Effects of a complex mixture of therapeutic drugs on unicellular algae *Pseudokirchneriella subcapitata*

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A B S T R A C T

Pharmaceutically-active compounds are regularly and widely released into the aquatic environment in an unaltered form or as metabolites. So far, little is known about their potential detrimental effects on algae populations which can ultimately impact nutrient cycling and oxygen balance. For our analysis, the common microalga *Pseudokirchneriella subcapitata* (*P. subcapitata*) was exposed to a mixture of 13 drugs found in Italian wastewaters and rivers. Traces of pharmaceuticals investigated were detected in treated algal cells, except for cyclophosphamide and ranitidine, indicating that these algae are able to absorb pharmaceutical pollutants from the environment. The effects of the treatment were investigated by Amplified Fragment Length Polymorphism (AFLP) assessment of DNA damage and 2-DE proteomic analysis. While no genotoxic effect was detected, proteomic analysis showed that algae are sensitive to the presence of drugs and that, in particular, the chloroplast is affected.

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1. Introduction

Pharmaceutically-active compounds (PACs) are widespread in the environment due to their extensive use in human and veterinary medicine. PACs are frequently excreted in an unmetabolized form or as a variety of different metabolites by humans and animals following medication. Moreover, improper disposal of expired PACs and industrial emissions contribute to environmental pollution. More than 80 PACs from various therapeutic classes have been detected up to $\mu g\,l^{-1}$ levels in sewage, surface, and ground waters (Kummerer, 2009). Conventional sewage treatments do not remove PACs, and some pharmaceuticals have a long half-life and may therefore accumulate in the environment.

PACs are specifically designed to elicit a biological response at very low levels and even small concentrations could have negative effects on target- and non-target organisms. Moreover, although PACs are designed to modify specific metabolic and molecular targets in humans and animals, they often have important and unexpected side-effects as well. The effects of different PAC mixtures have been reported on phyto-, zoo-plankton and other aquatic organisms (Richards et al., 2004; Cleuvers, 2004; Brain et al., 2004; Mimeault et al., 2005; Fent et al., 2006; Kim et al., 2009). Pomati et al. (2006) designed a mixture of 13 PACs to mimic the combination and the experimental concentrations of drugs found in an Italian environment (Zuccato et al., 2000; Calamari et al., 2003). This mixture is able to inhibit cell proliferation and activate stress-response proteins in the human embryonic cell line HEK293 and in Zebrafish liver cells (Pomati et al., 2006, 2007).

The aim of this work was to explore the effects of the same mixture on the unicellular green alga *Pseudokirchneriella subcapitata* (*P. subcapitata*). The rationale is that PACs can induce toxic effect even in non target organisms, due to the evolutionary conservation of pathways and receptor targets among different organisms. Homologous targets for fluoroquinolone (chloroplast replication), lincosamide (transcription and translation), sulphonamide (folate biosynthesis) antibiotics have been identified in plants (Brain et al., 2009). For other drugs as atenolol, carbamazepine, furosemide an indirect toxic effect on aquatic photosynthetic organisms has been shown (Escher et al., 2006; Isidori et al., 2006).

We chose *P. subcapitata* (Chlorophyceae; formerly known as *Selenastrum capricornutum* or as *Raphidocelis subcapitata*) because it is highly sensitive to the contamination of aquatic environments, and for this reason is recommended (by EPA and OECD) as a standard test organism in ecotoxicology. Moreover, this organism has a modest DNA content and grows rapidly with a duplication time of approximately 18 h. In non-target organisms classic toxicity

tests that use organism survival as the sole endpoint may overlook sublethal effects, such as subtle alterations of genome or proteome profile, which could have long-term ecological importance. For this reason, to have a more complete picture of the ecological risk, we analysed the potential physiological and genetic toxicity of the mixture. We selected the Amplified Fragment Length Polymorphisms (AFLP) approach as a suitable methodology for the evaluation of DNA damage. The specific changes in total soluble protein pattern induced by the PAC mixture were detected through 2-DE and MS/MS analysis, which is an appropriate approach for positive identification of proteins from an organism whose genome has yet to be sequenced.

2. Materials and methods

2.1. Preparation of PAC solution

The list of PAC used in this study is reported in Table 1. Pharmaceuticals were all purchased from Sigma–Aldrich Co. (Dorset, UK), apart from ciprofloxacin and ranitidine that were obtained from ICN Biochemicals (Meckenheim, Germany) and GlaxoSmithKline (Philadelphia, PA), respectively. Stock solutions were prepared in methanol as reported by Pomati et al. (2006), mixed and diluted in methanol to a concentration 100 times higher than the corresponding test level and stored at $-20\,^{\circ}$C. Appropriate aliquots of this concentrated test solution were evaporated to dryness and suspended in algal culture media, to final concentrations mimicking those measured in the environment ($1\times$ solution reported in Table 1; Pomati et al., 2006). A mixture 10 times more concentrated ($10\times$) was also assayed. The pharmaceuticals considered in this investigation are mostly hydrophilic, since they have $\log K$ value lower than 2 (except bezafibrate and ibuprofen) and water solubility always higher than $15\,\mathrm{mg\,l^{-1}}$ (Zuccato et al., 2004). The composition and the purity of the stock solutions were checked before use by mass spectrometric analysis using an analytical method described in detail in a previous publication (Castiglioni et al., 2005).

