Assessment of the anaerobic microbial potential for the bioremediation of gas condensate-contaminated groundwater

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Abstract. The gas condensate leakage incident took place in Bashkortostan (Russian Federation), which caused soil and groundwater pollution with hydrocarbons. After the incident, the emergency plan was started and kinds of measures were carried out immediately. But the residual subsurface source of pollution within ground is still present and contaminates groundwater. We investigated the capability of ground’s indigenous microbial community to degrade gas condensate hydrocarbons under anaerobic conditions. The enrichment microbial cultures of nitrate reducers, ferric reducers, sulphate reducers and methanogens, capable to degrade gas condensate hydrocarbons, were isolated at incident site from polluted ground. All cultures demonstrated the ability to degrade hydrocarbons under laboratory conditions. The enrichment culture of nitrate reducers was chosen as the most appropriated one to biotechnological application in situ (i.e. at incident site) — it held the capability to degrade gas condensate hydrocarbons after three consecutive aerobic incubations in the rich medium (meat-peptone broth) amended with glucose. To our mind, biomass of this culture being readily and quickly grown in aerobic conditions on cheap organic media could be directly used to remediate gas condensate-polluted groundwater and ground in situ.

1. Introduction

Hydrocarbons are common pollutants of the environment, which threaten to the public health and quality of potable groundwater resources. The low solubility of many hydrocarbons and their tendency to strongly sorb to aquifer materials enhances the persistence of hydrocarbon pollution in subsurface environments [1]. The ability of microorganisms to degrade a wide variety of hydrocarbons is well-known. These organisms are widespread in the environment and occur in fresh and salt water, soil, and groundwater. Biodegradation, or intrinsic bioremediation, is known to be the principal natural process for the removal of hydrocarbons from the environment [2-5].

The biodegradation of hydrocarbons is essentially an oxidation-reduction reaction where the hydrocarbon is oxidized (donate electrons) and an electron acceptor is reduced (accepts electrons). The role of electron acceptor can fulfil such compounds as oxygen (O2), nitrate ion (NO3–), iron oxides [e.g., Fe(OH)3], sulphate ion (SO42–), and carbon dioxide (CO2). Accordingly, the biodegradation of hydrocarbons could proceed both in aerobic and anaerobic conditions. During biodegradation of hydrocarbons microbial community utilizes electron acceptors in stepwise manner with preferential use of one that provide the most potential energy upon concomitant oxidation of hydrocarbons. Microorganisms gain more energy from aerobic reactions, so, oxygen is the most preferred electron acceptor and used first. In anaerobic condition other aforementioned compounds are used in listed order — carbon dioxide is the least preferred electron acceptor because microorganisms gain the least
energy from the usage of it [3, 6-10]. A variety of other metals, oxyanions, and even large molecular weight organic compounds have also been proposed to act as electron acceptors in anaerobic condition [11]. Some microorganisms are capable of multiple forms of anaerobic respiration, — they could use several electron acceptors in stepwise manner. However, the availability of the certain electron acceptors mainly dictates the physiological types of bacteria that can develop and proliferate in an environment and thus the types of metabolic activities that can be reasonably anticipated [12].

The basic concept behind intrinsic bioremediation is to use the capability of indigenous microorganisms to degrade pollutants and at the same time minimize risk to public health and the environment [6]. The intrinsic bioremediation of hydrocarbon-polluted environment is particularly beneficial, because it ultimately converts hydrocarbons to carbon dioxide, water, and methane. The full extent of hydrocarbon degradation by microorganisms is not completely catalogued, although indigenous microbial metabolism is now believed to be the most important process controlling the destruction of these contaminants in many environmental compartments, including groundwater [13]. To enhance natural degradation of hydrocarbons it was recommended to add mineral nutrients such as nitrogen and phosphorus, and corresponding electron acceptors to polluted environments [14-16]. Another techniques use the introduction of hydrocarbon-degrading bacteria or bacterial consortia, isolated from polluted environments, to such environments [14, 17].

