Supplementary materials

Development and application of a ratiometric nanosensor for measuring pH inside the gastrointestinal tract of zooplankton

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Number of figures: 9

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Methodology:

Step by step synthesis:

To synthesize FITC-APTS

1. 6.5 mg FITC dissolved in 20 mL ethanol in a glass vial
2. Then add 10 uL of APTS under vigorous stirring
3. React for 3 hours at room temperature in the dark
4. This yielded FITC-APTS

To synthesize RB-APTS

1. 0.96 g RB dissolved in 30 mL chloroform in a round bottom flask
2. Stir and heated to boiling point (61 degrees)
3. 0.465 mL APTS added and reaction was then allowed to continue for 30 mins
4. Evaporated chloroform and water formed during the reaction was then collected via distillation apparatus
5. After another 30 mins remaining chloroform was removed using vacuum distillation to yield a red-purple solid which was RB-APTS

Now using both above APTS conjugates the following steps were undertaken:

1. Transfer the 20 mL of ethanol containing FITC-APTS into 150 mL round bottom flask
2. Then add 1.6 mL of UPW containing 6.5 mg of RB-APTS
3. Add 250 uL of 2M HCl (which acts as a catalyst)
4. Add 9 mL TEOS (source of silica)
5. Reflux for 1 hour
6. Once cooled, dilute with water to desired nanosensor concentration

Characterisation of the nanosensors

Dynamic Light Scattering (DLS) was used to determine the hydrodynamic diameter of the silica nanosensors. DLS measurements were performed using a Malvern Zetasizer nano with the nanosensor samples placed in plastic low volume disposable sizing cuvettes (ZEN0112). Measurements were made using backscatter mode at an angle of 173^{o} and 20^{o} C with a sample equilibration time of 2 mins. To ensure reliability of the measurements a minimum of 3 runs were performed on each sample. Zeta potential measurements were also performed using a Malvern Zetasizer nano, the samples were placed into a folded-capillary cell and measurements taken using an SOP. Materials and dispersant properties contained within the SOP were consistent with those used for DLS measurements.

Differential centrifugal sedimentation (DCS) was used to determine the equivalent spherical volume of the FITC RB-doped silica nanosensors. Analytical centrifugation measurements were made using a CPS instruments disc centrifuge. Given the nanosensor particle density (Silica: 2.2g/mL) a high viscosity sucrose gradient was required for adequate particle separation. To prepare the sucrose gradient a stock solution of 8% sucrose (2.0 g sucrose with 23.0 mL ultrapure water) and a stock solution of 24% sucrose (6.0 g with 19 mL ultrapure water) were made. The stock solutions were then mixed in the ratios shown in Table S5. The first solution in the gradient series was 1.6 mL of 24% sucrose solution, this was injected in the disc prior to beginning centrifugation. TaTo build the gradient up, each sucrose solution was injected in turn, ending with 1.6 mL of 8% sucrose solution. To prevent evaporation of the sucrose gradient 0.5 mL of dodecane was injected as a fluid gradient cap. The disc was then allowed to spin for 1 hr prior to beginning measurements, to allow time for the gradient to build within the disc. A CPS instruments PVC calibration standard was ran prior to each sample measurement, the calibration standard was 0.239 μm which was dispersed in deionised water. The samples were then analysed using a standard operating procedure (SOP) with the following parameters: maximum diameter of

1 μm, minimum diameter of 0.003 μm, particle density of 2.2 g/mL, refractive index at 1.45, particle absorption of 0.001 K and a non- sphericity factor of 1; fluid density of 1.045 g/mL, fluid refractive index of 1.344 and fluid viscosity at 1.2 cps.

Transmission Electron Microscopy (TEM) images were collected and analysed to determine the size distribution and morphology of silica nanoparticles doped with various combinations of organic fluorescent dyes. Samples of silica nanosensors were prepared for TEM analysis by deposition of 20 μL of nanosensor stock suspension (as synthesised) onto a copper TEM grid coated with carbon film (Agar Scientific), and covered to prevent contamination with airborne particles. After 1 hr the suspension was removed and the TEM grid was washed twice by suspending the grid in ultrapure water (20 mL) for 30 secs each wash. The TEM grid was then re-covered and allowed to dry overnight, prior to analysis or storage in TEM grid box. The washing step is included to reduce the number of image artefacts introduced onto the TEM grid by the crystallisation of salts during the drying process. Samples were then analysed using a JEOL 1200EX at an operating voltage of 80 KeV. Images were recorded using Gatan microscopy suite software and subsequently analysed using the open source ImageJ/FIJI software.