2.2. Algal culture conditions and growth inhibition test

The *P. subcapitata* Hindák strain was obtained from the Collection of Algal Cultures, Göttingen, Germany (SAG 61.81, http://www.epsag.uni-goettingen.de/html/sag.html). Cultures were periodically checked for contamination on nutrient agar plates.

The algae were cultured in axenic condition in algal culture medium (Charles and Searle, 1991), under gentle and continuous shaking (100 cpm) conditions, controlled temperature ($23 \pm 2\,^{\circ}$C)

Table 1
Mixture of therapeutic drugs at $1\times$ experimental concentration (from Pomati et al., 2006).

Pharmaceutical	Terapeutic category	ng L^{-1} (environ. cond.)
Atenolol	Anti-hypertensive	241
Bezafibrate	Lipid regulating	57
Carbamazepine	Anticonvulsant/antidepressant	33
Ciprofloxacin	Antibacterial (fluoroquinolone)	26
Lincomycin	Antibacterial (lincosamyne)	249
Ofloxacin	Antibacterial (fluoroquinone)	150
Sulfamethoxazole	Antibacterial (sulphonamide)	46
Cyclophosphamide	Citotoxic (alkylating agent)	10
Furosemide	Diuretic	255
Hydrochlorothiazide	Diuretic	256
Ibuprofen	Anti-inflammatory (arylproprionic acid)	92
Ranitidine	Ulcer heating	39
Salbutamol	Bronchodilator	4,6

and constant light irradiance ($4000 \pm 500\,\mathrm{lx}$). Erlenmeyer flasks containing 150 ml of solution were inoculated to obtain an initial concentration of $2 \times 10^5\,\mathrm{cell\,ml^{-1}}$. Only cultures in logarithmic phases were used for inoculation and for each experiment six flasks were inoculated for untreated (control) and treated cells. Algae were treated with $1\times$ and $10\times$ PAC mixtures after 15 h from inoculation to allow cells to recover from the effect of the subculture before exposing them to the PAC treatment. Cell proliferation was recorded over three time points in a 72 h exposure period during which cells were subjected to a single initial treatment with PAC mixture. Algal density was determined at 750 nm using an UV–Visible recording spectrophotometer (JASCO, V-560, UV/VS spectrophotometer). Measures were repeated at least three times. After 72 h algal cells were collected and immediately used for pigment analysis. Cells for uptake, AFLP and 2-DE analysis were washed three times with MilliQ water and stored at $-80\,^{\circ}$C until their use.

2.3. PAC presence in algal cells

The presence of PACs in the algal cells exposed to the selected mixture of pharmaceuticals was evaluated by successive ultrasonic solvent extraction (USE), solid phase extraction (SPE) and high pressure liquid chromatography tandem mass spectrometry (HPLC–MS–MS) analysis. The method for USE was adapted from Ternes et al. (2005), while SPE and HPLC–MS–MS analysis were performed using a multiresidue method described in detail in a previous publication (Castiglioni et al., 2005). Algal pellets from $-80\,^{\circ}$C were lyophilised, weighed and minced in liquid nitrogen to break cell walls and be able to recover also the pharmaceuticals localized within the wall or inside the cells. Algal samples (about 20 mg dry weight) were spiked with 20 ng of deuterated standards (salbutamol-d3, atenolol-d7, ciprofloxacin-d8, carbamazepine-d10, ibuprofen-d3) and were extracted successively with 10 ml methanol (two times), 10 ml 2% ammonia solution in methanol and 10 ml acetone. In each extraction step, the samples were ultrasonicated for 10 min and then centrifuged at 6000 rpm. The supernatants were pooled, evaporated to a volume of about 1 ml, and diluted with MilliQ water to a final volume of 50 ml for solid phase extraction as a clean-up step. The SPE was performed using an Oasis HLB (60 mg, Waters Corp., Milford, MA) at pH 3.0 for ciprofloxacin and ofloxacin, and an Oasis MCX (60 mg, Waters Corp., Milford, MA) at pH 2.0 for all the other compounds (method slightly modified from Castiglioni et al., 2005). The Oasis MCX cartridges were eluted with 2 ml methanol and 2 ml 2% ammonia solution in methanol; the Oasis HLB cartridges were eluted with 2 ml methanol. Eluates were pooled, dried under nitrogen and redissolved in 200 µl of MilliQ water. Extracts were filtered on regenerated cellulose syringe filters, 0.2 µm (PHENEX, Phenomenex, Torrance, CA, USA) for further clean up before analysis.

