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Bacterial consortium as a model for studying the response of the microbial community of the Verkhnekamsk salt mining region to combined pollution

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The unique ecosystem of industrial development area of the Verkhnekamsk salt deposit (VSD) (Perm krai, Russia) is characterized by the combined effect of salinization and contamination by organic pollutants, including polycyclic aromatic hydrocarbons (PAH). The purpose of the present study was to examine the degradative potential in relation to naphthalene, as a model PAH, under different salinity of bacterial consortium SMB3, previously isolated from soil of the VSD region, as well as the effect of long-term exposure to high salinity on the taxonomic composition of the consortium. The consortium SMB3 was able to grow on naphthalene both in the presence of NaCl up to 90 g/L, and in its absence. With an increase in the concentration of NaCl to 90 g/L, the growth rate of the consortium decreased by 2.1 times (compared with that of the consortium in a salt-free medium), and the naphthalene utilization estimated after 72 hours of cultivation decreased by 22.9 times. As a result of long-term cultivation in a mineral medium with naphthalene in the presence of 70 g/L NaCl, moderately halophilic strains *Halomonas* sp. SMB31 and *Salinicola socius* SMB35^T, not using naphthalene as the sole source of carbon and energy, and naphthalene degraders *Rhodococcus* spp. SMB37 and SMB38 were shown to preserve in the consortium, while strains *Glutamicibacter* sp. SMB32, *Microbacterium* sp. SMB33, "*Thalassospira permensis*" SMB34^T, not growing on naphthalene, were eliminated. Thus, using the model experiments with the bacterial consortium SMB3, it has been shown that the soil autochthonous microbocenosis in the VSD salt-mining area is able to degrade persistent toxic organic compounds in a wide range of salinity, and prolonged exposure to a high salt concentration leads to a decrease in species richness.

Keywords: polycyclic aromatic hydrocarbons, naphthalene, degradation, salinity, soil.

УДК 57.042+579.222.2

Бактериальный консорциум как модель для изучения реакции микробного сообщества района разработки Верхнекамского месторождения солей на комбинированное загрязнение

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Уникальная экосистема района промышленной разработки Верхнекамского месторождения солей (ВМС) (Пермский край, Россия) характеризуется комбинированным действием засоления и загрязнения органическими поллютантами, в том числе полициклическими ароматическими углеводородами (ПАУ). Целью настоящего исследования являлось изучение деградативного потенциала бактериального консорциума SMB3, выделенного ранее из почвы района ВМС, в отношении нафталина, как модельного ПАУ, в условиях разной солёности среды, а также влияния длительного действия высокого засоления на таксономический состав консорциума. Консорциум SMB3 был способен расти на нафталине как в присутствии NaCl в концентрации до 90 г/л, так и в отсутствие соли. С увеличением концентрации NaCl до 90 г/л скорость роста консорциума снижалась в 2,1 раза (по сравнению с таковой при росте консорциума в среде без соли), а утилизация нафталина, оценённая через 72 ч культивирования, уменьшилась в 22,9 раз. Показано, что в результате длительного культивирования в минеральной среде с нафталином в присутствии 70 г/л NaCl в составе консорциума сохранялись умеренно галофильные штаммы *Halomonas* sp. SMB31 и *Salinicola socius* SMB35^T, не использующие нафталин в качестве единственного источника углерода и энергии, и деструкторы нафталина *Rhodococcus* spp. SMB37 и SMB38, а штаммы *Glutamicibacter* sp. SMB32, *Microbacterium* sp. SMB33, «*Thalassospira permensis*» SMB34^T, не растущие на нафталине, элиминировались. Таким образом, на примере модельных экспериментов с бактериальным консорциумом SMB3 показано, что автохтонный микробоценоз почвы района солеразработок ВМС обладает способностью к деструкции стойких токсичных органических соединений в широком диапазоне солёности среды, а длительное воздействие высокой концентрации соли приводит к уменьшению видового богатства.

