Supplemental information

Emerging investigator series: A multispecies analysis of the relationship between oxygen content and toxicity in graphene oxide

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Submitted to:

Environmental Science: Nano

Keywords: graphene oxide, surface chemistry, toxicity, multispecies.

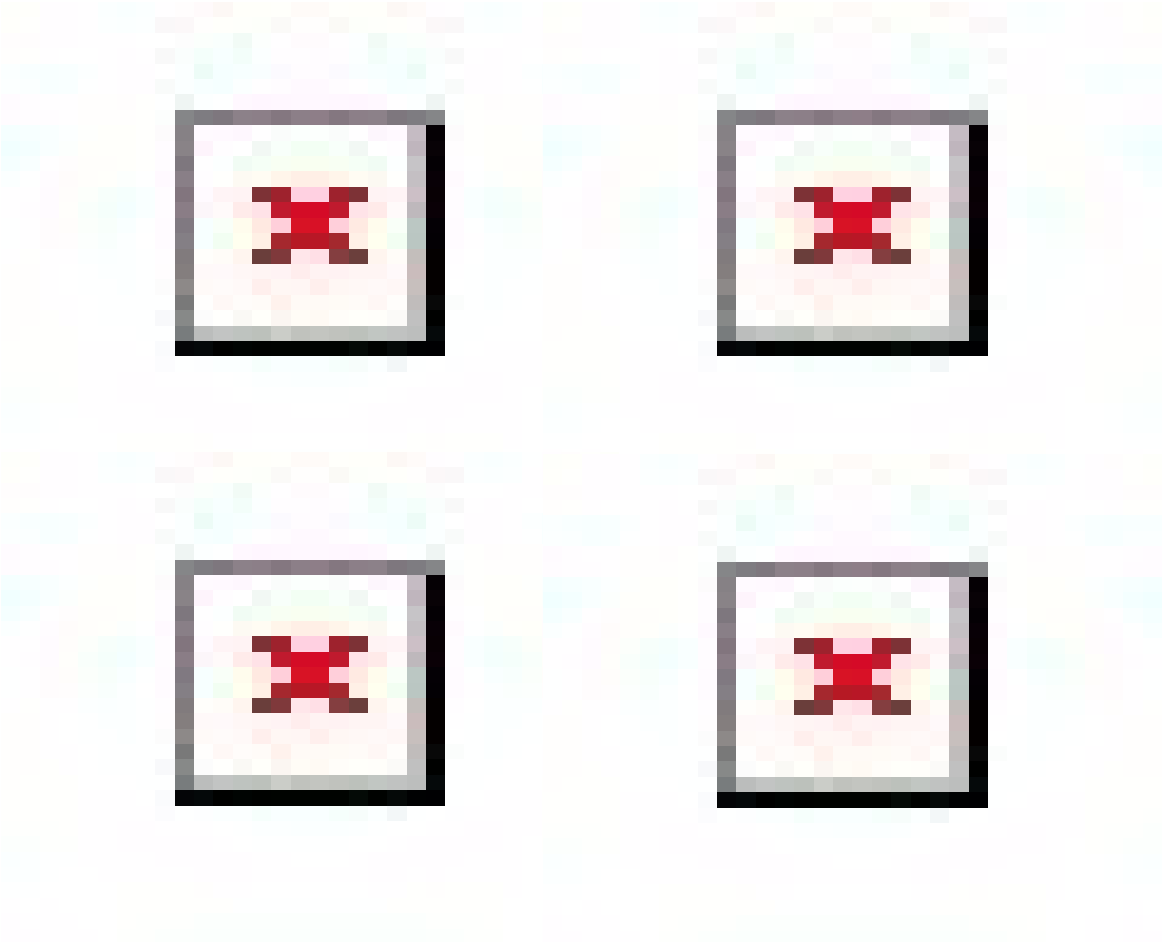

Figure S1. Dose response curves of *S. obliquus* after exposure to GBNMs at different concentrations after 96h (n=9). A sigmoidal fit was conducted on each dose-response curve but only ARGO had a successful fit and a calculated EC_{50} concentration.