The HPLC–MS–MS analysis was performed as described by Castiglioni et al. (2005). Briefly, a Luna C8 column 50 mm × 2 mm i.d., 3 µm particle size (Phenomenex, Torrance, CA, USA) was used for chromatographic separation and an Applied Biosystem–SCIEX API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray source (Applied Biosystems–SCIEX, Thornhill, Ontario, Canada) was used for analysis. Analysis was done in the selected reaction monitoring mode (SRM), in positive and negative ionisation mode, and quantification was performed by isotopic dilution.

Culture media from the growth inhibition tests and the respective controls were also analysed to check the presence/absence of the PACs under study. 50 ml aliquots of culture media were collected at the end of the experiments and were frozen at $-20\,^{\circ}$C until analysis that was performed by SPE and HPLC–MS–MS (Castiglioni et al., 2005).

Table 2
Sequences of adapters and primers used for AFLP analysis.

Type	Name	DNA sequence
EcoRI adapter		5′ -CTC GTA GAC TGC GTA CC-3′
		3′ -CAT CTG ACG CAT GGT TAA-5′
MseI adapter		5′ -GAC GAT GAG TCC TGA G-3′
		3′ -TAC TCA GGA CTC AT-5′
EcoRI + 1 primer	E00	5′ -GAC TGC GTA CCA ATT C-3′
MseI + 1 primer	M00	5′ -GAT GAG TCC TGA GTA A-3′
EcoRI + 3 primers	E32	5′ -GAC TGC GTA CCA ATT CAA C-3′
	E38	5′ -GAC TGC GTA CCA ATT CAC T-3′
MseI + 3 primers	M38	5′-GAT GAG TCC TGA GTA AAC T-3′
	M42	5′-GAT GAG TCC TGA GTA AAG T-3′

Method performance was checked within this study and was in line with the results reported in Castiglioni et al. (2005). Recoveries were greater than 70% and the overall variability of the method was below 8%. The instrumental quantification limit (IQL) was in the low pg/injected range, and the limits of quantification (LOQ) were in the low ng l^{-1} range. The Oasis HLB cartridges were used to optimize ciprofloxacin and ofloxacin extraction, and the recoveries were improved from 30% (Castiglioni et al., 2005) to $45 \pm 7\%$ and $58 \pm 5\%$ for ciprofloxacin and ofloxacin, respectively.

2.4. DNA extraction and AFLP analysis.

DNA was extracted using the Plant Genomic DNA Miniprep Kit (Sigma–Aldrich), starting from 100 mg of fresh algae material (*P. subcapitata* is diploid with modest DNA content: *C* value = 0.2 pg). DNA concentration was estimated by electrophoresis on 0.7% of agarose gel in TAE buffer for 1 h at 100 mV. The gel was stained in 0.5 mg/ml ethidium bromide and analysed with a Gel Doc 2000 (Biorad, USA). The DNA concentration of each sample was obtained by the comparison of ethidium bromide stained band intensities with λ DNA standard AFLP method is based on the selective amplification of a subset of restriction fragments from a complex mixture of DNA fragments obtained after digestion of genomic DNA with restriction endonucleases (Vos et al., 1995). Genomic DNA (100 ng) was digested (2 h) with EcoRI (1 U) and MseI (1 U). The DNA fragments were ligated (with T4-DNA ligase) to EcoRI (2.5 pmol) and MseI (25 pmol) adapters in a final volume of 40 μl. Ligation reaction was performed at 22 °C for 2 h. This mixture was used as the template in the preamplification reaction containing DNA primers E00 and M00 (Table 2) complementary to the core of the EcoRI and MseI adapters, respectively. The 50 μl amplification mixture contained 30 μl of DNA digested/ligated, 50 ng of the selected primers, 200 mM of each of the 4 dNTPs, 0.5 U Dynazyme II (Finnzymes, Finland) and 5 μl Dynazyme buffer. After 2 min at 94 °C, amplification was carried out for 20 cycles of denaturation (45 s at 94 °C), annealing (30 s at 50 °C) and extension (1 min at 72 °C). After a final elongation step (7 min at 72 °C) the preamplification product was diluted 1:20. The PCR products were amplified in a second round with primers containing selective bases (Table 2); this was carried out using primer pairs E32–M38, E32–M42, E38–M38, E38–M4. The EcoRI-primer was labelled by using fluorescent 6-carboxy fluorescein (6-FAM) on the 5′ nucleotide. The temperature profile for this step was the following: one cycle of 5 min at 94 °C, followed by 10 cycles of 30 s at 94 °C, 1 min at 65 °C (for the first cycle, subsequently reduced each cycle by 1 °C for the next nine cycles), and 1 min at 72 °C, followed by 25 cycles of 30 s at 94 °C, 1 min at 56 °C and 1 min at 72 °C. The PCR was terminated with a final incubation step of 10 min at 72 °C. The amplified fragments were fractionated and detected with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) with the data collection software 3.0 (ABI). AFLP fragment analysis was performed with GeneScan Analysis Software 4.0 (ABI) and the data were assembled in binary format. Fragments were resolved using capillary electrophoresis.

