D4.4 PATHOGENS DETECTION BY INNOVATIVE BIOSENSORS-BASED APPROACHES

[D4.4.1, D4.4.2, D4.4.3 &D 4.4.4]

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PROJECT AdSWiM

Work Package:	**4. Pathogens detection by innovative biosensors-based approaches**
Activity:	**4.4.1 microalgae exploitation in monitoring relevant seawater pollutants** **4.4.2 synthesis and characterization of biomimetic peptides for bio-sensing** **4.4.3 immobilization strategies** **4.4.4 optical biosensors optimization**
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Deliverable:	**Viviana Scognamiglio IC-CNR**

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CONTENTS

D4.4.1 Protocol assessing the microalgae capability to test water global toxicity

Analysis of water biotoxicity by changes in algae physiological parameters: samples from DP's in San Giorgio end Lignano were exploited to study their potential biotoxic effect on the green photosynthetic algae *Chlamydomonas reinhardtii*. Results evidenced a toxic effect of sample from San Giorgio DP on the algae growth and its capability in pigment production (i.e.chlorophylls). Further studies will be focused on the use of seawater algae to analyse seawatersamples from the same sites.

Inoculation

Inoculation from Petri plate to liquid culture of C. reinhardtii CC125, in agitation at 150 rpm, under controlled temperature 24°C and white light illumination (50 mmol photons m^{-1} s^{-1}).

Day 0

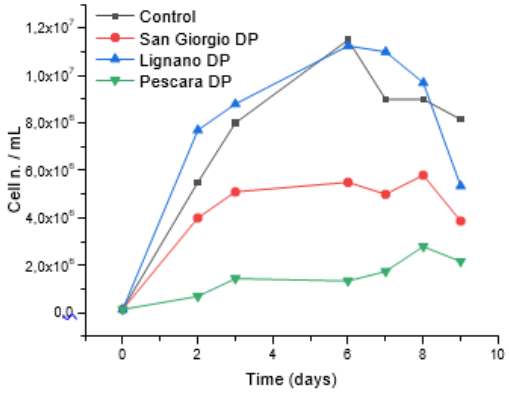
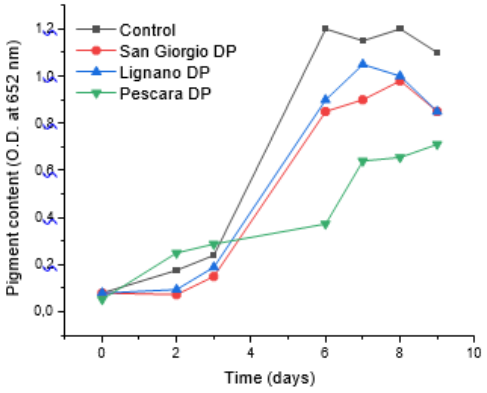
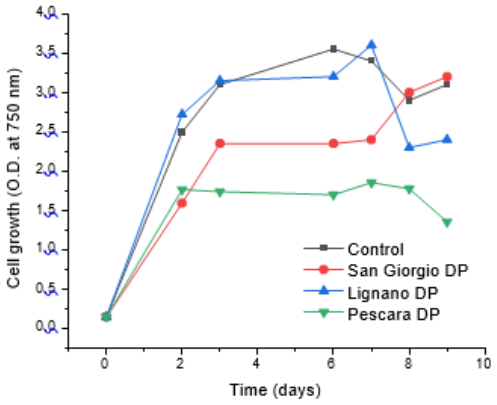
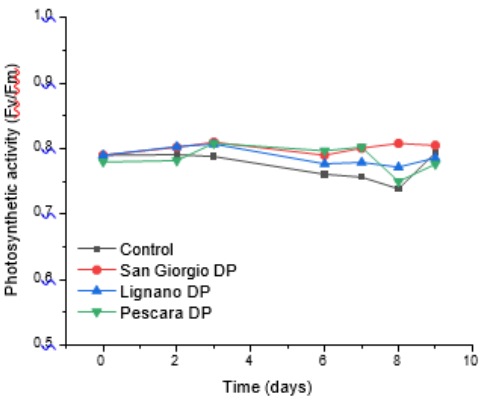
Refresh in TAP buffer 1:2 (200 mL), in agitation at 150 rpm, under controlled temperature set at 24 °Cand white light illumination (50 mmol photons m^{-1} s^{-1}). The days after, all the parameters are measured.

Cell number				
Day n.	Control in TAP	Lignano DP	San Giorgio DP	Pescara DP
0	1,5E5	1,5E5	1,5E5	1,5E5
2	5,5E6	4E6	7,7E6	0,7E6
3	8E6	5,1E6	8,8E6	1,4E6
6	1,15E7	5,5E6	1,125E7	1,3E6
7	9E6	5E6	1,1E7	1,7E6
8	9E6	5,8E6	9,7E6	2,8E6
9	8,15E6	3,87E6	5,35E6	2,2E6

Optical density at 750 nm				
Day n.	Control in TAP	Lignano DP	San Giorgio DP	Pescara DP
0	0,15	0,15	0,15	0,15
2	2,4	1,6	2,7	1,7
3	3,1	2,3	3,15	1,7
6	3,5	2,3	3,2	1,7
7	3,4	2,2	3,6	1,8
8	2,9	3	2,3	1,7
9	3,1	3,2	2,4	1,3

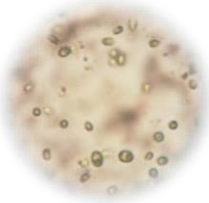
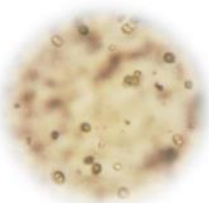
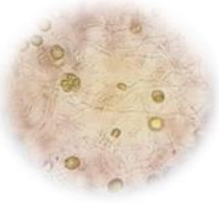
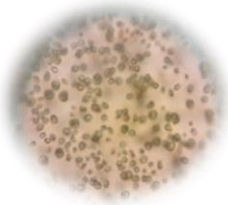
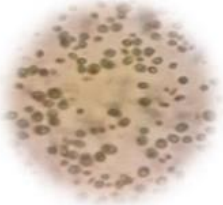
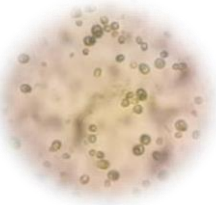
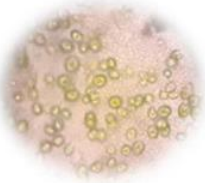
Chl content 652 nm				
Day n.	Control in TAP	Lignano DP	San Giorgio DP	Pescara DP
0	0,08	0,08	0,08	0,053
2	0,176	0,073	0,095	0,25
3	0,24	0,15	0,19	0,2875
6	1,2	0,85	0,9	0,373
7	1,15	0,9	1,05	0,64
8	1,2	0,98	1	0,655
9	1,1	0,85	0,85	0,711

Photosynthetic activity (FV/FM)				
Day n.	Control in TAP	Lignano DP	San Giorgio DP	Pescara DP
0	0,701	0,702	0,701	0,780
2	0,791	0,802	0,803	0,782
3	0,788	0,810	0,807	0,808
6	0,761	0,790	0,777	0,797
7	0,757	0,801	0,779	0,802
8	0,738	0,808	0,772	0,750
9	0,794	0,825	0,786	0,776

Cell number	Chlorophyll content
	
Cell growth	**Photosynthetic activity**
	

3° days

Photo of the pellet (13.000 rpm 3 min) and at the optical microscope to check the presence of bacteriain liquid culture.

day	TAP	Lignano DP	San Giorgio DP	Pescara DP
3				
3				
7				

6° day

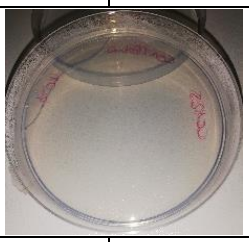
Photo of the petri dishes to check the presence of bacteria in liquid culture. 100 mL each colture in 900 mL TAP and make 4 serial dilutions 1:10

