Electronic Supplementary Information

Combined toxicity of graphene oxide and wastewater to the green alga *Chlamydomonas reinhardtii*

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CONTENTS:

Table S1. Wastewater characterization parameters (0.45 μm filtered samples).

Parameters	**value**	**Ions**	**(mg/L)**
pH	6.5*	Nitrate	0.38
Turbidity (NTU)	1.4	Chloride	125.5
Conductivity (mS/cm)	1.23 ± 0.03	Sulphate	143.7
COD (mg/L)	49.2 ± 1.2	Fluoride	<0.80
NPOC (mg/L)	17.2 ± 0.5	Nitrite	<0.10
Suspended solids (mg/L)	7.6	Bicarbonate	348.4
Alkalinity (mg $CaCO_3$/L)	286		
Total-P (mg/L)	0.1	Sodium	95.3
Total-N (mg/L)	2.3	Potassium	23.4
		Magnesium	14.3
		Calcium	49
		Ammonium	68.4

* Measured just before runs.

Table S2. Experimental design for determining toxicological interactions of GO and wastewater (WW) and their binary combinations for *C. reinhardtii* growth inhibition.

Constant ratio			**Non-constant ratio**			
Dilutions	Binary combinations GO+WW (1:0.3)		Dilutions GO	Dilutions WW	Binary combinations GO+WW ($WW = -0.3\,GO + 0.3$)	
	GO (mg L^{-1})	WW (dil.)			GO (mg L^{-1})	WW (dil.)
$\frac{1}{2.9}$ (EC_{50})	0.36	0.11	$\frac{1}{1.2}$ (EC_{50})	$\frac{1}{6}$ (EC_{50})	0.86	0.05
$\frac{1}{1.7}$ (EC_{50})	0.61	0.19	$\frac{1}{1.5}$ (EC_{50})	$\frac{1}{3}$ (EC_{50})	0.69	0.11
1 (EC_{50})	1.04	0.33	$\frac{1}{2}$ (EC_{50})	$\frac{1}{2}$ (EC_{50})	0.52	0.16
1.7 (EC_{50})	1.76	0.55	$\frac{1}{3}$ (EC_{50})	$\frac{1}{1.5}$ (EC_{50})	0.35	0.22
2.9 (EC_{50})	2.99	0.94	$\frac{1}{6}$ (EC_{50})	$\frac{1}{1.2}$ (EC_{50})	0.17	0.27

Table S3. Concentrations and physicochemical properties of pollutants detected in wastewater.

