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Effective removal of the rotifer *Brachionus calyciflorus* from a *Chlorella vulgaris* microalgal culture by homogeneous solar photo-Fenton at neutral pH

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ABSTRACT

In this study, a citrate-modified photo-Fenton process was successfully applied to decontaminate a *Chlorella vulgaris* microalgae culture spiked with the rotifer *Brachionus calyciflorus* (5 individuals mL^{-1}). The applied treatment (1 mg L^{-1} Fe^{2+}, 20 mg L^{-1} H_2O_2, 17.5 mg L^{-1} citric acid) had only moderate effects on viability and regrowth of the microalgae since, after a short post-treatment delay of a few days, they reached final cell densities similar to that obtained for microalgae cultures that were not spiked. The decontamination was effective as no regrowth of rotifers was observed in the microalgae cultures after treatment. The efficacy of the citrate-modified photo-Fenton treatment was also studied with a higher starting concentration of 20 rotifers mL^{-1} and was compared with a solar light/H_2O_2 treatment. Results show that both treatments had similar efficacies on the rotifer elimination, but that the citrate-modified photo-Fenton treatment had a lower negative impact on the regrowth of microalgae than the solar light/H_2O_2 treatment. However, when microalgae cultures were spiked with 20 rotifers mL^{-1}, rotifers were only partially inactivated and post-treatment regrowth occurred, which highlights the importance to apply the photo-Fenton process at an early stage of a contamination to achieve full rotifer elimination. In any case, a contamination with 5 rotifers mL^{-1} is already a significant threat as numbers above 1000 rotifers mL^{-1} were reached after 14 days and caused the microalgae culture to fail. Overall, our treatment suggests that the citrate-modified solar photo-Fenton process is an environmentally friendly solution to support the maintenance of contaminant-free microalgal cultures.

1. Introduction

Microalgae are photosynthetic organisms which are widely spread in aquatic and terrestrial environments and have interesting marketable applications. For example, microalgae are used as food supplements in human nutrition or to produce valuable compounds, such as natural carotenoids, antioxidants, and vitamins (Singh et al., 2019; Vaz et al., 2016). Moreover, they are used in aquacultures to feed zooplankton, bivalve mollusks, and larval stages of crustaceans and some fish species (Brown et al., 1997; Sirakov et al., 2015). Recently, microalgae raised interest for their potential applications in biofuels, in biopolymer production, and even in pharmaceutical biotechnology (Yan et al., 2016; Zanchetta et al., 2021; Zewdie and Ali, 2020). Additionally, their demand as fertilizers makes them a lucrative market, with high expected growth over the next decade (Barsanti and Gualtieri, 2018; Uysal et al., 2015). Hence, there is accumulated interest for developing stable and functional growth protocols.

Despite their high utility, the mass production of microalgae is hindered by several factors, such as their high culturing costs and challenges associated with the use and up-scaling of cultivations systems,

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such as photobioreactors (PBRs) or open ponds (Borowitzka and Vonshak, 2017; Tredici et al., 2016). While the cultivation of microalgae in open ponds under natural irradiance is usually cheaper and more scalable than the cultivation in PBRs (Davis et al., 2011; Slade and Bauen, 2013), open systems are more vulnerable to biological contamination by grazers transmitted for instance through the air. Indeed, in natural ecosystems, microalgae are at the base of the food chain and they are predated by grazing microzooplankton, which has been documented as occasional contaminant of industrial cultures of microalgae (Carney et al., 2016; Molina et al., 2019; Yuan et al., 2018). Grazers thus represent a threat for the production and quality of the algal biomass and there is a need for effective and easy applicable treatments to inactivate the growth of these contaminants, particularly at an early culturing stage (Day et al., 2017).

Among grazers, rotifers, such as *Brachionus calyciflorus*, are small animals with a length between 100 and 200 μm (Nandini et al., 2007), which are voracious predators of microalgae (Rico-Martínez and Dodson, 1992). They thrive by producing diploid and haploid eggs, via amictic and mictic reproduction, respectively (Fussmann et al., 2007). Diploid eggs from amictic females will develop into females, while haploid eggs from mictic females will develop into males, or if fertilized, into resting eggs (cysts). Rotifers can consume a large variety of microalgae at high rates and their predation can cause a complete collapse of microalgae cultures (Abou-Shanab et al., 2016).

The cultures of microalgae are particularly vulnerable to rotifers when the cultivation is carried out at low cell densities (Deruyck et al., 2019b), hence contamination has to be detected at an early stage after culture inoculation and corrective measures have to be taken to avoid the failure of the microalgal cultures. Moreover, rotifers are abundant in various aquatic or moist environments such as lakes and rivers but also marshes, moist soils, mosses, tree holes, etc. (Gilbert, 2018; Phan et al., 2021; Walsh et al., 2014). Therefore, the contamination of microalgae cultures by rotifers may increase if natural freshwater is used. When the cultivation is carried out in open systems, wind and rain could also carry particles into the cultures, which potentially contain rotifers or their eggs/cysts.

Effective approaches for the removal of rotifers from microalgae cultures remain relatively unexplored, even if some innovative methods have been investigated and successfully applied so far, such as the use of hydrodynamic cavitation, surfactants, or botanical pesticides (Deruyck et al., 2019a; Huang et al., 2014a, 2014b; Kim et al., 2017; Zhang et al., 2021). Sodium hypochlorite (0.45 to 0.6 mg Cl L^{-1}) was also successfully applied to inactivate rotifers in microalgae cultures, and to the best of our knowledge, this is the only study where a (plain) oxidative treatment was used for an *in-situ* inactivation of rotifers (Park et al., 2016). However, sodium hypochlorite and ozone treatments were used to produce bacteria-free cultures of rotifers, which shows that rotifers are equipped to face oxidative stress (Davis and Arnold, 1997; Douillet, 1998; Gonçalves and Gagnon, 2011). In our present study, we are aiming at the development of an effective oxidation process, which is fine-tuned to overcome the resistance of rotifers to oxidative damage and removes them from *Chlorella vulgaris* cultures while preserving the viability of the microalgae and allowing their regrowth after the decontamination treatment.

Advanced Oxidation Processes (AOPs) are a group of powerful and versatile treatments that have gathered significant interest within the water community over the last two decades (Giannakis et al., 2021). The common denominator between processes like the Fenton reaction, ozone (at neutral/basic pH) and UV/H_2O_2 are the generation of very oxidative hydroxyl radicals (*HO*$^\bullet$), second only to F^-. While the Fenton process has the advantage of requiring simple reagents (Fe, H_2O_2), it also has significant drawbacks, such as the operation at acidic pH or the generation of iron sludge (Pignatello et al., 2006). Nevertheless, recent advances in the field allow envisioning applications for matrices with neutral pH, by the use of chelators or sunlight (Ahile et al., 2020; Clarizia et al., 2017; O'Dowd and Pillai, 2020). The Fenton process has been used in (waste)water treatment to inactivate a wide array of microorganisms, such as bacteria (de la Obra Jiménez et al., 2020; Esteban García et al., 2018), viruses (Giannakis, 2018; Ortega-Gómez et al., 2015), fungi (Aguas et al., 2017), protozoa (Abeledo-Lameiro et al., 2019), even antibiotic resistant strains (Polo-López and Sánchez Pérez, 2021), and obtain purified effluents. It requires only Fe and H_2O_2, and is considered as an environmentally friendly method, which can be described by the following equations at near-neutral pH (Mosteo et al., 2020):

Equation 1. Production of HO$^\bullet$ via the Fenton reaction

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^\bullet + OH^-$$

Equation 2. Reduction of Fe^{3+} into Fe^{2+}

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^\bullet + H^+$$

Equation 3. Photo-catalytic reduction of Fe^{3+} into Fe^{2+}

$$Fe^{3+} + H_2O \xrightarrow{h\nu} Fe^{2+} + HO^\bullet + H^+$$

Equation 4. Reduction of Fe^{3+} into Fe^{2+} via ligand-to-metal charge transfer

$$\left[Fe^{3+}L\right] + h\nu \rightarrow \left[Fe^{3+}L\right]^* \rightarrow Fe^{2+} + L_{ox}^+$$

In a recent study from our group (Pulgarin et al., 2020), the application of the photo-Fenton (PF) process under (simulated) solar light with the help of a natural chelator (citrate) has effectively eliminated bacterial contaminants from a microalgae culture. Compared to existing oxidative decontaminating processes, this application has the advantage of performing well at neutral-basic pH thanks to citrate (Ruales-Lonfat et al., 2016), and utilizing solar light, a key component in algal cultures (Acién et al., 2012). Furthermore, despite the non-selective nature of hydroxyl radicals (Pignatello et al., 2006) and their capability of disrupting algae cells (Romero-Martínez et al., 2021; Torres et al., 2018), a selective action on bacteria was observed, leaving the microalgal culture relatively unharmed and with potential for post-treatment regrowth (Pulgarin et al., 2020). As such, it would make an excellent case if a similar effect was attained in eliminating rotifers from a microalgal culture, without significantly hampering microalgae growth.

