

SUPPLEMENTARY INFORMATION

Toxic interactions of silver species with freshwater green algae and cyanobacteria and their effects on mechanistic endpoints and the production of extracellular polymeric substances

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Table S1. AG1 in in MBL, CR⁻, SL⁻ and CR⁺ : TEM data Primary particle (d_{pp}) and cluster diameters (d_{AGG}), size ranges, aspect ratios of primary particles (S_{pp}) and clusters (S_{AGG}) and percentage of clusters in tightly bound form visually estimated.

Media	Time (h)	Conc. (mg/L)	d_{pp} (nm/no particles)	d_{pp} range (nm)	S_{pp} (range)	d_{AGG} (nm/no particles)	d_{AGG} range (nm)	S_{AGG} (range)	%Tightly Bound (no. particles)
MBL	0	10	8.43±3.71/102	2.43-23.4	1.14±0.26 (1.0-1.46)	51.22±26.24/17	22.89-96.08	1.32±0.252 (1.01-1.69)	58.8% (17)
	336	10	9.62±4.95/110	3.45-33.74	1.25±0.23 (1.0-2.08)	120.45±85.25/17	32.35-313.69	1.2±0.241 (1.01-1.90)	82.35% (17)
CR ⁻	0	10	7.77±3.34/106	4.94-15.2	1.22±0.48 (1.0-2.24)	32.11±11.28/40	12.32-57.56	1.28±0.23 (1.01-2.0)	37.5% (40)
	336	10	7.81±3.36/104	4.81-12.65	1.17±0.48 (1.0-2.25)	43.6±17.32/37	14.22-88.74	1.26±0.43 (1.0-3.3)	51.35% (37)
SL ⁻	0	10	9.45±4.31/102	4.49-22.98	1.21±0.45 (1.0-1.87)	63.31±56.35/31	16.8-333.73	1.23±0.29 (1.0-2.36)	58.07% (31)
	336	10	10.4±4.7/92	3.48-21.28	1.27±0.49 (1.0-2.15)	44.72±16.6/36	18.38-94.89	1.28±0.26 (1.02-2.05)	63.89% (36)
CR ⁺	0	10	9.17±3.59/107	4.64-19.01	1.30±0.46 (1.01-2.57)	48.33±35.96/37	17.53-189.37	1.21±0.23 (1.01-2.06)	48.65% (37)
	336	10	7.84±3.48/102	4.21-15.19	1.15±0.46 (1.0-1.52)	48.64±24.45/38	14.26-133.61	1.12±0.13 (1.0-1.6)	76.32% (38)

Table S2. Stock solutions used in the creation of MBL Woods Hole media and masses of chemical added to distilled water to create them. Media was prepared by adding 1 mL of each stock solution (1 -11) to 1 L of distilled water (dH₂O). pH was adjusted to 7.2 with HCl. Media was then autoclaved at 121°C (15 PSI) for 15 minutes.

Stock Solution	Constituent	Mass of constituent chemical added to 1L distilled water (dH ₂ O) (g)	
1	CaCl ₂ .2H ₂ O	36.76	
2	MgSO ₄ .7H ₂ O	36.97	
3	NaHCO ₃	12.60	
4	K ₂ HPO ₄	8.71	
5	NaNO ₃	85.01	
6	Na ₂ SiO ₃ .9H ₂ O	28.42	
7	Na ₂ EDTA	4.36	
8	FeCl ₃ .6H ₂ O	3.15	
9	Metal Mix	Additional instructions: Add each constituent chemical separately to ~750mL of dH ₂ O, fully dissolve between each addition. Finally make up to 1L with dH ₂ O.	
	CuSO ₄ .5H ₂ O		0.01
	ZnSO ₄ .7H ₂ O		0.022
	CoCl ₂ .6H ₂ O		0.01
	MnCl ₂ .4H ₂ O		0.18
	Na ₂ MoO ₄ .2H ₂ O		0.006
10	H ₃ BO ₃	28.4	
11	Tris stock	250.0	

Table S3. Nominal and Corrected EC₂₀, ₅₀, ₇₀ values for each toxicant exposed to both species estimated from log-logistic equation fitted to dose-response data sets comprised of independent grown inhibition ecotoxicology experiments. Corrected EC_x values converted using correction factor derived from difference between nominal and total measured silver values in analytics experiments. EC_x values given as nominal dose or corrected dose ± 1 standard error. CF is the identifier of correction factor (Table S7) used to convert nominal values.

N= number of experiments used to create curve, n=total number of data points used to create curve, R=replicates within each experiment. *C. reinhardtii* AG1: N=7, n=111, R=2. AG2: N=4, n=64, R=2, AgNO₃: N=6, n=48, R=2. *S. leopoliensis* AG1: N=6, n=94, R=2. AG2: N=6, n=93, R=2. AgNO₃: N=3, n=43, R=2

<i>C. reinhardtii</i>									
ECx	AG1 [µg/L]			AG2 [µg/L]			AgNO ₃ [µg/L]		
	Initial	CF	Corrected	Initial	CF	Corrected	Initial	CF	Corrected
20	970 ± 23.4	4	36.6 ± 0.9	1624 ± 180	5	27.7 ± 3.1	24 ± 5.8	2	25 ± 6
50	993 ± 7	4	37.5 ± 0.3	1819 ± 62	5	31.1 ± 1.1	43 ± 7.7	2	44.6 ± 8
70	1008 ± 7	4	38.1 ± 0.3	1950 ± 117	5	33.3 ± 2	63 ± 13	2	65.4 ± 13.5
<i>S. leopoliensis</i>									
ECx									
20	95 ± 23.3	8	16.1 ± 4	33 ± 19	10	1.8 ± 1.1	9.3 ± 5	6	11 ± 6
50	164 ± 27	8	27.8 ± 4.6	184 ± 59.8	10	10.2 ± 3.3	66.9 ± 65	7	38.2 ± 37.1
70	228 ± 53	9	17.9 ± 4.2	531 ± 267	11	27.9 ± 14.1	223 ± 342	7	127.4 ± 195.4

Table S4. Nominal concentrations, measured and dissolved silver levels from analytical measurements at 0 h and 72 h for plain media and AgNO₃, AG1 and AG2 *C. reinhardtii* exposures