Preparation of daphnia incubation media. High-Hardness Combo (HH Combo) medium is a well-known medium for culturing hard-water *Daphnia* such as *D. magna* over a long period of time (21 days). HH Combo is also known to be able to promote the growth of green algae *Chlorella vulgaris*, which is a source of food for *D. magna*. HH Combo medium is also a more realistic representation of water-hardness in natural waters found in the environment (Baer and Goulden, 1998). To prepare HH Combo medium 4 mL of each stock listed in Table S1 were added to 3.5 L of deionised water (15 MΩ), apart from sodium bicarbonate, of which 8 mL was added. Sodium selinate (200 μL) was then added and the solution made up to 4 L with deionised water. To saturate the medium with oxygen, the solution was then aerated for 12 hours. After

aeration had been completed, the medium was then pH adjusted to between pH 7.6 – 7.8 using 0.1 or 1 M HCl. A solution of Animate was prepared using 1 mL of the each stock solution listed in Table S6, and made up to 1 L with deionised water. A 50 mL vitamin stock solution containing Biotin (*d*-biotin) and B12 (cyanocobalamin) at 1.04 g/L and 1.12 g/L respectively was then prepared using deionised water. The vitamin stock solution was completed with the addition of 10 mg of thiamine hydrochloride. To complete the HH Combo medium 4 mL of Animate solution and 2 mL of vitamin solution were added. Bolds Basal growth medium (BBM, Table S7) was used to maintain an algal culture (*Chlorella vulgaris*) as sustenance for *Daphnia magna*.

Fluorescence Spectroscopy. Fluorescence Spectroscopy was used to measure the excitation and emission spectra of the indicator and reference dyes. Fluorescence measurements were performed using a Cary Varian Eclipse 3D fluorescence spectrophotometer; samples were contained in a quartz cuvette with a path length of 1 cm. Fluorescence measurements were made with an excitation range between 300-800 nm and a minimum excitation scan step size of 10 nm intervals. A Raman background was performed using a sealed water cell at an excitation wavelength of 370 nm at the beginning of each session, in order to compensate for variation in lamp intensity. Samples were measured neat with a slit width of 5 nm and an excitation voltage of between 500-725V.

Figure S1. Transmitted light image of a *Daphnia* gut dissected from an adult female. On the *left*, the paired intestinal ceca can be seen. The gut ends at the *right* side. The esophagus cannot be seen in this preparation. The dark material is partially digested gut content (Ebert, 2005).