Ключевые слова: полициклические ароматические углеводороды, нафталин, деградация, солёность, почва.

On the territory of the city of Berezniki (Perm krai, Russia) there is a complex of chemical industry enterprises (Azot branch of Uralchem JSC, BSZ JSC, Uralkali PJSC). More than 40% of industrial production in the city belongs to the enterprises of PJSC Uralkali, which are engaged in the extraction and processing of potassium-magnesium salts of the Verkhnekamsk deposit, which is accompanied by the transformation of the natural landscape [1, 2]. A large amount of halite waste, sludge and brine, produced by enterprises, pollutes the environment with compounds of sodium, potassium, magnesium and other elements, and leads to a change in the physicochemical characteristics of biotopes, in particular, their salinization [1, 3]. On the other hand, the raw materials and technological components, used in its processing, serve as a source of pollution of the ecosystem with persistent organic compounds, including polycyclic aromatic hydrocarbons (PAH) [4, 5], which are mutagenic and carcinogenic [6, 7].

Thus, anthropogenic transformation of biotopes leads to the formation of new abiotic factors affecting the autochthonous bacterial communities, such as salinity and persistent toxic organic compounds. It is known from the literature that salinization of biotopes of various genesis leads to succession of microbocenosises with a predominance of halotolerant and halophilic microorganisms in them, as well as a significant slowdown in remediation processes [8–12]. Thus, the need to study the degradative potential of soil autochthonous communities in the area of development of the Verkhnekamsk salt deposit (VSD) and their stability under salinity is obvious. The ongoing research in this area will contribute to the development of new and effective bioremediation strategies for saline soils contaminated with toxic organic compounds. The purpose of the present study was to examine the degradative potential of the consortium SMB3 isolated from the soil of the industrial development area of the VSD [13] in relation to naphthalene as a model PAH under different salinity of the environment, as well as the effect of prolonged exposure to high salinity on the taxonomic composition of the consortium.

Materials and methods

Consortium SMB3 isolated from soil of the Verkhnekamsk salt mining region (Russia) was able to grow on naphthalene (model compound of PAH) under 60 g/L NaCl [13]. The consortium consists of seven strains: SMB31, SMB32, SMB33, SMB34, SMB35, SMB37, and SMB38 [13]. Two strains have been described as type strains of new taxa: *Salinicola socius* SMB35^T [14] and "*Thalassospira permensis*" SMB34^T [15]; taxonomic position at the genus level of strains SMB38, SMB37 on the basis of the analysis of incomplete 16S rRNA gene nucleotide sequences with a length of about 500 bp was defined [16]. As a part of this work, the taxonomic position of the five strains SMB31, SMB32, SMB33, SMB37, and SMB38 based on the determination and analysis of almost complete 16S rRNA gene nucleotide sequences with a length of more than 1200 bp was clarified as described [17, 18]. The 16S rRNA gene nucleotide sequences of strains SMB31, SMB32, SMB33, SMB37, and SMB38 were deposited in the GenBank database at numbers: MH321816, MH327514, MH321886, MH321888, and MH321889, accordingly.

Growth of the bacterial consortium at different NaCl concentrations. Since an increase in bacterial biomass when using naphthalene as the sole source of carbon and energy indicates the ability to metabolize (to degrade) it, accompanied by the inclusion of carbon in newly synthesized cell structures, at the first stage the growth of the consortium in a mineral medium with naphthalene under conditions of different salinity was studied. Consortium SMB3 was precultured in liquid mineral Raymond medium (MRM) [15] containing 60 g/L NaCl with naphthalene (1 g/L) to late exponential growth phase; the conditions corresponded to those for the consortium isolation [13]. Then 1 mL of inoculum was transferred in 99 mL of MRM (without or with NaCl 60, 70, 90 g/L) with naphthalene crystalline powder (1 g/L) in 250 mL bottles. Cultivation was carried out on the thermostable rotary shaker at 100 rpm and 28 °C. The numbers of viable cells within the consortium were determined by the serial dilution method using nutrient Raymond medium (NRM) [15] agar plates with 60 g/L NaCl. The results were expressed as colony-forming units (CFU). Initial CFU value in consortium was approximately 10^5–10^6 CFU/mL.

