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1 **Linking mode of action of the model respiratory and**
2 **photosynthesis uncoupler 3,5-dichlorophenol to adverse**
3 **outcomes in *Lemna minor***

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43 Abstract

44 Standard chemical toxicity testing guidelines using aquatic plant *Lemna minor* have been
45 developed by several international standardisation organisations. Although being highly
46 useful for regulatory purposes as focusing on traditional adverse endpoints, these tests
47 provide limited information about the toxic mechanisms and modes of action (MoA). The
48 present study aimed to use selected functional assays in *L. minor* after exposure to 3,5-
49 dichlorophenol (3,5-DCP) as a model to characterize the toxic mechanisms causing growth
50 inhibition and lethality in primary producers. The results demonstrated that 3,5-DCP caused
51 concentration-dependent effects in chloroplast and mitochondria. Uncoupling of oxidative
52 phosphorylation (OXPHOS), reduction in chlorophyll (Chlorophyll *a* and *b*) content,
53 reproduction rate and frond size were the most sensitive endpoints, followed by formation of
54 reactive oxygen species (ROS) formation, lipid peroxidation (LPO), carotenoids reduction
55 and impairment of photosynthesis efficiency. Suppression of photosystem II (PSII) efficiency,
56 electron transport rate (ETR), chlorophylls (*a* and *b*), oxidative phosphorylation (OXPHOS)
57 was closely correlated while ROS production and LPO were negative correlated to ETR,
58 carotenoid content and growth parameters. A network of conceptual Adverse Outcome
59 Pathways (AOPs) was developed to decipher the causal relationships between molecular,
60 cellular, and apical adverse effects occurring in *L. minor* to form a basis for future studies
61 with similar compounds.

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63 **Keywords:** *Lemna minor*, 3,5-dichlorophenol, reactive oxygen species, growth, PSII,
64 lipid peroxidation, oxidative phosphorylation, Mode of action, adverse outcome pathway.

1. Introduction

Primary producers such as aquatic plants play a key role in aquatic system and trophic chain since they contribute to oxygen production and regulate the biogeochemical cycling of elements (Cloern et al., 2014; Elser et al., 2007). Their central function in the ecosystem makes these organisms ideal for monitoring the ecosystem health and assessing adverse impact of abiotic and biotic factors on the lower part of the food web. The aquatic vascular plant *L. minor*, a small freshwater aquatic plant that reproduce rapidly by thalli division to form plant carpets covering still waters worldwide (Mendiola, 1918; Einhellig et al., 1985; Landolt 1975; Landolt 1998), has been used with large success as animal fodder, a bioremediator, and in toxicity testing (Fairchild et al., 1997; Horvat et al., 2007; Kirby and Sheahan, 1994; Ziegler et al., 2016). The popularity of this species is predominantly due to rapid reproductive rates (Cowgill and Milazzo, 1989), ease of culturing under lab conditions and sensitivity to a number of stressors including chemical pollutants such as herbicides, pesticides and metals (Fairchild et al., 1997; Hartman and Martin, 1985; Hou et al., 2007) or environment stressors include changes in temperature, salinity and pH (Haller et al., 1974; Uysal and Taner, 2009). This combination of properties has led to the development of testing guidelines that allow standardised toxicity testing under controlled laboratory conditions (ISO20079, 2005; OECD, 2006). Such testing methods represent key components of chemical hazard assessment, by providing characterisation of the toxic properties of chemicals and identifying potential adverse effects of regulatory relevance. However, these standardised toxicity tests predominantly provide information of the adverse outcome (AO), whereas the toxic mode of action (MoA) are often not addressed in detail. To provide this information, biomarkers based on biochemical assays or physiological responses are frequently used for characterisation of the MoA (Fernandes et al., 2013; Gupta and Huang, 2014), whereas approaches such as Adverse Outcome Pathways (AOPs) has been proposed to

93 provide causal links between the MoA and adverse effects of the stressors (Ankley et al.,
94 2010).

95
96 The AOPs represent a framework platform to assemble, evaluate and visualize the chain of
97 events occurring from a molecular initiating event (MiE) occurring at a stressors biological
98 target site, to a series of key events (KEs) at different levels of biological organisation that
99 ultimately leads to the AO at the individual or population level (Villeneuve et al., 2014). The
100 AOPs can thus be used to improve the MoA knowledge and how chemicals cause adversity,
101 to assist bioassay development and develop suitable toxicity testing guidelines. Additionally,
102 AOPs can potentially inform regulatory processes by directing the use of testing resources;
103 perform species screening and prioritization of chemicals and support Integrated Approaches
104 to Testing and Assessment (IATA) (Tollefsen et al., 2014). Despite a substantial AOP
105 development worldwide, none of the over 250 AOPs submitted to the AOP repository,
106 AOPwiki (aopwiki.org, December 2017) focuses specifically on primary producers or address
107 chemicals known to specifically target these environmental keystone species.

108
109 Chlorophenols (CPs), a diverse group of chlorinated phenols, are commonly used as
110 pesticides, disinfectants, and as chemical intermediates in the production of more complex
111 chemicals (Igbiosa et al., 2013). The CPs exhibit a range of toxic MoAs in eukaryotes,
112 whereof some CPs are used as herbicides due to their high toxic potency to aquatic primary
113 producers (Michałowicz and Duda, 2007). The CPs cause growth inhibition in primary
114 producers by disrupting energy metabolism, either by uncoupling oxidative or photosynthetic
115 phosphorylation through inhibiting electron transport on inner membrane of mitochondria
116 and thylakoid (Escher et al., 1996). As a consequence of these toxic properties, CPs such as
117 3,5-dichlorophenol (3,5-DCP) are normally used as positive controls in toxicity testing

118 guidelines and studies with algae and aquatic plants (ISO8692:2012, 2012; OECD,
119 2006; OECD, 2011; Michel et al, 2004) or briefly study the toxicity in *Lemna* at growth
120 level (Baskar et al, 2016). Effort to characterise the different MoA of CPs in primary
121 producers is thus considered key to understand how other and similar compounds cause
122 effects in aquatic plants and algae.

123 The objectives of this study were to characterize the MoA of 3,5-DCP as a model CP in *L.*
124 *minor* and link these cellular perturbations to inhibition of growth and vegetative
125 reproduction. To achieve these goals, a combination of a 7d chronic toxicity test to assess
126 growth inhibition (reproduction, frond area and weight) and MoA studies to characterise
127 changes in key physiological processes (oxidative stress, photosynthetic capacity, oxidative
128 phosphorylation) were conducted. The resulting data were assembled into an AOP framework
129 to identify the key MiE and KEs for the adversity observed.

130 **2. Materials and Methods**

131 **2.1. Culture and exposure**

132 Test species *Lemna minor* (Strain ID: 5544, Rutgers Duckweed Stock Cooperative,
133 <http://www.ruduckweed.org>) was provided by Blases Biological Ltd (Cat.ID: LBA 041,
134 Edenbridge, UK) and cultured at Ghent University (Belgium) (De Schamphelaere et al., 2010;
135 Van Echelpoel et al., 2016) prior to transfer to the Norwegian Institute for water research
136 (NIVA). Upon arrival at NIVA, thalli were disinfected by immersion in 0.5% NaOCl (v/v) for
137 3 min and then rinsing with distilled water for 5 min to remove algae contamination. The *L.*
138 *minor* cultures were maintained in 200 ml Erlenmeyer flasks containing 100 ml of Steinberg
139 (SB) medium (with composition: 350 mg/L KNO₃, 295 mg/L Ca(NO₃)₂·4H₂O, 90 mg/L
140 KH₂PO₄, 12 mg/L K₂HPO₄, 100 mg/L MgSO₄·7H₂O, 0.12mg/L H₃BO₃, 0.18 mg/L
141 ZnSO₄·7H₂O, 0.044 mg/L Na₂MoO₄·2H₂O, 0.18mg/L MnCl₂·4H₂O, 0.76 mg/L FeCl₃·6H₂O,

142 0.15 mg/L EDTA disodium-dihydrate, pH 5.5.) (OECD, 2006). All cultures were kept in a
143 growth chamber for 24h under continuous white light in photosynthetic active radiation (PAR)
144 at $80 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ and temperature of $24 \pm 2 \text{ }^\circ\text{C}$ according to the OECD guidelines 221
145 (OECD, 2006), with stock thalli sub-cultured twice a week. The irradiance was measured by
146 a LI-COR quantum sensor Model LI-190 (Lincoln, Nebraska, USA) connected to a LI-COR
147 LI-250 photometer unit.

149 The test chemical 3,5-DCP (Purity 97%, Sigma-Aldrich) was dissolved in dimethylsulfoxide,
150 DMSO (Purity 99.7%, Sigma-Aldrich) and resulting stock solutions (10 mg/L) were stored in
151 the dark at $-18 \text{ }^\circ\text{C}$ until use. Before exposure, the fronds are pre-cultured in the test medium
152 for 14 days (Naumann et al., 2007). Exposure studies were conducted using independent
153 colonies (N=3, 3-4 green fronds each) in glass beakers (100 mL) containing diluted 3,5-DCP
154 (0.5, 1, 1.5, 2, 3, 4, 8 mg/L) with blank controls (SB Medium) and solvent controls (0.1%
155 DMSO) in 50 mL SB Medium. Exposures were performed under the same conditions as
156 culturing and was repeated 3 times for securing samples for analyses. Fronds were sampled
157 after 7 days exposure for the assessment of the toxicity endpoints.

159 2.2. Growth rate

160 The growth rate on basis of the fronds number (FN) was calculated according to the equation:

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$$\text{GR}_i = \frac{\ln N_{t_i} - \ln N_{t_0}}{t_i - t_0}$$
, where GR_i is the growth rate per day; N_{t_0} is the fronds number at day t_0

162 (the beginning of the experiment); N_{t_i} is the fronds number at day t_i ($i=7$); $t_i - t_0$ is the time
163 period between t_i and t_0 , expressed in days. The doubling time (T) of FN was calculated using

164 the equation $T_i = \frac{\ln 2}{\text{GR}_i}$, where GR_i is the growth rate determined as described before. The test

165 was considered valid when the growth rate of FN in the control groups were higher than

166 0.275 d⁻¹ (OECD, 2006).

167

168 For measurement of frond area (FA), the total area of floating fronds was determined

169 optically by a digital camera (FinePix S2500HD, Fujifilm, Japan) using a floating scale bar.

170 The frond area in each photograph was analysed using the Image-J software program version

171 1.48 (National Institutes of Health, Maryland, USA). The dry mass (DM) of the fronds was

172 determined gravimetrically after centrifugation of exposed fronds at 3000 rpm for 10 min at

173 room temperature to 20 °C in pre-weighted eppendorf tubes. The fronds were then dried in an

174 oven at 70 °C until constant weight was obtained and DM recorded.

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176 **2.3. Mitochondrial inner membrane potential**

177 The mitochondrial inner membrane potential (MMP) was determined using

178 tetramethylrhodamine methyl ester (TMRM, Life Technologies AS, Oslo, Norway) as an

179 indicator of OXPHOS in the fronds (Ehrenberg et al., 1988; Scaduto and Grotyohann, 1999).

180 In brief, stock solutions of TMRM (5 µM) were prepared in DMSO (Purity 99.7%, Sigma-

181 Aldrich) and stored under -18°C until use. The MMP assay was optimized for dye

182 concentration and incubation duration on the basis of recommendations for the green algae

183 *Chlamydomonas reinhardtii* and zebrafish cells (Jamers et al., 2009; Legradi et al., 2014).

