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

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Abstract

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

Keywords: *Lemna minor*, 3,5-dichlorophenol, reactive oxygen species, growth, PSII, 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

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

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

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

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

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

2. Materials and Methods

2.1. Culture and exposure

Test species *Lemna minor (Strain ID: 5544,* Rutgers Duckweed Stock Cooperative, *http://www.ruduckweed.org)* was provided by Blases Biological Ltd (Cat.ID: LBA 041, Edenbridge, UK) and cultured at Ghent University (Belgium) (De Schamphelaere et al.,2010; Van Echelpoel et al., 2016) prior to transfer to the Norwegian Institute for water research (NIVA). Upon arrival at NIVA, thalli were disinfected by immersion in 0.5% NaOCl (v/v) for 3 min and then rinsing with distilled water for 5 min to remove algae contamination. The *L. minor* cultures were maintained in 200 ml Erlenmeyer flasks containing 100 ml of Steinberg (SB) medium (with composition: 350 mg/L KNO_3, 295 mg/L $Ca(NO_3)_2.4H_2O$, 90 mg/L KH_2PO_4, 12 mg/L K_2HPO4, 100 mg/L $MgSO_4.7H_2O$, 0.12mg/L H_3BO_3, 0.18 mg/L $ZnSO_4.7H_2O$, 0.044 mg/L $Na_2MoO_4.2H_2O$, 0.18mg/L $MnCl_2.4H_2O$, 0.76 mg/L $FeCl_3.6H_2O$,

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

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

2.2. Growth rate

The growth rate on basis of the fronds number (FN) was calculated according to the equation: $GRi = \frac{\ln Nt_i - \ln Nt_0}{t_i - t_0}$, where GR_i is the growth rate per day; Nt_0 is the fronds number at day t0 (the beginning of the experiment); Nt_i is the fronds number at day t_i (i=7); t_i-t_0 is the time period between t_i and t_0, expressed in days. The doubling time (T) of FN was calculated using the equation $Ti = \frac{\ln 2}{GR_i}$, where GRi is the growth rate determined as described before. The test was considered valid when the growth rate of FN in the control groups were higher than

0.275 d^{-1} (OECD, 2006).

For measurement of frond area (FA), the total area of floating fronds was determined optically by a digital camera (FinePix S2500HD, Fujifilm, Japan) using a floating scale bar. The frond area in each photograph was analysed using the Image-J software program version 1.48 (National Institutes of Health, Maryland, USA). The dry mass (DM) of the fronds was determined gravimetrically after centrifugation of exposed fronds at 3000 rpm for 10 min at room temperature to 20 °C in pre-weighted eppendorf tubes. The fronds were then dried in an oven at 70 °C until constant weight was obtained and DM recorded.

2.3. Mitochondrial inner membrane potential

The mitochondrial inner membrane potential (MMP) was determined using tetramethylrhodamine methyl ester (TMRM, Life Technologies AS, Oslo, Norway) as an indicator of OXPHOS in the fronds (Ehrenberg et al., 1988; Scaduto and Grotyohann, 1999). In brief, stock solutions of TMRM (5 µM) were prepared in DMSO (Purity 99.7%, Sigma-Aldrich) and stored under -18^{o}C until use. The MMP assay was optimized for dye concentration and incubation duration on the basis of recommendations for the green algae *Chlamydomonas reinhardtii* and zebrafish cells (Jamers et al., 2009; Legradi et al., 2014). After exposure, 3 fronds for each replicate (N=3) were transferred to the glass flasks containing 2 mL SB medium prior to the addition of 200 µl TMRM (500 nM) diluted in the SB medium. The fronds were incubated with TMRM for 2h at room temperature. After incubation, the fronds were rinsed with SB medium for 5 minutes to remove free (unbound) TMRM and transferred to a Costar 96-Well Black Clear-Bottom microplate (Corning Incorporated, USA) containing 200 µl SB medium. The fluorescent intensity of TMRM was