Naphthalene degradation by the consortium at different salinity of the cultivation medium. The biodegradation experiments were carried out in 1.5 mL of MRM using 5 mL Balch tubes with teflon-lined stoppers. Naphthalene was added as a concentrated solution in acetone (stock solution 1.5 g/L) to a final concentration of 0.1 g/L. After that, the vials were kept open for 1 hour to evaporate the acetone. All the experiments were carried out in triplicate on three separate occasions.

Bacterial consortium SMB3 was grown on agarized MRM with 60 g/L NaCl in the vapour phase of naphthalene at 28 °C for 1 week. The biomass was washed off with MRM containing 60 g/L NaCl and transferred into eppendorf tube. Bacterial cells were harvested by centrifugation at 8000 g for 10 min at 28 °C. The biomass washing procedure was repeated 3 times. Next, the biomass was resuspended to a final concentration of approximately 10^6 CFU/mL. One hundred and fifty microliters of this suspension were inoculated into 1.5 mL MRM with NaCl (60 or 90 g/L) or without it. Variant without the addition of bacterial cells served as control. After 72 h of incubation at 28 °C on a rotary shaker at 100 rpm, residual naphthalene was twice extracted from the culture medium with hexane. Naphthalene was determined by gas chromatography-mass spectrometry as was described [19].

Naphthalene utilization by pure cultures. All strains of the consortium were tested for the ability to degrade naphthalene (0.1 g/L) in MRM with 60 g/L NaCl. Because among the bacteria of the consortium, there could be cultures not utilizing naphthalene, the strains were preliminarily grown on agarized NRM with 60 g/L NaCl. Then the experimental scheme described above was followed.

Selection of the consortium at 70 g/L NaCl. In order to study the resistance of the consortium SMB3 to the long-term of high salinity of the medium, 1 mL inoculum of the consortium grown to the late exponential growth phase in liquid MRM with 60 g/L NaCl and naphthalene (1 g/L), was transferred into 99 mL MRM with 70 g/L NaCl and naphthalene (1 g/L). The cultivation was carried out on a thermostable rotary shaker at 100 rpm and a temperature of 28 °C until the late exponential growth phase. In the next five passages, the consortium grown in liquid MRM containing 70 g/L NaCl and naphthalene (1 g/L) served as an inoculum.

The taxonomic composition of the consortium SMB3 selected at 70 g/L NaCl was studied using the denaturing gradient gel electrophoresis (DGGE) method. Total genomic DNA was prepared from bacterial biomass of consortium SMB3 grown on naphthalene in liquid MRM at 70 g/L NaCl (see above). Pure cultures of the consortium: SMB31, SMB32, SMB33, SMB34, SMB35, SMB37, and SMB38 were grown on NRM with 60 g/L NaCl agar plates. DNA was extracted from bacterial biomass according to described SDS-CTAB method [20].

The variable V1–V3 regions of 16S rDNA were amplified and subjected to denaturing gradient gel electrophoresis (DGGE) according to [21]. Electrophoresis was carried out for 4 h at 130 V and 60 °C on a 6% (wt/vol) polyacrylamide gel with a denaturing gradient ranging from 30 to 60% (where 100% denaturant contains 7M urea and 40% formamide) on Dcode™ Universal Mutation System ("Bio-Rad", USA). After electrophoresis gel was stained in an ethidium bromide solution (0.5 μg/mL) and documented with Gel Doc XR ("Bio-Rad", USA) system. The bands position in consortium DGGE-profile was compared with those of pure cultures.