<i>C. reinhardtii</i>					
Silver concentrations (µg/L)					
Toxicant	Nominal Concentration	Measured Total Silver		Measured Total Dissolved Silver	
		0 h	72 h	0 h	72 h
Plain media	n/a	0.065	n/a	n/a	n/a
	n/a	0.102	n/a	n/a	n/a
AgNO ₃	1	1.80	1.18	1.47	0.15
	1	1.79	1.69	1.63	0.10
	10	9.92	5.93	10.59	0.25
	10	11.02	6.52	10.99	0.27
	20	20.60	18.02	20.01	5.44
	20	20.92	17.14	20.72	5.81
AG1	100	7.50	1.50	1.21	2.10
	100	7.12	1.98	1.79	1.13
	500	24.04	27.09	2.22	2.22
	500	23.32	28.21	1.60	2.03
	700	27.60	27.88	2.54	1.66
	700	25.26	27.92	12.36	1.58
AG2	500	15.02	5.52	0.37	1.41
	500	28.69	4.61	0.16	2.07
	1500	28.84	27.28	0.95	1.28
	1500	27.96	23.68	0.85	6.25
	1700	28.91	22.20	0.77	1.77
	1700	29.14	26.11	0.90	4.94

Table S5. Nominal concentrations, measured and dissolved silver levels from analytical measurements at 0 h and 72 h for AgNO₃, AG1 and AG2 *S. leopoliensis* exposures

<i>S. leopoliensis</i>					
		Silver Concentrations (µg/L)			
Toxicant	Nominal Concentrations	Measured Total Silver		Measured Total Dissolved Silver	
		0 h	72 h	0 h	72 h
AgNO ₃	2	2.3585	1.4965	2.112	0.0875
	2	2.3795	1.3705	2.131	0.0665
	20	21.9835	19.314	21.6915	3.4515
	20	22.636	20.246	22.303	5.025
	50	28.3305	26.9265	28.2285	16.583
	50	28.799	27.5395	27.2015	16.153
AG1	20	0.514	1.984	0.514	0.989625
	20	0.8515	1.669	0.8515	0.75126
	100	17.1695	13.239	1.915869	1.995141
	100	16.7355	9.1705	2.096332	2.057427
	250	19.894	10.202	1.639329	1.95861
	250	19.3585	7.789	0.940856	1.90564
AG2	250	14.025	4.481	1.741068	2.645941
	250	13.653	10.367	2.115146	-0.16676
	500	26.877	15.43	1.202053	3.476291
	500	25.819	16.3285	2.14638	2.64101
	1100	26.1775	20.612	2.958099	2.500913
	1100	25.941	17.4825	2.670417	3.645613

Table S6. Particle size distribution, hydrodynamic/stokes diameter and zeta potential for AG1 and AG2 nanoparticles over a 2 week period using DLS, NTA, DCS and ZP.

Sample	Media	Conc. (mg/L)	Technique					ZP range (mV)	EPM range ($\mu\text{mcm/Vs}$)
			DLS	NTA		DCS			
			Z-average d_H (nm)	PSD (nm)	d_H (nm)	PSD (nm)	d_s (nm)		
AG1	MBL	10	145.7-169.2	~3-212	~30-35	~10-90	~15-25	-43 to -38.6	-2.64 to -0.27
	CR ⁺	10	185.9-217.1	~3-269	~56-51	~5-297	~35	-11.7 to -12.9	-1.01 to -0.91
	CR ⁻	10	117.3-121.6	~7-187	~32	~10-299	~30-45	-15 to -36.1	-2.83 to -1.19
	SL ⁺	10	123.7-134.2	~7-171	~32	~10-300	~30-40	-40.8 to -35.5	-3.20 to -2.70
AG2	MBL	1	68.1-77.1	0-60	~20	~20-100	~45	-24.6 to -21.1	-2.16 to -1.73
	CR ⁺	1	76.4-81.5	3-205	~18-22	~14-298	~50	-24.7 to -20.8	-1.94 to -1.63
	CR ⁻	1	71.1-77.3	3-183	~16-19	~14-298	~50	-25 to -17	-1.96 to -1.34
	SL ⁺	1	69.9-86.3	3-238	~17-20	~10-297	~50	-32 to -26	-2.51 to -2.04

Table S7. AG2 in in MBL, CR⁻, SL⁻ and CR⁺ : TEM data Primary particle (d_{pp}) and cluster diameters (d_{AGG}), size ranges, aspect ratios of primary particles (S_{pp}) and clusters (S_{AGG}) and percentage of clusters in tightly bound form visually estimated.

Media	Time (h)	Conc. (mg/L)	d_{pp} (nm/no particles)	d_{pp} range (nm)	S_{pp} (range)	d_{AGG} (nm/no particles)	d_{AGG} range (nm)	S_{AGG} (range)	%Tight Bound (no particles)
MBL	0	10	43.2 \pm 17.07/55	9.56-104.73	1.29 \pm 0.21 (1.01-1.87)	102.77 \pm 50.49/8	56.16-195.39	1.64 \pm 0.43 (1.06-2.22)	0% (8)
	336	10	47.0 \pm 17.65/45	19.13-86.37	1.28 \pm 0.2 (1.0-2.08)	71.03 \pm 16.51/5	52.13-90.55	1.81 \pm 0.52 (1.29-2.58)	0% (5)
CR ⁻	0	10	50.51 \pm 14.5/52	21.31-96.15	1.18 \pm 0.091 (1.02-1.39)	99.73 \pm 50.28/12	39.29-215.29	1.57 \pm 0.55 (1.13-2.84)	0% (12)
	336	10	60.92 \pm 20.82/56	26.74-130.08	1.19 \pm 0.14 (1.01-1.64)	154.08 \pm 98.51/7	72.38-365.93	1.59 \pm 1.59 (1.08-5.95)	0% (7)
SL ⁻	0	10	56.12 \pm 18.45/51	16.99-133.39	1.21 \pm 0.13 (1.02-1.7)	0/0	0	0 (0)	0% (0)
	336	10	75.73 \pm 53.49/39	35.08-313.12	1.28 \pm 0.24 (1.02-1.98)	0/0	0	0 (0)	0% (0)
CR ⁺	0	10	54.84 \pm 15.95/54	26.52-104.53	1.18 \pm 0.13 (1.0-1.48)	131.23 \pm 81.88/4	73.97-249.35	1.82 \pm 0.69 (1.13-2.71)	0% (4)
	336	10	54.29 \pm 13.42/51	32.62-109.67	1.2 \pm 0.13 (1.02-1.72)	91.09 \pm 47.4/7	55.1-191	1.97 \pm 0.47 (1.2-2.81)	0% (7)

Table S8. Calculation of EC_x correction factors. Total silver concentration of the experiment (at 0 h) and correction factor calculated through total measured silver concentrations over nominal concentrations applied. Correction factors are given an identifier to show which correction factor was used to convert nominal concentrations.