184 After exposure, 3 fronds for each replicate (N=3) were transferred to the glass flasks

185 containing 2 mL SB medium prior to the addition of 200 µl TMRM (500 nM) diluted in the

186 SB medium. The fronds were incubated with TMRM for 2h at room temperature. After

187 incubation, the fronds were rinsed with SB medium for 5 minutes to remove free (unbound)

188 TMRM and transferred to a Costar 96-Well Black Clear-Bottom microplate (Corning

189 Incorporated, USA) containing 200 µl SB medium. The fluorescent intensity of TMRM was

190 measured using VICTOR 3 fluorometer (PerkinElmer) with the excitation wavelength of
191 530nm and emission wavelength of 590 nm. The natural fluorescence of the exposure media
192 in combination with the dye (without presence of *Lemna* fronds) was also analyzed and the
193 resulting fluorescence subtracted. The relative fluorescence obtained was normalized by
194 weight of test frond and expressed as fold induction comparative to the control.

2.4. Reactive oxygen species (ROS) formation

197 2',7'-Dichlorofluorescein diacetate (H₂DCFDA) (Molecular Probes Inc., Eugene, OR, USA)
198 was used to quantify the ROS (H₂O₂, O₂⁻ and ¹O₂) in *L. minor* (Razinger et al., 2010). The
199 stock solutions of H₂DCFDA (10 mM) was prepared in DMSO and stored at -18 °C until use.
200 After exposure to 3,5-DCP, the fronds were immersed in H₂DCFDA diluted in the culture
201 medium to a final concentration of 50 μM (maximum DMSO concentration: 0.5%). After 2 h
202 incubation, the fronds were rinsed using clean growth medium for 5 minutes to remove
203 excess dye. Clean fronds were subsequently transferred to a black Costar 96-well polystyrene
204 microplate with clear-bottom (Corning Incorporated, USA) containing 200 μl of culture
205 medium. The fluorescence signal of H₂DCFDA was measured using a microplate reader
206 (VICTOR 3, PerkinElmer, Waltham, Massachusetts, USA) with excitation wavelength of 485
207 nm and emission wavelength of 538 nm. The natural fluorescence of the exposure media in
208 combination with the dye (without presence of fronds) was also analyzed and the resulting
209 fluorescence subtracted. The relative fluorescence obtained was normalized by weight of test
210 frond expressed as fold induction comparative to the control.

2.5. Lipid peroxidation

213 Lipid peroxidation of exposed fronds was assessed by determining malondiadehyde (MDA),

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214 an product of lipid peroxidation following decomposition of polyunsaturated fatty acid
215 peroxides, using the TBARs method (Zezulka et al., 2013). Around 5 mg of fresh mass of
216 exposed fronds were homogenized in 1 ml of 0.25% (w/v) 2-thiobarbituric acid (TBA,
217 Sigma-Aldrich) in 10% trichloroacetic acid (TCA, Sigma-Aldrich) and incubated at 95 °C for
218 30 min. The samples were then cooled to room temperature in an ice bath for 10 min. and
219 centrifuged at 10,000g at 4 °C for 10 min. The absorbance of the supernatant was recorded at
220 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. In
221 addition, absorbance at 400 nm was also recorded to avoid the signal interruption from
222 carbohydrates. A blank containing 0.25% TBA in 10% TCA was also analysed and subtracted
223 from each sample absorbance. The content of MDA was presented as $\mu\text{mol g}^{-1}$ using an
224 extinction coefficient of $155 \text{ nmol}^{-1} \text{ cm}^{-1}$ and calculated as a fold difference compared to the
225 control.

227 **2.6. Pigments content**

228 Pigment content was determined spectrophotometrically essentially as described by Ritchie et
229 al. (2006). In brief, 25 mg of fresh fronds were submerged in 2 ml methanol (Purity:99.9%,
230 Sigma-Aldrich) overnight, the absorbance of the extracts was determined by a UV-vis
231 spectrophotometer Lambda 40 (PerkinElmer, Waltham, USA) at wavelength of 652 nm
232 (chlorophyll *a*, A652), 665 nm (chlorophyll *b*, A665) and 470 nm (carotenoids, A470). The
233 individual levels of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids was
234 calculated by the following equations (Sumanta et al., 2014):

$$236 \text{ Chl } a (\mu\text{g/ml})=16.72\times A665 - 9.16\times A652 \quad (\text{eq. 1})$$

$$237 \text{ Chl } b (\mu\text{g/ml})=34.09\times A652 - 15.28\times A665 \quad (\text{eq. 2})$$

238 Total carotenoids ($\mu\text{g/ml}$)= $(1000 \times A_{470} - 1.63 \times \text{Chl } a - 104.96 \times \text{Chl } b) / 221$ (eq. 3)

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2.7. Chlorophyll fluorescence

241 Photosynthetic capability of *L. minor* was determined as Pulse-Amplitude-Modulated (PAM)
242 chlorophyll fluorescence kinetics using a PAM 2000 (Walz, Effeltrich, Germany). Plants
243 were first maintained in the dark for 30 min prior to allow complete oxidation of PSII centers
244 and initial fluorescence (F_o) was measured under weak modulated illumination
245 ($1 \mu\text{mol m}^{-2} \text{s}^{-1}$). The measurement of maximal fluorescence (F_m) was obtained by applying a
246 saturating light pulse ($5000 \mu\text{mol m}^{-2} \text{s}^{-1}$, 0.8 s), whereas the Minimal and Maximal
247 fluorescence yield of illuminated sample (F_t and F_m') were determined at the equilibrium
248 state of electron transport after 10 min of continuous illumination ($\text{PAR}=80 \mu\text{mol m}^{-2} \text{s}^{-1}$).
249 All fluorescence yields (F_o , F_o' , F_m , F_m' , and F_t) were used to calculate the maximum quantum
250 yield (F_v/F_m) and the quantum yield of photochemical energy conversion in PSII (F_v'/F_m')
251 essentially as described by Maxwell (2000). The photosynthetic electron transport rate (ETR)
252 was calculated using the formula $0.5 \times \text{PAR} \times \text{Abs} \times \Phi_{\text{PSII}}$. The multiplying factor of 0.5
253 assumed that 50% of the absorbed photosynthetically active radiation (PAR) is distributed to
254 PSII (Laisk and Loreto, 1996) and the absorbance (Abs) theoretically assumed that 84% of
255 the incident photons of photosynthetically active radiation is absorbed by a typical green leaf
256 (Perkins et al., 2002). The non-photochemical quenching (NPQ) was calculated according to
257 method by Bilger et al. (1995) under the exposure condition ($\text{PAR}=80 \mu\text{mol m}^{-2} \text{s}^{-1}$) using Eq.
258 4. All results of parameters were expressed as fold induction comparative to the control.

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260 $\text{NPQ}=(F_m-F_m')/F_m'$ (eq. 4)

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262 **2.8. Fluorescence microscopy imaging**

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3 263 The fluorescence from the photosynthetic chloroplast (red fluorescence) and H₂DCFDA
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6 264 loading in cells (yellow/green fluorescence) was observed under a fluorescence microscope
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8 265 (Olympus IX 71, Tokyo, Japan). The light was applied by a high intensity fluorescence
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11 266 illumination system (X-Cite 120, Excelitas Technologies Corp., Fremont, Canada) and
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13 267 images were captured using the software Cell[^]D (Olympus, Tokyo, Japan).
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19 269 **2.9. Statistical analysis**

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23 270 The results for each endpoint were calculated as fold difference compared to the control and
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25 271 presented as the mean of three replicates with standard error (Mean±SEM). Statistical
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28 272 significance between groups was determined using a one-way analysis of variance (ANOVA)
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30 273 followed by Tukey's post-hoc test after data were assessed for normality by the Shapiro-Wilk
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33 274 and KS normality testing. Concentration-response curves were calculated using GraphPad
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35 275 Prism version 6 (Graphpad Software, California, USA). Concentrations that caused 50%
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37 276 effect (EC₅₀), no-observed-effect-concentration (NOEC) and low-observed-effect-
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40 277 concentration (LOEC) were determined on basis of resulting concentration-response
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43 278 relationships. A principal component analysis (PCA) was applied to the overall data to assess
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45 279 relationships between parameters using XLStat2015 (Addinsoft, Paris, France). The same
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47 280 software was used to calculate Pearson's correlation to measure the strength of association
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50 281 between the endpoints determined in *L. minor*. Statistical significance was set at $p<0.05$.
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56 283 **3. Results**

60 284 **3.1 Growth inhibition**

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285 The fronds reproduction rate of *L. minor* in the solvent control and the blank control (FN)
286 was $0.372\pm 0.024\text{ d}^{-1}$ and $0.374\pm 0.035\text{ d}^{-1}$, respectively. No significant differences between
287 solvent and blank control were identified. In addition, reproduction rates in both solvent and
288 blank control were above the validity limit for the OECD 221 test guideline (average specific
289 growth rate of 0.275d^{-1} ; OECD, 2006). For all growth parameters, significant changes in
290 fronds number (FN) and fronds area (FA) were identified after 7d exposure to as low as 1
291 mg/L 3,5-DCP, while total fronds dry mass (DM) displayed large variation below 2 mg/L (Fig.
292 1). Inhibition of growth-related variables (FN, FA and DM) occurred in a concentration-
293 dependent manner with the NOECs typically observed at 0.5-1 mg/L and with EC_{50} values
294 for FN, FA and DM of 2.20 ± 0.01 , 1.45 ± 0.13 and 1.92 ± 0.27 mg/L (Table 2), respectively. Full
295 growth inhibition (100%) occurred at 4-8 mg/L of 3,5-DCP (Fig. 1).

3.2 Mitochondrial membrane potential

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298 Results from the TMRM assay showed clear concentration-dependent decrease in MMP in *L.*
299 *minor* fronds after 7-day exposure to 3,5-DCP (Fig. 2.A). Significant dissipation of MMP was
300 observed at higher 3,5-DCP concentrations (1.5-4 mg/L) with an EC_{50} of 1.41 ± 0.47 mg/L
301 (Table 2). The highest 3,5-DCP concentration (8 mg/L) resulted in 100% mortality and results
302 was thus excluded from the analyses.

3.3 oxidative stress

303
304 *3,5-DCP* caused a concentration-dependent induction of ROS formation from 0.5 to 3 mg/L
305 ($\text{EC}_{50}= 1.52$ mg/L) in *L. minor* (Fig. 2B, Table 2), with highest ROS levels observed at 2 and
306 3 mg/L. The levels of lipid peroxidation (LPO) also displayed a concentration-dependent
307 response (Fig. 2.C). Similarly, to ROS formation, the MDA levels increased at concentrations
308 higher than 1 mg/L 3,5-DCP ($\text{EC}_{50}= 1.41$ mg/L). Both ROS and MDA reached a plateau at

1.5-3 mg/L 3,5-DCP, with no significant increase in response at higher concentrations. Complete growth inhibition precluded the analysis of these two endpoints at the highest concentration tested (8 mg/L). Using fluorescence microscopy imaging, red photosynthetic fluorescence (Fig. 3.D) and green-yellow ROS probe (H₂DCFDA) fluorescence (Fig. 3E) was clearly observed under fluorescent microscope. After exposure to 3,5-DCP, increased ROS fluorescence at the distal (Fig.3B) and whole (Fig.3C) fronds were typically observed. The increase in ROS fluorescence was inversely proportional to the photosynthetic fluorescence, as seen in the shift between the distribution of red versus green-yellow fluorescence in the control (Fig. 3A) and exposed fronds. The ROS fluorescent appeared to be spatially distributed with the highest levels detected at the base of the frond (Fig. 3B).

3.3 Pigments

A concentration-dependent decrease in chl *a*, chl *b* and carotenoids was observed in *L. minor* after 7-days exposure to 3,5-DCP (Fig. 4). A significant reduction was already observed at 1 mg/L (LOEC) for both chl *a* and chl *b*, with EC₅₀ at 1.31 and 1.41 mg/L (Table 2), respectively. A significant decrease in on carotenoids content was detected at exposure concentrations over 2 mg/L (EC₅₀=2.60±0.42 mg/L). At the highest concentration tested, no chlorophyll (*a* or *b*) and carotenoids were measured due to complete necrosis and/or cell dead of most fronds.

