Photosynthesis Research
https://doi.org/10.1007/s11120-020-00756-5

ORIGINAL ARTICLE

Biodegradation of phenol by *Chlamydomonas reinhardtii*

Theocharis T. Nazos[1] · Leonidas Mavroudakis[1] · Spiros A. Pergantis[1] · Demetrios F. Ghanotakis[1]

Received: 22 January 2020 / Accepted: 22 April 2020

Abstract

The data presented in this particular study demonstrate that the biodegradation of phenol by *Chlamydomonas reinhardtii* is a dynamic bioenergetic process mainly affected by the production of catechol and the presence of a growth-promoting substrate in the culture medium. The study focused on the regulation of the bioenergetic equilibrium resulting from production of catechol after phenol oxidation. Catechol was identified by HPLC-UV and HPLC-ESI-MS/MS. Growth measurements revealed that phenol is a growth-limiting substrate for microalgal cultures. The *Chlamydomonas* cells proceed to phenol biodegradation because they require carbon reserves for maintenance of homeostasis. In the presence of acetic acid (a growth-promoting carbon source), the amount of catechol detected in the culture medium was negligible; apparently, acetic acid provides microalgae with sufficient energy reserves to further biodegrade catechol. It has been shown that when microalgae do not have sufficient energy reserves, a significant amount of catechol is released into the culture medium. *Chlamydomonas reinhardtii* acts as a versatile bioenergetic machine by regulating its metabolism under each particular set of growth conditions, in order to achieve an optimal balance between growth, homeostasis maintenance and biodegradation of phenol. The novel findings of this study reveal a paradigm showing how microalgal metabolic versatility can be used in the bioremediation of the environment and in potential large-scale applications.

Keywords Phenol · Catechol · Biodegradation · *Chlamydomonas reinhardtii* · Bioenergetics · HPLC-ESI-MS/MS

Abbreviations

HPLC-ESI-MS/MS	High-performance liquid chromatography-electrospray ionization tandem mass spectrometry
F_o	Minimum fluorescence that corresponds to the time that all photosynthetic reaction centers are open
F_{max}	Maximal fluorescence that corresponds to the time that all reaction centers are closed
F_v,	Variable fluorescence ($F_{max} - F_o$)
F_v/F_{max}	Photosynthetic efficiency
MRM	Multiple Reaction Monitoring
TAP	Tris-Acetate-Phosphate medium used for the growth of microalgae and in experimental cultures
TP	Tris-Phosphate medium used in experimental cultures

Introduction

Increased industrial activity has led to the release and accumulation of significant amounts of phenolic compounds in the environment (Stoilova et al. 2006). Phenol and its derivatives are common contaminants of terrestrial and aquatic environments and as a result, these compounds have been categorized as priority pollutants by the US Environmental Protection Agency (EPA) (Agarry et al. 2008; Du et al. 2009). An approach for the decrease of high concentrations of phenolic compounds from ecosystems relies on their biodegradation by microorganisms (Van Schie and Young 2000). Metabolic pathways, various bioreactor types, energetic approaches and a significant number of microorganisms (mainly bacteria and fungi) have been identified (Al-Khalid and El-Naas 2012).

The first step of the aerobic biodegradation of phenol includes the conversion of phenol to catechol by the enzyme phenol hydroxylase that uses molecular oxygen. The catechol aromatic ring can be cleaved either by *ortho* or *meta* pathways. In the *ortho* pathway (β-ketoadipate pathway), the enzyme 1,2-dioxygenase cleaves the bond between the two carbons connected with the hydroxide groups, to form

✉ Demetrios F. Ghanotakis
ghanotakis@uoc.gr

[1] Department of Chemistry, University of Crete, Vasilika Voutes, Heraklion, Crete 70013, Greece

Published online: 02 May 2020

cis, cis- muconic acid. This intermediate is subsequently metabolized through multiple steps to succinic acid and acetyl-CoA. In the *meta*-cleavage pathway, ring cleavage takes place in the adjacent carbon–carbon bond of the one hydroxide group by 2,3-dioxygenase. As a result, 2-hydroxy-muconic semialdehyde is formed, which is finally converted to acetaldehyde and pyruvate (Fig. 1). It appears that the strategy followed is initially to decrease the complexity of the mother compound followed by its conversion to products that can be utilized by microbial metabolism (Williams and Sayers 1994; Van Schie and Young 2000; Zhou et al. 2016). These pathways have been found to exhibit many similarities both in bacteria and unicellular microalgae.

Green microalgae are a class of well adapted microorganisms that can grow in unfavorable environmental conditions because they possess versatile metabolic networks (Mata et al. 2010; Satyanarayana et al. 2011; Koller et al. 2014). Microalgae have been successfully grown in municipal, agricultural and industrial waste-waters in order to reduce their phosphorous, nitrogen, micronutrient and organic content (Guldhe et al. 2017). The use of microalgae for wastewater bioremediation applications has been proposed as a win–win biorefinery paradigm, because the resulting biomass could be used for a series of applications such as biodiesel production (Delrue et al. 2016).

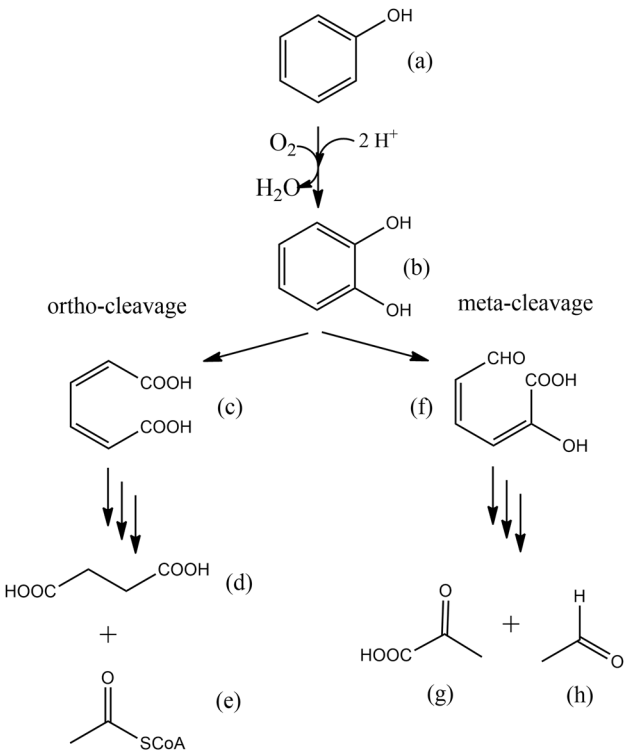

Fig. 1 Metabolic pathways for aerobic biodegradation of phenol. Bond-line structures **a** phenol; **b** catechol; **c** cis, cis-muconic acid; **d** succinic acid; **e** acetyl-CoA; **f** 2-hydroxymuconic semialdehyde; **g** pyruvate and **h** acetaldehyde

Phenol and its substituted derivatives inhibit growth of green microalgae (Shigeoka et al. 1988). The presence of phenol in cultures of marine microalgae upregulates genes attributed to the expression of antioxidant enzymes and biosynthesis of carotenoids (Zhou et al. 2017), production of reactive oxygen species (ROS) as well as a decrease in chlorophyll content (Duan et al. 2017). Despite these observations, several studies have proposed the efficient use of freshwater and marine microalgae for biodegradation of phenolic compounds and have provided a few mechanistic insights. Semple and Cain (1996) elucidated the mechanism of phenol biodegradation in the brown chrysophyte microalga *Ochromonas danika*. They showed that phenol was initially converted to catechol, which was subsequently catabolized through the *meta*-cleavage pathway. The cyanobacterium *Synechococcus* PCC 7002 was able to perform extracellular biodegradation of phenol via the *ortho* cleavage pathway. A significant finding was that phenol was only biodegraded in the dark, while under photoautotropic and photoheterotrophic conditions no biodegradation activity was observed (Wurster et al. 2003). Otto and Schlosser (2014) isolated and characterized an extracellular laccase, produced by the green microalga *Tetracystis aeria,* that is related to the oxidation of phenolic compounds. The freshwater green alga *Monoraphidium braunii* was able to degrade significant amounts of bisphenol-A, while at the same time physiological changes, such as a decrease of photosystem II activity and decreases in cell size, were also observed (Gattullo et al. 2012). The alga *Cyclotella caspia* was shown to biodegrade nonylphenol. At increased concentrations of this phenol, the chlorophyll *a* content and cell growth rate were significantly decreased. Biodegradation rates of nonylphenol were high after an adaptation period of 48 h, while after 144 h no further biodegradation of the substance was observed (Liu et al. 2013). The marine microalga *Tetraselmis marina* was found to detoxify and degrade 2,4-dichlorophenol using a glucose transfer followed by a malonyl transfer (Petroutsos et al. 2008). Recently, Das et al. (2015) discovered that *Chlorella pyrenoidosa* biodegraded phenol both via *ortho* and *meta*-cleavage pathways. The *ortho* pathway was found to be significantly more active than the *meta* pathway. A remarkable observation was the accumulation of catechol, cis-cis muconic acid and 2,3-hydroxy-muconic semialdehyde intermediates in the growth medium. *Spirulina maxima* was found to biodegrade phenol up to a concentration of 400 mg/L in 24 h and studies with ^{13}C-labeled carbon demonstrated that 30% of the phenol carbon was assimilated in biomass along with its mineralization to CO_2 (Lee et al. 2015). In the marine microalga *Lingulodinium polyedrum* phenol biotransformation was catalyzed by glutathione S-transferase, phenol hydroxylase and catechol

