Journals of Materials Chemistry A

Supporting Information

Enhanced Enzymatic and *Ex Situ* Biodegradation of Petroleum Hydrocarbons in Solutions using *Alcanivorax borkumensis* Enzymes in the Presence of Nitrogen, and Phosphorous Co-doped Reduced Graphene Oxide as a Bacterial Growth Enhancer

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Farzin Nekouei *, Shahram Nekouei

Total protein and Enzymes activities assays

Total protein assay: The procedure is summarized in the following steps: (1) preparation of a series of bovine serum albumin solutions (30 to 150 µg mL^{-1}) in the same buffer or solvent used to prepare the sample; (2) addition of 1.0 mL of the protein-containing sample to 0.90 mL of Hartree-Lowry reagent A (2 g $KNaC_4H_4O_6{\cdot}4H_2O$ + 100 g Na_2CO_3 +500 mL 1 N NaOH + 500 mL H_2O) in all tubes containing different concentrations of bovine serum albumin solutions; (3) Incubation of all samples in a water bath (50 °C) for 10 min; (4) cooling all tubes to room temperature and addition of Hartree-Lowry reagent B (2 g $KNaC_4H_4O_6{\cdot}4H_2O$ + 1 g $CuSO_4.5H_2O$ + 10 mL 1 N NaOH + 90 mL H_2O +) to each tube and incubation for 10 min at room temperature; (5) fast addition of 3 mL Hartree-Lowry reagent C (dilution of Folin Ciocalteau reagent with water by the ratio of 1:15) to each tube and mixing and incubation in a 50 °C for 10 min and cooling to room temperature (assay volume: 5 mL); (6) spectrophotometric measurements at 650 nm and

Young Researchers and Elites Club, Science and Research Branch, Islamic Azad University, Tehran, Iran
*F.nekouei@hotmail.com; Fa.nekouei@gmail.com
† Footnotes relating to the title and/or authors should appear here.
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determination of the protein concentration by interpolation from the plot (Abs. ***vs*** µg serum albumin).

Lipase assay: 40 mg of *p*NPP was dissolved in 12 mL of propane-2-ol and added to 9.5 mL of a solution containing 0.1 g of gum Arabic and 0.4 g of Triton X-100 in 90 mL of distilled water under rigorous stirring condition for 2 h. After incubating the mixture consisted of 0.9 mL of substrate solution, 0.1 mL of suitable buffer (0.5 M) and 0.1 mL of suitably diluted enzyme at 50 °C for 30 min, the released *p*-nitrophenol was determined at 410 nm in Spectronic-117 spectrophotometer. One unit of enzyme activity was defined as 1 µmol of *p*-nitrophenol released per minute.

Esterase assay: a reaction mixture was prepared by mixing 980 µL of *p*-nitrophenylbutyrate (0.56 mM) solution in 50 mM potassium phosphate buffer (pH 7) with 11.3 mM sodium cholate and 0.43 M tetrahydrofuran. 20 µL of sample was added to the reaction mixture and monitored against blank solution for 15 min. One unit of esterase activity was defined as the amount of esterase required to release 1 µmol of *p*-nitrophenol in one minute, under the specified conditions. All the enzymes were tested in triplicates.

Alkane hydroxylase assay: After diluting the supernatant containing the enzyme into a mixed solution containing 200 µL phosphate buffer (0.1 M, pH 8), alkane substrate (0.5-1 mM), and dimethyl sulfoxide (DMSO; 1%, v/v), alkanes (in DMSO) were added to the buffer. 300 µL NADPH (200 µM, as reducing agent) was added to the above mixture and the reaction was measured spectrophotometrically at 340 nm.

Table(s)

Table S1. Chemical compositions of synthesized materials using XPS analysis.

Sample	N level, at.%	O level, at.%	C level, at.%	P level, at.%
GO	0.50	30.47	68.05	-
N-rGO	6.85	10.45	82.35	-
N-P-rGO	6.70	9.1	81.49	1.05

Table S2. Summary of results related to the effect of nutrients (N and P) on *A. borkumensis* growth with hexadecane.

Bacteria environment	Average lag time, λ (h)	Average specific growth rate (cells/h)[a]	Max bacteria concentration (cells/mL)
N-P-rGO	11	4.35×10^7	5.59×10^8
N-rGO	15	3.70×10^7	6.35×10^8
GO	38	2.32×10^7	9.62×10^8
Only bacteria	40	2.28×10^7	9.92×10^8

[a] All experiments were Run two times with three replicates each. Error was calculated according to the standard deviation of all six replicates. An error of 20% would be surmised for all calculated growth rates.

Table S3. Summary of results corresponded to the effect of N-P-rGO dose on *A. borkumensis* growth with hexadecane.

