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Characterization of multiple biomarker responses using flow cytometry to improve environmental hazard assessment with the green microalgae *Raphidocelis subcapitata*

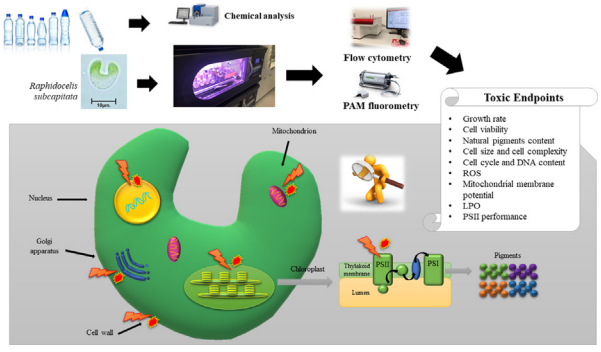
Ana Catarina Almeida [a,*], Tânia Gomes [a], Mirna Habuda-Stanić [b], Jose Antonio Baz Lomba [a], Željka Romić [c], Jelena Vešligaj Turkalj [c], Adam Lillicrap [a]

[a] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, Oslo 0349, Norway
[b] Josip Juraj Strossmayer University of Osijek, Faculty of Food Technology Osijek, Franje Kuhača 20, 31000 Osijek, Croatia
[c] Vodovod-Osijek d.o.o., Poljski put 1, 31000 Osijek, Croatia

HIGHLIGHTS

- Sub-lethal toxicity of bottled waters to *Raphidocelis subcapitata*
- Reactive oxygen species formation, lipid peroxidation, effects in pigments, cell size, complexity and cell cycle observed
- Ca, Na, Mg, and NH_4 primarily responsible for the observed effects
- The methodological approach effectively revealed the observed mechanistic effects.

GRAPHICAL ABSTRACT



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ABSTRACT

Microalgal toxicity tests using integrative endpoints as algal growth are regularly required to analyse the toxicity of potentially hazardous substances in the aquatic environment. However, these do not provide mechanistic information on the toxic mode of action by which contaminants may affect algae. Bottled waters can be used as a substitute for culturing media and should not impose any stress to the cultured organisms. However, certain chemical components can interfere with specific cell targets which are not revealed by general toxicity assays. The present study investigated the sensitivity of flow cytometry (FCM) to analyse sub-lethal effects of different bottled waters to the freshwater microalgae *Raphidocelis subcapitata*. Several endpoints were analysed including growth rate, natural pigments content, cell size, complexity, viability and cycle, Reactive Oxygen Species (ROS) formation, mitochondrial membrane potential and Lipid Peroxidation (LPO). Additionally, photosystem II (PSII) performance was analysed by PAM fluorometry, to provide further information on the absorption, distribution and use of energy in photosynthesis. Results indicated that the most sensitive endpoints were the oxidative stress related endpoints ROS formation and LPO, pigment content, morphological endpoints as cell size, complexity and cycle, with growth rate being one of the least sensitive. Although being essential macronutrients for algal growth, the chemical elements Ca, Na, Mg, and NH_4 were identified as being primarily responsible for the observed toxicological effects to exposed algae. The applied methodology proved to be of high throughput, simultaneously assembling information on morphological, biochemical, and physiological status of algal cells. FCM also

* Corresponding author at: Norwegian Institute for Water Research (NIVA), Section of Ecotoxicology and Risk Assessment, Gaustadalléen 21, N-0349 Oslo, Norway.
E-mail address: aal@niva.no (A.C. Almeida).

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showed potential to reveal mechanistic information on the toxic mode of action of the bottled waters before any effects on algal growth was observed. The used approach demonstrated its potential for being integrated into future microalgal toxicity bioassays for testing chemicals to improve the hazard information obtained from currently approved internationally accepted test guidelines.

1. Introduction

Microalgae are one of the required model organisms to investigate the effects of contaminants in aquatic ecosystems. Since they are at the base of the aquatic food chain, changes in these organisms may disturb the productivity of an entire ecosystem (Martinez et al., 2014). Being in direct contact with the aquatic environment, only separated by a cell wall, and with short generation times, these organisms respond rapidly to environmental changes (Debenest et al., 2010; Franqueira et al., 2000; Prado et al., 2009).

Raphidocelis subcapitata is one of the species recommended for ecotoxicological bioassays by the Organization for Economic Cooperation and Development (OECD, 2011). This unicellular green alga is often more sensitive to various substances than other species (Rojíčková-Padrtová and Maršálek, 1999), is commonly found in freshwater, has a short generation time with higher growth rates, is easy to culture and its genome has been sequenced (OECD, 2011; Suzuki et al., 2018).

Currently, most toxicity bioassays with microalgae use integrative endpoints such as growth and cell viability. These population-based parameters are ecologically relevant, providing general information on the consequences of the presence of contaminants at the cellular and population level that can potentially affect higher trophic levels. However, these tests do not provide any mechanistic information on the toxic mode of action by which contaminants can disturb biological processes in algal cells. Presently, research has been applying a suite of more specific molecular, biochemical and physiological endpoints that can provide early warning signals for the possible hazardous effects of contaminants to unicellular algal species (Adler et al., 2007; Esperanza et al., 2015; Prado et al., 2009).

Flow cytometry (FCM) can be used as a diagnostic tool to analyse the metabolic status of algal cells. This technique enables rapid data acquisition with multiparametric analyses of individual cells, where cell functions can be quickly determined at conditions close to the *in vivo* state. The physiological status of the algae can be characterized through analyses of single cells with different fluorometric and light-scattering parameters. Some useful parameters can be measured directly including cell size, cell complexity and natural pigments content, being possible to differentiate between live and dead cells. The use of fluorescence markers (or probes) can further improve the detection of modifications in algal metabolic processes. These specific probes enable differentiation of diverse biochemical processes that become fluorescent when a certain process occurs, such as cell viability, cell cycle, formation of reactive oxygen species (ROS), occurrence of lipid peroxidation (LPO), changes in mitochondrial membrane potential and DNA content (Adler et al., 2007; Franklin et al., 2001; Hyka et al., 2013; Jamers et al., 2009; Stauber et al., 2002).

Besides the natural pigments' fluorescence analysed by FCM, another significant endpoint to further complement the effects of contaminants in microalgae is based on alterations in chlorophyll *a* fluorescence. This provides important information on the absorption, distribution and use of energy in photosynthesis, giving an integrated measurement of the efficiency of energy conversion at photosystem II (PSII) reaction centers. One of the most informative endpoints that can be analysed, related to PSII, is the analysis of the PSII performance (Ralph et al., 2007).

Recently, several studies showed the applicability of FCM to characterise the toxic effects of contaminants to microalgae, such as metals and herbicides (Adler et al., 2007; Esperanza et al., 2015; Franklin et al., 2001; Franqueira et al., 2000; Jamers et al., 2009; Ortega-Villasante et al., 2016; Prado et al., 2009). This technique has been shown to be sensitive and allows for high-throughput to detect the mechanisms involved in the toxicity of several contaminants that are known for being highly toxic to microalgae.

According to OECD test guidelines, for example OECD 202 for *Daphnia magna* acute ecotoxicity testing and OECD 211 for *D. magna* reproduction test (OECD, 2004, 2012), natural waters are acceptable media for culturing and use as dilution water purposes. Several microalgae species are recommended as a food source for *D. magna* cultures and testing, with *R. subcapitata* one of the most commonly used (OECD, 2004, 2012). Given that the successful outcome of these tests is dependent on the quality of food source, the use of these waters, with specific chemical composition defined by the type and *characteristics* of their *source* (Medema et al., 2003), should not impose any stress to the cultured organisms and the provided algal food. The use of bottled waters as a substitute for OECD culturing media have become popular due its simplicity, constant availability and quality (*e.g.* Manar et al., 2012), but their chemical components may interfere with certain cell targets not necessarily shown in the present OECD test guidelines. Therefore, the present study aimed to investigate the sensitivity of FCM methods to analyse the possible sub-lethal toxicity of bottled waters to the freshwater microalgae *R. subcapitata*. To compare bottled waters from different sources in Europe, commercially available bottled waters from Norway and Croatia were tested and compared. Exponentially growing microalgae were exposed to the different brands of water in batch cultures over 72 h. The waters were spiked with nutrients specified in ISO 8692 media (ISO 8692, 2012), allowing the full expression of system response to the effects (optimal sensitivity), and making sure that cultures grew under nutrient sufficient conditions. Several endpoints were analysed: growth rate, natural pigments content, cell size, cell complexity, cell viability, cell cycle, formation of ROS, mitochondrial membrane potential, LPO, and PSII performance. Furthermore, the applicability of these endpoints to further improve the understanding on the mechanistic effects of compounds rather than the conventional and integrative endpoints such as algal growth, was evaluated.

2. Material and methods

2.1. Microalgae cultures

The unicellular freshwater microalgae *R. subcapitata* (previously called *Pseudokirchneriella subcapitata* and *Selenastrum capricornutum*, NIVA-CHL 1, Norwegian Institute for Water Research, Oslo, Norway), was used for all tests. Algal cells were cultured in glass flasks with an initial number of 5×10^6 cells/l in ISO 8692 medium (ISO 8692, 2012), prepared at least 24 h before use to allow equilibrium of media components. Culture flasks were incubated for 3 to 4 days in an orbital shaker incubator (Innova 1, 44R, incubator shaker series, New Brunswick Scientific, Eppendorf AG, Germany) at 22 ± 2 °C, with orbital shaking at 90 rpm and under continuous illumination from day light-type fluorescent tubes (60.61–61.48 $\mu mol.s^{-1}.m^{-2}$). All the glassware used for media preparation, algal cultures and exposure experiments were properly washed and autoclaved prior to use to avoid any microbial contamination. Culture samples were regularly checked under the microscope to detect the presence of microbial contamination.

2.2. Water samples collection and chemical analysis

Four brands of noncarbonated bottled waters from Norway and Croatia were randomly selected in stores in Norway and Croatia for this study, namely Voss, Isklar, Imsdal, Olden, Studena, Jana, Kala and Cetina (Table A1, Supplementary Information [SI]).