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

2.4. Reactive oxygen species (ROS) formation

2′,7′-Dichlorofluorescein diacetate (H_2DCFDA) (Molecular Probes Inc., Eugene, OR, USA) was used to quantify the ROS (H_2O_2, O_2^- and $^1O_2$) in *L. minor* (Razinger et al., 2010). The stock solutions of H_2DCFDA (10 mM) was prepared in DMSO and stored at -18 °C until use. After exposure to 3,5-DCP, the fronds were immersed in H_2DCFDA diluted in the culture medium to a final concentration of 50 µM (maximum DMSO concentration: 0.5%). After 2 h incubation, the fronds were rinsed using clean growth medium for 5 minutes to remove excess dye. Clean fronds were subsequently transferred to a black Costar 96-well polystyrene microplate with clear-bottom (Corning Incorporated, USA) containing 200 µl of culture medium. The fluorescence signal of H_2DCFDA was measured using a microplate reader (VICTOR 3, PerkinElmer, Waltham, Massachusetts, USA) with excitation wavelength of 485 nm and emission wavelength of 538 nm. The natural fluorescence of the exposure media in combination with the dye (without presence of fronds) was also analyzed and the resulting fluorescence subtracted. The relative fluorescence obtained was normalized by weight of test frond expressed as fold induction comparative to the control.

2.5. Lipid peroxidation

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

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

2.6. Pigments content

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

Chl *a* (µg/ml)=16.72×A665 – 9.16×A652 (eq. 1)

Chl *b* (µg/ml)=34.09×A652 – 15.28×A665 (eq. 2)

Total carotenoids (µg/ml)=(1000×A470 – 1.63×Chl *a* – 104.96×Chl *b*)/221 (eq. 3)

2.7. Chlorophyll fluorescence

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

$NPQ=(F_m-F_m')/F_m'$ (eq. 4)

2.8. Fluorescence microscopy imaging

The fluorescence from the photosynthetic chloroplast (red fluorescence) and H_2DCFDA loading in cells (yellow/green fluorescence) was observed under a fluorescence microscope (Olympus IX 71, Tokyo, Japan). The light was applied by a high intensity fluorescence illumination system (X-Cite 120, Excelitas Technologies Corp., Fremont, Canada) and images were captured using the software Cell^D (Olympus, Tokyo, Japan).

2.9. Statistical analysis

The results for each endpoint were calculated as fold difference compared to the control and presented as the mean of three replicates with standard error (Mean±SEM). Statistical significance between groups was determined using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test after data were assessed for normality by the Shapiro-Wilk and KS normality testing. Concentration-response curves were calculated using GraphPad Prism version 6 (Graphpad Software, California, USA). Concentrations that caused 50% effect (EC_{50}), no-observed-effect-concentration (NOEC) and low-observed-effect-concentration (LOEC) were determined on basis of resulting concentration-response relationships. A principal component analysis (PCA) was applied to the overall data to assess relationships between parameters using XLStat2015 (Addinsoft, Paris, France). The same software was used to calculate Pearson's correlation to measure the strength of association between the endpoints determined in *L. minor*. Statistical significance was set at $p<0.05$.

3. Results

3.1 Growth inhibition

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

3.2 Mitochondrial membrane potential

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

3.3 oxidative stress

3,5-DCP caused a concentration-dependent induction of ROS formation from 0.5 to 3 mg/L (EC_{50}= 1.52 mg/L) in *L. minor* (Fig. 2B, Table 2), with highest ROS levels observed at 2 and 3 mg/L. The levels of lipid peroxidation (LPO) also displayed a concentration-dependent response (Fig. 2.C). Similarly, to ROS formation, the MDA levels increased at concentrations higher than 1 mg/L 3,5-DCP (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 (H_2DCFDA) 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 EC_{50} 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 (EC_{50}=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 (EC_{50}=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

observed in both maximum quantum yield of PSII (F_v/F_m, EC_{50}=1.81±0.05 mg/L) and ETR (EC_{50}=1.42±0.04 mg/L). Compared to F_v/F_m (LOEC=1.5 mg/L), ETR (LOEC=1 mg/L) was identified as the more sensitive endpoint when exposed to 3,5-DCP (Table.1). A significant and enhanced NPQ yield was observed in *L. minor* after exposure to 1 mg/L 3,5-DCP, followed by a reduction at higher concentrations. A clear concentration-dependent inhibition of the photosynthetic electron transport rate (ETR) was also identified (EC_{50}= 1.42±0.04 mg/L). The different parameters of chlorophyll *a* fluorescence were below the threshold for quantification at concentrations above 4 mg/L due to complete inhibition of growth.