N-P-rGO dose (g/ L)	Average lag time, λ (h)	Average specific growth rate (cells/h)[a]	Maximum bacteria concentration (cells/mL)
0	40	2.28×10^7	9.92×10^8
0.25	33	2.74×10^7	9.84×10^8
0.5	25	3.26×10^7	8.95×10^8
0.75	17	3.80×10^7	7.26×10^8
1.0	11	4.35×10^7	5.59×10^8

[a] All experiments were Run two times with three replicates each. Error was calculated according to the standard deviation of all six replicates. An error of 20% would be surmised for all calculated growth rates.

Table S4. Recovery efficiency of N-P-rGO for hexadecane (5%, 4000 ppm) after 7 days. Culture conditions: 31 °C, pH 8.0, 120 rpm.

Cycle no.	Removal (%)	N content (at.%)	P content (at.%)
1	96.7	6.40	1.00
2	94.5	6.00	0.94
3	90.8	5.10	0.85
4	78.6	2.10	0.35

Figure(s)

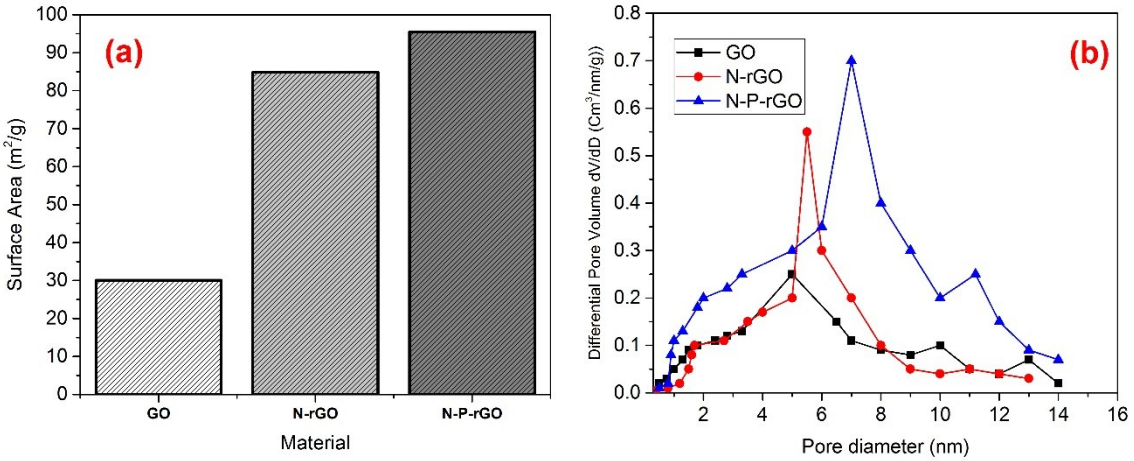


Fig. S1. (a) Specific surface areas of GO, N-rGO, and N-P-rGO; **(b)** Pore size distribution of GO, N-rGO, and N-P-rGO.

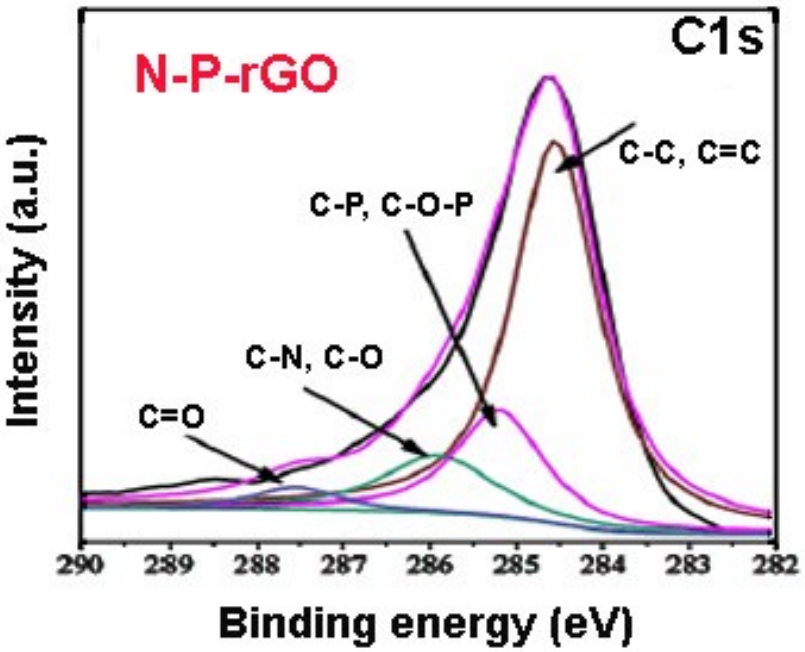


Fig. S2. High resolution spectrum of C 1s for N-P-rGO.