Considering the above, in the present study, two solar-based treatment methods, solar light/H_2O_2 and PF, as well as corresponding control experiments, were applied on microalgae cultures that were experimentally contaminated with rotifers. In general, we address photoautotrophic culturing systems with our solar-driven decontamination process, because they are most commonly used for microalgae mass production and their commercial use (Udayan et al., 2022). In addition, our approach is also meant to be potentially applicable for closed photo-bioreactors that are increasingly used for the production of high value products and pharmaceuticals (Jha et al., 2017).

A further adaptation of our methodology to a mixotrophic growth mode in open plants or closed photobioreactors might be foreseeable. In mixotrophic cultivation microalgae simultaneously combine photosynthesis under light irradiation and organic carbon assimilation, which often results in higher microalgae biomass yields as compared to strict photoautotrophic cultivation (Zhan et al., 2017). The ultimate objective is to determine if the homogeneous citrate-modified PF method developed against bacterial contamination could also be used to remove a bigger and more complex organism that predates microalgae. To attain this goal, we studied i) the individual effects that the solar-based treatments exert on microalgae (*Chlorella vulgaris*) and rotifers (*Brachionus calyciflorus*) and ii) we tried to adapt the process parameters to completely eliminate rotifers from contaminated cultures while concomitantly achieve a good survival rate of microalgae. For this purpose, the effects on microalgae and rotifers were assessed during and immediately after different solar-based treatments as well as for up to two weeks later to assess long-term effects on microalgae growth and decontamination efficacy. Ultimately, the study aims to contribute to

the development of a cheap and environmentally friendly in-situ oxidative treatment for the protection against biological contaminants in microalgae cultures.

2. Materials and methods

2.1. Preparation of synthetic freshwater

A moderately hard synthetic freshwater was prepared with the following concentrations (mg L^{-1}): 122.85 $MgSO_4{\cdot}7H_2O$, 60 $CaSO_4{\cdot}2H_2O$, 4 KCl, 96 $NaHCO_3$ in ultrapure water (milli-Q, Merck) (EPA, 2002). This solution was stored in the dark at ambient temperature and used as liquid medium for the cultivation of rotifers and the preparation of microalgae suspensions.

2.2. Microalgae methods

2.2.1. Cultivation of microalgae

Chlorella vulgaris (SAG 211-11b, Göttingen University, Germany) was cultivated to feed rotifers and to prepare standardized microalgae cultures, i.e., cultures with a dry weight of 100 mg L^{-1} and a cell density of $\approx 10^7$ cell mL^{-1}, which were used to carry out experiments. The methods used to cultivate microalgae and to assess their viability, as well as the setup of glass-column PBRs, were largely identical to what was described in our previous study (Pulgarin et al., 2020). Briefly, the setup of PBRs consists of glass columns (height: 50 cm, internal diameter 3.0 cm), a temperature controller, DULUX cool white lamps for illumination and mass flow controllers for CO_2 and air sparging. The following modifications were introduced in the methods employed in the present work:

i) Tap water was replaced by synthetic freshwater as culture medium.
ii) When microalgae were mixed with rotifers, samples to determine the cultivability of microalgae were filtered with a 40 μm mesh to remove rotifers prior to analysis.
iii) Microalgae cultivation in glass-column PBRs was slightly altered: a) the nutrient concentration of the liquid mineral medium was doubled, b) instead of a gradually increasing light intensity, a constant intensity of 250 μmol m^2 s^{-1} of photosynthetically active radiation was used, c) the cultivation period was increased from 6 to 7 days, and d) liquid mineral medium was added to the culture after 3 days to increase the volume from 100 to 200 mL.
iv) The microalgae used to carry out the experiments were always harvested after 7 days of growth and used fresh on the same day. About 100 mL of the microalgae culture was required to prepare the experiment and the stock to feed rotifers. Therefore, the remaining 100 mL were adjusted to 200 mL with liquid mineral medium if necessary, and the cultivation in a glass-column PBR was extended to 14 days. The microalgae harvested during this extended period were not used in experiments but to feed rotifers.

2.2.2. Preparation of microalgae stocks to feed rotifers

Synthetic freshwater was used for every step of the following procedure: microalgae were harvested, washed two times (4000 rcf for 3 min), and re-suspended in their initial volume. The cell count was determined, and microalgae were diluted to a cell density of $2{\cdot}10^8$ cells mL^{-1}. This stock was stored up to 4 days at 4°C and used daily to feed rotifers.

2.2.3. Cell cultivability of microalgae on agar plates

The method is described in detail in our previous study (Pulgarin et al., 2020), hence it is only briefly summarized here. A 1-mL sample was mixed with 30 μL of a catalase suspension (3 mg mL^{-1}) to remove residual H_2O_2. Then, 0.1 mL of sample was then spread on agar plates prepared with mineral medium. When microalgae were mixed with rotifers, the sample was filtered with a 40 μm mesh before the addition of catalase. Agar plates were incubated at room temperature with a constant illumination of 60 μmol m^{-2} s^{-1} for 9 days. The number of cultivable cells of *C. vulgaris* was determined by manual counting.

2.3. Rotifer methods

2.3.1. Cultivation, harvesting and counting of rotifers

Brachionus calyciflorus was cultivated in synthetic freshwater in a 1-L culture and the population size was adjusted daily to 60 individuals mL^{-1}. The detailed protocols and methods to hatch eggs, to scale up and maintain the culture, and to enumerate the rotifers are described in the accompanying MethodsX article of the current submission (*A comprehensive method for the stable and continuous production of Brachionus calyciflorus rotifers in a 1L culture using live microalgae feed*).

2.3.2. Assessment of the rotifer viability during experiments

Viability of rotifers was determined at regular time points during the experiments by assessing rotifer growth in duplicate 1-mL samples, which were incubated up to 7 days (at 25°C and under a low light intensity) in Petri dishes filled with 30 mL of synthetic water and microalgae (10^7 cells mL^{-1}). Additionally, 30 μL of a 3-mg mL^{-1} bovine catalase solution (2000-5000 units mg^{-1}, Sigma-Aldrich) was added to neutralize the residual H_2O_2. Rotifers were counted every 24 h and monitoring was stopped one day after their concentration surpassed 100 rotifers per Petri dish (shown as a red line in figures, *vide infra)*.

2.4. Experimental procedures and analytical methods

2.4.1. Practical experimental details of solar-based oxidative treatments

The decontamination reactors used in the solar-based treatments consisted of 250-mL beakers with floating magnetic stirring bars (DS6630, Thermo Scientific), both of which were acid-washed, rinsed, and autoclaved before carrying out the experiments. The setup of the solar simulator, light intensity (700 W m^{-2}), and temperature (25 ± 0.5°C) were the same as described in our previous study (Pulgarin et al., 2020), but the stirring was decreased to 150 rpm. 200 mL of a microalgae and/or rotifers suspension was poured into each reactor and samples were retrieved to assess the viability of microalgae and rotifers at t=0. Reagents were then added to the reactors, according to the treatment, in the following order: 1) citric acid, 2) Fe^{2+}, and 3) H_2O_2, with two-minute intervals in between. The lamp was switched on and the experiment was carried out for 2 h. The first sample for the analyses of dissolved Fe^{2+}/Fe^{3+} and H_2O_2 was taken 30 s after the addition of reagents.

Samples were taken regularly to determine the viability of rotifers and microalgae, concentrations of dissolved Fe^{2+}/Fe^{3+} and H_2O_2, and pH. For all the rotifer experiments, the average pH value at t=0 was 8.0 ± 0.1 for synthetic freshwater alone and 7.6 ± 0.1 when microalgae were added; it dropped to 6.9 ± 0.1 in both cases when Fe^{2+} was added. However, the average pH value increased during experiments to reach 7.4 ± 0.1 in synthetic freshwater and 7.7 ± 0.2 in microalgae cultures at the end of the 2-h treatment.

If a treated culture was cultivated after the 2-h treatment, H_2O_2 was neutralized in the reactor by addition of 1.4 mL of a bovine catalase solution (3 mg mL^{-1}). Then, the stirring bar was removed, and nutrients were added to reach 1/4 of the concentration of the liquid mineral medium. The reactor was placed under a lamp (Easy LED Universal Full Spectrum, Aquatlantis) with a light intensity of 100 μmol m^{-2} s^{-1} in a water bath at 25 ± 1.5°C. A mass flow controller provided to the culture a constant air flow of 0.1 L_N min^{-1} (2 % CO_2 (v/v)), and 200 μL of a 1 M NaOH solution were added after 30 min to stabilize the pH value around 7. The algal cell and rotifer concentrations were determined daily by counting under a light microscope (in duplicate) or a zoom stereomicroscope (in triplicate), respectively. Water evaporation was compensated daily by addition of synthetic freshwater. The stability of the pH

value was verified every three days.

