Supplementary material

MICROAEROBIC BIODEGRADATION OF AROMATIC HYDROCARBON MIXTURES: STRATEGIES FOR EFFICIENT NITRATE AND OXYGEN DOSAGE

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Supplementary Data 1: BTEXIeIaN mixture

Table S1. Volumes added to achieve equal mass of 100 mg of each compound for the preparation of the pure BTEXIeIaN mixture.

Compound	Density ($g\ mL^{-1}$)	Amount (mg)	Volume added (µL)
Benzene	0.879	100	114
Toluene	0.862	100	116
Ethylbenzene	0.867	100	115
o-Xylene	0.880	100	114
m-Xylene	0.860	100	116
p-Xylene	0.860	100	116
Indene	0.965	100	104
Indane	0.996	100	100
Naphthalene	1.160	100	Solid (0.1 g)

Concentrations of the pure mixture of BTEXIeIaN measured in HPLC

Once that the pure mix was added to the batch bottles, the mass ratio was found to be not equally distributed (5:2:1:2:1:2:1:2, B:T:E:o-X:m/p-X:Ie:Ia:N). Initial concentrations for each compound at the beginning of the experiment is given in Table S2. It is hypothesized that the uneven distribution is due to headspace-liquid distribution in the batch bottles. As the analysis were done only by monitoring the liquid phase, it was assumed that compounds were also present in gas phase or absorbed to the sediment resulting to variable ratios of the compounds in the bottles. Since all bottles were prepared within the same procedure, and had similar initial BTEXIeIaN concentrations and ratios, this issue was disregarded as it would not affect the experimental design. This issue was by-passed by comparing the active bottles to the control bottles.

Table S2. Initial concentrations (mean of five bottles: 3 active and 2 control) for each compound at the beginning of the experiment for set up I, II and III.

Initial concentration (mg L^{-1})

	Set up I	Set up II	Set up III
Benzene	2.56 ±0.09	2.54 ±0.16	3.58 ±0.09
Toluene	1.22 ±0.06	1.26 ±0.15	2.10 ±0.06
Ethylbenzene	0.60 ±0.02	0.64 ±0.13	1.19 ±0.05
o-Xylene	0.85 ±0.02	0.91 ±0.18	1.80 ±0.05
m/p-Xylene	0.63 ±0.03	0.68 ±0.13	1.15 ±0.07
Indene	1.16 ±0.02	1.27 ±0.23	2.41 ±0.10
Indane	0.54 ±0.02	0.60 ±0.15	1.38 ±0.05
Naphthalene	0.91 ±0.02	1.03 ±0.24	1.54 ±0.30

±; standard deviation

Supplementary Data 2: Oxygen calculations

Absolute oxygen values added to the bottles

On day 1, 4, 7, 14, 21, 42 (set up I) and 60, 67 (set up II) oxygen was added to the bottles. For this 8.5 mL air was injected. First, the amount of 8.5 mL air was converted to mol based on the ideal gas law (Eq.S1) and further to mg of oxygen (Eq.S2). For this the atmospheric pressure of each day of was considered (Table S3).

Table S3. Pressure (P) of the atmospheric air on the days of oxygen addition.

	Date	day	Hg	mbar	atm
Set up I	24-02-2022	1	29.7	1005.8	1.01
	03-03-2022	7	30.1	1019.3	1.02
	10-03-2022	14	30.2	1022.7	1.02
	17-03-2022	21	30.4	1029.5	1.03
	07-04-2022	42	29.0	982.10	0.98
Set up II	25-04-2022	60	29.9	1012.53	1.01
	02-05-2022	67	30.2	1022.69	1.02

For the calculation of the added oxygen (n), Equation S1 and Equation S2 were used based on the values given in Table S4.