3.4. Chlorophyll *a* fluorescence

A concentration-dependent decrease in initial fluorescence (F_o) of PSII was demonstrated in *L. minor* after exposure to 3,5-DCP (EC₅₀=2.53±0.25 mg/L), whereof significant differences from control was observed at 3 mg/L and maximal fluorescence (F_m) was significantly decreased at 1.5 mg/L (Fig. 4 and Table 2). Concentration-dependent decrease was also

333 observed in both maximum quantum yield of PSII (F_v/F_m , $EC_{50}=1.81\pm0.05$ mg/L) and ETR
334 ($EC_{50}=1.42\pm0.04$ mg/L). Compared to F_v/F_m (LOEC=1.5 mg/L), ETR (LOEC=1 mg/L) was
335 identified as the more sensitive endpoint when exposed to 3,5-DCP (Table.1). A significant
336 and enhanced NPQ yield was observed in *L. minor* after exposure to 1 mg/L 3,5-DCP,
337 followed by a reduction at higher concentrations. A clear concentration-dependent inhibition
338 of the photosynthetic electron transport rate (ETR) was also identified ($EC_{50}= 1.42\pm0.04$
339 mg/L). The different parameters of chlorophyll *a* fluorescence were below the threshold for
340 quantification at concentrations above 4 mg/L due to complete inhibition of growth.

341 **3.5. Principal component analysis**

342 A PCA was applied to the data to decipher potential causal relationships between the
343 determined endpoints (Fig. 7). The two highest concentrations used in this study (4 and 8
344 mg/L 3,5-DCP) were excluded from the PCA analysis due to complete necrosis and/or cell
345 dead of most fronds. The PC1 shows a clear separation between the 3 lowest and the 3
346 highest concentrations of 3,5-DCP in the first axis that explained 84.2% of the total variance.
347 Photosystem II performance, pigments content (Chl *a*, *b* and carotenoids), oxidative
348 phosphorylation and frond number, area and DM were the variables best explaining PC1 and
349 most sensitive at the lower 3,5-DCP concentrations. At concentrations higher than 1.0 mg/L,
350 ROS and LPO levels were more responsive endpoints and parameters were directly
351 associated, showing the capacity of 3,5-DCP to inflict oxidative stress in *L. minor*. At these
352 concentrations, the remaining variables were significantly inhibited, especially at
353 concentration of 3 mg/L and higher. PC1 also displayed a negative association between ROS
354 formation and frond number and DM, ETR, pigments content (carotenoids, Chl *a* and *b*) and
355 OXPHOS. A similar association was found for LPO, especially with frond number, Chl *a* and
356 Chl *b* and ETR. The PC2, explaining only 11.2% of total variance, distinguished between the

1 357 responses obtained for photosystem performance, pigments content, growth inhibition
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3 358 parameters and oxidative phosphorylation, particularly those associated with the two lowest
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5 359 3,5-DCP concentrations used in this study. The two main groups separated by PC2 were NPQ,
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7 360 frond area, F_o and F_v/F_m from that of frond number and DM, OXPHOS, F_m , Chl *a* and Chl *b*
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9 361 and ETR.

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13 362 The Pearson correlation analysis showed several significant positive and negative correlations
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15 363 between the endpoints measured in *L. minor* exposed to 3,5-DCP (Table 1). Similarly, to the
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18 364 PCA analysis, the two highest concentrations of 3,5-DCP were excluded from this analysis.
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20 365 Frond number was positively correlated with DM of fronds, pigments contents (Chl *a*, *b* and
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23 366 carotenoids), F_m , ETR and OXPHOS and negatively correlated with LPO and ROS formation.
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25 367 Positive correlations were found between frond area, carotenoids content, F_o , F_v/F_m and
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28 368 OXPHOS. DM was positively correlated with all endpoints except for frond area, F_o and
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30 369 NPQ. ROS formation was negatively correlated with frond's DM, pigments content, F_m ,
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33 370 ETR and OXPHOS and positively correlated with LPO. The Chl *a* and Chl *b* contents were
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35 371 positively correlated with carotenoids content, F_m , ETR and OXPHOS, but negatively
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38 372 correlated with LPO. Carotenoids content was positively correlated with all variables related
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40 373 to PSII performance and OXPHOS. F_o was positively correlated with F_v/F_m while F_m was
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43 374 correlated with F_v/F_m , ETR and OXPHOS. A positive and significant correlation was found
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45 375 between ETR and OXPHOS, as well as F_v/F_m with NPQ and OXPHOS. A negative
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47 376 correlation was also found between ETR and LPO.

51 377 **4. Discussion**

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54 378 In most phytotoxicity tests, growth inhibition, as well as parameters such as frond size,
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57 379 pigment content, chlorosis and necrosis are evaluated to provide regulatory relevant
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60 380 information (ISO20079, 2005; Kumar and Han, 2010; OECD, 2006). However, the MoA

381 associated with the adverse effects observed are not always provided, and results in lack of
382 knowledge of toxicity mechanisms triggered by a stressor. This study provided a
383 comprehensive assessment of potential MoAs of the chemical 3,5-DCP in terms of the
384 uncoupling capacity and oxidative stress in *L. minor* as a representative aquatic primary
385 producer. The studies conducted were aiming at providing mechanistic insight into how
386 OXPHOS uncouplers interfere with key processes in the mitochondria and chloroplasts to
387 cause growth inhibition.

388 The results from the studies clearly showed that 3,5-DCP caused a concentration-dependent
389 change in all parameter studied (Table 2), and that many of these processes were strongly
390 correlated (Fig.7, Table 2). In-depth review of the different parameters and their internal
391 dependence are presented in subsequent sections to characterise the causal relationship
392 between potential MoA and adversity.

393 **4.1 Growth inhibition**

394 In this study, the adverse effects of 3,5-DCP on survival, growth and associated physiological
395 variables in *L. minor* were identified using a standard 7-days toxicity testing protocol
396 (OECD,2006). Growth rate of *L. minor* in the control groups (Fig. 1) demonstrated that the
397 plants were cultured under satisfactory conditions and that the use of solvents did not affect
398 the outcome of the studies. 3,5-DCP was found to be a potent inhibitor of *L. minor* growth by
399 reducing fronds number (frond production as a measure of reproductive rate), fronds size and
400 DM (Fig. 1). Frond area were identified as the most sensitive growth-related endpoint in this
401 test when comparing EC₅₀ values, closely followed by fronds number and DM (Table 2).
402 Albeit fronds area was the more sensitive parameter in this study, fronds number has been
403 recommended as the better toxicity indicator in *L. minor* exposed to metals and organics
404 (Mitsou et al., 2006), likely due to the fact that chemically-induced changes in cell volume

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405 may also affect the fronds area (Severi and Fornasiero, 1983; Tsukaya, 2003). Good
406 coherence between the CRCs of frond number and DM, and the observed higher sensitivity
407 of fronds area support that this was also the case for 3,5-DCP in *L. minor*.

408 **4.2 Uncoupling of OXPHOS and photophosphorylation**

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409 Oxidative phosphorylation and photophosphorylation produce ATP as a primary source of
410 energy in photosynthetic organism by the mitochondria and the chloroplast. Respiratory
411 uncoupler such as chlorophenols, nitrophenols, and catechol chlorocatechols have been
412 reported to inhibit ATP by interfere with the coupling between electron transport and
413 phosphorylation reactions (Michel et al., 2004; Penttinen, 1995). Based on this mechanism,
414 3,5-DCP could be an inhibitor of mitochondrial respiration as well as photosynthesis by
415 interfering with the energy-transduction by the inner mitochondrial membrane and thylakoids
416 through uncoupling of OXPHOS and photophosphorylation (Plengvidhya and Burris, 1965).
417 The observations that the MMP was among the most sensitive endpoints analysed (Fig. 2A),
418 support that uncoupling of OXPHOS and photophosphorylation may be key to understanding
419 3,5-DCP toxicity. Additionally, mitochondrial dysfunction due to ROS activated programmed
420 cell death (Murphy, 2013; Wang et al., 2013) may enhance the toxicity as seen by an increase
421 in mortality at high 3,5-DCP (>4 mg/L) concentrations (Fig.2, A and Table.1). These
422 observations seem to cohere with previously reported effects of the potent uncoupler carbonyl
423 cyanide *m*-chlorophenyl hydrazine (CCCP) in plants cells (Dzyubinskaya et al., 2006).

424
425 From PCA results (Table.1), the positive correlations found between the reduction of MMP,
426 pigment content, NPQ and growth in *L. minor*, indicated that besides the direct action of 3,5-
427 DCP on these organelles, mitochondrial dysfunction may interfere with the light energy
428 pathway (NPQ) in thylakoid. In consequence, the dysfunction of both organelles may lead to

429 growth inhibition due to reduction of ATP synthesis.

430 **4.4 Oxidative stress and cellular damage**

431 Endogenous ROS are usually produced as a consequence of aerobic metabolic processes in
432 plants, such as photosynthesis and respiration and rapidly eliminated by the antioxidant
433 defence mechanism (Gamble and Burke, 1984; Gechev et al., 2006; Rabilloud et al., 2001).
434 Excessive formation of ROS by exposure to toxicants may lead to cellular oxidative stress,
435 DNA damage and programmed cell death (Agarwal and Said, 2005; Barzilai and Yamamoto,
436 2004). This study confirmed that exposure to 3,5-DCP caused induction of ROS formation in
437 *L. minor* (Fig. 2B and Fig. 3). This induction of ROS formation may be directly caused by
438 uncoupling of OXPHOS and photophosphorylation or indirectly enhanced by a malfunction
439 in the PSII, in which high energy triplet chlorophyll can enhance the formation of singlet
440 oxygen and increase the chance of O₂ to receive electrons from PS I and form superoxide
441 radicals (Asada, 2006). These superoxide radicals can rapidly form hydrogen peroxide (H₂O₂)
442 via the action of the antioxidant enzyme superoxide dismutase, but if not rapidly detoxified,
443 it can give rise to the highly reactive hydroxyl radical (Asada, 2006; Mittal et al., 2012). As a
444 phenolic compound, 3,5-DCP likely caused direct ROS formation and potentially caused
445 oxidative damage as observed in primary producers elsewhere (Luo et al., 2005; Michałowicz
446 et al., 2009; Michałowicz et al., 2010).

447 When the levels of ROS exceed the capacity of the antioxidant system to counteract them,
448 oxidative damage can occur in several cellular components as lipids, proteins, and DNA
449 (Jambunathan, 2010; Salmon et al., 2004). Lipid peroxidation (LPO) in particular, is a
450 process by which oxyradicals attack polyunsaturated fatty acids present in the cellular
451 membrane, causing a chain reaction during which the lipid will be further degraded into lipid
452 hydroperoxides (Halliwell and Gutteridge, 2015; Repetto et al., 2012). Accordingly, the

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453 determination of LPO levels (or its by-products) can be used as an indicator of oxidative
454 damage and assess the overall efficiency of the antioxidant system of organisms exposed to
455 different stressors. This association between ROS formation and LPO was further confirmed
456 by the PCA, where a positive correlation between both endpoints confirms the oxidative
457 stress caused by 3,5-DCP.

458 **4.5 Pigments**

459 Alterations in photosynthetic pigments content (Chlorophyll *a*, *b* and carotenoids) have been
460 indicated as reliable indicators of pollutant toxicity in plants (Einhellig and Rasmussen, 1979;
461 Wang and Freemark, 1995). Chlorophyll contents were reduced by 3,5-DCP at concentrations
462 as low as 1 mg /L (Fig. 4 A, B and Table.1). Chlorophyll plays an important role in light
463 harvest complex where chlorophyll *b* is a part of antenna pigments and chlorophyll *a* is
464 known as the core pigments (Thomber 1975). Decreased chlorophylls concentration indicate
465 a reduction of light energy absorbing capacity in LHC and consequently reduced ETC
466 activity that lead to the reduction of light reaction active. (Flagella et al., 1994; Jiang et al.,
467 2008; Yusuf et al., 2010).