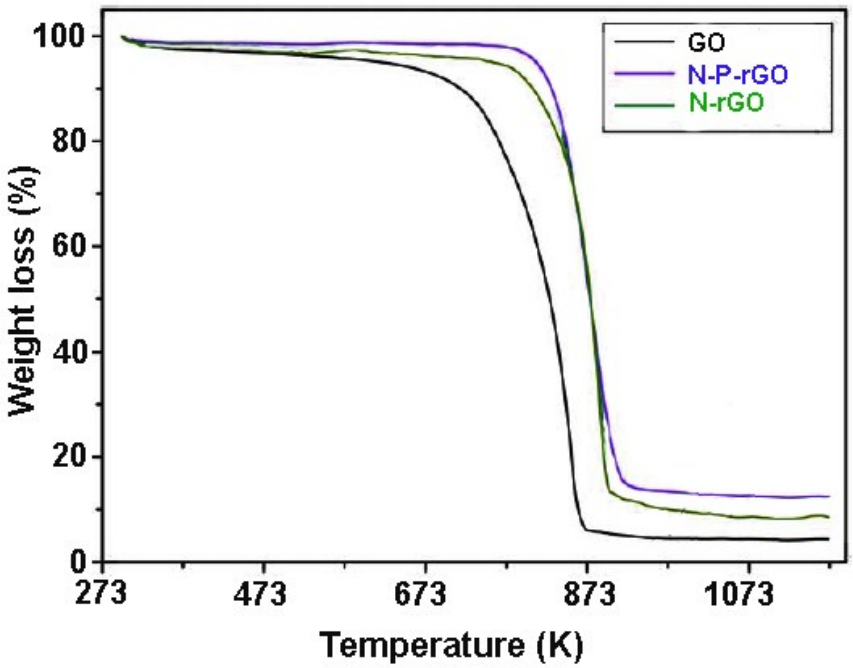


Fig. S3. TGA curves of GO, N-rGO, and N-P-rGO.

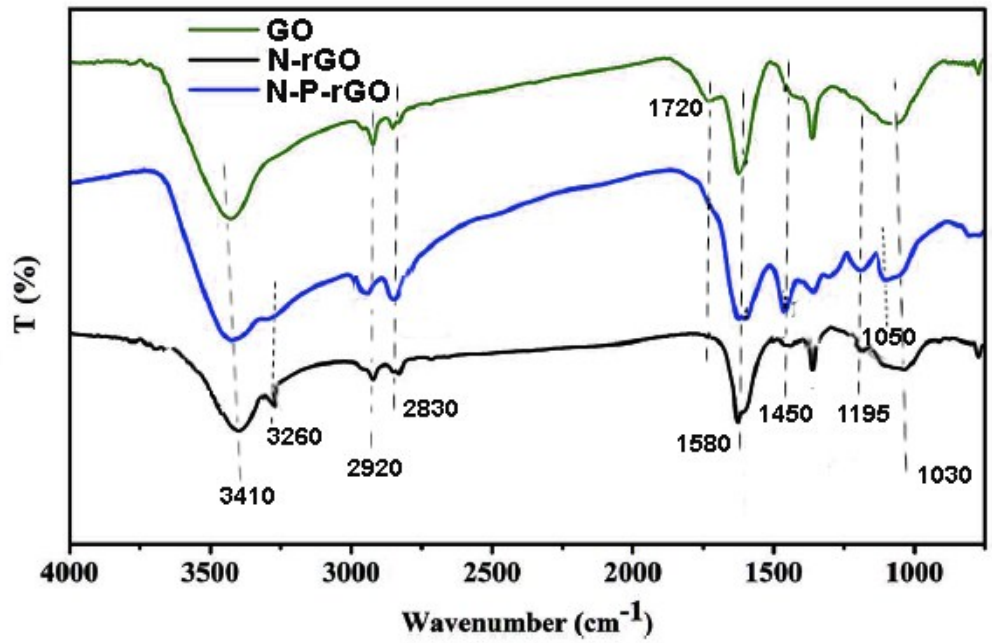


Fig. S4. IR spectra of GO, N-rGO, and N-P-rGO.