Toxicant	Nominal Concentrations (µg/L)	Measured Average Total Silver Concentration (0 h)	Correction Factor (measured / nominal concentration)	Correction Factor Identifier
<i>C. reinhardtii</i>				
AgNO₃	10	10.47375	1.047375	1
	20	20.76175	1.038088	2
AG1	100	7.31375	0.073138	3
	700	26.42775	0.037754	4
AG2	1700	29.0245	0.017073	5
<i>S. leopoliensis</i>				
AgNO₃	2	2.369	1.1845	6
	50	28.56475	0.571295	7
AG1	100	16.9525	0.169525	8
	250	19.62625	0.078505	9
AG2	250	13.839	0.055356	10
	500	26.348	0.052696	11

Table S9. Nominal and Corrected concentrations for each toxicant in EPS experiments. Corrected EC_x values converted using correction factor derived from difference between nominal and total measured silver values in analytics experiments. CF is the identifier of correction factor (Table S7) used to convert nominal values.

<i>C. reinhardtii</i>								
AG1 [µg/L]			AG2 [µg/L]			AgNO ₃ [µg/L]		
Nominal	CF	Corrected	Nominal	CF	Corrected	Nominal	CF	Corrected
50	3	3.7	1624	5	27.7	5	1	5.2
969	4	36.6	3500	5	59.8	23	2	24.1
<i>S. leopoliensis</i>								
300	9	23.6	600	11	31.6	9.2	6	10.9

Table S10. Total EPS detected as DOC per dry weight [mg/mg] produced by *C. reinhardtii* within 72 h in control cultures or when exposed to nominal concentrations of AG1, AG2, or AgNO₃. Each line represents one replicate.

	AG1		AG2		AgNO₃	
Control	50 µg/L	969 µg/L	1624 µg/L	3500 µg/L	5 µg/L	23 µg/L
0.0503	0.0378	0.0476	0.0189	0.0237	0.0369	0.0449
0.0519	0.0373	0.0275	0.0486	0.0148	0.0402	0.0587
0.0484	0.0391	0.0284	0.0360	0.0178	0.0579	0.0313

Table S11. Percentage of biopolymers of EPS detected as DOC per dry weight [mg/mg] produced by *C. reinhardtii* within 72 h in control cultures or when exposed to nominal concentrations of AG1, AG2, or AgNO₃.

	AG1		AG2		AgNO₃	
Control	50 µg/L	969 µg/L	1624 µg/L	3500 µg/L	5 µg/L	23 µg/L
62.7742	65.3284	14.3012	19.5643	11.4372	44.3222	16.4550
66.2854	61.2603	16.9474	23.3519	21.3451	61.0834	14.4921
68.3537	63.5068	15.5916	18.8535	59.9493	19.4811	18.9951

Table S12. Percentage of building blocks of humic substances of EPS detected as DOC per dry weight [mg/mg] produced by *C. reinhardtii* within 72 h in control cultures or when exposed to nominal concentrations of AG1, AG2, or AgNO₃.

	AG1		AG2		AgNO₃	
Control	50 µg/L	969 µg/L	1624 µg/L	3500 µg/L	5 µg/L	23 µg/L
12.9051	11.2910	18.7251	19.9382	18.0025	9.2403	19.1310
11.6171	11.3852	19.7658	16.8085	17.5492	11.1245	20.2411
10.6347	12.1131	20.1097	18.6824	11.6142	18.2799	24.5197

Table S13. Percentage of low molecular weight acids of EPS detected as DOC per dry weight [mg/mg] produced by *C. reinhardtii* within 72 h in control cultures or when exposed to nominal concentrations of AG1, AG2, or AgNO₃.

	AG1		AG2		AgNO₃	
Control	50 µg/L	969 µg/L	1624 µg/L	3500 µg/L	5 µg/L	23 µg/L
14.4672	13.8517	25.2617	25.7078	25.1097	11.4593	26.5819
13.1457	14.3313	26.7416	24.2158	22.5802	14.5322	27.8564
11.2992	14.8051	26.8568	24.6405	14.7896	25.9487	29.3520

Table S14. Percentage of neutral/amphiphilic compounds of EPS detected as DOC per dry weight [mg/mg] produced by *C. reinhardtii* within 72 h in control cultures or when exposed to nominal concentrations AG1, AG2, or AgNO₃.

Control	AG1 50 µg/L	969 µg/L	AG2 1624 µg/L	3500 µg/L	AgNO ₃ 5 µg/L	23 µg/L
9.8535	9.5289	41.7120	34.7898	45.4506	34.9782	37.8321
8.9518	13.0232	36.5451	35.6237	38.5255	13.2600	37.4103
9.7124	9.5750	37.4419	37.8236	13.6470	36.2903	27.1331

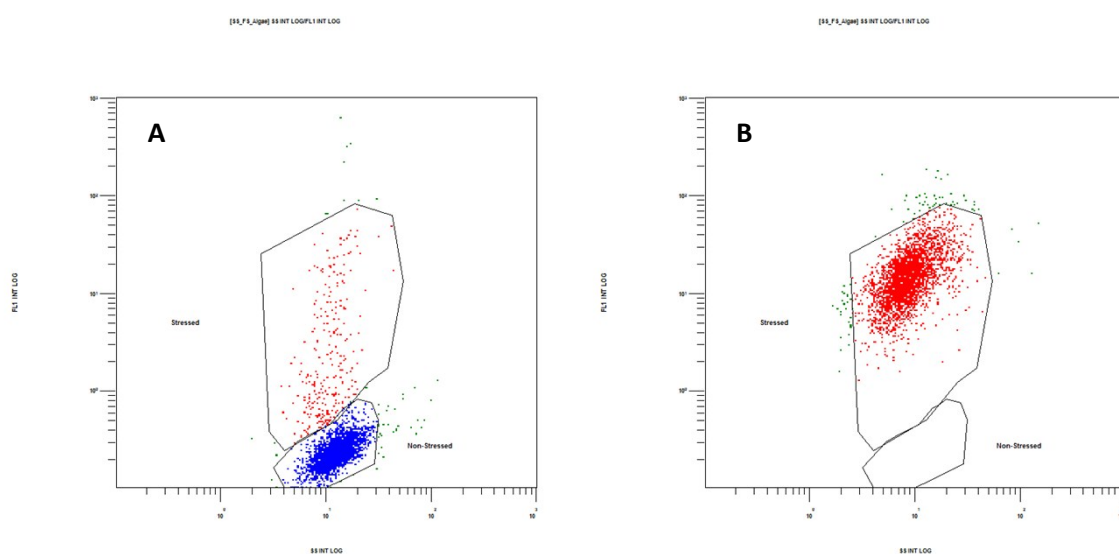


Figure S1. Dot plots of FL1 detected auto-fluorescence vs. Side scatter (SS) obtained by flow cytometry showing a control population stained using FDA (A) and the effect of H₂O₂ on the population (B).