2.2.1. Chemical analysis on element levels in the bottled waters

Dissolved anions bromide (Br), chloride (Cl), fluoride (F), nitrate (NO_3), nitrite (NO_2), phosphate (PO_4) and sulphate (SO_4) were analysed according to ISO 10304-1 (2009). Dissolved cations lithium (Li), ammonia (NH_3), potassium (K), magnesium (Mg), calcium (Ca) and sodium (Na) were analysed according to ISO 14911 (1998). Both anions and cations were determined by liquid chromatography using a Dionex ICS 3000 modular ion chromatography system comprising a high-pressure pump, eluent generator module filled with KOH cartridge, IonPac AG 19 guard column and IonPac AS 19 analytical column, suppressor and conductivity detector. The concentrations of total iron and manganese were determined by UV–Vis spectrometric method (Lambda 20, Perkin Elmer) according to ISO 6332 (1998). Concentrations of total As (As(tot)) were determined by anodic stripping voltammetry using the scTRACE Gold sensor (Computrace 797 VA, Metrohm). The accuracy of the method was evaluated by samples spiked with known amounts of As, using internal standards and by analysing standard reference materials and blanks. The accuracy of all analytical methods was checked daily using internal standards and by analysing standard reference materials and blanks obtained from Fisher Scientific (ion chromatography), Fluka (UV–Vis spectrometry) and Carlo Erba (anodic stripping voltammetry). All analyses were performed in triplicate and results are given as average values. The analytical methods and detection limits for determined parameters are given in Table A2 in the SI.

2.2.2. High-resolution mass spectrometry analysis of the bottled waters

The analysis of the different bottled water samples was performed in accordance with a pre-established screening method (Baz-Lomba et al., 2016). Briefly, 500 ml of drinking water were first extracted using Waters Oasis HLB (1 g/20 cc cartridges) (Waters Corporation, Milford, MA, USA). The HLB sorbent, with a mixed-mode cation exchange sorbent, offers the possibility to extract a wide range of compounds with different psychochemical characteristics, enabling the simultaneous analysis of the widest range of chemicals in one single extraction. The water samples were loaded, extracted with 10 ml of methanol, evaporated with nitrogen and finally reconstituted in 100 μl of methanol for a preconcentration factor of 5000, before analysis.

The compounds were chromatographically separated on a Waters Acquity UPLC system (Milford, MA, USA) fitted with an Acquity UPLC HSS C18 column (1.8 μm, 2.1 mm × 150 mm) (Waters, Milford, MA, USA). A Xevo G2-S Q-TOF mass spectrometer (Waters, Milford, MA USA) with electrospray ionization (ESI) probe, operated in the positive ion mode, was used for acquisition using MSe, enabling the simultaneous acquisition of precursor and fragment ions from a single analysis. Gradient elution was performed at a constant flow of 0.4 ml min^{-1} using 5 mM ammonium formate, pH 3.0 (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient elution starts with 87% A and then increasing B to 95% in 15 min: Solvent A, held for 0.5 min; 0.5–10 linear rate to 50% B, 10–10.75 linear rate to 95% B, held for 0.5 min; reconditioning with a linear rate to 87% A, 12.50–15 min. The analytical column and the guard column were kept at 50 °C and the sampler manager at 5 °C.

Data processing was performed using UNIFI screening platform (Waters Corporation, Milford MA, USA) and the study consisted of suspect screening of the different drinking water samples against a broad list of 1442 compounds (mainly pharmaceuticals, pesticides and illicit drugs). For suspect screening, UNIFI automatically assesses the candidate values with the fragments, mass defect, isotope pattern and adducts. The suspect screening database (including mass spectra, assigned fragments and retention time) was applied under the same acquisition parameters as above.

2.3. 72 h algal toxicity assays and multiple endpoint analysis

Exponentially growing microalgae were exposed to the different water brands in batch cultures over 72 h. ISO 8692 media was used as the control. Preliminary studies were made comparing the algal growth of algae growing in the analysed bottled waters without further addition of nutrients (data not shown). The obtained results showed that the algae did not have sufficient nutrient concentration to grow, especially after 48 h and 72 h exposure. Therefore, in this study all bottled waters were spiked with the stock solutions from the ISO 8692 media (Table A1). This allowed the full expression of system response to the effects (optimal sensitivity) and ensured that cultures grew under sufficient nutrient conditions. All exposure solutions were sterilized by filtering through a 0.2 μm membrane filter before use.

After 48 h and 72 h exposure, several endpoints were analysed to determine the effects of the different brands of bottled water to the freshwater microalgae *R. subcapitata*: growth inhibition, cell size and complexity, natural pigments content, formation of ROS, mitochondrial membrane potential, LPO, cell viability and DNA content. PSII performance was only analysed after 72 h exposure due to limitation of cell density in the exposure conditions. Six replicates for the control and triplicates for the exposure samples were incubated in 25 ml glass flasks with 15 ml of test volumes. Experiments were performed twice.

2.3.1. Flow cytometry (FCM) analysis

FCM analysis of *R. subcapitata* cells were performed in an Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, USA). The instrument was monitored and maintained, with routine quantification using reference beads, as provided by the manufacturer. Non-algal particles were excluded from all analyses by fluorescence, displaying FL3-A (laser exicator 488 nm, filter >670 nm; an estimation of chlorophyll content) *versus* FL4-A (laser exicator 640 nm, filter 6705/25 nm), and setting an acquisition threshold positioned to the left of the distribution of healthy control cells. This allowed the elimination of false data and improved a clear separation of algal particles, positioned to the top right of the non-algal particles. The acquisition threshold was set on FSC-H so that both increases and decreases in autofluorescence could be observed. All endpoints were measured using this threshold, where algal cells were gated using the chlorophyll *a*utofluorescence of control algal cells analysed in the FL3 channel (488 nm excitation, >670 nm emission). 10,000 gated cells were collected per sample and analysed using BD AccuriTM C6 software version 1.0.264.21. For each sample, the median fluorescence value was acquired and divided by the respective number of events per μl. Data were then expressed as fold induction compared to the control, and the mean ± SEM was used to graphically represent the obtained data.

The 488 nm argon-ion laser was used as excitation source for all the probes assayed. Preliminary studies were performed to optimize all the procedures including concentration of cells, concentration of probes and incubation conditions (data not shown). All probe stock solutions were prepared in dimethyl sulfoxide (DMSO ≥ 99.9% purity; Sigma-Aldrich, United Kingdom) upon arrival, except for PicoGreen that is already supplied in DMSO. Aliquots were then stored at −20 °C until further use. Further dilutions were made in the media to a final concentration of DMSO of <0.01%. The same concentration of DMSO (0.01% v/v) was added to the controls, as preliminary tests confirmed no effects of the solvent on the toxicity of the compounds or algal growth.

2.3.1.1. Absolute cell counting. Algal growth was quantified from measurements of absolute cell counting by FCM as a function of time, recorded at 48 h and 72 h and compared with control values (algal

cultures in ISO 8692 media). Preliminary experiments comparing the flow cytometer counts with direct counts using a multisizer counter (Beckman-Coulter Multisizer 3 Coulter Counter; Miami, FL, USA) were performed (data not shown). The procedure was based on OECD Guideline 201 (OECD, 2011). The average growth rate (μ) for each test concentration was calculated from the initial cell concentration (0 h) and cell concentration at each time point (48 h and 72 h) of the cell count using the equation:

$$\mu_{n-0} = \frac{\ln\,(N_n) - \ln(N_0)}{t_n - t_0} \times 24\left(day^{-1}\right)$$

where μ_{n-0} is the average specific growth rate from time 0 to n, N_n is the cell density at time n and N_0 is the cell density at time 0. The growth rate was expressed as fold induction compared to control.

2.3.1.2. Cell size, complexity and natural pigments content. Cell size and complexity data were collected by displaying cell counts *versus* FSC-A (forward scatter) and SSC-A (side scatter), respectively. Natural pigments content (chlorophyll *a* and *b*, carotenoids, xanthophyll and peridinin) were measured by using natural autofluorescence of algae (Table A3 in the SI; BD Accuri™, 2016).

2.3.1.3. Cell viability. Effects on cell membrane and on esterases activity (*i.e.*, enzymes involved in phospholipid turnover) were determined by the inhibition of fluorescein diacetate (FDA; Invitrogen, ThermoFisher Scientific, Eugene, OR, USA). This is a cell-permeant esterase substrate that can be used as a viability probe to measure both enzymatic activity, which is required to activate its fluorescence, and cell-membrane integrity, which is needed for intracellular retention of the fluorescent product (Jamers et al., 2009). The FDA non-fluorescent, lipophilic molecule is taken up by cells and cleaved by nonspecific esterases inside viable cells, producing fluorescent fluorescein that can then be detected by fluorescence measurements. The excitation and emission wavelengths of fluorescein are 488 nm and 530 nm, respectively. Stock solutions of 12.5 mM were prepared on arrival from the supplier. Subsequently, a working solution (25 μM) was prepared by dilution in the exposed algae (1 ml). Thirty minutes after incubation in the dark at room temperature, fluorescent fluorescein was analysed in the FL1 channel (488 nm excitation, 533/30 emission).

Cell viability index (adapted from Berglund and Eversman, 1988; Gala and Giesy, 1990) was calculated as:

$$\frac{(\textit{FDA fluorescence median}) \times (\textit{number of stained events}/\mu l)}{(\textit{Green autofluorescence median}) \times (\textit{number of stained events}/\mu l)}$$

The percentage of viable and non-viable cells was also calculated from histograms of cell number (count) *versus* FL1-A, according to Gala and Giesy (1990).

2.3.1.4. ROS formation. ROS formation was quantified by the ability of free radicals and other ROS to oxidize the non-fluorescent probe carboxy-H_2DFFDA (Invitrogen, ThermoFisher Scientific, Eugene, OR, USA) to a fluorescent product that can be measured fluorometrically (Almeida et al., 2017, 2019). When entering the cells, cellular esterases hydrolyse the probe to the non-fluorescent H_2DFF, which then reacts with cellular ROS to produce the highly fluorescent DFF (difluorofluorescein; 488 nm excitation, 520 nm emission). Specificity of this probe to H_2O_2, NO, $ONOO^-$, O_2 and OH has been reported (Gunawan et al., 2013). Stock solutions of 2.5 mM of carboxy-H_2DFFDA were prepared in DMSO upon arrival. A working solution was then prepared by diluting the probe (5 μM) in the final exposure solution along with algae (1 ml). Thirty minutes after incubation at room temperature in ambient light, ROS formation was analysed in the FL1 channel (488 nm excitation, 533/30 emission).

2.3.1.5. Mitochondrial membrane potential. The mitochondrial membrane potential was measured using two different complementary probes, DHR 123 (dihydrorhodamine 123; Invitrogen, ThermoFisher Scientific, Eugene, OR, USA) and tetramethylrhodamine, methyl ester, perchlorate (TMRM; Invitrogen, ThermoFisher Scientific, Eugene, OR, USA; Cid et al., 1996; Jamers et al., 2009).