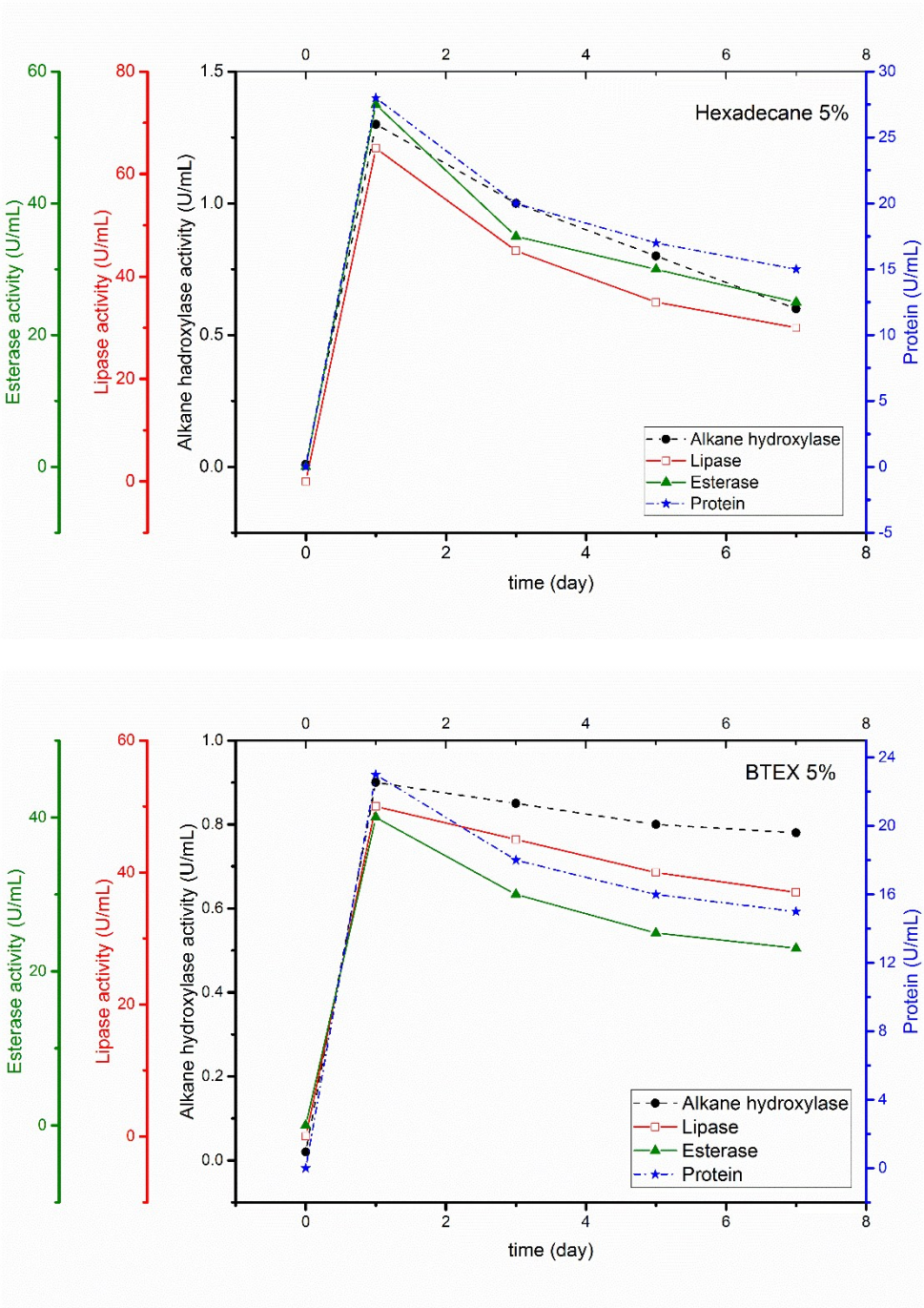
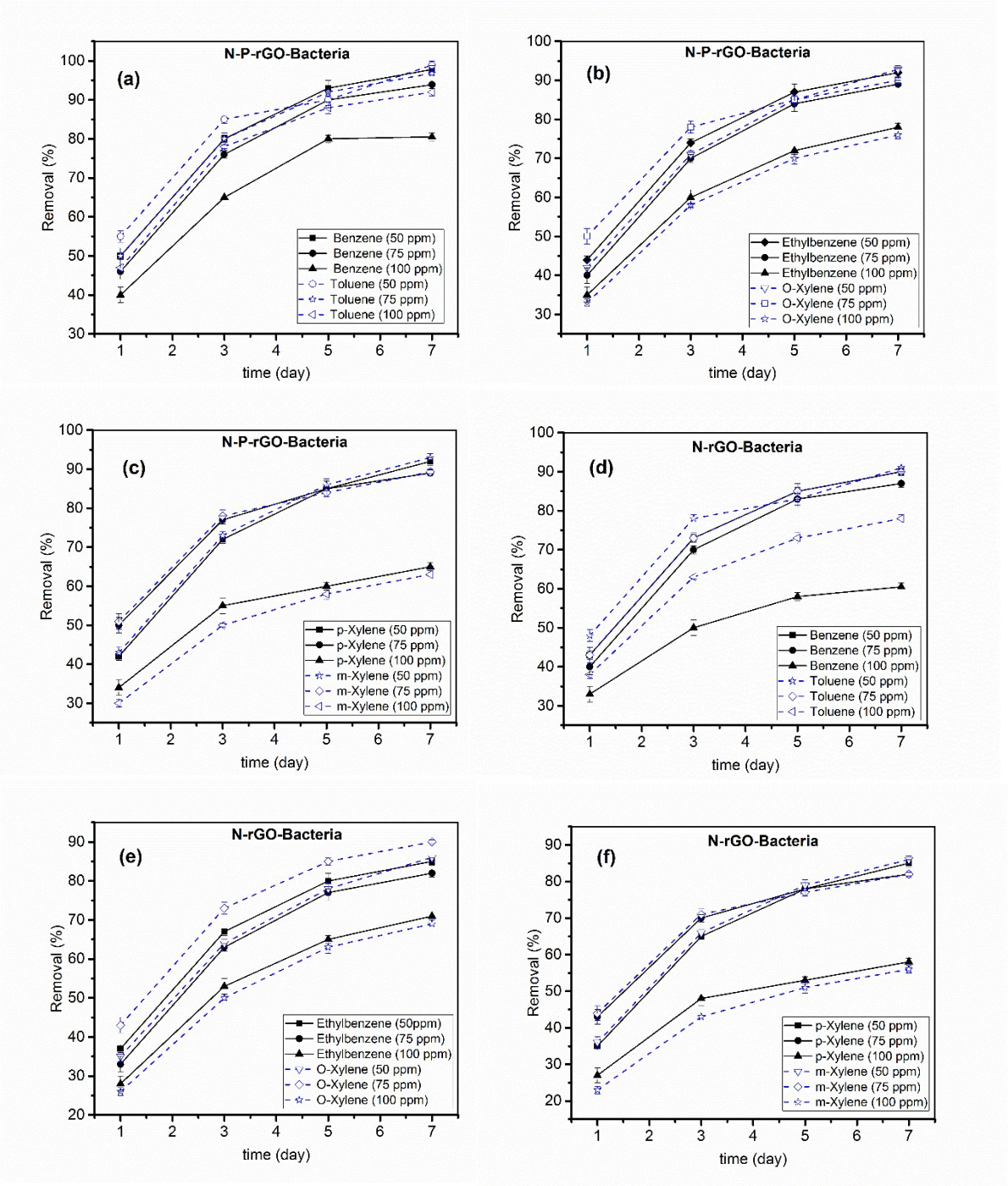