3.5. Principal component analysis

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

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

The Pearson correlation analysis showed several significant positive and negative correlations between the endpoints measured in *L. minor* exposed to 3,5-DCP (Table 1). Similarly, to the PCA analysis, the two highest concentrations of 3,5-DCP were excluded from this analysis. Frond number was positively correlated with DM of fronds, pigments contents (Chl *a*, *b* and carotenoids), F_m, ETR and OXPHOS and negatively correlated with LPO and ROS formation. Positive correlations were found between frond area, carotenoids content, F_o, F_v/F_m and OXPHOS. DM was positively correlated with all endpoints except for frond area, F_o and NPQ. ROS formation was negatively correlated with frond´s DM, pigments content, Fm, ETR and OXPHOS and positively correlated with LPO. The Chl *a* and Chl *b* contents were positively correlated with carotenoids content, F_m, ETR and OXPHOS, but negatively correlated with LPO. Carotenoids content was positively correlated with all variables related to PSII performance and OXPHOS. Fo was positively correlated with F_v/F_m while F_m was correlated with F_v/F_m, ETR and OXPHOS. A positive and significant correlation was found between ETR and OXPHOS, as well as F_v/F_m with NPQ and OXPHOS. A negative correlation was also found between ETR and LPO.

4. Discussion

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

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

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

4.1 Growth inhibition

In this study, the adverse effects of 3,5-DCP on survival, growth and associated physiological variables in *L. minor* were identified using a standard 7-days toxicity testing protocol (OECD,2006). Growth rate of *L. minor* in the control groups (Fig. 1) demonstrated that the plants were cultured under satisfactory conditions and that the use of solvents did not affect the outcome of the studies. 3,5-DCP was found to be a potent inhibitor of *L. minor* growth by reducing fronds number (frond production as a measure of reproductive rate), fronds size and DM (Fig. 1). Frond area were identified as the most sensitive growth-related endpoint in this test when comparing EC_{50} values, closely followed by fronds number and DM (Table 2). Albeit fronds area was the more sensitive parameter in this study, fronds number has been recommended as the better toxicity indicator in *L. minor* exposed to metals and organics (Mitsou et al., 2006), likely due to the fact that chemically-induced changes in cell volume

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

4.2 Uncoupling of OXPHOS and photophosphorylation

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

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

growth inhibition due to reduction of ATP synthesis.

4.4 Oxidative stress and cellular damage

Endogenous ROS are usually produced as a consequence of aerobic metabolic processes in plants, such as photosynthesis and respiration and rapidly eliminated by the antioxidant defence mechanism (Gamble and Burke, 1984; Gechev et al., 2006; Rabilloud et al., 2001). Excessive formation of ROS by exposure to toxicants may lead to cellular oxidative stress, DNA damage and programmed cell death (Agarwal and Said, 2005; Barzilai and Yamamoto, 2004). This study confirmed that exposure to 3,5-DCP caused induction of ROS formation in *L. minor* (Fig. 2B and Fig. 3). This induction of ROS formation may be directly caused by uncoupling of OXPHOS and photophosphorylation or indirectly enhanced by a malfunction in the PSII, in which high energy triplet chlorophyll can enhance the formation of singlet oxygen and increase the chance of O_2 to receive electrons from PS I and form superoxide radicals (Asada, 2006). These superoxide radicals can rapidly form hydrogen peroxide (H_2O_2) via the action of the antioxidant enzyme superoxide dismutase, but if not rapidly detoxified, it can give rise to the highly reactive hydroxyl radical (Asada, 2006; Mittal et al., 2012). As a phenolic compound, 3,5-DCP likely caused direct ROS formation and potentially caused oxidative damage as observed in primary producers elsewhere (Luo et al., 2005; Michałowicz et al., 2009; Michałowicz et al., 2010).

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

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

4.5 Pigments

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

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

modulating enzymes involved in carotenoids synthesis (Chang et al., 2013), whereas the resulting increase in ROS-related hydrogen peroxide (H_2O_2) can oxidize a wide range of molecules inside the chloroplast (Borisova et al., 2012), including the carotenoids themselves (Lopez-Serrano and Ros Barceló, 1999). In addition, reduced content of carotenoids may enhance ROS formation, as some carotenoids (such as lutein, violaxanthin, β-carotene and etc.) can quench ROS or the triplet state of chlorophyll to prevent singlet oxygen formation (Dreuw et al., 2005). The reduced content of carotenoids may thus indicate potential accumulation of damage from oxidative stress. A close relationship between pigments content (Chl *a* and *b* and carotenoids), oxidative stresses (ROS and LPO) was also detected in the PCA, where a strong negative correlation was observed between these endpoints, thus reinforcing the negative impact of 3,5-DCP in the photosynthetic apparatus of *L. minor* and its relation to oxidative stress.

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

4.3 Interference with photosynthetic capacity

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

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

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

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

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

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

5. Conclusion

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

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Figure legends

Fig. 1. Inhibition of growth measured as frond number (A), frond area (B) and frond dry mass (C) in L. minor after 7d exposure to 3,5-dichlorophenol (3,5-DCP). The experiment results (Mean ± SEM) represented 3 independent studies.

