# The phage-driven microbial loop in petroleum bioremediation

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## Summary

**During the drilling process and transport of crude oil, water mixes with the petroleum. At oil terminals, the water settles to the bottom of storage tanks. This drainage water is contaminated with emulsified oil and water-soluble hydrocarbons and must be treated before it can be released into the environment. In this study, we tested the efficiency of a continuous flow, two-stage bioreactor for treating drainage water from an Israeli oil terminal. The bioreactor removed all of the ammonia, 93% of the sulfide and converted 90% of the total organic carbon (TOC) into carbon dioxide. SYBR Gold staining indicated that reactor 1 contained $1.7 \times 10^8$ bacteria and $3.7 \times 10^8$ phages per millilitre, and reactor 2 contained $1.3 \times 10^8$ bacteria and $1.7 \times 10^9$ phages per millilitre. The unexpectedly high mineralization of TOC and high concentration of phage in reactor 2 support the concept of a phage-driven microbial loop in the bioremediation of the drainage water. In general, application of this concept in bioremediation of contaminated water has the potential to increase the efficiency of processes.**

## Introduction

Bioremediation is an area of applied environmental microbiology. As such, it can benefit from principles of microbial ecology. One such principle, the microbial loop has been largely overlooked in bioremediation studies. The term 'microbial loop' was originally coined by Azam and colleagues (1983) to describe the contribution of bacteria to the turnover of carbon in the sea. The loop results from the fact that a substantial fraction of the organic matter produced by microscopic photosynthetic microorganisms is released into the water as dissolved compounds, and that these compounds are used by bacteria and protists for growth. Thus, rather than going only up the food chain, from photosynthetic microorganisms to protozoa to large animals, as previously believed (Steele, 1974), a loop is formed involving released organic matter and microorganisms. The major role of the microbial loop is that it accelerates carbon dioxide production (mineralization) and recycles nitrogen and phosphorus compounds (Caron, 2004) which are rate limited in an oligotrophic environment. Some of the N and P is immobilized in viruses.

The discovery of abundant populations of bacteriophages (phages, for short) in the sea (Hennes and Suttle, 1995; Noble and Fuhrman, 1998) expanded the concept of the microbial loop (Azam, 1998; Wilhelm and Suttle, 1999). When phages lyse bacteria, most of the cell constituents, including nucleic acids and proteins, are released into the water as dissolved organic carbon. There are two fundamental differences between protozoan grazing on bacteria and phages-lysing bacteria. The first is that with phage lysis the carbon goes 'down' the food chain, whereas with protozoan grazing, most of the carbon goes 'up' the food chain. The second is that, unlike protozoa, phages are host-specific, often attacking only a specific bacterial strain. Because phage : host dynamics predicts that the most abundant bacteria (the ones that would outcompete the others in the absence of predators/parasites) lyse much more rapidly than less abundant bacterial species, phage lysis results in a change in the bacterial community, the so-called 'killing the winner' hypothesis (Thingstad and Lignell, 1997). It has recently been shown that bacterial growth rate also affects the rate of lysis by phage (Winter *et al.*, 2010).

In this article, we shall demonstrate how phages and the microbial loop can play an important role in reducing the total organic carbon (TOC) in drainage water from an oil terminal facility. Drainage water forms whenever crude oil is allowed to stand. The water originates from the drilling process and transportation via oil tankers and slowly settles to the bottom of storage tanks.

## Results

### Chemical analyses of microbiologically treated drainage water in pilot reactors

The first step in the treatment of contaminated water at oil facilities is gravitational separation, generally with an American Petroleum Institute (API) separator. Following API separation at the Eilat-Ashkelon Pipeline (EAPC), the water is termed 'before treatment' (BT). The BT water is pumped continuously and sequentially into reactor 1 (vol. 1 m$^3$) and reactor 2 (vol. 2 m$^3$) at 50 l h$^{-1}$. The process is initiated with aeration and inoculation of 5 l of oil-degrading bacteria (see *Experimental procedures*).