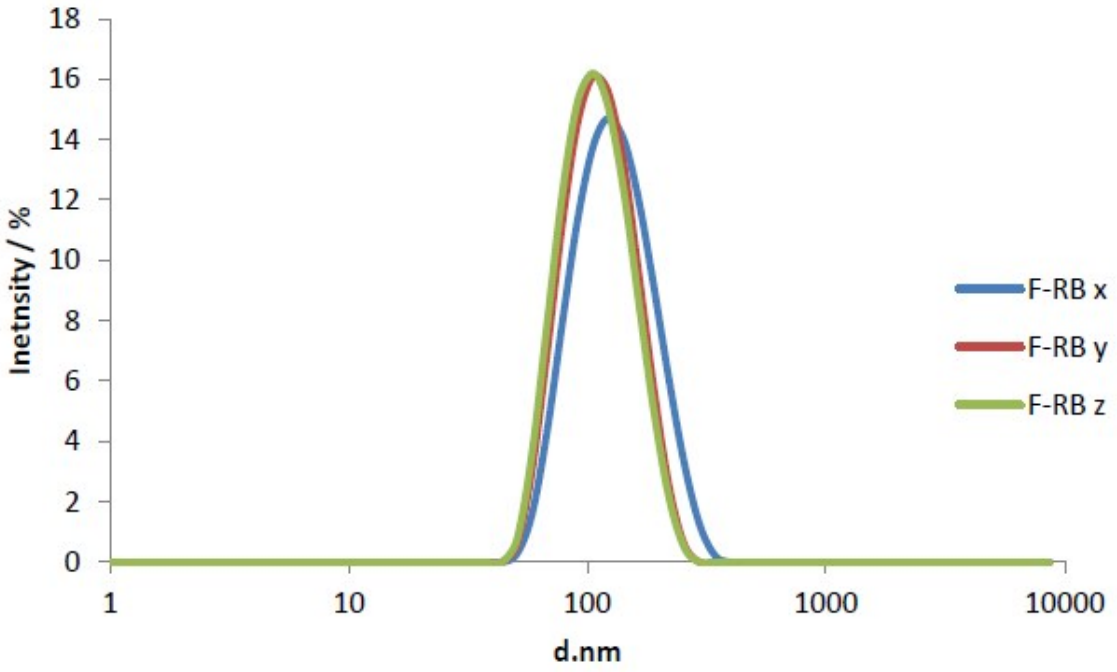

Figure S2. DLS of FITC RB nanosensors prepared from FITC RB sol after 24 hrs

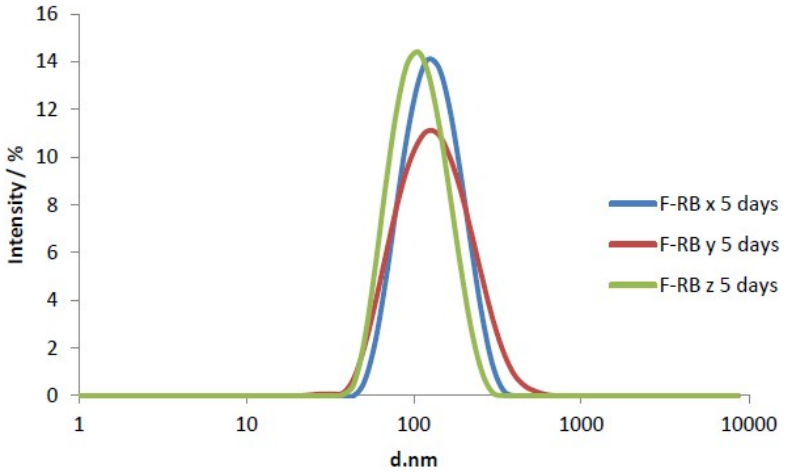

Figure S3. DLS of FITC RB nanosensors prepared from FITC RB sol after 5 days.

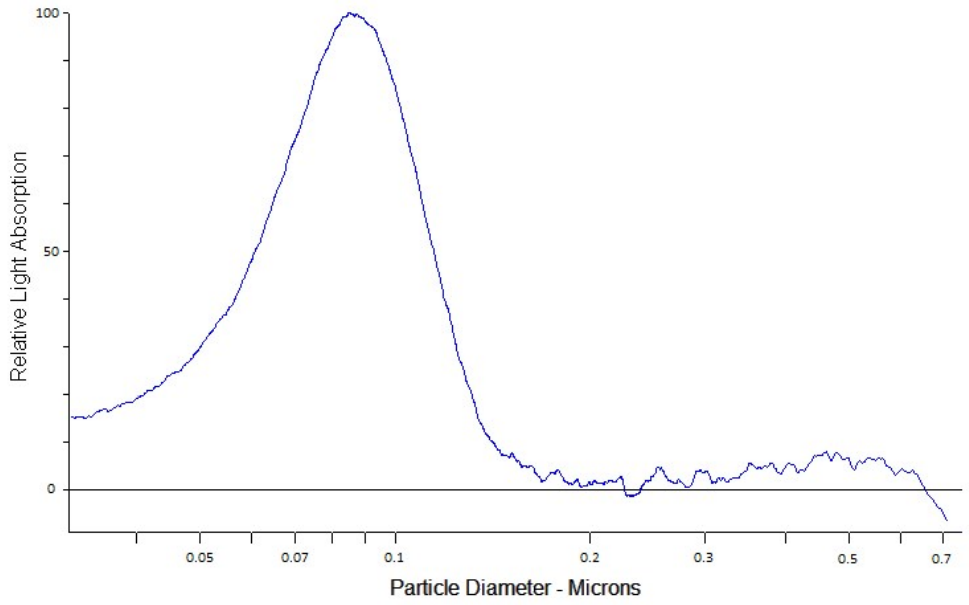

Figure S4. DCS results for F-RB x prepared without Pluronic F127.