2.4.2. General experimental outline of solar-based oxidative treatments

Four different sets of experiments were carried out in microalgae cultures or synthetic freshwater with different treatments and concentrations of rotifers, always at a simulated solar light intensity of 700 W/m^2. The first set of experiments focused on microalgae cultures (dry weight: 100 mg L^{-1}, cell density ≈10^7 cells mL^{-1}) without rotifers and aimed to assess the effect of i) simulated solar light alone, ii) solar light and Fe^{2+} with citric acid (1 mg L^{-1} Fe^{2+}, 17.5 mg L^{-1} citric acid), iii) solar light and H_2O_2 (20 mg L^{-1} H_2O_2), and iv) the homogeneous citrate-modified PF (1 mg L^{-1} Fe^{2+}, 20 mg L^{-1} H_2O_2, 17.5 mg L^{-1} citric acid). Further sets of experiments consisted of the same treatments but used an inoculum of 20 rotifers per mL, in synthetic freshwater without microalgae (second set) and in a microalgae culture (third set), respectively. The fourth set of experiments consisted of 5 rotifers mL^{-1} in a microalgae culture to which a citrate-modified PF treatment (1 mg L^{-1} Fe^{2+}, 20 mg L^{-1} H_2O_2, 17.5 mg L^{-1} citric acid) was applied. Two biological replicates were carried out for each experimental condition.

Microalgae cultures with 20 rotifers mL^{-1} were recultivated after their treatment with solar light/H_2O_2 and citrate-modified PF. Microalgae cultures with 5 rotifers mL^{-1} were also recultivated after the treatment with citrate-modified PF. Additionally, microalgae cultures with and without rotifers, which were not treated, were cultivated under the same conditions and their data were used as a reference.

2.4.3. H_2O_2 and dissolved iron measurements

1 mL of a filtered sample was mixed with 10-20 μL of titanium (IV) oxysulfate to measure the concentration of H_2O_2 by colorimetry (DIN°38.402 H15) at 410 nm with a spectrophotometer (UV-1800, Shimadzu). The concentration of H_2O_2 was determined with a calibration curve of seven points between 0 and 25 mg mL^{-1} ($R^2 > 0.99$). The Ferrozine method was used to measure spectrophotometrically at 562 nm the dissolved Fe^{2+} and Fe^{3+} (Viollier et al., 2000). 1.6 mL of a filtered sample was mixed with 0.2 mL of a 4.9 mM ferrozine solution, 0.2 mL of hydroxylamine hydrochloride solution (10 % w/w) and 0.5 mL of an acetate buffer (pH 4.66). A calibration curve of six points between 0 and 2 mg mL^{-1} was used as a reference to determine the concentration of dissolved Fe^{2+}/Fe^{3+} and the limit of quantification (LOQ) was 0.01 mg L^{-1}. Filtrations were carried out with syringe filters (0.45 μm). Error bars represent the standard error from the mean.

2.4.4. Data treatment

Data on the viability of microalgae, from the plating on agar of samples collected during the treatment time, were standardized to the initial concentration and are given as colony forming units (CFU), which was expressed graphically as the inactivation of cells in logarithmic units (logU) per mL (logCFU/mL). Data on algal cell counts, from the enumeration under a light microscope of samples collected during the post-treatment recultivation, were also standardized to the initial concentration and are given as the concentration of cells per mL in logU (log cells/mL). Rotifer counts are given as the number of swimming female rotifers per Petri dish or per mL. Errors bars represent the standard error of the mean. Swimming female rotifers were the focus of this study, and therefore, eggs, male rotifers and motile/inactive female rotifers are not shown in the results. Male rotifers were excluded from the study because they accounted for only 10 to 20 % of the population, thanks to the precise daily maintenance routine. Moreover, male rotifers do not produce eggs, hence they do not contribute to population regrowth after treatment, and had a neglectable weight, hence their contribution to consumption of reagents was negligible.

The limits of detection for the microalgae cultivability on agar plates and cell enumeration under the light microscope were 10 CFU mL^{-1} and 10^4 cells mL^{-1}, respectively. The limits of detection for the enumeration of rotifers were 1 unit (rotifer) per Petri dish for viability tests carried out during the treatments, and 1 rotifer mL^{-1} for the monitoring carried out during the cultivation of treated cultures.

3. Results and discussion

3.1. Moderate inhibitory effects of solar-based oxidative processes on microalgae cultures

First experiments were carried out with cultures of *C. vulgaris* without addition of rotifers to assess inhibitory effects of two different solar-based processes (solar light/H_2O_2, and homogeneous citrate-modified photo-Fenton) and their controls (solar light alone, solar light/Fe^{2+} with citric acid) on the cultivability of algal cells during the treatment and to determine the associated consumption of reagents (Fig. 1). The results show that the exposure to solar light alone had not detrimental effect on microalgae viability and neither had the addition of Fe^{2+} and citric acid. Indeed, iron is an important nutrient that plays a key role in many metabolic processes of microalgae, such as photosynthesis, respiration and nitrogen uptake (Marchetti and Maldonado, 2016). Moreover, the concentration of iron used in this study was in the range, or below, the concentration usually used to cultivate *C. vulgaris* (Doušková et al., 2010; Golub et al., 2018). Therefore, a solar light intensity of 700 W m^{-2}, which is in the range of ambient irradiation, and the presence of a low concentration of iron, was unlikely to have a significant negative impact on the microalgae viability. However, the solar light/H_2O_2 and citrate-modified PF treatments inactivated 2.46 ± 0.13 and 1.83 ± 0.31 logU of microalgae per mL of culture after 2 h, respectively (Fig. 1A). These results corroborate observations made in our previous study (Pulgarin et al., 2020), i.e., that the solar light/H_2O_2 treatment is more efficient than the citrate-modified PF in inactivating the growth of microalgae. However, in the present study, the loss of microalgae was moderate for both treatments due to an adapted H_2O_2 concentration, which was decreased to 20 mg L^{-1} compared to our previous work. Indeed, a residual, post-treatment concentration of viable microalgae in the range of 10^5 cells mL^{-1} is expected to be high enough to enable successful regrowth of the culture (Bohutskyi et al., 2016).

The consumption curves of reagents during treatment of microalgae (Fig. 1B) were compared with curves of the same treatments carried out in absence of microalgae in ultrapure water and synthetic freshwater (Supplementary Fig. S1). The results show that microalgae induce the high consumption of H_2O_2 during solar light/H_2O_2 and citrate-modified PF treatments (i.e., this is not a matrix effect). The consumption of H_2O_2 in the PF reaction was 6 or 8 mg L^{-1} when the reaction was carried out in microalgae cultures or synthetic water, respectively. Most likely, H_2O_2 is initially consumed by reaction with Fe^{2+}, followed by reaction with algal cells. In terms of iron, it is also likely that a fraction of the dissolved Fe^{2+}/Fe^{3+} was consumed by interaction with microalgae (binding or uptake). However, the iron concentration of the culture medium was also affected using freshwater, which is most likely due to the near-neutral pH value that accelerates its precipitation. Our data show that the citrate-modified homogeneous PF reaction occurred mainly during the first 30 min, most probably followed by a considerably milder heterogeneous reaction (in terms of $HO^{\bullet}$ generation), driven by the iron precipitates. The photo-Fenton process following this step should be slower due to the heterogeneous nature of precipitated iron (iron oxides).

3.2. Inhibitory effects of different solar-based processes on the regrowth of rotifer cultures in the absence of microalgae

The same solar-based processes as detailed above were applied to a rotifer culture with 20 individuals mL^{-1} in synthetic freshwater, to assess the effects of the different treatments on their viability and to determine the consumption of reagents during the treatment period (Fig. 2). Rotifers were neither adversely impacted by solar light in the range of ambient irradiation (Fig. 2A), nor by a concentration of 1 mg

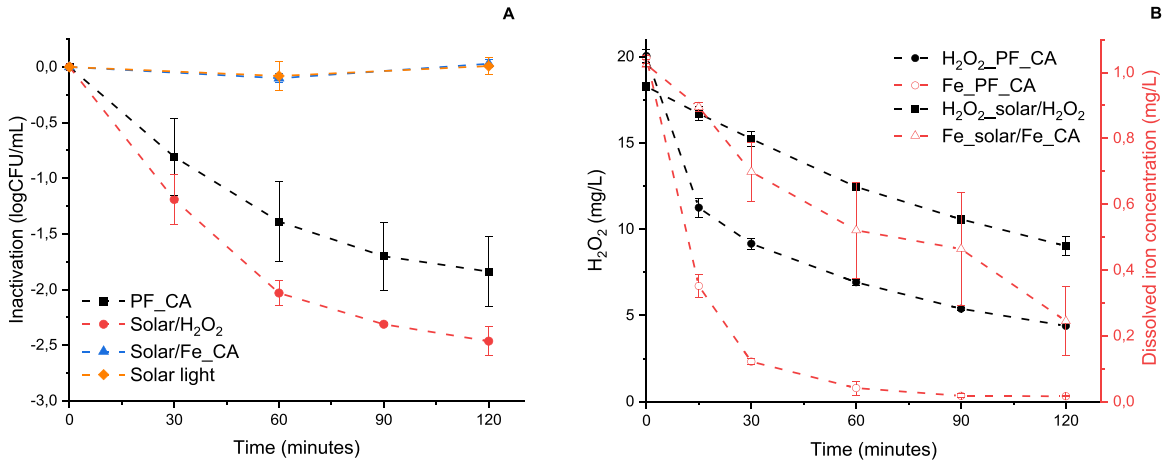

Fig. 1. (A) Inhibitory effects of two solar-based processes (solar light/H_2O_2 and citrate-modified PF) as well as corresponding controls (solar light and solar light/Fe^{2+} with citric acid) on the cell cultivability of cultures of *C. vulgaris* during treatments. $[Microalgae]_0 = 10^7$ CFU mL^{-1}. (B) Consumption of H_2O_2 and concentration of dissolved iron during the solar light/Fe^{2+} with citric acid, solar light/H_2O_2 and citrate-modified PF processes/treatments in microalgae cultures.