10^{-1}

10^{-2}

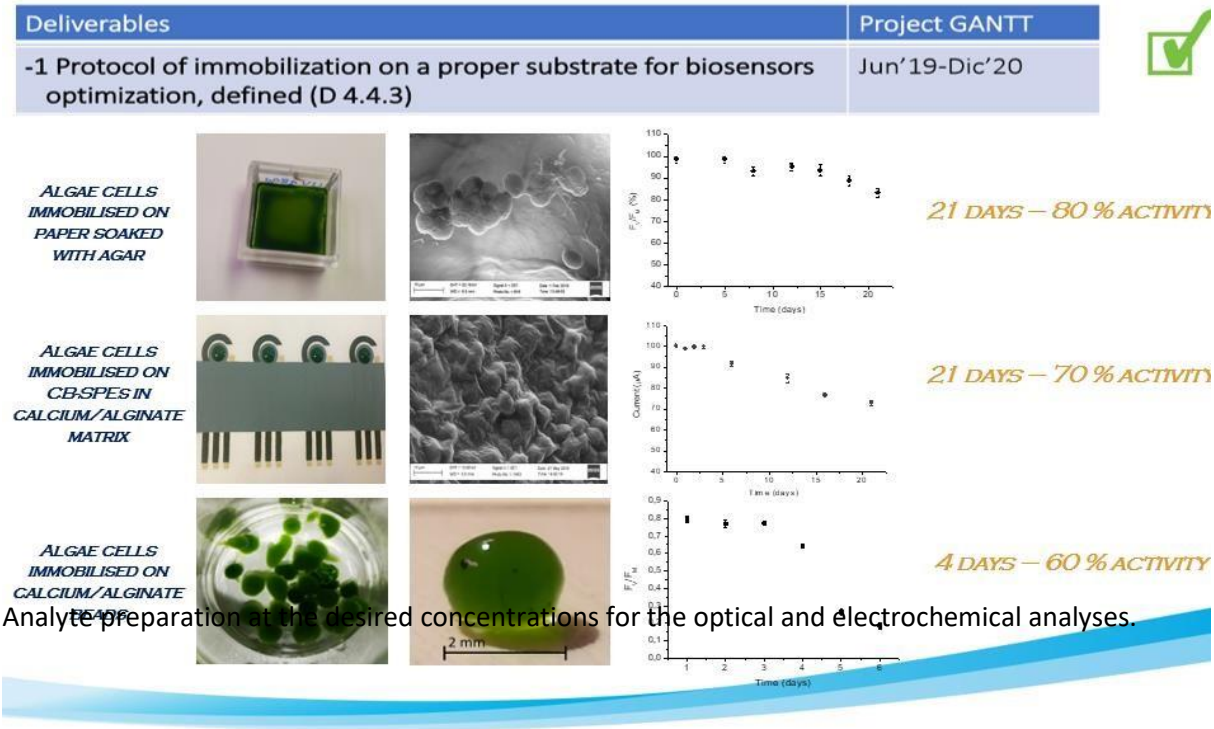
10^{-3} -> 100 µL on TAP agar plate for each sample 10-4 -> 100 µL on TAP agar plate for each sample 24 hours at 37°C in dark

7° day

TAP		Lignano DP		San Giorgio DP		Pescara DP	
10^{-3}	10^{-4}	10^{-3}	10^{-4}	10^{-3}	10^{-4}	10^{-3}	10^{-4}
							
0 colonies	0 colonies	0 colonies	0 colonies	0 colonies	0 colonies	0 colonies	0 colonies

Development of algae-based optical and electrochemical biosensors for pathogen detection: once explored different immobilisation protocols for algae on different supports (e.g. absorption on paper, entrapment in calcium/alginate beads, and drop-casting on screen-printed electrodes), two different biosensing configuration have been projected based on optical and electrochemical detection. The latter biosensor was capable to sense the presence of Escherichia coli by means of changes in current signal due to higher oxygen evolution from algae when co-cultived with bacteria. Further studies will be focused on sample pre-concentration protocols to enhance the biosensor sensitivity.

Immobilisation of algae on appropriate supports:



Analyte preparation at the desired concentrations for the optical and electrochemical analyses.

Inoculation

Inoculation *E. coli* BL21DE3 Plys S, A and B in 5 mL LB medium 37°C ON at 200 rpm

Day 0

Inoculation diluted and growth on LB agar plates (stock stored at 4°C) Dilution: 100 mL each colture in 900 mL LB and make 5 serial dilutions 1:10 10-1

10^{-2}

10^{-3}

10^{-4} -> 100 mL on LB agar plate for each sample A and B in duplicate 10^{-5} -> 100 mL on LB agar plate for each sample A and B in duplicate

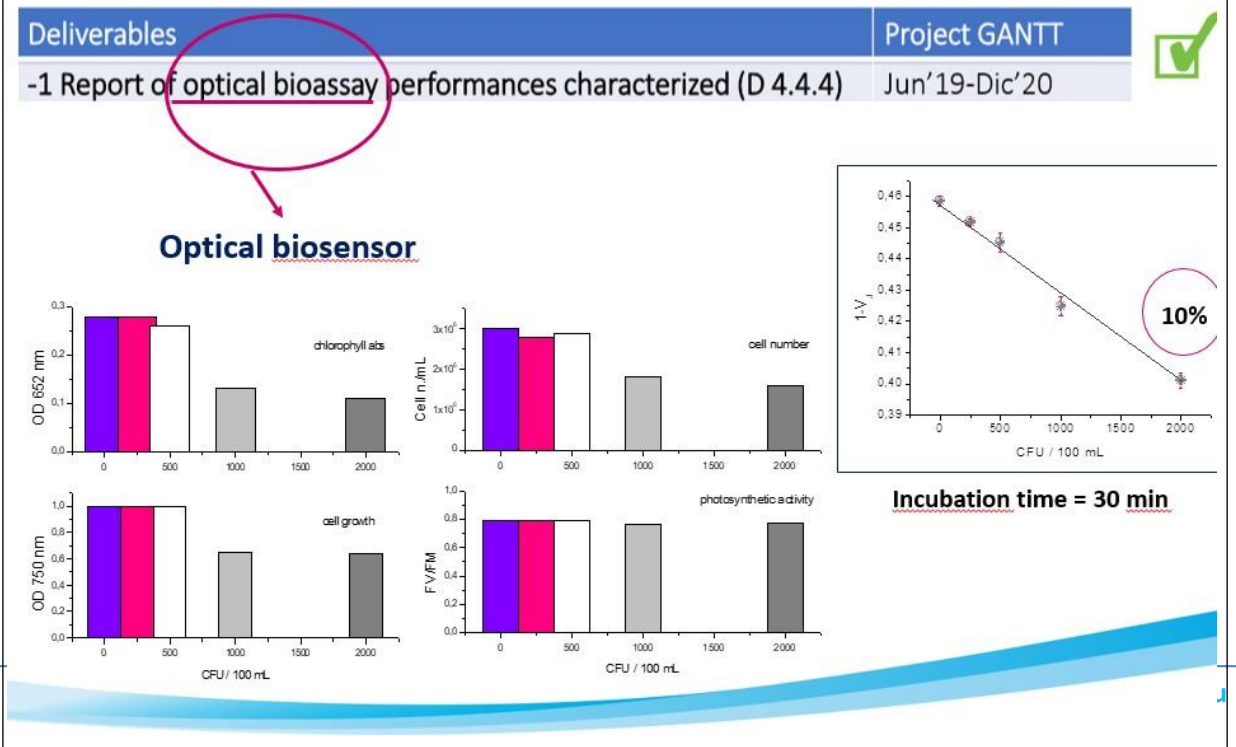
24 hours at 37°C in dark

- Refresh of *C. reinhardtii* CC125

Day 1

Refresh in 1:2 (50 mL) light 50 mmol photons m^{-1} s^{-1}

TAP 2x 25 mL	Water 25 mL			Stock 50 CFU / 100 µL
TAP2x	Water	+ *E. coli* 50 CFU/100mL	0,05 CFU / 100 µL	0,05 mL
TAP2x	Water	+ *E. coli* 100 CFU/100mL	0,1 CFU / 100 µL	0,1 mL
TAP2x	Water	+ *E. coli* 500 CFU/100mL	0,5 CFU / 100 µL	0,5 mL
TAP2x	Water	+ *E. coli* 1000 CFU/100mL	1 CFU / 100 µL	1 mL