Fig. 2. Inhibition of oxidative phosphorylation (OXPHOS) measured as MMP (A), reactive oxygen species (ROS) formation (B) and lipid peroxidation (LPO) measured as MDA content (C) in L. minor after 7 days exposure to 3,5-dichlorophenol (3,5-DCP). The experiment results (Mean ± SEM) represent 3 independent studies and calculated as fold increase compare to control groups (0 mg/L). Asterisks (*) represent significant statistical differences ($p<0.05$). Complete growth inhibition at concentrations above 4 mg/L precluded analysing of these endpoints.

Fig. 3. In vivo visualization of ROS formation in L. minor exposed to different concentrations of 3,5-DCP: (A) Control, (B) Low concentration (1.5 mg/L) and (C) high concentration (3 mg/L). (D) Healthy cell in fronds. (E) ROS formation in fronds cells. Complete growth inhibition at concentrations above 4 mg/L precluded analysing of these endpoints.

Fig. 4. Reduction of chlorophyll a (A), chlorophyll b (B) and Carotenoids (C) content in L. minor after 7d exposure to 3,5-DCP. The experiment results (Mean ± SEM) represented 3 independent studies and were displayed as normalised (fold) increase compared to the control groups. 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. 5. Maximal photosystem II efficiency parameters Fo (A), Fm(B), Fv/Fm (C), W (D) and ETR (E) in L. minor after 7d exposure to 3,5-dichlorophenol (3,5-DCP). The experiment results (Mean ± SEM) represented 3 independent studies and calculated as fold increase

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-dichorophenol 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 b–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.

Table(s)

Table 1. Pearson correlation matrix obtained from all the endpoints determined in L. minor exposed to 3,5-dichorophenol for 7 days. 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**

* ***FN – frond number, FA – frond area, DM – dry mass, ROS – reactive oxygen species, Chl a – Chlorophyll a, Chl b – Chlorophyll b, Car – carotenoids, Fo – initial fluorescence yield, Fm – maximal fluorescence yield, Fv/Fm – maximum quantum yield of PS II, ETR – electron 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 (EC_{50}) of selected endpoints in L. minor after 7-day exposure to 3,5-dichlrorophenol (3,5-DCP). (R^2: 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
OXPHOS 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 *a* content	0.5	1	1.31±0.29	0.965
Chl *b* content	0.5	1	1.41±0.24	0.960
Car content	1.5	2	2.60±0.42	0.932

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

Figure(s)