Calculation of the oxygen (in mol) based on the ideal gas law:

$$n = P*V / R*T \quad \textbf{(Equation S1)}$$

Calculation of oxygen (mol to mg):

$$n*32 \text{ (oxygen in g)} * 1000 \text{ (to mg)} *21/100 \text{ (21\% oxygen in air)} \quad \textbf{(Equation S2)}$$

Table S4. Theoretical calculation of oxygen (in mg) added to the bottles for set up I and II.

Set up	day	P (atm)	V (L)	R (L atm K^{-1} mol^{-1})	T (°K)	n (mol)	n (g)	n (mg)	*21 (21% oxygen)
I	1	1.01	0.0085	0.082	293.15	0.00	0.01	11.38	2.39
	7	1.02	0.0085	0.082	293.15	0.00	0.01	11.53	2.42
	14	1.02	0.0085	0.082	293.15	0.00	0.01	11.57	2.43
	21	1.03	0.0085	0.082	293.15	0.00	0.01	11.65	2.45
	42	0.98	0.0085	0.082	293.15	0.00	0.01	11.11	2.33
									Total: 12.02 mg
II	60	1.01	0.0085	0.082	293.15	0.00	0.01	11.46	2.39
	67	1.02	0.0085	0.082	293.15	0.00	0.01	11.57	2.42
									Total: 4.82 mg

Table S5. Oxygen percentage measured in GC (in blue) and their calculations to mg (in orange) for each bottle of set up I (SI) and set up II (SII). C1 and C2 are duplicates of the control bottles.

day 1	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SI-1	1.14	100	0.1	0.0821	293	0.00	0.15	151.65	1.14		1.73	
SI-2	1.26	100	0.1	0.0821	293	0.01	0.17	167.61	1.26		2.11	
SI-3	1.17	100	0.1	0.0821	293	0.00	0.16	155.64	1.17		1.82	
SI-C1	1.51	100	0.1	0.0821	293	0.01	0.20	200.87	1.51		3.03	
SI-C2	1.31	100	0.1	0.0821	293	0.01	0.17	174.27	1.31		2.28	
Active (mean)									*1.19*	*±0.05*	*1.89*	*±0.16*
Control (mean)									*1.41*	*±0.10*	*2.66*	*±0.38*
day 7	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SI-1	1.39	102	0.102	0.0821	293	0.01	0.19	188.61	1.39		2.62	
SI-2	1.52	102	0.102	0.0821	293	0.01	0.21	206.24	1.52		3.13	
SI-3	1.46	102	0.102	0.0821	293	0.01	0.20	198.10	1.46		2.89	
SI-C1	1.49	102	0.102	0.0821	293	0.01	0.20	202.17	1.49		3.01	
SI-C2	1.24	102	0.102	0.0821	293	0.01	0.17	168.25	1.24		2.09	
Active (mean)									*1.46*	*±0.05*	*2.88*	*±0.21*
Control (mean)									*1.37*	*±0.13*	*2.55*	*±0.46*
day 14	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SI-1	1.42	104	0.104	0.0821	293	0.01	0.20	196.45	1.42		2.79	
SI-2	1.55	104	0.104	0.0821	293	0.01	0.21	214.44	1.55		3.32	