Deliverables	Project GANTT
-1 Report of optical bioassay performances characterized (D 4.4.4)	Jun'19-Dic'20

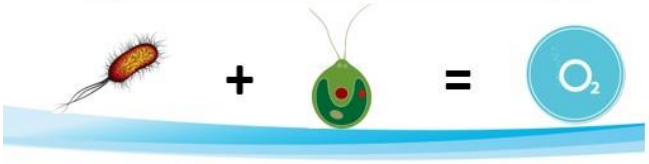
electrochemical biosensor

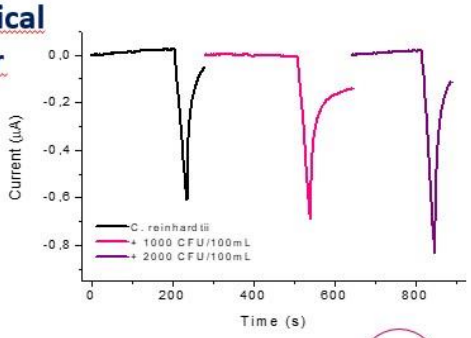
Aerobic bacteria can **promote algal growth** by reducing the photosynthetic oxygen tension within the microenvironment of the algal cells.

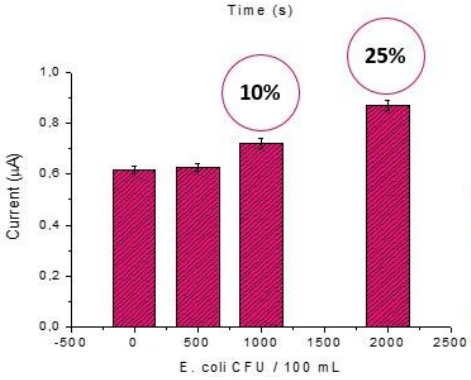
FEMS Microbiology Ecology, 18(1), 35-43.

Moderate bacterial concentration did not influence the growth of algae in the co-culture significantly, with **increased oxygen production**.

Materials Science Energy Technologies, 2(1), 1-7.



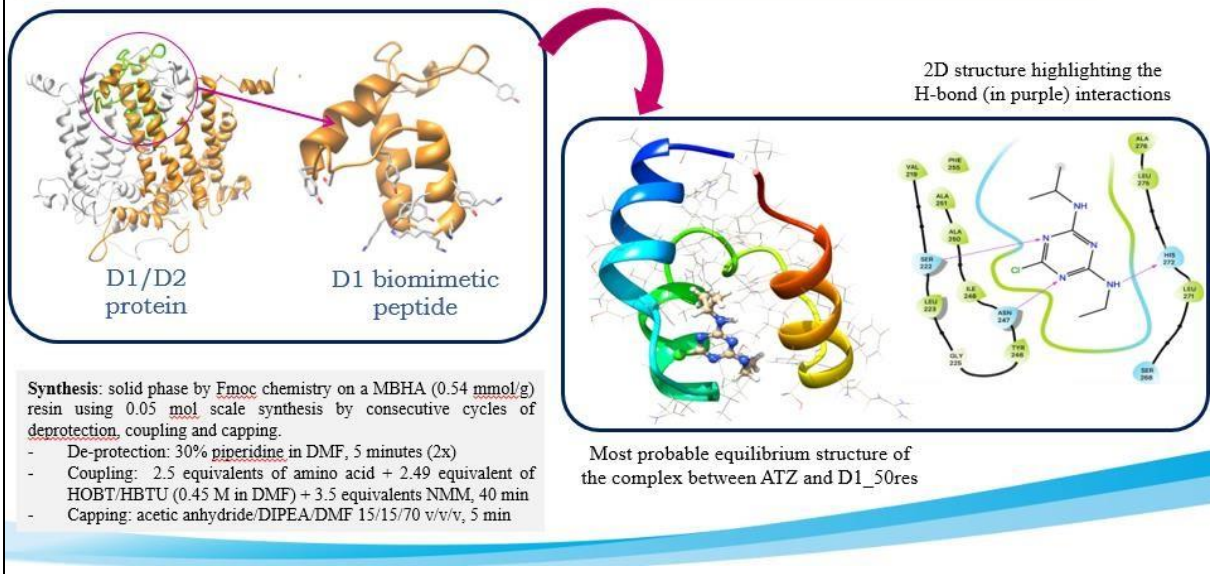




D4.4.2 Protocol of synthesis of mini-proteins and/or biomimetic peptide and their characterization

Design of novel artificial molecules bioinspired to natural ones for herbicide detection: bioinformatics studies helped providing docking and modelling simulation of a novel mini-protein bioinspired to the D1 protein from the photosystem II of *Chlamydomonas reinhardtii*. According to simulation data, this novel molecule was able to fold in a secondary structure and bind atrazine. The mini-protein was thus synthesised by automated synthesis and further studies will be conducted for its structural and functional characterisation by means of fluorescence spectroscopy and circular dichroism.

Deliverables	Project GANTT
-1 Protocol of synthesis of mini-proteins and/or biomimetic peptide structurally and functionally characterized (D4.4.2)	Jan'19-Dic'20



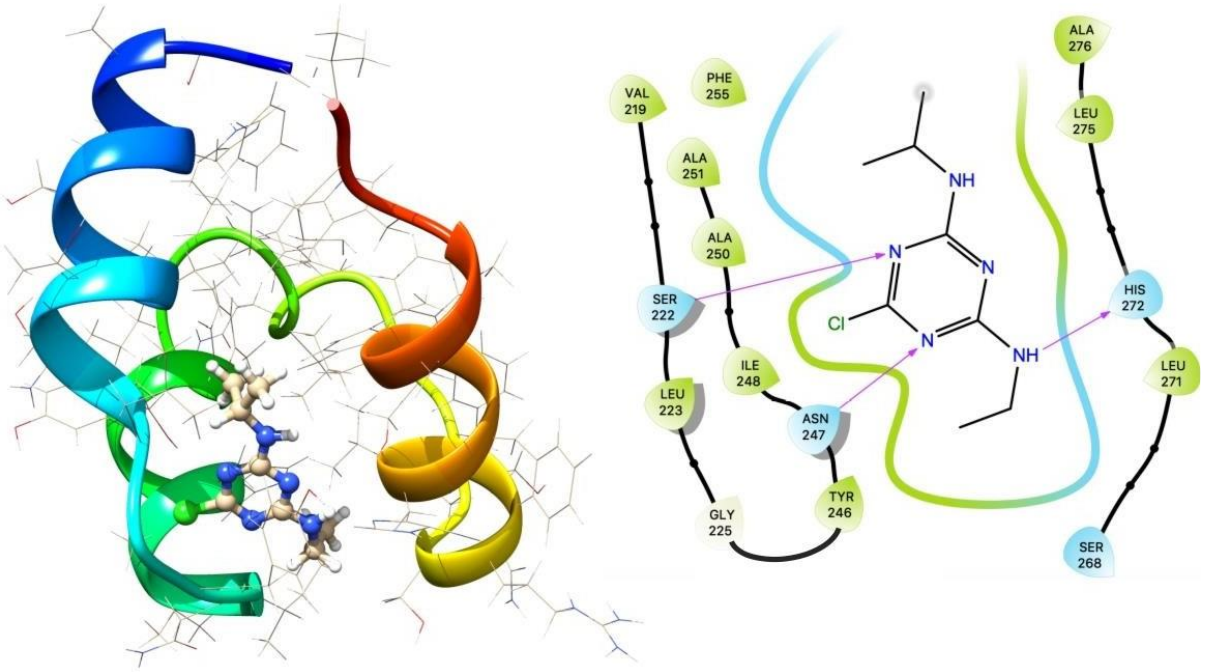
The structure of *Chlamydomonas reinhardtii* Photosystem II (PSII) D1/D2 was taken as template for the design of the following peptide:

FSAMHGSLVTSSLGGYNIVAAHGYFGRLIFQYASFNNSRSLHFFLAAWPV

Molecular dynamic simulations were performed using the GROMACS 5.1.1 package[i]. Interactions were described using an all-atoms CHARMM27 force field[ii]. The simulations for the various systems were performed using a cubic box of NaCl 150mM in explicit TIP3P water solution. Periodic boundary conditions were applied. Force field parameter files and initial

configuration for the polypeptides were created by GROMACS utilities programs. Force fieldparameters for atrazine (ATZ) was taken from the ATB repository[iii]. The equilibrationprocedure was done in several steps, starting from a NVT simulation at 300K with the polypeptides heavy atom positions restrained to equilibrate the solvent around it, followed bya NPT run at 300 K and pressure at 1 bar, for a 10 ns run. After the equilibration phase, the system was run for a total of 300 ns for a NVT production run; the trajectory was saved at a frequency of 10 ps to evaluate dynamical and structural properties. The simulations were always checked versus the root mean square displacement (RMSD) and the energy profile. During the production runs for the temperature coupling, we used a velocity rescalingthermostat[iv] (with a time coupling constant of 0.1 ps), while for the pressure coupling, we useda Parrinello–Rahman barostat[v] (1 ps for the relaxation constant). The Leap-Frog algorithm with a 2 fs time step was used for integrating the equations of motion. Cut-offs for the Lennard-Jones and real space part of the Coulombic interactions were set to 10 Å. For the electrostaticinteractions, the Particle Mesh Ewald (PME) summation method[vi] was used, with an interpolation order of 4 and 0.16 nm of FFT grid spacing. Selected images and polypeptide manipulation were done using Maestro[vii] and VMD[viii].

The MD simulation performed on the ATZ/D1_50res complex showed an equilibratedstructure. In this complex, ATZ is tightly inserted in the D1_50res pocket and makes three H-bonds with residues SER222, ASN247 and HIS272. Noteworthy, ATZ seems to make a stronger binding with the shorter than with the longer polypeptide.

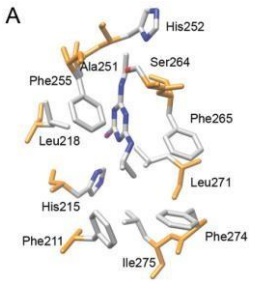
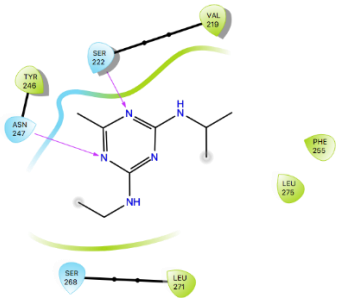
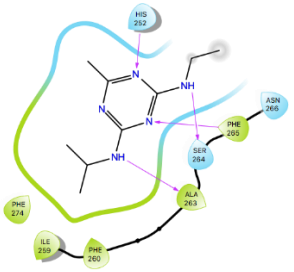


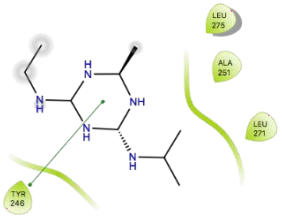
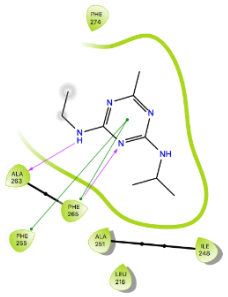
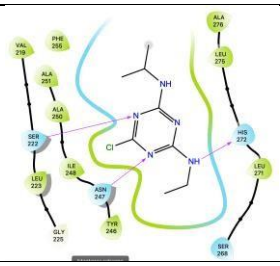
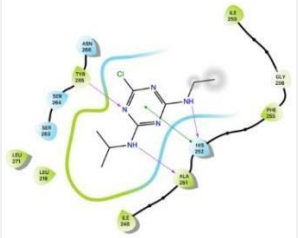
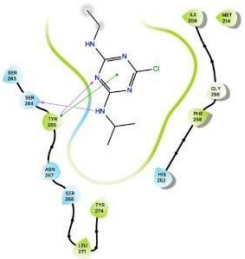
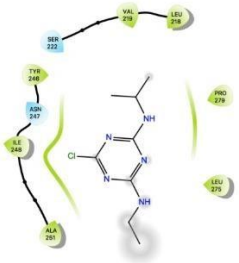
Most probable equilibrium structure of the complex between ATZ and D1_50res (left). The 2D structure, highlighting the H-bond (in purple) interactions, is also shown (right)

The peptide was also mutated to provide higher hydrophilicity in water solutions, being the natural protein enclosed into a membrane, thus very hydrophobic. A new peptide was then designed, with the following sequence:

YSSMHGSLVTSSLGGYNIVSAHGYFGRLIYQYSSYNNSRSLHYYLAAWPV

Molecular simulations were performed also on this novel peptide, all the data reported in thefollowing table:

	H-bond	Interactions	Figure
D1 protein WT	H 215 S 264 F 265	F 211 L 218 A 251 H 252 F 255 G 256 S 268 L 271 F 274	
D1Pep50 wt	N 247 S 222		
D1Pep50 wt	F 265 A 263 S 264		

D1Pep50 wt		T 246	
D1Pep50 wt	F 255 F 265	A 263	
D1pep50 provab2 mutated	S 222 N 247 H 272	---	
D1pep50Mut New_atz1 mutated	Y 265 A 251 H 252	H 252	
D1pep50Mut New_atz2 mutated	Y 265 S 264	Y 265	
D1pep50Mut New_provab2 mutated	---	---	

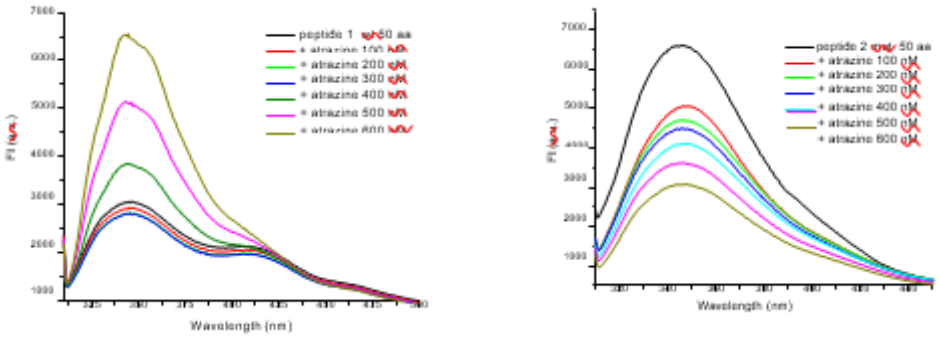
The peptides were thus synthesised using the following protocol:

The D1_50res peptide was thus synthesised on solid phase by Fmoc chemistry on a MBHA (0.54 mmol/g) resin using 0.05 mol scale synthesis by consecutive cycles of deprotection, coupling and capping.