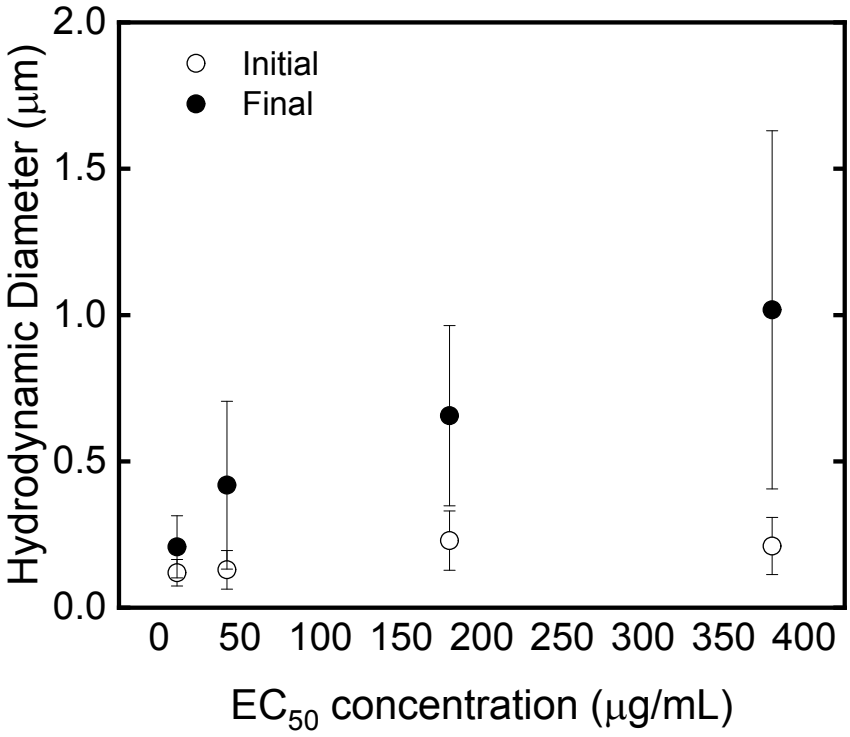

Figure S2. Relationship between GBNMs' hydrodynamic diameter and EC_{50} concentration after the initial and final aggregation measurements for each media. The ARGO EC_{50} concentrations used were 11, 42, 180, and 380 μg/mL for *M. aeruginosa*, *S. obliquus*, *E. coli*, and *D. magna*, respectively. Data is shown as average ± standard deviation of three measurements.

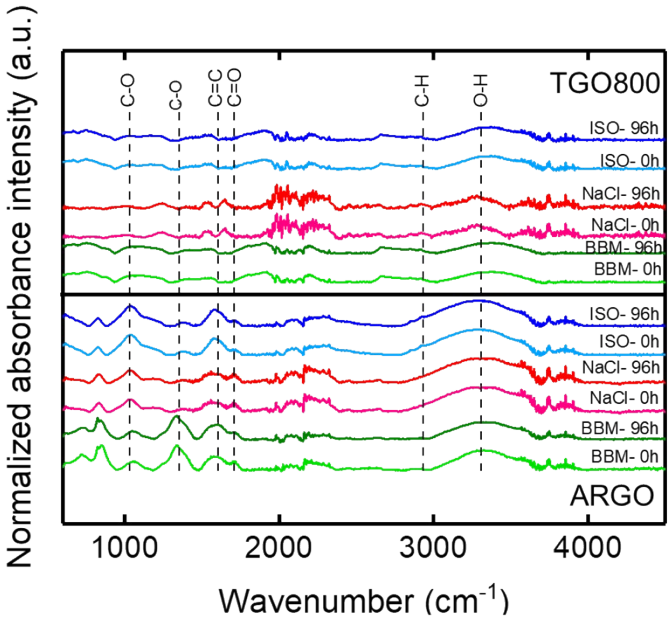

Figure S3. Fourier transform infrared spectrometry (FTIR) of ARGO (bottom panel) and TGO800 (top panel) immediately (0h) and after 96h of exposure to BBM, 0.9% NaCl, and ISO media. The appearance of a broad peak in the 3300 cm^{-1} confirms the presence of O-H bonds. The peaks that appear in the 1707 cm^{-1}, 1351.4 cm^{-1} and 1031.2 cm^{-1} area, which are representative of carboxyl, epoxy, and carbonyl functional groups, confirm further oxidation of the material. Similarly, the decrease in intensity in these peaks confirm a reduction as is shown in the TGO800 samples (top panel).