No.	Compound	Concentration (ng/L)	CAS Number	Molecular formula	log K_{ow}	pK_a	Acid/Base	log D_{ow} (pH 6.5)	Charge (+/-)	Main use
1	4-FAA*	6597 ± 326	1672-58-8	$C_{12}H_{13}N_3O_2$	-0.41	5.0	Weakly basic	-0.42	0.0	Metabolite of aminopyrine
2	Acesulfame	110 ± 2	33665-90-6	$C_4H_5NO_4S$	-1.33	5.7	Acidic	-1.39	-1.0	Sweetener
3	Amitriptyline	56 ± 9	50-48-6	$C_{20}H_{23}N$	4.92	9.4	Basic	2.00	1.0	Antidepressant
4	Antipyrine	2690 ± 337	60-80-0	$C_{11}H_{12}N_2O$	0.38	1.4	Weakly basic	0.38	0.0	Analgesic
5	Azithromycin	206 ± 9.4	83905-01-5	$C_{38}H_{72}N_2O_{12}$	4.02	8.7	Basic	1.76	1.0	Antibiotic
6	Bezafibrate	140 ± 12	41859-67-0	$C_{19}H_{20}ClNO_4$	4.25	3.61	Acidic	1.38	-1.0	Antilipemic
7	Carbamazepine	297 ± 5.1	298-46-4	$C_{15}H_{12}N_2O$	2.45		Neutral	2.45	0.0	Antiepileptic
8	Carbamazepine epoxide	67 ± 1	36507-30-9	$C_{15}H_{12}N_2O_2$	1.58		Neutral	1.58	0.0	Metabolite of carbamazepine
9	Ciprofloxacin	177 ± 34	85721-33-1	$C_{17}H_{18}FN_3O_3$	0.28	6.1/8.7	Zwitterionic	n.a.	-1.0	Antibiotic
10	Citalopram	214 ± 2.4	59729-33-8	$C_{20}H_{21}FN_2O$	3.74	9.7	Basic	0.52	1.0	Antidepressant
11	Clarithromycin	164 ± 25	81103-11-9	$C_{38}H_{69}NO_{13}$	3.16	9.0	Basic	0.65	1.0	Antibiotic
12	Diazepam	24 ± 2	439-14-5	$C_{16}H_{13}ClN_2O$	2.82	3.4	Weakly basic	2.82	0.0	Anxiolytic
13	Erythromycin	134 ± 2	114-07-8	$C_{37}H_{67}NO_{13}$	3.06	8.9	Basic	0.64	1.0	Antibiotic
14	Fenofibric acid	172 ± 7	42017-89-0	$C_{17}H_{15}ClNO_4$	4.00	3.1	Acidic	0.62	-1.0	Metabolite of fenofibrate
15	Furosemide	984 ± 30	54-31-9	$C_{12}H_{11}ClN_2O_5S$	2.03	3.8	Acidic	-0.65	-1.0	Antihypertensive
16	Gemfibrozil	2522 ± 49	25812-30-0	$C_{15}H_{22}O_3$	4.77	4.5	Acidic	2.79	-1.00	Antilipemic
17	Hydrochlorothiazide	2510 ± 85	58-93-5	$C_7H_8ClN_3O_4S_2$	-0.07	7.9/9.2	Basic	-4.23	1.00	Antihypertensive
18	Indomethacin	26 ± 5	53-86-1	$C_{19}H_{16}ClNO_4$	4.27	4.5	Acidic	2.29	-1.00	Analgesic
19	Lidocaine	447 ± 64	137-58-6	$C_{14}H_{22}N_2O$	2.44	7.8	Basic	1.15	1.00	Anesthetic

Table S3 (cont.). Concentrations and physicochemical properties of pollutants contained in wastewater.

No.	Compound	Concentration (ng/L)	CAS Number	Molecular formula	log K_{ow}	pK_a	Acid/Base	log D_{ow}**	Charge (+/-)	Main use
20	Mepivacaine	40 ± 4	96-88-8	$C_{15}H_{22}N_{20}$	1.95	7.7	Basic	0.70	1.00	Anesthetic
21	Metoclopramide	49 ± 2	364-62-5	$C_{14}H_{22}ClN_3O_2$	2.62	9.3	Basic	-0.17	1.00	Antiemetic
22	Metoprolol	38 ± 2	37350-58-6	$C_{15}H_{25}NO_3$	1.88	9.7	Basic	-1.34	1.00	β-blocker
23	Ofloxacin	472 ± 39	82419-36-1	$C_{18}H_{20}FN_3O_4$	-0.39	6.1/8.2	Zwitterionic	n.a.	-1.0	Antibiotic
24	Paraxanthine	251 ± 36	611-59-6	$C_7H_8N_4O_2$	-0.22	10.8	Weakly basic	-0.22	0.00	Metabolite of caffeine
25	Pentoxifylline	275 ± 20	06/05/6493	$C_{13}H_{18}N_4O_3$	0.29	0.3	Basic	0.29	0.00	Vasodilator
26	Primidone	370 ± 3	125-33-7	$C_{12}H_{14}N_2O_2$	0.91	12.3	Weak acid	0.91	0.00	Antiepileptic
27	Ranitidine	857 ± 43	66357-35-5	$C_{13}H_{22}N_4O_3S$	0.27	2.3/8.2	Diprotic base	-1.46	1.00	Antiacid
28	Sucralose	2117 ± 157	56038-13-2	$C_{12}H_{19}Cl3O_8$	-1.00	11.8	Neutral	11.8	0.0	Sweetener
29	Sulfamethoxazole	576 ± 15	723-46-6	$C_{10}H_{11}N_3O_3S$	0.89	1.8/5.6	Amphiprotic	-0.04	-1.0	Antibiotic
30	Sulfapyridine	313 ± 34	144-83-2	$C_{11}H_{11}N_3O_2S$	0.35	2.3/8.4	Amphiprotic	0.35	-1.0	Antibiotic
31	Theophylline	235 ± 31	58-55-9	$C_7H_8N_4O_2$	-0.02	8.8	Basic	-2.35	1.0	Bronchodilator/ Vasodilator
32	Trimethoprim	602 ± 21	738-70-5	$C_{14}H_{18}N_4O_2$	0.91	7.1	Basic	0.20	1.0	Antibiotic
33	Venlafaxine	559 ± 51	93413-69-5	$C_{17}H_{27}NO_2$	3.20	9.4	Basic	0.28	1.0	Antidepressant