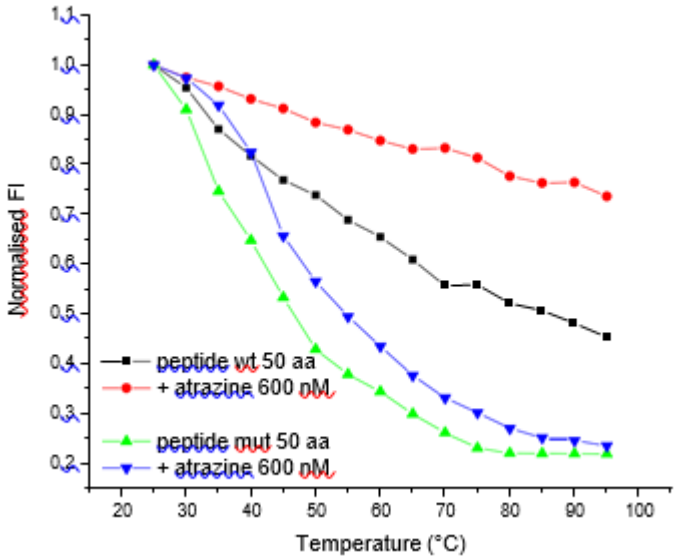
- De-protection: 30% piperidine in DMF, 5 minutes (2x)
- Coupling: 2.5 equivalents of amino acid + 2.49 equivalent of HOBT/HBTU (0.45 M in DMF) + 3.5 equivalents NMM, 40 minutes
- Capping: acetic anhydride/DIPEA/DMF 15/15/70 v/v/v, 5 minutes

The peptide was cleaved off the resin and deprotected by treatment of the resin with a solution of TFA/TIS/H2O 95/2.5/2.5 v/v/v, 90 minutes. TFA was concentrated and the peptide was precipitated in cold ethylic ether. Analysis of the crudes was performed by LC-MS using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 30 to 70% in 30 minutes. Purification was performed by semipreparative RP-HPLC using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 30 to 70% in 30 minutes.

The obtained peptides were characterised by fluorescence spectroscopy. Peptide 1 (50 aa wt) and peptide 2 (50 aa mut) were exploited for the analysis of atrazine binding in the range from 100 to 600 nM:



Peptide 1 (50 aa wt) and peptide 2 (50 aa mut) were subjected to denaturation by means of temperature rise from 25 to 95 °C, in the absence and in the presence of atrazine 600 nM:



In particular, peptide 2 showed a folded structure especially in the presence of the atrazine, which helped the peptide to obtain a melting temperature higher (45 °C) than the peptide in the absence of atrazine (28 °C). In addition, with the rise of the temperature both a decrease of the fluorescence intensity and the red shift of the wavelength underline that peptide 2 (50 aa mut) had a folded structure which was subjected to an unfolding mechanism due to the temperature increase.

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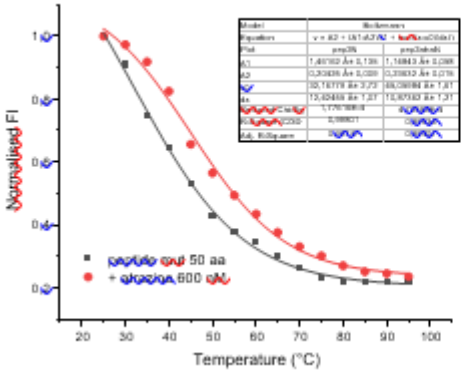

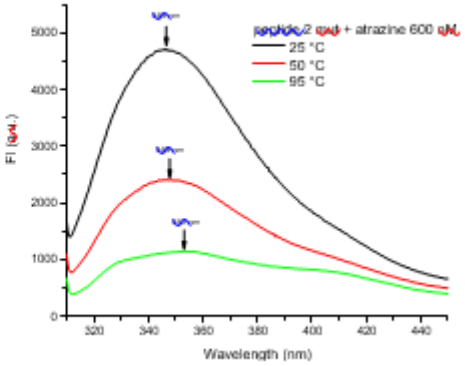

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Immobilisation of algae on appropriate supports.

Growth conditions of the algal liquid cultures

All the strains of C. reinhardtii were maintained under continuous light (50 µmol photons m- 2 s-1), at 25 °C, and on tris-acetate-phosphate (TAP) agar culture medium plates. Before each experiment, each strain was collected with a sterile loop and used to inoculate stock culture made in 100 mL flasks with 50 mL of liquid TAP closed with bacteriological cotton. Flasks were placed into an orbital shaker at 25 °C, stirring at 150 rpm and under continuous light (50 µmol photons m-2 s-1). All the materials and media used for the inoculation were sterilized in the autoclave at 120 °C and 1 bar pressure for 20 minutes. The inoculation was performed under the biological fume hoods to avoid any contamination. After 72 hours from inoculation, the optical density of each strain culture was checked by spectrophotometer analysis at a 750 nm wavelength in TAP medium. After this measurement, the algae cultures were diluted in TAP to an optical density of 0.15 OD750 in a final volume of 200 mL. Then the refreshed cultures were mixed on an orbital shaker under the same conditions above reported for all the periods of the physiological characterization.

Algae physiological characterization

All experiments were performed under continuous light (50 µmol photons m-2 s-1) and agitation (150 rpm) at 25 °C, starting with cell cultures in an early mid-exponential growth phase, with Abs750 0.5 O.D., 106 cells/mL, and 5 µg/mL chlorophyll content. Cell culture growth was spectrophotometrically evaluated measuring the absorbance (O.D.) at 750 nm wavelength. Cell number was quantified using a Bio-Rad TC-10 automated counter (Hemel Hempstead, UK), using a 10 µL-volume cell counting slide. Pigment content was spectrophotometrically measured by quantifying the absorbance (O.D.) of the chlorophylls a and b at 652 nm wavelength, once extracted with 80% acetone. Chlorophyll extraction: 1 mL of cell culture was harvested and centrifuged at 13000 rpm for 5 min to destroy all the phospholipidic membranes and preserve the pigments. 800 µL of the supernatant was removed and the remaining pellet and 200 µL supernatant was diluted by adding 800 µL of 100% aqueous acetone. The solution is vortexed for 2 minutes in dark and then centrifuged for 3 minutes. The total chlorophyll content was determined spectrophotometrically measuring the absorbance at 652 nm (80% acetone is used as blank). The calculation of the total chlorophyll concentration expressed as µg/mL was performed by the equation: (O.D.652 × 1000)/34.5. The photosynthetic profile was assessed by the chlorophyll a fluorescence induction (Kautsky) curves, recorded with a Plant Efficiency Analyzer (PEA) at room temperature after 10 min of dark adaptation and with a 5 s saturating pulse excitation light (3500 µmol photons m-2 s-1) using an array of six red light-emitting diodes (650 nm peak). Kautsky curves or OJIP curves are defined by a polyphasic fluorescence rise in time, with O as the minimal dark-acclimated fluorescence level (indicating that all QA are oxidised) and P as the maximal level (indicating that all PSII quinone acceptors are fully reduced). The difference in the fluorescence signal of these distinct states helps to evaluate the PSII functionality through the following parameters calculated by the fluorimeter:

- F0 or fluorescence in initial state: minimum fluorescence intensity in the state acclimated to the darkness, recorded when all PSII reaction centres are open (oxidized quinones).