2,3-dihydroxygenase metabolic pathways, while at the same time phenol was found to induce the activity of antioxidant enzymes as a response to oxidative stress (Martins et al. 2015).

Apart from the mechanistic studies presented above, several published studies have focused on the role of bioenergetic conditions (such as the presence of an alternative carbon source and light intensity that provide sufficient energy to microalgal cells) in the biodegradation of phenolic compounds. Priyadharshini and Bakthavatsalam (2016) modeled and optimized phenol biodegradation by *Chlorella pyrenoidosa* and found out that it was affected by the interaction of various factors, such as phenol concentration, initial algal concentration and reaction time. It has been reported in the literature that the presence of an alternative organic carbon source plays an important role in the alleviation of toxicity effects of phenolic compounds on green microalgae (Megharaj et al. 1992). Theoretical studies suggest that the presence of an alternative carbon source that promotes growth could inhibit the process of biodegradation because both substrates require sufficient amounts of oxygen in order to be metabolized (Lika and Papadakis 2009). Papazi and Kotzabasis (2007) showed that the biodegradability of different types of monosubstituted phenols by *Scenedesmus obliquus* was based on the selection of appropriate culture conditions as well as the type of phenolic compound tested. Based on their data, it was proposed that monosubstituted halogenated phenols were biodegraded initially by dehalogenation followed by fission of the phenolic ring. When cultures were supplemented with glucose or CO_2 as carbon sources, an increased biodegradability took place, while the biodegradation process was found to be photosynthetically dependent. On the other hand, low concentrations of phenol and monosubstituted methylphenols exhibited an increased biodegradability in the absence of an alternative carbon source in the culture medium (Papazi and Kotzabasis 2007, 2008). Biodegradation of *para*-cresol by *Scenedesmus* cells was found to be a two-step process. Removal of the methyl group from the phenolic ring led to the production of phenol, which was further metabolized (Papazi et al. 2012). Regarding the biodegradation of dichlorophenols, *Scenedesmus* cells were found to follow a rational strategy, based on a bioenergetic balance between the type of the compound, growth, as well as the presence of glucose in the culture medium (Papazi and Kotzabasis 2013). All these data prove the metabolic versatility of photosynthetic microorganisms and demonstrate that they can be efficient systems for the biodegradation of phenols.

Chlamydomonas reinhardtii is a well-studied model microorganism that has provided many insights in the elucidation of the photosynthetic mechanism and genetics in microalgae (Goodenough et al. 1995; Rochaix et al. 1998; Harris 2009). This microalga possesses versatile metabolic networks regarding the carbon uptake and utilization, and moreover it can grow in autotrophic, heterotrophic and mixotrophic conditions (Hippler et al. 1998; Johnson and Alric 2013). Depending on growth conditions, *Chlamydomonas* cells exhibit a different response to exogenous conditions that induce stress (Endo and Asada 1996). In a recent publication we showed that the green microalga *Chlamydomonas reinhardtii* biodegraded significant amounts of phenol (Nazos et al. 2017). This work was focused on the bioenergetics of the biodegradation process under conditions affecting photosynthesis. It was shown that biodegradation of phenol by *Chlamydomonas reinhardtii* is a photoregulated, aerobic process. High concentrations of phenol were found to induce higher levels of biodegradation as a response to stress, while at the same time the presence of an alternative organic carbon source (acetic acid) had a prominent role in the alleviation of stress effects.

The research presented here provides new results on biodegradation of phenol by axenic cultures of the green microalga *Chlamydomonas reinhardtii,* including characterization of a catechol molecule that is produced as metabolite. In addition, the role of the presence or absence of an alternative organic carbon source on the regulation of the bioenergetic equilibrium of the biodegradation of phenol by *Chlamydomonas reinhardtii* is characterized.

Materials and methods

Organism and growth conditions

The CC-125 strain (wild type) of *Chlamydomonas reinhardtii* was used in all experiments. Mother cultures were grown photoheterotrophically in Tris- Acetate- Phosphate (TAP) medium (Harris 2009) at 25 °C, under a continuous light intensity of 70–80 μmol photons·m^{-2}·s^{-1} for five days in Erlenmeyer flasks shaken at a rate of 140 min^{-1}. For the preparation of experimental cultures, cells were first collected by centrifugation at 1000 *g* for 3 min., washed twice by resuspension in fresh medium (depending on each particular experimental condition); 50 mL aliquots were transferred into 100 mL Erlenmeyer flasks closed tightly with septa. Initial cell concentration in the experimental cultures was adjusted to be equal to 3.633 ($\pm$0.528) ·10^7 cells·mL^{-1}.

Two different experimental culture conditions were tested; one with added acetic acid (1.048 g/L) as an alternative carbon source and another containing only phenol. In the first experimental condition, TAP was used as growth medium while in the second cells were cultivated in Tris–phosphate (TP) medium. The final phenol concentration in the experimental cultures was 4.0 mM (376.4 mg/L). Phenol was dissolved in double distilled water, and the solution was filtered through a 0.2 μm syringe filter. The phenol

stock solution's concentration was 0.5 M. Parallel cultures of *Chlamydomonas* cells in TAP and TP mediums were the corresponding controls for the two experimental conditions described.

All experiments were repeated at least three times. All nutrient media and glassware were autoclaved at 120 °C for 20 min, in order to avoid contamination. The experiments were performed in a temperature-controlled room (25 °C) under a continuous light intensity of 70–80 µmol photons·m^{-2}·s^{-1}. Cultures were shaken at 140 min^{-1} on a rotary shaker. Experiments were carried out in a laminar flow hood fitted with a UV lamp and sterilized with ethanol.

High-performance liquid chromatography (HPLC) for the analysis of phenol and catechol

For phenol and catechol analysis, culture samples were centrifuged for 5 min at 1000 *g*. The supernatants were filtered through a 0.2 µm syringe filter and were diluted with the mobile phase and then injected through a 20 µL loop into the HPLC system. Analyses were performed using an HPLC system equipped with a high precision pump (LC-10AD, Shimadzu), a UV–Vis detector (SPD 10AV, Shimadzu) and a C18 column (Grace Smart RP18, 250 mm l and 4.6 mm ID, 5 µm particle size). Analyses were performed at 25 °C. An isocratic HPLC method, using as a mobile phase methanol: water: acetic acid (50:49:1), and a flow rate of 1.0 mL· min^{-1} according to the protocol of Lovell et al. (2002). Detection was carried out by measuring absorbance at 279 nm and quantification by integration of known quantities of phenol and catechol.

Catechol identification with electrospray ionization mass spectrometry coupled with high-performance liquid chromatography (HPLC-ESI-MS/MS) using the multiple reaction monitoring (MRM) method

For the identification of the catechol product, a 500 µg L^{-1} catechol standard and a culture sample collected after 5 days of incubation, diluted 100 times in methanol: water: acetic acid (50:49:1), were infused in a TSQ Quantum triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA) through a syringe pump at a flow rate of 20 µL·min^{-1}. Mass spectra were obtained with an electrospray source in the negative ionization mode through tandem mass spectrometry (MS/MS) in order to obtain the fragmentation pattern of catechol. Having acquired the fragmentation pattern of catechol, the method was coupled with a High-performance liquid chromatography system (HPLC–ESI–MS/MS). For the reversed phase HPLC–ESI–MS/MS method, a C18 column (Grace Smart RP18, 250 mm l and 4.6 mm ID, 5 µm particle size) was used. The mobile phase was methanol: water: acetic acid (50:49:1) at a flow rate of 0.5 mL· min^{-1}. Technique was operated in the negative Multiple Reaction Monitoring (MRM) mode and identification of catechol in the sample was carried out by comparing the retention time of the selected reaction of catechol fragmentation products in the culture sample with those obtained for the catechol standard. All measurements were performed using the following operation parameters: electrospray voltage – 3.5 kV, sheath gas pressure 35 (arbitrary units), auxiliary gas pressure 10 (arbitrary units), ion transfer capillary temperature 320 °C and source collision- induced dissociation (CID) 12 eV.