DHR 123 is a non-fluorescent molecule that, by oxidation, produces rhodamine 123, a fluorescent cationic and lipophilic probe (505 nm excitation, 529 nm emission). The probe passively diffuses across most cell membranes where it is oxidized to cationic rhodamine 123 in mitochondria by the presence of ROS. This probe is most often used for measuring $ONOO^-$ (Gunawan et al., 2013). Stock solutions of 2.5 mM of DHR 123 were prepared on arrival. A working solution was prepared by diluting the probe (final concentration of 5 μM) with the exposed algae (1 ml) for staining. Incubation was thirty minutes in the dark at room temperature. Probe fluorescence was analysed in the FL1 channel (488 nm excitation, 533/30 emission).

Tetramethylrhodamine, methyl ester (TMRM) is a cell-permeant probe that accumulates in active mitochondria with intact membrane potentials. If the cells are healthy and have functioning mitochondria, the probe signal is bright. Upon loss of the mitochondrial membrane potential, TMRM accumulation ceases and the signal dims or disappears. TMRM signal can be detected with FCM (535 nm excitation filter, 600 nm emission). Stock solutions of 0.1 mM TMRM were firstly prepared and stored. Working solutions (final concentration of 0.2 μM) were prepared by diluting the probe with exposed algae (1 ml) for staining. Incubation was thirty minutes in the dark at room temperature. Probe fluorescence was analysed in the FL2 channel (488 nm excitation, 585/40 emission).

2.3.1.6. Lipid peroxidation (LPO). Oxyl-radical induced lipid oxidation (LPO) was determined using the lipophilic fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid or C_{11}-BODIPY$^{581/591}$ (Invitrogen, ThermoFisher Scientific, Eugene, OR, USA). This is a fluorescent radio-probe for indexing lipid peroxidation and antioxidant efficacy in model membrane systems and living cells (Almeida et al., 2017, 2019). When excited with blue light at 488 nm wavelength, the molecule emits fluorescence with a maximum at 595 nm. Following oxyl-radical induced oxidation, the fluorescence emission shifts to shorter wavelengths with a maximum emission at 520 nm. Due to its lipophilic properties, the molecules easily enter the lipid bilayer and once inside, the cellular membranes are subject to oxidation by oxyl-radicals together with endogenous fatty acids. Stock solutions of 2.5 mM probe were prepared and stored upon arrival. Working solutions with a final concentration of 5 μM were then prepared by diluting the probe with exposed algae (1 ml) for staining. Incubation was thirty minutes in the dark at room temperature. Probe fluorescence was analysed in the FL1 channel (488 nm excitation, 533/30 emission).

2.3.1.7. DNA content and cell cycle. PicoGreen (Invitrogen, ThermoFisher Scientific, Eugene, OR, USA) was used to quantify the DNA content by measuring the absorbance of double-stranded DNA (dsDNA). PicoGreen enables distinguishing DNA peaks corresponding to cells with different DNA content, where three cell cycle phases can be clearly defined: G1 (Gap phase), S (Synthesis - DNA replication), and G2 phases (growth) (Marie et al., 1996). Histograms of cell number *versus* fluorescence (FL1 channel; 488 nm excitation, 533/30 emission) were used to define these three fluorescence states (G1, S, and G2) relative to the distribution of healthy control cells (Veldhuis et al., 2001).

PicoGreen was supplied as a 1 ml concentrated probe solution in DMSO. On the day of the experiment, an aqueous working solution was prepared by making a 1:200 dilution of the concentrated DMSO solution in 10 mM Tris-HCl, 1 mM EDTA at a pH of 7.5. First, 20 μ1 of 1% Triton X-100 (to facilitate stain penetration and to increase probe fluorescence emission; Marie et al., 1996) was added to 200 μ1 of exposed

algae, vortex mixed, and incubated at room temperature for 10 min in the dark, at room temperature. Thereafter, 220 μl of PicoGreen working solution was added to each sample. The samples were incubated for thirty minutes in the dark at room temperature. Probe fluorescence was analysed in the FL1 channel (488 nm excitation, 533/30 emission).

DNA content in the different phases was calculated as:

$$\frac{(Picogreen\ fluorescence) \div (number\ of\ stained\ events/\mu l)}{\%of\ plot}$$

Being the % of plot indicative of the % of the different phases of the cell cycle.

2.3.2. Photosystem II performance

PSII performance in exposed algae was determined using a pulse amplitude modulated (PAM) fluorometer (Underwater Fluorometer DIVING-PAM, Heinz Walz GmbH, Effeltrich, Germany), according to the method described in Almeida et al. (2017) and adapted to the experimental conditions used in this study. Given that PAM fluorometry is an optical method, the intensity of the measured signal is of great importance, and algal cell density should be high enough to be detected by the PAM fluorometer (Ralph et al., 2007). For this reason, PSII performance was only analysed after 72 h exposure to the bottled waters to ensure sufficient cell density of *R. subcapitata* in the experiments. To up-concentrate *R. subcapitata* cells after the 72 h exposure, 15 ml of the algal suspension was centrifugated at 4200 rpm for 15 min at room temperature. The resulting supernatant was decanted, and the pellet resuspended in 600 μl of ISO media and/or bottled waters. 200 μl of each replicate was transferred to a 96-well black microplate (Corning Incorporated, Costar®, NY, USA) in duplicate for PSII performance analyses. Algal cells were dark acclimated for 30 min to allow complete oxidation of PSII reaction centres, after which the minimum and maximum fluorescent yields of PSII (F_0 and F_m, respectively) were determined and used to calculate the maximum quantum yield (F_v/F_m) and the efficiency of oxygen-evolving complex (OEC) of PSII (Ralph et al., 2007; Kriedemann et al., 1985). Subsequently, dark-acclimated cells were illuminated by an actinic light at an intensity equivalent to the incubation light (~60 $\mu mol.m^{-2}.s^{-1}$) and the current fluorescence yield (F_t), the minimum fluorescence yield in the light (F'_0) and the maximum fluorescence yield in the light (F'_m) values recorded. With these yields, the effective quantum yield (Φ_{PSII}), the rate of electrons flow (ETR), the coefficients of photochemical (qP), the coefficients of non-photochemical quenching (qN), and the non-photochemical quenching (NPQ) were calculated (Genty et al., 1989; Juneau and Popovic, 1999). The relative dissipation of energy through PSII was also investigated by estimating the relative photochemical ($qP_{(rel)}$), the relative non-photochemical quenching $qN_{(rel)}$ and the relative unquenched fluorescence ($UQF_{(rel)}$) (Buschmann, 1995; Juneau et al., 2005). The PSII performance fluorescence parameters were calculated according to the formulas expressed in Table A4 of the SI.

2.4. Statistical analysis

All experimental data obtained from both independent experiments were pooled and expressed as fold induction and presented as mean ± standard error of mean (SEM). XLStat2018® software (Addinsoft, Paris, France) was used for all statistical analyses and GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA) was used for graphical representation. Data was checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test), after which the parametric test one- and/or two-way ANOVA and the non-parametric test Kruskall-Wallis were used to test significant differences between test waters. Multiple comparisons were performed using the Tukey or the Dunn's test. A Redundancy Analysis (RDA) was used to distinguish among the tested bottled waters, which chemical components explained as much variance as possible in the determined toxicological endpoints. A Pearson correlation analysis was performed to measure the strength of association between all variables. A p value <0.05 was considered as statistically significant.

3. Results

3.1. Chemical analysis on element levels in the bottled waters

The chemical analysis of the different elements present in the bottled waters is shown in Table I. There was a clear difference between the Norwegian and Croatian bottled waters, notably in the concentration ranges of Ca (Norwegian: 1.658–9.873 mg/l; Croatian: 61.799–86.31 mg/l) and Mg (Norwegian: 0.162–0.497 mg/l; Croatian: 2.957–32.06 mg/l), which indicates that the Norwegian bottled waters can be classed as soft, and the Croatian bottled waters as moderately hard/hard. Concentrations of Na also varied significantly. In the Norwegian bottled waters, Na ranged from 0.927 to 1.793 mg/l, whilst the Croatian bottled waters ranged from 1.87 to 11.10 mg/l. NH_4 was detected in one Norwegian bottled water (0.001 mg/l), whilst it ranged between 0.030 and 0.430 mg/l in all the Croatian bottled waters. Differences were also noted in the concentration ranges of Cl (Norwegian: 0.280–1.961 mg/l; Croatian: 1.526–9.817 mg/l) and NO_3 (Norwegian: <D.L. - 0.276 mg/l; Croatian: 0.128–4.333 mg/l). Mn was detected only in one Norwegian bottled water (0.018 mg/l), whereas it was present in all the Croatian bottled waters (0.008–0.017 mg/l).

3.2. Results from the chemical suspect screening of the bottled waters

Bottled water samples were analysed in triplicate and processed using the UNIFI screening platform. The 24 chromatograms were matched against the scientific database following these criteria: mass accuracy of the parent ion (H^+) below 3 mDa, chromatographic retention time accuracy of ±0.5 min, at least two fragments in the high energy channel and a minimal detector response of 200 counts. The qualitative analysis of these samples against the 1442 chemicals present in the library (Table A5 in SI) did not find any positive match and therefore no pharmaceuticals, pesticides or drugs were present in any of the bottled waters analysed.

3.3. Results from the algal toxicity assays

The fluorescence data and the number of events per μl obtained for each measured parameter in the FC is presented in Table A6 in SI.

Table I
Analytical results of different elements in Norwegian and Croatian bottled waters (LoD – detection of limit).

Nutrient (unit)	A	B	C	D	E	F	G	H
F (mg/l)	0.024	0.026	0.130	0.050	0.022	0.027	0.049	0.166
NO_2 (mg/l)	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD
NO_3 (mg/l)	<LoD	0.276	1.464	0.127	3.086	1.048	4.333	0.128
Br (μg/l)	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD
SO_4 (mg/l)	2.896	7.212	5.975	0.647	3.197	5.286	10.88	1.221
PO_4 (μg/l)	<LoD	36.322	<LoD	30.696	<LoD	<LoD	<LoD	<LoD
Cl (mg/l)	0.280	1.961	1.266	1.535	2.927	1.526	9.817	3.095
K (mg/l)	0.727	4.214	0.873	0.516	0.575	0.945	1.795	1.413
Li (mg/l)	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD
Mg (mg/l)	0.427	0.497	0.334	0.162	2.957	32.06	19.29	27.14
Ca (mg/l)	9.873	7.944	4.570	1.658	78.270	61.799	86.310	78.920
Na (mg/l)	0.927	1.271	1.793	1.329	1.870	1.920	8.092	11.100
Mn (mg/l)	<LoD	<LoD	<LoD	0.018	0.017	0.011	0.008	0.011
NH_4 (mg/l)	0.001	<LoD	<LoD	<LoD	0.030	0.184	0.073	0.430
Fe (μg/l)	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD
As (μg/l)	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD	<LoD

3.3.1. Growth rate and pigments content

Cell counts (mean ± SEM), volume of sample read (mean ± SEM; μl), cells per ml (mean ± SEM) and growth rate (mean ± SEM; d^{-1}) obtained for each bottled water and control at 48 h and 72 h exposure are presented in Table A7 in the SI. The data obtained for the algal growth (Fig. 1) showed a clear distinction between the Norwegian and Croatian bottled waters. After 48 h and 72 h exposure, the Croatian waters (from E to H, Fig. 1) affected the microalgae growth, presenting a growth rate (μ) significantly lower than the control at both timepoints. The lowest growth rate was obtained for the microalgae grown in the Croatian water H at both 48 h and 72 h exposure, with a 1.7-fold decrease compared to control. No significant differences existed between the control cultures and those from the Norwegian bottled waters (from A to D, Fig. 1). Microalgae grown in the Norwegian waters C and D presented the highest growth rate, but not significantly higher than the control. No significant differences were obtained between the 48 h and 72 h exposures for each tested water.