* NFAA = N-formyl-4-aminoantipyrine
** pH 6.5

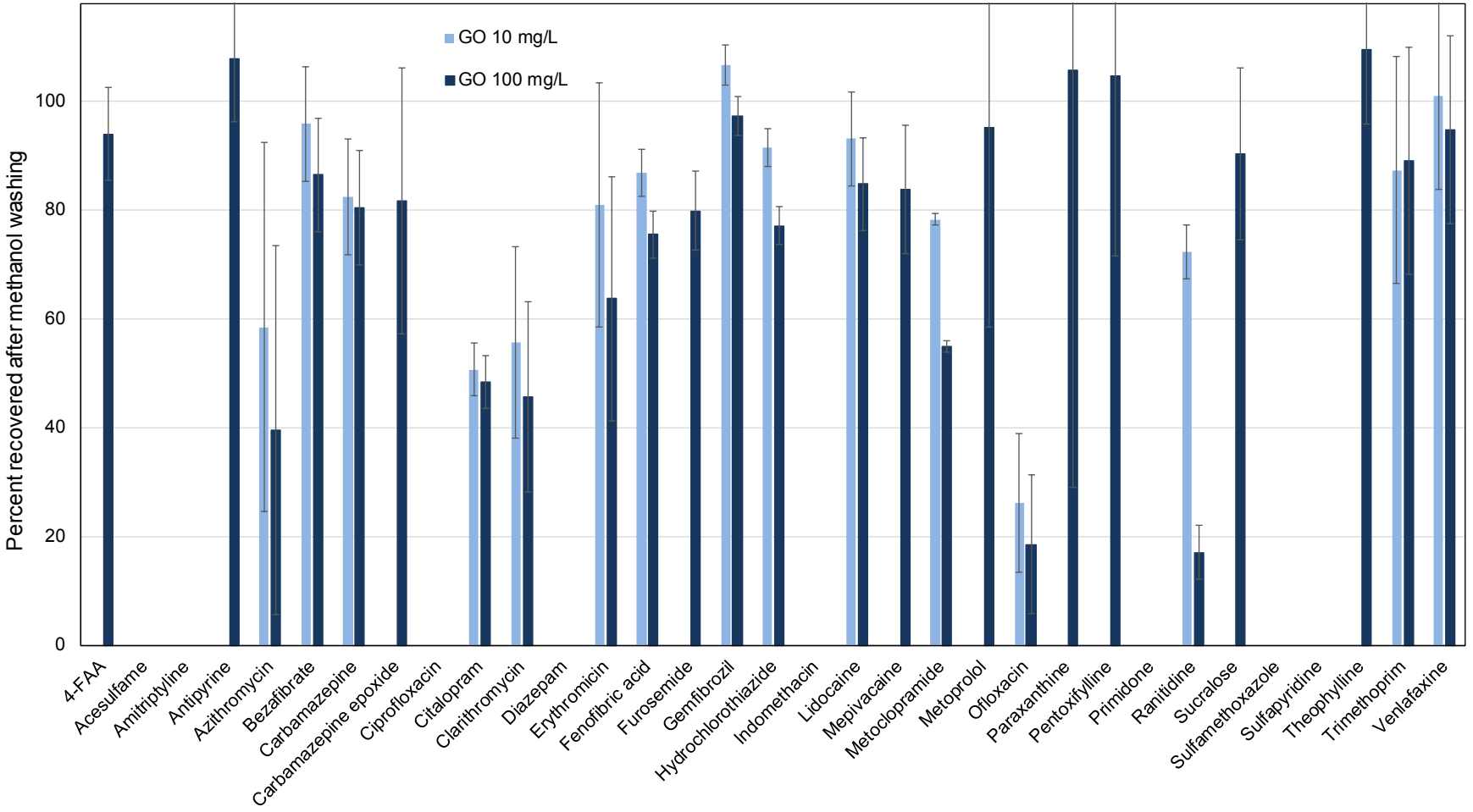
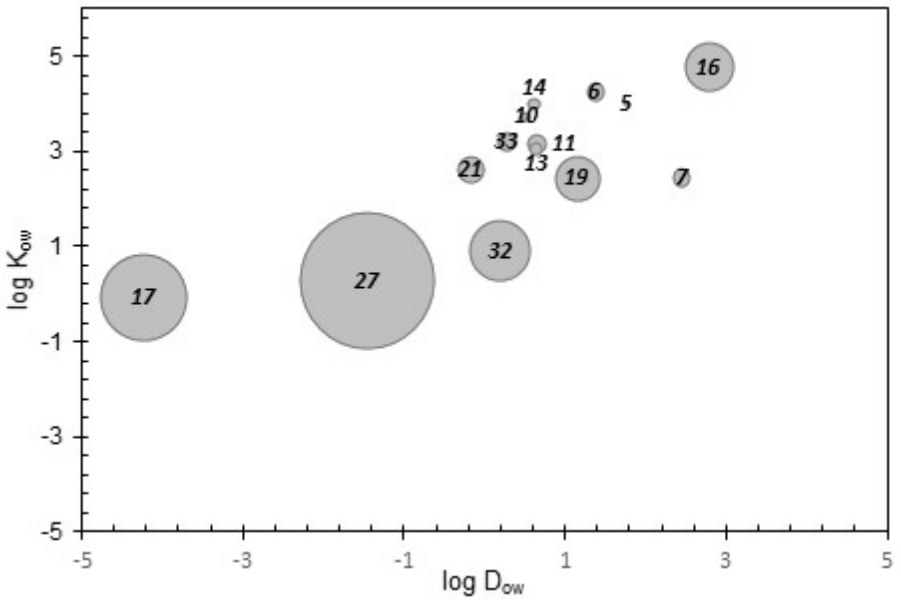