The gas condensate leakage incident took place in Bashkortostan (Russian Federation) at gas condensate pipeline in spring of 2017, which caused soil and groundwater pollution. After the incident, the emergency plan was started and kinds of measures were carried out immediately, such as plugging leakage point, cleaning up the contaminated soil and setting sorption barriers across the ravine’s thalweg and the dam across the surface stream at the end of the ravine. But the residual subsurface source of pollution is still present and contaminates groundwater. This paper reports the results of an examination of the capability of polluted ground’s indigenous microbial community to degrade gas condensate hydrocarbons under anaerobic conditions.

2. Materials and methods

2.1. Study area
The site of research is located in the western part of Bashkortostan (Russian Federation), 700 m northwest of Sergeevka village in Ufa Region, where the gas condensate leakage incident took place and caused soil and groundwater pollution (figure 1). The groundwater flows from northwest to southeast. The water table is usually 6.7-16.4 m below the land surface. The precipitation infiltration is the main source of groundwater recharge, and the main discharges are groundwater evaporation and numerous seeps into the ravine located in immediate vicinity of the leakage site. The depth of the ravine is 4-6 m, the width is 2-3 m; it is directed towards the village and flattens out — the depth is decreased and the width is increased as the ravine approaches the village.

Gas condensate chemical content is presented in table 1. Leaked gas condensate has spreaded into the ravine, saturated soil and infiltrated to groundwater. The discharges of polluted groundwater into the ravine have caused the pollution of soils located downstream of discharges and formed the surface stream of polluted water.

2.2. Sample collection
Three monitoring wells were placed within the gas condensate-impacted groundwater aquifer. One-litre samples of groundwater were collected from each well in sterile glass bottles, filled to capacity, sealed without a headspace, stored on ice, and transported back to the laboratory. The location of monitoring wells and depths of sampling below water table (in meters) are shown in figure 1. Groundwater samples were used for chemical analyses immediately upon return to the laboratory.

The core of hydrocarbon-contaminated ground was sampled from the depth of 1.0 m with hand corer, placed in nitric acid-rinsed PVC container and stored on ice before using. Sampling point is
Figure 1. The schematic representation of polluted site location and points of sampling. Depth of groundwater sampling below water table (in meters) is shown in brackets.

Table 1. Chemical characteristic of gas condensate contents (Bystrykh, pers. commun.).

| Hydrocarbon      | Content (% of total hydrocarbons) |
|------------------|----------------------------------|
| Methane          | <0.01                            |
| Ethane           | <0.01                            |
| Propane          | 0.02                             |
| iso-Butane       | 0.11                             |
| n-Butane         | 0.85                             |
| iso-Pentane      | 4.39                             |
| n-Pentane        | 4.84                             |
| Hexanes and higher homologues | 89.79 |
shown in figure 1. The inner part of the sample was used in microbiological investigations immediately upon return to the laboratory.

2.3. Chemical analyses
Groundwater samples were filtered through a Whatman 0.45-µm membrane filter prior to analyses. All quantifications were performed in the laboratory according to The Standarts of Chemical Measurements in Russian Federation. Concentrations of HCO$_3^-$ and CO$_3^{2-}$ ions were determined spectrophotometrically; total dissolved Fe was determined spectrophotometrically with sulfosalicylic acid; Cl$^-$, SO$_4^{2-}$, NO$_3^-$, NO$_2^-$, Na$^+$, K$^+$, Mg$^{2+}$, Ca$^{2+}$ and NH$_4^+$ ions were quantified using capillary electrophoresis system; hydrocarbon content in groundwater was determined spectrophotometrically; chemical oxygen demand (COD) was determined titrimetrically with potassium bichromate; pH was measured on-site with a pH electrode after pH 4 and 7 calibrations.

2.4. Microbiological investigations

2.4.1. Isolation of enrichment cultures of anaerobic groups of microorganisms. A hydrocarbons mineralization assay for the assessment of microbial biodegradation potential was performed on ground sample. To isolate microorganisms capable to anaerobic destruction of gas condensate hydrocarbons the enrichment culture technique was used [18]. As the basic solution for media preparation we used Phosphate-Saline Buffering solution (pH 7.8) supplemented with 0.3 g L$^{-1}$ NH$_4$Cl as a nitrogen source. The following chemicals were added to solutions as terminal electron acceptors for distinct anaerobic groups of microorganisms:

- KNO$_3$, 1 g L$^{-1}$, for nitrate reducers;
- FeCl$_3$, 1 g L$^{-1}$, for ferric reducers;
- Na$_2$SO$_4$, 1 g L$^{-1}$, for sulfate reducers;
- no acceptors addition was for methanogens, but the medium was supplemented with 0.1 g L$^{-1}$ glucose as additional electron donor to initiate process only.