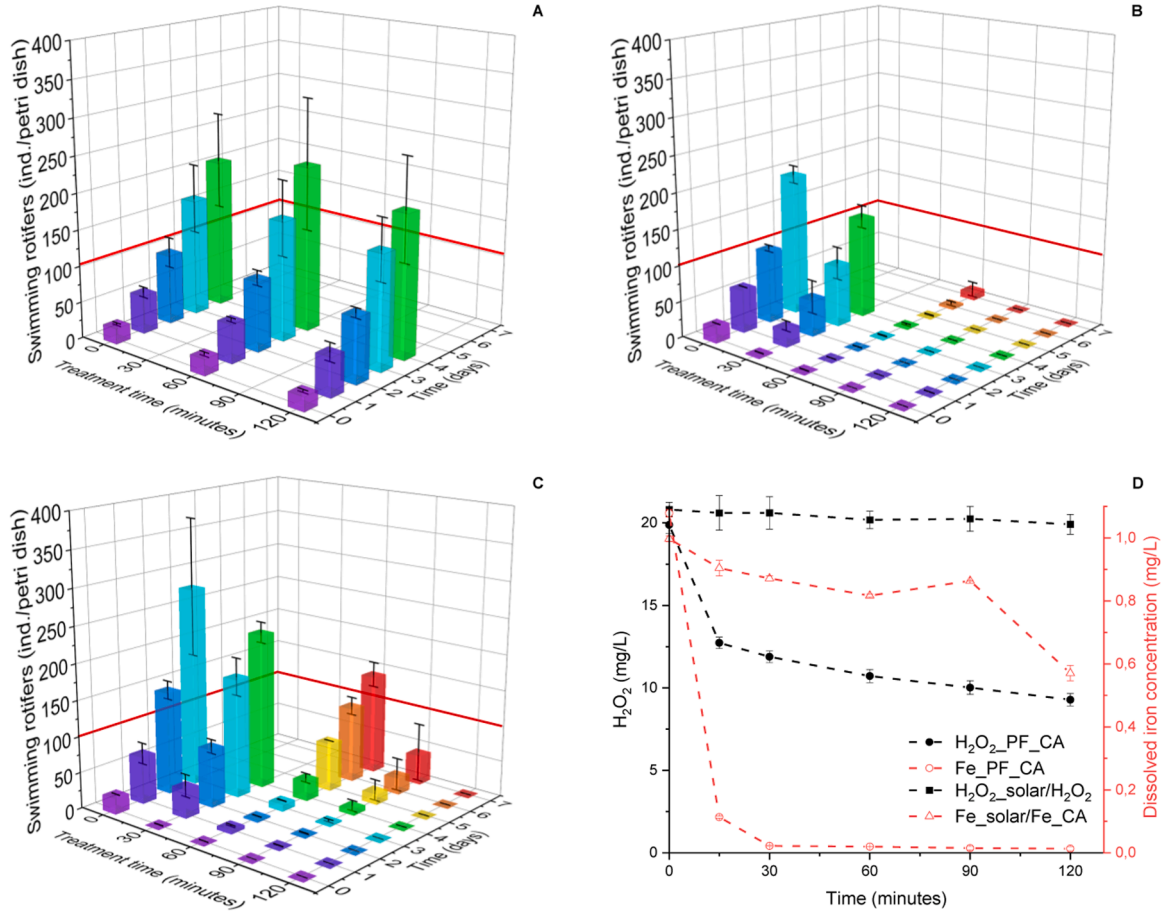

Fig. 2. Inhibitory effects of (A) solar light (control), (B) solar light/H_2O_2 and (C) citrate-modified PF on the regrowth of rotifers at different time points of the treatments. Rotifer cultures (20 individuals mL^{-1}) were prepared in synthetic freshwater. Conditions: 7-day incubation or 1 day after $[rotifer]_{t\ (days)}$ >100 per Petri dish (red line). (D) Consumption of H_2O_2 and concentration of dissolved iron during the solar light/Fe^{2+} process with citric acid, solar light/H_2O_2 and citrate-modified PF treatments of rotifer cultures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

L^{-1} of Fe^{2+} and 17.5 mg L^{-1} of citric acid (Supplementary Fig. S2). Indeed, adverse effects of iron on the rotifer growth were previously only reported for a much higher concentration (Han et al., 2021). Therefore, the rotifer inactivation observed during the solar light/H_2O_2 and citrate-modified PF (Fig. 2B, 2C) resulted predominantly from oxidation of rotifers by the solar/H_2O_2 process or the generated radicals, such as $HO^{\bullet}$, produced during the photo-Fenton reaction (Eqs. 1-4).

The results in Fig. 2B show that rotifers were fully inactivated by the solar light/H_2O_2 treatment within 90 min. Moreover, the regrowth was already strongly reduced after 60 min of treatment with an average value of only 11 ± 11 rotifers per Petri dish after 7 days of incubation. As far as the efficacy of the PF process is concerned, rotifers were fully

inactivated by the citrate-modified PF treatment in 120 min and their regrowth was already severely impacted after 90 min of treatment, as an average of only 42 ± 42 rotifers were counted in Petri dishes after 7 days of incubation (Fig. 2C). Comparatively, the growth rates of untreated rotifers (i.e., samples taken at t=0) were much higher and the incubation was stopped after 3 days since the average value reached 196 ± 12 and 277 ± 95 rotifers.

These results show that rotifers can be fully inactivated, and their subsequent regrowth prevented by applying certain oxidative treatments (i.e., H_2O_2 and citrate modified PF). The solar light/H_2O_2 treatment appeared to be the main driving force of the rotifer inactivation, being more efficient than the citrate-modified PF in yielding more pronounced inactivation, as it has also been observed in the treatment of microalgae alone, and quicker regrowth inhibition. Rotifers are bigger in size than microalgae, and, therefore, it is possible that they are also more resistant to a strong, but short, external oxidative stress burst induced by the citrate-modified PF reaction. However, the efficacy difference observed between the two treatments remains relatively narrow (only one sampling time-step difference; 90 vs. 120 min in treatment, 60 vs. 90-min effect in regrowth).

A population of 20 rotifers mL^{-1} did not consume much of H_2O_2 and dissolved Fe^{2+}/Fe^{3+} when compared to the corresponding curves obtained for microalgae cultures (Fig. 2D). This is likely due to the comparatively lower total biomass and contact surface in rotifer cultures. Dry weight of a single rotifer is estimated between 0.11 and 0.47 μg (Dumont et al., 1975), which results in 2.2 to 9.4 mg L^{-1} for a concentration of 20 rotifers mL^{-1}. Dry weight of microalgae was 100 mg L^{-1} for a higher number of organisms (≈10^7 cells mL^{-1}), hence the specific surface for reagent interaction was higher for microalgae cultures. These results indicate that the inactivation of rotifers may be feasible in a microalgae culture without leading to a faster reagent consumption than observed for cultures of microalgae alone, and therefore, without potentially impacting operation and cost-related factors.

3.3. *Treatment of highly contaminated microalgae cultures with solar light/H_2O_2 and citrate-modified photo-Fenton processes, and subsequent regrowth of treated cultures*

Microalgae cultures were contaminated with a rather high starting concentration of 20 rotifers mL^{-1} and then treated with solar light/H_2O_2 or citrate-modified PF treatments. Solar light and solar light/Fe^{2+} (with citric acid) processes were carried out as controls. In all experiments, the cultivability of microalgae, regrowth of rotifers, and concentrations of H_2O_2 and dissolved Fe^{2+}/Fe^{3+} were monitored during the treatment (Fig. 3 and Supplementary Fig. S3).