Determination of growth

Cell growth was determined by cell counting using an improved Neubauer cell counting chamber (Milliscience) and a Nikon Eclipse E800 optical microscope. Cell density was expressed as number of cells · mL^{-1}. Culture samples were collected every 24 h with a sterile syringe. Sample collection was carried out in a laminar flow hood with a UV lamp sterilized with ethanol.

Fluorescence induction measurements

The fluorescence induction measurements were carried out using the Handy Plant Efficiency Analyser (Hansatech Instruments, Kings' Lynn, Norfolk, UK). The parameter that was measured was the maximal photosynthetic efficiency (F_v/F_{max}) according to the method of Strasser and Strasser (1995). This method is based on the measurement of a fast fluorescence transient with a 10 µs resolution for 1 s after a 10 min dark sample adaptation and yields a dynamic measurement of a photosynthetic sample at a given physiological state. Fluorescence was measured at 12-bit resolution and excited by three light-emitting diodes providing a saturating red (650 nm) light intensity of 3000 µmol photons·m^{-2}·s^{-1}.

Sample preparation and observation with optical and fluorescence microscopy

Culture samples were collected after a cultivation period of five days, using a sterile pipette tip. The observation of the cells was performed using a Nikon Eclipse E800 optical microscope and an optical oil-immersion Plan Apo 60× lens and a CCD ProgRes CF camera. For the fluorescence microscopy, excitation was produced by a UV lamp; fluorescence detection utilized a B-2A filter that allows only wavelengths in the region of 450–490 nm to pass. The chloroplast photosynthetic units exhibit red color fluorescence emission.

Results and discussion

Identification of the production of catechol by cultures of *Chlamydomonas reinhardtii* grown in the absence of acetic acid

Samples from cultures of *Chlamydomonas reinhardtii* cultivated in TP medium were collected after an incubation period of 10 days. After centrifugation the supernatant from cultures that had been incubated with 4.0 mM initial phenol concentration had a brownish color, a fact that could be attributed to the production of one or more metabolites by algal cells after phenol biotransformation (Fig. 2). No such color was observed for the samples of the corresponding cultures in TP medium where phenol was not present. This macroscopic observation was accompanied by changes in the chromatographic profile of the samples collected from the cultures (Fig. 3). More specifically, chromatographic profiles of culture samples collected at the beginning of the experiments and after a 10-day growth period exhibited significant differences. The peak that corresponds to phenol (retention time of 4.73 min) was found to decrease after 10 days of incubation, while at the same time appearance of three new peaks with retention time of 2.02, 2.32 and 3.15 min were observed. These peaks could be attributed to the production of possible metabolites. A comparison with the chromatograms of control cultures and the TAP medium indicated that the peak with a retention time 1.78 min corresponds

Fig. 2 Supernatant and pellet of ten-day old culture samples after centrifugation at 1000 *g* for 5 min. The left tube, control culture; right tube, culture incubated with 4.0 mM initial phenol concentration in the absence of an alternative carbon source (TP medium)

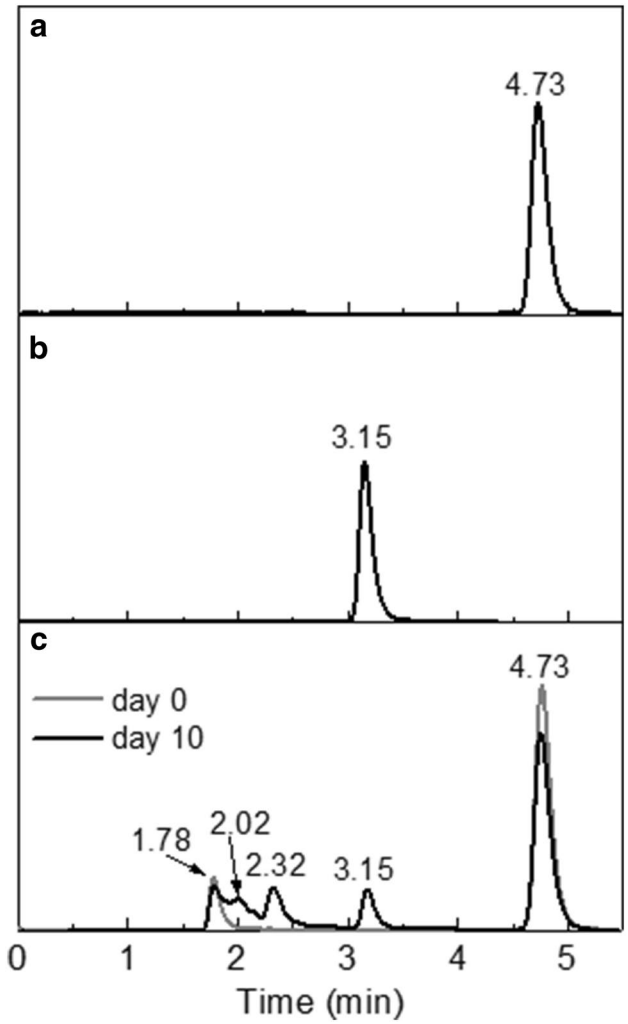

Fig. 3 HPLC–UV profiles of **a** standard phenol sample, **b** standard catechol sample and **c** culture supernatant from the initial (gray line) and from the 10th (black line) day of the experiment, using methanol: water: acetic acid (50:49:1) mobile phase with a flow rate of 1.0 mL/min. The retention time of each peak is shown with the corresponding number. Peak intensity was measured using the detector's arbitrary units

to one or more components of the TAP medium (data not shown). The peak with a retention time of 3.15 min probably corresponds to catechol, as shown by a comparison with the elution profiles of a catechol standard under the same HPLC operating conditions. To clarify these observations, further analysis with tandem mass spectrometry was carried out.

Figure 4 shows the comparison of the MS/MS spectra of the peak with *m/z* 109 of the standard catechol sample, which corresponds to the deprotonated ion of catechol, $[M–H]^-$, and the filtered supernatant of the sample collected from the algal cultures after five days of incubation. The fragmentation patterns (Fig. 4a, b, respectively) of the two MS/MS spectra, exhibit similar fragment ions with *m/z* values of 108, 91, 81, 65, and 53. These fragments are in agreement