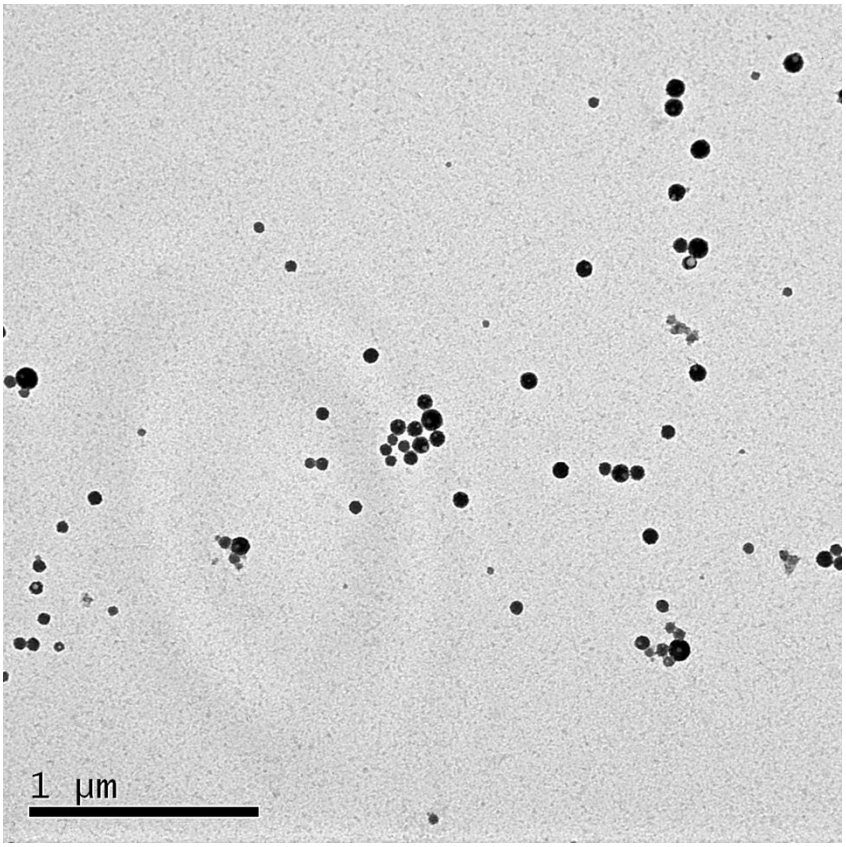

Figure S5. Typical TEM images (A and B) of FITC RB-doped silica nanoparticles

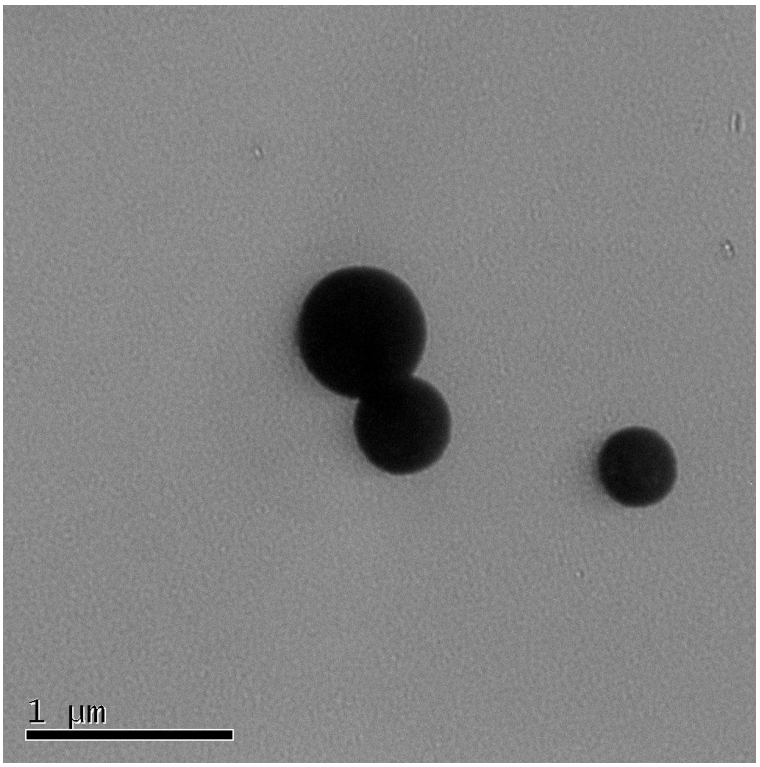

Figure S6. A typical TEM images of unlabelled silica nanoparticles, as a negative control for comparing with labelled silica nanoparticles, which contain a core .