Extraction and analysis of osmoprotective compounds. Not all strains of the consortium were able to use naphthalene as a source of carbon and energy, and NRM contains compounds that can be uptaken by cells and used as osmoprotectors; therefore, in the experiment to study synthesized *de novo* osmoprotectors by cells, it

was decided to use glucose as a substrate. The concentration of sodium chloride used in the experiment corresponded to that in the enrichment culture for the isolation of the consortium SMB3. Pure bacterial cultures were grown on glucose (1 g/L) in 100 mL MRM with 60 g/L NaCl on rotary shaking at 28 °C until late exponential growth phase. Ectoine extraction and high performance liquid chromatography using a Separon SGX 100 NH2, 4.6 ×150 mm, 5 μm (Dr. Maisch, Germany) on a Shimadzu prominence LC-20AD device (Shimadzu Corporation, Japan) equipped with a CPD-20A UV/VIS detector (Shimadzu Corporation, Japan) were carried out according to protocols described in [22].

Statistical data processing. The experiments were carried out in three replicates in three independent experiments. The results were processed using Microsoft Office Excel 2003.

Results and discussion

Growth and naphthalene degradation at different concentrations of NaCl by the consortium SMB3. The consortium SMB3 was capable of growth on naphthalene both in the presence of NaCl up to 90 g/L, and its absence. However, the *lag*-phase increased from 2 to 11 days and the growth rates of the consortium decreased from 0.103±0.033 h^{-1} to 0.048±0.008 h^{-1} with increased NaCl concentrations in culture medium (Fig. 1). Study of naphthalene degradation (0.1 g/L) after 72 h of cultivation showed almost complete destruction of the substrate (87±6%) in a medium without sodium chloride and utilization of about 50% (47±13%) from the added naphthalene in the presence of 60 g/L NaCl (Fig. 1).

The decrease in the amount of naphthalene in the presence of 90 g/L NaCl was insignificant in relation to the control at $p < 0.05$; this observation can be explained by the fact that the bacteria were in the *lag*-growth phase – the stage of adaptation to cultivation conditions. However, it should be noted that the bacteria of the consortium were able to use naphthalene as the sole source of carbon and energy in the presence of 90 g/L NaCl, as evidenced by an increase in their number to $9.6 \cdot 10^7$ CFU/mL in the culture medium (Fig. 1).

There are few reports in the literature on pure bacterial cultures or consortia able to efficient growth in presence of 1 g/L of naphthalene [23–26] or almost complete degradation of 0.1 g/L in a few days [27, 28]. Moreover, only one bacterial consortium, able to degrade naphthalene in presence of sodium chloride up to 60 g/L [29], and one pure culture *Pseudomonas* sp. LZ-Q, degrading naphthalene within medium salinity limits 75 g/L NaCl, are known [30]. It is known halophilic bacteria and their bacterial consortia utilizing PAH (naphthalene, phenanthrene) at higher concentrations of sodium chloride (up to 250 g/L), but not able to grow in absence of salt in medium [31–33].

Study of the ability to degrade naphthalene by pure strains of the consortium. Phylogenetic analysis of 16S rRNA gene sequences from of all isolates showed their affiliated to the bacterial genera *Salinicola,* and *Thalassospira* [14, 15], *Rhodococcus* [16], *Glutamicibacter*, *Microbacterium* and *Halomonas* (Table). In degradation experiments in presence of 60 g/L NaCl within 72 h reliable naphthalene decrease was shown to occur when using the strains *Rhodococcus* spp. SMB37 and SMB38 (Table). Previously it was established the strains *Rhodococcus* spp. SMB37 and SMB38 can degrade naphthalene without NaCl [16]. The strain *Rhodococcus* sp. SMB38 was shown to be able to grow on naphthalene in the presence of up 90 g/L NaCl [16]. Thus, salt range of the consortium SMB3 coincided with that of strain-degrader *Rhodococcus* sp. SMB38. The obtained results indicate the leading role of *Rhodococcus* genus bacteria in naphthalene degradation by the consortium SMB3.