2.5. Protein extraction and 2-D gel electrophoresis

Total soluble proteins were extracted from control and 10× sample after 72 h of treatment in extraction buffer (500 mM Tris–HCl pH 8, 700 mM sucrose, 10 mM EDTA, 4 mM ascorbic acid, 0.4% 2-mercaptoethanol, 0.2% Triton×-100, 1 mM phenylmethanesulfonylfluoride (PMSF, Sigma, St. Louis, MO, USA), 1 μM Leupeptin (Fluka, Stenheim, Germany), 0.1 mg ml^{-1} Pefabloc (Fluka, Stenheim, Germany). After centrifugation at $13,000 \times g$ for 20 min at 4 °C, an equal volume of phenol saturated with Tris–HCl 0.1 M pH 8 was added to the supernatant. The proteins were precipitated overnight by adding five volumes of cold 0.1 M ammonium acetate in methanol to the phenol phase. The precipitates were recovered by centrifuging at $13,000 \times g$ for 30 min, washed with ammonium acetate in methanol and with acetone 80%, and then resuspended in the IEF solubilization buffer [7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 50 mg ml^{-1} dithiothreitol (DTT)]. After sample clarification at $13,000 \times g$ for 10 min, protein concentration was measured by the Bio-Rad protein assay (Hercules, CA, USA). The samples were directly loaded for isoelectrofocusing (IEF) or stored in aliquots at −80 °C until use. Three independent protein extractions were performed, and the typical yield was 450 μg protein g^{-1} FW from both samples.

IEF was carried out with 600 μg of soluble protein extract by using an immobilized 4–7 pH gradient (Immobiline DryStrip, 13 cm; Amersham Biosciences, Uppsala, Sweden). The strips were rehydrated in the IPGphor system (Amersham Biosciences, Bucks, UK) for 1 h at 0 V, 20 °C and 10 h at 30 V, 16 °C with the solubilization buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 50 mg/ml DTT, 0.5% of carrier ampholyte (4–7 NL IPG buffer; Amersham Biosciences, Uppsala, Sweden), bromophenol blue 0.005% and the protein extracts. IEF was performed at 16 °C in the IPGphor system (Amersham Biosciences, Uppsala, Sweden) for 4 h at 200 V, from 200 to 3500 V in gradient during 30 min, 3 h at 3500 V, from 3500 to 8000 V in gradient during 30 min, after which the run was continued at 8000 V to give a total of 70 kVh.

Each focused strip was equilibrated for 30 min against 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris–HCl pH 8.8, 2% DTT and then a further 30 min with the substitution of the DTT with 2.5% iodoacetamide in the equilibration buffer. The equilibrated strips were placed on top of vertical 12.5% polyacrylamide gels. The molecular weight markers, covering a 10–250 kDa range, were run on the acidic side of each gel. Electrophoresis was performed at 4 °C in a Laemmli running buffer (25 mM Tris–HCl pH 8.3, 192 mM glycine, 0.1% SDS) for 30 min at 15 mA/gel then at 45 mA/gel until the dye front reached the bottom of the gel.

2.6. Staining and analysis of 2-D gels

High resolution 2-DE analysis was used to separate total soluble proteins of 72 h-treated and non-treated cells in three independent experiments. For each sample three gels were analysed, and the data showed a high level of reproducibility. Approximately 800 protein spots were resolved and detected by CBB staining over a pH range of 4–7 and a size range of 10–250 kDa. The analysis of the gels scanned by ImageScanner (Amersham Bioscience, Uppsala, Sweden) was performed by using the Image Master 2D Platinum software. Data were normalised by expressing protein abundance as percent spot volume relative to volume of total protein in the gel (%vol). 2D gel replicates for each condition tested were averaged and the resulting gels contain only spots present in all the replicates. The two average-gels (control and 10× PACs mixture) were

compared and only proteins with a fold change of ±1.5, significant in Student's t-test at a level of 95%, were accepted as differentially expressed. These spots were selected for MS/MS analysis.