Chemical analysis of the water in reactors 1 and 2 was preformed after steady state equilibrium was achieved, based on constant TOC values in the reactors (day 4). The values are summarized in Table 1. The TOC decreased in reactor 2, compared with before treatment, by 90% and 88% based on direct TOC and (TC – TCi) (total carbon minus total inorganic carbon) analyses respectively. By the end of the process (reactor 2), essentially all of ammonia was consumed, with a small amount being converted to nitrate. Likewise, the sulfide decreased by 93%. Interestingly, the viable bacterial counts increased by 1.9 times in reactor 1 and then decreased by 5.2 times in reactor 2, compared with the BT value. The total microscopic bacterial counts were 3.8 times higher in reactors 1 and 2 than BT. There was a one unit pH increase during the process.

In addition to the analyses shown in Table 1, volatile organic compounds (VOC) and carbon dioxide were measured. VOC was less than 1% in the reactors and BT waters indicating that it did not contribute significantly to the reduction in TOC in the reactors. The amount of carbon dioxide produced in the reactors ($CO_2$ released minus the net decrease in the reactor waters compared with BT water) accounted for 91% of TOC in the BT water, confirming the high value of mineralization in the reactors.

### Bacterial communities based on 16S rRNA gene sequence of uncultured bacteria

There were different abundant bacterial ribotypes in the before treatment, in reactor 1 and in reactor 2 waters (Table 2). In fact, none of the abundant ribotypes in the before treatment water were abundant in the reactors, and only one of the abundant ribotypes was present in both reactor 1 and reactor 2. The before treatment water was dominated by three strains of *Thiomicrospira*, composing 52% of the sample. This species is a sulfur-oxidizing bacterium and probably reflects the presence of sulfide in the drainage water. The main ribotype in reactor 1 was an *Achromobacter*, a group that is known to degrade aromatic hydrocarbons, such as biphenyl (Furukawa *et al.*, 1989). Two dominant bacteria that were reported as cultured in reactor 2 were *Cyclobacterium linum* and *Nitromonas halophila*. The former was previously isolated in the South China Sea (Ying *et al.*, 2006) and the latter is a marine ammonia-oxidizing bacterium (Pommerening-Roeser *et al.*, 1996).

### SYBR gold staining of phages and bacteria

Figure 1 shows a typical picture of the fluorescently stained phages and bacteria in the reactors. Quantitative enumeration revealed reactor 1 contained $1.7 \pm 0.2 \times 10^8$ bacteria per millilitre and $3.7 \pm 0.3 \times 10^8$ phages per millilitre, and reactor 2 contained $1.3 \pm 0.1 \times 10^8$ bacteria per millilitre and $1.7 \pm 0.2 \times 10^9$ phages per millilitre. Thus, ratios of phages to bacteria were 2.2 and 13.1 in reactors 1 and 2 respectively. There were fewer bacteria and more phages in reactor 2 than reactor 1.

**Table 1.** Analyses of microbiologically treated EAPC water.[a]

| Measurement (units)[b] | Before treatment | Reactor 1 | Reactor 2 |
|---|---|---|---|
| TOC (ppm) | 675 ± 3 | 103 ± 4 | 68 ± 0.1 |
| TC (ppm) | 1204 ± 3 | 370 ± 2 | 302 ± 1.5 |
| TCi (ppm) | 475 ± 1 | 258 ± 7 | 212 ± 7.5 |
| TC – TCi (ppm) | 730 ± 6 | 112 ± 6 | 90 ± 7.8 |
| Ammonia (ppm) | 183 ± 1 | 5.2 ± 0.2 | 0.2 ± 0.02 |
| Nitrate (ppm) | 2.3 ± 0.3 | 2.1 ± 0.2 | 4.7 ± 0.2 |
| Inorganic phosphorus (ppm) | 4.8 ± 0.1 | 1.4 ± 0.1 | 2.9 ± 0.03 |
| Sulfide (ppm) | 0.7 ± 0.1 | 0.1 ± 0.003 | 0.05 ± 0.005 |
| Viable bacteria (CFU per millilitre) | $3.8 \pm 0.2 \times 10^7$ | $7.2 \pm 0.3 \times 10^7$ | $7.3 \pm 0.4 \times 10^6$ |
| Total bacteria (microscope) | $1.2 \pm 0.1 \times 10^8$ | $4.5 \pm 0.3 \times 10^8$ | $4.5 \pm 0.4 \times 10^7$ |
| pH | 7.7 | 8.6 | 8.7 |
| Conductivity (mS) | 43 | 44.6 | 42.7 |