The structure of the consortium SMB3 selected in a medium with 70 g/L NaCl. Influence of increasing sodium chloride concentration in culture medium on composition of consortium SMB3 was studied. As increase of sodium chloride concentration from 60 to 90 g/L led to significant decrease of consortium SMB3 growth parameters on naphthalene (Fig. 1), in further experiments we used concentration of 70 g/L NaCl. Five successive passages of consortium SMB3 to MRM with naphthalene and 70 g/L NaCl were performed.

PCR-DGGE analysis of selected consortium SMB3 composition revealed that selection at 70 g/L NaCl had led to elimination of strains "*T. permensis*" SMB34^T, *Glutamicibacter* sp. SMB32, *Microbacterium* sp. SMB33 and preservation of halophilic bacteria *S. socius* SMB35^T and *Halomonas* sp. SMB31, and also naphthalene degrading strains *Rhodococcus* spp. SMB38 and SMB37 (Fig. 2).

It is known that one of the mechanisms of bacterial adaptation to high medium osmolarity is *de novo* synthesis or consumption of low-molecular weight organic compounds from the medium that provides bacteria survival under

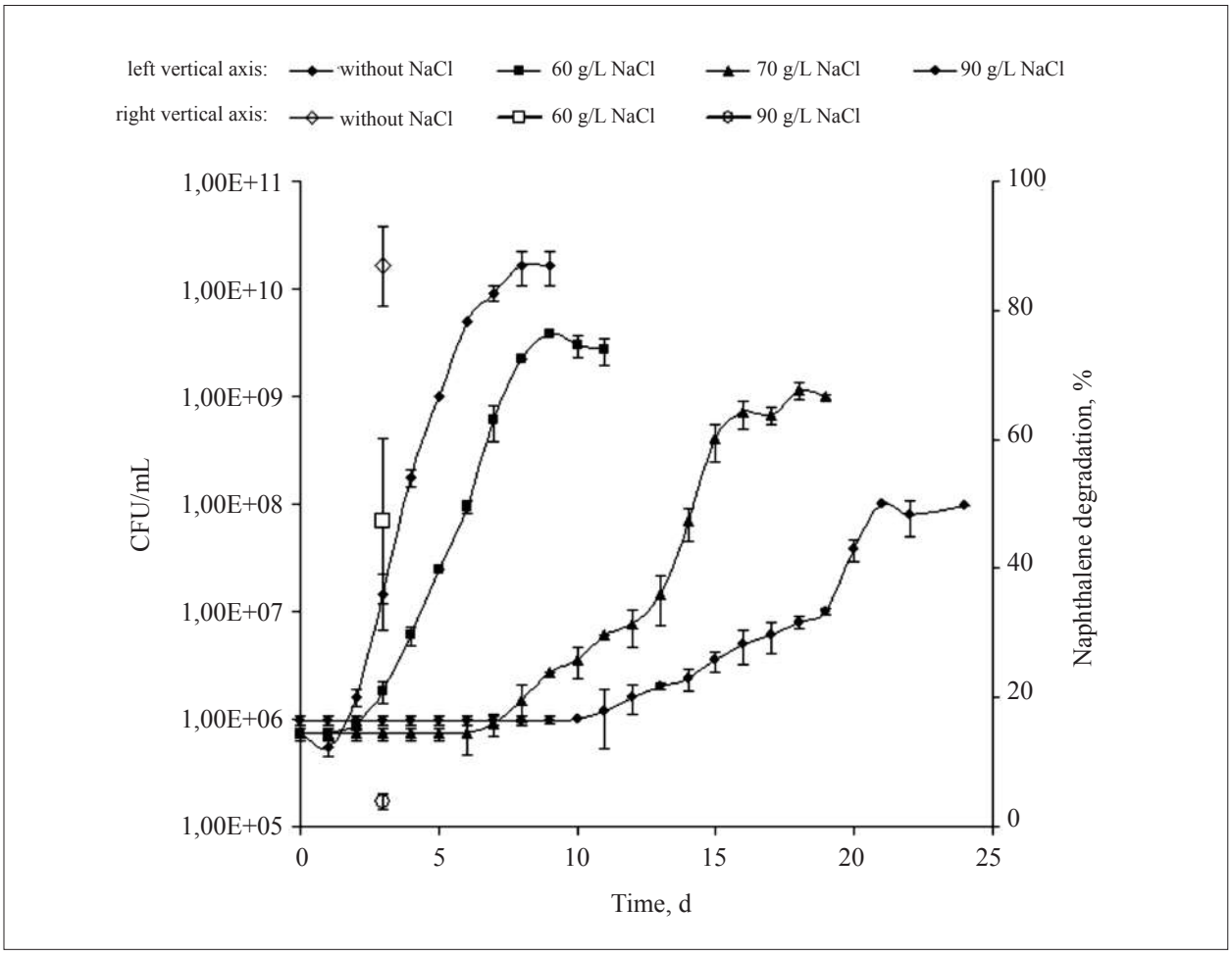