2.7. In gel digestion and mass spectrometric analysis

Selected spots were manually excised from the 2D-gels, washed twice and stored in 50% ethanol at 4 °C until digestion. Spot digestion was performed as previously described (Marsoni et al., 2008). The extracted tryptic fragments were analysed by MS/MS after reverse phase separation of peptides (Liquid Chromatography-ElectroSpray Ionization Mass tandem Spectrometry, LC–ESI–MS/MS). For all experiments, a Finningan LXQ linear ion trap mass spectrometer, equipped with a Finningan Surveyor MS plus HPLC system (Thermo Electron Corporation, CA, and USA) was used. Chromatographic separations were conducted on a BioBasic C18 column (150 μm I.D. × 150 mm length and 5 μm particle size; Thermo Electron Corporation, USA), using a linear gradient from 5 to 75% Acetonitril, containing 0.1% formic acid with a flow of 2 μl/min. Acquisitions were performed in the data-dependent MS/MS scanning mode (full MS scan range of 400–1400 m/z followed by Zoom scan for the most intense ion from the MS scan and full MS/MS for the most intense ion from the zoom scan), thus enabling a dynamic exclusion window of 3 min. Protein identification was performed by searching in the National Center for Biotechnology Information (NCBI) viridiplantae and/or EST-viridiplantae protein database using the MASCOT program (http://www.matrixscience.com). The following parameters were adopted for database searches: complete carbamidomethylation of cysteines, partial oxidation of methionines, peptide mass tolerance 1.2 Da, fragment mass tolerance 0.8 Da and missed cleavage 1. For positive identification, the score of the result of $[-10 \times \log(P)]$ had to be over the significance threshold level ($p > 0.05$) and we make a point of a minimum of two matched peptides.

Unsuccessful protein identifications were submitted to de novo analysis by PepNovo software using default parameters (http://peptide.ucsd.edu/pepnovo.py). Peptide sequences with a mean probability score of at least 0.5 were edited according to MS BLAST rules and MS BLAST search was performed against NCBI non redundant database at http://www.dove.embl-heidelberg.de/Blast2/msblast.html. Statistical significance of hits was evaluated according to MS BLAST scoring scheme. For the subcellular localization we used CELLO v.2.5 (Yu et al., 2006).

2.8. Pigment analysis

Algal suspensions were harvested by two centrifugations at $5000 \times g$ for 15 min. The pellet was resuspended in 1 ml of algal culture medium, transferred in an Eppendorf tube and centrifuged at $9000 \times g$ for 3 min. The cells were resuspended and incubated overnight in 1 ml of N,N-dimethylformamide (DMF, Sigma). After centrifugation at $9000 \times g$ for 3 min, the surnatants were used to absorption spectra measurement. All steps were performed in the dark at 4 °C. The absorption spectra were recorded from 350 to 800 nm, with 0.5-nm band width and a scan rate of 200 nm s^{-1}. Pigment concentration was measured by the following equations (Porra et al., 1989):
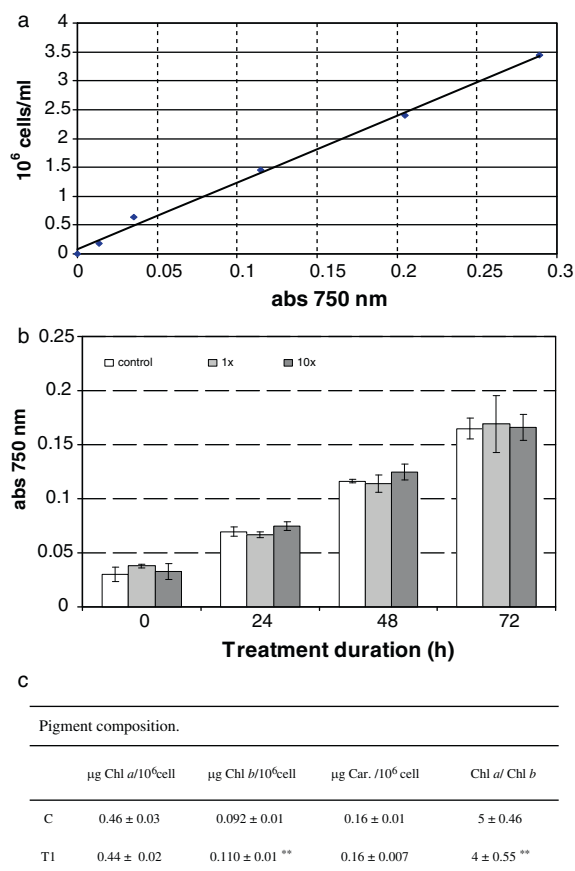
Chlorophyll a (μg/ml)

$$= 12.00(Abs664 - Abs750) - 3.11(Abs647 - Abs750)$$

Chlorophyll b (μg/m)

$$= 20.78(Abs647 - Abs750) - 4.88(Abs664 - Abs750)$$

Carotenoids (μg/ml) = (Abs461 − 0.046Abs664)4

Fig. 1. (a) Relationship between the value of turbidity (as absorbance at 750 nm) and cell number of *P. subcapitata*. (b) Algal growth of PAC-treated vs. control samples. Cells were collected at 24 h intervals after treatment with control (white bar), 1× (grey bar), or 10× (black bar). (c) Pigment composition. C, control; T1, cells treated with 10× PAC. Values shown are mean ± S.D. ($n = 3$), ** $p < 0.01$ (referred to change between C/T1).