SI-3	1.50	104	0.104	0.0821	293	0.01	0.21	207.52	1.50		3.11	
SI-C1	1.40	104	0.104	0.0821	293	0.01	0.19	193.69	1.40		2.71	
SI-C2	1.22	104	0.104	0.0821	293	0.01	0.17	168.78	1.22		2.06	
Active (mean)									*1.49*	*±0.05*	*3.08*	*±0.22*
Control (mean)									*1.31*	*±0.09*	*2.39*	*±0.33*
day 21	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SI-1	1.79	106	0.106	0.0821	293	0.01	0.25	252.41	1.79		4.52	
SI-2	1.89	106	0.106	0.0821	293	0.01	0.27	266.51	1.89		5.04	
SI-3	1.72	106	0.106	0.0821	293	0.01	0.24	242.53	1.72		4.17	
SI-C1	1.59	106	0.106	0.0821	293	0.01	0.22	224.20	1.59		3.56	
SI-C2	1.30	106	0.106	0.0821	293	0.01	0.18	183.31	1.30		2.38	
Active (mean)									*1.80*	*±0.07*	*4.58*	*±0.36*
Control (mean)									*1.45*	*±0.15*	*2.97*	*±0.59*
day 42	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SI-1	1.47	108	0.108	0.0821	293	0.01	0.21	211.19	1.79		3.78	
SI-2	1.68	108	0.108	0.0821	293	0.01	0.24	241.36	1.89		4.56	
SI-3	1.64	108	0.108	0.0821	293	0.01	0.24	235.62	1.72		4.05	
SI-C1	1.26	108	0.108	0.0821	293	0.01	0.18	181.02	1.59		2.88	
SI-C2	1.07	108	0.108	0.0821	293	0.00	0.15	153.73	1.3		2.00	
Active (mean)									*1.80*	*±0.07*	*4.13*	*±0.32*
Control (mean)									*1.45*	*±0.15*	*2.44*	*±0.44*

day 60	P (in bottles)	V (mL)	V(L)	R	T	n (mol O_2)	g O_2	mg O_2	O_2 in GC %		mg O_2	
SII-1	1.12	112	0.112	0.0821	293	0.01	0.17	166.87	1.12		1.87	
SII-2	1.19	112	0.112	0.0821	293	0.01	0.18	177.30	1.19		2.11	
SII-3	1.12	112	0.112	0.0821	293	0.01	0.17	166.87	1.12		1.87	
SII-C1	1.28	112	0.112	0.0821	293	0.01	0.19	190.71	1.28		2.44	
SII-C2	1.34	112	0.112	0.0821	293	0.01	0.20	199.65	1.34		2.68	
Active (mean)									*1.14*	*±0.03*	*1.95*	*±0.11*
Control (mean)									*1.31*	*±0.03*	*2.56*	*±0.12*
day 67	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SII-1	1.42	114	0.114	0.0821	293	0.01	0.22	215.19	1.31		2.82	
SII-2	1.41	114	0.114	0.0821	293	0.01	0.21	214.43	1.34		2.87	
SII-3	1.36	114	0.114	0.0821	293	0.01	0.21	205.49	1.28		2.63	
SII-C1	1.40	114	0.114	0.0821	293	0.01	0.21	211.70	1.24		2.63	
SII-C2	1.40	114	0.114	0.0821	293	0.01	0.21	211.70	1.37		2.90	
Active (mean)									*1.31*	*±0.02*	*2.77*	*±0.10*
Control (mean)									*1.31*	*±0.07*	*2.76*	*±0.14*

74

Table S6. Dissolved oxygen (DO) concentrations measured with oxygen sensor for each sampling day. Mean of DO concentrations of active bottles (triplicate) and controls (duplicate) are given with the standard deviations (±) for set up I and II. Oxygen additions are shown in blue.