- FM or maximum fluorescence: maximum fluorescence intensity reached after 10 minutes of darkness and a subsequent saturating light pulse, recorded when all reaction centres of the PSII are closed (reduced).
- FV/FM: maximum fluorescence yield of PSII photochemical reaction expressed as a ratio of variable fluorescence (FM-F0) and maximum fluorescence, calculated according to the equation:

$$FV/FM = (FM-F0)/FM$$

where FV represents the maximum variable fluorescence calculated as FM-F0, FM corresponds to the maximum fluorescence emission and F0 is the minimum fluorescence emission.
It reflects the efficiency of PSII in using light for photochemical conversion and its value is usually at 0.8 in physiological conditions or decreased values under stress.

Algae immobilization protocol

C. reinhardtii cell cultures in an early mid-exponential growth phase, with Abs750 0.7 O.D., 10^7 cells/mL, and 10 µg/mL chlorophyll content, were exploited for the immobilization on screen-printed electrodes nanomodified with carbon black (CB-SPEs) purchased from Sens4Med (Rome, Italy). A volume of 14 mL of cell cultures was harvested by 10 min centrifugation at 2000 × g and 15 °C to obtain a final amount of cells equal to 1.2×10^7. The cell pellet was re-suspended in 50 µL of 50 mM tricine pH 7.2, and mixed with 100 µl of a 1 % (w/v) sodium alginate solution in the same buffer, obtaining a final cell concentration of 0.08×10^6 cells/µL. 5 µl of this suspension, containing ~ 4×10^5 cells, were deposited over a carbon black nanomodified working electrode surface (diameter 3.0 mm) of a three-electrodes system printed on paper, with a carbon and an Ag/AgCl reference electrode. 5 µl of 200 mM $CaCl_2$ in 50 mM Tricine-NaOH were drop cast on the deposited algae to allow the physical gelation of the alginate and the consequent entrapment of cells on the working electrode, keeping in mind that this step needs to be done more quickly to avoid damage to the algae cells due to running dry. Finally, algae/CB-SPEs were stored into 50 mM Tricine, 20 mM $CaCl_2$, 5 mM $MgCl_2$, 50 mM NaCl, 70 mM sucrose pH 7.2 and incubated for 2 h under continuous light (50 µmol photons m-2 s-1) and 25 °C.

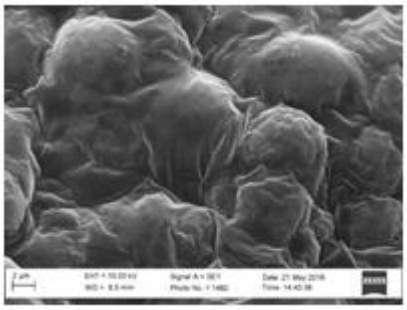

Figure 2. SEM microphotographs of algae whole cells entrapped into the calcium/alginate matrix.

D 4.4.4 Report of optical/electrochemical bioassayperformances characterized

Chemicals
All reagents were purchased as high purity grade. Tris-acetate-phosphate, tricine, sucrose, methanol, sodium alginate, sodium chloride, calcium chloride, atrazine, catechol, bisphenol A were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Copper (Cu^{2+}) and arsenic (AsIII)were purchased from Carlo Erba. Carbon black nanomodified screen-printed electrodes (CB-SPEs) were delivered by SENSE4MED, Italy.

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Algae immobilization protocol

C. reinhardtii cell cultures in an early mid-exponential growth phase, with Abs750 0.7 O.D., 107 cells/mL, and 10 µg/mL chlorophyll content, were exploited for the immobilization on screen-printed electrodes nanomodified with carbon black (CB-SPEs) purchased from Sens4Med (Rome, Italy). A volume of 14 mL of cell cultures was harvested by 10 min centrifugation at 2000 × g and 15 °C to obtain a final amount of cells equal to 1.2 × 107. The cell pellet was re-suspended in 50 µL of 50 mM tricine pH 7.2, and mixed with 100 µl of a 1
% (w/v) sodium alginate solution in the same buffer, obtaining a final cell concentration of 0.08 × 106 cells/□L. 5 µl of this suspension, containing ~ 4 × 105 cells, were deposited over a carbon black nanomodified working electrode surface (diameter 3.0 mm) of a three-electrodes system printed on paper, with a carbon and an Ag/AgCl reference electrode. 5 µl of 200 mM CaCl2 in 50 mM Tricine-NaOH were drop cast on the deposited algae to allow the physical gelation of the alginate and the consequent entrapment of cells on the working electrode, keeping in mind that this step needs to be done more quickly to avoid damage to the algae cells due to running dry. Finally, algae/CB-SPEs were stored into 50 mM Tricine, 20 mM CaCl2, 5 mM MgCl2, 50 mM NaCl, 70 mM sucrose pH 7.2 and incubated for 2 h under continuous light (50 µmol photons m-2 s-1) and 25 °C.

Biosensor prototype

A dual electro-optical transduction prototype was projected and realized to furnish both optical and electrochemical analysis by the company Biosensor Srl (Figure 1A). The instrument is a portable prototype consisting of 6 module chambers for the insertion of the algal CB-SPEs (Figure 1B). The chamber is equipped with a LED system (of 350 µmol photons m-2 s-1 of red light at a 650 nm wavelength) which provide algae illumination. The electrochemical set-up is constituted of a DC voltage supply, which provides a bias potential in the range of ±0.800 V between the working and the reference electrodes, and an amperometer to detect the current intensity variation deriving from the algae oxygen evolution process. The biological module, perfectly sealed, hosts the samples under test. Both static and dynamic operations are allowed thanks to an automatically controlled fluidic system equipped with inlet and outlet connections for the electrolytic/washing solution and sample flow.

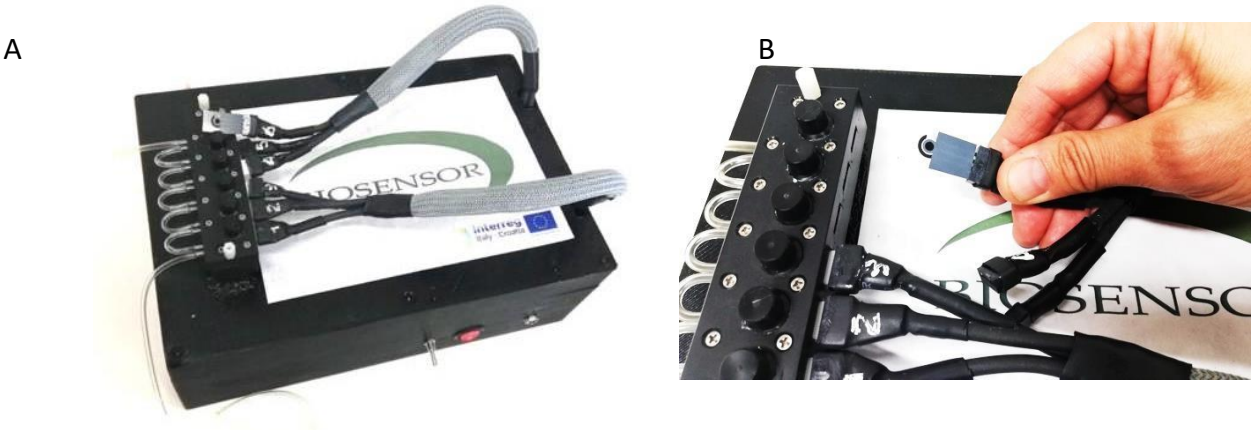

Figure 1. A) Algal biosensor prototype equipped with 6 measurement cells, fluidics, and the opto-electrochemical set-up, B) Screen-printed electrode ready for the insertion into the measurement cell.