Fig. 1. Growth (closed symbols) of the consortium SMB3 exposed to different NaCl concentrations into liquid MRM and naphthalene degradation (open symbols)

Table

Characterization of strains isolated from the consortium SMB3

Characteristic	SMB31	SMB32	SMB33	SMB34^T	SMB35^T	SMB37	SMB38
Type strain of most similar valid species (16S rDNA similarity, %)	*Halomonas taeanensis* BH539^T (99.8)	*Glutamicibacter halophytocola* KLBMP 5180^T (99.8)	*Microbacterium oxydans* DSM 20578^T (99.6)	*"Talassospira permensis"* [a]	*Salinicola socius* [b]	*Rhodococcus artemisiae* YIM 65754^T (98.8)	*Rhodococcus jostii* IFO 16295^T (99.4)
Residual naphthalene (%)[c] after 72 h of incubation at 60 g/L NaCl	96.0±17.0	91.5±24.8	98.0±15.8	99.1±21.6	93.0±14.4	52.1±16.4[d]	68.5±8.5[d]
Intracellular ectoine content (μmol/mg CDW)	0.535± 0.018[f, g]	nd	nd	nd	nd	0.075± 0.026[f]	0.088± 0.024[g]

Note: [a] according to [15], [b] according to [14]. [c] Value is given as the mean percentage of residual naphthalene ± sd. [d] Difference between control and experimental variant is reliable under $p < 0.05$. [f, g] Difference between experimental variants is reliable under $p < 0.05$. nd – not determined.