Regarding the pigments content in *R. subcapitata*, a difference between the Norwegian and Croatian bottled waters was again observed. The Croatian water H showed generally the highest increase in the analysed pigments, especially after the 72 h exposure. Significant differences were obtained between 48 h and 72 h exposures for the Croatian waters G and H, with a decrease in pigments content for the first, and an increase for the last. For the Norwegian bottled waters (from A to D, Fig. 1), no significant differences were observed compared to the control. Interestingly, a clear inverse correlation between pigments content and growth rate was observed.

3.3.2. Cell size, complexity and viability

For cell size and cell complexity endpoints (Fig. 2), only significant differences from the control were observed for the Croatian bottled waters with the highest values obtained for microalgae exposed to water H at 72 h exposure (7.1 and 6.7-fold increase, respectively). The Croatian waters G and H showed significant differences between 48 h and 72 h exposures, with a decrease in both cell features for water G (from 3.5 to 2.5-fold for cell size and 4.0 to 2.4-fold for cell complexity) and an increase for H (from 4.8 to 7.1-fold for cell size and 5.0 to 6.7-fold for cell complexity).

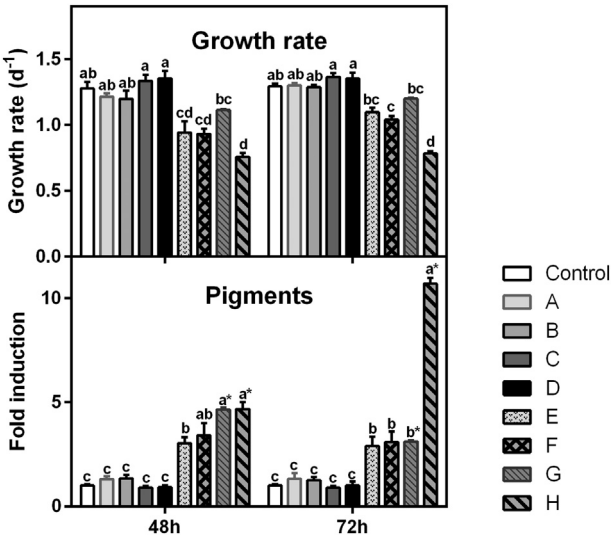

Fig. 1. Growth rate (d^{-1}) and pigments content in *Raphidocelis subcapitata* exposed to the different bottled waters. The data (mean ± SEM) represent 2 independent studies. Similar letters indicate a lack of statistical significant differences between bottled waters within the same time point (48 h and 72 h; $p > 0.05$). Different letters indicate significant differences between bottled waters within the same time point (48 h and 72 h; $p < 0.05$). Significant differences between 48 h and 72 h for each bottled water are indicated with * ($p < 0.05$).

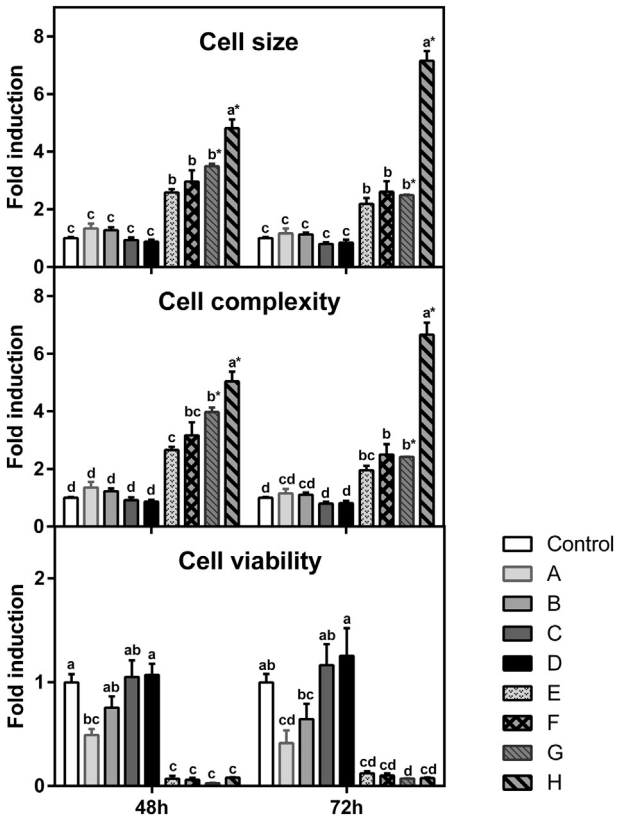

Fig. 2. Cell size, cell complexity and cell viability of *Raphidocelis subcapitata* exposed to the different bottled waters. The data (mean ± SEM) represent 2 independent studies. Similar letters indicate a lack of statistical significant differences between bottled waters within the same time point (48 h and 72 h; $p > 0.05$). Different letters indicate significant differences between bottled waters within the same time point (48 h and 72 h; $p < 0.05$). Significant differences between 48 h and 72 h for each bottled water are indicated with * ($p < 0.05$).

Cell viability index (mean ± SEM) and percentage of viable and non-viable cells obtained for algae cells exposed to each bottled water and control at 48 h and 72 h exposure is presented in Table A8 in SI. For the cell viability index (Fig. 2), significant differences from the control were observed not only for the Croatian waters but also for some of the Norwegian waters. At both 48 h and 72 h, cell viability for microalgae growing in the Norwegian water A was significantly lower than the control (2.0 to 2.4-fold decrease, respectively). However, the highest differences were again observed for the Croatian waters, where all resulted in a significant decrease in cell viability compared to control, with the lowest value obtained for Croatian water G at 48 h exposure (37.5-fold decrease compared to control). The highest cell viability was observed for microalgae in the Norwegian water D at 72 h, although this was not significantly different from the control. No significant differences were obtained between 48 h and 72 h exposures for each tested water. Regarding the percentage of non-viable cells (Table A8 in SI), for control cells at 48 h and 72 h exposure, values were between 5% and 6%, almost in the same order of magnitude as that for the algae exposed to the Norwegian bottled waters (4–13%). For the algae exposed to the Croatian bottled waters the values were significantly higher, with a percentage of non-viable cells between 40% and 73%. Both measures of cell viability show a similar trend between the control and analysed bottled waters, even though in opposite directions.

3.3.3. ROS, LPO and mitochondrial membrane potential

For all the endpoints related to ROS formation, LPO and mitochondrial membrane potential (DHR 123 and TMRM; Fig. 3), significant differences from the controls were only observed for microalgae exposed

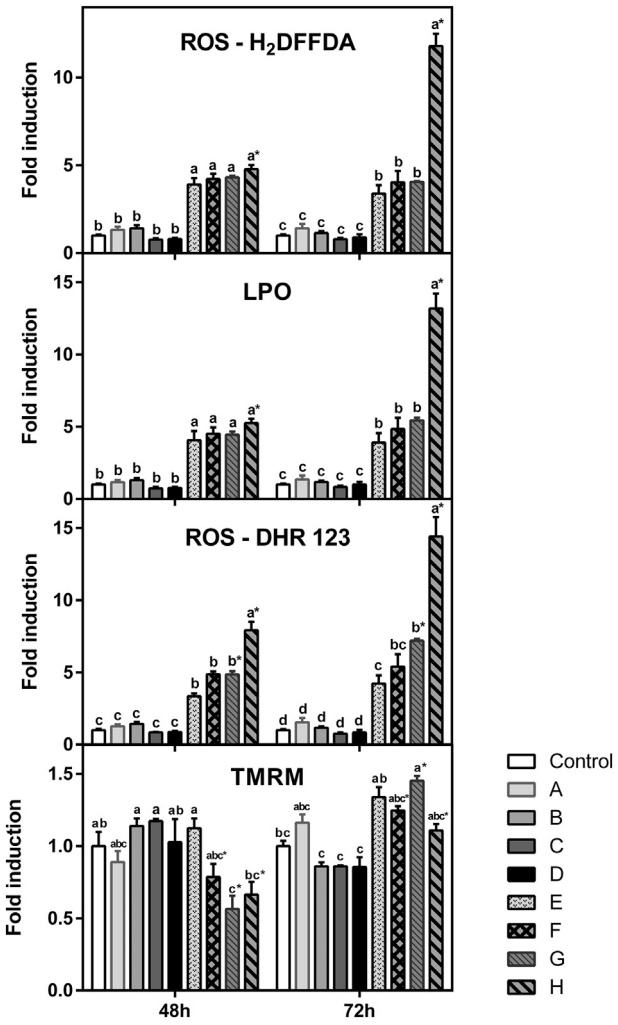

Fig. 3. Reactive oxygen species (ROS) formation, lipid peroxidation (LPO) and mitochondrial membrane potential indicated by DHR 123 (dihydrorhodamine 123; indicative of ROS formation in mitochondria) and TMRM (tetramethylrhodamine) in *Raphidocelis subcapitata* exposed to the different bottled waters. The data (mean ± SEM) represent 2 independent studies. Similar letters indicate a lack of statistical significant differences between bottled waters within the same time point (48 h and 72 h; $p > 0.05$). Different letters indicate significant differences between bottled waters within the same time point (48 h and 72 h; $p < 0.05$). Significant differences between 48 h and 72 h for each bottled water are indicated with * ($p < 0.05$).