Figure S1. Percent recovery of wastewater pollutants after washing twice with methanol. (The results are shown only for compounds significantly adsorbed on GO nanoparticles; error bars represent standard deviation.)

a)



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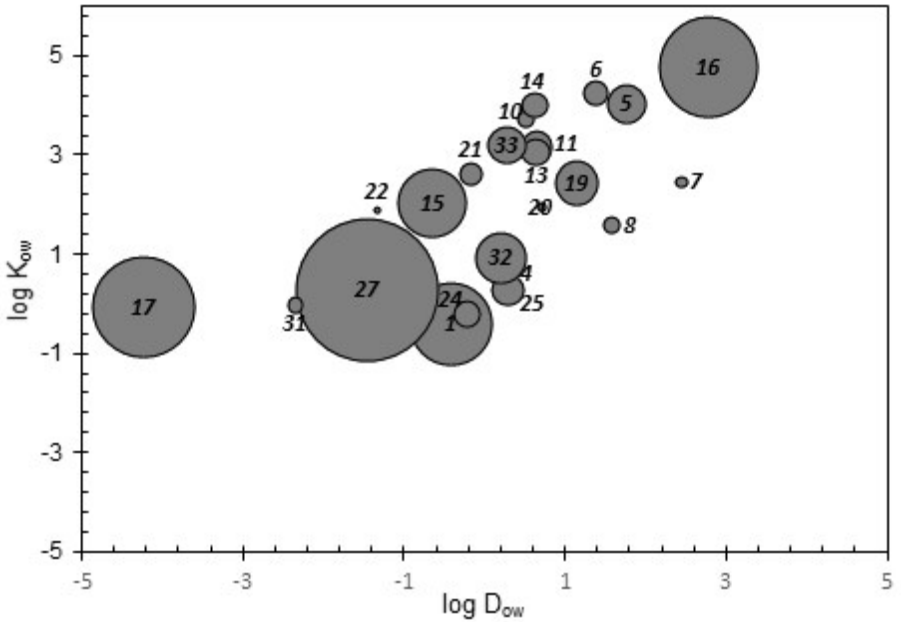

Figure S2. Amount adsorbed (size of bubble proportional to the amount adsorbed in μg/g) as a function of D_{ow}, and K_{ow} (Table S3). The results are shown for compounds significantly adsorbed with respect to the experimental error. Compounds numbered as in Table S3. (a) 10 mg L^{-1} GO, (b) 100 mg L^{-1} GO.

