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S-metolachlor promotes oxidative stress in green microalga *Parachlorella kessleri* - A potential environmental and health risk for higher organisms

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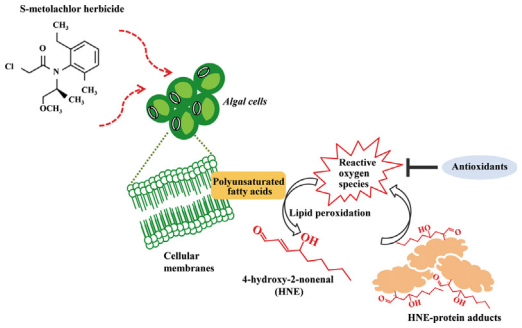
HIGHLIGHTS

- Oxidative stress response was assessed in *P. kessleri* following S-MET exposure.
- S-MET alters algal growth and antioxidant mechanisms inducing lipid peroxidation.
- HNE-protein adducts accumulate in *P. kessleri* during longer S-MET treatment.
- Algae with increased HNE content could represent environmental toxic factor.

GRAPHICAL ABSTRACT



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ABSTRACT

The estimation of the toxic influences of herbicide products on non-target aquatic organisms is essential for evaluation of environmental contamination. We assessed the effects of the herbicide S-metolachlor (S-MET) on unicellular green microalga *Parachlorella kessleri* during 4–72 in vitro exposure to concentrations in the range 2–200 μg/L. The results have shown that S-MET had a significant effect on algae, even in doses 10 and 20 times lower than the EC50 values obtained for *P. kessleri* (EC50-72 h = 1090 μg/L). It generates reactive oxygen species in algae, decreases their growth and photosynthetic pigment concentration, changes their ultrastructure and alters the cellular antioxidant defence capacities. The levels of protein adducts with the reactive aldehyde 4-hydroxy-2-nonenal (HNE), the end-product of lipid peroxidation, were significantly elevated in S-MET treated cells revealing the insufficient effectiveness of *P. kessleri* antioxidant mechanisms and persistent lipid peroxidation. Since algae are fundamental aquatic food component, the damaged algal cells, still capable of dividing while having

Abbreviations: AA, ascorbic acid; APX, ascorbate peroxidase; BBM, Bold's Basal Medium; BSA, bovine serum albumin; CAT, catalase; Chl*a*, chlorophyll *a*; Chl*b*, chlorophyll *b*; Car, carotenoid; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DCFH, 2′,7′-dichlorfluorescein; DCF, 2′,7′-dichlorofluorescein; DNPH, dinitrophenyl hydrazine; EDTA, ethylenediaminetetraacetic acid; GAE, gallic acid equivalent; GR, glutathione reductase; GSH, reduced glutathione; HBSS, Hanks' Balanced Salt Solution; HNE, 4-hydroxy-2-nonenal; HNE-His, HNE-histidine; H_2O_2, hydrogen peroxide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; $O_2^{\bullet -}$, superoxide anion; OD_{750}, optical density at 750 nm; $^1O_2$, singlet oxygen; •OH, hydroxyl radical; PBS, phosphate buffered saline; PHE, phenolic; PUFA, polyunsaturated fatty acid; RO•, alkoxyl radical; ROO•, peroxyl radical; ROS, reactive oxygen species; S-MET, S-metolachlor; TEM, transmission electron microscopy.

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Reactive oxygen species
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Lipid peroxidation
Antioxidants

persistently increased content of HNE upon S-MET contamination could represent an important environmental toxic factor that might further affect higher organisms in the food chain.