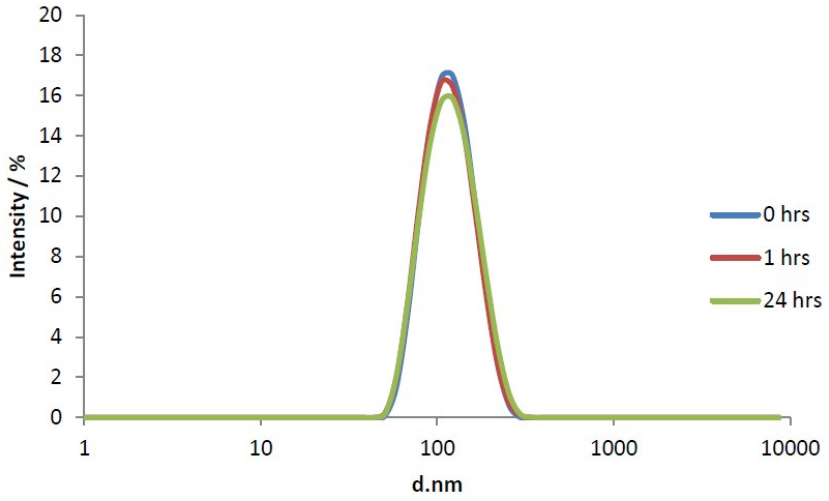

Figure S7. Average DLS result for FITC RB nanosensors in HH Combo medium over 24 hrs.

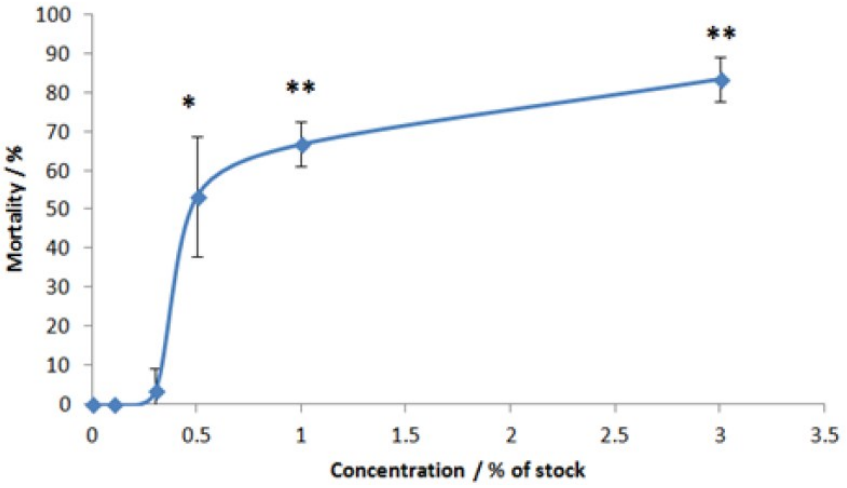

Figure S8. FITC RB-doped silica nanoparticles, toxicity towards *D. magna*, significance is noted using * (95% confidence) and ** (99% confidence).

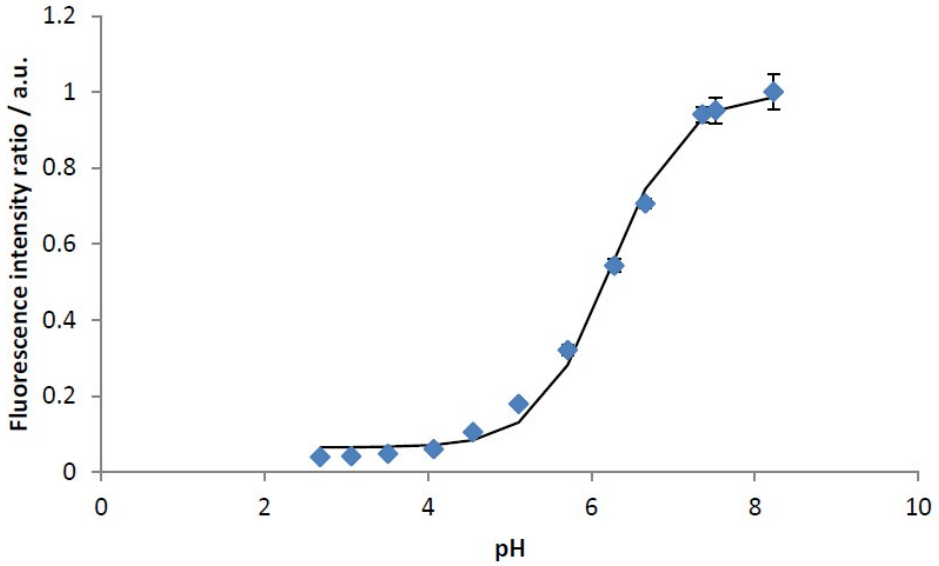

Figure S9. FITC RB nanosensors, bulk calibration in buffer, normalised fluorescence intensity ratio of I_{FITC}/I_{RB} with varying pH. Error bars represent one standard deviation.