**a.** Water from the API oil/water separator (before treatment) was transferred continuously into reactor 1 (vol. 1 m$^3$, 50 l h$^{-1}$) and then to reactor 2 (vol. 2 m$^3$, 50 l h$^{-1}$).
**b.** Measurements were preformed as described in *Experimental procedures*. The average value + SE ($n = 3$) are presented.

**Table 2.** Most abundant bacterial ribotypes in untreated and treated EAPC water.[a]

| Closest match in BLASTn (% identity)[b] | Relative abundance of ribotype in | | |
| --- | --- | --- | --- |
| | BT | Reactor 1 | Reactor 2 |
| *Thiomicrospira* sp. CVO (97.2%) | 26.1% | | |
| Thiomicrospira sp. CVO (97.7%) | 17.4% | | |
| *Thiomicrospira* sp. CVO (97.4%) | 8.7% | | |
| UC gamma proteobacterium 162 t0h-oil (97%) | 8.7% | | |
| *Marinobacter* sp. SCSWE03 (99.8%) | 4.3% | | |
| UC *Achromobacter* sp. 2SN (98.3%) | | 20.8% | |
| UC bacterium GZKB128 (98.5%) | | 12.5% | 13.6% |
| *Halomonas alimentaria* (99.9%) | | 8.3% | |
| *Thiomicrospira* sp. JB-A1F (98.8%) | | 4.2% | |
| *Marinobacterium* sp. IC961 (100%) | | 4.2% | |
| UC bacterium 91-151 (99.6%) | | 4.2% | |
| *Legionella yabuuchiae* strain OA1-3 (94.4%) | | 4.2% | |
| *Cyclobacterium linum* strain RW54 (96.5%) | | | 9.1% |
| UC delta proteobacterium PENS_4 (100%) | | | 4.5% |
| *Nitrosomonas halophila* (99.7%) | | | 4.5% |

**a.** 16S rRNA genes were cloned and sequenced from the before treatment (BT), reactor 1 and reactor 2 waters described in Table 1. The numbers of clones sequenced are 23 (BT), 24 (reactor 1) and 22 (reactor 2).
**b.** Clones which showed > 99.5% sequence identity were clustered. UC stands for uncultured.

### Cultured bacteria and phages from reactor 2

Based on culturing on MB agar, the most abundant bacteria in reactor 2 were *Marinobacter* sp., *Winogradskyella proferorum* and *Pseudomarina* sp. (Table 3). *Marinobacter* sp., which was also found by molecular (culture-independent) techniques (Table 2), is a ubiquitous Gram-negative, non-spore forming bacterium that has been isolated from oil-producing wells (Nguyen *et al.*, 1999). *Winogradskyella poriferorum* is a marine bacterium of the family *Flavobacteriaceae* that is able to degrade polymers, such as protein and DNA (Lau *et al.*, 2005).
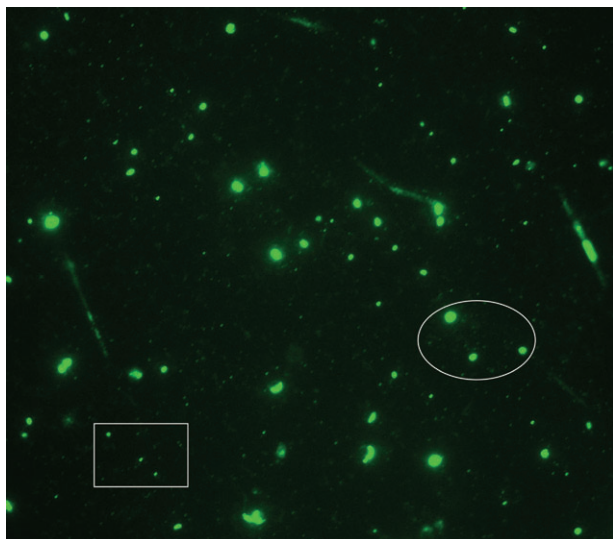


**Fig. 1.** Epifluoresecence-microscopy image of a sample from reactor 2 that was filtered onto a Whatman 0.02 μm Anodisc filter and stained with SYBR gold. The square shows three bacteria, and the circle shows > 20 virus-like particles.