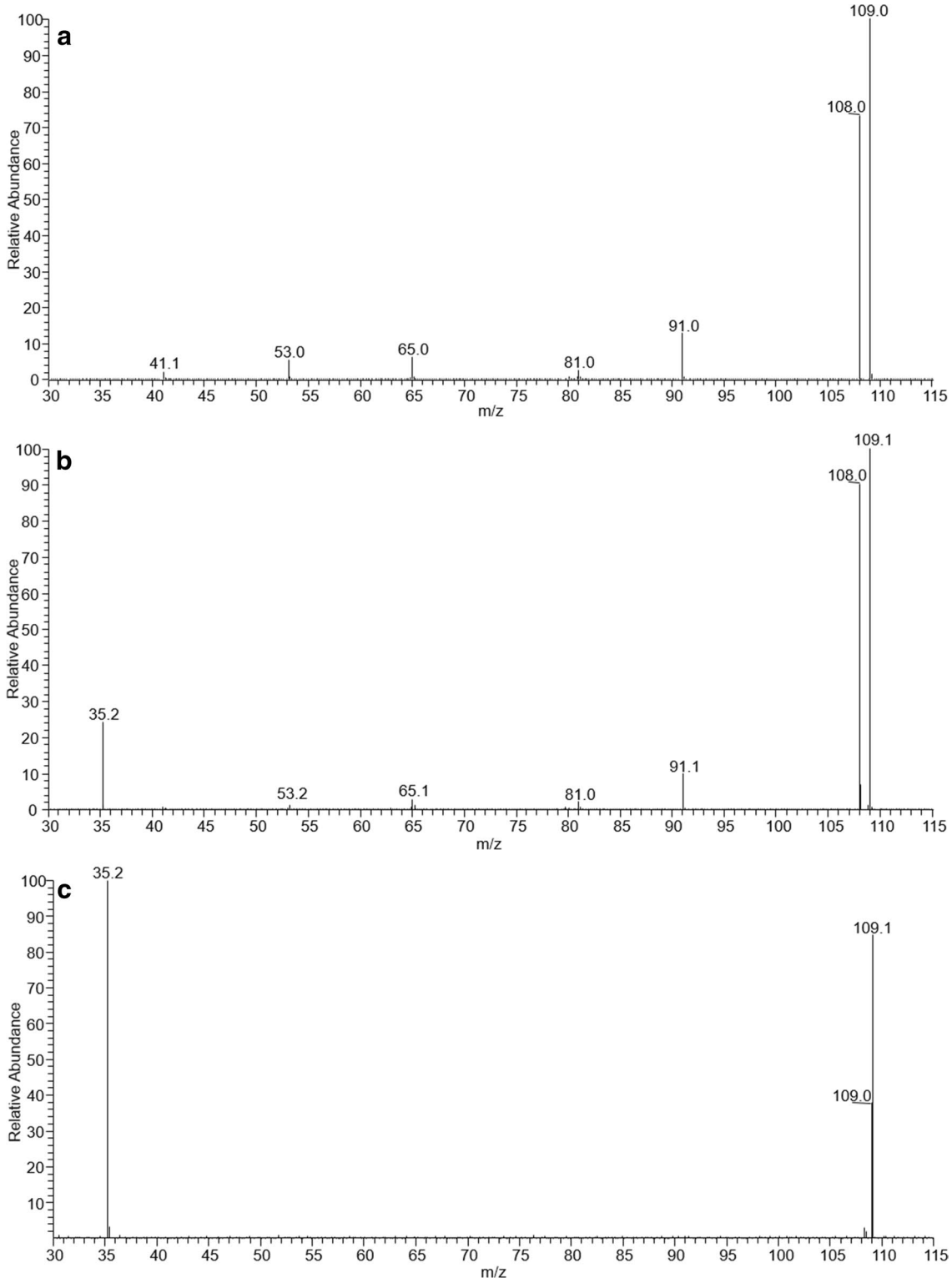

Fig. 4 ESI–MS/MS spectra of the peak with m/z = 109 observed in the negative ion mode. **a** 500 µg L^{-1} standard catechol sample, **b** culture sample collected after a five-day incubation period in TP medium with an initial phenol concentration equal to 4.0 mM, **c** culture sample collected from the after an incubation period of 5 days in TP medium. Cells were grown in TP medium with an initial phenol concentration of 4.0 mM under continuous light intensity of 70–80 µmol photons·m^{-2}·s^{-1}

with those of the fragmentation patterns of catechol previously described in the literature (Binkley et al. 1994). A difference between the two MS/MS spectra is that the spectrum of the standard catechol sample shows a low intensity peak with *m/z* 41, which is not present in the culture sample. This could be due to the lower concentration of catechol in the culture sample, compared to the standard catechol solution; in such a case, ions that are not abundant enough cannot reach the detection limits. In addition, another difference between the two mass spectra is the presence of a fragment with *m/z* 35 in the culture sample. This peak is attributed to the fragmentation of a background ion with *m/z* value of 109, as shown in the MS/MS spectrum of the control culture sample from the same experiment (Fig. 4c). HPLC-ESI-MS/MS analysis was used in order to identify the presence of catechol in the culture medium. The multiple reaction monitoring (MRM) method was used for the fragmentation reactions of 109 → 108 and 109 → 91, because of the high abundance of the corresponding ions in the MS/MS spectra (Fig. 4a, b). Figure 5 shows the chromatographic elution profiles acquired for both the catechol standard and the culture sample. In all cases only one peak with a retention time of 6.1 min was observed, which clearly proves the presence of catechol in the culture medium. Thus, it can be concluded that catechol is produced by the metabolism of phenol by *Chlamydomonas reinhardtii* cells. At this point, it is important to note that in experiments carried out under the same conditions in the absence of the cells, neither a decrease of phenol concentration nor of catechol production were recorded (data not shown).

Corresponding HPLC-UV profiles received at the beginning of the experiment and on the fifth and tenth day of incubation (Fig. 6) clearly show the accumulation of catechol in the culture medium with a respective decrease of the phenol peak. The same behavior was also observed for the other two uncharacterized peaks.

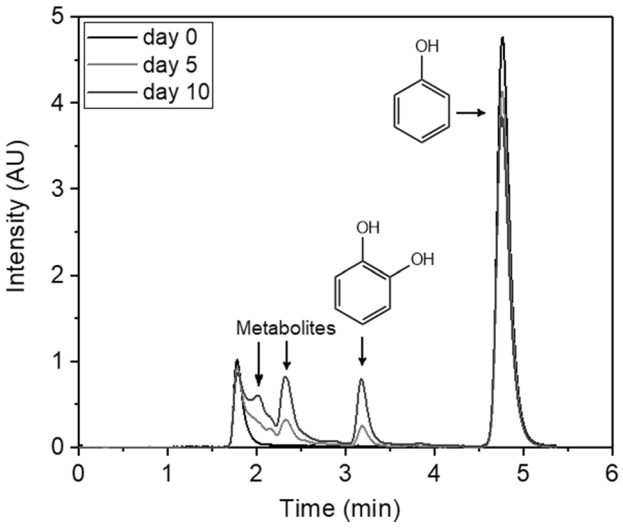

Fig. 6 HPLC–UV profile of the culture supernatant at the beginning of the experiment (black line), after an incubation period of five (light gray line) and ten (dark gray line) days, using the methanol: water: acetic acid (50:49:1) mobile phase with a flow rate of 1.0 mL/min. Peak intensity is given in the detector's arbitrary units. Cells were grown in TP medium with an initial phenol concentration of 4.0 mM under continuous light intensity of 70–80 µmol photons·m^{-2}·s^{-1}

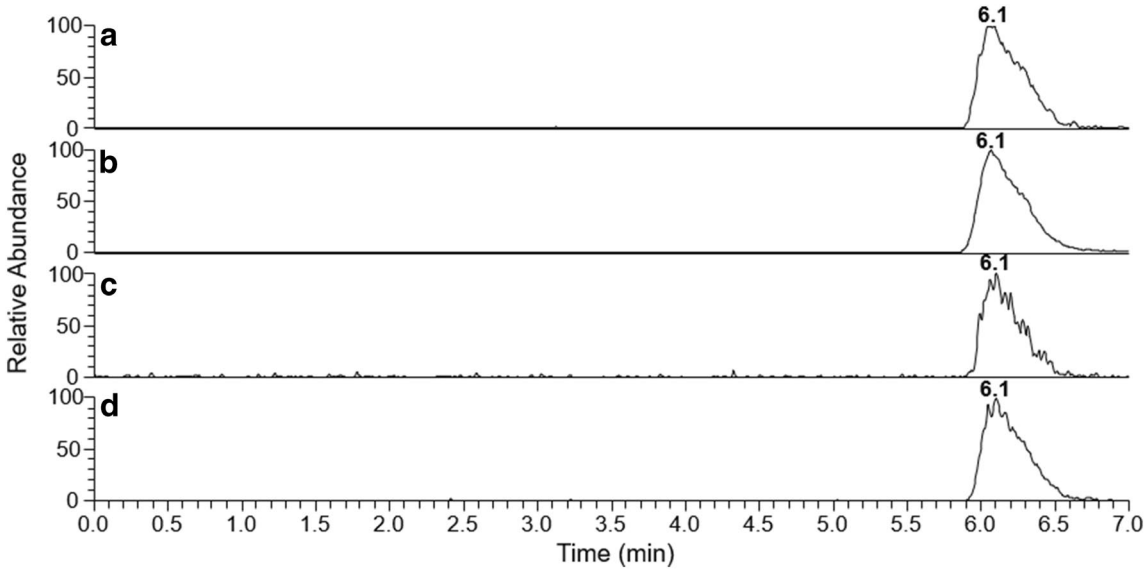

Fig. 5 HPLC–ESI–MS/MS chromatographic profiles, a mobile phase methanol: water: acetic acid (50:49:1) mobile phase at a flow rate of 0.5 mL/min. Monitoring of the reaction **a** 109 → 91 and **b** 109 → 108 in a 500 µg L^{-1} standard catechol sample. Monitoring of the reaction **c** 109 → 91 and **d** 109 → 108 of samples collected from the algal culture after an incubation period of five days. Cells were grown in TP medium with an initial phenol concentration of 4.0 mM under continuous light intensity of 70–80 µmol photons·m^{-2}·s^{-1}