	Set up I – DO (mg L^{-1})				Set up II – DO (mg L^{-1})			
Day	Active		Control		Active		Control	
1	**0.70**	±0.11	**0.90**	±0.04	**0.17**	±0.05	**0.11**	±0.01
4B	**0.15**	±0.08	**0.76**	±0.01	**0.23**	±0.03	**0.12**	±0.01
4A	**0.18**	±0.09	**0.82**	±0.01	**0.21**	±0.03	**0.15**	±0.01
7B	**0.16**	±0.08	**0.74**	±0.00	**0.19**	±0.04	**0.15**	±0.01
7A	**0.20**	±0.09	**0.78**	±0.00	**0.20**	±0.04	**0.18**	±0.00
7O2	**0.60**	±0.24	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
11B	**0.17**	±0.09	**0.71**	±0.01	**0.20**	±0.02	**0.16**	±0.01
11A	**0.18**	±0.09	**0.73**	±0.00	**0.23**	±0.04	**0.18**	±0.00
14B	**0.17**	±0.09	**0.70**	±0.00	**0.20**	±0.03	**0.17**	±0.01
14A	**0.20**	±0.09	**0.77**	±0.01	**0.25**	±0.08	**0.20**	±0.00
14O2	**0.76**	±0.11	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
18B	**0.18**	±0.09	**0.71**	±0.00	**0.27**	±0.02	**0.20**	±0.01
18A	**0.19**	±0.09	**0.74**	±0.00	**0.22**	±0.03	**0.210**	±0.00
21B	**0.24**	±0.12	**0.83**	±0.01	**0.35**	±0.01	**0.29**	±0.00
21A	**0.28**	±0.10	**0.86**	±0.00	**0.36**	±0.05	**0.31**	±0.00
21O2	**0.78**	±0.19	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
25B	**0.25**	±0.09	**0.80**	±0.01	**0.32**	±0.03	**0.30**	±0.01
25A	**0.27**	±0.09	**0.86**	±0.01	**0.33**	±0.05	**0.33**	±0.01
42B	**0.25**	±0.10	**0.80**	±0.01	**0.34**	±0.02	**0.31**	±0.00
42A	**0.26**	±0.10	**0.80**	±0.01	**0.34**	±0.02	**0.32**	±0.01
42O2	**0.71**	±0.30	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

46B	**0.42**	±0.18	**0.79**	±0.00	**0.39**	±0.04	**0.33**	±0.01
46A	**0.42**	±0.17	**0.80**	±0.00	**0.40**	±0.02	**0.34**	±0.01
54*	**0.34**	±0.15	**0.78**	±0.02	**0.34**	±0.07	**0.33**	±0.02
56B	**0.33**	±0.12	N.A.	N.A.	**0.38**	±0.09	**0.3**	±0.01
56A	**0.33**	±0.12	**0.51**	±0.02	**0.36**	±0.07	**0.3**	±0.01
60	**0.36**	±0.12	**0.51**	±0.01	**0.35**	±0.03	**0.31**	±0.01
60O2	N.A.	N.A.	N.A.	N.A.	**0.84**	±0.08	**0.68**	±0.15
61	**0.33**	±0.11	**0.49**	±0.02	**0.59**	±0.07	**0.72**	±0.00
63B	**0.36**	±0.11	**0.51**	±0.01	**0.38**	±0.10	**0.75**	±0.02
63A	**0.36**	±0.11	N.A.	N.A.	**0.38**	±0.10	**0.77**	±0.01
67B	**0.34**	±0.10	**0.49**	±0.02	**0.31**	±0.04	**0.68**	±0.02
67A	**0.35**	±0.10	**0.51**	±0.01	**0.3**	±0.06	**0.72**	±0.02
67O2	N.A.	N.A.	N.A.	N.A.	**0.79**	±0.10	N.A.	N.A.
70B	**0.39**	±0.11	**0.59**	±0.01	**0.35**	±0.04	**0.75**	±0.03
70A	**0.41**	±0.11	**0.57**	±0.02	**0.33**	±0.07	**0.77**	±0.03
78B	**0.36**	±0.10	N.A.	N.A.	**0.28**	±0.06	**0.69**	±0.02
78A	**0.39**	±0.10	**0.56**	±0.03	**0.37**	±0.02	**0.74**	±0.02
98B	**0.36**	±0.10	**0.53**	±0.02	**0.27**	±0.05	**0.66**	±0.03
98A	**0.38**	±0.09	**0.54**	±0.02	**0.29**	±0.07	**0.7**	±0.03

B: before sampling, A: after sampling, O2: After oxygen addition, N.A. No addition, or no measurement, *;gas exchange

Supplementary data 3: Distribution of oxygen in gas and liquid phase based on Henry's Law

The relation between the concentration in the gas phase and the concentration in the liquid phase can be described by the dimension less Henry coefficient (K_H) by using Eq.S3.