*Pseudomarina* sp. is a non-motile heterotrophic aerobic, marine bacterium (Jean *et al.*, 2009).

Using pure cultures of the cultured bacteria, phages were isolated from reactor 2 water for three of the bacteria (Table 3). Electron micrographs of the isolated phages are shown in Fig. 2.

### Discussion

Chemical analysis based on direct TOC, TC minus TCi and carbon dioxide production all indicated that TOC was reduced by 90% in the two-stage pilot reactor system. This reduction in TOC was much higher than expected because bacteria generally convert 40–60% of substrate carbon into cell carbon (Wodzinski and Johnson, 1968; Chistykova *et al.*, 2005). For example, bacteria can convert 1 g hydrocarbon into 1 g dry cells. Because

**Table 3.** Cultured bacteria from reactor 2.[a]

| Closest match in BLAST (% identity) | Frequency (%) |
| --- | --- |
| *Marinobacter* sp. SCSWE03 (99.7%) | 42.1%[b] |
| *Winogradskyella poriferorum* (98.5%) | 15.8% |
| *Pseudidiomarina* sp. P63 (99.4%) | 10.5%[b] |
| Rhodobacteraceae bacterium CL-GR66 (99.3%) | 5.3% |
| *Oceanicola* sp. W11-2B 16S (97.5%) | 5.3% |
| *Rhodobacter* group bacterium LA5 (98.7%) | 5.3% |
| *Acinetobacter lwoffii* strain BA49 (99.4%) | 5.3% |
| Bacterium K2-69 (99.7%) | 5.3% |
| *Pseudidiomarina taiwanensis* strain PIT1 (98.5%) | 5.3%[b] |

**a.** Water from reactor 2 was diluted and plated on MB agar. Nineteen colonies were randomly selected, obtained in pure culture and classified by their 16S rRNA gene sequences as described in Table 2.
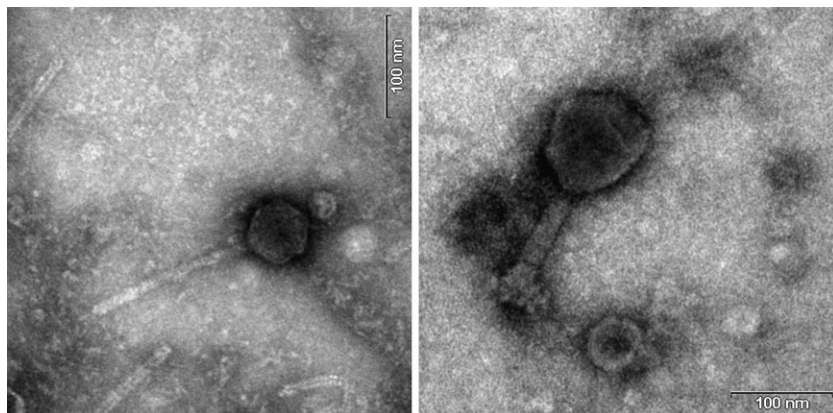**b.** Phages were isolated from pure cultures of these strains.

**Fig. 2.** Electron micrographs of bacteriophages isolated from reactor 2. Right – ΦMarinobacter; Left – ΦP63.

hydrocarbons and bacterial cells contain approximately 87% and 50% carbon, respectively, the expected reduction in TOC is 43%. With a more oxidized substrate, such as glucose, the cell yield is approximately 25%. However, glucose contains only 40% carbon, so that the expected reduction in TOC is 69%.