Pathogen detection

The electrochemical detection of Escherichia coli (E. coli BL21), exploited as a case study pathogen, was provided by following algae oxygen evolution capacity at an applied potential of -0.6 V, using a dual electro-optical transducer prototype (Biosensor Srl, Via degli Olmetti, Rome, Italy). Algae were illuminated by a 350 µmol photons m-2 s-1 light with repeated cycles of 30 s light excitation and 10 min dark. An applied potential of -0.6 V was used with an acquirement interval of 0.5 s. Pathogens were added into the electrochemical chamber (200 µL volume containing 50 mM Tricine, 20 mM CaCl2, 5 mM MgCl2, 50 mM NaCl, 70 mM sucrose pH 7.2) in a concentration range from 100 to 2000 CFU/100 mL and the current signals, due to oxygen production on the CB-SPE working electrode, were recorded in dependence to the target analyte concentrations.

Results and discussion

Effect of wastewater samples on the alga C. reinhardtii

With the aim to design an algal biosensor for pathogen detection, the first step entailed the study of the effect of wastewater samples on the algal physiological parameters including the photosynthetic activity, the growth rate, and the pigment content. The green photosynthetic alga C. reinhardtii was thus grown in different water samples from 3 selected sites in the Adriatic region i.e. Lignano, San Giorgio, and Pescara depuration plants (DPs). In detail, optical density, cell number, chlorophyll *a* fluorescence, and the total chlorophyll content were measured. Results on growth (absorption at 750 nm and cell number / mL, Figure2A and B) and pigment content (Figure 2C) evidenced a slight influence of Lignano water sample on algae cell grow in terms of altered vital processes and variations in the physiologicalparameters (e.g. cell duplication). On the contrary, a toxic effect from San Giorgio and Pescarawater samples was evidenced on both algae growth (Figure 2A and 2B) and pigment content production (i.e. chlorophylls) (Figure 2C). The photochemical efficiency of PSII was also evaluated through Kautsky curves as described in Section 2.3 “Algae physiological characterization”, following the maximum fluorescence yield of Photosystem II F_V/F_M duringthe analyzed period of 9 days (Figure 2D). In this case, no effect was registered regarding themaximum fluorescence yield which remains constant during the time.

Optical microscopy images also evidenced a slowdown of the cell growth in the time for algaeincubated in San Giorgio and Pescara water samples, in comparison with the control, while any effect was observed in the presence of Lignano water (Figure 3).

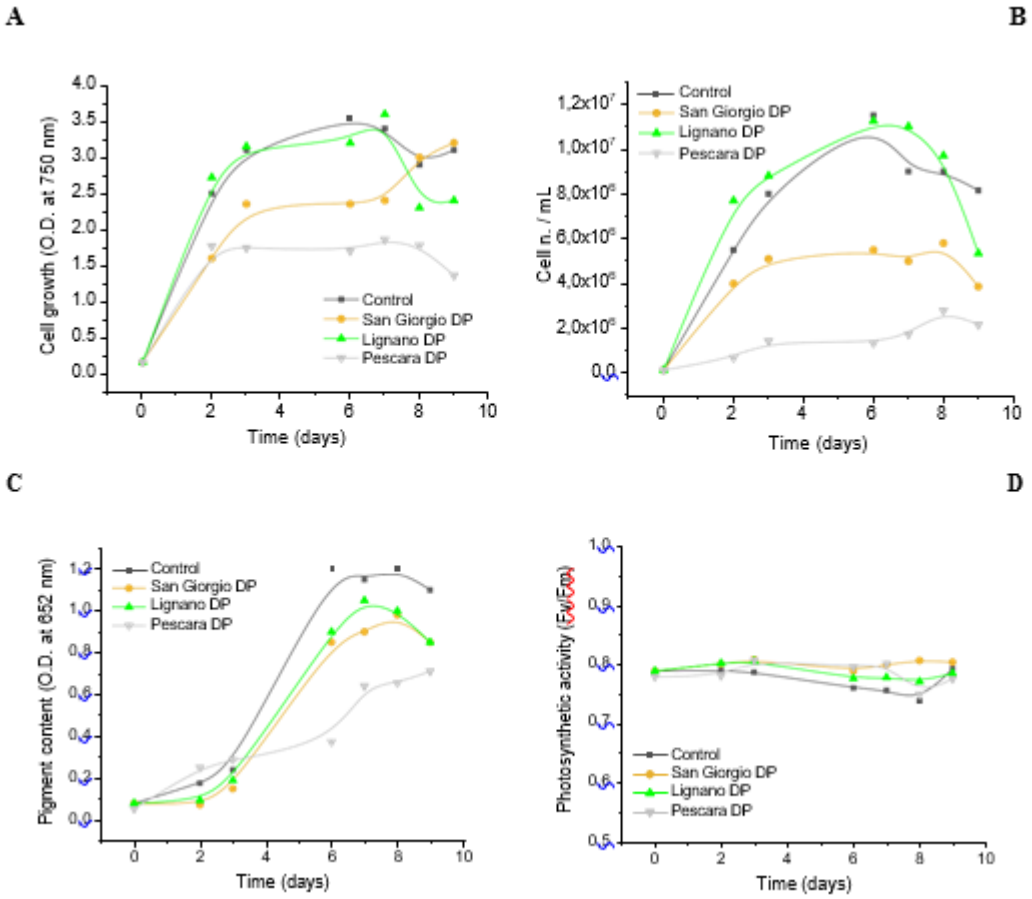

Figure 2. *C. reinhardtii* physiological characterization. Cell culture growth reported as absorbance at750 nm (A) and cell number / mL (B). C) Chlorophyll content. D) Maximum fluorescence yield F_V/F_M calculated on each Kautsky curve. Incubation time: 9 days under continuous light (50 µmol photons m^{-2} s^{-1}). Average values ± SE (n=3)

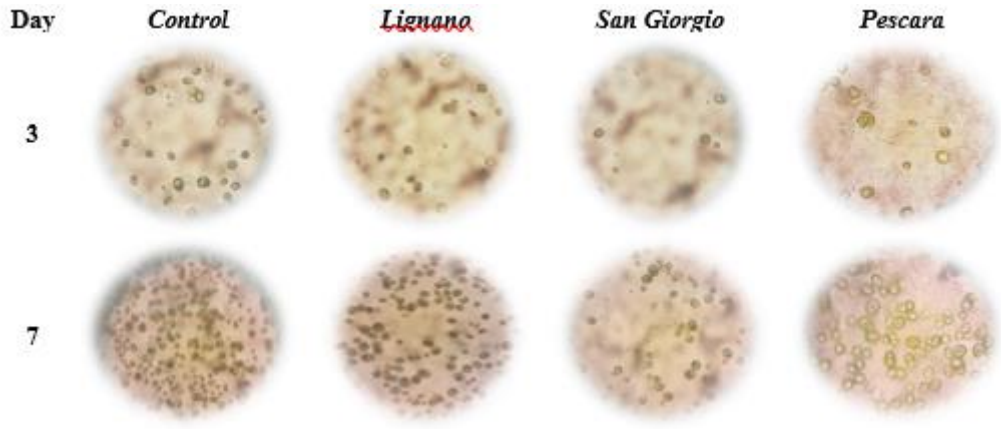

Figure 3. Optical microscopy images of algae cells grown in control medium and wastewater samplesfrom depuration plants in Lignano, San Giorgio, and Pescara (Italy).

Set-up of the algal biosensor and assessment of the analytical parameters

C. reinhardtii whole cells were suspended in 50 µL of tricine and 100 µL of 1% sodium alginate and drop cast on a CB-SPE working electrode, successively cross-linked using 5 µL of 200 mM $CaCl_2$ in the same buffer, to obtain a final number of cells on the working electrodeof 4×10^5. The algal CB-SPEs were stored in 200 mM $CaCl_2$ in 50 mM Tricine, 20 mM $CaCl_2$,5 mM $MgCl_2$, 50 mM NaCl, 70 mM sucrose, pH 7.2 under continuous light at 50 µmol photonsm^{-2} s^{-1} for storage and detection analysis. The algal CB-SPEs were observed at Scanning Electron Microscopy (SEM) as reported in Figure 4, which show microphotographs of algae whole cells entrapped into the calcium/alginate matrix at a magnification of 2 µm Figure 4. SEM microphotographs of algae whole cells entrapped into the calcium/alginate matrix.