Pigment content was normalised by dividing for cell number measured using a Bürker chamber by light microscopy and expressed as number of cells ml^{-1} medium. Measures were repeated three times for each sample.

2.9. Statistical analysis

For all analysis three technical replicates were performed for controls and PAC treatments, each with a pool of six culture flasks. Measurements of algal density and pigment were used to calculate mean values ± standard deviations ($n = 3$). In the 2-DE analysis, three replicate gels were run for each technical replica. In total, nine gels were performed for control and for treated sample. Analysis of the relative abundance of proteins was calculated as mean values ± standard deviations ($n = 9$).

Student's t-test was used to make pair-wise comparisons between control and PAC-treated samples. Unless otherwise noted, $p < 0.05$ was used as the threshold for statistical significance.

3. Results

3.1. Algal proliferation assay, pigment analysis and PAC presence in algal cells.

To ensure that the turbidity accurately measured the cell density, a relationship between turbidity and cell number was generated. As shown in Fig. 1a, the value of turbidity (as absorbance
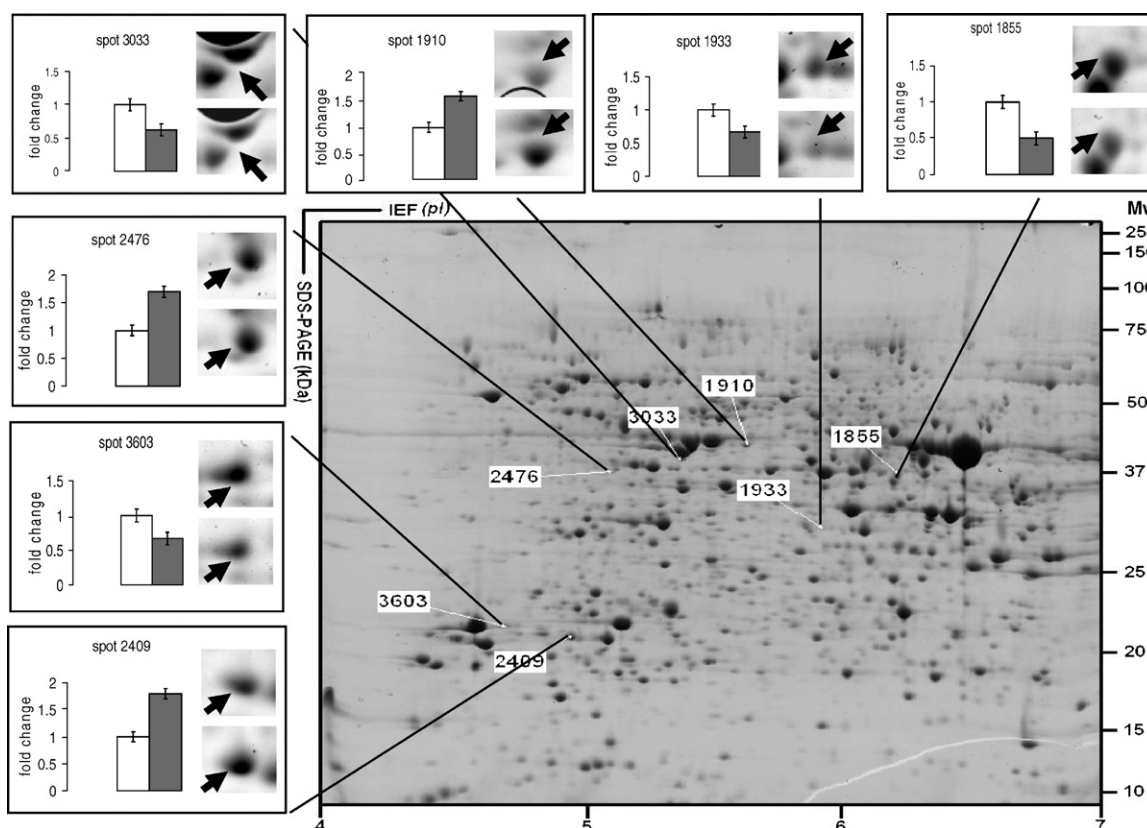
Fig. 2. A representative image of 2-DE protein separation visualized by CBB staining. In the boxes are reported the magnified areas of the selected spots and bar graphs showing the fold-change expression of drug-exposed vs. control algal proteins. White bar = control cells, grey bar = 10× PAC-treated cells.

at 750 nm) within the range of 0.01–0.29 linearly correlated with the cell number. *P. subcapitata* cells treated with the mixture of 13 PACs at both concentrations did not show any appreciable difference in cell concentrations relative to control samples at any time point (Fig. 1b). Based on these data, further measurements were performed at 10× PAC concentration. To assess the effects of the PAC mixture on the algal photosynthetic apparatus, the pigment content (Chl *a*, Chl *b* and carotenoids) was monitored by spectrophotometry. As reported in Fig. 1c, pigment analysis showed a significant increase of the Chl *b* in treated vs. control cells.