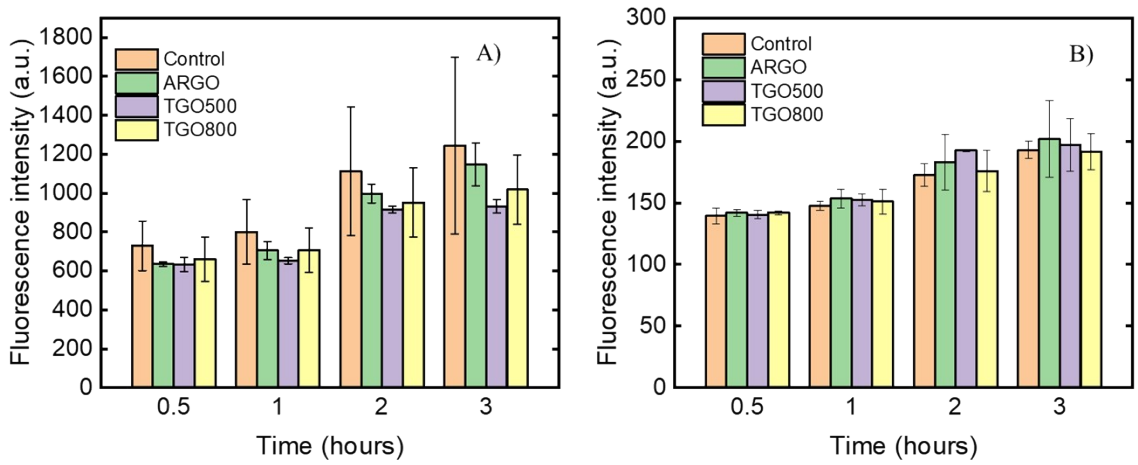

Figure S4. Fluorescence intensity of A) FDA dye and B) H_2DCFDA dye over a 3h period. ARGO, TGO500, and TGO800 were suspended in nanopure water at their EC_{50} concentrations and spiked with 10 µL of either dye. The control treatment has no material present. Results are shown as average ± standard deviation of triplicate samples per material.

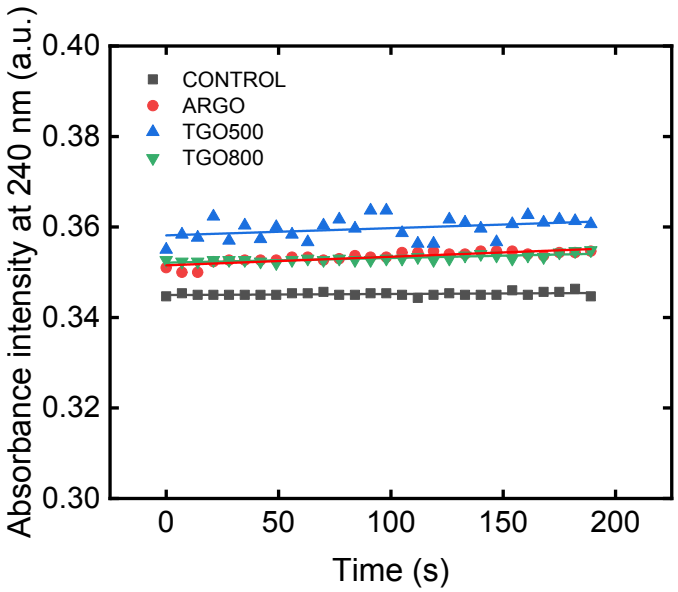

Figure S5. Absorbance intensity at 240 nm measured over 3 minutes to assess H_2O_2 decomposition. The GO-containing treatments have ARGO, TGO500, or TGO800 at their EC_{50} concentrations suspended in nanopure water and spiked with 30 mM H_2O_2. The control treatment is nanopure water spiked with 30 mM H_2O_2 (no GO). Results are the average of triplicate samples for each time point.