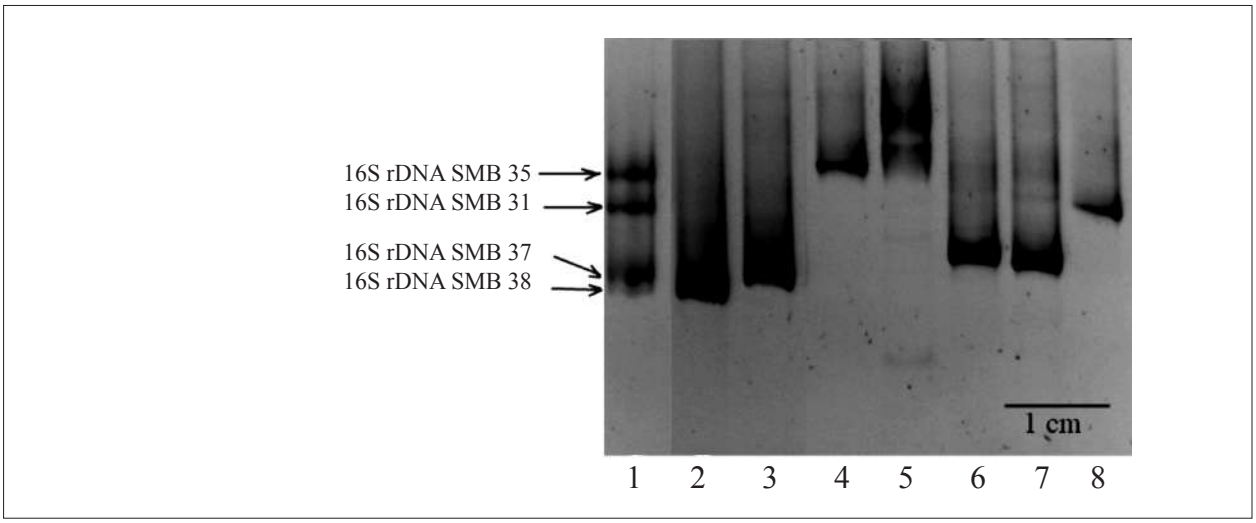

Fig. 2. Denaturing gradient gel electrophoresis of amplified bacterial 16S rRNA gene fragments from genomic DNA of: 1 – the consortium SMB3 exposed to 70 g/L NaCl concentration into liquid MRM with naphthalene, 2 – a strain SMB38, 3 – a strain SMB37, 4 – a strain SMB35, 5 – a strain SMB34, 6 – a strain SMB33, 7 – a strain SMB32, 8 – a strain SMB31. The scale is 1 cm

high salinity [34]. Currently the molecular aspects of osmoadaptation are studied in detail by the example of moderately halophilic bacteria of *Halomonadaceae* family (including strains of species *Halomonas elongata*) for which, as a response to hyperosmotic stress it is typical to synthesize and accumulate in cells predominantly osmoprotector – ectoine in the concentrations from 0.26 to 0.98 μmol/mg cell dry weight (CDW) [35, 36]. At the same time, the information on the ectoine accumulation by rhodococci cells is rather scarce. Ectoine was detected in cells of *Rhodococcus opacus* strain PD630 under water stress, its content was 34.5 nmol/mg CDW [37]. Our studies on intracellular ectoine content of moderately halophilic strain *Halomonas* sp. SMB31 and halotolerant strains *Rhodococcus* spp. SMB37 and SMB38 are in agreement with previously published data (Table). Under conditions of hyperosmotic stress, the intracellular amount of ectoine of strain-degraders was shown to be approximately 10 times less than in the cells of the moderately halophilic bacterium *Halomonas* sp. SMB31. Based on the above, it could be suggested that moderately halophilic strains of the *Halomonadaceae* family are the source of osmoprotectors for strains-degraders (*Rhodococcus* spp. SMB37, SMB38) improving the viability and maintaining the function under high salinity.

Conclusion

Using the consortium SMB3 as a model system, it was shown that soil autochthonous bacterial communities of Verkhnekamsk salt mining region have a high degradative potential with respect to the polycyclic aromatic hydrocarbon – naphthalene maintaining it in a wide range of salinity. Meanwhile, salinization of the environment has a negative effect on microbial community, leading to a slowdown in the degradation of naphthalene and a decrease in the species richness. Nevertheless, consortium retains both naphthalene-degrading strains and concomitant halophilic strains possessing mechanisms of salt tolerance due to the synthesis of an osmoprotective solute – ectoine. While halotolerant naphthalene degrading bacteria were noted to accumulate less ectoine. Finally it should be noted that the given work may indicate existence of interactions between moderately halophilic bacteria and bacteria-degraders in the consortium during naphthalene degradation under high salinity, the molecular mechanisms of which require further study. These observations provide hope for the establishment of effective methods for bioremediation as applied in PAH-polluted and saline environments.

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