468 Unlike chlorophyll, reduction of carotenoids content was only observed at 3,5-DCP
469 concentration over 2 mg/L (Fig.4C and Table.1). Carotenoids, which primary role is to act as
470 accessory light-harvesting pigments, may also protect the photosynthetic apparatus from
471 oxidative damage (Young and Britton, 1990). Many studies reported that oxidative stress is
472 one of the most important regulatory mechanisms in photosynthesis (Triantaphylidès and
473 Havaux, 2009; Tripathy and Oelmüller, 2012). However, excessive ROS formation in the
474 chloroplast can enhanced degradation of chlorophyll through oxidation (Vass, 2012).
475 Additionally, phenolic compounds can reduce chlorophyll content by interfering with the
476 chlorophyllase activity that regulate the degradation of chlorophyll (Yang et al., 2002).
477 Increased oxidative stress can also interrupt carotenoid formation and degradation by

1 478 modulating enzymes involved in carotenoids synthesis (Chang et al., 2013), whereas the
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3 479 resulting increase in ROS-related hydrogen peroxide (H₂O₂) can oxidize a wide range of
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5 480 molecules inside the chloroplast (Borisova et al., 2012), including the carotenoids themselves
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7 481 (Lopez-Serrano and Ros Barceló, 1999). In addition, reduced content of carotenoids may
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10 482 enhance ROS formation, as some carotenoids (such as lutein, violaxanthin, β-carotene and
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12 483 etc.) can quench ROS or the triplet state of chlorophyll to prevent singlet oxygen formation
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15 484 (Dreuw et al., 2005). The reduced content of carotenoids may thus indicate potential
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17 485 accumulation of damage from oxidative stress. A close relationship between pigments content
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20 486 (Chl *a* and *b* and carotenoids), oxidative stresses (ROS and LPO) was also detected in the
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22 487 PCA, where a strong negative correlation was observed between these endpoints, thus
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25 488 reinforcing the negative impact of 3,5-DCP in the photosynthetic apparatus of *L. minor* and
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27 489 its relation to oxidative stress.

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30 490 Moreover, decreased chlorophyll content may also be caused by the reduction of chlorophyll
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32 491 biosynthesis due to inhibition of ATP production. In the chlorophyll biosynthetic reaction
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34 492 processes in chloroplast, ATP is essential for conversion of glutamate to glutamyl-transfer
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37 493 RNA (tRNA) and the production of Mg-protoporphyrin IX which ultimately form
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39 494 chlorophylls (Willows, 2006).

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43 44 496 **4.3 Interference with photosynthetic capacity**

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47 497 In recent years, the use of rapid and sensitive bioindicators of plant stress in response to
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50 498 different type of stressors has been growing, especially those related to photosystem
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52 499 performance (Kumar and Han, 2010). The most frequently used parameter to assess PSII
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55 500 performance is the maximum quantum efficiency of primary photochemistry, but other
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57 501 parameters such as the operational plant capacity to convert light energy into chemical energy
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60 502 such as NPQ are very useful to assess the health state of plants (Eullaffroy and Vernet, 2003;

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503 Mallick and Mohn, 2003). However, there is lack of knowledge about the effects of
504 OXHPOS and photophosphorylation uncouplers in *L. minor*. In this study, maximal quantum
505 yield (F_v/F_m) was significantly suppressed after 7d exposure to 3,5-DCP. In photosynthetic
506 organisms, the formation of ROS and oxidative damage to chloroplast and thylakoids
507 membranes can also lead to a decrease in PSII photosynthetic efficiency and the content of
508 chlorophyll and carotenoids (Nishiyama et al., 2006). Several studies also showed that
509 oxidative stress in chloroplast can cause PSII inhibition as high ROS content in chloroplast
510 reduce D1 protein synthesis and inhibit repair of PSII (Nishiyama et al., 2006). This
511 reduction was also in agreement with the reduction in relative ETR, which normally
512 represents the rate of electrons pumped via PSII into the photosynthetic chain (Schreiber et
513 al., 2012). The decrease in ETR was potentially also due to inhibition of the PSII donor sites,
514 as phenols can bind to hydroxylamine and interaction with the 41-KDa protein (Pfister and
515 Schreiber, 1983; Strasser, 1997). For example, some phenolic herbicides such as Ioxynil had
516 been reported to inhibit ATP synthesis due to their direct binding to the major protein
517 complexes in the ETC and block the electron flow (Schreiber et al., 2007). Minimal and
518 maximal fluorescence intensity (F_o and F_m) are strongly relative to excitation rate of the
519 photoreaction centres which are associated with antenna pigments complexes (Baker, 2008).
520 The damage of PSII, especially in D1 protein due to ROS enhancement might have caused
521 the reduction of F_m (Guenther and Melis, 1990; Roach and Krieger-Liszkay, 2014) at low 3,5-
522 DCP concentration (1mg/l), as the F_o value was not affected at this concentration. Thus, PSII
523 inhibition is likely caused by the concentration-dependent reduction of the F_m value at low to
524 intermediate concentrations, whereas changes to F_o only occurred at higher concentrations.
525 This was supported by the PCA results, where PSII parameters were strongly negative
526 correlated to ROS formation and LPO.

527 Among all antioxidant actions in the chloroplast, non-photochemical quenching (NPQ) of

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528 chlorophyll fluorescence is thought to be the key regulatory and photoprotective mechanism
529 against oxidative stress in photosynthetic organisms (Lambrev et al., 2012). In this study,
530 increased NPQ observed at low 3,5-DCP concentrations (0.5-1.5 mg/L) may be protective by
531 either scavenging ROS or quenching the excess energy which can convert ROS to heat
532 (Carbonera et al., 2012; Dall'Osto et al., 2006; Müller et al., 2001). At high 3,5-DCP
533 concentration (over 2 mg/L), reduction of NPQ might be explain by the direct oxidative
534 damage to PSII reaction centres (Lawlor and Tezara, 2009).

535 **4.4 Identification of the main MoAs and correlation between different parameters.**

536 The responses and effects observed in the current study suggest that 3,5-DCP display both
537 concentration-dependent and target-specific MoAs that seem to be causally related. Some
538 endpoints including growth, ETR, chlorophyll content and OXPHOS were observed at low
539 concentration (0.5-1 mg/L), which indicate that these endpoints were directly associated with
540 the respiratory and photosynthesis uncoupling activity of 3,5-DCP. High concentration (>1
541 mg/L) effects such as ROS formation, LPO, reduction of carotenoid content and modulation
542 of PSII efficiency, indicated that these endpoints were associated with excessive ROS
543 formation and oxidative damage to key cellular components in *L. minor*. The current data was
544 used to assemble an initial AOP network connecting the different MoAs and toxicity
545 pathways to the apical (adverse) effect (Fig. 7). The putative network of AOPs were
546 submitted to the AOP repository AOPwiki (<https://aopwiki.org>) as an initial effort to compile
547 AOPs for the diverse group of CPs in primary producers, to propose a suite of bioassays for
548 future implementation in IATA approaches (Tollefsen et al., 2014) and support development
549 of more mechanistically-focussed hazard and risk assessment initiatives.

57 **5. Conclusion**

1 551 The present study exposed *L. minor* to a model toxicant 3,5-DCP to investigate potential
2 552 MoA and adverse endpoints in a representative aquatic primary producer. The results
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4 553 indicated that 3,5-DCP cause a number of concentration-dependent MoAs in *L. minor*,
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7 554 whereof uncoupling of OXPHOS and photooxidative phosphorylation were proposed to be
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10 555 the main MoAs leading to reduction in ATP synthesis and growth inhibition at low 3,5-DCP
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12 556 concentrations. ROS induced oxidative stress and damage were proposed to occur at higher
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14 557 3,5-DCP concentration and indirectly affect a number of endpoints such as pigments content
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17 558 and PSII efficiency. A network of AOPs were subsequently proposed and submitted to the
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19 559 AOPwiki (AOP: 245, <https://aopwiki.org/aops/245>) as an initial effort to develop AOPs for
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22 560 CPs, identify suitable bioassays for IATA approaches, and support future hazard and risk
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24 561 assessment initiatives for CPs.

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7. References

- 1
2
3
4 Agarwal, A., Said, T.M., 2005. Oxidative stress, DNA damage and apoptosis in male
5 infertility: a clinical approach. *BJU international* 95, 503-507.
6
- 7 Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D.,
8 Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrano, J.A., Tietge, J.E.,
9 Villeneuve, D.L., 2010. Adverse outcome pathways: a conceptual framework to support
10 ecotoxicology research and risk assessment. *Environ Toxicol Chem* 29, 730-741.
11
- 12
13 Asada, K., 2006. Production and scavenging of reactive oxygen species in chloroplasts and
14 their functions. *Plant physiology* 141, 391-396.
15
- 16
17 Azcón-Bieto, J., Osmond, C.B., 1983. Relationship between photosynthesis and respiration
18 the effect of carbohydrate status on the rate of CO₂ production by respiration in darkened and
19 illuminated wheat leaves. *Plant Physiology* 71, 574-581.
20
- 21
22 Baker, N.R., 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annu. Rev.*
23 *Plant Biol.* 59, 89-113.
24
- 25
26 Baskar, K., Sudha, V., Tamilselvan, V., 2016. Growth Inhibitory Effect of 3,5-dichlorophenol
27 on *Lemna gibba* (L.). *Asian Journal of Biological Sciences*, 9: 47-52.
28
- 29
30 Barzilai, A., Yamamoto, K.-I., 2004. DNA damage responses to oxidative stress. *DNA repair*
31 3, 1109-1115.
32
- 33
34 Bennett, J., 1981. Biosynthesis of the light-harvesting chlorophyll a/b protein. Polypeptide
35 turnover in darkness. *European journal of biochemistry* 118, 61-70.
36
- 37
38 Bennett, J., 1983. Regulation of photosynthesis by reversible phosphorylation of the light-
39 harvesting chlorophyll a/b protein. *Biochemical Journal* 212, 1.
40
- 41
42 Bilger, W., Schreiber, U., Bock, M., 1995. Determination of the quantum efficiency of
43 photosystem II and of non-photochemical quenching of chlorophyll fluorescence in the field.
44 *Oecologia* 102, 425-432.
45
- 46
47 Borisova, M.M.M., Kozuleva, M.A., Rudenko, N.N., Naydov, I.A., Klenina, I.B., Ivanov,
48 B.N., 2012. Photosynthetic electron flow to oxygen and diffusion of hydrogen peroxide
49 through the chloroplast envelope via aquaporins. *Biochimica et Biophysica Acta (BBA)-*
50 *Bioenergetics* 1817, 1314-1321.
51
- 52
53 Carbonera, D., Gerotto, C., Posocco, B., Giacometti, G.M., Morosinotto, T., 2012. NPQ
54 activation reduces chlorophyll triplet state formation in the moss *Physcomitrella patens*.
55 *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1817, 1608-1615.
56
- 57
58 Chang, H.-L., Hsu, Y.-T., Kang, C.-Y., Lee, T.-M., 2013. Nitric oxide down-regulation of
59 carotenoid synthesis and PSII activity in relation to very high light-induced singlet oxygen
60 production and oxidative stress in *Chlamydomonas reinhardtii*. *Plant and cell physiology* 54,
61 1296-1315.
62
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64 Cloern, J.E., Foster, S., Kleckner, A., 2014. Phytoplankton primary production in the world's
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estuarine-coastal ecosystems. *Biogeosciences* 11, 2477-2501.

Cowgill, U. M., Milazzo, D. P., 1989. The culturing and testing of two species of duckweed. In *Aquatic Toxicology and Hazard Assessment: 12th Volume*. ASTM International. ISBN-EB: 978-0-8031-5088-1. 427p.