Fig.1

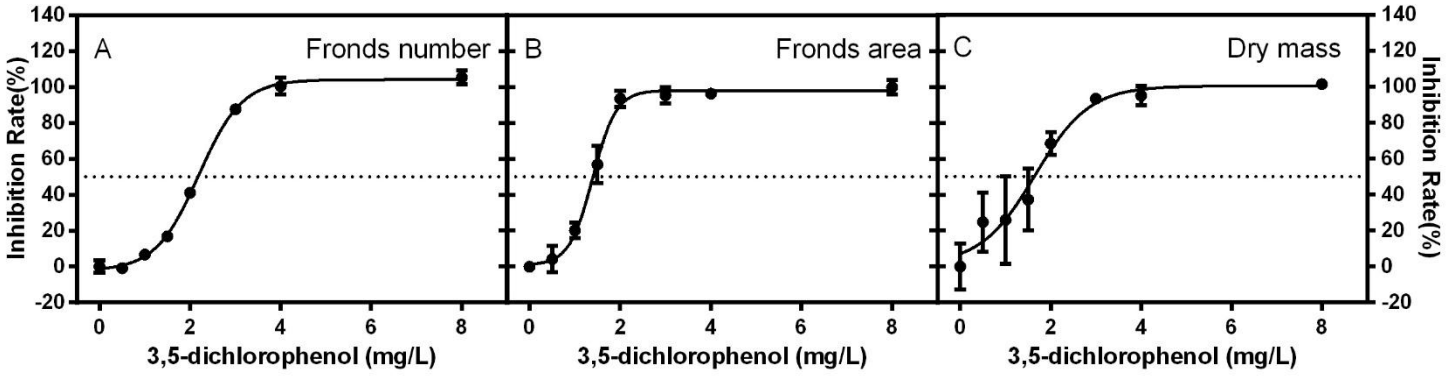


Fig.2

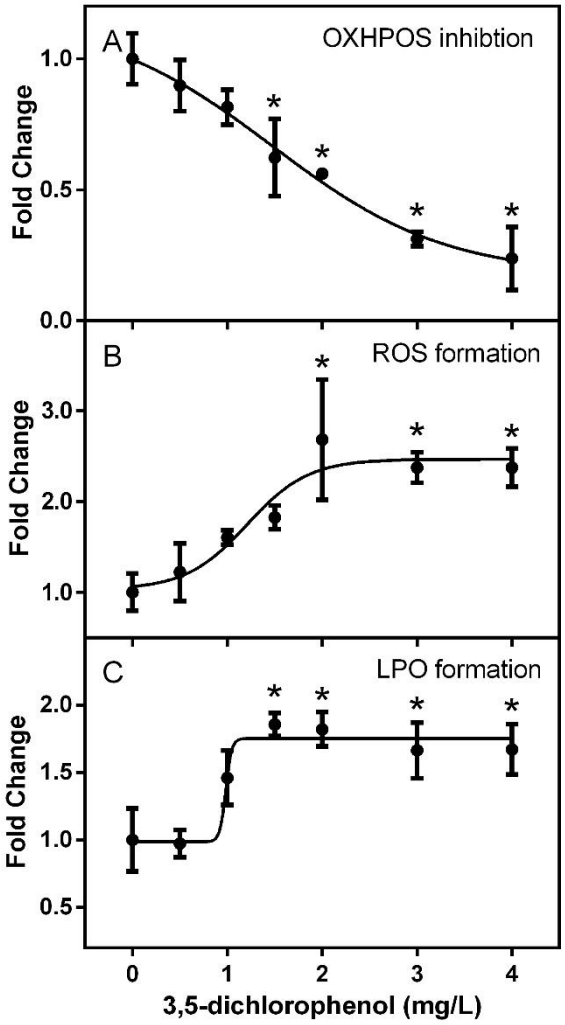

A
OXHPOS inhibtion
Fold Change
1.0
0.5
0.0
B
ROS formation
Fold Change
3.0
2.0
1.0
C
LPO formation
Fold Change
2.0
1.5
1.0
0.5
0
1
2
3
4
3,5-dichlorophenol (mg/L)