Microalgae were inactivated by 1.8 ± 0.13 and 2.00 ± 0.01 logU per mL of culture by the citrate-modified PF and solar light/H_2O_2 treatments, respectively (Fig. 3A), which was similar to the results obtained with microalgae cultures that were not contaminated with rotifers. However, the inactivation of rotifers upon treatment with citrate-modified PF (Fig. 3B) and solar light/ H_2O_2 (Fig. 3C) was lower when

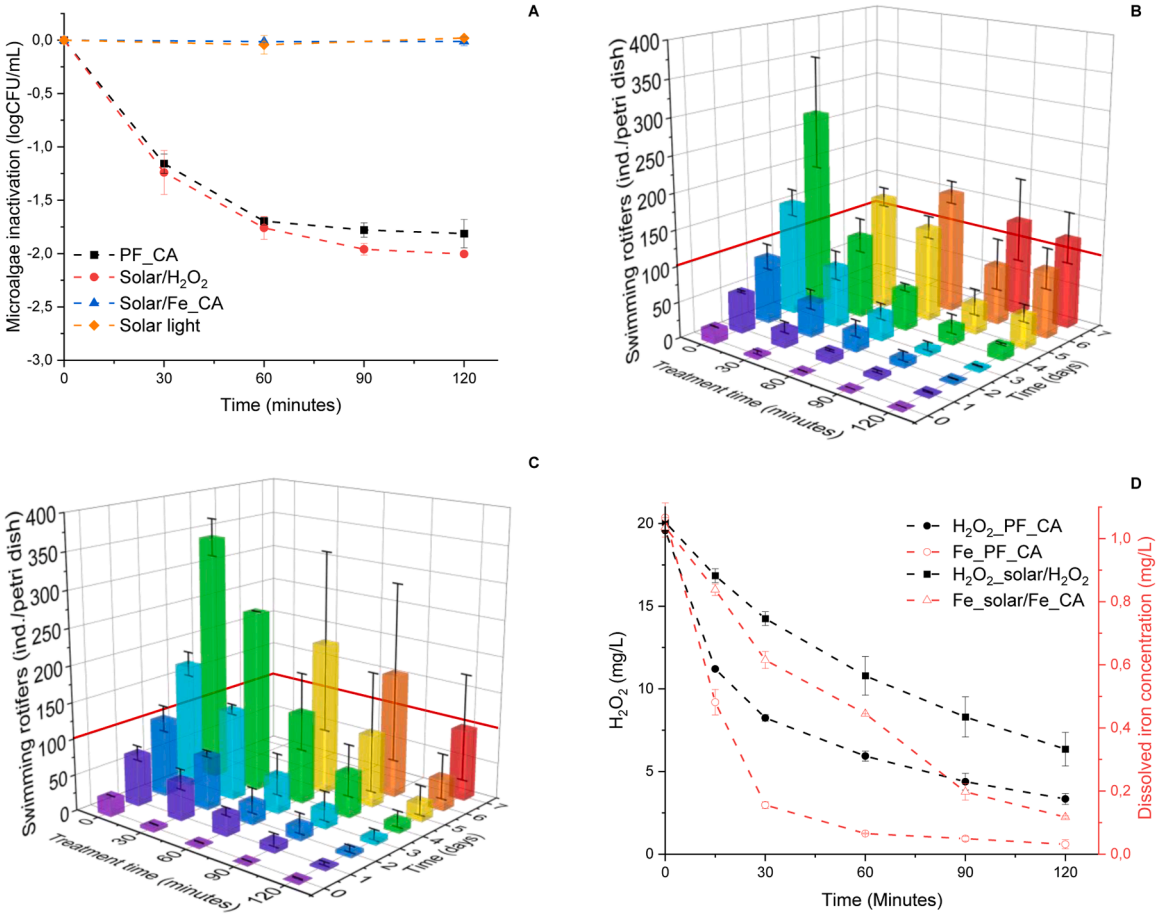

Fig. 3. (A) Inhibitory effect of two solar-based processes (solar light/ H_2O_2, and citrate-modified PF) as well as the corresponding controls (solar light, solar light/ Fe^{2+} with citric acid) on the cell cultivability of microalgae in cultures contaminated with rotifers. $[Microalgae]_0 = 10^7$ CFU mL^{-1}; $[Rotifers]_0 = 20$ mL^{-1}. Regrowth of rotifers sampled at regular time points in mixed cultures during the (B) citrate-modified PF and (C) solar light/ H_2O_2 treatments and incubated in Petri dishes. Conditions: 7-day incubation or 1 day after $[rotifer]_{t\ (days)}$ >100 per Petri dish (red line). (D) Consumption of H_2O_2 and dissolved iron concentration in all four processes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compared to their treatment in the absence of microalgae. As a result, the rotifer population recovered during the 7-day incubation, even when cultures were treated for 2 h (contrary to their full inactivation in absence of microalgae, Fig. 2B, 2C). This indicates that treatment efficacy was mainly impacted by the presence of microalgae, because they dominated the consumption of H_2O_2 (Fig. 3D), and therefore, decreased its oxidative effect on rotifers. However, recovery of rotifers was carried out in Petri dishes under ideal culturing conditions adapted for rotifers and results may differ under conditions ideal for microalgae growth, i.e., in liquid mineral medium, illuminated, and bubbled with an air/CO_2 flow. Hence, the results obtained here represent a worst-case scenario for a field-application.

After treatment with oxidative solar-based processes, the treated cultures were cultivated for 7 days and the ability of microalgae and rotifers to recover from the different treatments was assessed by the monitoring of algal cell and rotifer concentrations (Fig. 4). Regrowth was compared to microalgae cultures with and without rotifer contamination (20 rotifers mL^{-1}) that were not treated. Regrowth of rotifers was, on average, not prevented by the treatments, but only delayed, indicating that not all rotifers were killed during the treatment. Indeed, rotifers reached moderate concentrations of 9 ± 8 and 16 ± 16 rotifers mL^{-1} after 7 days of cultivation in cultures treated with solar light/H_2O_2 and citrate-modified PF, respectively. However, these concentrations were much lower than the concentration of rotifers of untreated contaminated microalgae cultures, where rotifers reached an average concentration of 145 ± 26 individuals mL^{-1}.

While microalgae cultures recovered from both solar-based treatments, a negative effect is visible. Cell concentration decreased during the first four days, most probably due to the residual effect of the inflicted oxidative damages, and then increased until the end of the cultivation. Finally, cultures reached cell density of 7.03 ± 0.06 and 7.70 ± 0.05 logU mL^{-1} for the solar light/H_2O_2 and citrate-modified PF treatments after 7 days, respectively, which was lower than the 8.46 ± 0.02 logU mL^{-1} reached by untreated cultures without rotifers, in 5 days. Microalgae cultures showed better regrowth when they were treated with citrate-modified PF instead of solar light/H_2O_2, which is consistent with the results of their cultivability on agar plates.

In comparison to untreated microalgae cultures, with and without rotifers, we demonstrate the considerable impact that rotifers have on algal growth. Cell densities of contaminated cultures fluctuated between

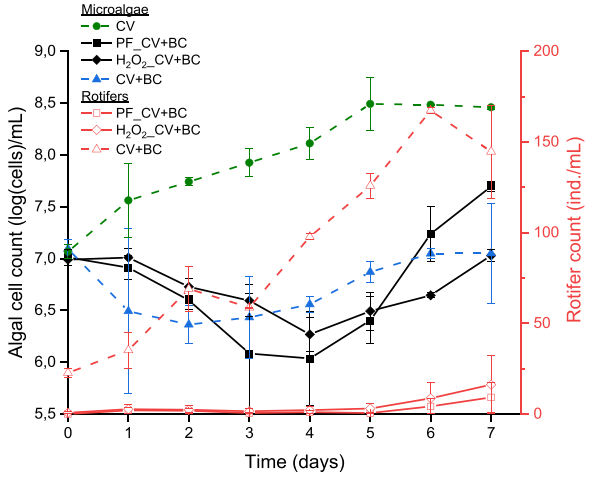

Fig. 4. Number of algal cells and rotifers during the cultivation of contaminated microalgae cultures ([Rotifers]$_0$ = 20 mL^{-1}), that were either treated for 2 h with solar light/H_2O_2 (H_2O_2_CV+BC) and citrate-modified PF (PF_CV+BC) or left untreated (CV+BC). A further control consisted of microalgae cultures that were neither contaminated with rotifers nor treated (CV). [Microalgae]$_0$ = 10^7 cells mL^{-1}. Continuous and dash lines between points are only used as a visual help.

6.36 and 7.09 logU mL^{-1}, which was clearly below those of cultures that were not contaminated with rotifers (8.46 LogU mL^{-1}). Additionally, the increasing population of rotifers (145 rotifers mL^{-1} after 7 days) indicated that their impact was likely increasing and that microalgae were most probably destined to be consumed.

3.4. Treatment of low-contaminated microalgae cultures with citrate-modified photo-Fenton

Our results indicate that the intensity of the oxidative treatments is appropriate to largely preserve the viability of *C. vulgaris* cultures. It was, however, not sufficient to fully inactivate a contamination of 20 rotifers per mL^{-1} in a microalgae culture. Therefore, the citrate-modified PF reaction, which affected microalgae less than the solar/H_2O_2 process, was applied to a lower concentration of only 5 rotifers mL^{-1}. This corresponds to a measure taken at an earlier stage of contamination in a field application (Yuan et al., 2018).

The inactivation of the microalgae and rotifers, and the concentration of reagents were monitored to assess the impact of the PF treatment (Fig. 5). The intensity of the treatment is modulated by the biomass of microalgae: their inactivation and the consumption of reagents were similar to the results obtained previously for microalgae cultures with and without rotifers. Microalgae were moderately affected and most of the reagents were consumed at the end of the 2-h treatment (Fig. 5A). Rotifers, however, were fully inactivated by the citrate-modified PF treatment in 90 min (Fig. 5B). This indicates that a rotifer contamination that is detected at an earlier stage can be successfully treated.

Treated cultures were cultivated for 14 days to confirm that the inactivation of rotifers was permanent and to monitor the regrowth of microalgae (Fig. 6). The successful inactivation of rotifers was confirmed, as no regrowth was observed during the 14 days of post-treatment cultivation. Treatment delayed growth of microalgae by two days only: microalgae of treated cultures were able to reach their stationary phase after only nine days of cultivation (8.23 ± 0.06 logU mL^{-1}) while non-contaminated microalgae cultures reached their stationary phase (8.27 ± 0.03 logU mL^{-1}) in seven days.