Dall'Osto, L., Lico, C., Alric, J., Giuliano, G., Havaux, M., Bassi, R., 2006. Lutein is needed for efficient chlorophyll triplet quenching in the major LHCII antenna complex of higher plants and effective photoprotection in vivo under strong light. *BMC Plant Biology* 6, 32.

De Schamphelaere, K. A. C., Stubblefield, W., Rodriguez, P., Vleminckx, K., & Janssen, C. R., 2010. The chronic toxicity of molybdate to freshwater organisms. I. Generating reliable effects data. *Science of the total environment*, 408(22), 5362-5371.

Dreuw, A., Fleming, G., Head-Gordon, M., 2005. Role of electron-transfer quenching of chlorophyll fluorescence by carotenoids in non-photochemical quenching of green plants. Portland Press Limited.

Dzyubinskaya, E.V., Kiselevsky, D.B., Lobysheva, N.V., Shestak, A.A., Samuilov, V.D., 2006. Death of stoma guard cells in leaf epidermis under disturbance of energy provision. *Biochemistry. Biokhimiia* 71, 1120-1127.

Ehrenberg, B., Montana, V., Wei, M., Wuskell, J., Loew, L., 1988. Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. *Biophysical journal* 53, 785-794.

Einhellig, F.A., Leather, G.R., Hobbs, L.L., 1985. Use of *Lemna minor* L. as a bioassay in allelopathy. *Journal of chemical Ecology* 11, 65-72.

Einhellig, F.A., Rasmussen, J.A., 1979. Effects of three phenolic acids on chlorophyll content and growth of soybean and grain sorghum seedlings. *Journal of Chemical Ecology* 5, 815-824.

Elser, J.J., Bracken, M.E., Cleland, E.E., Gruner, D.S., Harpole, W.S., Hillebrand, H., Ngai, J.T., Seabloom, E.W., Shurin, J.B., Smith, J.E., 2007. Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecology letters* 10, 1135-1142.

Escher, B.I., Snozzi, M., Schwarzenbach, R.P., 1996. Uptake, speciation, and uncoupling activity of substituted phenols in energy transducing membranes. *Environmental Science & Technology* 30, 3071-3079.

Eullaffroy, P., Vernet, G., 2003. The F684/F735 chlorophyll fluorescence ratio: a potential tool for rapid detection and determination of herbicide phytotoxicity in algae. *Water research* 37, 1983-1990.

Fairchild, J., Ruessler, D., Haverland, P., Carlson, A., 1997. Comparative sensitivity of *Selenastrum capricornutum* and *Lemna minor* to sixteen herbicides. *Archives of Environmental Contamination and Toxicology* 32, 353-357.

Fernandes, T.C., Pizano, M.A., Marin-Morales, M.A., 2013. Characterization, modes of action and effects of trifluralin: a review, *Herbicides-Current Research and Case Studies in*

1
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4
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6
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64
65

Use. InTech.

Flagella, Z., Pastore, D., Campanile, R., Di Fonzo, N., 1994. Photochemical quenching of chlorophyll fluorescence and drought tolerance in different durum wheat (*Triticum durum*) cultivars. *The Journal of Agricultural science* 122, 183-192.

Gamble, P.E., Burke, J.J., 1984. Effect of water stress on the chloroplast antioxidant system I. Alterations in glutathione reductase activity. *Plant Physiology* 76, 615-621.

Gechev, T.S., Van Breusegem, F., Stone, J.M., Denev, I., Laloi, C., 2006. Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *Bioessays* 28, 1091-1101.

Guenther, J.E., Melis, A., 1990. The physiological significance of photosystem II heterogeneity in chloroplasts. *Photosynthesis research* 23, 105-109.

Gupta, B., Huang, B., 2014. Mechanism of Salinity Tolerance in Plants: Physiological, Biochemical, and Molecular Characterization. *International Journal of Genomics* 2014, 18.

Haller, W.T., Sutton, D.L., Barlowe, W.C., 1974. Effects of salinity on growth of several aquatic macrophytes. *Ecology* 55, 891-894.

Halliwell, B., Gutteridge, J.M., 2015. *Free radicals in biology and medicine*. Oxford University Press, USA.

Hartman, W.A., Martin, D.B., 1985. Effects of four agricultural pesticides on *Daphnia pulex*, *Lemna minor*, and *Potamogeton pectinatus*. *Bulletin of environmental contamination and toxicology* 35, 646-651.

Horvat, T., Vidaković-Cifrek, Ž., Oreščanin, V., Tkalec, M., Pevalek-Kozlina, B., 2007. Toxicity assessment of heavy metal mixtures by *Lemna minor* L. *Science of the total environment* 384, 229-238.

Hou, W., Chen, X., Song, G., Wang, Q., Chang, C.C., 2007. Effects of copper and cadmium on heavy metal polluted waterbody restoration by duckweed (*Lemna minor*). *Plant physiology and biochemistry* 45, 62-69.

Igbinosa, E.O., Odjadjare, E.E., Chigor, V.N., Igbinosa, I.H., Emoghene, A.O., Ekhaise, F.O., Igiehon, N.O., Idemudia, O.G., 2013. Toxicological profile of chlorophenols and their derivatives in the environment: the public health perspective. *The Scientific World Journal* 2013.

ISO8692:2012, 2012. Water quality -- Fresh water algal growth inhibition test with unicellular green algae. ISO TC.

ISO20079, 2005. Water quality – determination of the toxic effect of water constituents and waste water to duckweed (*Lemna minor*) – Duckweed growth inhibition test. ISO TC.

Jambunathan, N., 2010. Determination and detection of reactive oxygen species (ROS), lipid peroxidation, and electrolyte leakage in plants. *Plant stress tolerance: methods and protocols*, 291-297.

1 Jamers, A., Lenjou, M., Deraedt, P., Bockstaele, D.V., Blust, R., Coen, W.d., 2009. Flow
2 cytometric analysis of the cadmium-exposed green alga *Chlamydomonas reinhardtii*
3 (Chlorophyceae). *European Journal of Phycology* 44, 541-550.

4 Jiang, H.-X., Chen, L.-S., Zheng, J.-G., Han, S., Tang, N., Smith, B.R., 2008. Aluminum-
5 induced effects on Photosystem II photochemistry in Citrus leaves assessed by the
6 chlorophyll a fluorescence transient. *Tree Physiol* 28, 1863-1871. Kirby, M., Sheahan, D.,
7 1994. Effects of atrazine, isoproturon, and mecoprop on the macrophyte *Lemna minor* and the
8 alga *Scenedesmus subspicatus*. *Bulletin of environmental contamination and toxicology* 53,
9 120-126.

10 Kumar, K.S., Han, T., 2010. Physiological response of Lemna species to herbicides and its
11 probable use in toxicity testing. *Toxicology and Environmental Health Sciences* 2, 39-49.

12 Laisk, A., Loreto, F., 1996. Determining photosynthetic parameters from leaf CO₂ exchange
13 and chlorophyll fluorescence (ribulose-1, 5-bisphosphate carboxylase/oxygenase specificity
14 factor, dark respiration in the light, excitation distribution between photosystems, alternative
15 electron transport rate, and mesophyll diffusion resistance. *Plant Physiology* 110, 903-912.

16 Lambrev, P.H., Miloslavina, Y., Jahns, P., Holzwarth, A.R., 2012. On the relationship between
17 non-photochemical quenching and photoprotection of Photosystem II. *Biochimica et*
18 *Biophysica Acta (BBA)-Bioenergetics* 1817, 760-769.

19 Landolt, E., 1975. Morphological differentiation and geographical distribution of the *Lemna*
20 *gibba-Lemna minor* group. *Aquatic botany*, 1, 345-363.

21 Landolt, E., 1998. *Lemnaceae*. In *Flowering Plants, Monocotyledons*. Springer Berlin
22 Heidelberg, 264-267.

23 Lawlor, D.W., Tezara, W., 2009. Causes of decreased photosynthetic rate and metabolic
24 capacity in water-deficient leaf cells: a critical evaluation of mechanisms and integration of
25 processes. *Annals of botany*.

26 Legradi, J., Dahlberg, A.-K., Cenijn, P., Marsh, G.r., Asplund, L., Bergman, Å., Legler, J.,
27 2014. Disruption of oxidative phosphorylation (OXPHOS) by hydroxylated polybrominated
28 diphenyl ethers (OH-PBDEs) present in the marine environment. *Environmental science &*
29 *technology* 48, 14703-14711.

30 Lopez-Serrano, M., Ros Barceló, A., 1999. H₂O₂-mediated pigment decay in strawberry as a
31 model system for studying color alterations in processed plant foods. *Journal of agricultural*
32 *and food chemistry* 47, 824-827.

33 Luo, Y., Su, Y., Lin, R.-z., Shi, H.-h., Wang, X.-r., 2006. 2-Chlorophenol induced ROS
34 generation in fish *Carassius auratus* based on the EPR method. *Chemosphere* 65, 1064-1073.

35 Luo, Y., Wang, X.R., Shi, H.H., Mao, D.q., Sui, Y.X., Ji, L.L., 2005. Electron paramagnetic
36 resonance investigation of in vivo free radical formation and oxidative stress induced by 2, 4
37 - dichlorophenol in the freshwater fish *Carassius auratus*. *Environmental toxicology and*
38 *chemistry* 24, 2145-2153.

39 Mallick, N., Mohn, F., 2003. Use of chlorophyll fluorescence in metal-stress research: a case
40 study with the green microalga *Scenedesmus*. *Ecotoxicology and environmental safety* 55,

64-69.

1
2 Maxwell, K., Johnson, G.N., 2000. Chlorophyll fluorescence—a practical guide. Journal of
3 experimental botany 51, 659-668.

4
5 Mendiola, N.B., 1918. Variation and selection within clonal lines of *Lemna minor*, [Ithaca, N.
6 Y.].

7
8
9 Michałowicz, J., Duda, W., 2007. Phenols--Sources and Toxicity. Polish Journal of
10 Environmental Studies 16.

11
12 Michałowicz, J., Posmyk, M., Duda, W., 2009. Chlorophenols induce lipid peroxidation and
13 change antioxidant parameters in the leaves of wheat (*Triticum aestivum* L.). Journal of plant
14 physiology 166, 559-568.

15
16
17 Michałowicz, J., Urbanek, H., Bukowska, B., Duda, W., 2010. The effect of 2, 4-
18 dichlorophenol and pentachlorophenol on antioxidant system in the leaves of *Phalaris*
19 *arudinacea*. *Biologia plantarum* 54, 597-600.

20
21
22 Michel, A., Johnson, R.D., Duke, S.O., Scheffler, B.E., 2004. Dose - response relationships
23 between herbicides with different modes of action and growth of *Lemna paucicostata*: An
24 improved ecotoxicological method. *Environmental toxicology and Chemistry* 23, 1074-1079.

25
26
27 Mitsou, K., Koulianou, A., Lambropoulou, D., Pappas, P., Albanis, T., Lekka, M., 2006.
28 Growth rate effects, responses of antioxidant enzymes and metabolic fate of the herbicide
29 Propanil in the aquatic plant *Lemna minor*. *Chemosphere* 62, 275-284.

30
31
32 Mittal, S., Kumari, N., Sharma, V., 2012. Differential response of salt stress on *Brassica*
33 *juncea*: photosynthetic performance, pigment, proline, D1 and antioxidant enzymes. *Plant*
34 *Physiology and Biochemistry* 54, 17-26.

35
36
37 Müller, P., Li, X.-P., Niyogi, K.K., 2001. Non-photochemical quenching. A response to excess
38 light energy. *Plant physiology* 125, 1558-1566.

39
40
41 Murphy, M.P., 2013. Mitochondrial dysfunction indirectly elevates ROS production by the
42 endoplasmic reticulum. *Cell metabolism* 18, 145-146.