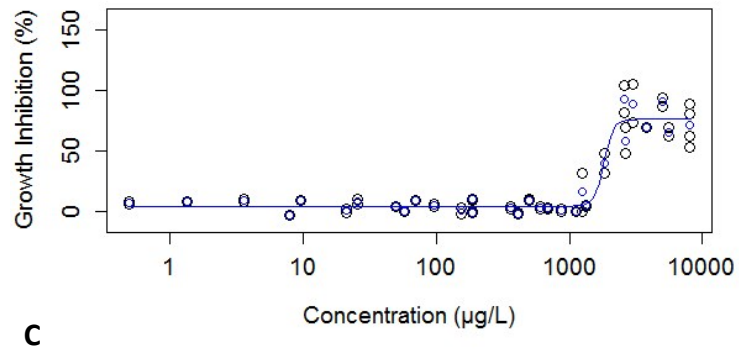
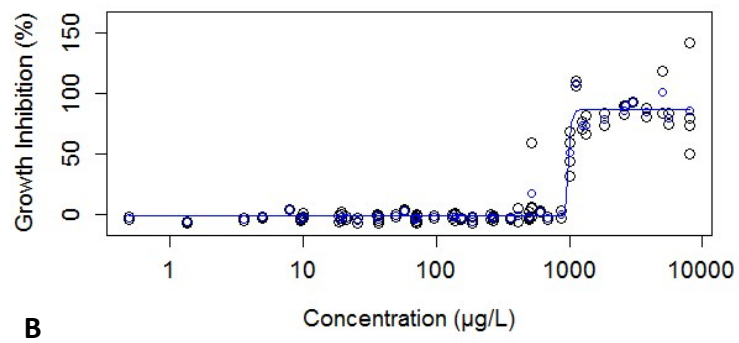
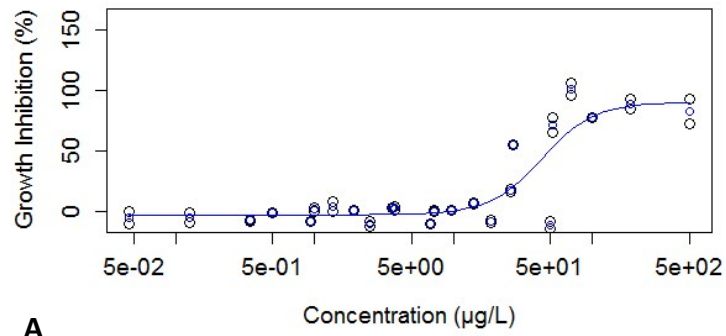
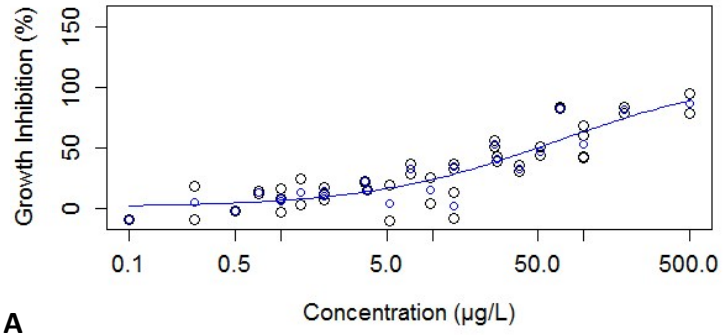
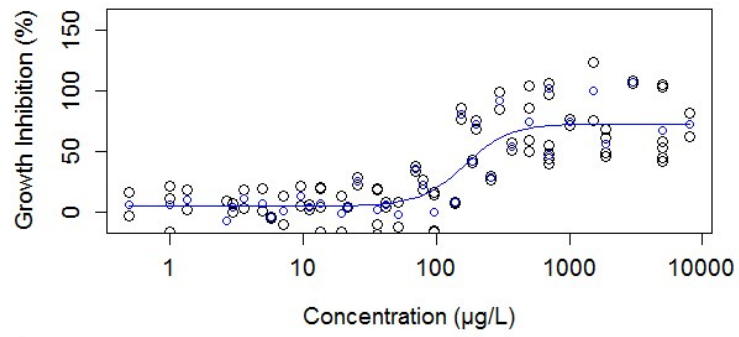


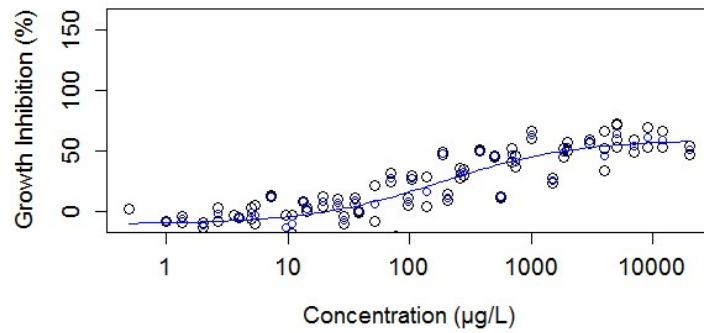
Figure S2. *C. reinhardtii* dose-response curves with log-logistic (4 parameter) curve fit A) AgNO₃ B) AG1 C) AG2



A



B



C

Figure S3. *S. leopoliensis* dose-response curves with log-logistic (4 parameter) curve fit A) AgNO₃ B) AG1 C) AG2