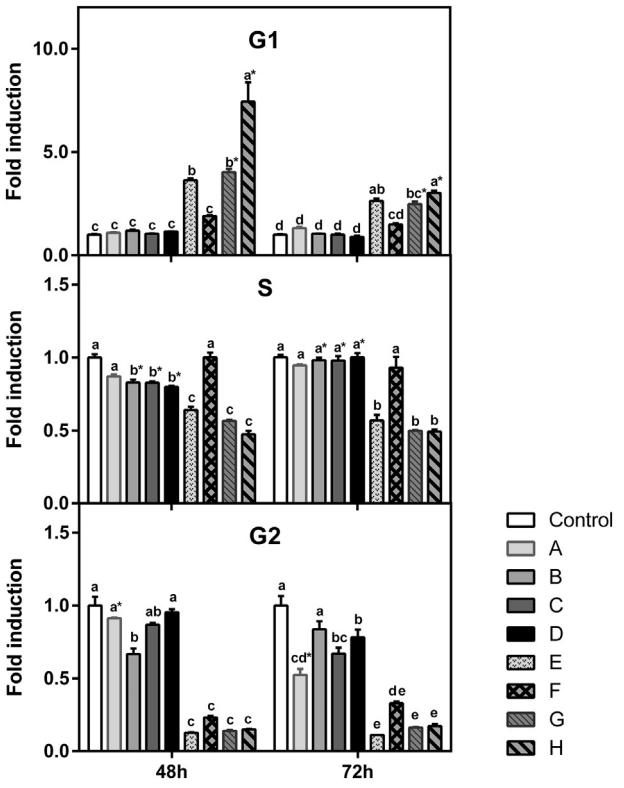

Fig. 4. DNA content indicated by PicoGreen in *Raphidocelis subcapitata* exposed to the different bottled waters. DNA content was then used to differentiate three cell cycle phases, G1 (Gap phase), S (Synthesis - DNA replication), and G2 phases (growth). The data (mean ± SEM) represent 2 independent studies. Similar letters indicate a lack of statistical significant differences between bottled waters within the same time point (48 h and 72 h; $p > 0.05$). Different letters indicate significant differences between bottled waters within the same time point (48 h and 72 h; $p < 0.05$). Significant differences between 48 h and 72 h for each bottled water are indicated with * ($p < 0.05$).

to the Croatian bottled waters. ROS was highest for Croatian water H at both 48 h (4.8-fold increase compared to control) and 72 h (11.8-fold increase). No significant differences were observed between the control and the Norwegian waters at both time-points. At 72 h exposure, Croatian water H induced a significant increase in ROS formation when compared to the 48 h data. A similar pattern was observed for LPO, with the highest increase for Croatian water H at both 48 h (5.3-fold) and 72 h (13.2-fold). For the ROS formation using DHR 123, the highest increase was again observed for microalgae exposed to the Croatian water H at 48 h (7.9-fold) and 72 h (14.4-fold). For TMRM, at 48 h exposure a significant decrease from the control was observed for Croatian water G (1.8-fold) and a decrease for water H (1.3-fold), but not significantly different from control. At 72 h only, a significant increase from the control was observed for water G (1.5-fold increment).

3.3.4. DNA content and cell cycle

The three cell cycle phases, G1, S and G2 were differentiated based on the DNA content (Fig. 4). The % of plot (mean ± SEM), indicative of the % of the different phases of the cell cycle (G1, S and G2) obtained for each bottled water and control at 48 h and 72 h exposure are presented in Table A9 in the SI. For the G1 phase, only the microalgae exposed to the Croatian waters showed significant differences from the control. At 48 h exposure, microalgae exposed to the Croatian water H showed the highest DNA content, with a 7.4-fold increase from the control. Croatian waters E and G were also significantly higher than the control, with a 3.6 and 4.0-fold increase, respectively. No significant differences existed between the control and the Norwegian waters, along with the Croatian water F. At 72 h exposure, the same waters E, G and H were still significantly higher than the control but with lower fold increments (2.6, 2.5 and 3.0-fold, respectively). When comparing the 48 h and 72 h exposures for this phase, a significant decrease in DNA content was observed for microalgae exposed to the Croatian waters G and H.

At the S phase, for both 48 h and 72 h exposures, Croatian waters E (1.6 and 1.8-fold decrease from control, respectively), G (1.8 and 2.0-fold decrease from control, respectively) and H (2.1 and 2.0-fold decrease from control, respectively) presented the lowest DNA content. Norwegian waters B, C and D at 48 h were also significantly lower ($p < 0.05$) than the control but to a lesser extent (1.2 to 1.3-fold decrease). At 72 h exposure, DNA content of microalgae exposed to the Norwegian waters B, C and D significantly increased to levels similar to the control when compared to the 48 h data.

For the last identified phase G2, a clear distinction between Norwegian and Croatian waters was again observed, with the latter presenting significantly lower DNA content. At 48 h exposure, the lowest values were obtained for the Croatian water E (7.9-fold decrease from control), followed by waters G (7.2-fold), H (6.7-fold) and F (4.3-fold). Norwegian water B was also significantly lower than the control (1.5-fold decrease from control). After 72 h exposure, significant differences were

observed for the Norwegian waters, with A (1.9-fold), C (1.5-fold) and D (1.3-fold) presenting significantly lower DNA content than the control. All Croatian waters were significantly lower than the control, from 3.0 to 9.1-fold for water F and E, respectively.

3.3.5. PSII performance

Results obtained for PSII performance showed a clear distinction between the bottled waters (Figs. 5 to 7). No significant differences were found between the control and the Norwegian waters in terms of F_v/F_m (Fig. 5). On the other hand, F_v/F_m decreased in waters E, G and H, to a relatively low extent (up to 1.3-fold for water E). The efficiency of the oxygen-evolving complex (OEC) increased slightly for waters A and B when compared to the control (1.1-fold). A higher increase was detected for waters E, G and H, with a maximum of 2.0-fold increase

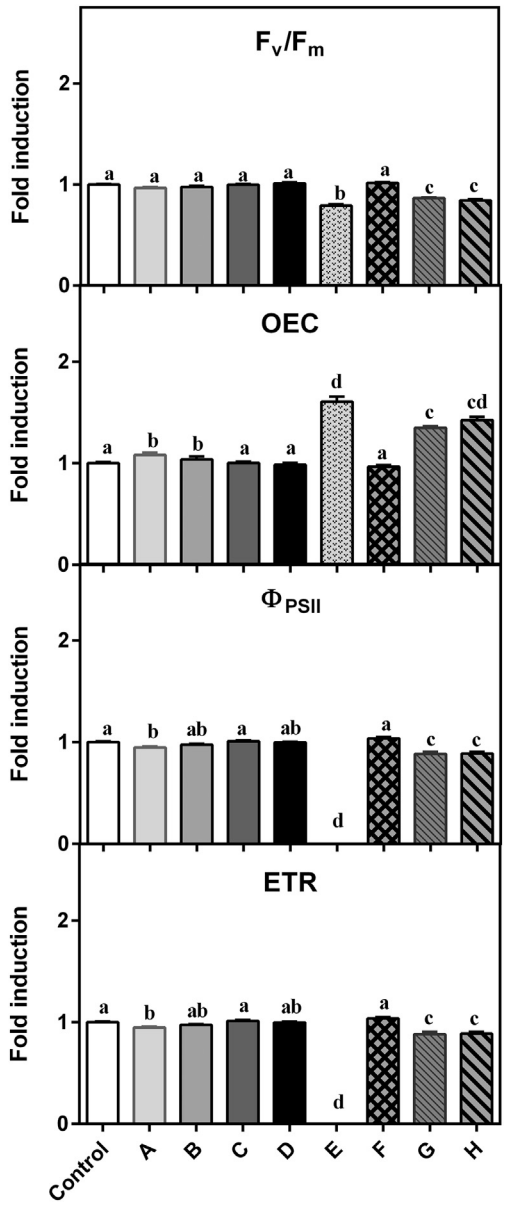

Fig. 5. Fluorescent parameters of *Rhaphidocelis subcapitata* in the dark- acclimated and light-acclimated states exposed to the different bottled waters for 72 h. F_v/F_m — Maximum quantum yield, OEC — Efficiency of oxygen-evolving complex, Φ_{PSII} – Quantum efficiency of PSII photochemistry, ETR – Electron transfer rate. The data (mean ± SEM) represent 2 independent studies. Similar letters indicate a lack of statistical significant differences between bottled waters within the same time point (48 h and 72 h; $p > 0.05$). Different letters indicate significant differences between bottled waters within the same time point (48 h and 72 h; $p < 0.05$).

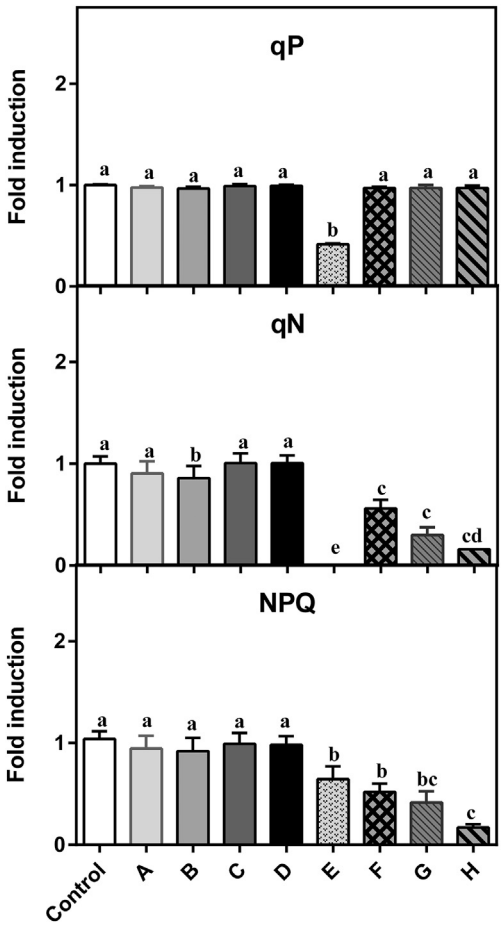

Fig. 6. Coefficient of photochemical quenching (qP), coefficient of non-photochemical quenching (qN) and non-photochemical quenching (NPQ) of PSII in *Rhaphidocelis subcapitata* exposed to the different bottled waters for 72 h. The data (mean ± SEM) represent 2 independent studies. Similar letters indicate a lack of statistical significant differences between bottled waters within the same time point (48 h and 72 h; $p > 0.05$). Different letters indicate significant differences between bottled waters within the same time point (48 h and 72 h; $p < 0.05$).

for water E compared to the control. In terms of quantum efficiency of PSII photochemistry (Φ_{PSII}), water A caused a small decrease compared to control (1.1-fold), followed by a 1.2-fold decrease for waters G and H. A complete inhibition of ΦPSII was recorded for water E. A similar effect was seen for PSII electron transport (ETR), with decreases in waters A, G and H and a complete inhibition for water E.