This short delay in growth after treatment is a modest drawback compared to an untreated culture, as complete restart of the cultivation likely takes considerably longer. The threat of rotifers, even at a low concentration of 5 individuals per mL, was demonstrated in untreated, but contaminated, microalgae cultures. Microalgae were first moderately impacted by rotifers during the first eight days and their cell density approached the value of the uncontaminated culture by reaching up to 8.07 ± 0.1 logU mL^{-1}. However, during the second week of cultivation, rotifer numbers reached 1521 ± 412 rotifers mL^{-1} and caused cultures to fail. This demonstrates the efficacy of the citrate-modified PF treatment to stop rotifer contamination in a microalgae culture, but also the dramatic decay of microalgae and strong propagation of rotifers, if no countermeasures are applied.

With the citrate-modified PF treatment (1 mg Fe^{2+}, 20 mg L^{-1} H_2O_2, 17.5 mg L^{-1} citric acid), a rotifer contamination was successfully removed from a microalgae culture, while microalgae were able to regrow. The performance achieved was comparable to other known chemical methods such as botanical pesticides, surfactants, or disinfectants, but without the associated disinfection by-products (Farinelli et al., 2021). For example, a complete inactivation of 5 rotifers mL^{-1} was achieved with 3 μM of cetyltrimethylammonium bromide in *C. vulgaris* cultures (biomass dry weight: 160 mg L^{-1}) (Deruyck et al., 2019a), and with 1.8 μg L^{-1} of toosendanin in *Chlorella* sp. cultures (cell density $2{\cdot}10^{-7}$ cells mL^{-1}) (Huang et al., 2014a). However, the citrate-modified PF process has the additional advantage of not being persistent in the water after the treatment. Sodium hypochlorite (0.45 to 0.6 mg Cl L^{-1}) supplied every two hours during 1 to 3 days inactivated a concentration of 7 rotifers mL^{-1} in *Chlorella kessleri* cultures (biomass dry weight: 60 to 90 mg L^{-1}) (Park et al., 2016). However, this treatment time was much longer than for the citrate-modified PF process, which, in our conditions,

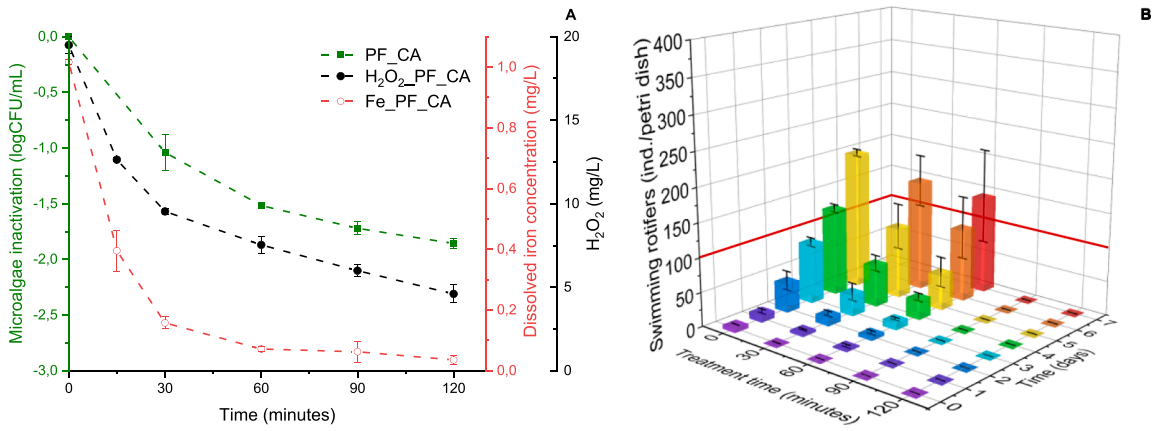

Fig. 5. (A) Inactivation of microalgae and associated consumption of reagents during the citrate-modified PF treatment of contaminated microalgae cultures ($[Rotifers]_0 = 5\ mL^{-1}$). $[Microalgae]_0 = 10^7$ CFU mL^{-1}. (B) Growth of rotifers during their incubation in Petri dishes for regular time points of the treatment. Conditions: 7-day incubation or 1 day after $[rotifer]_{t\ (days)}$ >100 per Petri dish (red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

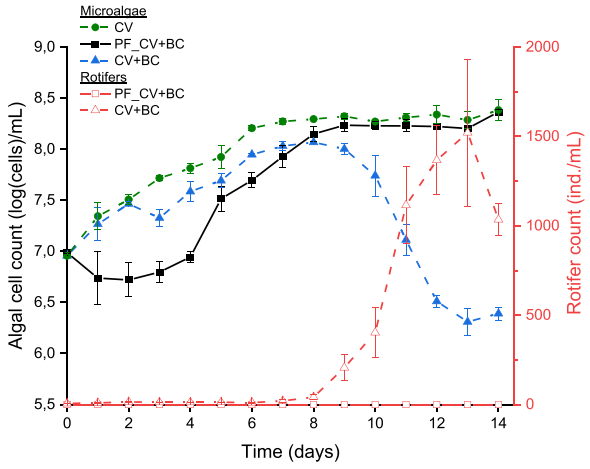

Fig. 6. Number of algal cells and rotifers during the cultivation of contaminated microalgae ($[Rotifers]_0 = 5\ mL^{-1}$) treated for 2 h with citrate-modified PF (PF_CV+BC). Control experiments included the cultivation of untreated cultures with microalgae alone (CV) or microalgae with rotifers (CV+BC). $[Microalgae]_0 = 10^7$ cells mL^{-1}. Continuous and dash lines between points are only used as a visual help.

required only 90 min. To summarize, the homogeneous, citrate-modified solar PF process presents a promising alternative treatment method that should be evaluated further.

4. Conclusions

A citrate-modified photo-Fenton process was used to treat microalgae cultures (*C. vulgaris*) that were artificially contaminated with rotifers (*B. calyciflorus*). The time-controlled treatment aimed to reach a full inactivation of rotifers and their eggs while minimizing harm to microalgae and, thus, allowing their continued cultivation. The treatment was effectively applied to a concentration of 5 rotifers mL^{-1} in the microalgae culture, corresponding to an intervention occurring at an early contamination stage.

In cultures containing both rotifers and microalgae, rotifers were fully inactivated after a short (90 min) oxidative treatment and no regrowth was observed during the following 14 days of cultivation. In contrast to rotifers, microalgae recovered from the oxidative stress and reached stationary phase with similar cell densities as obtained for non-contaminated microalgae cultures with a delay of two days.

Both the citrate-modified photo-Fenton and solar light/H_2O_2 processes have similar efficacies for the inactivation of rotifers in contaminated microalgae cultures. Nevertheless, the citrate-modified photo-Fenton tends to inflict less damage on *C. vulgaris* and offers a better control on the reagent removal as more of the H_2O_2 and all dissolved Fe^{2+}/Fe^{3+} were consumed at the end of a 2-h treatment.

The study shows the importance of applying treatment at an early stage of contamination. Indeed, citrate-modified photo-Fenton and solar light/H_2O_2 treatments failed to fully inactivate rotifers in microalgae cultures at a concentration of 20 rotifers mL^{-1}. The results demonstrate the successful application of an oxidative treatment for the *in-situ* inactivation of microalgae predators in an environmentally friendly way. The next step of the project would be to apply the treatment at larger scale in a commercial PBR designed to produce microalgae, and to continue assessment of its potential against other biological (e.g., ciliates, fungi, or invading microalgae) and chemical contaminants of microalgae cultures (Yang et al., 2021; Zhu et al., 2020).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.119301.

References

Abeledo-Lameiro, M.J., Polo-López, M.I., Ares-Mazás, E., Gómez-Couso, H., 2019. Inactivation of the waterborne pathogen Cryptosporidium parvum by photo-Fenton process under natural solar conditions. Appl. Catal. B 253, 341–347. https://doi.org/10.1016/j.apcatb.2019.04.049.

Abou-Shanab, R.A.I., Singh, M., Rivera-Cruz, A., Power, G., Bagby-Moon, T., Das, K., 2016. Effect of Brachionus rubens on the growth characteristics of various species of microalgae. Electron. J. Biotechnol. 22, 68–74. https://doi.org/10.1016/j.ejbt.2016.06.005.

Acién, F.G., Fernández, J.M., Magán, J.J., Molina, E., 2012. Production cost of a real microalgae production plant and strategies to reduce it. Biotechnol. Adv. 30, 1344–1353. https://doi.org/10.1016/j.biotechadv.2012.02.005. Special issue on ACB 2011.

Aguas, Y., Hincapie, M., Fernández-Ibáñez, P., Polo-López, M.I., 2017. Solar photocatalytic disinfection of agricultural pathogenic fungi (Curvularia sp.) in real urban wastewater. Sci. Total Environ. 607–608, 1213–1224. https://doi.org/10.1016/j.scitotenv.2017.07.085.

Ahile, U.J., Wuana, R.A., Itodo, A.U., Sha'Ato, R., Dantas, R.F., 2020. A review on the use of chelating agents as an alternative to promote photo-Fenton at neutral pH: Current trends, knowledge gap and future studies. Sci. Total Environ. 710, 134872 https://doi.org/10.1016/j.scitotenv.2019.134872.