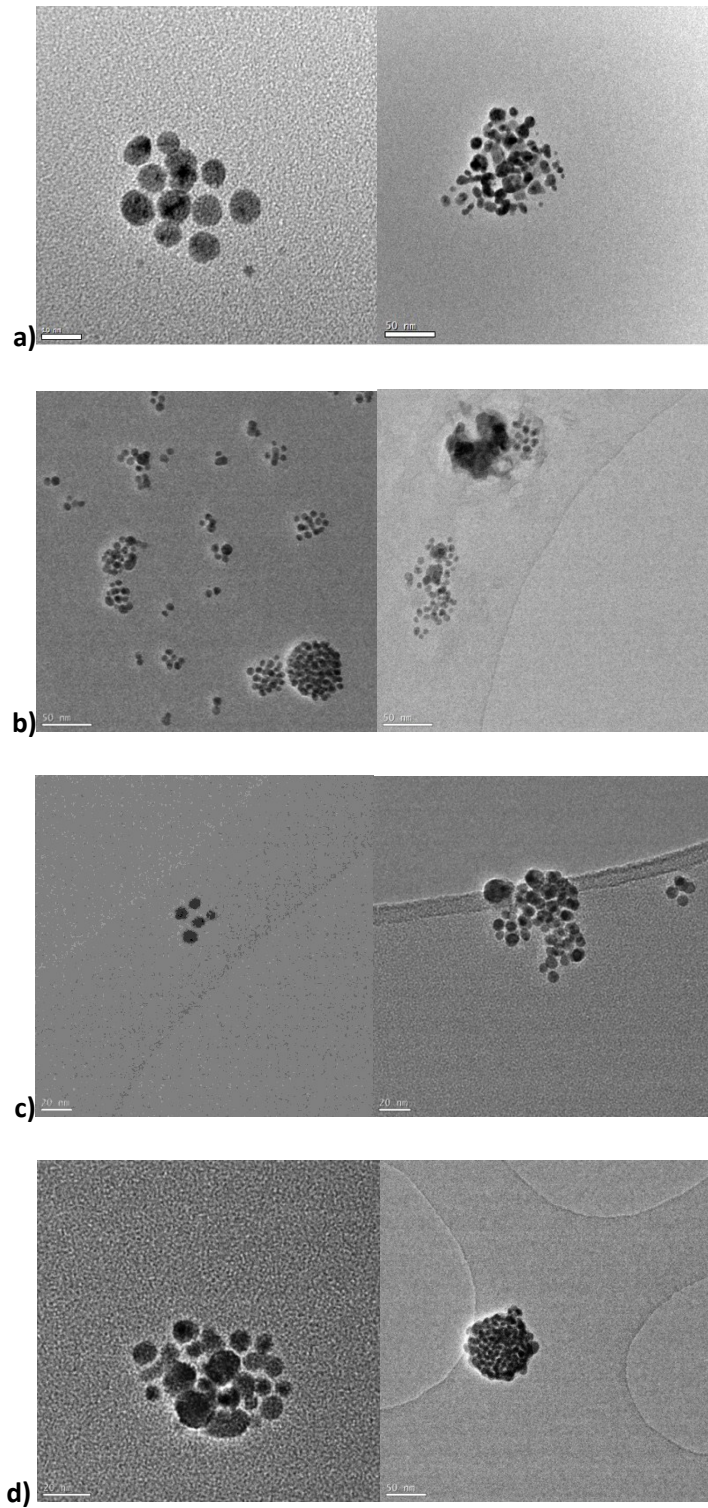


Figure. S4. TEM image of 10mg/L AG1 in different media at 0h and 2 weeks – a) MBL medium b) CR⁻ c) CR⁺ d) SL⁻

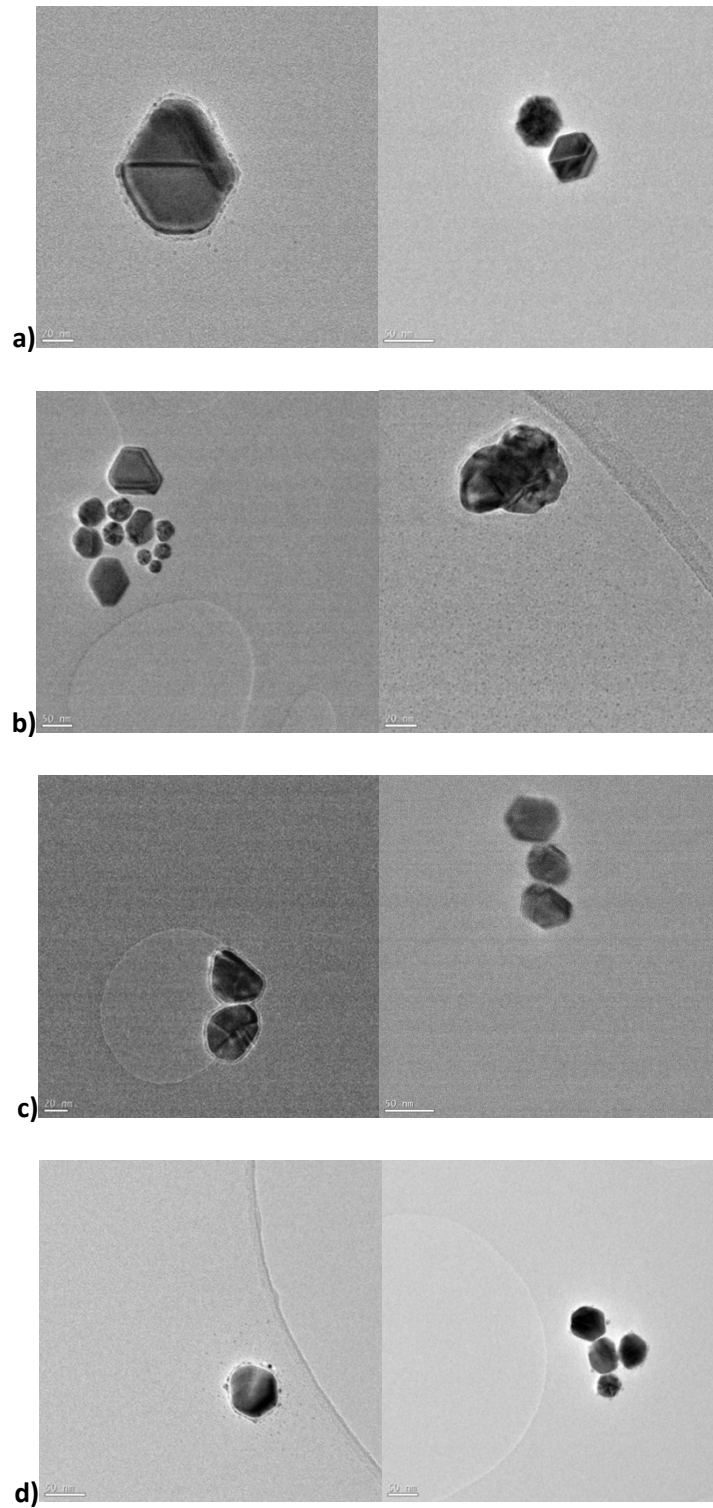


Figure. S5. TEM image of 1mg/L AG2 in different media at 0h and 2 weeks – a) MBL medium b) CR⁻ c) CR⁺ d) SL⁻