The media were distributed into 250 ml Erlenmeyer flasks, autoclave sterilized, inoculated with 20 g of contaminated soil, and filled to flasks’ necks with corresponding sterile media to minimize residual volume of air in the flasks. Ferrous clips were added to the medium for sulphate reducers to decrease oxidative-reductive potential of the medium as recommended [19]. Gas-condensate, 3 ml, was added into each flask as an electron donor and a carbon source. Flasks were sealed with rubber stopper, and incubated at room temperature during 1 month. All manipulations were done aseptically in UV-sterilized table microbiological glove box.

The development of corresponding anaerobic groups of microorganisms was concluded from the emulsifying of gas condensate and the simultaneous occurrence of characteristic ions in the media:

- NO$_3^-$ for nitrate reducers (Griss-Ilosvay method with sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride);
- Fe$^{2+}$ for ferric reducers (probe with K$_3$[Fe(CN)$_6$]);
- HS$^-$ for sulphate reducers (probe with Cd-acetate) [18, 19];
- the development of methanogens was concluded from the emulsifying of gas condensate and gas bubbles occurred in the medium.

Twenty ml of each developed enrichment culture was transferred to the corresponding fresh medium with gas condensate and incubated as described above to test the ability of cultures to be cultivated and to degrade gas condensate hydrocarbons under laboratory conditions.

2.4.2. The study of nitrate reducing bacteria enrichment culture capabilities. The capability of the enrichment culture of nitrate reducing bacteria to degrade gas condensate hydrocarbons after aerobic growth in the rich medium was tested also. The matter is the bacterial capability to degrade hydrocarbons could be encoded in plasmid DNA, and such plasmid(s) could be lost after bacterial growth in rich media. Ten ml of the enrichment culture was transferred to 250 ml conical flask with
100 ml of sterilized meat-peptone broth medium supplemented with glucose, 1 g L\(^{-1}\), and incubated aerobically in rotary shaker at room temperature and 100 rpm during 5 days, after that 10 ml of this “aerobic” culture was used to inoculate the same fresh medium. This procedure was twice repeated (three transfers in total) and that 10 ml of the last “aerobic” culture was transferred to the medium for nitrate reducers supplemented with gas condensate as described above, and incubated at room temperature during 1 month. The development of nitrate reducers capable to degrade gas condensate was concluded as described above.

3. Results and discussion

3.1. Groundwater pollution

The results of groundwater samples chemical analyses are presented in table 2. Groundwater had the HCO\(_3\)–Ca hydrochemical type, and its mineralization was up to 950 mg L\(^{-1}\). Water samples #1 and #2 were transparent and exhibited weak odor of hydrocarbons, but an iridescent film of hydrocarbons on the surface of the samples was not detected. These samples contained hydrocarbons in amount exceeded the maximum admissible concentration for drinking water in Russian Federation. Water sample #3 was transparent, had not the hydrocarbons odor, and contained hydrocarbons in amount not exceeded the maximum admissible concentration for drinking water.

Thus, the near-surface hydrosphere in the area of gas condensate spill is characterized by local pollution. At present, the main source of pollutants entering groundwater is a residual contamination of subsurface ground at the site of gas condensate leaking, as well as soil and subsoil of ravine, soaked in gas condensate.

