 days the number of *Rh. jialingia* species representatives became equal for all the three samples. The number of representatives of *Ps. gessardii* in the sample with no introduction changed considerably during the remediation and remained within the limits of $1.17 \times 10^6$ to $3.05 \times 10^6$ gene copy number g$^{-1}$. The first and the second inoculations induced the growth of the introduced strain as compared to the control sample. Thus, in sample II after the first inoculation the number of *Ps. gessardii* representatives increases from $4.71 \times 10^6$ gene copy number g$^{-1}$ on the first day up to $1.63 \times 10^7$ gene copy number g$^{-1}$ on day 28 of the experiment. The second inoculation in sample III induced the growth of the number from $9.09 \times 10^6$ gene copy number g$^{-1}$ on day 28 up to $2.45 \times 10^7$ gene copy number g$^{-1}$ on day 49, whereas the third inoculation did not have any significant effect. After 70 days, when the content of oil products used as a substrate decreases, the number of *Ps. gessardii* representatives decreases in all the samples. The reduction of the number of the introduced strains after 70 days can be explained with the reduction of oil product concentration being the main source of carbon in oily wastes [14,17]. As for the third strain, *S. rhizophila*, the introduction did not lead to the increase of its copy numbers in the inoculated variants as compared with the control sample which can be explained by competitive exclusion of this strain by the species of the indigenous community.

Absence of hydrocarbon decomposition rate on the background of introduced decomposers growth may be a sign that the indigenous microbial community was interrupted. Indeed, the maximum bacterial gene copy number was observed in the non-inoculated waste ($4.06 \times 10^9$ gene copy number g$^{-1}$), while in the inoculated samples it ranged between $1.11 \times 10^8$ and $2.35 \times 10^9$ gene copy number g$^{-1}$. This can be explained by the fact that the introduced strains, despite their low relative abundance in the whole waste microbial community, destabilized it and inhibited indigenous species [30].

Then, the influence of the introduced biopreparation on the functional diversity of the wastes’ microflora was estimated. Community level physiological profiling reflecting a number of carbon substrates that are decomposed by the heterotrophic members of the community was analyzed by means of BIOLOG® EcoPlates method [31–33]. Levels of AWCD are presented in Figure 2.

![AWCD Plot](image_url)

**Figure 2.** AWCD Values Estimated For Microbial Communities of the Remediated Waste Samples (I, II, III – Variants of Bioremediation Methods).

It can be concluded, that single introduction on day 1 lead to a certain reduction of AWCD value, however, on days 7 and 14 AWCD in sample II was higher than in the control sample. The second and the third inoculation also help to increase AWCD value, but it is not significant as compared with the control sample (I) and the sample with single inoculation (II). A similar regularity was witnessed by Mansur et al. (2016) in bioaugmented samples [34].
Functional diversity of microbial communities calculated based on Shannon index is presented in Table 2.

**Table 2. Dynamics of Functional Diversity of the Microbial Communities in Wastes**

| Method of Bioremediation | Day 3 | Day 7 | Day 14 | Day 31 | Day 59 | Day 120 |
|--------------------------|-------|-------|--------|--------|--------|---------|
| I                        | 3.15  | 3.14  | 3.12   | 3.22   | 3.11   | 3.02    |
| II                       | 2.92  | 3.01  | 3.08   | 3.09   | 3.17   | 3.11    |
| III                      | 2.86  | 3.10  | 3.17   |        |        |         |

It can be observed that the introduction of destruction preparations in both variants (II and III) led to short term declination of Shannon index as compared with the control sample. As in the case of total bacterial copy numbers, it may be explained by the destabilization of the indigenous community of the wastes by the introduced microbes, and partial inhibition of the indigenous species. Interestingly, in the literature we found a description of an opposite effect – Dejonghe with co-authors (2001) demonstrated the deviation of Shannon index in the bioaugmented samples [35]. Indeed, the effects of biopreparation inoculations may vary strongly depending on many factors such as indigenous community structure, physical and chemical characteristics of the remediated waste, survivability of the introduced strains, etc. Therefore, these effects should be estimated in each specific case of bioremediation. However, the effect observed in our study lasted only for a short time – for several days after the introductions, Shannon index increased in the inoculated samples and later remained on the level of the control sample.

The analysis of using substrate groups has shown that in all the three samples carbons and carboxylic acids recover the most effectively, and substrates belonging to phenol compounds and amine group recover the least effectively, which was also observed in the study by Alisi et al. (2009). It was found out that the lowest number of oxidized substrates contained in the control sample (I) [36]. It was proved by other authors as well, thus, Teng et al. (2010) notice that the control samples are characterized by a lower number of oxidized substrates than the bioaugmented ones [37].

**Conclusions**

It can be concluded, that despite the high survival rate of two of the three introduced strains – *Rh. jialingiae* and *Ps. gessardii*, the introduction of the biopreparation did not lead to the improvement of petroleum hydrocarbon degradation in the remediated oily waste. This may be due to the deviation of total bacterial counts and short term alteration of physiological community profile as compared with the control sample. The poor efficacy of bioaugmentation may be also connected with relatively low abundance of the introduced strains as compared with total bacterial counts in the remediated waste.