No significant differences were found for photochemical quenching (qP) between the control and water brands, except for water E, where a 2.4-fold decrease was observed (Fig. 6). The coefficient of non-photochemical quenching (qN) was mostly affected after exposure to Croatian waters, where a 1.8, 3.3 and 6.3-fold decrease was detected for waters F, G and H, respectively. A total inhibition of this process was seen after exposure to water E. This decrease in qN was accompanied by a similar decrease in non-photochemical quenching (NPQ), being the highest decrease observed for water H (6.0-fold compared to control).

The relative distribution of the energy dissipation processes through the PSII (Fig. 7) also showed different patterns between the Norwegian and Croatian bottled waters. For the Norwegian waters, only exposure to water B caused an alteration in these processes, where a small increase was recorded for the relative photochemical quenching ($qP_{(rel)}$) and the relative unquenched fluorescence ($UQF_{(rel)}$), plus a small decrease for the relative non-photochemical quenching ($qN_{(rel)}$) (1.1-, 1.4- and 1.2-fold, respectively). For the Croatian waters, water E showed a distinct response compared to the other brands, with the highest

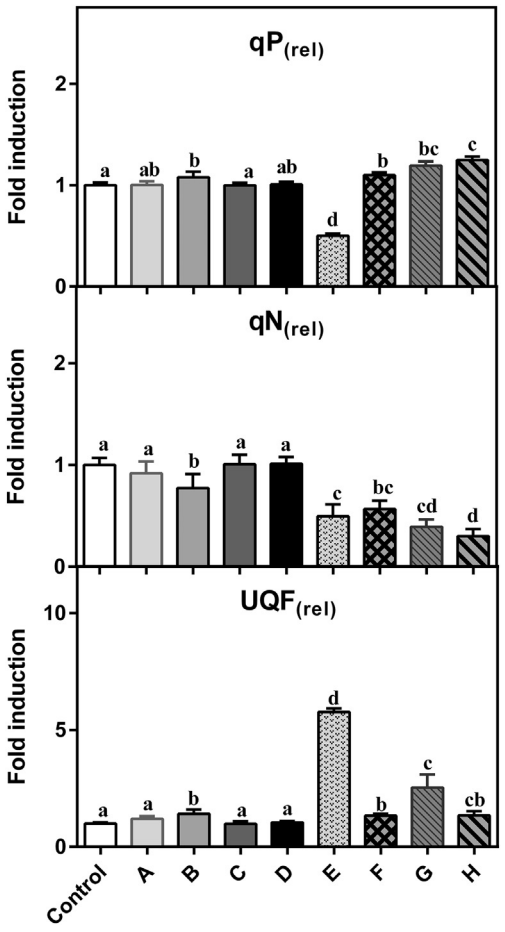

Fig. 7. Relative distribution of dissipation energy processes through the PSII of *Rhaphidocelis subcapitata* exposed to the different bottled waters for 72 h. $qP_{(rel)}$ – Relative photochemical quenching, $qN_{(rel)}$ – Relative non-photochemical quenching, $UQF_{(rel)}$ – Relative unquenched fluorescence. The data (mean ± SEM) represent 2 independent studies. Similar letters indicate a lack of statistical significant differences between bottled waters within the same time point (48 h and 72 h; $p > 0.05$). Different letters indicate significant differences between bottled waters within the same time point (48 h and 72 h; $p < 0.05$).

decrease (2.0-fold) and increase (5.8-fold) in $qP_{(rel)}$ and ($UQF_{(rel)}$), respectively. For the remaining Croatian waters, a significant increase was detected for $qP_{(rel)}$ (1.2-fold for water H) and $UQF_{(rel)}$ (2.5-fold for water G) in comparison to the control, followed by a decrease in $qN_{(rel)}$ (up to 3.3-fold for water H).

3.4. Relation between endpoints

The relationship between the biological endpoints and the chemical characterization of the bottled waters was evaluated using RDA (Fig. 8) for the 48 h and 72 h exposures (Tables A10 and A11, SI). For the 48 h exposure, the two axes accounted for 98.21% of total data variance (Fig. 8A). F1, explaining 94.18% data (eigenvalue 25.62), showed a clear discrimination between the Norwegian and Croatian bottled waters, with the control clearly associated with the responses obtained for the Norwegian waters. The Norwegian bottled waters (C, D, A and B) and control were closely related with cell viability, cell cycle phase G2, growth, mitochondrial membrane potential (measured by TMRM), and cell cycle phase S. Conversely, the Croatian bottled waters (H, G, F and E) were associated with DHR 123 (ROS formation in mitochondria), cell cycle phase G1, LPO, ROS (carboxy-H_2DFFDA), chlorophylls, cell complexity and size. Associated with these endpoints were the chemical elements Ca, Na, NH_4 and Mg. For the 72 h exposure a similar pattern was obtained, with the two axes accounting for 98.87% (Fig. 8B). F1 accounted for 95.06% of the variance of data (eigenvalue 73.47), again associating the Norwegian bottled waters with the control and separated from the Croatian bottled waters. The Norwegian bottled waters (C, D, B and A) and control were closely related with cell viability, PSII efficiency parameters qN, NPQ and $qN_{(rel)}$, cell cycle phases G2 and S, and growth. Conversely, the Croatian bottled waters (H, G, F and E) were linked to ROS (DHR 123 and carboxy-H_2DFFDA), LPO, pigments content, and cell size and complexity. These last bottled waters were also mainly associated with the chemical elements NH_4, Na, Mg and *Ca.*

A correlation analysis between biological endpoints and chemical characterization was also performed, in which the r^2 and *p*-values obtained are shown in Tables A12–A15 in the SI. For the 48 h data, positive correlations were obtained between cell viability, cell cycle phase G2, growth and mitochondrial membrane potential (measured by TMRM). These endpoints were negatively correlated with pigments content, ROS (carboxy-H_2DFFDA and DHR 123), LPO, cell cycle phase G1, cell size and complexity. Regarding the chemical elements, Ca, Mg, Na and NH_4 were positively correlated with the toxicological endpoints ROS (carboxy-H_2DFFDA and DHR 123), LPO, pigments content, cell size and complexity, while cell cycle phase G1 was positively correlated with Na, NH_4 and *Ca.* For the 72 h data, positive correlations were obtained between cell viability, qN and $qN_{(rel)}$, NPQ, cell cycle phases S and G2 and growth. qN was also positively correlated with the other PSII parameters F_v/F_m, Φ_{PSII} and ETR. Cell viability was negatively correlated with LPO, ROS (indicated by DHR 123), cell cycle G1 and mitochondrial membrane potential (measured by TMRM), while qN, NPQ and $qN_{(rel)}$ showed a negative correlation not only with these endpoints but also with the pigments content, ROS, cell size and complexity. The chemical elements NH_4, Na, Mg and Ca were positively correlated with the pigments content, ROS (carboxy-H_2DFFDA and DHR 123), LPO and cell size and complexity, while cell cycle phase G1 was positively correlated with Ca, Na and NH_4.

4. Discussion

In the present study, the growth rate of *R. subcapitata* exposed to different bottled waters was assessed and clear differences between Norwegian and Croatian waters were observed. While no differences existed between growth rates of the control and the microalgae exposed to the Norwegian waters, Croatian waters elicited a significant decrease in growth after 48 h and 72 h exposure. Nonetheless, by only analysing growth as an endpoint, no information could be obtained regarding the physiological processes affecting the microalgae. Moreover, by being a population-based endpoint, growth rate does not give information on the response of individual cells, which can lead to a less sensitive response as seen in the present and other studies comparing growth rate with other toxicological endpoints (*e.g.* Esperanza et al., 2015; Zhou et al., 2009).

FCM can be used to evaluate the toxicological effects of contaminants in individual cells under near *in vivo* conditions (Esperanza et al., 2015). However, the potential of this technique as a tool in microalgal toxicity bioassays has not yet been fully explored. Herein, not only growth rate but several other endpoints were analysed using FCM, namely natural pigments content, cell size, complexity and viability, ROS formation, LPO, mitochondrial membrane potential, and cell cycle. Due to the importance of photosynthesis in microalgae, PSII performance was also analysed.

For pigments content, clear differences between the Norwegian and Croatian waters were observed. While the microalgae exposed to the Norwegian waters did not show any differences from the controls, algae exposed to the Croatian waters showed a significant increase in pigments content. A clear inverse relationship between this endpoint and growth rate was observed. An increase in pigments has previously been observed in microalgae exposed to metals (Jamers et al., 2009). An increase in chlorophyll *a* fluorescence is known to be due to the inhibition of electron flow in the PSII reaction center (Franqueira et al.,