$K_H = m = C_g/C_l$ **(Equation S3)**

K_H = dimensionless Henry constant (-)

m = distribution coefficient (-)

C_g = concentration contamination in the gas phase (mol/m^3)

C_l = concentration contamination in the liquid phase (mol/m^3)

The dimensionless Henry coefficient (K_H) can be derived from the ideal gas law as shown in Eq.S4.

$K_H = m = H / R*T$ **(Equation S4)**

H = Henry coefficient ($Pa\ m^3/mol$)

R = gas constant (J/mol/K)

T = temperature (K)

Table S7. Mean of the Henry's coefficients calculated for set up I (day 1) and set up II (day 60) bottles based on Eq.S3. and Eq.S4.

Set up	C_g	C_l	K_H	**H***
I	21.96 ± 4.64	0.78 ± 0.13	28.13 ± 2.57	688.16 ± 62.82
II	21.93 ± 3.20	0.77 ± 0.13	29.79 ± 8.98	728.91 ± 219.82

*For water in equilibrium with the atmosphere, the concentration is governed by Henry's Law, and KH for O_2 is 769.23 L atm mol^{-1} at 298 K.

Supplementary Data 4: Oxygen concentrations in set up III

Table S8. Oxygen percentage measured in GC (in blue) and their calculations to mg (in orange) for each bottle of set up III (SIII). C1 and C2 are duplicates of the control bottles.

Set up III - Oxygen												
day 1	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SIII-1	1.14	100	0.100	0.0821	293	0.00	0.15	151.65	16.97		25.74	
SIII-2	1.26	100	0.100	0.0821	293	0.01	0.17	167.61	17.19		28.81	
SIII-3	1.17	100	0.100	0.0821	293	0.00	0.16	155.64	16.88		26.27	
SIII-C1	1.51	100	0.100	0.0821	293	0.01	0.20	200.87	16.86		33.87	
SIII-C2	1.31	100	0.100	0.0821	293	0.01	0.17	174.27	16.83		29.33	
Active (mean)									*17.01*	*±0.13*	*26.94*	*±1.34*
Control (mean)									*16.85*	*±0.02*	*31.60*	*±2.27*
day 3	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SIII-1	1.14	101	0.101	0.0821	293	0.00	0.15	153.17	16.01		24.52	
SIII-2	1.26	101	0.101	0.0821	293	0.01	0.17	169.29	15.59		26.39	
SIII-3	1.17	101	0.101	0.0821	293	0.00	0.16	157.20	15.30		24.05	
SIII-C1	1.51	101	0.101	0.0821	293	0.01	0.20	202.88	19.10		38.75	
SIII-C2	1.31	101	0.101	0.0821	293	0.01	0.18	176.01	19.07		33.56	
Active (mean)									*15.63*	*±0.29*	*24.99*	*±1.01*

Control (mean)									*19.09*	*±0.02*	*36.16*	*±2.59*
day 8	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SIII-1	1.14	102	0.102	0.0821	293	0.00	0.15	154.68	11.78		18.22	
SIII-2	1.26	102	0.102	0.0821	293	0.01	0.17	170.97	11.27		19.27	
SIII-3	1.17	102	0.102	0.0821	293	0.00	0.16	158.75	10.55		16.75	
SIII-C1	1.51	102	0.102	0.0821	293	0.01	0.20	204.89	19.19		39.32	
SIII-C2	1.31	102	0.102	0.0821	293	0.01	0.18	177.75	19.09		33.93	
Active (mean)									*11.20*	*±0.50*	*18.08*	*±1.03*
Control (mean)									*19.14*	*±0.05*	*36.63*	*±2.69*
day 15	**P (in bottles)**	**V (mL)**	**V(L)**	**R**	**T**	**n (mol O_2)**	**g O_2**	**mg O_2**	**O_2 in GC %**		**mg O_2**	
SIII-1	1.14	103	0.103	0.0821	293	0.00	0.16	156.20	7.30		11.40	
SIII-2	1.26	103	0.103	0.0821	293	0.01	0.17	172.64	7.33		12.65	
SIII-3	1.17	103	0.103	0.0821	293	0.01	0.16	160.31	6.91		11.08	
SIII-C1	1.51	103	0.103	0.0821	293	0.01	0.21	206.90	18.36		37.99	
SIII-C2	1.31	103	0.103	0.0821	293	0.01	0.18	179.49	18.35		32.94	
Active (mean)									*7.18*	*±0.19*	*11.71*	*±0.68*
Control (mean)									*18.36*	*±0.00*	*35.46*	*±2.52*