1. Introduction

Continuous reliance on herbicides in extensive agriculture has resulted in frequent contamination of surface and ground waters, becoming one of the major ecological issues in the past few decades. Chloroacetanilide herbicides are among the most frequently used chemicals in agriculture worldwide (Abigail et al., 2015). Among them, S-metolachlor (S-MET) is listed as one of the most applied herbicides in the European Union (Eurostat, 2007). Due to its extensive use over the last 20 years, and strong leaching potential, S-MET and its metabolites are usually being detected in environmental waters at nanogram-per-litre levels (see Copin et al., 2016). However, in the United States, concentrations can reach 143 μg/L during post-application runoff events in Midwestern streams and rivers (Battaglin et al., 2000). The most recent data available for Europe report concentrations up to 0.5 μg/L (Leyre River, France) (Roubeix et al., 2012). Even at environmentally relevant concentrations, S-MET may pose a potential hazard to aquatic organisms (Mai et al., 2013). Its toxic action in algae and plants is exerted by the imbalanced pattern of very-long-chain fatty acids in vital cellular constituents, especially membranes and cell wall compartments, altering their integrity and function (Schmalfuss et al., 1998; Böger, 2003). It can inhibit the biosynthesis of proteins, terpenoids, and phenolics and bind to nucleophiles like glutathione (GSH) and cysteine, thus having the potential to alkylate enzymes (see Böger et al., 2000). Moreover, its toxicity in plant and algal species is related to strong oxidation of cell components (Liu and Xiong, 2009; Liu et al., 2017). It elevates production of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydroxyl (•OH), alkoxyl (RO•) and peroxyl (ROO•) radicals, hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). In excess, ROS can alter the cells' normal metabolism, damaging lipids, proteins, nucleic acids and photosynthetic pigments leading to lipid peroxidation, membrane damage and inactivation of enzymes, thereby affecting many physiological processes as well as cell viability (Arora et al., 2002; Jaganjac et al., 2016). In order to overcome oxidative stress, plant cells respond by activating enzymatic antioxidants, including catalase (CAT), and the enzymes of ascorbate-glutathione cycle, such as ascorbate peroxidase (APX) and glutathione reductase (GR), as well as by increasing the content of the most potent non-enzymatic antioxidative substances: phenolics (PHE), carotenoids (Car) and ascorbic acid (AA) (Corpas et al., 2015). When antioxidative defences fail, the free radicals attack bisallylic site of polyunsaturated fatty acids (PUFA) triggering a chain reaction of lipid peroxidation. Eukaryotic algae are the main primary producers of PUFAs, especially of the very long chain type, and consequently the source of many biologically active compounds which derive from oxylipin development pathways (Harwood and Guschina, 2009). Peroxidation of lipids in plants and algae yields formation of phytoprostanes and reactive aldehydes, including 4-hydroxy-2-nonenal (HNE). By the action of lipases or fragmentation, HNE can be released from membranes (Mueller and Berger, 2009) and diffuse to targets remote from the initial oxidative injury (Andreou et al., 2009). HNE is associated with the development of numerous diseases (Brown and Goldstein, 1983; Poli and Parola, 1997; Butterfield, 2002) including cancer (Hu et al., 2002; Jaganjac et al., 2012). HNE also acts as important signalling molecule in proliferation (Žarković et al., 1993), differentiation (Borović Šunjić et al., 2005; Elrayess et al., 2017; Jaganjac et al., 2017), transformation (Sharma et al., 2004) and apoptosis (Awasthi et al., 2008). Moreover, it modulates gene expression (Elrayess et al., 2017), interacts with membrane receptors, transcription factors and repressors (Liu et al., 1999; Sharma et al., 2008), and promoters (Jaganjac et al., 2010). Studies on HNE in plant cells and tissues remain far behind research on their animal counterparts. In vivo HNE presence was reported in soybean preparations (Gardner et al., 1991), *Arabidopsis* cell cultures (Winger et al., 2005), and seaweed species (Paul and Fenical, 1980; Pflugmacher et al., 2007; Box et al., 2008). We have reported for the first time the development and accumulation of HNE-modified proteins in the cells of green microalgae (Špoljarić et al., 2011). According to their nutritional requirements, physiology and their position at the base of aquatic food chains, algae represent highly suitable biological indicators of ecosystem condition, but also strongly affect organisms at higher trophic levels (Adolph et al., 2004; Ruocco et al., 2016). Also, as a high-quality food source, microalgae are used as the primary live feed for zooplankton, molluscs, crustaceans and fish in aquaculture (Hemaiswarya et al., 2011). Given the environmental relevance of herbicide contamination and bioaccumulation, we utilised a unicellular microalga *P. kessleri* as a model system for physiological, biochemical and morphological studies on herbicide S-MET stress response. Hence, the special reference was given to the presence of lipid peroxidation marker HNE in unicellular green algae as a consequence of herbicide application, as well as to the efficiency of algal antioxidant defence machinery in herbicide-induced stress.

2. Materials and methods

2.1. Culture conditions and treatment

The culture of unicellular green alga *Parachlorella kessleri* (strain LARG/1) was purchased from the Culture Collection of Autotrophic Organisms, Institute of Botany CAS (Trebon, Czech Republic). Axenic cells were pre-cultured to exponential phase and tested in Bold's Basal Medium (BBM, Bischoff and Bold, 1963) (25 ± 2 °C, 16/8 photoperiod, illumination of 100 μmol photons m^{-2} s^{-1}).

Agricultural herbicide formulation of S-MET (96% purity) was used for the treatment. Tests were performed in sterile 250-mL glass Erlenmeyer flasks containing 120 mL of BBM. Three replicates were used as control and for each of the tested nominal herbicide concentrations (S-MET 2, 50, 100 and 200 μg/L). The growth was monitored by spectrophotometric measurements (Lambda 2, Perkin-Elmer) of the optical density at 750 nm (OD_{750}) after 4, 24, 48 and 72 h exposure. The average specific growth rate (μ) for every period was calculated from absorbance increase per day as the logarithmic increase in biomass from the Eq. (1) for every single flask of controls and treatments (OECD, 2011).

$$\mu_{i-j} = \ln X_j - \ln X_i / t_j - t_i \left(\text{day}^{-1}\right) \quad (1)$$

where μ_{i-j} represents the average specific growth rate from time i to j, X_i represents biomass at time i and X_j represents biomass at time j.

The preliminary tests were used to check the relationship between the densities of algal cells (direct counts) pretreated with 4% formaldehyde performed in Bürker-Türk chamber using an inverted microscope (Axiovert 25, Zeiss) and OD_{750}. Values of OD_{750} were positively correlated with cell counts ($r = 0.989$; $p = 0.000$). According to the calibration curve, the initial density of *P. kessleri* for each replicate was 5×10^5 cells/mL (equal to an OD_{750} of 0.045).

The percent inhibition of growth rate ($\%I_r$) was calculated from Eq. (2) as the ratio between the average specific growth rates of different S-MET treatments (μ_t) and that of the control (μ_c) (OECD, 2011):

$$\%I_r = (\mu_c - \mu_r) \times 100/\mu_c \quad (2)$$