To better evaluate the metabolic response of algae on PAC exposure, the presence of the different drugs in *P. subcapitata* was analysed. PACs were not detected in the culture medium in the control, which were not spiked with pharmaceuticals. In the culture medium containing 10× PAC mix without algal inoculation, PAC concentrations coincide with the concentrations we put in the medium as reported in Table 1. About PAC detection in *P. subcapitata*, algal cell numbers were not enough to obtain reliable quantitative data to provide a significant correlation with PAC concentrations inoculated in the medium. The results were therefore expressed qualitatively as presence/absence in algal cells and indicated that only traces of the PACS were detected inside the cells, except for cyclophosphamide and ranitidine, which were completely absent, suggesting little or no uptake. Despite this methodological bias, the detection of PACs in algal cells was nonetheless useful for correlating the molecular changes in *P. subcapitata* with the selected mixture of pharmaceuticals in the culture medium.

3.2. DNA damage in response to PACs

The potential genotoxic effects of the PAC mixture on *P. subcapitata* were evaluated by AFLP analysis which was performed on DNA

extracted from controls and 10× PAC-treated algal samples (3 control samples and 3 treated algae obtained from each independent experiments). Altogether, the analysis revealed a total of 574 DNA bands, 27 (4.7%) of which were polymorphic. Details are given in Table 3. Generally, the number of polymorphic bands (%p < 5) was very low and compatible with the basal level of genetic variation in a pool of unicellular organisms obtained by vegetative propagation (Karp et al., 1998; Labra et al., 2007).

3.3. 2-DE analysis

A representative 2-DE gel from a control algal sample is shown in Fig. 2. All spots were matched by gel comparison, and the difference in the relative abundance (%volume) of each spot was analysed. We focused our attention on the spots whose abundance in the PAC-treated cells was significantly different (by at least 1.5-fold) from that in non-treated cells. Using this criterion, we selected 7 proteins which are marked on the representative gel in Fig. 2. These spots were excised from the gels and 6 were successfully identified by LC–MS/MS analysis (Table 4). Since the *P. subcapitata* genome is not yet completely sequenced it was necessary to perform cross-species protein identification using the EST-database and BLASTP (http://www.ncbi. nlm.nih.gov/BLAST) (Carpentier et al., 2008).

Table 3
Primer combination used in AFLP analysis; Number of total and polymorphic bands and percent of polymorphism (%) detected for each primer combination.

Primer combinations	Total bands	Polymorphic bands	(%)
E32–M38	165	5	3.0
E32–M42	151	8	5.3
E38–M38	132	7	5.3
E38–M42	126	7	5.6

Table 4
Results of protein identification by MS/MS.

Spot	NCBI accession no.	Protein name (and localization)	Organism	Fold change	MASCOT Score (peptides)	MW (kDa)/pI	
						Theor.	Exp.
Proteins involved in metabolism							
3033	gi|4519272	ATP synthase beta-subunit (Chl)	*V. aureus*	0.63 ± 0.1	93(2)	40.8/5.5	41.0/5.4
1910	gi|81157953[b]	Enolase (cyt)	*C. pyrenoidosa*	1.6 ± 0.1	70(2)	34.0/5.0	33.0/5.9
1933	gi|2494745[a]	Glutamine synthetase, chloroplast precursor (Chl)	*C. reinhardtii*	0.67 ± 0.01		41.6/6.8	30.0/5.9
Proteins involved in photosynthesis							
2409	gi|60391855[a]	Chlorophyll *a/b* binding protein CP29 (Chl)	*C. reinhardtii*	1.8 ± 0.2		30.0/6.2	21.0/4.9
3603	gi|75218995[a]	Chlorophyll *a/b*-binding protein (Chl)	*M. crystallinum*	0.67 ± 0.05		28.4/5.3	22.0/4.7
2476	gi|159487493[b]	DegP-type protease like (Chl)	*C. reinhardtii*	1.7 ± 0.1	71(1)	41.5/5.3	55.0/7.0
1855		Unidentified		0.63 ± 0.1			37.0/6.2

Chl, chloroplast; cyt, cytoplasm.
[a] proteins identified only by PepNovo/MS BLAST.
[b] Borderline MASCOT hits independently validated by PepNovo/MS BLAST.

The proteins identified were classified into two groups according to their presumed biological function. The first group includes three proteins involved in metabolism: a subunit of ATP synthase (spot 3033), an enolase (spot 1910) and a glutamine synthase (spot 1933). Three proteins are involved in the photosynthesis: two chlorophyll *a/b* binding proteins (spots 2409 and 3603) and a DegP-type protease like (spot 2476). All spots showed quantitative changes: three proteins (spots 2409, 2476, and 1910) were significantly up-regulated while four proteins (spots 3603, 3033, 1855, 1933) were down-regulated with respect to the control. The range of increase/decrease of the proteins is between 0.63 (down-regulation) and 1.8 (up-regulation).