Table S4. Dose-effect parameters for the growth inhibition of *C. reinhardtii* after 72 h of exposure.

	GO (mg L^{-1})				WW (effluent dilution)			
	EC_x	**SD**	**CI_L 95%**	**CI_L 95%**	**EC_x**	**SD**	**CI_L 95%**	**CI_L 95%**
EC_{20}	0.035	0.004	0.027	0.042	0.109	0.006	0.097	0.121
EC_{50}	1.036	0.055	0.926	1.145	0.325	0.007	0.311	0.340
EC_{80}	30.9	3.1	24.8	36.9	0.969	0.040	0.888	1.050

Table S5. Ecotoxicological effects reported in the literature for GO on photosynthetic microalgae.

Organism	**Size range**	**Exposure conditions**	**Toxicological effects**	**Reference**
Chlorella vulgaris	Thickness: 0.8–1 nm Diameter: 1–5 µm Size distribution: 465–486 nm	0.01, 0.1, 1, and 10 mg L^{-1} for 8 days.	- Cell growth inhibition and decrease in chlorophyll content as the concentrations of GO increased. - Generation of ROS and disrupted antioxidant enzymes. - GO enveloped and entered algal cells, and damaged organelles (especially via plasmolysis and an increase in the starch grain number).	Hu *et al*.[1]
	Thickness: 0.8–1.2 nm Lateral length: 0.5–5 µm Hydrodynamic diameter: 295–825 nm	0.01, 0.1, 1, and 10 mg L^{-1} for 96 h.	- Cell division was promoted at 24 h and then inhibited at 96 h. - GO promoted the generation of ROS, loss of mitochondrial membrane potential, plasmolysis and increases in the number of starch grains and the number of lysosome. - GO covered cell surface.	Hu *et al*.[2]
	Thickness: 0.1-1 nm Lateral length: 1.5 µm	0.01, 0.1, 1, and 10 mg L^{-1} for 96 h.	- Inhibition of cell division. - Increased intracellular ROS and mitochondrial membrane potential. - Internalization of GO, damaged the cell ultrastructure, reduced cell permeability, and plasmolysis.	Ouyang *et al*.[3]
	Thickness: 1.02 ± 0.15 nm Lateral length: 0.5–5 µm	0.1, 1 and 10 mg L^{-1} for 96 h	- Inhibition of cell division and chlorophyll *a* biosynthesis. - Enhancement of ROS. Oxidative stress-induced membrane damage, and nutrient depletion. - Cell plasmolysis.	Hu *et al*.[4]
Chlorella pyrenoidosa	Thickness: 2.1 nm	50, 100, 150, 200 mg	- Growth inhibition (EC_{50} 37.3 mg L^{-1}).	Zhao *et al*.[5]

	Lateral size: 2 µm	L^{-1} for 96 h	- Increase of intracellular ROS and membrane damage.	
Picochlorum sp.	Thickness: 3-6 nm	0.5, 1, 2.5, and 5.0 mg L^{-1} for 32 days	- Decrease in viable cell number and chlorophyll *a* concentration. - GO formed a coating layer around algal cells and penetrated the cells without a significant change in their structure.	Hazeem *et al*.[6]
Raphidocelis subcapitata	Thickness: 3.5 nm. Hydrodynamic diameter: 110 nm	0.5, 2, 5, 10, 20, 50, 70, and 100 mg L^{-1} for 96 h.	- Growth inhibition (EC_{50} 20 mg L^{-1}). - Decrease in percentage of the chlorophyll autofluorescence intensity. - Oxidative stress and membrane integrity damage.	Nogueira *et al*.[7]
Scenedesmus obliquus	Thickness: 0.7 nm. Size: 1-10 µm.	1, 2, 4, 8, 16, 32, and 64 mg L^{-1} for 96 h (Combined exposure to GO and Cu^{2+} for 96 h and 12 days.	- Growth inhibition (EC_{50} 21.2 ± 0.5 mg L^{-1}). - Antagonistic effects between GO and copper. GO reduce ecotoxicity of Cu^{2+} at low and environmentally relevant concentrations.	Hu *et al*.[8]