The possible explanations for the higher than expected decrease in TOC in the two-stage reactor are (i) uncoupled substrate degradation without cell growth and (ii) bacteria cell lysis followed by degradation of released bacterial compound, i.e. the phage-driven microbial loop. Uncoupled substrate degradation occurs during unbalanced growth and other stress conditions (Forrest, 1967). However, the data presented here appear to more easily be explained by a phage-driven microbial-loop. This latter hypothesis is supported by the large concentration of phages, especially in reactor 2, and the change in bacterial communities from the input to reactor 1 and then to reactor 2. Part of the increase in phage in the reactors could be due to induction of the lytic cycle in lysogens (Cochran *et al.*, 1998). It should be pointed out that the estimated number of phages is probably an underestimation because not all viruses are in the same focal plane and those that are out of focus may not be visible (Patel *et al.*, 2007). Protozoan predation of bacteria did not appear to play a major role in the reactors because they were in very low abundance, possibly because of the toxicity of hydrocarbons and hydrogen sulfide in the input water.

Withey and colleagues (2005) have discussed the potential applications of bacteriophages in wastewater treatment and indicated the need for a better understanding of wastewater microbial community dynamics. Recently it has been proposed that phage play a role in modifying the bacterial composition of a membrane reactor treating industrial wastewater (Shapiro *et al.*, 2010). There are at least two possible applications for the principle of the phage-driven microbial loop in treatment of polluted water. In facilities that provide large quantities of polluted water, such as oil refineries which treat up to 1000 m$^3$ h$^{-1}$ of oily water, TOC is reduced by flocculating the microorganisms and disposing of the of the resulting sludge at a disposal site. By lysing the bacteria with phages the quantity of sludge should be greatly reduced, saving part of the cost of disposal. In the treatment of lower volumes of polluted water, such as the drainage water at oil farms and bilge water on ships, flocculation is not practical. In such cases, properly controlling the phage-driven microbial loop may reduce the TOC to levels that meet international standards for direct disposal in the sea.

The study reported here was carried out at only one set of conditions. Further research should be carried out on the effect of parameters, such as temperature and residence time in the reactors, on yield of bacteria and phage and reduction of TOC. However, it is clear that phages can have a positive effect on bioremediation when using mixed cultures, whereas they have a detrimental effect when using pure cultures.

## Experimental procedures

### Continuous flow two-stage reactor

A two-stage continuous flow reactor system based on the Automated Chemostat Technology (ACT) developed by Bio-PetroClean (BPC) was installed next to the API separator at the EAPC. After the contaminated drainage water was fractionated by gravitational separation, the water was fed into reactor 1 (vol. 1 m$^3$) at 50 l h$^{-1}$. The efflux water was then fed into reactor 2 (vol. 2 m$^3$). The water in the reactor was maintained at 27 ± 1°C. The reactors were aerated with spargers so that dO$_2$ was maintained at 5 ± 2 mg l$^{-1}$. To initiate the microbial process, reactor 1 was inoculated with 5 l of a mixed bacterial culture that had been enriched on drainage water from EAPC. The inoculum included bacteria that had been previously isolated from the Haifa, Israel refinery.

## Chemical analysis

Total organic carbon, total carbon (TC) and total inorganic carbon (TCi) were determined using a Shimadzu (Kyoto) analyser according to standard methods, BS EN 1484:1997, EN 13137 and EPA 415.1 respectively. Ammonia was determined with an electrode (Thermo Fisher Scientific, Waltham, MA). Nitrate, phosphate and sulfide were determined photometrically, using kits provided by Merck, Darmstadt, according to standard methods ISO 7890/1, EPA 965.2+3 and EPA 376.2 respectively. VOC and carbon dioxide were determined by A.S. Reasearch Services, Givat Yearim, Israel (Certified by ISO-17025), using GC-MS.