4. Discussion

The PAC presence in the environment could have adverse effects against a range of wildlife species because the PAC molecular targets are evolutionarily well conserved. Recently, Gunnarsson et al. (2008) showed that green algae have orthologs to 35% of the drug targets.

The aim of this work was to analyse if the PAC mixture selected by Pomati et al. (2006) was able to induce negative effects on photosynthetic organisms, as it does for human and zebrafish cell lines. We chose to test a mixture of pharmaceutical rather than the individual chemicals separately because this is the form in which the chemicals are found in nature. In fact, some reports indicate that a classical toxicology approach relying on the study of single substances can under- or over-estimate their real environmental impact (Khetan and Collins, 2007; Silva et al., 2002; Pomati et al., 2008).

The results show that, although traces of almost all the pharmaceuticals investigated were found inside the algal cells, the algal cell proliferation was not reduced at the tested PAC concentrations.

However, the absence of an effect on growth of non-target organisms is not enough to exclude other effects at the cellular or physiological level. To check this, we analysed the effects of the PAC mixture on the genome and total soluble proteome of *P. subcapitata.*

However, AFLP also demonstrated that the used PACs do not have any significant genotoxic potential on the alga used. As a matter of fact, the action of these drugs even on target cells is not directed towards the DNA nucleus. However, it is possible that an indirect DNA damage is exerted through the production of Reactive Oxygen Species (ROS) (Roldan-Arjona and Ariza, 2009). The effects of ROS on DNA are base modification, primarily the formation of 8-oxoguanine, base adducts of carbon-centered radicals, and single- or double-strand breaks in the phospho-sugar backbone of DNA (Labra et al., 2007; Roldan-Arjona and Ariza, 2009). These mutations can easily be detected by molecular analysis such as AFLP. In our case, no mutations were detected by AFLP, suggesting that PACs do not induce alteration in cellular oxidative metabolism resulting in DNA mutations.

Proteomic analysis showed that although only seven proteins were differentially expressed in the presence of PACs, five of them were localized in the chloroplast, and three of these are directly involved in photosynthetic apparatus, suggesting that the chloroplast is the main target of the pharmaceuticals used.

The alteration in the two chlorophyll a/b binding proteins suggests an adaptation of the Light-Harvesting Complex (LHC) in order to avoid damage to the photosynthetic apparatus, as also confirmed by the decrease of the Chl *a/b* ratio, detected by spectrophotometry. The DegP-type protease showed high homology with DegP1 of *Arabidopsis*. This protein is tightly associated on the luminal side of the thylakoid membrane. Recent works have suggested that DegP1 is involved in PSII repair both through the degradation of D1 protein and assisting the assembly of the PSII complex (Kapri-Pardes et al., 2007; Sun et al., 2010; Nixon et al., 2010). The accumulation of DegP1 could be indicative of photo-oxidative damage caused by PAC exposure.

The lower Chl *a/b* ratio in the treated algal cells suggests that the treatment does not affect the synthesis of the core complexes, but the synthesis of the external antenna due to the fact that core complexes contain only Chl *a* (Jennings et al., 1996). In the future, the Clarke type O_2-based photosynthesis ability and/or chlorophyll a fluorescence (Fv/Fm, Fv′/Fm′, NPQ) would be determined for the confirmation of photosynthesis acclimation to PAC challenges Also the observed inhibition of the ATP synthase beta-subunit (spot 3033) could be related to a change in photosynthesis in the treated cells.

In PAC-treated algae, we also found a lower amount of the Glutamine Synthetase (GS) chloroplast precursor (spot 1933). GS catalyses the synthesis of glutamine, an important precursor for amino acid biosynthetic pathways. Palatnik et al. (1999) showed that chloroplastic GS is particularly prone to degradation under conditions of oxidative stress. The pharmaceutical impact on chloroplast could be explained by the presence of a number of targets for antibiotics due largely to the bacterial ancestry of the plastid organelles. In particular, homologous targets for fluoroquinolones (chloroplast replication) and lincosamides (transcription and translation), have been identified in photosynthetic organisms (Brain et al., 2009).

In conclusion, our proteomic data showed that the chloroplast is the main target of the PAC mix used. However, the PAC concentration tested was within the homeostatic range of the algae, as no

lethal effect was observed. Moreover, a PAC concentration 10 fold higher than the environmental one induced only minor changes in *P.subcapitata* proteome indicating that the investigated mixture of drugs could be not so dangerous in an ecological context.

It should be noted that algae were grown under optimal growing conditions for light intensity, temperature, and other factors. In nature, algae often grow under suboptimal conditions that affect their physiology and may make them more sensitive to external agents, producing additive or synergistic effects. Further analyses are therefore required to explore the real exposure risks of algal cells in contaminated aquatic environments.

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