Table S1. Compiled literature comparing graphene-based nanomaterials' characteristics, experimental conditions, and toxicity effects to *Escherichia coli, Daphnia magna, Scenedesmus obliquus*, and *Microcystis aeruginosa*.

	Organism	Material	C/O	Another characteristic	Time of exposure (h)	EC_{50} (mg/L)	Other toxicity endpoint	Reference
Bacteria	*E. coli*	GO	2-4*	D/G ratio: 1	2	100		[1]
	E. coli	rGO		D/G ratio: 1.9	2	100		[1]
	E. coli	GO	2-4*	Thickness: 1.1nm, 1.0 nm	2		98.5% viability loss at 85 mg/L**	[2]
	E. coli	rGO		Thickness: 1.1nm, 1.0 nm	2		90% viability loss at 85 mg/L**	[2]
	E. coli	GO	2-4*	D/G ratio: 1.36	2	30		[3]
	E. coli	rGO			2	40		[3]
	E. coli	GO	5.1		2	5		[4]
	E. coli	GO	2-4*	Size: 0.01 μm^2; D/G ratio: 1.03	3		20% viability loss at 200 mg/L**	[5]
	E. coli	GO	2-4*		24		MIC = 100 mg/L	[6]
	E. coli	GO	2-4*	D/G ratio: 0.92	48		65% viability loss at 65 mg/L	[7]
	E. coli	GO	2-4*		3	38		[8]
	E. coli	GO	1.9		12		MIC = 35 mg/L	[9]
	E. coli	GO	2-4*		2		20% viability loss at 100 mg/L	[10]
	E. coli	GO	2-4*		2.5		15% viability loss at 5 mg/L	[11]
	E. coli	GO	2.23		3		25% viability loss at 100 mg/L	[12]
	E. coli	GO	2-4*	Size: 205 µm; Thickness: 1 nm	2.5	25		[13]
	E. coli	GO	2.0	Thickness: 1.08 nm	3		75% inhibition at 50 mg/mL	[14]
	E. coli	dfGO	3.1	Thickness: 3.09 nm	3	50		[14]
	E. coli	GO 1:3	33.1[a]	D/G ratio: 1.00	2		14.7% colony reduction at 40 mg/L**	[15]
	E. coli	GO 1:6	44.4[a]	D/G ratio: 0.99	2		30.3% colony reduction at 40 mg/L**	[15]
	E. coli	GO 1:9	54.5[a]	D/G ratio: 1.00	2		34.1% colony reduction at 40 mg/L**	[15]
	E. coli	GO 1:12	58.6[a]	D/G ratio: 1.04	2		41.2% colony reduction at 40 mg/L**	[15]
	E. coli	rGO 1:3	27.2[a]	D/G ratio: 0.96	2		32.2% colony reduction at 40 mg/L**	[15]
	E. coli	rGO 1:6	31.2[a]	D/G ratio: 0.94	2		38.4% colony reduction at 40 mg/L**	[15]
	E. coli	rGO 1:9	33.0[a]	D/G ratio: 0.92	2		41.7% colony reduction at 40 mg/L**	[15]
	E. coli	rGO 1:12	34.7[a]	D/G ratio: 0.94	2		48.8% colony reduction at 40 mg/L**	[15]
	E. coli	GO	2.0	Size: 1 µm	3	183		[16]
	E. coli	TGO200	4.4	Size: 1.1 µm	3	143		[16]
	E. coli	TGO500	6.5	Size: 1.1 µm	3	127		[16]
	E. coli	TGO800	7.1	Size: 1.1 µm	3	86.3		[16]
Invertebrate	*D. magna*	TPGO	2.44		24, 48		No effect at 200 mg/L	[17]
	D. magna	GO	2.11	D/G ratio: 1.04	96	0.58		[18]
	D. magna	GO	2-4*	D/G ratio: 1.02; Size: 1108 nm	48	84.3		[19]
	D. magna	GO	3.27	Size: 0.9-5.2 µm; Thickness: 0.72 nm	48	150.8		[20]
	D. magna	GO	1.2		72	44.3	LC_{50} = 45.4 mg/L	[21]
	D. magna	GO-PEG	2-4*		48		No effect at 1 mg/L	[22]
	D. magna	GO	2.4	D-G ratio: 1.3; Size: 1.1 µm; Thickness: 1.02 nm	48	75	LC_{50} = 145 mg/L	[23]
	D. magna	GO	2-4*	Thickness: 1.0-1.77 nm	72			[24]