43
44
45 Naumann, B., Eberius, M., & Appenroth, K. J., 2007. Growth rate based dose–response
46 relationships and EC-values of ten heavy metals using the duckweed growth inhibition test
47 (ISO 20079) with *Lemna minor* L. clone St. *Journal of plant physiology*, 164(12), 1656-1664.

48
49
50 Nishiyama, Y., Allakhverdiev, S.I., Murata, N., 2006. A new paradigm for the action of
51 reactive oxygen species in the photoinhibition of photosystem II. *Biochimica et Biophysica*
52 *Acta (BBA)-Bioenergetics* 1757, 742-749.

53
54
55 OECD, 2006. Test No. 221: *Lemna* sp. Growth Inhibition Test. OECD Publishing.

56
57
58 OECD, 2011. Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test.
59 OECD Publishing.

60
61
62 Penttinen, O.-P., 1995. Chlorophenols in aquatic environments: Structure-activity correlations,
63 *Annales Zoologici Fennici*. JSTOR, pp. 287-294.

1 Perkins, R., Oxborough, K., Hanlon, A., Underwood, G., Baker, N., 2002. Can chlorophyll
2 fluorescence be used to estimate the rate of photosynthetic electron transport within
3 microphytobenthic biofilms? Marine Ecology Progress Series 228, 47-56.

4 Pfister, K., & Schreiber, U., 1984. Comparison of diuron-and phenol-type inhibitors:
5 Additional inhibitory action at the photosystem II donor site. Zeitschrift für Naturforschung
6 C, 39(5), 389-392

7
8
9 Plengvidhya, P., Burris, R., 1965. Inhibitors of photophosphorylation and photoreduction.
10 Plant physiology 40, 997.

11
12 Rabilloud, T., Heller, M., Rigobello, M.P., Bindoli, A., Aebersold, R., Lunardi, J., 2001. The
13 mitochondrial antioxidant defence system and its response to oxidative stress. Proteomics 1,
14 1105-1110.

15
16
17 Razinger, J., Drinovec, L., Zrimec, A., 2010. Real - time visualization of oxidative stress in a
18 floating macrophyte *Lemna minor* L. exposed to cadmium, copper, menadione, and AAPH.
19 Environmental toxicology 25, 573-580.

20
21
22 Repetto, M., Semprine, J., Boveris, A., 2012. Lipid peroxidation: chemical mechanism,
23 biological implications and analytical determination, Lipid peroxidation. InTech.

24
25
26 Ritchie, R.J., 2006. Consistent sets of spectrophotometric chlorophyll equations for acetone,
27 methanol and ethanol solvents. Photosynthesis research 89, 27-41.

28
29
30 Roach, T., Krieger-Liszkay, A.K., 2014. Regulation of Photosynthetic Electron Transport and
31 Photoinhibition. Current Protein & Peptide Science 15, 351-362.

32
33
34 Salmon, T.B., Evert, B.A., Song, B., Doetsch, P.W., 2004. Biological consequences of
35 oxidative stress-induced DNA damage in *Saccharomyces cerevisiae*. Nucleic Acids Research
36 32, 3712-3723.

37
38
39 Scaduto, R.C., Grotyohann, L.W., 1999. Measurement of mitochondrial membrane potential
40 using fluorescent rhodamine derivatives. Biophysical journal 76, 469-477. Schreiber, U.,
41 Klughammer, C., Kolbowski, J., 2012. Assessment of wavelength-dependent parameters of
42 photosynthetic electron transport with a new type of multi-color PAM chlorophyll
43 fluorometer. Photosynthesis research, 1-18.

44
45
46 Schreiber, U., Quayle, P., Schmidt, S., Escher, B.I., Mueller, J.F., 2007. Methodology and
47 evaluation of a highly sensitive algae toxicity test based on multiwell chlorophyll
48 fluorescence imaging. Biosensors and Bioelectronics 22, 2554-2563.

49
50
51 Severi, A., Fornasiero, R.B., 1983. Morphological variations in *Lemna minor* L. and possible
52 relationships with abscisic acid. Caryologia 36, 57-64.

53
54
55 Strasser, B. J., 1997. Donor side capacity of photosystem II probed by chlorophyll *a*
56 fluorescence transients. Photosynthesis Research, 52(2), 147-155. Terada, H., 1990.
57 Uncouplers of oxidative phosphorylation. Environmental Health Perspectives 87, 213.

58
59
60 Thornber, J. P., 1975. Chlorophyll-proteins: light-harvesting and reaction center components
61 of plants. Annual Review of Plant Physiology, 26(1), 127-158.

1 Tollefsen, K.E., Scholz, S., Cronin, M.T., Edwards, S.W., de Knecht, J., Crofton, K., Garcia-
2 Reyero, N., Hartung, T., Worth, A., Patlewicz, G., 2014. Applying adverse outcome pathways
3 (AOPs) to support integrated approaches to testing and assessment (IATA). *Regulatory*
4 *Toxicology and Pharmacology* 70, 629-640.

5
6 Triantaphylidès, C., Havaux, M., 2009. Singlet oxygen in plants: production, detoxification
7 and signaling. *Trends in plant science* 14, 219-228.

8
9 Tripathy, B.C., Oelmüller, R., 2012. Reactive oxygen species generation and signaling in
10 plants. *Plant signaling & behavior* 7, 1621-1633.

11
12 Tsukaya, H., 2003. Organ shape and size: a lesson from studies of leaf morphogenesis.
13 *Current opinion in plant biology* 6, 57-62.

14
15 Uysal, Y., Taner, F., 2009. Effect of pH, temperature, and lead concentration on the
16 bioremoval of lead from water using *Lemna minor*. *International journal of phytoremediation*
17 11, 591-608.

18
19 Vass, I., 2012. Molecular mechanisms of photodamage in the Photosystem II complex.
20 *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1817, 209-217.

21
22 Van Echelpoel, W., Boets, P., & Goethals, P. L., 2016. Functional Response (FR) and Relative
23 Growth Rate (RGR) Do Not Show the Known Invasiveness of *Lemna minuta* (Kunth). *PloS*
24 *one*, 11(11), e0166132.

25
26 Villeneuve, D.L., Crump, D., Garcia-Reyero, N., Hecker, M., Hutchinson, T.H., LaLone, C.A.,
27 Landesmann, B., Lettieri, T., Munn, S., Nepelska, M., 2014. Adverse outcome pathway (AOP)
28 development I: strategies and principles. *Toxicological Sciences* 142, 312-320.

29
30 Wang, C.-H., Wu, S.-B., Wu, Y.-T., Wei, Y.-H., 2013. Oxidative stress response elicited by
31 mitochondrial dysfunction: implication in the pathophysiology of aging. *Experimental*
32 *Biology and Medicine* 238, 450-460.

33
34 Wang, W., Freemark, K., 1995. The use of plants for environmental monitoring and
35 assessment. *Ecotoxicology and environmental safety* 30, 289-301.

36
37 Willows, R.D., 2006. Chlorophyll Synthesis, in: Wise, R.R., Hooper, J.K. (Eds.), *The*
38 *Structure and Function of Plastids*. Springer Netherlands, Dordrecht, pp. 295-313.

39
40 Yang, C.-M., Lee, C.-N., Chou, C.-H., 2002. Effects of three allelopathic phenolics on
41 chlorophyll accumulation of rice (*Oryza sativa*) seedlings: I. Inhibition of supply-orientation.
42 *Botanical Bulletin of Academia Sinica* 43.

43
44 Young, A.J., Britton, G., 1990. Carotenoids and oxidative stress, *Current research in*
45 *photosynthesis*. Springer, pp. 3381-3384.

46
47 Yusuf, M.A., Kumar, D., Rajwanshi, R., Strasser, R.J., Tsimilli-Michael, M., Sarin, N.B.,
48 2010. Overexpression of γ -tocopherol methyl transferase gene in transgenic *Brassica juncea*
49 plants alleviates abiotic stress: physiological and chlorophyll a fluorescence measurements.
50 *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1797, 1428-1438.

51
52 Ziegler, P., Sree, K. S., Appenroth, K. J. (2016). Duckweeds for water remediation and
53
54
55
56
57
58
59
60
61
62
63
64
65

toxicity testing. *Toxicological & Environmental Chemistry*, 98(10), 1127-1154.

Zezulka, Š., Kummerová, M., Babula, P., Váňová, L., 2013. *Lemna minor* exposed to fluoranthene: growth, biochemical, physiological and histochemical changes. *Aquatic toxicology* 140, 37-47.

1
2
3
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5
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Figure legends

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2 *Fig. 1. Inhibition of growth measured as frond number (A), frond area (B) and frond dry mass*
3 *(C) in L. minor after 7d exposure to 3,5-dichlorophenol (3,5-DCP). The experiment results*
4 *(Mean ± SEM) represented 3 independent studies.*
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10 *Fig. 2. Inhibition of oxidative phosphorylation (OXPHOS) measured as MMP (A), reactive*
11 *oxygen species (ROS) formation (B) and lipid peroxidation (LPO) measured as MDA content*
12 *(C) in L. minor after 7 days exposure to 3,5-dichlorophenol (3,5-DCP). The experiment*
13 *results (Mean ± SEM) represent 3 independent studies and calculated as fold increase*
14 *compare to control groups (0 mg/L). Asterisks (*) represent significant statistical differences*
15 *(p<0.05). Complete growth inhibition at concentrations above 4 mg/L precluded analysing of*
16 *these endpoints.*
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28 *Fig. 3. In vivo visualization of ROS formation in L. minor exposed to different concentrations*
29 *of 3,5-DCP: (A) Control, (B) Low concentration (1.5 mg/L) and (C) high concentration (3*
30 *mg/L). (D) Healthy cell in fronds. (E) ROS formation in fronds cells. Complete growth*
31 *inhibition at concentrations above 4 mg/L precluded analysing of these endpoints.*
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39 *Fig. 4. Reduction of chlorophyll a (A), chlorophyll b (B) and Carotenoids (C) content in L.*
40 *minor after 7d exposure to 3,5-DCP. The experiment results (Mean ± SEM) represented 3*
41 *independent studies and were displayed as normalised (fold) increase compared to the*
42 *control groups. Asterisk (*) represent significant statistical differences (p<0.05) compare to*
43 *control. Complete growth inhibition at concentrations above 4 mg/L precluded analysing of*
44 *these endpoints.*
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55 *Fig. 5. Maximal photosystem II efficiency parameters Fo (A), Fm(B), Fv/Fm (C), W (D) and*
56 *ETR (E) in L. minor after 7d exposure to 3,5-dichlorophenol (3,5-DCP). The experiment*
57 *results (Mean ± SEM) represented 3 independent studies and calculated as fold increase*
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compare to control groups (0 mg/L). Asterisk (*) represent significant statistical differences ($p < 0.05$) compare to control. Complete growth inhibition at concentrations above 4 mg/L precluded analysing of these endpoints.

Fig. 6. Principal component analysis (PCA) of the endpoints determined in Lemna minor exposed to 3,5-dichlorophenol for 7 days. ROS–reactive oxygen species, LPO–lipid peroxidation, NPQ–non-photochemical quenching, Fo–minimal fluorescence yield, Fv/Fm–maximum quantum yield of PSII, OXPHOS – oxidative phosphorylation, Fm–maximal fluorescence yield, ETR–electron transfer rate, Chl a–Chlorophyll a, Chl b–Chlorophyll b.

Fig. 7. Tentative network of Adverse Outcome Pathway (AOP for uncoupling of oxidative phosphorylation, OXPHOS (mitochondria) and photophosphorylation (chloroplast) at low concentrations (blue arrows) as the main molecular initiating events (MIEs). A series of key events (KEs) including reduction of ATP due to ETC inhibition (Terada, 1990) were proposed to cause growth inhibition as the adverse outcome (AO). Reduction of cellular ATP were also proposed to cause reduction in photosynthetic pigments synthesis and light harvesting (Bennett, 1981; Bennett, 1983) which ultimately will reduced photosynthetic energy production and glucose production thus reduce respiration in mitochondria and ultimately lead to growth inhibition (Azcón-Bieto and Osmond, 1983). Oxidative stress at high 3,5-DCP concentration (red arrows) was proposed to enhance OXPHOS and photophosphorylation by increasing membrane associated peroxidase activity and/or damage the D1 protein and result in PSII inhibition, NPQ enhancement and reduction in pigment production that reduce photosynthesis and respiration. Endpoints measured in this study were marked in red text.