Impact of the presence or absence of an alternative carbon source, acetic acid, in the culture medium on the bioenergetic equilibrium of catechol production by cultures of *Chlamydomonas reinhardtii*

As shown above, catechol is produced in the culture medium during phenol biodegradation by *Chlamydomonas reinhardtii*. The origin of this result was investigated. We have already shown that biodegradation of phenol by *Chlamydomonas reinhardtii* is a dynamic bioenergetic process affected by a set of factors including light intensity, oxygen availability, presence or absence of an alternative growth-promoting organic substrate as well as phenol concentration (Nazos et al. 2017). Two parallel sets of experiments were carried out, one in the presence of acetic acid as an extra carbon source, apart from phenol, in the culture medium (TAP medium) and another where phenol was the only carbon source for *Chlamydomonas* cells (TP medium). Figure 7a presents the growth of *Chlamydomonas* cultures in these conditions. In the absence of acetic acid, zero to negligible biomass increase was observed. During experiment, flasks were sealed with septum and as a result no CO_2 from the atmosphere was getting inside. Cells consumed the CO_2 that existed in the air and this resulted to negligible growth. On the other hand, in the presence of acetic acid phenol was found to cause inhibition of microalgal growth when compared to the corresponding cultures grown in TAP medium without phenol. This result is in agreement with our previous findings, which demonstrated that high concentrations of phenol inhibited algal growth (Nazos et al. 2017).

Removal of phenol and production of catechol in the absence or presence of acetic acid in the culture medium (Fig. 8) emphasizes the importance of an alternative carbon source in the culture medium. In the absence of acetic acid, an increased uptake of phenol by *Chlamydomonas* cells is observed in the first 48 h of incubation. This could be attributed to the fact that phenol is the only carbon source in the medium; cells consume phenol in order to synthesize carbon reserves. Compared to phenol removal rates in the absence of acetic acid (Fig. 9a), the maximum rate was recorded 48 h after the initiation of the experiment. Phenol removal was found to take place in cycles, with the maxima recorded on days 2, 5, and 8. For every maximum the value of the rate appears to be lower than the previous one, which indicates that after each cycle the ability of *Chlamydomonas* to take up phenol decreases. An interesting fact is that at the same time the catechol production rate starts to increase on the third incubation day and reaches its maximum value on the sixth experimental day, where phenol removal rate is almost equal to zero; catechol is produced with an almost steady rate until the end of the experiment. Phenol consumption after the sixth day is significantly lower compared to the consumption observed at the beginning of the experiment. It is interesting to note that the amount of catechol produced in the medium was approximately 20% of the amount of total phenol removed. These results show that catechol ring cleavage is the most energetically demanding step during phenol biodegradation by *Chlamydomonas* cells. This is supported by the fact that no growth of the cultures was observed (Fig. 7a) despite the consumption of phenol when an alternative carbon source was not present in the medium. Taking into account previous observations (Nazos et al. 2017), we conclude that phenol drives microalgal metabolism to "maintenance". The observed increase in the peak intensity of the other metabolites during the experiment (Fig. 6)

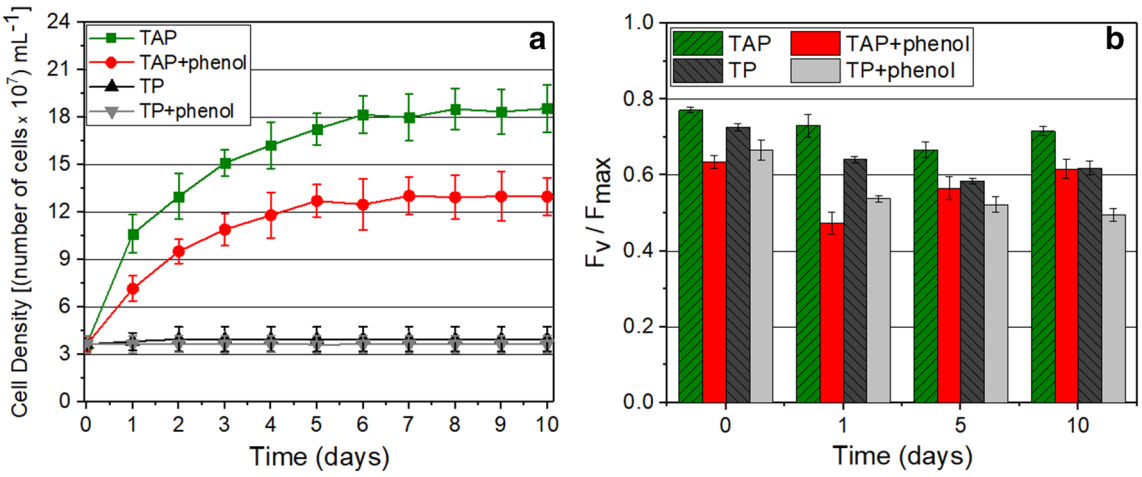

Fig. 7 Graphs presenting the growth curves (**a**) and maximal photosynthetic efficiency (**b**) of *Chlamydomonas reinhardtii* cultures, during experiments, grown in TAP and TP medium in the absence and in the presence of 4.0 mM initial phenol concentration under continuous light intensity of 70–80 μmol photons·m^{-2}·s^{-1}. Biomass was expressed in number of cells·(mL culture)$^{-1}$

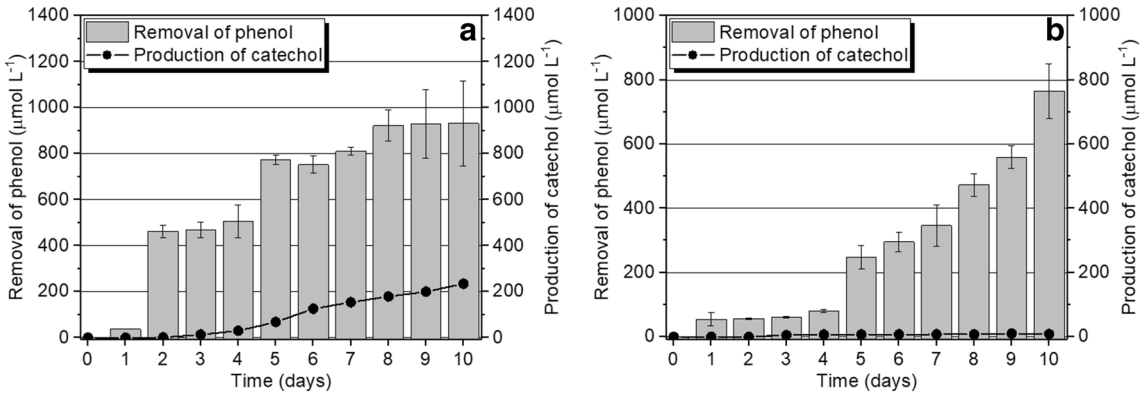

Fig. 8 Graphs presenting phenol removal and catechol production in the culture medium by *Chlamydomonas reinhardtii* cultures **a** in the absence and **b** in the presence of acetic acid as an alternative carbon source, with initial phenol concentration of 4.0 mM. Experiments were carried out under a continuous light intensity of 70–80 μmol photons·m^{-2}·s^{-1}. Phenol removal and catechol production were expressed in μmol·L^{-1}