	D. magna	rGO	8.33		48		20% immobilization at 30 mg/L	[25]
	D. magna	GO	2-4*	Size: 0.5-3 µm Thickness: 0.55-1.20 nm	48	21		[26]
	D. magna	GO	2-4*		24	0.18 (MHF); 0.31 (HF)		[27]
	D. magna	GO	2-4*		48		No effect at 100 mg/L	[28]
	D. magna	GO	2.5	Size: 1 µm	48	383		This study
	D. magna	TGO200	4.7	Size: 1.1 µm	48	187		This study
	D. magna	TGO500	7.5	Size: 1.1 µm	48	320		This study
	D. magna	TGO800	12	Size: 1.1 µm	48	263		This study
Algae/ Cyanobacteria	*S. obliquus*	GO	2-4*	D/G ratio: 1.02; Size: 1108 nm	72	20.6		[19]
	S. obliquus	GO	2-4*	Size: 0.5-3 µm; Thickness: 0.55-1.20 nm	96	11		[19]
	S. obliquus	GO	2	Size: 3-8 µm; Thickness: 1.34 nm	96	10		[29]
	S. obliquus	rGO	2.8	Size: 0.2-5 µm; Thickness: 0.5-3 nm	72	148		[30]
	S. obliquus	GO	2.1	Size: 1-10 µm; Thickness: 0.7 nm	96	21.2		[31]
	S. obliquus	G	-	Size: 0.5-2 µm; Thickness: 0.8-1.2 nm	96	8.2		[32]
	S. obliquus	GO	2-4*	Size: 0.5-5 µm; Thickness: 0.8-1.2 nm	96	20.6		[32]
	S. obliquus	GO	2.0		96	42.4		This study
	S. obliquus	TGO200	4.6		96		EC_{50} of the TGO200 was not reached at 100 mg/L	This study
	S. obliquus	TGO500	7.6		96		EC_{50} of the TGO500 was not reached at 100 mg/L	This study
	S. obliquus	TGO800	12		96		EC_{50} of the TGO800 was not reached at 100 mg/L	This study
	M. aeruginosa	GO	2.0	Size: 3-8 µm; Thickness: 1.34 nm	96		35% growth inhibition at 10 mg/L	[29]
	M. aeruginosa	GO	2-4*	Size: 0.5-5 µm; Thickness: 0.8-1.2 nm	96		20% chl-*a* reduction at 50 mg/L	[33]
	M. aeruginosa	GO	2.0		96	52.3		[34]
	M. aeruginosa	GO	2.0	Size: 1.0 µm	96	11.1		This study
	M. aeruginosa	TGO200	4.6	Size: 1.1 µm	96	16.3		This study
	M. aeruginosa	TGO500	7.6	Size: 1.1 µm	96	126		This study
	M. aeruginosa	TGO800	12	Size: 1.1 µm	96		EC_{50} of TGO800 was not reached at 100 mg/L	This study

Abbreviations:

E. coli: Escherichia coli
D. magna: Daphnia magna
S. obliquus: Scenedesmus obliquus
M. aeruginosa: Microcystis aeruginosa
G: graphene
GO: graphene oxide
rGO: reduced graphene oxide
dfGO: debris-free graphene oxide
TGO: thermally-annealed graphene oxide
TPGO: thermal and pH dual-sensitive graphene oxide
PEG-GO: pegylated graphene oxide
MHF: moderately hard freshwater
HF: hard freshwater
C/O: carbon to oxygen ratio
EC_{50}: Effective concentration at 50%
MIC: Minimum inhibitory concentration
* an estimated C/O value between 2-4 was used for GO prepared using the Modified Hummers method and a value of 8-246 for rGO [35,36]
**Cell viability tested at only one concentration.
[a] Study used O/C ratio

Table S2. Compiled XPS data representing the atomic percent of the carbon and oxygen content and relative atomic percentage of carbon-oxygen functional groups determined from the component fitting of the C 1s envelope for all GBNMs. A new batch was synthesized per species (n=3) and three measurements were done per material for each batch.

