Algal Research 31 (2018) 1–13

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Non-steroidal anti-inflammatory drugs initiate morphological changes but inhibit carotenoid accumulation in *Haematococcus pluvialis*

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ARTICLE INFO

Keywords:
Cyst formation
Astaxanthin
Diclofenac
Diflunisal
Mefenamic-acid

ABSTRACT

The economic role of certain types of cysts is unquestionable, since the production of several valuable biomolecules is connected to the resting stages of algae, including the red ketocarotenoid astaxanthin. It is relatively well known, how adverse environmental conditions induce cyst formation and astaxanthin accumulation. In the contrary, there is very limited information about stressors inhibiting these processes. An undesirable consequence of increasing drug use of the human and veterinary medicine is the appearance of the drugs both in natural and in mains water. Therefore, to study the effects of micro-contaminants, e.g. pharmaceuticals to non-target aquatic organisms is a recent issue both from ecological and economical point of view. In this study, the effects of three non-steroidal anti-inflammatory drugs (NSAIDs: diclofenac, diflunisal and mefenamic acid) on growth, cyst formation and astaxanthin accumulation of the flagellated green alga *Haematococcus pluvialis* were investigated. All three drugs inhibited growth, inhibition ranged from 29 to 81% on the basis of vegetative cell numbers on the 14th day of the experiments. Higher concentrations of the drugs led to higher proportion of cysts, which exceeded 60% of total cell number to the 14th day in diclofenac and diflunisal treatments. On the contrary, astaxanthin contents of treated cultures were lower with the increasing drug concentration, the pigment was undetectable in the presence of 0.075 and 0.05 mg ml^{-1} diclofenac. Results of carotenoid and chlorophyll content analysis suggest more specific processes behind the observed phenomena than membrane damage. Furthermore, the different phenomena or different extents of the same phenomena suggest that NSAIDs with diverse chemical structures may have different target points in physiological processes. Our results clearly show that NSAIDs could have much wider effective spectra than expected, long-term effects on microalgae might have unexpected ecological or economical consequences due to continuous exposure to these chemicals.

1. Introduction

The presence of a long-lived resting stage (cyst) is a common feature in the case of many planktonic organisms, different algae groups among them (e.g. chrysophytes, diatoms, dinoflagellates and green algae). The economic role of certain types of cysts is unquestionable, since the production of several valuable biomolecules is connected to the resting stages of different algae [1, 2]. Astaxanthin, the red ketocarotenoid is one of the high-value microalgal products of the future, mainly because it has higher antioxidant activity than most of the known hydrophobic antioxidants [3]. This pigment has important applications in food and foraging industry, in cosmetics and even in pharmaceutical industry [4]. Although astaxanthin is produced by several organisms (bacteria, yeasts, microalgae, plants; [4]), astaxanthin accumulation of the unicellular freshwater microalga *Haematococcus pluvialis* exceeds any other known sources [5].

The life cycle of *H. pluvialis* is comprehensively reviewed by Shah et al. [4]. Briefly, the flagellated fast-dividing vegetative cells start losing flagella and expand their cell size under unfavorable environmental or culture conditions, developing into non-motile spherical

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https://doi.org/10.1016/j.algal.2018.01.007
Received 4 August 2017; Received in revised form 9 January 2018; Accepted 11 January 2018

palmella stage and becoming resting cells [4, 6]. Palmella transform into non-dividing aplanospores (cysts) under continued environmental stress (i.e., nutrient deprivation, high light irradiance, high salinity), which accumulate astaxanthin [4]. Some strains are capable to accumulate astaxanthin in the flagellated vegetative stage (without cyst formation; [7]). Although the exact role of astaxanthin in the defense process of the producer cells is strongly contentious until now, the fact that astaxanthin accumulation excessively increases cell tolerance to adverse environmental conditions is generally accepted [8–10]. One main role of astaxanthin is thought to be a sunscreen: absorbtion of excessive light and shielding the vulnerable cell structures from photo-oxidative damage [11–14]. Astaxanthin containing lipid droplets in the cytoplasm are also suggested as protectors of the nucleus and the chloroplast from reactive oxygen species (oxidative stress; [8, 15]), although results of physiological studies suggest that protection of the cell from reactive oxygen species is mainly performed by the "classic" antioxidant enzymes (catalase, peroxidase and superoxide dismutase); at least at the initial phase of stress-induced astaxanthin accumulation [16]. It is expected, that antioxidative role of astaxanthin could be significant in the lipid droplets, where astaxanthin protects unsaturated fatty acids from oxidation [17]. It is also suggested, that astaxanthin synthesis itself serves as protection system against reactive oxygen species, since the transformation of β-carotene to astaxanthin consumes oxygen and the biosynthesis of astaxanthin and fatty acids (astaxanthin esthers) provides a potent sink for the photosynthetic products that cannot be utilized for cell growth and division under stress conditions [17].