Barsanti, L., Gualtieri, P., 2018. Is exploitation of microalgae economically and energetically sustainable? Algal Res. 31, 107–115. https://doi.org/10.1016/j.algal.2018.02.001.

Bohutskyi, P., Kligerman, D.C., Byers, N., Nasr, L.K., Cua, C., Chow, S., Su, C., Tang, Y., Betenbaugh, M.J., Bouwer, E.J., 2016. Effects of inoculum size, light intensity, and dose of anaerobic digestion centrate on growth and productivity of Chlorella and Scenedesmus microalgae and their poly-culture in primary and secondary wastewater. Algal Res. 19, 278–290. https://doi.org/10.1016/j.algal.2016.09.010.

Borowitzka, M.A., Vonshak, A., 2017. Scaling up microalgal cultures to commercial scale. Eur. J. Phycol. 52, 407–418. https://doi.org/10.1080/09670262.2017.1365177.

Brown, M.R., Jeffrey, S.W., Volkman, J.K., Dunstan, G.A., 1997. Nutritional properties of microalgae for mariculture. In: Aquaculture, Fish Nutrition and Feeding Proceedings of the Sixth International Symposium on Feeding and Nutrition in Fish, 151, pp. 315–331. https://doi.org/10.1016/S0044-8486(96)01501-3.

Carney, L.T., Wilkenfeld, J.S., Lane, P.D., Solberg, O.D., Fuqua, Z.B., Cornelius, N.G., Gillespie, S., Williams, K.P., Samocha, T.M., Lane, T.W., 2016. Pond Crash Forensics: Presumptive identification of pond crash agents by next generation sequencing in replicate raceway mass cultures of Nannochloropsis salina. Algal Res. 17, 341–347. https://doi.org/10.1016/j.algal.2016.05.011.

Clarizia, L., Russo, D., Di Somma, I., Marotta, R., Andreozzi, R., 2017. Homogeneous photo-Fenton processes at near neutral pH: a review. Appl. Catal. B 209, 358–371. https://doi.org/10.1016/j.apcatb.2017.03.011.

Davis, D.A., Arnold, C.R., 1997. Tolerance of the rotifer Brachionus plicatilis to ozone and total oxidative residuals. Ozone: Sci. Eng. 19, 457–469. https://doi.org/10.1080/01919512.1997.10382871.

Davis, R., Aden, A., Pienkos, P.T., 2011. Techno-economic analysis of autotrophic microalgae for fuel production. Appl. Energy Spec. Issue Energy Algae 88, 3524–3531. https://doi.org/10.1016/j.apenergy.2011.04.018.

Day, J.G., Gong, Y., Hu, Q., 2017. Microzooplanktonic grazers – A potentially devastating threat to the commercial success of microalgal mass culture. Algal Res. 27, 356–365. https://doi.org/10.1016/j.algal.2017.08.024.

de la Obra Jiménez, I., Giannakis, S., Grandjean, D., Breider, F., Grunauer, G., Casas López, J.L., Sánchez Pérez, J.A., Pulgarin, C., 2020. Unfolding the action mode of light and homogeneous vs. heterogeneous photo-Fenton in bacteria disinfection and concurrent elimination of micropollutants in urban wastewater, mediated by iron oxides in Raceway Pond Reactors. Appl. Catal. B 263, 118158. https://doi.org/10.1016/j.apcatb.2019.118158.

Deruyck, B., Nguyen, T.K.H., Praveenkumar, R., Muylaert, K., 2019a. Low doses of the quaternary ammonium salt Cetyltrimethylammonium bromide can be used as a pesticide to control grazers in microalgal cultures. Algal Res. 41, 101570 https://doi.org/10.1016/j.algal.2019.101570.

Deruyck, B., Thi Nguyen, K.H., Decaestecker, E., Muylaert, K., 2019b. Modeling the impact of rotifer contamination on microalgal production in open pond, photobioreactor and thin layer cultivation systems. Algal Res. 38, 101398 https://doi.org/10.1016/j.algal.2018.101398.

Douillet, P., 1998. Disinfection of rotifer cysts leading to bacteria-free populations. J. Exp. Mar. Biol. Ecol. 224, 183–192. https://doi.org/10.1016/S0022-0981(97)00200-1.

Doušková, I., Kaštánek, F., Maléterová, Y., Kaštánek, P., Doucha, J., Zachleder, V., 2010. Utilization of distillery stillage for energy generation and concurrent production of valuable microalgal biomass in the sequence: Biogas-cogeneration-microalgae-products. Energy Convers. Manage. 51, 606–611. https://doi.org/10.1016/j.enconman.2009.11.008.

Dumont, H.J., Van de Velde, I., Dumont, S., 1975. The dry weight estimate of biomass in a selection of Cladocera, Copepoda and Rotifera from the plankton, periphyton and benthos of continental waters. Oecologia 19, 75–97. https://doi.org/10.1007/BF00377592.

EPA, 2002. Method for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organims.

Esteban García, B., Rivas, G., Arzate, S., Sánchez Pérez, J.A., 2018. Wild bacteria inactivation in WWTP secondary effluents by solar photo-fenton at neutral pH in raceway pond reactors. In: Catalysis Today, 5th European Conference on Environmental Applications of Advanced Oxidation Processes, 313, pp. 72–78. https://doi.org/10.1016/j.cattod.2017.10.031.

Farinelli, G., Giagnorio, M., Ricceri, F., Giannakis, S., Tiraferri, A., 2021. Evaluation of the effectiveness, safety, and feasibility of 9 potential biocides to disinfect acidic landfill leachate from algae and bacteria. Water Res. 191, 116801 https://doi.org/10.1016/j.watres.2020.116801.

Fussmann, G.F., Kramer, G., Labib, M., 2007. Incomplete induction of mixis in Brachionus calyciflorus: patterns of reproduction at the individual level. Hydrobiologia 593, 111–119. https://doi.org/10.1007/s10750-007-9041-1.

Giannakis, S., 2018. Analogies and differences among bacterial and viral disinfection by the photo-Fenton process at neutral pH: a mini review. Environ. Sci. 25, 27676–27692. https://doi.org/10.1007/s11356-017-0926-x.

Giannakis, S., Samoili, S., Rodríguez-Chueca, J., 2021. A meta-analysis of the scientific literature on (photo)Fenton and persulfate advanced oxidation processes: Where do we stand and where are we heading to? Curr. Opin. Green Sustain. Chem. 29, 100456 https://doi.org/10.1016/j.cogsc.2021.100456.

Gilbert, J.J., 2018. Morphological variation and its significance in a polymorphic Rotifer: environmental, endogenous, and genetic controls. Bioscience 68, 169–181. https://doi.org/10.1093/biosci/bix162.

Golub, N.B., Tsvetkovych, M., Levtun, I.I., Maksyn, V.I., 2018. Nanostructured ferric citrate effect on chlorella vulgaris development. Biotechnol. Acta 11, 47–54. https://doi.org/10.15407/biotech11.06.047.

Gonçalves, A.A., Gagnon, G.A., 2011. Ozone application in recirculating aquaculture system: an overview. Ozone: Sci. Eng. 33, 345–367. https://doi.org/10.1080/01919512.2011.604595.

Han, C., Kim, H.-J., Lee, J.-S., Sakakura, Y., Hagiwara, A., 2021. Species-specific effects of iron on temperate and tropical marine rotifers in reproduction, lipid and ROS metabolisms. Chemosphere 277, 130317. https://doi.org/10.1016/j.chemosphere.2021.130317.

Huang, Y., Li, L., Liu, J., Lin, W., 2014a. Botanical pesticides as potential rotifer-control agents in microalgal mass culture. Algal Res. Progr. Perspect. Microalgal Mass Cult. 4, 62–69. https://doi.org/10.1016/j.algal.2013.08.001.

Huang, Y., Liu, J., Wang, H., Gao, Z., 2014b. Treatment potential of a synergistic botanical pesticide combination for rotifer extermination during outdoor mass cultivation of Spirulina platensis. Algal Res. 6, 139–144. https://doi.org/10.1016/j.algal.2014.11.003.

Jha, D., Jain, V., Sharma, B., Kant, A., Garlapati, V.K., 2017. Microalgae-based pharmaceuticals and nutraceuticals: an emerging field with immense market potential. ChemBioEng Rev. 4, 257–272. https://doi.org/10.1002/cben.201600023.

Kim, D., Kim, E.K., Koh, H.G., Kim, K., Han, J.-I., Chang, Y.K., 2017. Selective removal of rotifers in microalgae cultivation using hydrodynamic cavitation. Algal Res. 28, 24–29. https://doi.org/10.1016/j.algal.2017.09.026.

Marchetti, A., Maldonado, M.T., 2016. Iron. In: Borowitzka, M.A., Beardall, J., Raven, J. A. (Eds.), The Physiology of Microalgae, Developments in Applied Phycology. Springer International Publishing, Cham, pp. 233–279. https://doi.org/10.1007/978-3-319-24945-2_11.

Molina, D., de Carvalho, J.C., Júnior, A.I.M., Faulds, C., Bertrand, E., Soccol, C.R., 2019. Biological contamination and its chemical control in microalgal mass cultures. Appl. Microbiol. Biotechnol. 103, 9345–9358. https://doi.org/10.1007/s00253-019-10193-7.