Table S9. Dissolved oxygen (DO) concentrations measured with oxygen sensor for each sampling day. Mean of DO concentrations of active bottles (triplicate) and controls (duplicate) are given with the standard deviations (±) for set up III.

Set up III – DO (mg L^{-1})								
Bottle \ Day	0	1B	3B	3A	8B	8A	15B	15A
Active (mean)	**8.43**	**6.36**	**4.32**	**4.42**	**2.85**	**2.92**	**2.10**	**2.18**
	±0.74	±0.29	±0.19	±0.19	±0.04	±0.04	±0.10	±0.08
Control (mean)	**10.90**	**7.51**	**6.18**	**6.26**	**5.33**	**5.44**	**4.91**	**5.27**
	±0.91	±0.42	±0.32	±0.31	±0.32	±0.28	±0.31	±0.27

B: before sampling, A: after sampling

Supplementary Data 5: BTEXIeIaN biodegradation under fully aerobic and fully anaerobic conditions

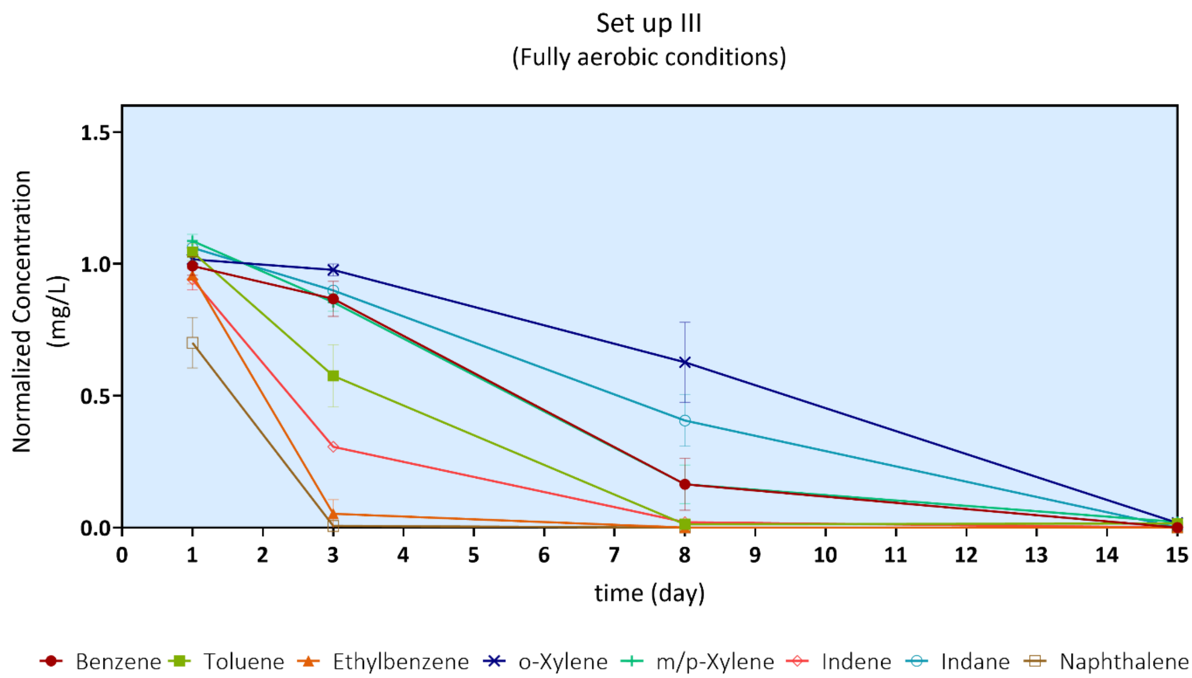

Fig. S1 BTEXIeIaN concentrations in set up III, under fully aerobic conditions.