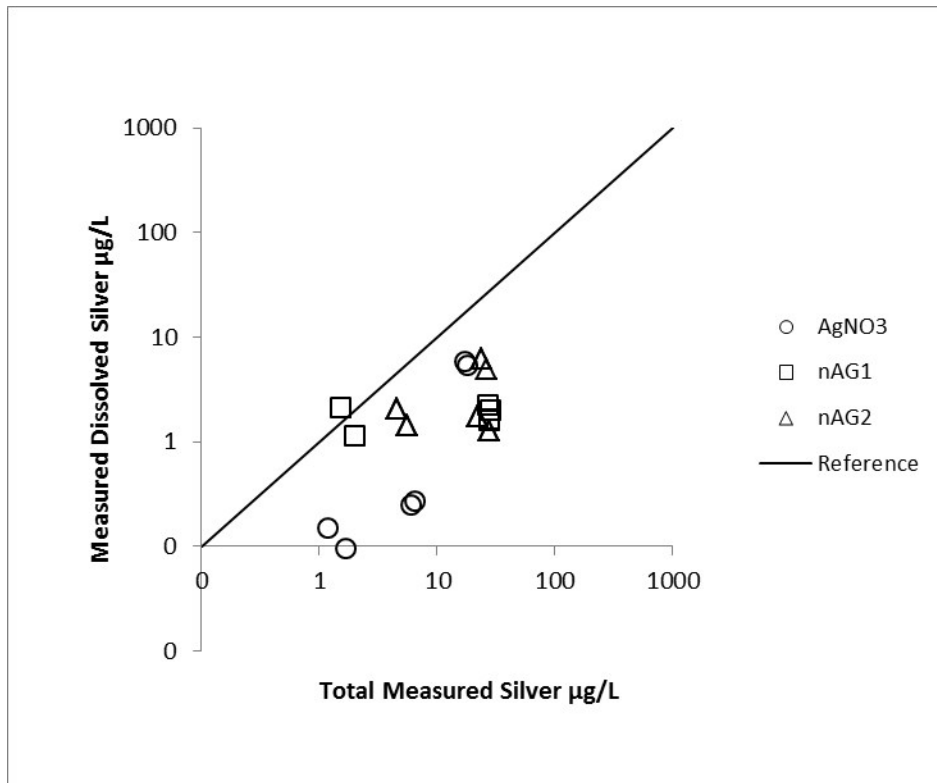


Figure S6. Total vs. Dissolved silver for AgNO₃, AG1 and AG2 *C. reinhardtii* exposures

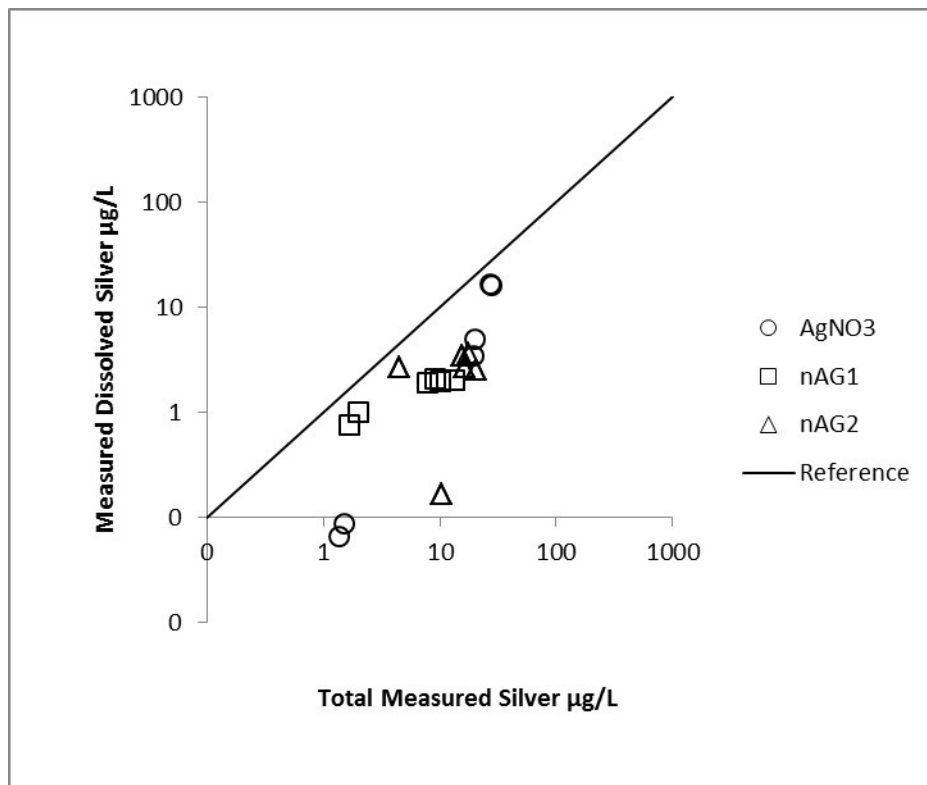


Figure S7. Total vs. Dissolved silver for AgNO₃, AG1 and AG2 *S. leopoliensis* exposures.