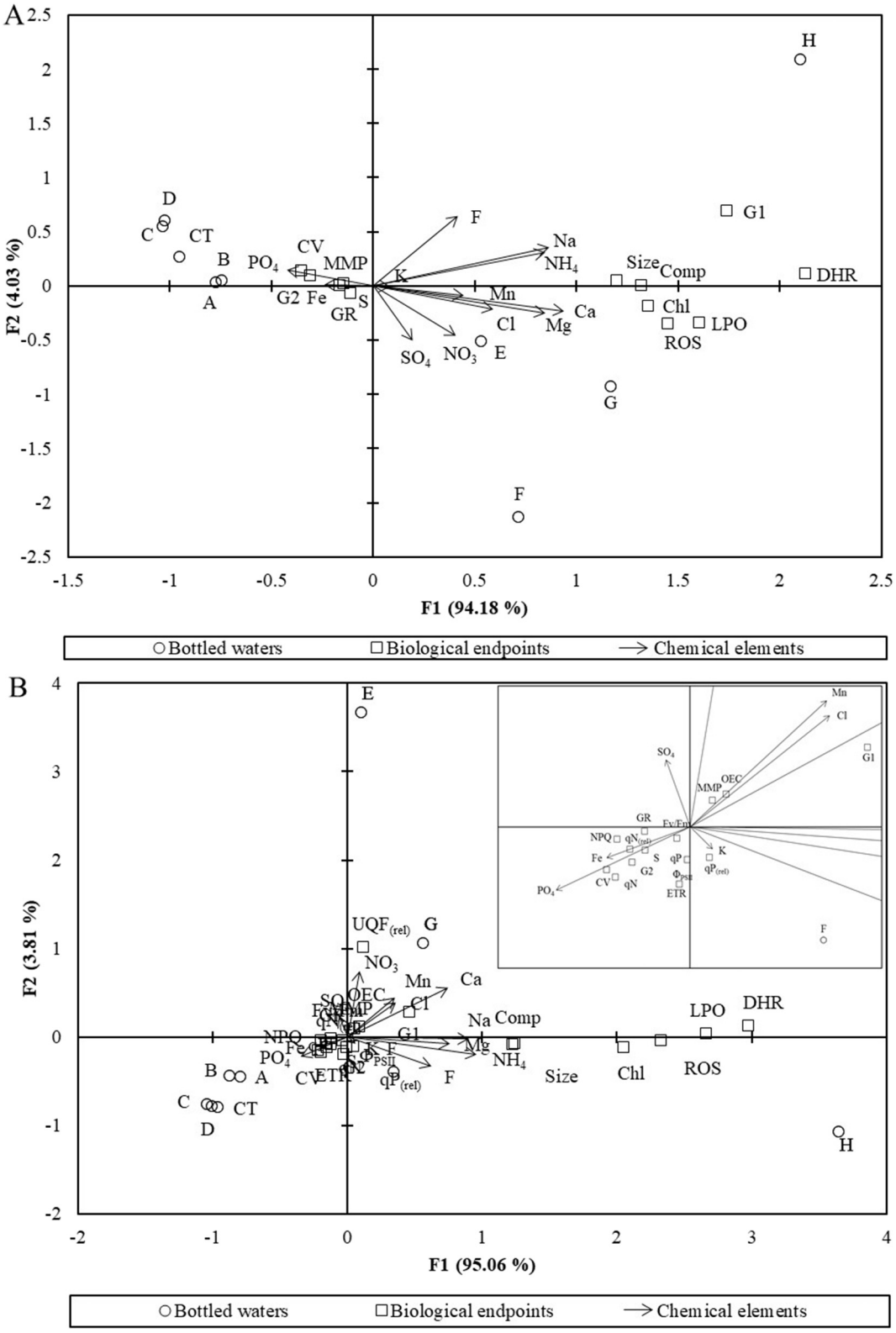

Fig. 8. Redundancy analysis (RDA) in algae *Rhaphidocelis subcapitata* exposed to the different bottled waters for 48 h (A) and 72 h (B). GR – Growth rate, Chl – Chlorophyll a and b, carotenoids, xanthophyll, peridinin, ROS – Reactive oxygen species detected by carboxy-H_2DFFDA probe, DHR - Reactive oxygen species detected by DHR 123 probe, LPO – Lipid peroxidation, CV – Cell viability, G1 – Gap phase in the cell cycle; S – Synthesis - DNA replication phase in the cell cycle, G2 – Growth phase in the cell cycle, Size – Cell size, Comp – Cell complexity, MMP – Mitochondrial membrane potential, F_v/F_m – Maximum quantum yield, OEC – Efficiency of the oxygen-evolving complex, Φ_{PSII} – Quantum efficiency of PSII photochemistry, ETR – Electron transfer rate, qP – Coefficient of photochemical quenching, qN – Coefficient of non-photochemical quenching, NPQ – Non-photochemical quenching, $qP_{(rel)}$ – Relative photochemical quenching, $qN_{(rel)}$ – Relative non-photochemical quenching, $UQF_{(rel)}$ – Relative unquenched fluorescence. Fluoride – F, Nitrate - NO_3, Sulphate – Sulphate, Phosphate – PO_4, Chloride – Cl, Potassium – K, Magnesium – Mg, Calcium – Ca, Sodium – Na, Manganese – Mn, Ammonia – NH_4, Iron – Fe.

2000), while an increase in pigments such as carotenoids is a response of cells to protect the photosynthetic apparatus (Juneja et al., 2013). This endpoint showed a slightly higher sensitivity than growth rate to differentiate the effects of the different waters in *R. subcapitata*.

Both cell size and complexity demonstrated significant differences from the control for microalgae exposed to Croatian waters which has previously been observed for microalgae exposed to metals (Franklin et al., 2001; Hyka et al., 2013; Jamers et al., 2009). Increases in size may be related to an increase in cell membrane permeability, and uncoupling of cell growth and cell division (Franqueira et al., 2000; Jamers et al., 2009; Juneja et al., 2013). Changes in cell complexity and granularity reveal ultrastructural differences that can be attributed to the presence of detoxification mechanisms with immobilization of toxic elements in non-toxic deposits within cells (Franklin et al., 2001; Jamers et al., 2009).

Regarding cell viability, significant differences were obtained for microalgae exposed to the Croatian bottled waters, which generally exhibited lower values than those exposed to the Norwegian bottled waters. This endpoint can be dependent on exposure duration, as membrane integrity is only affected after other cellular parameters, such as ROS levels or mitochondrial dysfunction, have already been strongly affected (Esperanza et al., 2015).

To detect the formation of ROS, three probes were used, carboxy-H_2DFFDA (ROS), DHR 123 (ROS) and C_{11}-BODIPY$^{581/591}$ (LPO). These presented similar trends and differences between the different waters, with higher ROS levels only observed for microalgae exposed to the Croatian waters. The small differences between DHR 123 and the other two probes are related to their specificity. Carboxy-H_2DFFDA is activated by H_2O_2, NO, $ONOO^-$, O_2 and OH (Gunawan et al., 2013), while C_{11}-BODIPY$^{581/591}$ reacts to oxyl-radicals such as HO•, ROO•, RO• and peroxynitrite responsible for LPO (Cheloni and Slaveykova, 2013). Although carboxy-H_2DFFDA and DHR 123 have similar structural features, and are oxidized by similar oxidative/radical mechanisms, carboxy-H_2DFFDA is generally associated with ROS formation in chloroplasts. This is because PSI and PSII reaction centers in thylakoids are the major production sites of ROS in photosynthetic organisms (Asada, 2006; Almeida et al., 2017; Knauert and Knauer, 2008). Conversely, DHR 123 is known for indicating ROS formation in the mitochondria (Gunawan et al., 2013; Ortega-Villasante et al., 2016).

In addition to DHR 123, TMRM was also used to indicate alterations in mitochondrial membrane potential. Few differences were obtained for this probe, resulting in less sensitivity than DHR 123. At 48 h, a significant decrease from the control was only observed for Croatian water G, with an increase at 72 h. An increase was also obtained from 48 h to 72 h for waters F, G and H. This may be related to an increase in the probe uptake due to membrane hyperpolarization (Franklin et al., 2001; Jamers et al., 2009). This observation has previously been reported in other studies, where it was demonstrated that membrane permeability can change due to an increase in respiration (Esperanza et al., 2015), and also due to the presence of small cations, as for example Na (Jamers et al., 2009).

PicoGreen enabled the distinction of DNA peaks correspondent to cells with different DNA content (Marie et al., 1996). Three cell cycle phases, G1 (Gap phase), S (Synthesis - DNA replication) and G2 (growth) phases, were defined (Fig. A1 SI). In general, for the G1 phase only the microalgae exposed to the Croatian waters E, G and H showed higher DNA content, with higher values after 48 h. High DNA values at this phase are known to be related to apoptosis associated with fractional DNA content (Alberts et al., 2002). On the S phase, and at both time-points, algae exposed to the same Croatian waters had the lowest DNA content. Microalgae exposed to the Norwegian waters B, C and D also had lower DNA content than the control at 48 h exposure, but at 72 h the DNA content increased to levels similar to the control. At the S phase, DNA replication occurs in cells that are in a good condition and capable of passing the previous checkpoint in the G1 phase (Alberts et al., 2002). Therefore, contrary to the preceding G1 phase, higher DNA values in the S phase correspond to the existence of more viable cells. For G2, a clear distinction between the Norwegian and Croatian waters was again observed, with the latter presenting the lowest DNA content at both time-points. These data indicate that the DNA of the microalgae exposed to Croatian bottled waters E, G and H was damaged and hence the microalgae was not able to properly replicate.

Similarly to what was seen with FCM, the chlorophyll fluorescence parameters obtained by PAM fluorometry showed an effect of the bottled waters on microalgal photosynthetic capacity. A complete inhibition in photosynthetic processes was only detected upon exposure to water E. No alterations in F_v/F_m were observed in the microalgae exposed to Norwegian waters, indicating that cells were maintained in a good physiological state throughout exposure. Conversely, F_v/F_m decreased in microalgae exposed to waters E, G and H, suggesting an impact in the photosynthetic capacity of PSII. A similar effect was detected for Φ_{PSII}, which was affected to a stronger degree than F_v/F_m for algae exposed to Croatian waters, especially for water E, where a complete inactivation of Φ_{PSII} was observed. This can be explained by a significant decrease in qP, indicating a reduction in the fraction of open PSII reaction centers that limit the proportion of captured light energy (Juneau and Popovic, 1999). The assessment of dissipation energy pathways demonstrated that in the microalgae exposed to water E, light energy was not used in photochemical and non-photochemical processes, as showed by the decrease in both $qP_{(rel)}$ and $qN_{(rel)}$, but mainly dissipated *via* $UQF_{(rel)}$. The predominance of $UQF_{(rel)}$ seems to indicate that PSII reaction centers were kept in a reduced efficiency state, impairing electron transport between photosystems (Juneau et al., 2005). For the remaining bottled waters, $qP_{(rel)}$ was the predominant energy dissipation pathway, as illustrated by the increase in $qP_{(rel)}$ and decrease in $qN_{(rel)}$ for waters C, F, G and H. The impact on photosynthetic performance was further supported by an increase in OEC and a reduction in ETR, especially for waters A, E, G and H.

Chemical analysis revealed a clear difference between the Norwegian and Croatian waters, with the later having higher concentrations of Ca, Mg, Na, NH_4, Cl, NO_3 and Mn. The RDA and correlation analyses indicated that Ca, Na, Mg, and NH_4 were positively correlated with the Croatian waters and possibly were responsible for the observed biological effects in the microalgae. Although essential elements for microalgal growth, these can have deterimental effects when present at high concentrations as observed in other studies (Bartolomé et al., 2009; Hopkins and Hüner, 2009; Mera et al., 2016; Perez-Garcia et al., 2011; Shimamatsu, 2004).

5. Conclusions

The present study indicated that the most sensitive endpoints to reflect the effects of the tested bottled waters to *R. subcapitata* were oxidative stress related endpoints, namely the formation of ROS (indicated by carboxy-H_2DFFDA and DHR 123) and LPO, pigment content, the morphological endpoints size and complexity, and cell cycle. Interestingly, the growth rate was one of the least sensitive endpoints. This study identified that toxicity tests based only on algal growth are less sensitive and provide limited information regarding the underlying toxic mechanisms. The applied methodology provided several advantages including relatively high throughput, greater sensitivity, and the ability to simultaneously gather information on the morphological, biochemical, and physiological status of the algae. Therefore, FCM has a great potential to be used for algal toxicity tests, giving not only information on effects in growth rate, but also mechanistic information on the toxic mode of action of contaminants. Consequently, the methodological approaches used in the present study are a good example of how these techniques could be integrated into future microalgal toxicity bioassays for testing chemicals and contaminants with the aim of improving the current hazard assessment strategies that are required by regulatory authorities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.06.124.