1. X. Hu, K. Lu, L. Mu, J. Kang and Q. Zhou, *Carbon*, 2014, **80**, 665-676.
2. X. Hu, S. Ouyang, L. Mu, J. An and Q. Zhou, *Environmental Science & Technology*, 2015, **49**, 10825-10833.
3. S. Ouyang, X. Hu and Q. Zhou, *ACS Applied Materials & Interfaces*, 2015, **7**, 18104-18112.
4. X. Hu, Y. Gao and Z. Fang, *Carbon*, 2016, **109**, 65-73.
5. J. Zhao, X. Cao, Z. Wang, Y. Dai and B. Xing, *Water Research*, 2017, **111**, 18-27.
6. L. J. Hazeem, M. Bououdina, E. Dewailly, C. Slomianny, A. Barras, Y. Coffinier, S. Szunerits and R. Boukherroub, *Environmental Science and Pollution Research*, 2017, **24**, 4144-4152.
7. P. F. M. Nogueira, D. Nakabayashi and V. Zucolotto, *Aquatic Toxicology*, 2015, **166**, 29-35.
8. C. Hu, N. Hu, X. Li and Y. Zhao, *Ecotoxicology and Environmental Safety*, 2016, **132**, 360-365.

Table S6. Dose-effect relationship parameters of GO, and wastewater (WW) individually and of their binary combinations on *C. reinhardtii* after 72 h of exposure.

	Drug combo	Dose-Effect parameters		
		D_m	m	r
Constant ratio	GO	1.31	0.61	0.953
	WW	0.34	1.04	0.970
	GO+WW	1.35	1.34	0.976
Non-constant ratio	GO	1.64	0.64	0.923
	WW	0.62	0.54	0.932
	GO+WW	1.16	2.48	0.964

The parameters m, D_m and r are the antilog of x-intercept, the slope and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose-effect curve, the potency (EC_{50}), and conformity of the data to the mass-action law, respectively. m was the Hill coefficient used to determine the shape of the dose-response curve, hyperbolic ($m = 1$), sigmoidal ($m > 1$) or negative sigmoidal ($m < 1$); also shown in the table, linear regression correlation coefficients (r-values) of the median-effect plots were > 0.90 in all cases, indicating the conformity of the data to the median-effect principle.