Generally, cyst formation and secondary metabolite accumulation – as it is already discussed above – occurs in microalgal cells under adverse conditions, when cell division and photosynthesis are slowed down (e.g., under excessive irradiance, nutrient deficiency, extreme temperatures, salinity, and their combinations; [8, 14]. There are many data in the literature, how high light intensity or UV-irradiance [18, 19], nitrate limitation [20, 21], or increase of pH [22] induce cyst formation and astaxanthin accumulation. Effects of small organic compounds on these processes also were investigated: addition of organic carbon sources such as acetate or sugars acts as inducer of astaxanthin production [23, 24]. Plant hormones associated with stress response mechanisms (e.g. abscisic acid, jasmonic acid, methyl jasmonate gibberellic acid, salicylic acid, or brassinosteroids) also increased astaxanthin accumulation in *H. pluvialis* [25–30]. However, there is very limited information about stressors inhibiting astaxanthin production. It is known that astaxanthin content decreased at high salinity (1–2%; [22] Sarada et al. 2002), but there are almost no data (at least to our knowledge) how certain micro-contaminants (potentially occurring both in nature and in artificial cultivating systems, especially in open ponds) affect the cyst formation and valuable product accumulation.

The non-steroidal anti-inflammatory drugs (NSAIDs) can be ranked among the most common analgesic products in the world [31]. As a consequence of increasing drug consumption, these drugs appear with increasing frequency in the environment due to their partial metabolization in human and animal body and to the not fully efficient wastewater treatment methods [32–34]. Appearance of NSAIDs can be expected even in piped water, although data evince very low concentrations [34]. It is known that these organic micro-contaminants can act as stressors among aerobic photosynthetic microorganisms (cyanobacteria and eukaryotic algae), causing growth inhibition [35–43], or structural changes of natural assemblages [42, 44, 45]. According to the results of our previous work, *H. pluvialis* showed a moderate sensitivity to NSAIDs among eukaryotic algae [42]. Based on our previous data concerning eukaryotic algal assemblages and isolated laboratory strains [42], a more detailed analysis of the toxicity of diclofenac, diflunisal and mefenamic acid was aimed in this study to gain a better understanding between micro-contaminants (NSAIDs) and growth, cyst formation and storage accumulation of a model organism with high economical importance. Our hypotheses were the followings:

- Since concentrations of 0.1 mg ml^{-1} of the drugs caused serious growth inhibition [42], we assumed that these drugs could be toxic already at lower concentrations.
- We hypothesized that NSAIDs as stress factors may induce morphological changes of flagellated vegetative cells, at least in a part of the exposition time.
- We assumed that NSAIDs as stress factors may cause changes in pigment composition of *H. pluvialis* cells.

2. Materials and methods

2.1. The studied NSAIDs

Diclofenac (Dic), 2-(2,6-dichloranilino)phenylacetic acid belongs to the family of aryl-alkanoic acids. The molecule contains a chlorinated aromatic group (xenobiotic structure), so its environmental degradation is presumably slow or incomplete [46–50]. It has the classic triple effects of the NSAIDs, such as anti-inflammatory, analgesic and antipyretic effects. Dic inhibits COX-2 more strongly than COX-1. Dic is largely excreted with bile and faeces [51].

Diflunisal (Dif), 5-(2,4-difluorophenyl)salicylic acid belongs to the family of salicylic acid derivatives. It has a fluoro-phenyl structure (xenobiotic structure), suggesting slow or incomplete environmental degradation [50]. Dif effectively reduces pain, swelling and joint stiffness caused by arthritis. In addition, it also has antipyretic effects [52].

Mefenamic acid (Mef), 2-[(2,3-dimetylphenyl)amino]-benzoic acid belongs to the family of antranylic acid derivatives. The molecule has a biphenyl-amine structure (xenobiotic structure), which likely makes its environmental degradation slow or incomplete [53]. Mef is mainly used to treat mild to moderate pain and acute inflammatory diseases [54].

2.2. Algal strain, culturing conditions and experimental setup

Monoalgal cultures of the cosmopolitan flagellated green alga *Haematococcus pluvialis* are maintained in Optimized Haematococcus Medium (OHM) [55] at 24 °C, under 14 h light (~80 μmol m^{-2} s^{-1} fluorescent light) – 10 h dark cycles in the algal culture collection of the Department of Hydrobiology, University of Debrecen (ACCDH-UD1205).

The experiments were carried out in triplicates, in standing cultures in 100 ml Erlenmeyer flasks with a final volume of 50 ml, under 14 h light – 10 h dark cycles, at 24 °C. The cultures were hand-shaken daily. Dic, Dif and Mef NSAIDs were applied in 0.025; 0.05; 0.075 and 0.1 mg ml^{-1} concentration during the treatments. The drugs were dissolved in 10 g l^{-1} sodium-carbonate buffer. Control cultures were prepared without the addition of NSAID solutions. Cultures containing sodium-carbonate buffer without NSAIDs were also applied to check the possible effects of the buffer used for the dissolution of the drugs. To prove the inducible astaxanthin production ability of the used *H. pluvialis* strain, induced cultures were also evaluated. Astaxanthin production was induced by adding H_2O_2 and sodium-acetate in final concentrations of 0.01 and 45 mM, respectively [23].

2.3. Measuring the growth and morphological changes of the cultures

Growth of the control and treated cultures was followed by counting cell numbers (vegetative cells and cysts separately) at every 48 h for 14 days. It has to be emphasized that all non-motile cell types (from the green palmella stage to the red mature aplanospores) were considered as cysts in this study. Cell numbers were counted from 10 μl samples in a Bürker chamber on 400 × magnification using an Olympus BX50F-3 fluorescent microscope. To give EC_{50} values to a certain exposition time (96 h, 7 or 14 days) for all three NSAIDs, the extents of growth inhibitions (%, considered control or buffer as 100% on the given day) were plotted as functions of NSAID concentrations and trend lines were fitted, quadratic regressions showed the best fit (R^2 values ranged from