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**References**

[1] Xu L, Ravnskov S, Larsen J, Nilsson RH, and Nicolaisen M 2012 *Soil Biol Biochem*. 46 26–32
[2] Mrayyan B, Battikhi MN. 2005 *J Hazard Mater*. 120 127–134
[3] O’Rourke D and Connolly S 2003 *Annu Rev Environ Resour*. 28 587–617
[4] Elektorowicz M 1994 *Environ Technol (United Kingdom)*. 15 373–380
[5] Swoboda-Colberg N 1995 *Microbial Transformations and Degradation of Toxic Organic Chemicals*. New York: Wiley-Liss Inc. Young L, Cerniglia C, editors. 27–74.
[6] Propst TL, Lochmiller RL, Qualls CW and McBee K. 1999 *Chemosphere*. 38 1049–1067
[7] Zappi ME, Rogers BA, Teeter CL, Gunnison D and Bajpai R 1996 *J Hazard Mater*. 46 1–12
[8] Galitskaya P, Gumerova R, Ratering S, Schnell S, Blagodatskaya E and Selivanovskaya S 2015 *J Plant Nutr Soil Sci*. 178 825–833
[9] Galitskaya P, Biktasheva L, Saveliev A, Ratering S, Schnell S and Selivanovskaya S 2015 Biogeosciences 12 3681–3693
[10] Jacques RJS, Okeke BC, Bento FM, Teixeira AS, Peralba MCR and Camargo FAO 2008 Bioresour Technol. 99 2637–2643
[11] da Silva LJ, Alves FC and de França FP 2012 Waste Manag Res. 30 1016–1030
[12] Galitskaya P, Akhmetzyanova L and Selivanovskaya S 2016 Biogeosciences 13 5739–5752
[13] Wu M, Dick WA, Li W, Wang X, Yang Q and Wang T 2016 Int Biodeterior Biodegrad. 107 158–164
[14] Hassanshahian M, Bayat Z, Cappello S, Smedile F and Yakimov M 2016 J Environ Sci (China). 43 136–146
[15] Lladó S, Covino S, Solanas AM, Viñas M, Petruccioli M and D’annibale A 2013 J Hazard Mater. 248-249 407–414
[16] Cerqueira VS, Peralba M do CR, Camargo FAO and Bento FM 2014 Int Biodeterior Biodegrad. 95 338–345
[17] Pacwa-Płociniczak M, Plaza GA and Piotrowska-Seget Z 2016 Appl Soil Ecol. 105 76–85
[18] Cerqueira VS, Hollenbach EB, Maboni F, Vainstein MH, Camargo FAO and Peralba M do CR 2011 Bioresour Technol. 102 11003–11010
[19] Das K and Mukherjee AK 2007 Bioresour Technol. 98 1339–1345
[20] Mukred AM, Hamid AA, Hamzah A and Yusoff WMW 2008 Online J Biol Sci. 8 73–79
[21] Dellagnezze BM, Vasconcellos SP, Angelim AL, Melo VMM, Santisi S and Cappello S, 2016. Mar Pollut Bull. 107 107–117
[22] Andreoni V, Cavalca L, Rao MA, Nocerino G, Bernasconi S and Dell’Amico E, 2004 Chemosphere 57 401–412
[23] Mao J, Luo Y, Teng Y and Li Z 2012 Int Biodeterior Biodegrad. 70 141–147
[24] Kruglov Y V 2016 Microbial community of the soil: physiological diversity and methods of research. Agric Biol. 51 46–59 (IN RUSSIAN).
[25] Yakushev AV 2015 Pedology. 4 429–446 (IN RUSSIAN).
[26] Heuer H, Krsek M, Baker P, Smalla K and Wellington EMH 1997 Appl Environ Microbiol. 63 3233–3241
[27] Nübel U, Engelen B, Felsre A, Snaidr J, Wieshuber A and Amann RI, 1996 J Bacteriol. 178 5636–5643
[28] White TJ, Bruns S, Lee S and Taylor J. 1990 PCR Protocols: A Guide to Methods and Applications. 315–322
[29] Team R. R Development Core Team. 2013 R A Lang Environ Stat Comput. 55 275–286.
[30] Cunliiffe M and Kertesz MA 2006 Environ Pollut. 144 228–237
[31] Choi KH and Dobbs FC. 1999 J Microbiol Methods. 36 203–213
[32] Garland JL 1997 FEMS Microbiol Ecol. 289–300
[33] Insam H and Goberna M 2004 Mol Microb Ecol Manual. 853–860
[34] Mansur AA, Taha M, Shahsavari E, Haleyur N, Adetutu EM and Ball AS 2016 Int Biodeterior Biodegrad. 115 179–185
[35] Dejonghe W, Boon N, Seghers D, Top EM and Verstraete W 2001 Environ Microbiol. 3 649–657
[36] Alisi C, Musella R, Tasso F, Ubaldi C, Manzo S and Creminisi C 2009 Sci Total Environ. 407 3024–3032
[37] Teng Y, Luo Y, Sun M, Liu Z, Li Z and Christie P 2010 Bioresour Technol. 101 3437–3443