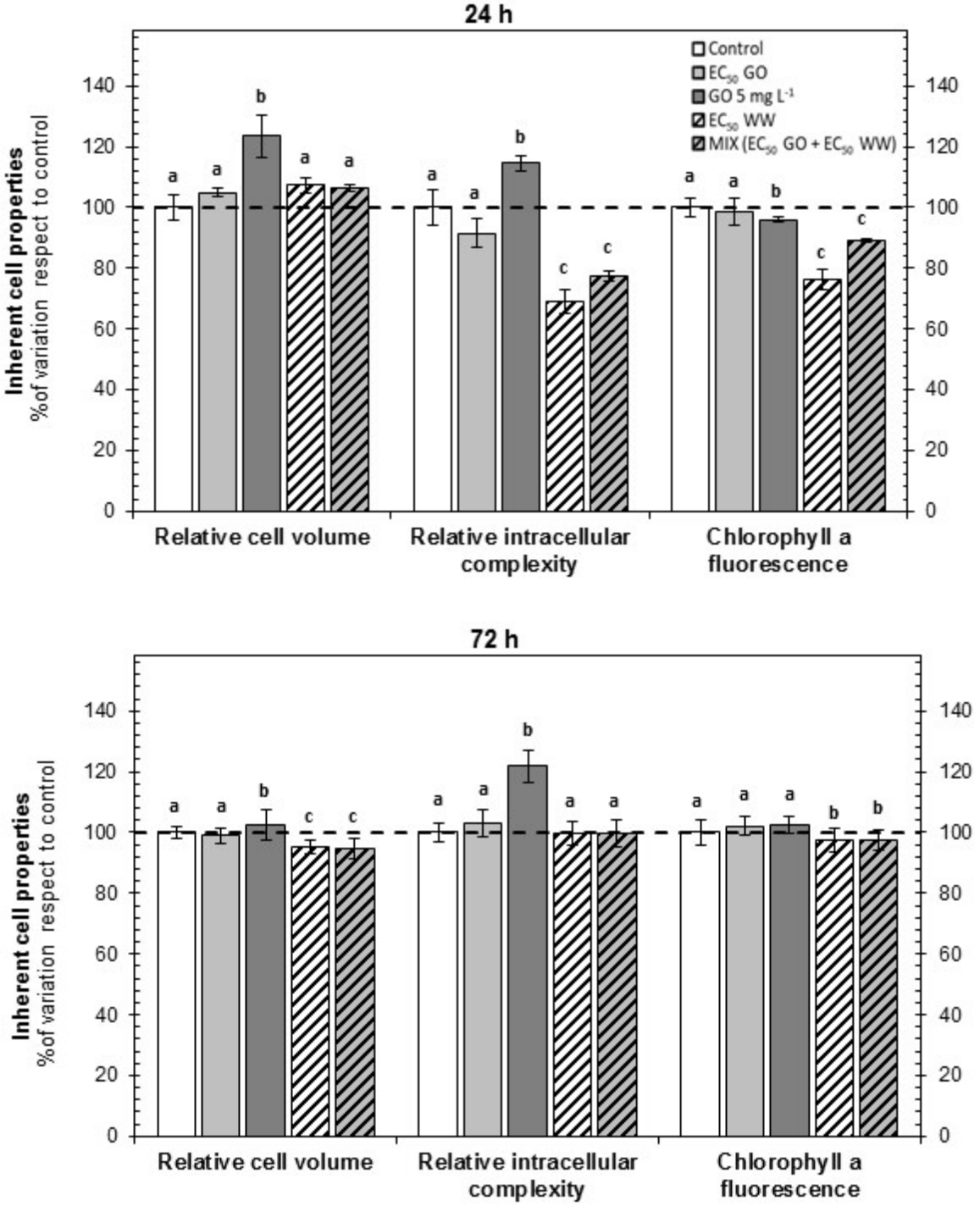

Figure S3. Variations in inherent cell properties of *C. reinhardtii* cells after 24 and 72 h of exposure. Results are shown as percentage of variation of cell volume, intracellular complexity, and chlorophyll *a* fluorescence ± SD with respect to control (assigned a value of 100%, indicated by the dashed line). Treatments with different letters are significantly different (Tukey's´ HSD, $p < 0.01$).