1 *Table 1. Pearson correlation matrix obtained from all the endpoints determined in L. minor exposed to 3,5-dichlorophenol for 7 days.*
 2 *Significant positive or negative correlatives are labelled in bold.*

Variables	FN	FA	DM	ROS	Chl a	Chl b	Car	Fo	Fm	Fv/Fm	ETR	NPQ	OXPHOS	LPO
FN	1	0.676	0.980	-0.945	0.981	0.988	0.923	0.689	0.984	0.801	0.968	0.648	0.962	-0.866
FA	0.676	1	0.750	-0.543	0.699	0.710	0.836	0.990	0.790	0.947	0.694	0.716	0.826	-0.344
DM	0.980	0.750	1	-0.939	0.949	0.961	0.978	0.764	0.986	0.883	0.957	0.756	0.957	-0.758
ROS	-0.945	-0.543	-0.939	1	-0.939	-0.940	-0.890	-0.594	-0.924	-0.716	-0.967	-0.542	-0.882	0.853
Chl a	0.981	0.699	0.949	-0.939	1	0.998	0.900	0.725	0.980	0.789	0.985	0.541	0.973	-0.899
Chl b	0.988	0.710	0.961	-0.940	0.998	1	0.912	0.734	0.987	0.800	0.988	0.568	0.981	-0.885
Car	0.923	0.836	0.978	-0.890	0.900	0.912	1	0.851	0.961	0.952	0.919	0.812	0.936	-0.636
Fo	0.689	0.990	0.764	-0.594	0.725	0.734	0.851	1	0.807	0.941	0.738	0.666	0.843	-0.378
Fm	0.984	0.790	0.986	-0.924	0.980	0.987	0.961	0.807	1	0.880	0.976	0.680	0.991	-0.802
Fv/Fm	0.801	0.947	0.883	-0.716	0.789	0.800	0.952	0.941	0.880	1	0.793	0.854	0.874	-0.447
ETR	0.968	0.694	0.957	-0.967	0.985	0.988	0.919	0.738	0.976	0.793	1	0.540	0.966	-0.861
NPQ	0.648	0.716	0.756	-0.542	0.541	0.568	0.812	0.666	0.680	0.854	0.540	1	0.630	-0.208
OXPHOS	0.962	0.826	0.957	-0.882	0.973	0.981	0.936	0.843	0.991	0.874	0.966	0.630	1	-0.793
LPO	-0.866	-0.344	-0.758	0.853	-0.899	-0.885	-0.636	-0.378	-0.802	-0.447	-0.861	-0.208	-0.793	1

3 * *FN – frond number, FA – frond area, DM – dry mass, ROS – reactive oxygen species, Chl a – Chlorophyll a, Chl b – Chlorophyll b, Car –*
 4 *carotenoids, Fo – initial fluorescence yield, Fm – maximal fluorescence yield, Fv/Fm – maximum quantum yield of PS II, ETR – electron*
 5 *transfer rate, NPQ – non-photochemical quenching, OXPHOS – oxidative phosphorylation, LPO – lipid peroxidation.*

Table 2. No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and half maximal effective concentration (EC₅₀) of selected endpoints in *L. minor* after 7-day exposure to 3,5-dichlorophenol (3,5-DCP). (R²: coefficient of determination, N/A: not observed)

Parameters	NOEC (mg/L)	LOEC (mg/L)	EC ₅₀ (mg/L)	R ²
Fronds number	0.5	1	2.20±0.01	0.996
Fronds size	0.5	1	1.45±0.13	0.976
Dry weight	1	1.5	1.92±0.27	0.881
Fo	1.5	2	2.53±0.25	0.911
Fm	0.5	1	1.63±0.10	0.982
Fv/Fm	1	1.5	1.81±0.05	0.953
ETR	N/A	0.5	1.42±0.04	0.996
OXPPOS inhibition	0.5	1	1.41±0.47	0.955
ROS formation	1.5	2	1.52±0.46	0.714
LPO formation	1	1.5	1.12±0.26	0.848
Chl <i>a</i> content	0.5	1	1.31±0.29	0.965
Chl <i>b</i> content	0.5	1	1.41±0.24	0.960
Car content	1.5	2	2.60±0.42	0.932

* *Fo* – initial fluorescence yield, *Fm* – maximal fluorescence yield, *Fv/Fm* – maximum quantum yield of PS II, *ETR* – electron transfer rate, *OXPPOS* – oxidative phosphorylation, *ROS* – reactive oxygen species, *LPO* – lipid peroxidation, *Chl a* – Chlorophyll *a*, *Chl b* – Chlorophyll *b*, *Car* – carotenoids.