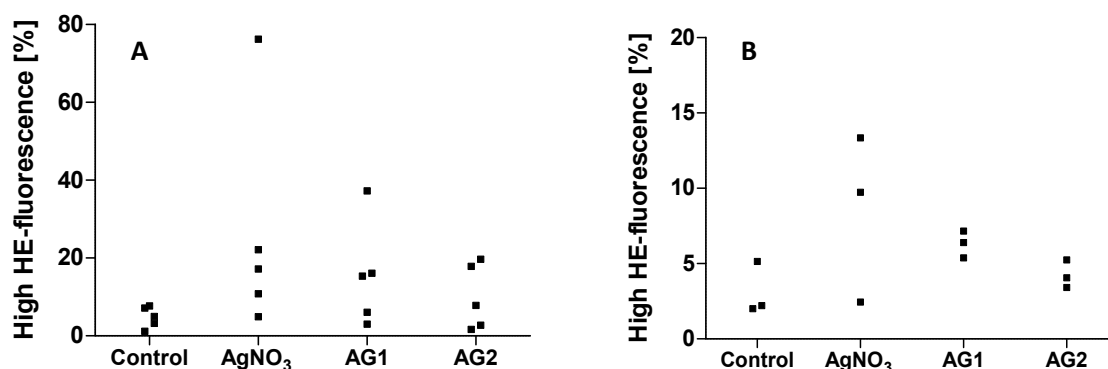


Figure S8. Reactive Oxygen Species production: HE Stain. Percentage of high HE fluorescent cells (detection of H₂O₂) when exposed to EC₇₀ of AgNO₃, AG1 and AG2 in A) *C. reinhardtii* and B) *S. leopoliensis*. Means +/- 1SD for the means of 3 replicates of 5 experiments (*C. reinhardtii*) and 3 experiments (*S. leopoliensis*) respectively. 'a' indicates significant difference compared to the control (p value ≤0.05) derived from fitting a standard linear model to ratios of stressed/unstressed cells. Control value deviates from 0 % because even in healthy cultures some cells fall into the stressed gate.

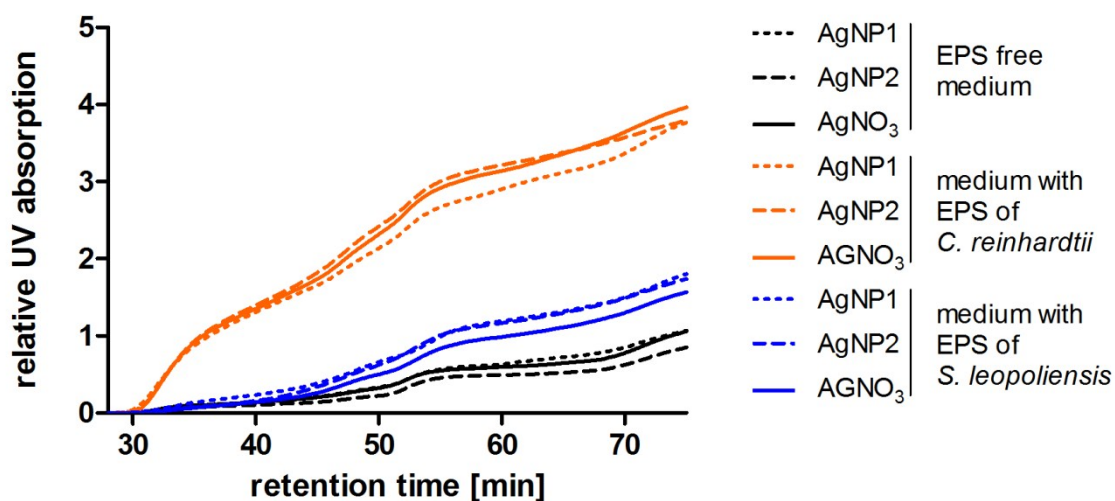


Figure S9. Representative UV-signals of DOC in algae growth medium integrated from 28 to 75 min retention time. Algae growth medium contained either no EPS or EPS produced within 72 h of culturing of either *C. reinhardtii* or *S. leopoliensis*. AG1, AG2, or AgNO₃ was diluted in each type of medium to yield the respective nominal concentration of Ag and incubated for 72 h as during the toxicity assays.

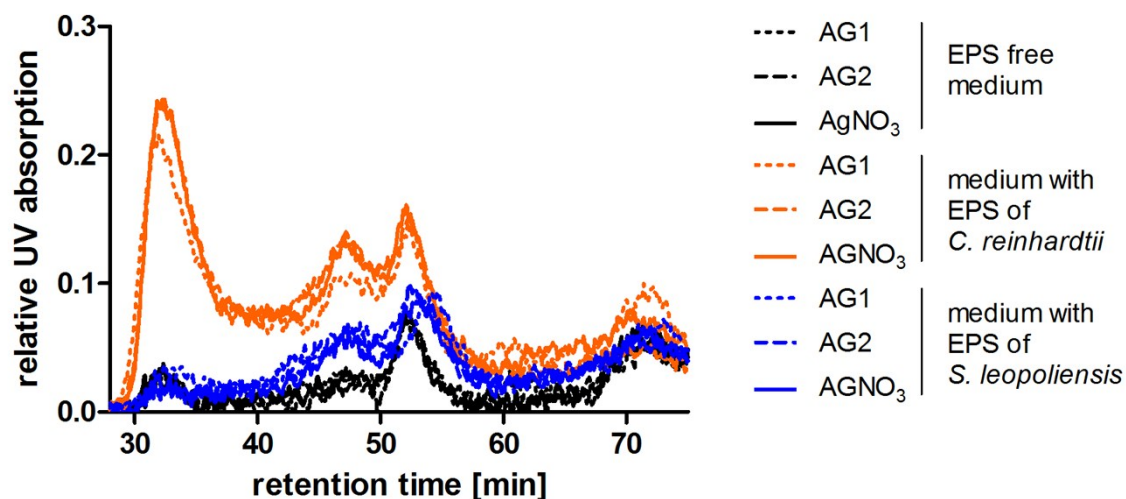


Figure S10. Representative UV-signals of DOC in algae growth medium between 28 to 75 min retention time. Algae growth medium contained either no EPS or EPS produced within 72 h of culturing of either *C. reinhardtii* or *S. leopoliensis*. AG1, AG2, or AgNO₃ was diluted in each type of medium to yield the respective EC₇₀ and incubated for 72 h as during the toxicity assays