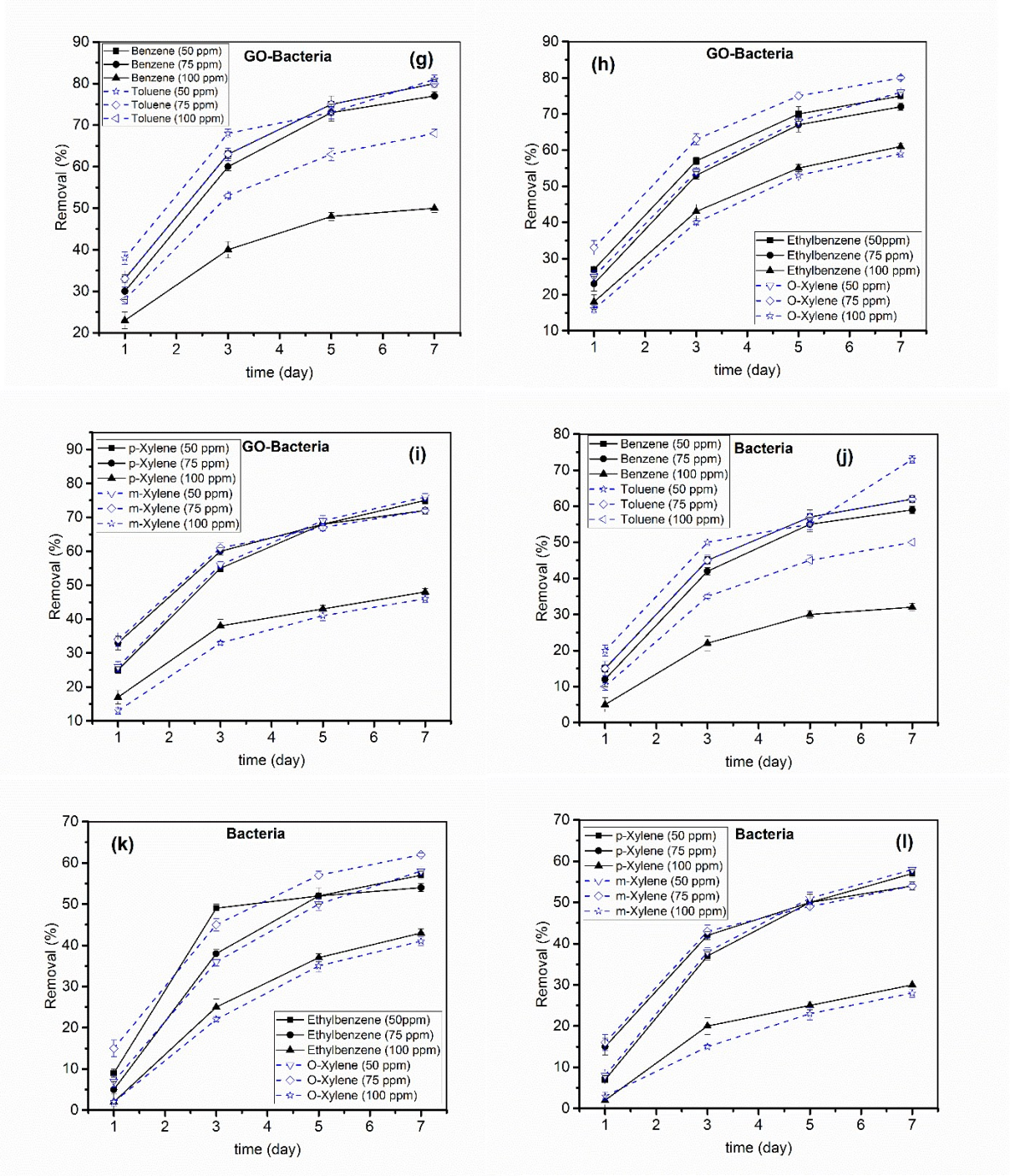