Fig.3

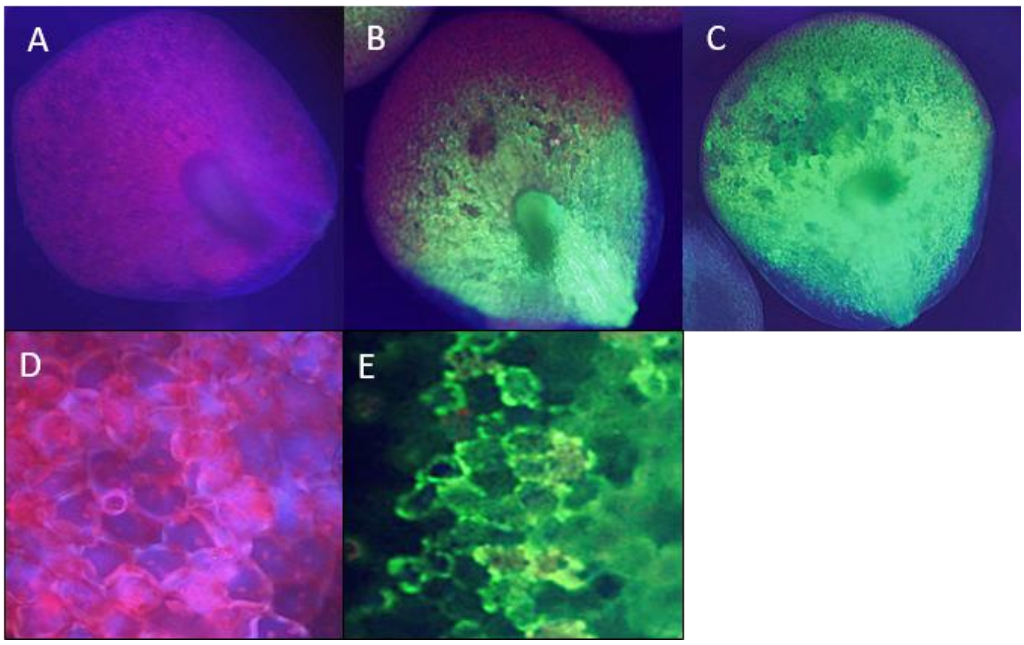


Fig.4

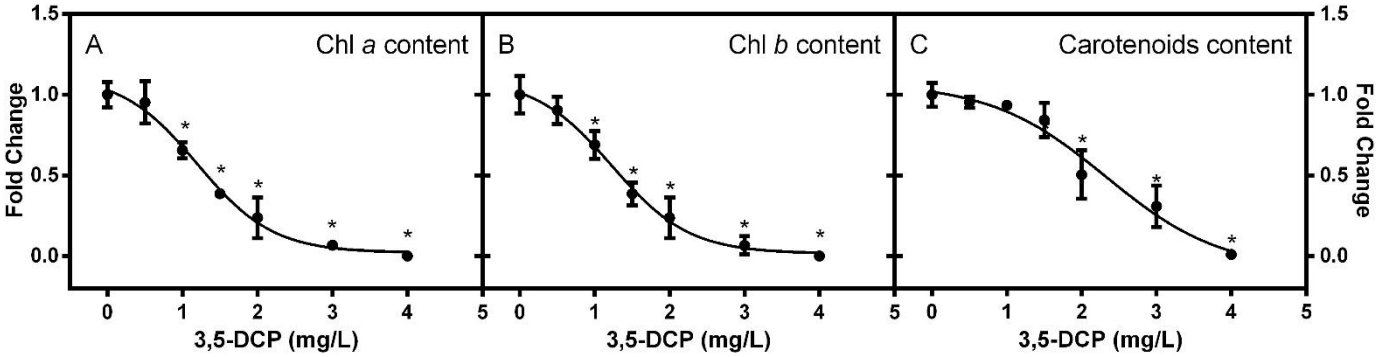

A
Chl a content
B
Chl b content
C
Carotenoids content
Fold Change
1.5
1.0
0.5
0.0
0
1
2
3
4
5
3,5-DCP (mg/L)