The algal/CB-SPEs were inserted into the measurement chamber of the biosensor prototype (Figure 1) and amperometric measurements were accomplished for the detection of E. coli, a case study pathogen that can be found in wastewater. Algal/CB-SPEs were incubated in dark with E. coli at a concentration of 1000 CFU / 100 mL from 5 to 60 minutes, to evaluate the incubation time at which a higher algae oxygen production occurs due to the presence of bacteria, which reduce the photosynthetic oxygen tension within the microenvironment of the algal cells. Indeed, within short incubation time from 5 and 15 min, an increase of the current signals was observed (Figure 5A), while at higher incubation time a balance of algal oxygen evolution and oxygen sequestration by bacteria was observed, thus providing current signals comparable to algae oxygen production in the absence of bacteria. Considering the results reported in Figure 5A, an incubation time of 15 min was selected. Thus, algal/CB-SPEs were incubated for 15 min in dark with E. coli in a concentration range from 100 to 2000 CFU / 100 mL in a reaction volume of 200 µL of measuring buffer; then, a light flash of 30 s of red LEDs, optically filtered to a peak wavelength of 650 nm at an intensity of 315 µmol photons m-2 s-1, was applied to periodically stimulate the photosynthetic activity and provide light-induced oxygen evolution. An increase of the oxygen evolution and thus of the current signals was registered in the presence of increasing of pathogen concentration (Figure 5B). A linear response was obtained by serial additions of E. coli in the selected concentration range, allowing for the construction of a calibration curve using the linear regression reported by the equation y = 1.530 (±0.059) - 0.00060 (±0.00005) x, with an R2 = 0.985 (Figure 5C). A detection limit of 92 CFU / 100 mL was achieved (LOD = 3×sd/slope). The linear range and the LOD found can be considered coherent with the maximum E. coli concentration suggested by Italian law for wastewater (less than 5000 CFU/100 ml) [25].

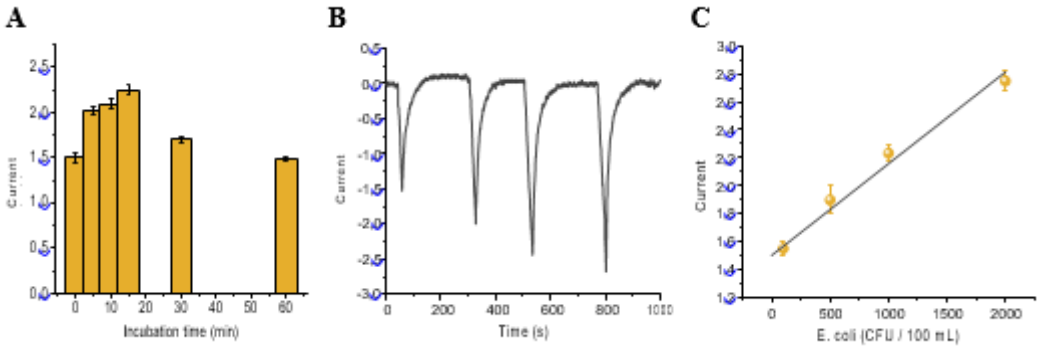

Figure 5. A) Incubation time, B) Algal CB-SPEs amperogram after addition of increasing amount of E. coli, and C) corresponding calibration plot. Applied potential -0.6 V, repeated cycles of 30 s light and 10 min dark, n=3. Measurement volume: 200 µL of measuring buffer.

With the aim to evaluate the capability of the proposed biosensor in wastewater samples, some chemicals including metals, pesticides, and phenolic compounds were analyzed as interferents at legal limits established by the European legislations for surface water (where present) [26].

In detail, algal/CB-SPEs were incubated in 200 µL of measuring buffer fortified with standard solutions of 10 ppb arsenic, 1.3 ppb copper, 5 ppb cadmium, 10 ppb lead, 10 ppb bisphenol A, 1 ppb paraoxon, and 5 µM atrazine as well as with a solution spiked with all the above listed compounds, to evaluate the synergistic effect of different chemicals in a mixture. Results reported in Figure 6A highlights that the interfering species did not affect the analysis of E. coli at the tested concentrations, unless atrazine, which is, as a photosynthetic herbicide, the specific target of the alga. However, the presence of such herbicides can be analyzed also by chlorophyll fluorescence, thus supporting the amperometric analysis of bacteria.
To investigate the suitability of the proposed biosensor in real samples, matrix effect was studied. Algal/CB-SPEs were incubated in 200 µL of 2X measuring buffer diluted 1:2 (v:v) in wastewater fortified with E. coli in a concentration range from 100 to 2000 CFU / 100 mL. A calibration curve was obtained for each wastewater sample analyzed, described by the equations y = 1.49 (±0.05) - 0.0005 (±0.00004) x (R2 = 0. 989), y = 1.49 (±0.01) - 0.0001 (±0.00001) x (R2 = 0. 975), y = 1.48 (±0.01) - 0.0002 (±0.00001) x (R2 = 0. 992) for Lignano, San Giorgio, and Pescara, respectively (Figure 6B). The ratio between slopes of the calibration curves obtained in standard solutions and real samples was equal to 0.83, 0.16, 0.33, indicating a ~ 17, 84, and 67 % dependence from Lignano, San Giorgio, and Pescara wastewater matrices, respectively. The calibration curve obtained for the algal biosensor in real samples was further used to calculate the recovery values of the surface water samples. Recovery values of 105 ±8, 83 ± 7, and 88 ± 7 % were obtained for 1000 CFU / 100 mL of E. coli for Lignano, San Giorgio, and Pescara wastewater samples, respectively.

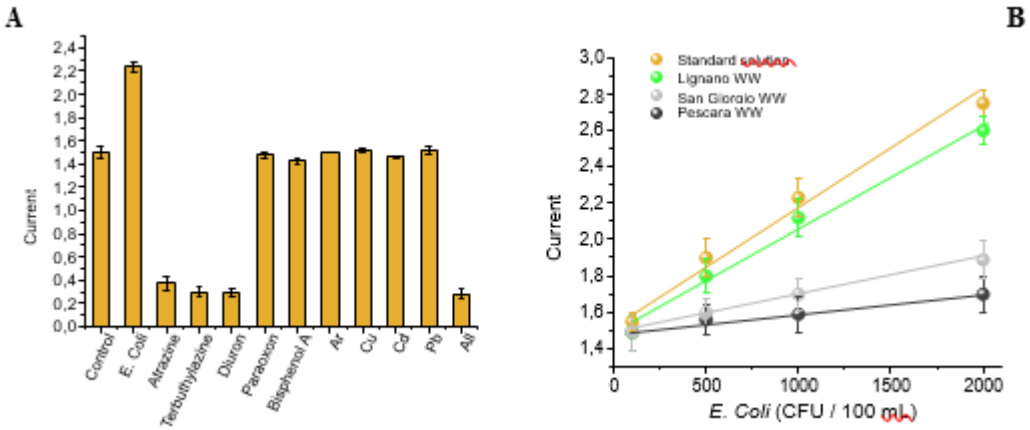

Figure 6. A) Algal/CB-SPE interference study using 10 ppb arsenic, 1.3 ppb copper, 5 ppb cadmium, 10 ppb lead, 10 ppb bisphenol A, 1 ppb paraoxon, and 5 µM atrazine as well as a solution spiked with all the above listed compounds. B) Matrix effect using Lignano, San Giorgio, and Pescara wastewater samples. Applied potential -0.6 V, repeated cycles of 30 s light and 10 min dark, n=3. Measurement volume: 200 µL of measuring buffer.