Fig. S5. Evolution of enzymes activities and protein contents during the biodegradation processes for hexadecane and BTEX. Culture conditions: 31°C, pH 8.0, 120 rpm, 7 days.


N-P-rGO-Bacteria
(a)
Removal (%)
time (day)
Benzene (50 ppm)
Benzene (75 ppm)
Benzene (100 ppm)
Toluene (50 ppm)
Toluene (75 ppm)
Toluene (100 ppm)
N-P-rGO-Bacteria
(b)
Removal (%)
time (day)
Ethylbenzene (50 ppm)
Ethylbenzene (75 ppm)
Ethylbenzene (100 ppm)
O-Xylene (50 ppm)
O-Xylene (75 ppm)
O-Xylene (100 ppm)
N-P-rGO-Bacteria
(c)
Removal (%)
time (day)
p-Xylene (50 ppm)
p-Xylene (75 ppm)
p-Xylene (100 ppm)
m-Xylene (50 ppm)
m-Xylene (75 ppm)
m-Xylene (100 ppm)
N-rGO-Bacteria
(d)
Removal (%)
time (day)
Benzene (50 ppm)
Benzene (75 ppm)
Benzene (100 ppm)
Toluene (50 ppm)
Toluene (75 ppm)
Toluene (100 ppm)
N-rGO-Bacteria
(e)
Removal (%)
time (day)
Ethylbenzene (50ppm)
Ethylbenzene (75 ppm)
Ethylbenzene (100 ppm)
O-Xylene (50 ppm)
O-Xylene (75 ppm)
O-Xylene (100 ppm)
N-rGO-Bacteria
(f)
Removal (%)
time (day)
p-Xylene (50 ppm)
p-Xylene (75 ppm)
p-Xylene (100 ppm)
m-Xylene (50 ppm)
m-Xylene (75 ppm)
m-Xylene (100 ppm)



S6. Removal percentage of BTEX compounds in different concentrations in the presence of: **(a-c)** N-P-rGO; **(d-f)** N-rGO; **(g-i)** GO; **(j-l)** only bacteria within 7 days using the crude enzyme produced by *A. borkumensis.* Culture conditions: 31 °C, pH 8.0, 120 rpm.

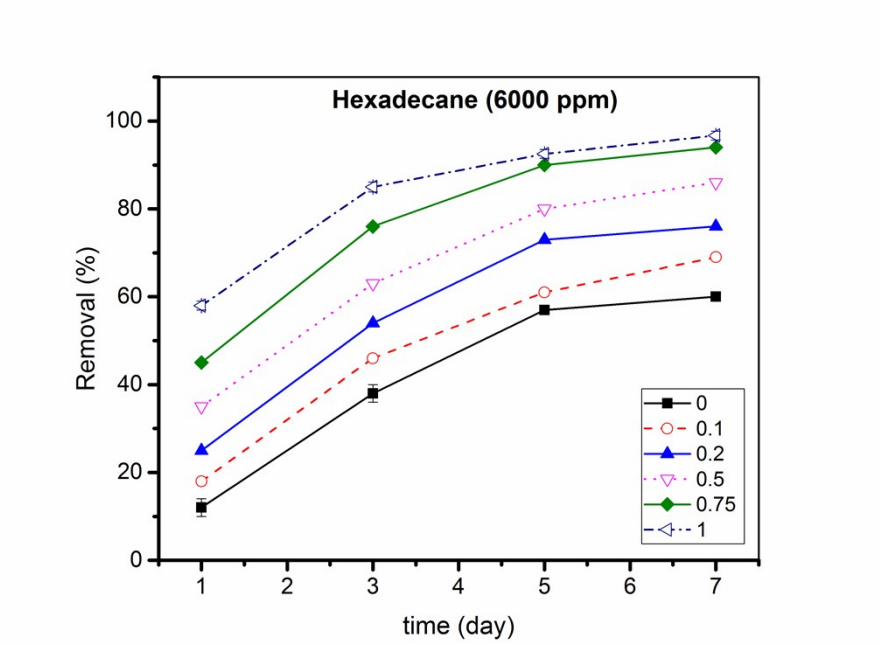


Fig. S7. The effect of N-P-rGO dose on the hexadecane removal within 7 days using the crude enzyme produced by *A. borkumensis.* Culture conditions: 31 °C, pH 8.0, 120 rpm.