Fig.5

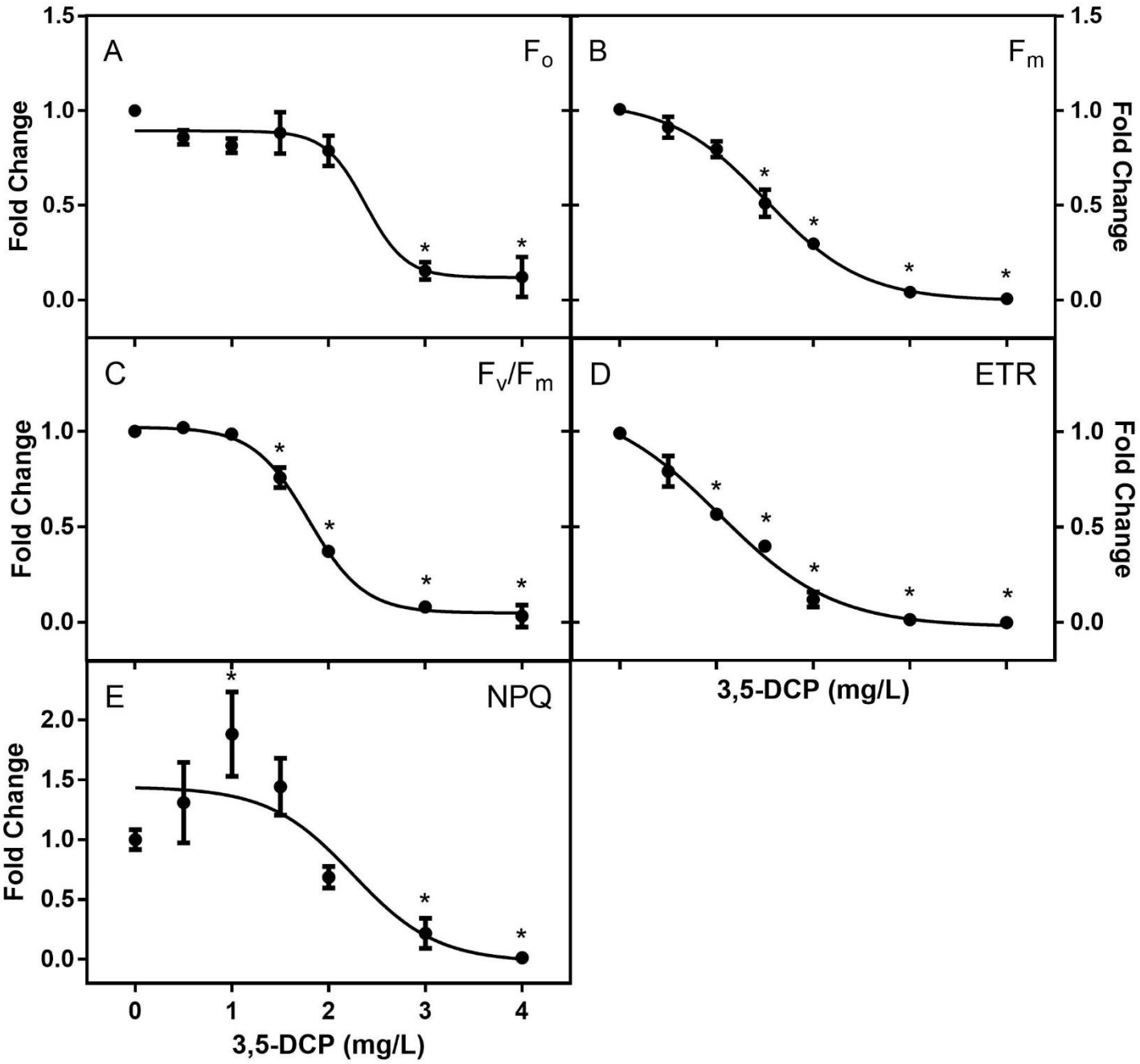

A
F_o
B
F_m
C
F_v/F_m
D
ETR
E
NPQ
Fold Change
3,5-DCP (mg/L)
0.0
0.5
1.0
1.5
2.0
0
1
2
3
4
*