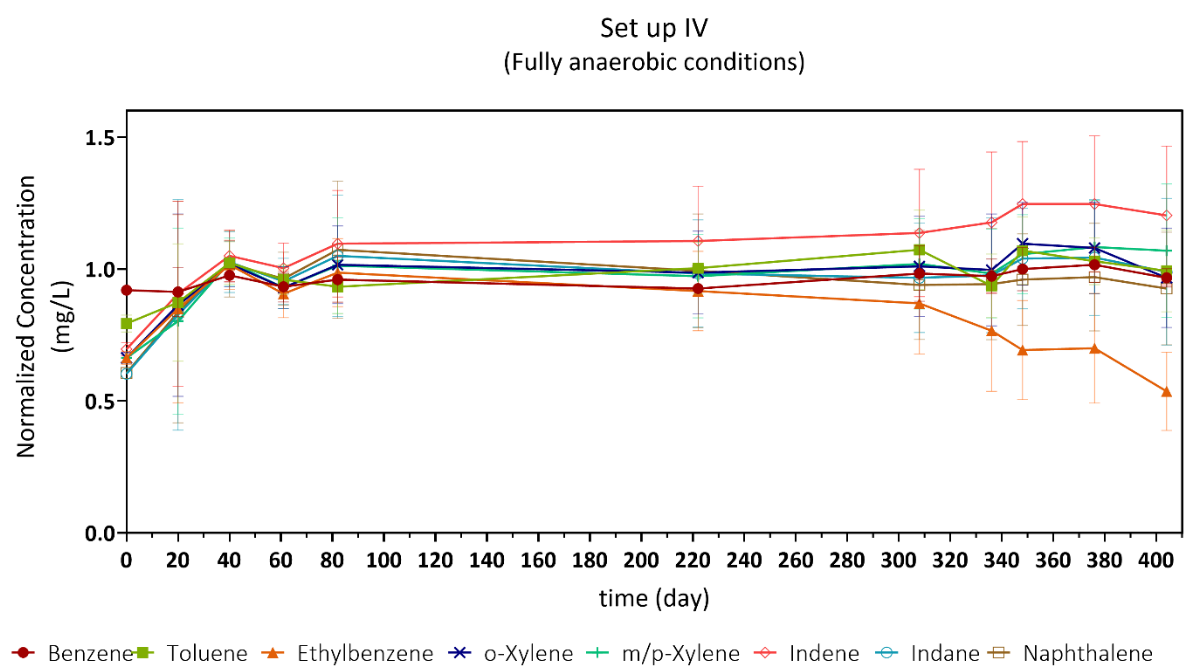

Fig. S2 BTEXIeIaN concentrations in set up IV, under nitrate-reducing conditions without any oxygen addition.

Supplementary Data 6: Stochiometric calculations

Table S10. Theoretical calculations for electron acceptor (EA) needed per mg of compound.

	mg compound / mg EA			
	mol		**mg**	
	oxygen	**nitrate**	**oxygen**	**nitrate**
Benzene	7.5	6.0	3.07	4.76
Toluene	9.0	7.2	3.13	4.85
Ethylbenzene	10.5	8.4	3.16	4.91
o-xylene	10.5	8.4	3.17	4.91
m/p-xylene	10.5	8.4	3.17	4.91
Indene	11.0	8.8	3.03	4.70
Indane	11.5	9.2	3.11	4.83
Naphthalene	12.0	9.6	3.00	4.64
Total (BTEXIeIaN)	82.5	66	24.83	38.49