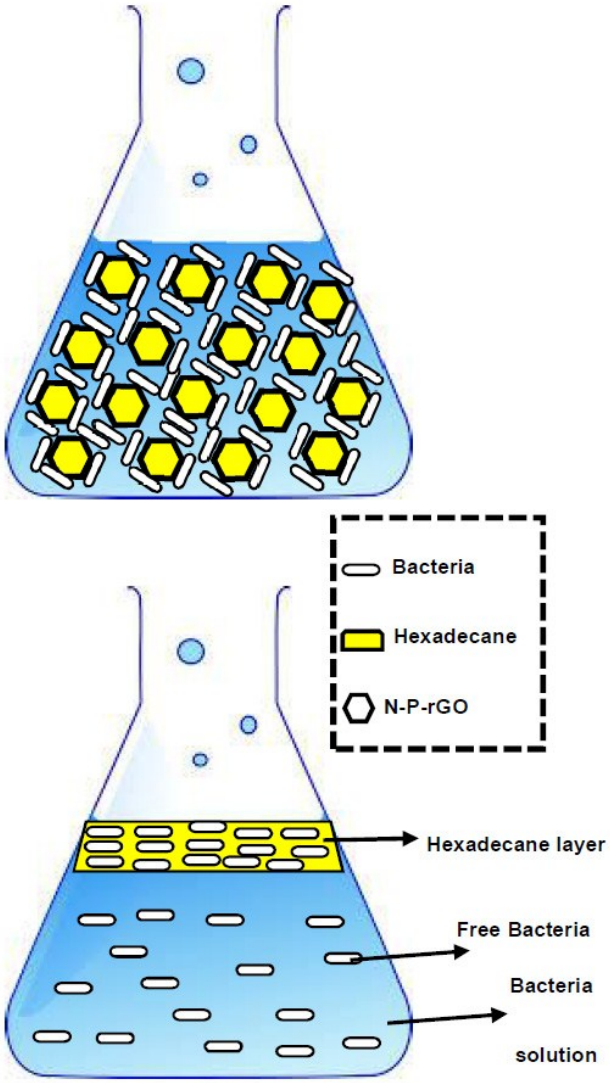


Fig. S8. Schematic illustration the effects of higher interfacial area and hydrophobic adsorption of hexadecane in the presence of N-P-rGO.

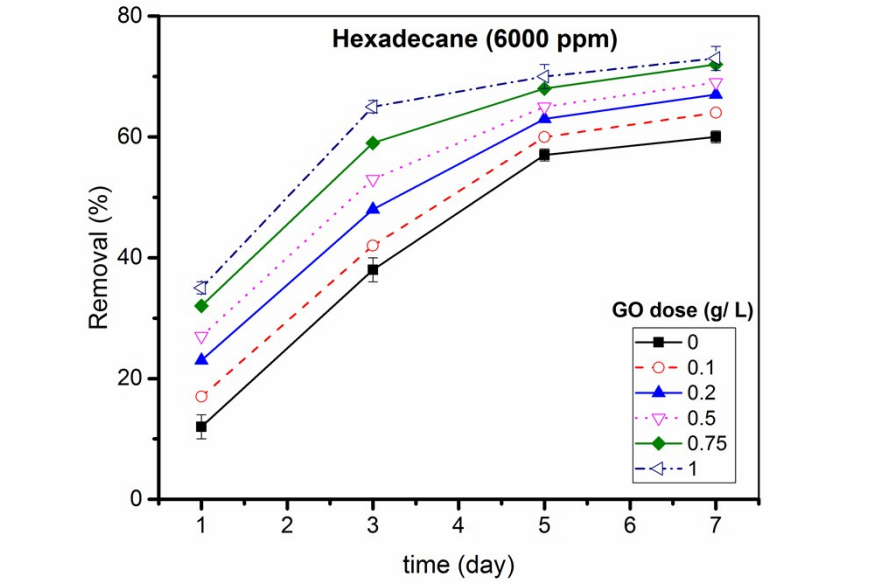


Fig. S9. The effect of GO dose on the hexadecane removal within 7 days using the crude enzyme produced by *A. borkumensis.* Culture conditions: 31 °C, pH 8.0, 120 rpm.