	Bacteria				Algae/ Cyanobacteria				Invertebrate			
Material	ARGO	TGO200	TGO500	TGO800	ARGO	TGO200	TGO500	TGO800	ARGO	TGO200	TGO500	TGO800
%C	65.85 ± 0.80	80.36 ± 0.21	84.76 ± 1.39	83.30 ± 0.44	66.13 ± 0.78	81.69 ± 0.51	88.38 ± 0.33	91.45 ± 1.27	70.80 ± 1.82	81.98 ± 0.42	87.81 ± 1.49	90.15 ± 0.77
%O	32.53 ± 0.54	18.23 ± 0.10	13.16 ± 1.09	11.59 ± 0.18	32.83 ± 0.67	17.60 ± 0.25	11.62 ± 0.33	7.89 ± 1.11	27.97 ± 2.20	17.44 ± 0.38	11.78 ± 0.77	7.54 ± 0.60
%C-C/C-H	37.23 ± 1.14	70.76 ± 0.01	75.75 ± 1.43	84.71 ± 0.22	45.32 ± 1.82	73.77 ± 0.63	80.26 ± 0.44	86.69 ± 2.53	55.53 ± 2.84	75.11 ± 0.91	79.86 ± 0.36	85.10 ± 0.48
%C-O	41.89 ± 0.66	18.35 ± 0.30	16.67 ± 1.62	9.09 ± 0.36	35.81 ± 2.16	15.02 ± 0.09	11.77 ± 0.58	8.18 ± 2.64	26.49 ± 4.36	13.83 ± 1.27	12.11 ± 0.50	9.75 ± 0.41
%C=O	15.66 ± 2.09	5.22 ± 0.08	5.06 ± 0.18	4.58 ± 0.37	13.82 ± 0.77	5.81 ± 0.31	5.53 ± 0.32	3.53 ± 0.14	12.91 ± 1.23	6.16 ± 0.24	5.38 ± 0.25	3.67 ± 0.09
%COOH	5.24 ± 0.29	5.68 ± 0.23	2.53 ± 0.01	1.62 ± 0.24	5.06 ± 0.51	5.40 ± 0.45	2.44 ± 0.12	1.60 ± 0.44	5.05 ± 0.30	4.90 ± 0.45	2.64 ± 0.12	1.49 ± 0.05

Table S3. Compiled XPS data representing the relative atomic percent of trace elements found in GBNMs. Trace amounts of impurities were also found and shown as atomic percent of sulfur, nitrogen, sodium, and calcium. A new batch was synthesized per species (n=3) and three measurements were done per material for each batch.

	Material	S%	N%	Na%	Ca%
Bacteria	ARGO	0.52 ± 0.04	0.76 ± 0.15	0.23 ± 0.07	nd
	TGO200	0.51 ± 0.03	0.59 ± 0.14	0.24 ± 0.07	nd
	TGO500	0.17 ± 0.01	1.13 ± 0.65	1.28 ± 0.28	nd
	TGO800	0.29 ± 0.17	nd	4.92 ± 0.34	nd
Algae	ARGO	1.04 ± 0.11	nd	nd	nd
	TGO200	0.54 ± 0.04	0.52 ± 0	nd	nd
	TGO500	nd	nd	nd	nd
	TGO800	nd	0.63 ± 0	0.22 ± 0.05	nd
Invertebrate	ARGO	0.79 ± 0.05	nd	nd	0.64 ± 0.12
	TGO200	0.58 ± 0.06	nd	nd	nd
	TGO500	0.62 ± 0.01	nd	nd	nd
	TGO800	1.16 ± 0.12	nd	0.39 ± 0.08	0.62 ± 0.05

nd = not detected

Table S4. Responses of *D. magna* immobilization after exposure to GBNMs at different concentrations after 48h (n=3). The EC_{50} concentrations were calculated using the Trimmed Spearman-Karber method. Data is shown as mean ± standard deviation.