| Characteristics | Sample #1 | Sample #2 | Sample #3 |
|-----------------|-----------|-----------|-----------|
| pH              | 7.4       | 7.2       | 7.3       |
| HCO\(_3\), mg L\(^{-1}\) | 591       | 657       | 699       |
| CO\(_3^{2-}\), mg L\(^{-1}\) | b.d.l.\(^{a}\) | b.d.l.\(^{a}\) | b.d.l.\(^{a}\) |
| Cl\(^{-}\), mg L\(^{-1}\) | 3.02      | 5.0       | 7.46      |
| SO\(_4^{2-}\), mg L\(^{-1}\) | 12.0      | 12.3      | 9.23      |
| NO\(_3^{-}\), mg L\(^{-1}\) | <0.2      | <0.2      | <0.2      |
| NO\(_2^{-}\), mg L\(^{-1}\) | <0.2      | <0.2      | <0.2      |
| Ca\(^{2+}\), mg L\(^{-1}\) | 177.2     | 177.8     | 180.0     |
| Mg\(^{2+}\), mg L\(^{-1}\) | 15.0      | 19.9      | 25.6      |
| NH\(_4^{+}\), mg L\(^{-1}\) | <0.5      | <0.5      | <0.5      |
| Na\(^{+}\), mg L\(^{-1}\) | 2.92      | 2.26      | 2.36      |
| K\(^{+}\), mg L\(^{-1}\) | 1.24      | 1.90      | 1.73      |
| Fe(total), mg L\(^{-1}\) | >2.0      | >2.0      | >2.0      |
| Mineralization, mg L\(^{-1}\) | 804       | 867       | 949       |
| Chemical oxygen demand, mg O\(_2\) L\(^{-1}\) | 78        | 77        | 21.7      |
| Hydrocarbons, mg L\(^{-1}\) | 0.63      | 2.15      | 0.04      |

\(^{a}\) Below detection limit.
3.2. Microbiological investigations

Techniques of polluted subsurface horizons in situ bioremediation could use both aerobic and anaerobic microbial processes. Aerobic process requires regular delivery of oxygen, that is expensive and sometimes difficult [20]. Moreover, aerobic microbial processes accompanied quite often with biofouling of subsurface. Anaerobic microbial processes have not such kind disadvantages and become sometimes the only possible solution to in situ bioremediation [16]. So, the capability of anaerobic bacterial cultures isolated from gas condensate-contaminated groundwater to degrade hydrocarbons was studied, aiming to use them in in situ bioremediation in future.

3.2.1. Bacterial media formulations. Such environmental factors as acid-alkaline conditions and the presence of nitrogen and phosphorus sources available to microorganisms influence on the development of hydrocarbon-degrading bacteria [17, 21]. We used Phosphate-Saline Buffering solution (pH 7.8) as the basic solution for media preparation, as its acid-alkaline conditions are appropriate to most bacteria, and the buffering capacity of the solution allows to support these conditions during a long-time interval [18, 19]. Moreover, the solution contains phosphates, which could be used by bacteria as a source of phosphorus. Ammonium chloride, 0.3 g L\(^{-1}\), was added to the media as a nitrogen source.

The glucose addition was used as the measure to stimulate the growth of methanogenic culture. In fact, methanogenic bacteria are incapable to use glucose in their metabolic reactions but fermentative bacteria do, and the development of methanogens in natural environment proceeds in the closest association with the fermenters [22]. Fermentative bacteria can also use aromatic compounds, but usually, complete biodegradation becomes energetically feasible when accompanying methanogens or sulphate reducers use metabolic end products, such as hydrogen or acetate, that are generated by the fermenters [21, 23]. So, methanogenic enrichment culture contains actually the association of fermentative and methanogenic bacteria, capable to degrade hydrocarbons of gas condensate. It is known that a wide variety of hydrocarbon contaminants can be degraded by sulphate-reducing bacteria directly [8, 24], so the addition of glucose to the medium for sulphate reducers was not necessary.

The strict anaerobic technique to isolate enrichment cultures of anaerobic bacteria was not used. The matter is the amount of gas condensate added to media — the thickness of gas condensate layer was about 2 mm in the necks of flasks that, it was suggested, prevented entry of air oxygen into the media. Indeed, some amount of water dissolved oxygen was present in the media, but it was consumed by aerobic microorganisms in oxidative reactions, so the anaerobic condition was formed and maintained in the cultures.