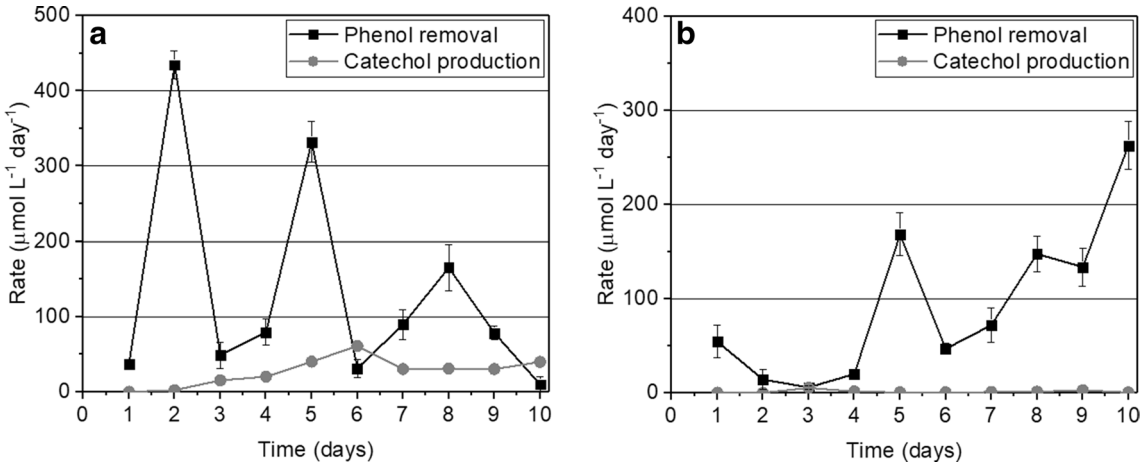

Fig. 9 Change in the phenol consumption rate (black squares) and in the catechol production rate (gray circles) by *Chlamydomonas reinhardtii* cultures incubated with an initial phenol concentration of 4.0 mM, under a light intensity of 70–80 μmol photons·m^{-2}·s^{-1} in the absence (**a**) and in the presence (**b**) of acetic acid

shows that *Chlamydomonas reinhardtii* follows a strategy that has as a main target decreasing the concentration of phenolic compounds in the culture medium rather than complete oxidation of the other metabolites. Cells perform the oxidation of phenol to catechol and then release catechol that they cannot further degrade. As complete metabolism of phenol is an energetically demanding process, under conditions where phenol is the only carbon source present in the culture medium, cells biodegrade only the amount of phenol that is necessary for the maintenance of cellular components.

This hypothesis is supported by the comparative light and fluorescence microscopy studies (Fig. 10) of control cultures incubated for five days in the absence of a carbon source (Fig. 10a, b) and cultures where phenol was the only carbon source present in the medium (Fig. 10c, d). Compared to the cells treated with phenol, the control cell cultures were found to exhibit empty spaces that include small vesicles. These empty spaces are located in chloroplasts, as shown clearly by fluorescence microscopy images, and could be attributed to the physiological phenomenon of autophagy. Autophagy is an intracellular catabolic process that allows cells to recycle useless or destroyed cellular material for maintenance of homeostasis (Couso et al. 2018). These mechanisms have been extensively studied in the literature (He and Klionsky 2009; Mizushima et al. 2011; Li and Vierstra 2012; Liu and Bassham 2012). The process includes formation of autophagosomes that transfer the "useless" cellular material into specific vacuoles. In the *Chlamydomonas* genus it has been observed that this process takes place under deprivation of nutrients such as carbon and nitrogen (Goodson et al. 2011; Perez-Perez et al. 2012; Goodenough et al. 2014; Davey et al. 2014). In our case this result is expected if we

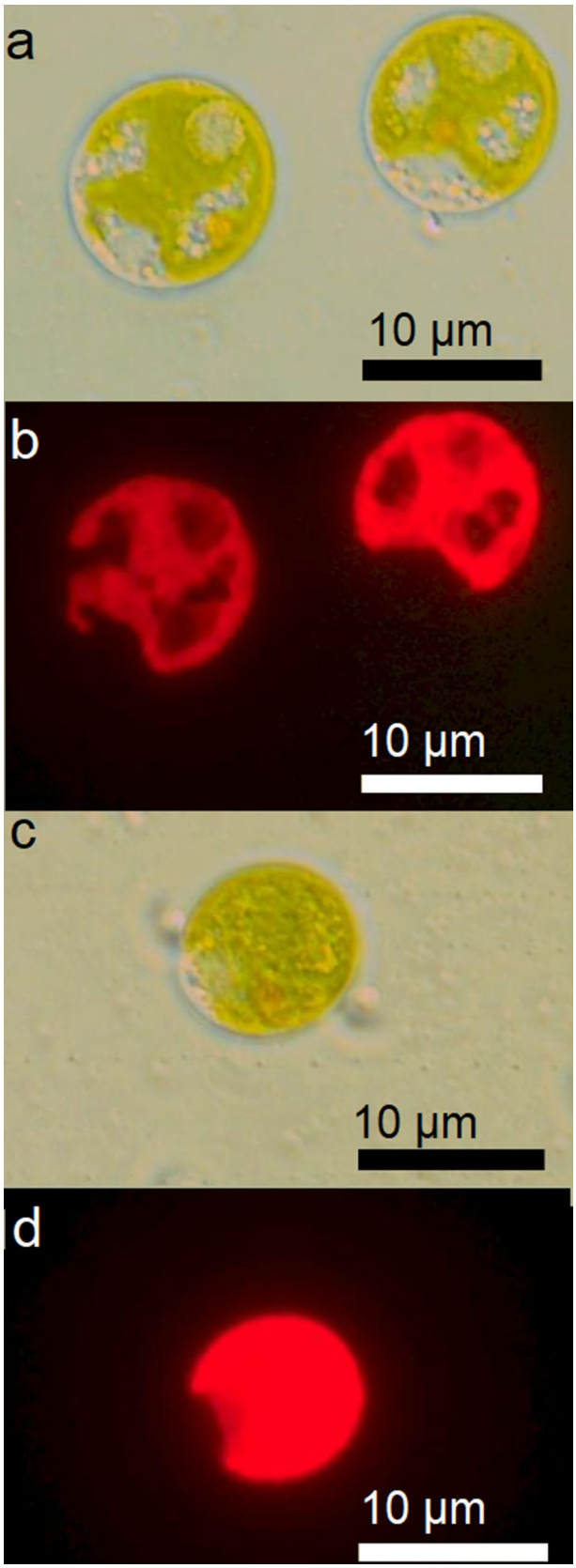

Fig. 10 Optical (**a**, **c**) and fluorescence (**b**, **d**) microscopy images of ▶ *Chlamydomonas reinhardtii* cells. Cells were grown in TP medium in the absence (**a**, **b**) and in the presence (**c**, **d**) of 4.0 mM initial phenol concentration. Samples were collected after an incubation period of five days. Cells were grown in TP medium with an initial phenol concentration of 4.0 mM under continuous light intensity of 70–80 μmol photons·m^{-2}·s^{-1}

take into consideration the fact that cells cultivated in TP medium have remained for many days in the sealed flask without a supply of a carbon source. On the other hand, in cultures where phenol was present, the autophagy phenomenon was not observed. If we also take into account the fact that removal of phenol takes place in the first hours of incubation, we could conclude that the *Chlamydomonas reinhardtii* cells biodegrade phenol in order to cover their carbon requirements for the preservation of their homeostasis and cellular structure. In order to compare cell viability, cells from all the examined experimental conditions (TAP and TP in the presence and absence of phenol) were collected 10 days after the end of the experiments, washed twice with fresh TAP medium and diluted into fresh TAP medium in order to obtain initial cell concentration equal to 4.5 ($\pm$0.4)·10^5 cells·mL^{-1}. Cells were left to grow under a light intensity of 70–80 μmol photons·m^{-2}·s^{-1} for 3 days. After this period cell counts were made. In all cases similar growth was observed; difference was less than 5%.

The behavior is different in the presence of acetic acid in the culture medium. In the beginning of the experiment cells take up a small amount of phenol, and no phenol degradation is observed until the fifth day (Fig. 8b). Significant metabolic activity is recorded after that day, when phenol removal increases continuously until the last day of the experiment. It is worth mentioning that in that day, cultures treated with phenol and acetic acid enter the stationary phase of growth and as a result all available reserves of acetic acid have been exhausted from the culture medium. With a double substrate in the medium, cells choose to biodegrade the growth-promoting substrate (acetic acid) first, consistent with the growth of the cultures, and then degrade the growth-limiting substrate (phenol). This strategy is followed by microalgae in order to gain energy reserves. An important observation is that catechol production in the culture medium was found to be negligible (less than 2% of the phenol removed). The profile of the rate of phenol removal during the experiment (Fig. 9b) is significantly different, when compared to that observed in the absence of acetic acid (Fig. 9a). After the cells reach the stationary phase, a maximum rate is observed on the fifth experimental day, while after the sixth experimental day cells exhibit continuous increase in the rate of phenol removal. This fact underscores the importance of the presence of an organic substrate in the culture medium that provides cells with sufficient energy reserves. In addition

to these data, measurements of the maximum photosynthetic efficiency revealed that the presence of acetic acid assists microalgae in overcoming stress effects caused by the presence of phenol in the culture medium. In the presence of acetic acid, the values of the F_v/F_{max} parameter exhibit a decrease during the first 24 h (acute stress caused by the presence of phenol), while maximal photosynthetic efficiency returns to high levels throughout the incubation period. In contrast, in the absence of an alternative organic carbon source, maximal photosynthetic efficiency gradually decreases during the experiment (Fig. 7b).