Table S1. Stock solutions for HH Combo medium

Stock solution	Chemical formula	Stock (g/L)
Calcium Chloride, Dihydrate	$CaCl_2 \cdot 2H_2O$	110.28
Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	113.50
Potassium phosphate dibasic	K_2HPO_4	1.74
Sodium nitrate	$NaNO_3$	17.00
Sodium metasilicate nonahydrate	$Na_2SiO_3 \cdot 9H_2O$	28.42
Boric acid	H_3BO_3	24.00
Potassium chloride	KCl	5.96
Sodium Bicarbonate	$NaHCO_3$	63.00

Table S2. Preparation of sodium phosphate/citric acid buffers

pH of buffer	Volume of Sodium Phosphate / mL	Volume of Citric Acid / mL
2.5	2.16	17.84
3.0	4.08	15.92
3.5	6.04	13.96
4.0	7.72	12.28
4.5	9.00	10.90
5.0	10.28	9.72
5.5	11.36	8.64
6.0	12.84	7.16
6.5	14.20	5.80
7.0	17.44	2.60
7.5	17.98	2.03
8.0	19.53	0.48

Table S3. Summary of DLS results for samples F-RB x, y and z immediately after preparation after 24 hrs and after 5 days.

Sample	Z average / d.nm	Pdi	Count rate /kcps
F-RB x	119.9	0.123	294.3
F-RB y	105.9	0.093	209.8
F-RB z	103.2	0.104	216.7
F-RB after 24 hrs	120.2	0.112	224.7
F-RB after 24 hrs	109.1	0.137	446.6
F-RB after 24 hrs	102.4	0.112	458.5
F-RB x after 5	118.2	0.122	416.4
F-RB y after 5	117.2	0.184	303.5
F-RB z after 5	100.0	0.128	311.0

Table S4. A summary of the Zeta potential values obtained for FITC RB nanosensors

Sample	Concentration / %	Zeta Potential / mV	Zeta Deviation / mV	Count rate /kcps
F-RB x	0.3	-4.3	7.07	53.0
F-RB y	0.3	-3.8	5.56	26.7
F-RB z	0.3	-4.5	6.65	14.9
F-RB x	3.0	13.2	9.68	633.9
F-RB y	3.0	13.4	9.79	159.7
F-RB z	3.0	12.2	8.58	161.0

Table S5. Sucrose gradient build

Volume 8% sucrose solution / mL	Volume 24% sucrose solution / mL
0.0	1.6
0.2	1.4
0.4	1.2
0.6	1.0
0.8	0.8
1.0	0.6
1.2	0.4
1.4	0.2
1.6	0.0

Table S6. Stock solutions for Animate

Stock solution	Chemical formula	Stock (g/100 ml)
Lithium chloride	LiCl	31
Rubidium chloride	RbCl	7
Strontium chloride	$SrCl_2 \cdot 6H_2O$	15
Sodium bromide	NaBr	1.6
Potassium iodide	KI	0.33

Table S7. Stocks solutions for Bolds Basal growth medium

Stock solution	Chemical formula	Stock (g/L)	Final (mg/L)
di-Potassium hydrogen	K_2HPO_4	7.5	75.0
Potassium di-hydrogen	KH_2PO_4	17.5	175.0
Magnesium sulfate	$MgSO_4.7H_2O$	7.5	75.0
Sodium nitrate	$NaNO_3$	25.0	250.0
Calcium chloride	$CaCl_2.2H_2O$	2.5	25.0
Sodium chloride	NaCl	2.5	25.0
EDTA tetrasodium	EDTA - $4Na^+$	50.0	50.0
Potassium hydroxide	KOH	31.0	31.0
Iron (II) sulfate & Sulfuric acid (0.1%)	$FeSO_4.7H_2O$	4.9	5.0
Boric acid	H_3BO_3	11.4	11.4
Zinc sulfate	$ZnSO_4.7H_2O$	14.1	1.41
Manganese chloride	$MnCl_2.4H_2O$	23.2	0.23
Cupric sulfate	$CuSO_4.5H_2O$	25.2	0.25