Mosteo, R., Varon Lopez, A., Muzard, D., Benitez, N., Giannakis, S., Pulgarin, C., 2020. Visible light plays a significant role during bacterial inactivation by the photo-fenton process, even at sub-critical light intensities. Water Res. 174, 115636 https://doi.org/10.1016/j.watres.2020.115636.

Nandini, S., Sarma, S.S.S., Amador-López, R.J., Bolaños-Muñioz, S., 2007. Population growth and body size in five rotifer species in response to variable food concentration. J. Freshwater Ecol. 22, 1–10. https://doi.org/10.1080/02705060.2007.9664139.

O'Dowd, K., Pillai, S.C., 2020. Photo-Fenton disinfection at near neutral pH: process, parameter optimization and recent advances. J. Environ. Chem. Eng. 8, 104063 https://doi.org/10.1016/j.jece.2020.104063.

Ortega-Gómez, E., Ballesteros Martín, M.M., Carratalà, A., Fernández Ibañez, P., Sánchez Pérez, J.A., Pulgarín, C., 2015. Principal parameters affecting virus inactivation by the solar photo-Fenton process at neutral pH and μM concentrations of H2O2 and Fe2+/3+. Appl. Catal. B 174–175, 395–402. https://doi.org/10.1016/j.apcatb.2015.03.016.

Park, S., Ginkel, S.W.V., Pradeep, P., Igou, T., Yi, C., Snell, T., Chen, Y., 2016. The selective use of hypochlorite to prevent pond crashes for algae-biofuel production. Water Environ. Res. 88, 70–78. https://doi.org/10.2175/106143015X14362865227670.

Phan, N.-T., Duong, Q.H., Tran-Nguyen, Q.A., Trinh-Dang, M., 2021. The species diversity of tropical freshwater rotifers (Rotifera: Monogononta) in relation to environmental factors. Water 13, 1156. https://doi.org/10.3390/w13091156.

Pignatello, J.J., Oliveros, E., MacKay, A., 2006. Advanced oxidation processes for organic contaminant destruction based on the fenton reaction and related chemistry. Crit. Rev. Environ. Sci. Technol. 36, 1–84. https://doi.org/10.1080/10643380500326564.

Polo-López, M.I., Sánchez Pérez, J.A., 2021. Perspectives of the solar photo-Fenton process against the spreading of pathogens, antibiotic-resistant bacteria and genes in the environment. Curr. Opin. Green Sustain. Chem. 27, 100416 https://doi.org/10.1016/j.cogsc.2020.100416.

Pulgarin, A., Giannakis, S., Pulgarin, C., Ludwig, C., Refardt, D., 2020. A novel proposition for a citrate-modified photo-Fenton process against bacterial contamination of microalgae cultures. Appl. Catal. B 265, 118615. https://doi.org/10.1016/j.apcatb.2020.118615.

Rico-Martínez, R., Dodson, S.I., 1992. Culture of the rotifer Brachionus calyciflorus Pallas. Aquaculture 105, 191–199. https://doi.org/10.1016/0044-8486(92)90130-D.

Romero-Martínez, L., Rivas-Zaballos, I., Moreno-Andrés, J., Moreno-Garrido, I., Acevedo-Merino, A., Nebot, E., 2021. Improving the microalgae inactivating efficacy of ultraviolet ballast water treatment in combination with hydrogen peroxide or peroxymonosulfate salt. Mar. Pollut. Bull. 162, 111886 https://doi.org/10.1016/j.marpolbul.2020.111886.

Ruales-Lonfat, C., Barona, J.F., Sienkiewicz, A., Vélez, J., Benítez, L.N., Pulgarín, C., 2016. Bacterial inactivation with iron citrate complex: A new source of dissolved iron in solar photo-Fenton process at near-neutral and alkaline pH. Appl. Catal. B 180, 379–390. https://doi.org/10.1016/j.apcatb.2015.06.030.

Singh, D.P., Khattar, J.S., Rajput, A., Chaudhary, R., Singh, R., 2019. High production of carotenoids by the green microalga Asterarcys quadricellulare PUMCC 5.1.1 under optimized culture conditions. PLoS One 14, e0221930. https://doi.org/10.1371/journal.pone.0221930.

Sirakov, I., Velichkova, K., Stoyanova, S., Staykov, Y., 2015. The importance of microalgae for aquaculture industry. Review. Int. J. Fisher. Aquat. Stud. 2 (4), 81–84.

Slade, R., Bauen, A., 2013. Micro-algae cultivation for biofuels: cost, energy balance, environmental impacts and future prospects. In: Biomass and Bioenergy, 20th European Biomass Conference, 53, pp. 29–38. https://doi.org/10.1016/j.biombioe.2012.12.019.

Torres, M.de A., Liz, M.V.de, Martins, L.R.R., Freitas, A.M., 2018. Does the photo-Fenton reaction work for microalgae control? A case study with Desmodesmus subspicatus. Photochem. Photobiol. Sci. 17, 517–521. https://doi.org/10.1039/C7PP00443E.

Tredici, M.R., Rodolfi, L., Biondi, N., Bassi, N., Sampietro, G., 2016. Techno-economic analysis of microalgal biomass production in a 1-ha Green Wall Panel (GWP®) plant. Algal Res. 19, 253–263. https://doi.org/10.1016/j.algal.2016.09.005.

Udayan, A., Sirohi, R., Sreekumar, N., Sang, B.-I., Sim, S.J., 2022. Mass cultivation and harvesting of microalgal biomass: Current trends and future perspectives. Bioresour. Technol. 344, 126406 https://doi.org/10.1016/j.biortech.2021.126406.

Uysal, O., Uysal, F.O., Ekinci, K., 2015. Evaluation of microalgae as microbial fertilizer. Eur. J. Sustain. Dev. 4 https://doi.org/10.14207/ejsd.2015.v4n2p77, 77–77.

Vaz, B., da, S., Moreira, J.B., Morais, M.G.de, Costa, J.A.V., 2016. Microalgae as a new source of bioactive compounds in food supplements. Current Opinion in Food Science. Food Chem. Biochem. Food Bioprocess. 7, 73–77. https://doi.org/10.1016/j.cofs.2015.12.006.

Viollier, E., Inglett, P.W., Hunter, K., Roychoudhury, A.N., Van Cappellen, P., 2000. The ferrozine method revisited: Fe(II)/Fe(III) determination in natural waters. Appl. Geochem. 15, 785–790. https://doi.org/10.1016/S0883-2927(99)00097-9.

Walsh, E.J., Smith, H.A., Wallace, R.L., 2014. Rotifers of temporary waters. Int. Rev. Hydrobiol. 99, 3–19. https://doi.org/10.1002/iroh.201301700.

Yan, N., Fan, C., Chen, Y., Hu, Z., 2016. The potential for microalgae as bioreactors to produce pharmaceuticals. Int. J. Mol. Sci. 17, E962 https://doi.org/10.3390/ijms17060962.

Yang, W., Gao, X., Wu, Y., Wan, L., Lu, C., Huang, J., Chen, H., Yang, Y., Ding, H., Zhang, W., 2021. Chemical- and species-specific toxicity of nonylphenol and octylphenol to microalgae Chlorella pyrenoidosa and Scenedesmus obliquus. Environ. Toxicol. Pharmacol. 81, 103517 https://doi.org/10.1016/j.etap.2020.103517.

Yuan, D., Zhan, X., Wang, M., Wang, X., Feng, W., Gong, Y., Hu, Q., 2018. Biodiversity and distribution of microzooplankton in Spirulina (Arthrospira) platensis mass cultures throughout China. Algal Res. 30, 38–49. https://doi.org/10.1016/j.algal.2017.12.009.

Zanchetta, E., Damergi, E., Patel, B., Borgmeyer, T., Pick, H., Pulgarin, A., Ludwig, C., 2021. Algal cellulose, production and potential use in plastics: Challenges and opportunities. Algal Res. 56, 102288 https://doi.org/10.1016/j.algal.2021.102288.

Zewdie, D.T., Ali, A.Y., 2020. Cultivation of microalgae for biofuel production: coupling with sugarcane-processing factories. Energy Sustain. Soc. 10, 27 https://doi.org/10.1186/s13705-020-00262-5.

Zhan, J., Rong, J., Wang, Q., 2017. Mixotrophic cultivation, a preferable microalgae cultivation mode for biomass/bioenergy production, and bioremediation, advances and prospect. Int. J. Hydrogen Energy 42, 8505–8517. https://doi.org/10.1016/j.ijhydene.2016.12.021.

Zhang, A., Wen, X., Wang, K., Huo, Y., Geng, Y., Ding, Y., Li, Y., 2021. Using surfactants for controlling rotifer contamination in mass cultivation of Chlorella pyrenoidosa. Algal Res. 53, 102166 https://doi.org/10.1016/j.algal.2020.102166.

Zhu, Z., Jiang, J., Fa, Y., 2020. Overcoming the biological contamination in microalgae and cyanobacteria mass cultivations for photosynthetic biofuel production. Molecules 25, 5220. https://doi.org/10.3390/molecules25225220.