The results presented here and in a previous publication of our research group (Nazos et al. 2017), show that *Chlamydomonas reinhardtii* cells adapt their metabolism in order to achieve an optimal bioenergetic equilibrium between growth, stress and phenol biodegradation. This equilibrium is affected significantly by the production of catechol and the presence or absence of a substrate for energy production in the culture medium.

Conclusion

The results of the experiments reported here provide further evidence for the hypothesis that biodegradation of phenol by *Chlamydomonas reinhardtii* is a dynamic bioenergetic process by focusing on regulation of the bioenergetic equilibrium by the production of catechol by the microalgae. Figure 11 summarizes schematically the proposed bioenergetic model of the present study's findings. When microalgae lack sufficient energy reserves, phenol oxidation to catechol was found to take place; the amount of catechol that could not be further biodegraded was released into the culture medium. In the presence of acetic acid, the amount of catechol found in the medium, compared to the amount of phenol consumed, was negligible. Phenol is a growth-limiting substrate that requires significant amounts of energy to be biodegraded. It was biodegraded only when *Chlamydomonas* cells required carbon reserves for the maintenance of their homeostasis. *Chlamydomonas reinhardtii* was proven to act as a versatile bioenergetic machine by regulating its metabolism to achieve the best balance between growth and biodegradation of phenol. These data extend previous findings and provide new unique metabolic and bioenergetic insights in the biodegradation of phenol by microalgae.

Acknowledgements This research was co-financed by Greece and the European Union (European Social Fund- ESF) through the Operational Programme "Human Resources Development, Education and Lifelong Learning" in the context of the project "Scholarships programme for post-graduate studies -2nd Study Cycle" (MIS-5003404), implemented by the State Scholarships Foundation (IKY).

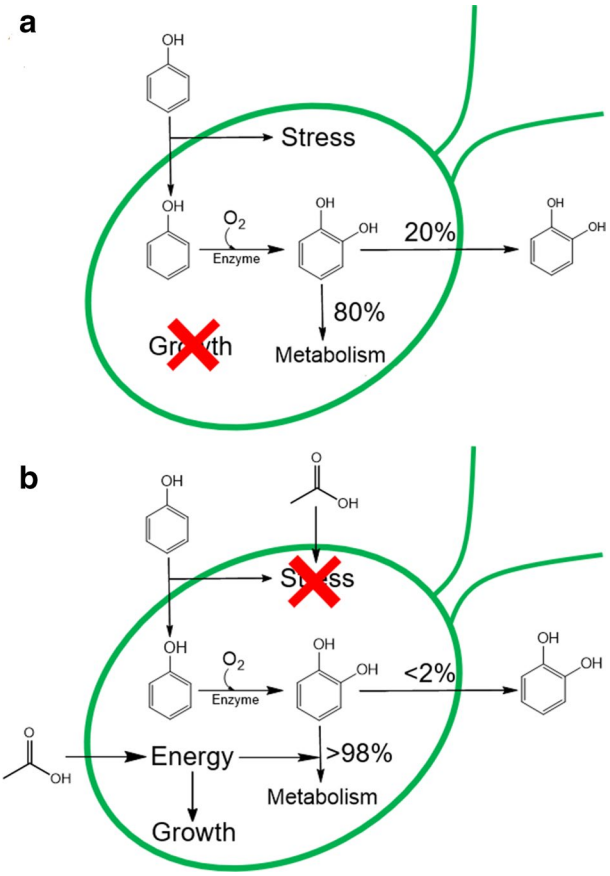

Fig. 11 Proposed bioenergetic models of phenol biodegradation by *Chlamydomonas reinhardtii.* **a** In the absence of acetic acid in the growth medium, phenol uptake leads to pronounced stress in *Chlamydomonas* cultures that lasts throughout the experimental period. No growth of the cultures is observed and approximately 20% of the consumed phenol is released as catechol into the culture medium. **b** In the presence of acetic acid growth of *Chlamydomonas* cultures is observed and the acute stress caused by the presence of phenol is decreased throughout the experiment. The amounts of catechol released in the culture medium are less than 2% of the total phenol amount consumed by *Chlamydomonas* cells

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Agarry SE, Solomon BO, Layokun SK (2008) Optimization of process variables for the microbial degradation of phenol by *Pseudomonas aeruginosa* using response surface methodology. Afr J Biotechnol 7(14):2409–2416

Al-Khalid T, El-Naas MH (2012) Aerobic biodegradation of phenols: a comprehensive review. Crit Rev Env Sci Tech 42(16):1631–1690. https://doi.org/10.1080/10643389.2011.569872

Binkley RW, Dillow GW, Flechtner TW, Winnik W, Tevesz MJS (1994) Fragmentation of collisionally activated hydroxyphenoxide anions