Fig.1

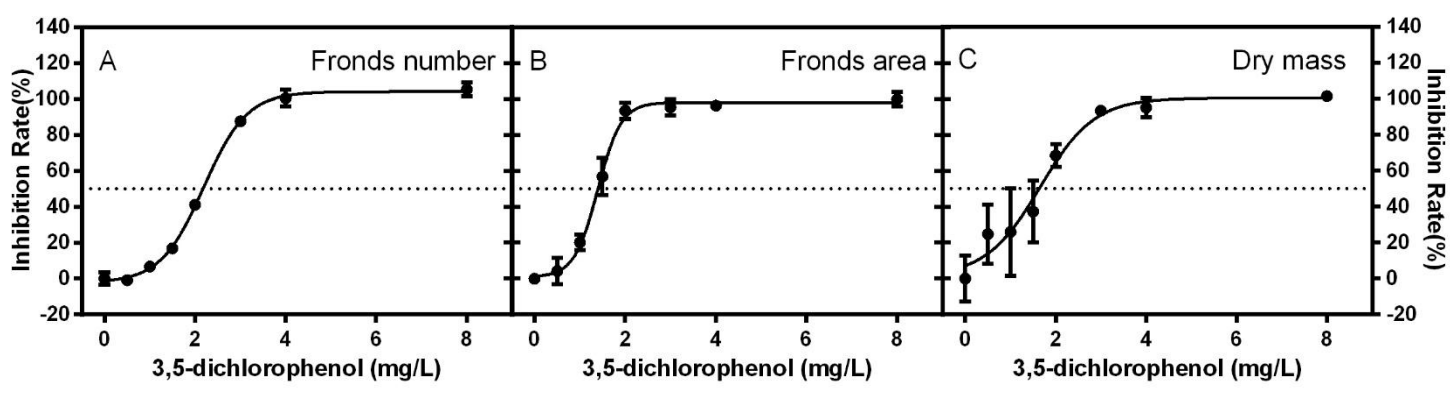


Fig.2

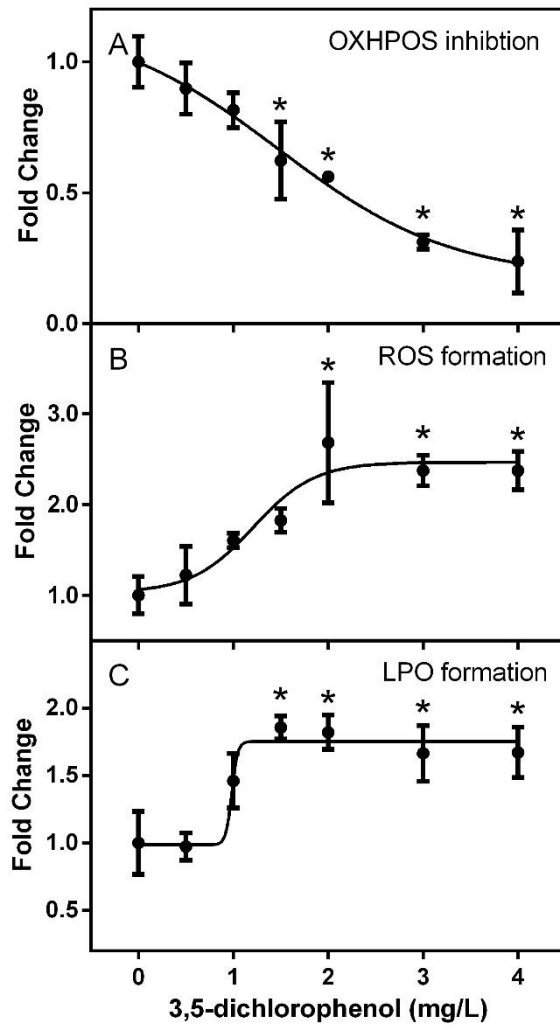


Fig.3

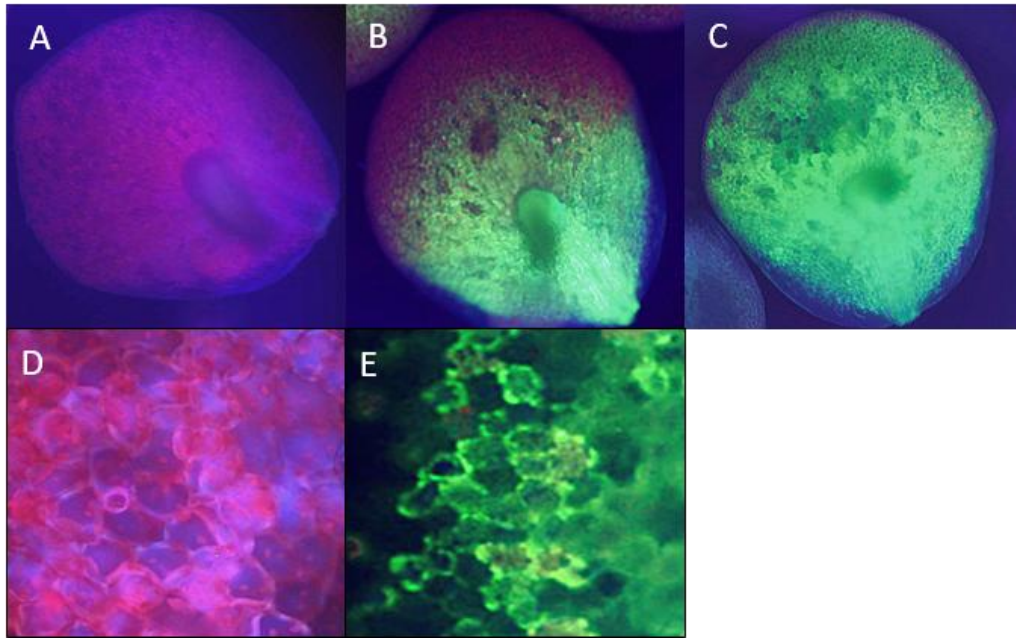


Fig.4

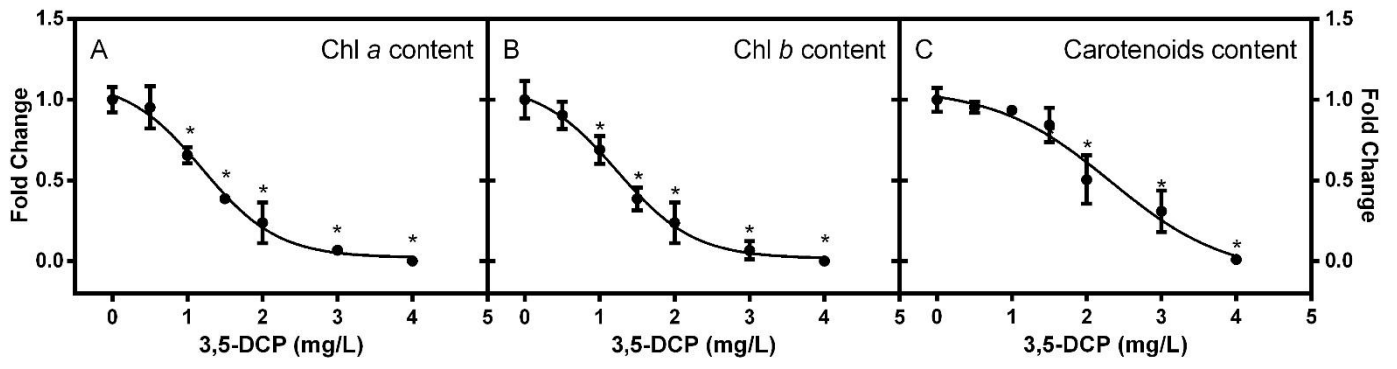


Fig.5

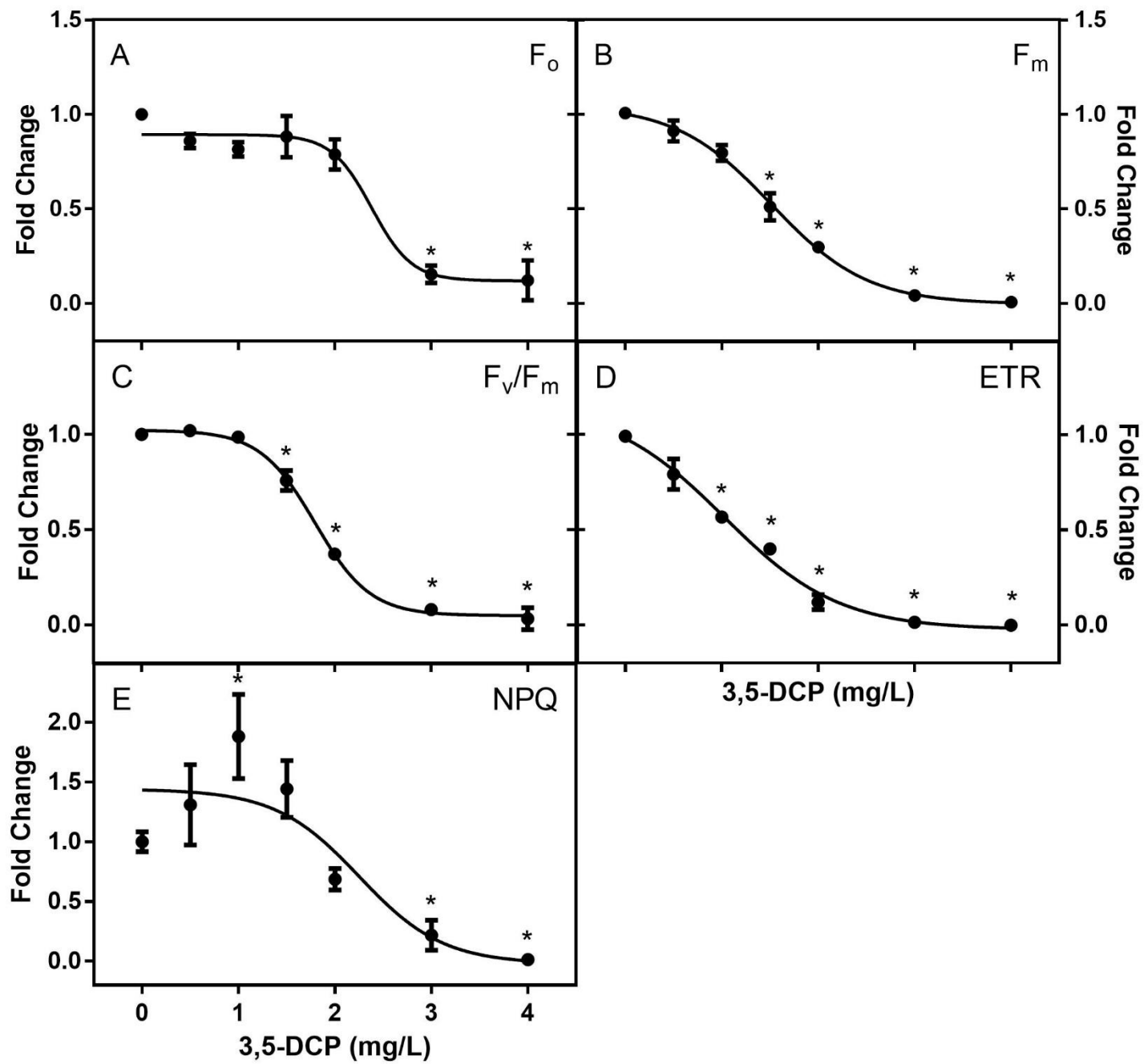


Fig.6

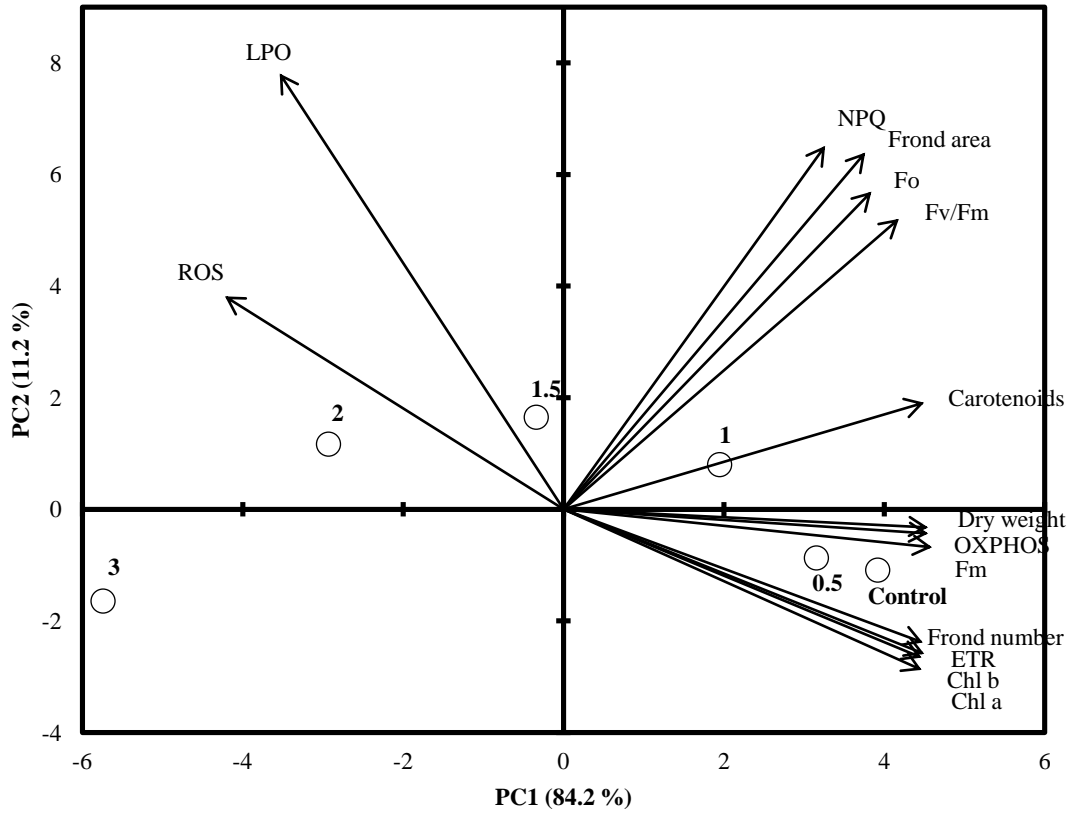
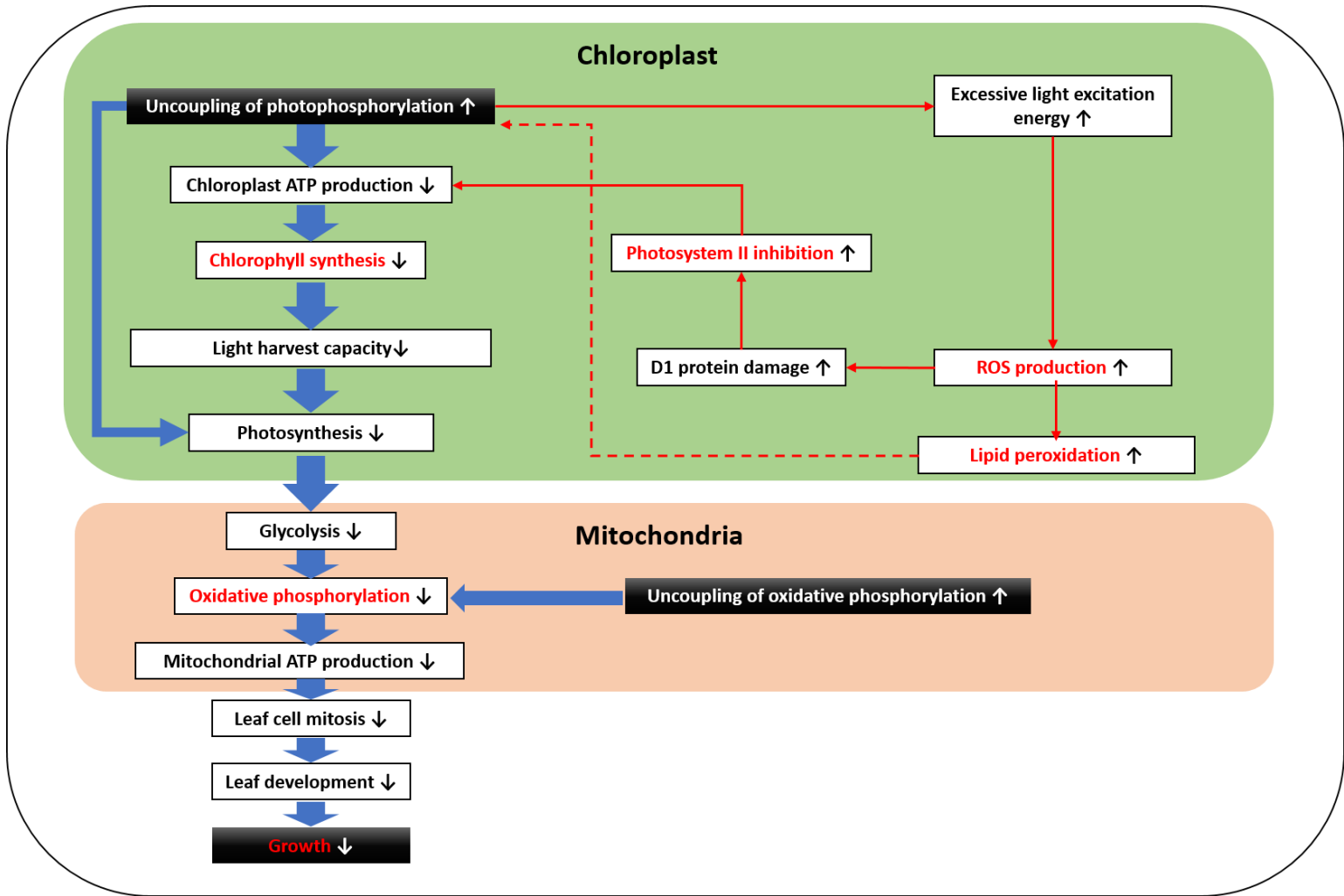


Fig.7



Supplementary Material

[Click here to download Supplementary Material: Supplementary information.docx](#)

Highlights:

- Mode of action and adversity of 3,5-dichlorophenol (3,5-DCP) was determined in the aquatic plant *Lemna minor*.
- Uncoupling of oxidative phosphorylation and photophosphorylation were the main modes of action.
- ROS formation and cellular damage were secondary effects of 3,5-DCP.
- Linkage between mode of action and adverse outcomes were developed into a network of Adverse Outcome Pathways.