References

Adler, N.E., Schmitt-Jansen, M., Altenburger, R., 2007. Flow cytometry as a toll to study phytotoxic modes of action. Environ. Toxicol. Chem. 26 (2), 297–306.
Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. Molecular Biology of the Cell. 4th edition. Garland Science, New York, p. 1464.
Almeida, A.C., Gomes, T., Langford, K., Thomas, K.V., Tollefsen, K.E., 2017. Oxidative stress in the algae *Chlamydomonas reinhardtii* exposed to biocides. Aquat. Toxicol. 189, 50–59.
Almeida, A.C., Gomes, T., Langford, K., Thomas, K.V., Tollefsen, K.E., 2019. Oxidative stress potential of the herbicides bifenox and metribuzin in the microalgae Chlamydomonas reinhardtii. Aquat. Toxicol. 210, 117–128.
Asada, K., 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol. 141, 391–396.
Bartolomé, M.C., D'ors, A., Sánchez-Fortún, S., 2009. Toxic effects induced by salt stress on selected freshwater prokaryotic and eukaryotic microalgal species. Ecotoxicology 18, 174–179.
Baz-Lomba, J.A., Reid, M.J., Thomas, K.V., 2016. Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF. Anal. Chim. Acta 914, 81–90.
BD Accuri™, 2016. C6 Plus Flow Cytometer. Flow cCytometry within Reach. (23-17667-00, 20 p). Bdbiosciences.com.
Berglund, D.L., Eversman, S., 1988. Flow cytometric measurement of pollutant stresses on algal cells. Cytometry 9, 150–155.
Buschmann, C., 1995. Variation of the quenching of chlorophyll fluorescence under different intensities of the actinic light in wildtype plants of tobacco and in an aurea mutant deficient of light harvesting-complex. J. Plant Physiol. 145, 245–252.
Cheloni, G., Slaveykova, V.I., 2013. Optimization of the C11-BODIPY$^{581/591}$ dye for the determination of lipid oxidation in *Chlamydomonas reinhardtii* by flow cytometry. Cytometry A 83A, 952–961.
Cid, A., Fidalgo, P., Herrero, C., Abalde, J., 1996. Toxic action of copper on the membrane system of a marine diatom measured by flow cytometry. Cytometry 25, 32–36.
Debenest, T., Gagné, F., Petit, A.-N., Kohli, M., Eullafroy, P., Blaise, C., 2010. Monitoring of a flame retardant (tetrabromobisphenol a) toxicity on different microalgae assessed by flow cytometry. J. Environ. Monit. 12, 1918–1923.
Esperanza, M., Cid, Á., Herrero, C., Rioboo, C., 2015. Acute effects of a prooxidant herbicide on the microalga *Chlamydomonas reinhardtii*: screening cytotoxicity and genotoxicity endpoints. Aquat. Toxicol. 165, 210–221.
Franklin, N., Stauber, J.L., lim, R.P., 2001. Development of flow cytometry-based algal bioassays for assessing toxicity of copper in natural waters. Environ. Toxicol. Chem. 20 (1), 160–170.
Franqueira, D., Orosa, M., Torres, E., Herrero, C., Cid, A., 2000. Potential use of flow cytometry in toxicity studies with microalgae. Sci. Total Environ. 247, 119–126.
Gala, W.R., Giesy, J.P., 1990. Flow cytometry techniques to assess toxicity to algae. Am. Soc. Test. Mater. 1096, 237–246.
Genty, B., Briantais, J.-M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta 990, 87–92.
Gunawan, C., Sirimanoonphan, A., Teoh, W.Y., Marquis, C.P., Amal, R., 2013. Submicron and nano formulations of titanium dioxide and zinc oxide stimulate unique cellular toxicological responses in the green microalga *Chlamydomonas reinhardtii*. J. Hazard. Mater. 260, 984–992.
Hopkins, W.G., Hüner, N.P., 2009. Introduction to Plant Physiology. J. Wiley, New York.
Hyka, P., Lickova, S., Přibyl, P., Melzoch, K., Kovar, K., 2013. Flow cytometry for the development of biotechnological processes with microalgae. Biotechnol. Adv. 31, 2–16.
ISO 10304-1, 2009. Water Quality - Determination of Dissolved Anions by Liquid Chromatography of Ions - Part 1: Determination of Bromide, Chloride, Fluoride, Nitrate, Nitrite, Phosphate and Sulfate (ISO 10204-1:2007) (ICS: 13.060.50, 28 p.).
ISO 14911, 1998. Water quality - determination of dissolved li^{+}, Na^{+}, NH_4^{+}, K^{+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+} and Ba^{2+} using ion chromatography – Method for water and waste water. ISO/TC 147/SC 2 Physical, Chemical and Biochemical Methods, ICS 13.060.50 (18 p).
ISO 6332, 1998. Water quality - determination of iron - spectrometric method using 1,10-phenanthroline. ISO/TC 147/SC 2 Physical, Chemical and Biochemical Methods, ICS 13.060.50 (4 p.).
ISO 8692, 2012. Water quality – Fresh water algal growth inhibition test with unicellular green algae. ISO/TC 147/SC 5 Biological Methods, ICS 13.060.70 (21 p).
Jamers, A., Lenjou, M., Deraedt, P., Bockstaele, D.V., Blust, R., de Coen, W., 2009. Flow cytometric analysis of the cadmium-exposed green alga *Chlamydomonas reinhardtii* (Chlorophyceae). Eur. J. Phycol. 44 (4), 541–550.
Juneau, P., Popovic, R., 1999. Evidence for the rapid phytotoxicity and environmental stress evaluation using the PAM fluorometric method: importance and future application. Ecotox 8, 449–455.
Juneau, P., Green, B.R., Harrison, P.J., 2005. Simulation of pulse-amplitude-modulated (PAM) fluorescence: limitations of some PAM-parameters in studying environmental stress effects. Photosynthetica 43 (19), 75–83.
Juneja, A., Ceballos, R.M., Murthy, G.S., 2013. Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production: a review. Energies 6, 4607–4638.
Knauert, S., Knauer, K., 2008. The role of reactive oxygen species in copper toxicity to two freshwater green microalgae. J. Phycol. 44, 311–319.
Kriedemann, P.E., Graham, R.D., Wiskich, J.T., 1985. Photosynthetic dysfunction and in vivo changes in chlorophyll a fluorescence from manganese-deficient wheat leaves. Aust. J. Agric. Res. 36, 157–169.
Manar, R., Vasseur, P., Bessi, H., 2012. Chronic toxicity of chlordane to *Daphnia magna* and Ceriodaphnia *dubia*: a comparative study. Environ. Toxicol. 27 (2), 90–97.
Marie, D., Vaulot, D., Partensky, F., 1996. Application of the novel nucleic acid dyes YOYO-1, YO-PRO-1, and PicoGreen for flow cytometric analysis of marine prokaryotes. J. Appl. Environ. Microbiol. 62 (5), 1649–1655.
Martinez, R.S., Di Marzio, W.D., Saenz, M.E., 2014. Genotoxic effects of commercial formulations of Chlorpyrifos and Tebuconazole on green algae. Ecotoxicology 24, 45–54.
Medema, G.J., Shaw, S., Waite, M., Snozzi, M., Morreau, A., Grabow, W., 2003. Catchment characterisation and source water quality. In: Dufour, A., Snozzi, M., Köster, W., Bartram, J., Ronchi, E., Fewtrell, L. (Eds.), Assessing Microbial Safety of Drinking Water: Improving Approaches and Methods. IWA Publishing, London, pp. 111–158.
Mera, R., Torres, E., Abalde, J., 2016. Effects of sodium sulfate on the freshwater microalga *Chlamydomonas moewusii*: implications for the optimization of algal culture media. J. Phycol. 52, 75–88.
OECD, 2004. OECD Guidelines for Testing of Chemicals. 202. *Daphnia* Sp., Acute Immobilisation Test. Organisation for Economic Co-operation and Development, Paris, France.
OECD, 2011. OECD guidelines for testing of chemicals. Freshwater Alga and Cyanobacteria, Growth Inhibition Test. vol. 201. Organisation for Economic Co-operation and Development, Paris, France.
OECD, 2012. OECD Guidelines for Testing of Chemicals. 211. *Daphnia magna* Reproduction Test. Organisation for Economic Co-operation and Development, Paris, France.
Ortega-Villasante, C., Burén, S., Barón-Sola, Á., Martínez, F., Hernández, L.E., 2016. *In vivo* ROS and redox potential fluorescent detection in plants: present approaches and future perspectives. Methods 109, 92–104.
Perez-Garcia, O., Escalante, F.M.E., de-Bashan, L.E., Bashan, Y., 2011. Heterotrophic cultures of microalgae: metabolism and potential products. Water Res. 45 (1), 11–36.
Prado, R., García, R., Rioboo, C., Herrero, C., Abalde, J., Cid, A., 2009. Comparison of the sensitivity of different toxicity test endpoints in a microalga exposed to the herbicide paraquat. Environ. Int. 35, 240–247.
Ralph, P.J., Smith, R.A., Macinnis-Ng, C.M.O., Seery, C.R., 2007. Use of fluorescence based ecotoxicological bioassay in monitoring toxicants and pollution in aquatic systems: review. Toxicol. Environ. Chem. 84 (4), 589–607.
Rojíčková-Padrtová, R., Maršálek, B., 1999. Selection and sensitivity comparisons of algal species for toxicity testing. Chemosphere 38 (14), 3329–3338.
Shimamatsu, H., 2004. *In*: Ang Jr., P. (Ed.), Asian Pacific Phycology in the 21st Century: Prospects and Challenges. Springer, Netherlands, pp. 39–44.
Stauber, J.L., Franklin, N.M., Adams, M.S., 2002. Applications of flow cytometry to ecotoxicity testing using microalgae. Trends Biotechnol. 20 (4), 141–143.
Suzuki, S., Yamaguchi, H., Nakajima, N., Kawachi, M., 2018. Raphidocelis subcapitata (= Pseudokirchneriella subcapitata) provides an insight into genome evolution and environmental adaptations in Sphaeropleales. Sci. Rep. 8, 8058.
Veldhuis, M., Kraay, G., Timmermans, K., 2001. Cell death in phytoplankton: correlation between changes in membrane permeability, photosynthetic activity, pigmentation and growth. Eur. J. Phycol. 36 (2), 167–177.
Zhou, L., Chen, H., Jiang, X., Fang, L., Zhou, Y., Yin, W., Ji, X., 2009. Modification of montmorillonite surfaces using a novel class of cationic Gemini surfactants. J. Colloid Interface Sci. 332, 16–21.