3.2.2. Enrichment cultures of anaerobic degraders of gas condensate hydrocarbons. According to the methodological guideline, the conclusion about the growth and the development of hydrocarbon-degrading bacteria must be confirmed by visual observations of the turbidity of aqueous phase, the appearance of pigments, the formation of bacterial pellets, the acidification of aqueous medium, and the emulsification of substrates [18, 19]. However, the use of a quite large amount of finely dispersed ground for inoculation of media does not allow to reliably detect the turbidity of aqueous phase of the media and the bacterial pellets formation during the development of microorganisms. Buffer capacity of the media based on Phosphate-Saline Buffering solution do not allow tracing the change in acid-base properties of the media. So, the development of anaerobic hydrocarbon-degrading microorganisms was judged by emulsifying the hydrocarbon film on the surface of the media and the occurring of characteristic ions in the media (see Materials and methods).

A month incubation of the anaerobic enrichment cultures with gas condensate hydrocarbons showed the following results:

- Nitrate reducers. The emulsification of the hydrocarbon film was observed, gas bubbles accumulated under the emuligated film, \(\text{NO}_2^-\) ions were detected in the medium;
- Ferric reducers. The emulsification of the hydrocarbon film was observed, \(\text{Fe}^{2+}\) ions were detected in the medium;
3.2.3. Biotechnological aspects. As a remediation strategy, bioremediation reduces both the contamination of groundwater, as well as the residual soil-bound contamination [26]. The rate of hydrocarbons degradation depends on terminal electron acceptor used by microorganisms — it is higher under the nitrate reduction conditions than under the sulphate reduction [27], and it is higher under the sulphate reduction condition than during methanogenesis [28]. The addition of more energetically favourable to microorganisms alternative electron acceptors to polluted aquifers causes the microbial community to “switch” to their use for the degradation of hydrocarbons [10]. The anaerobic treatment of hydrocarbon contaminated underground horizons by stimulating the vital activity of nitrate-reducing or iron-reducing microorganisms was considered as a possible strategy for remediation purposes [16].

We supposed, the use of nitrate-reducing destructors of hydrocarbons could be more economically, technologically, and ecologically acceptable for the remediation of polluted groundwater, as a) the rate of decomposition of hydrocarbons by nitrate-reducing bacteria is higher than in other anaerobic processes; b) no toxic substances are formed during the reduction of nitrates; c) intermediate metabolite (NO$_2^-$ ions) and an excess of electron acceptor (NO$_3^-$ ions) are easily metabolized by the microbial community; d) nitrate is water soluble, not costly, and not react with other inorganic species present; e) nitrate is not retarded and hence will migrate at groundwater velocity, therefore, nitrate can be applied behind the plume of contaminants, and the processes of advection and dispersion will mix the added nitrate into the plume; f) the cultivation of nitrate-reducing bacteria in laboratory is possible in aerobic conditions, that is technically easy, since they are facultative anaerobes, g) the biomass of these microorganisms can be rapidly accumulated during growth on easily microbe-accessible substrates (for example, glucose) [8, 10, 11, 16, 22-24]. The capability of the enrichment culture of nitrate reducers to degrade gas condensate hydrocarbons after the aerobic growth in the rich medium was confirmed in our study. The culture holds the capability after three consecutive incubations in the rich medium (meat-peptone broth) amended with glucose.

Pure bacterial strains from the enrichment culture of hydrocarbon-degrading nitrate reducing bacteria were not isolated because numerous studies have shown that a single microbial species can degrade only one or two classes of hydrocarbons and a consortia of microorganisms were required to significantly biodegrade a large fraction of hydrocarbon spill [5]. So, biomass of the enrichment culture of hydrocarbon-degrading nitrate reducing bacteria being readily and quickly grown in aerobic conditions on cheap organic media could be directly used to remediate gas condensate-polluted groundwater and ground.
4. Conclusions
The ability of indigenous microbial community to degrade hydrocarbons under anaerobic conditions at the site of gas condensate leakage was investigated. The enrichment microbial cultures of nitrate reducers, ferric reducers, sulphate reducers, and methanogens isolated from polluted ground were capable to degrade hydrocarbons of gas condensate. These cultures demonstrated the ability to grow under laboratory conditions. The enrichment culture of nitrate reducing bacteria held the capability to degrade gas condensate hydrocarbons after three consecutive aerobic incubations in the rich medium, so it could be directly used to remediate gas condensate-polluted groundwater and ground in situ.

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