## Isolation and enumeration of bacteria and phage

Total bacteria and phage counts were performed with SYBR gold staining (Molecular Probes) according to the SYBR green protocol (Noble and Fuhrman, 1998). Oil-degrading bacteria were enriched, using water from reactor 2 as the inoculum, and a medium containing artificial sea water (ASW, Instant Ocean) saturated with hydrocarbons from gasoline, according to Saeed and Al-Mutairi (1998). The medium was supplemented with 20 ml $l^{-1}$ urea and 5 ml $l^{-1}$ potassium phosphate as nitrogen and phosphate sources, respectfully. The enrichment procedure was repeated four times and then streaked on both MB agar (1.8% Marine Broth Difco 2216, 0.9% NaCl and 1.8% agar) and minimal agar plates (1.8% agar, 2% urea, 0.5% potassium phosphate, in ASW). Plates were incubated 'inverted' at 30°C for 5 days with a Whatman 114 filter containing gasoline on the lid. Twenty of the colonies that appeared at the highest dilutions were isolated and restreaked on MB agar and grown as pure culture on the gasoline minimal medium. Isolates were subsequently used for phage isolation and 16S rRNA gene analysis.

Phages were isolated by inoculating 10 ml of MBT medium (1.8% Marine Broth 2216, 0.9% NaCl and 0.5% Difco Tryptone) with 0.1 ml of a pure culture from reactor 2 and incubating at 30°C with shaking (160 r.p.m.). After 4 h, 10 ml of filtered (0.22 μm Filter, Millipore) water from reactor 2 was added and incubation was continued. After 24 h, the culture was again filtered and dilutions plated by the soft agar overlay technique (Adams, 1959), and incubated for 24 h at 30°C. Several rounds of plaque purifications were performed to ensure a pure phage stock. High titre phage lysates were negatively stained with 1% uranyl acetate and examined with IEOL 840A electron microscope at 80 kV.

## DNA extraction, PCR amplification of 16S rRNA genes, clone library construction and sequence analysis

Water samples were centrifuged at $16\,000\times g$ for 15 min, and DNA was extracted from the pellets with the UltraClean Soil DNA kit (MoBio, Carlsbad, CA). For culturable bacteria, colony DNA was extracted with the Wizard Genomic DNA purification kit (Promega Corp., Madison, WI). Primers 16S8F and 16S1492R (Lane, 1991) were used for amplification of the 16S rRNA genes from isolated bacterial genomic DNA and environmentally extracted DNA. 16S rRNA genes of the isolated strains were amplified in 25 μl of reaction mixture consisting of 2.5 μl of 10× buffer, 0.5 μl of 2.5 mM total deoxy-

nucleoside triphosphate mixture, each primer at 5 μM, 10 ng of template DNA, and 1.25 U of Ex Taq DNA polymerase (TaKaRa Bio, Shiga, Japan). Amplification conditions for the PCR included an initial denaturation step of 94°C for 3 min, followed by 30 cycles of 94°C for 35 s, 56°C for 35 s and 72°C for 45 s and a final extension step of 72°C for 3 min. Reaction products were checked for size and purity on 1% agarose gel.

Amplified DNA from water samples was ligated into the pGEM-T Easy vector by the protocol of the manufacturer (Promega Corp., Madison, WI). The ligated vector and insert were transformed into competent *Escherichia coli* TG1 cells. Each clone was amplified by colony PCR with M13 forward and reverse primers. Amplification conditions for the colony PCR included an initial denaturation step of 95°C for 4.5 min, followed by 30 cycles of 95°C for 0.5 min, 59.5°C for 0.5 min and 72°C for 1 min and a final extension step of 72°C for 10 min. Reaction products were checked for size and purity on 1% agarose gel.

PCR products were cleaned with ExoSAP-IT (USB Corporation, USA). DNA sequencing was performed by the chain termination method in an ABI Prism (model 377, version 2.1.1) automated sequencer. Primer used for the sequencing reaction was complementary to the conserved region of the 16S rRNA genes (16S8F).

Sequences were aligned with ClustalX (Thompson *et al.*, 1997), and a DNA distance matrix was created with BioEdit. Sequences that had > 99.5% identity were clustered together with DOTUR (Schloss and Handelsman, 2005). BLASTN (Altschul *et al.*, 1990) (http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi) was then used to characterize each sequence cluster.

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