in the gas phase. Org Mass Spectrom 29(9):491–495. https://doi.org/10.1002/oms.1210290908
Couso I, Pérez-Pérez ME, Martínez-Force E, Kim H-S, He Y, Umen JG, Crespo JL (2018) Autophagic flux is required for the synthesis of triacylglycerols and ribosomal protein turnover in *Chlamydomonas*. J Exp Bot 69(6):1355–1367
Das B, Mandal TK, Patra S (2015) A comprehensive study on *Chlorella pyrenoidosa* for phenol degradation and its potential applicability as biodiesel feedstock and animal feed. Appl Biochem Biotechnol 176(5):1382–1401. https://doi.org/10.1007/s12010-015-1652-9
Davey MP, Horst I, Duong GH, Tomsett EV, Litvinenko AC, Howe CJ, Smith AG (2014) Triacylglyceride production and autophagous responses in *Chlamydomonas reinhardtii* depend on resource allocation and carbon source. Eukaryot Cell 13(3):392–400. https://doi.org/10.1128/EC.00178-13
Delrue F, Álvarez-Díaz P, Fon-Sing S, Fleury G, Sassi J-F (2016) The environmental biorefinery: using microalgae to remediate wastewater, a win-win paradigm. Energies 9:132–151. https://doi.org/10.3390/en9030132
Du W, Zhao F, Zeng B (2009) Novel multiwalled carbon nanotubes-polyaniline composite film coated platinum wire for headspace solid-phase microextraction and gas chromatographic determination of phenolic compounds. J Chromatogr A 1216(18):3751–3757. https://doi.org/10.1016/j.chroma.2009.03.013
Duan W, Meng F, Lin Y, Wang G (2017) Toxicological effects of phenol on four marine microalgae. Environ Toxicol Pharmacol 52:170–176. https://doi.org/10.1016/j.etap.2017.04.006
Endo T, Asada K (1996) Dark Induction of the non-photochemical quenching of chlorophyll fluorescence by acetate in *Chlamydomonas reinhardtii*. Plant Cell Physiol 37(4):551–555. https://doi.org/10.1093/oxfordjournals.pcp.a028979
Gattullo CE, Bahrs H, Steinberg CE, Loffredo E (2012) Removal of bisphenol A by the freshwater green alga *Monoraphidium braunii* and the role of natural organic matter. Sci Total Environ 416:501–506. https://doi.org/10.1016/j.scitotenv.2011.11.033
Goodenough U, Blaby I, Casero D, Gallaher SD, Goodson C, Johnson S, Lee JH, Merchant SS, Pellegrini M, Roth R, Rusch J, Singh M, Umen JG, Weiss TL, Wulan T (2014) The path to triacylglyceride obesity in the sta6 strain of *Chlamydomonas reinhardtii*. Eukaryot Cell 13(5):591–613. https://doi.org/10.1128/EC.00013-14
Goodenough UW, Armbrust EV, Campbell AM, Ferris PJ (1995) Molecular-genetics of sexuality in *Chlamydomonas*. Annu Rev Plant Phys 46:21–44
Goodson C, Roth R, Wang ZT, Goodenough U (2011) Structural correlates of cytoplasmic and chloroplast lipid body synthesis in *Chlamydomonas reinhardtii* and stimulation of lipid body production with acetate boost. Eukaryot Cell 10(12):1592–1606. https://doi.org/10.1128/EC.05242-11
Guldhe A, Kumari S, Ramanna L, Ramsundar P, Singh P, Rawat I, Bux F (2017) Prospects, recent advancements and challenges of different wastewater streams for microalgal cultivation. J Environ Manage 203(Pt 1):299–315. https://doi.org/10.1016/j.jenvman.2017.08.012
Harris E (2009) The *Chlamydomonas* sourcebook: introduction to *Chlamydomonas* and its laboratory use. In: The *Chlamydomonas* sourcebook, vol I. 2nd edn. Academic Press, Canada.
He C, Klionsky DJ (2009) Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 43:67–93. https://doi.org/10.1146/annurev-genet-102808-114910
Hippler M, Redding K, Rochaix JD (1998) *Chlamydomonas* genetics, a tool for the study of bioenergetic pathways. Biochem Biophys Acta 1367(1–3):1–62
Johnson X, Alric J (2013) Central carbon metabolism and electron transport in *Chlamydomonas reinhardtii:* metabolic constraints for carbon partitioning between oil and starch. Eukaryot Cell 12(6):776–793. https://doi.org/10.1128/EC.00318-12
Koller M, Muhr A, Braunegg G (2014) Microalgae as versatile cellular factories for valued products. Algal Res 6:52–63. https://doi.org/10.1016/j.algal.2014.09.002
Lee H-C, Lee M, Den W (2015) Spirulina maxima for Phenol removal: study on its tolerance, biodegradability and phenol-carbon assimilability. Water Air Soil Poll 226(12):395–405. https://doi.org/10.1007/s11270-015-2664-3
Li F, Vierstra RD (2012) Autophagy: a multifaceted intracellular system for bulk and selective recycling. Trends Plant Sci 17(9):526–537. https://doi.org/10.1016/j.tplants.2012.05.006
Lika K, Papadakis IA (2009) Modeling the biodegradation of phenolic compounds by microalgae. J Sea Res 62:135–146
Liu Y, Bassham DC (2012) Autophagy: pathways for self-eating in plant cells. Annu Rev Plant Biol 63:215–237. https://doi.org/10.1146/annurev-arplant-042811-105441
Liu Y, Dai X, Wei J (2013) Toxicity of the xenoestrogen nonylphenol and its biodegradation by the alga *Cyclotella caspia*. J Environ Sci 25(8):1662–1671
Lovell CR, Eriksen NT, Lewitus AJ, Chen YP (2002) Resistance of the marine diatom *Thalassiosira* sp. to toxicity of phenolic compounds. Mar Ecol Prog series 229:11–18
Martins PL, Marques LG, Colepicolo P (2015) Antioxidant enzymes are induced by phenol in the marine microalga *Lingulodinium polyedrum*. Ecotoxicol Environ Saf 116:84–89. https://doi.org/10.1016/j.ecoenv.2015.03.003
Mata TM, Martins AA, Caetano NS (2010) Microalgae for biodiesel production and other applications: a review. Renew Sust Energ Rev 14(1):217–232. https://doi.org/10.1016/j.rser.2009.07.020
Megharaj M, Pearson HW, Venkateswarlu K (1992) Effects of phenolic compounds on growth and metabolic activities of *Chlorella vulgaris* and *Scenedesmus bijugatus* isolated from soil. Plant Soil 140(1):25–34. https://doi.org/10.1007/bf00012803
Mizushima N, Yoshimori T, Ohsumi Y (2011) The role of ATG proteins in autophagosome formation. Annu Rev Cell Dev Biol 27:107–132. https://doi.org/10.1146/annurev-cellbio-092910-154005
Nazos TT, Kokarakis EJ, Ghanotakis DF (2017) Metabolism of xenobiotics by *Chlamydomonas reinhardtii*: phenol degradation under conditions affecting photosynthesis. Photosynth Res 131(1):31–40. https://doi.org/10.1007/s11120-016-0294-2
Otto B, Schlosser D (2014) First laccase in green algae: purification and characterization of an extracellular phenol oxidase from *Tetracystis aeria*. Planta 240(6):1225–1236. https://doi.org/10.1007/s00425-014-2144-9
Papazi A, Assimakopoulos K, Kotzabasis K (2012) Bioenergetic strategy for the biodegradation of p-cresol by the unicellular green alga *Scenedesmus obliquus*. PLoS ONE 7(12):e51852. https://doi.org/10.1371/journal.pone.0051852
Papazi A, Kotzabasis K (2007) Bioenergetic strategy of microalgae for the biodegradation of phenolic compounds—exogenously supplied energy and carbon sources adjust the level of biodegradation. J Biotechnol 129(4):706–716. https://doi.org/10.1016/j.jbiotec.2007.02.021
Papazi A, Kotzabasis K (2008) Inductive and resonance effects of substituents adjust the microalgal biodegradation of toxical phenolic compounds. J Biotechnol 135(4):366–373. https://doi.org/10.1016/j.jbiotec.2008.05.009
Papazi A, Kotzabasis K (2013) "Rational" management of dichlorophenols biodegradation by the microalga Scenedesmus obliquus. PLoS ONE 8(4):e61682. https://doi.org/10.1371/journal.pone.0061682
Perez-Perez ME, Couso I, Crespo JL (2012) Carotenoid deficiency triggers autophagy in the model green alga *Chlamydomonas reinhardtii*. Autophagy 8(3):376–388. https://doi.org/10.4161/auto.18864

Petroutsos D, Katapodis P, Samiotaki M, Panayotou G, Kekos D (2008) Detoxification of 2,4-dichlorophenol by the marine microalga *Tetraselmis marina*. Phytochemistry 69(3):707–714. https://doi.org/10.1016/j.phytochem.2007.09.002

Priyadharshini DS, Bakthavatsalam AK (2016) Optimization of phenol degradation by the microalga *Chlorella pyrenoidosa* using Plackett–Burman design and response surface methodology. Bioresour Technol 207:150–156. https://doi.org/10.1016/j.biortech.2016.01.138

Rochaix J, Goldschmidt- Clermont A, Merchand S (1998) The molecular biology of chloroplasts and mitochondria in *Chlamydomonas*. In: Advances in Photosynthesis. Kluwer Academic Publishers, Dordrecht, Netherlands, p 7

Satyanarayana KG, Mariano AB, Vargas JVC (2011) A review on microalgae, a versatile source for sustainable energy and materials. Int J Energ Res 35(4):291–311. https://doi.org/10.1002/er.1695

Semple KT, Cain RB (1996) Biodegradation of phenols by the alga *Ochromonas danica*. Appl Environ Microbiol 62(4):1265–1273

Shigeoka T, Sato Y, Takeda Y, Yoshida K, Yamauchi F (1988) Acute toxicity of chlorophenols to green algae, *Selenastrum capricornutum* and *Chlorella vulgaris*, and quantitative structure-activity relationships. Environ Toxicol Chem 7(10):847–854. https://doi.org/10.1002/etc.5620071007

Stoilova I, Krastanov A, Stanchev V, Daniel D, Gerginova M, Alexieva Z (2006) Biodegradation of high amounts of phenol, catechol, 2,4-dichlorophenol and 2,6-dimethoxyphenol by *Aspergillus awamori* cells. Enzyme Microb Tech 39:1036–1041

Strasser BJ, Strasser RJ (1995) Measuring fast fluorescence transients to address environmental questions: the JIP-test. In: Mathis P (ed) Photosynthesis: from light to biosphere. Kluwer Academic Press, Dordrecht

Van Schie PM, Young LY (2000) Biodegradation of Phenol: Mechanisms and Applications. Bioremediat J 4(1):1–18. https://doi.org/10.1080/10588330008951128

Williams PA, Sayers JR (1994) The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas*. Biodegradation 5(3–4):195–217

Wurster M, Mundt S, Hammer E, Schauer F, Lindequist U (2003) Extracellular degradation of phenol by the cyanobacterium *Synechococcus* PCC 7002. J Appl Phycol 15(2):171–176. https://doi.org/10.1023/a:1023840503605

Zhou L, Cheng D, Wang L, Gao J, Zhao Q, Wei W, Sun Y (2017) Comparative transcriptomic analysis reveals phenol tolerance mechanism of evolved Chlorella strain. Bioresour Technol 227:266–272. https://doi.org/10.1016/j.biortech.2016.12.059

Zhou WG, Guo WB, Zhou HB, Chen XH (2016) Phenol degradation by *Sulfobacillus acidophilus* TPY via the meta-pathway. Microbiol Res 190:37–45. https://doi.org/10.1016/j.micres.2016.05.005