Material:	**ARGO**	**TGO200**	**TGO500**	**TGO800**
Concentration (mg/L)	Mean immobilization (%)	Mean immobilization (%)	Mean immobilization (%)	Mean immobilization (%)
12.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
25	1.7 ± 2.9	8.3 ± 2.9	0.0 ± 0.0	8.3 ± 2.9
50	6.7 ± 2.9	13.3 ± 2.9	6.7 ± 2.9	11.7 ± 2.9
100	15.0 ± 5.0	25.0 ± 10.0	13.3 ± 2.9	16.7 ± 2.9
200	23.3 ± 7.6	38.3 ± 10.4	23.3 ± 2.9	28.3 ± 7.6
400	51.7 ± 2.9	81.7 ±17.6	63.3 ± 5.8	78.3 ± 2.9
EC_{50}	383 ± 29.9	187 ± 18.3	320 ± 14.4	263 ± 17.0

Table S5. Chemical composition and concentrations to prepare stock solutions for each media. A 0.9% NaCl media was used for bacteria experiments, ISO media for invertebrate studies (D. magna) according to the International Organization for Standardization, and Bold's Basal Media (BBM) for green alga and cyanobacteria studies. All medias were prepared in DI water and their pH was adjusted with HCl or KOH as required. Aggregation studies were done using the GBNMs suspended in each media, with no organisms present.

0.9% NaCl		ISO*		BBM**	
Chemical	Concentration (g/L)	Chemical	Concentration (g/L)	Chemical	Concentration (g/L)
NaCl	9.0	$CaCl_2•2H_2O$	11.8	KH_2PO_4	17.5
		$MgSO_4•7H_2O$	4.93	$CaCl_2•2H_2O$	25
		$NaHCO_3$	2.59	$MgSO_4•7H_2O$	75
		KCl	0.23	$NaNO_3$	250
				K_2HPO_4	75
				NaCl	25
				$Na_2EDTA•2H_2O$[a]	
				$FeSO_4•7H_2O$[b]	
				Trace metal solution (TMS)[c]	(see below)
				H_3BO_3	11.5 g/L
				TMS[c]	
				H_3BO_3	2.86
				$MnCl_2•4H_2O$	1.81
				$ZnSO_4•7H_2O$	0.22
				$Na_2MoO_4•2H_2O$	0.39
				$CuSO_4•5H_2O$	0.08
				$Co(NO_3)_2•6H_2O$	0.05

*Mix 25 mL of each of the four stock solutions and make up to 1 L with DI water.

**Mix 1 mL of each of the stock solutions (10 mL for KH_2PO_4) in the order shown above to avoid salt precipitation. Make up to 1 L with DI water. Adjust pH and then autoclave.

[a] The $Na_2EDTA•2H_2O$ solution was prepared in 1 L of a KOH (6.2 g/L).

[b] The $FeSO_4•7H_2O$ solution was prepared in 1 L of concentrated H_2SO_4 (1 mL/L).

[c] The Trace Metal Solution (TMS) was prepared separately, and all salts were added to 1 L of water. Then, 1 mL of the TMS was added to 1 L of BBM media.

Table S6. Solution chemistry characteristics of the different media. Bacteria experiments were done in 0.9% NaCl, alga experiments in BBM and invertebrates' studies were done according to the International Organization for Standardization (ISO).

Media	pH	Ionic Strength (mM)	Divalent Cations (mM)	Monovalent Cations (mM)
0.9% NaCl	7.0	147.90	0.00	154.00
BBM	6.8	7.10	0.30	5.68
ISO	7.8	6.74	2.00	0.68

Table S7. Intercepts and slopes of each linear fit according to the H_2O_2 decomposition over 3 minutes with the formula y = a + bx. Linear fitting was done using the OriginPro Software version 2018.

Material	Intercept (a)	Slope (b)
Control	0.34 ± 1.45E-4	2.33E-6 ± 1.32E-6
ARGO	0.35 ± 2.61E-4	1.90E-5 ± 2.37E-6
TGO 500	0.36 ± 8.22E-4	1.59E-5 ± 7.47E-6
TGO 800	0.35 ± 1.50E-4	1.03E-5 ± 1.36E-6

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