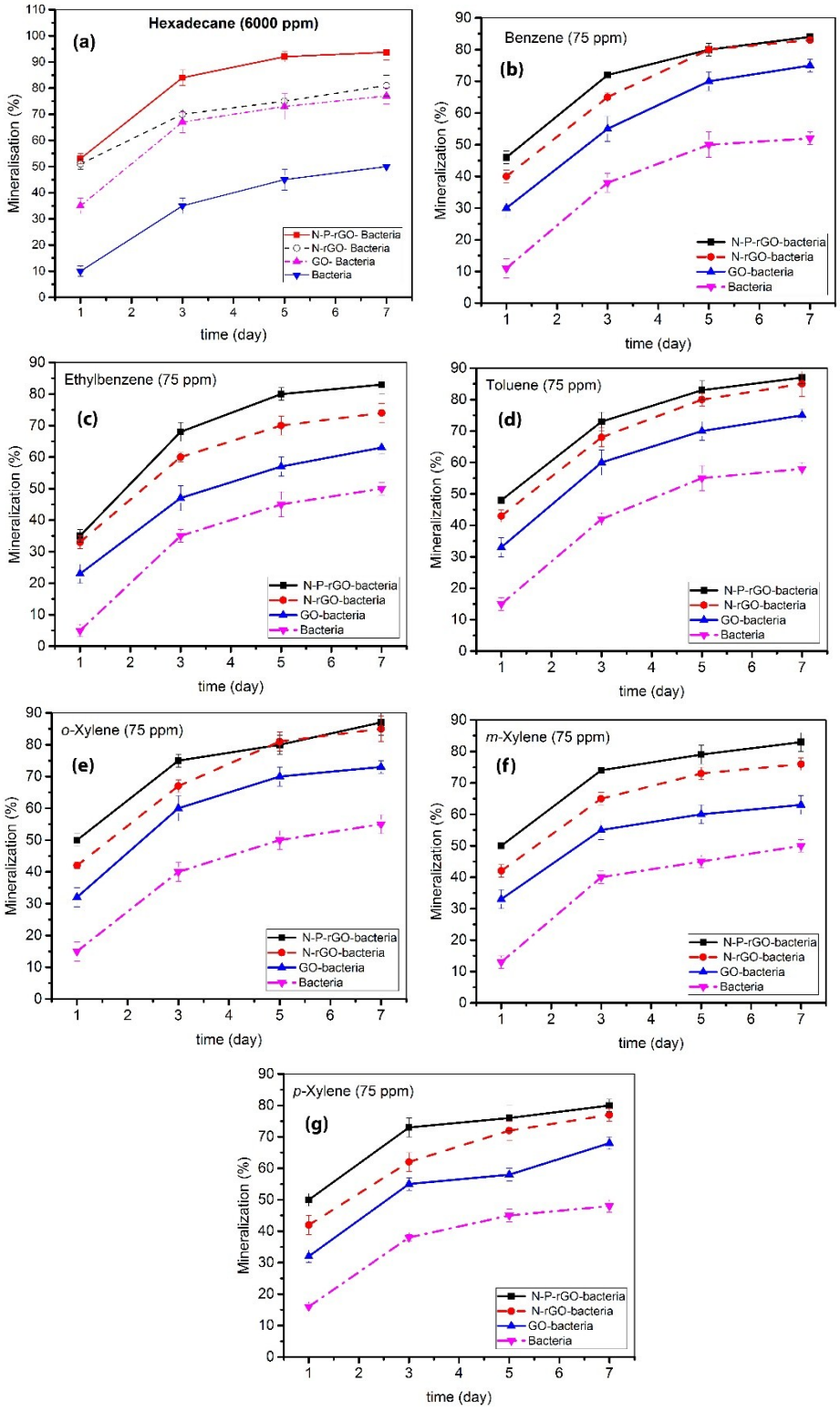
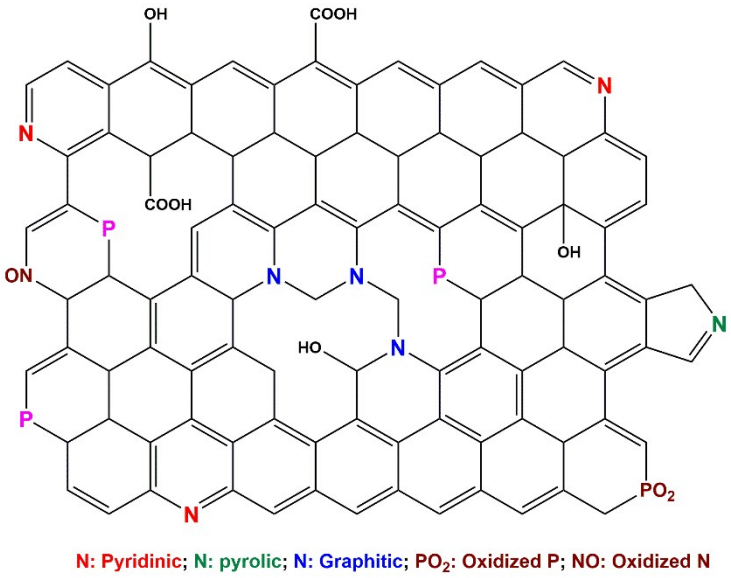


Fig. S10. Mineralisation assays for the ^{14}C-labelled of **(a)** hexadecane (6000 ppm) and **(b-g)** BTEX compounds (75 ppm).



Scheme S1. Schematic structure of N-P-rGO is presented in **Scheme S1**.