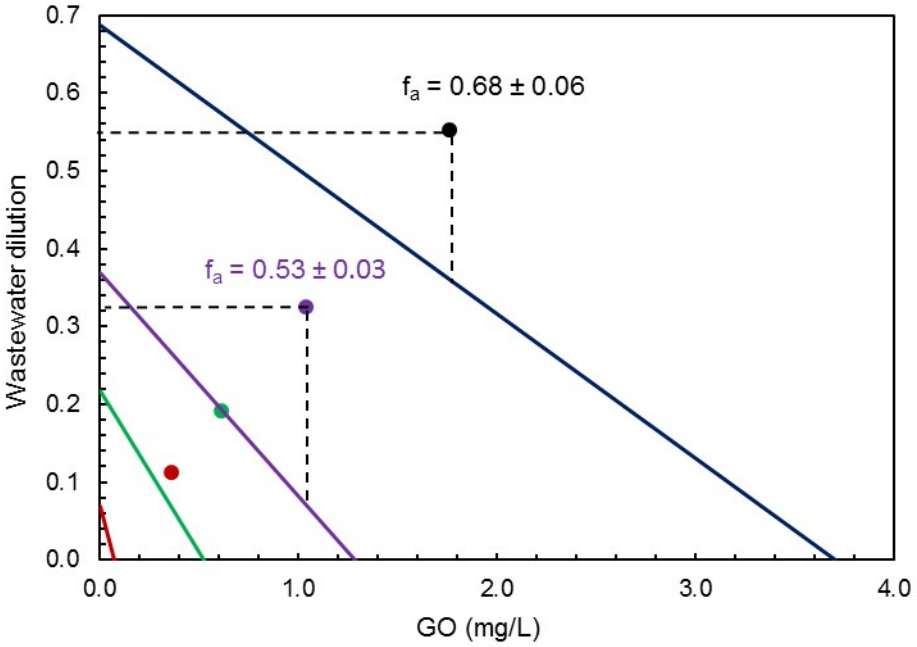

Figure S4. Isobolograms representing constant ratio binary combinations for different joint affected fraction (f_a). Vertical distances represent the wastewater dilution theoretically required to reach additivity. Points with the same colour represent experimental toxicity and error.

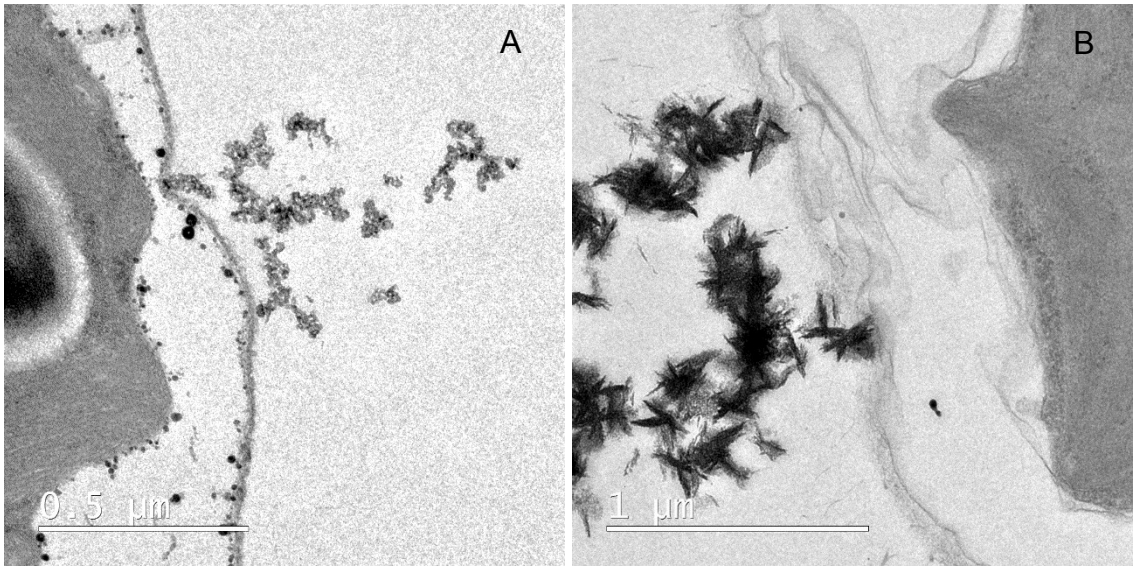

Figure S5. TEM micrographs of *C. reinhardtii* (A) exposed to 5 mg L^{-1} GO in Tris-minimal phosphate (TP) medium at pH 6.5 and (B) in wastewater at EC_{50} GO + EC_{50} wastewater for 72 h.

Table S7. Experimental results related to the data points shown in Fig. 1.

Constant ratio					
Data point	GO (mg/L)	WW dil.	f_a	CI	CI SD
1	0.358	0.112	0.173	5.20	0.71
2	0.609	0.191	0.379	1.96	0.40
3	1.036	0.325	0.499	1.73	0.31
4	1.761	0.552	0.685	1.16	0.25
5	2.994	0.939	0.799	0.98	0.23
Non-constant ratio					
Data point	GO (mg/L)	WW dil.	f_a	CI	CI SD
1	0.173	0.271	0.087	37.8	4.9
2	0.345	0.217	0.123	18.9	4.8
3	0.518	0.163	0.192	6.96	1.37
4	0.691	0.108	0.271	3.08	0.43
5	0.863	0.054	0.350	1.56	0.21