Fig.6

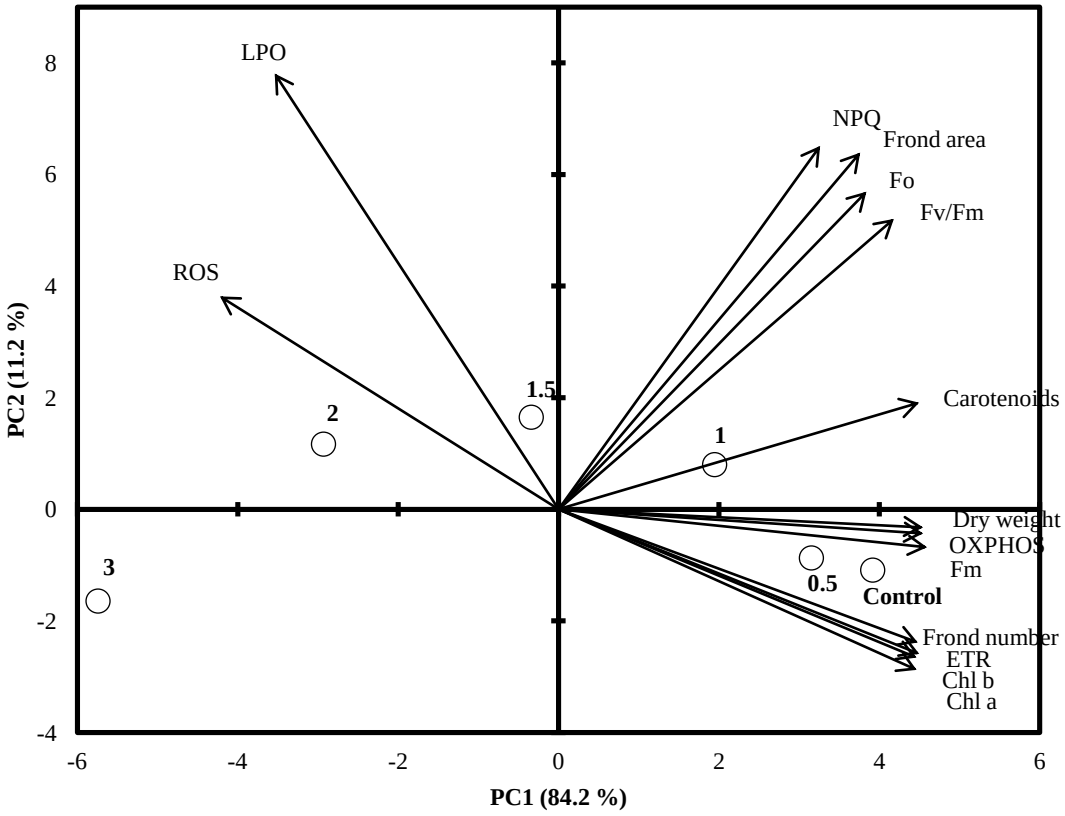
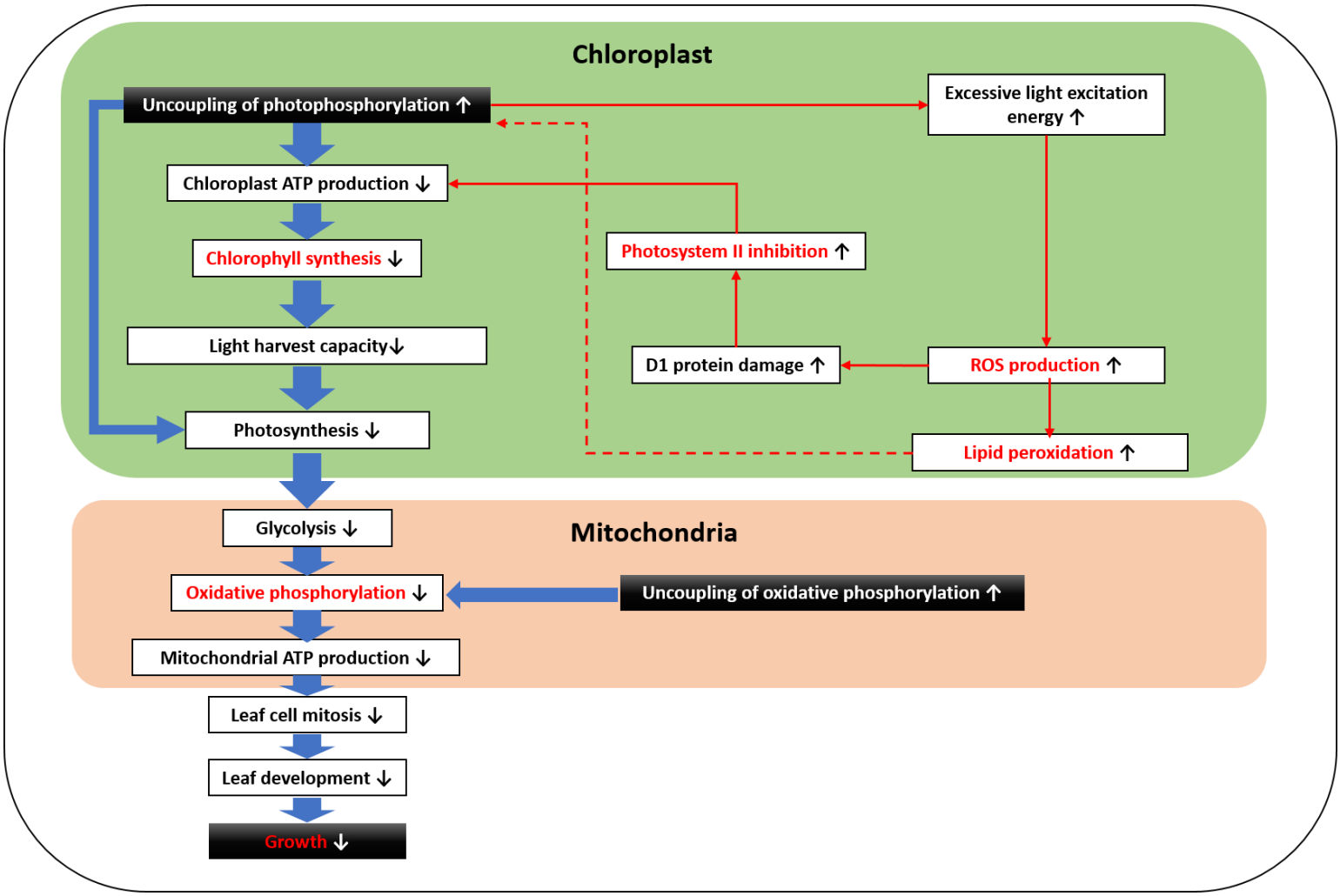

LPO
NPQ
Frond area
Fo
Fv/Fm
ROS
Carotenoids
1.5
2
1
Dry weight
OXPHOS
Fm
3
0.5
Control
Frond number
ETR
Chl b
Chl a
PC2 (11.2 %)
PC1 (84.2 %)
8
6
4
2
0
-2
-4
-6
-4
-2
0
2
4
6

Fig.7


Chloroplast
Uncoupling of photophosphorylation ↑
Excessive light excitation energy ↑
Chloroplast ATP production ↓
Chlorophyll synthesis ↓
Photosystem II inhibition ↑
Light harvest capacity↓
D1 protein damage ↑
ROS production ↑
Photosynthesis ↓
Lipid peroxidation ↑
Glycolysis ↓
Mitochondria
Oxidative phosphorylation ↓
Uncoupling of oxidative phosphorylation ↑
Mitochondrial ATP production ↓
Leaf cell mitosis ↓
Leaf development ↓
Growth ↓

Supplementary Material

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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.