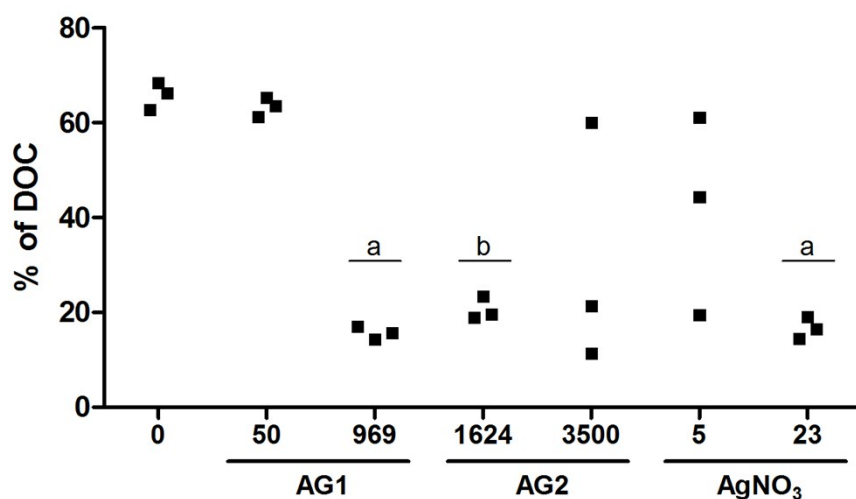


Figure S11. Percentage of biopolymers of total EPS detected as DOC per dry weight produced by *C. reinhardtii* within 72 h in control cultures or when exposed to indicated concentrations [$\mu\text{g/L}$] of AG1, AG2, or AgNO₃. a: **, b: *, means are significantly different from control, one way ANOVA, $p=0.0005$, $F=8.505$, Bonferroni's post-test.

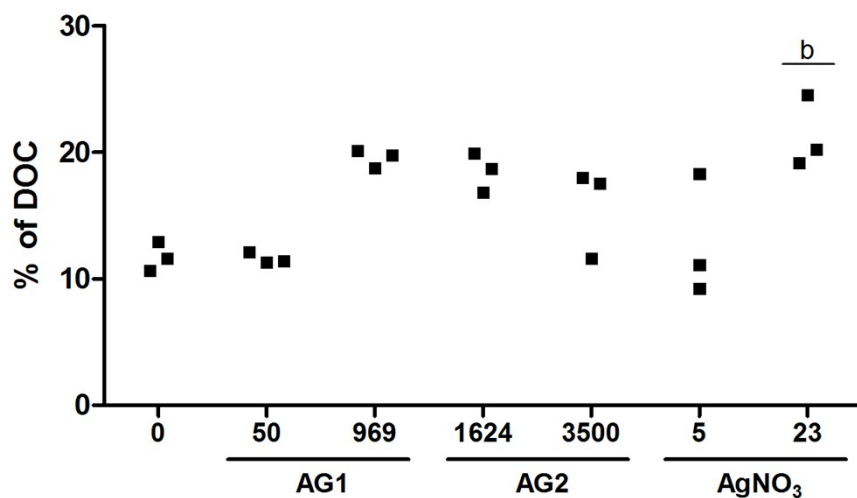


Figure S12. Percentage of building blocks of humic substances of total EPS detected as DOC per dry weight produced by *C. reinhardtii* within 72 h in control cultures or when exposed to indicated concentrations [$\mu\text{g/L}$] of AG1, AG2, or AgNO_3 . b: *, mean is significantly different from control, one way ANOVA, $p=0.0015$, $F=6.856$, Bonferroni's post-test.

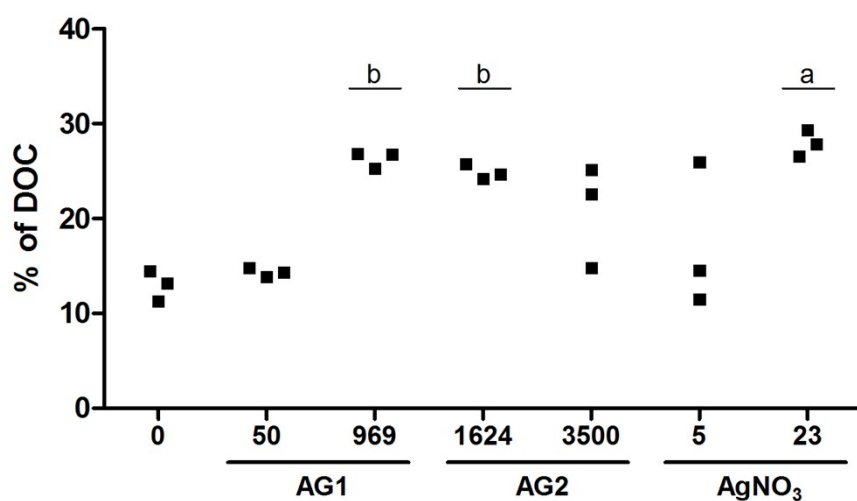


Figure S13. Percentage of low molecular weight acids of total EPS detected as DOC per dry weight produced by *C. reinhardtii* within 72 h in control cultures or when exposed to indicated concentrations [$\mu\text{g/L}$] of AG1, AG2, or AgNO_3 . a: **, b: *, means are significantly different from control, one way ANOVA, $p=0.0007$, $F=7.974$, Bonferroni's post-test.

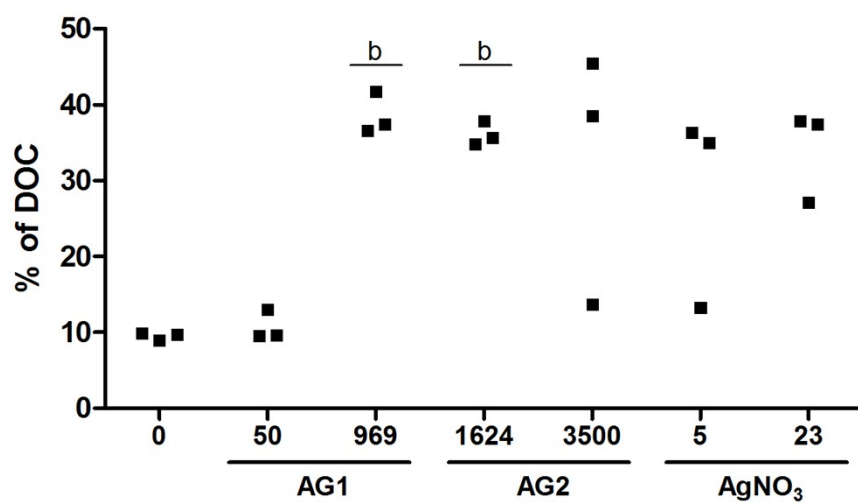


Figure S14. Percentage of neutral/amphiphilic compounds of total EPS detected as DOC per dry weight produced by *C. reinhardtii* within 72 h in control cultures or when exposed to indicated concentrations [$\mu\text{g/L}$] of AG1, AG2, or AgNO_3 . b: *, means are significantly different from control, one way ANOVA, $p=0.0025$, $F=6.117$, Bonferroni's post-test.