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Impact of synthetic hormone 17α-ethinylestradiol on growth of microalgae *Desmodesmus communis*

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Abstract. Microalgae has recently attracted much attention as a feedstock for biogas. Using wastewater as microalgae nutrition is a way how to produce algal biomass with low cost and minimum impact on environment. However, wastewater often is polluted with chemicals like pharmaceuticals which are among the commonly used chemicals in everyday life. The present study was aimed at the toxicity evaluation of a commonly used synthetic hormone, 17α-ethinylestradiol, using freshwater green algae *Desmodesmus communis* as a biotest organism. Parameters like healthy cell number and photosynthetic activity were determined and used to assess the toxicity. Lowest Observed Effect Concentration (LOEC) and 50% Effective Concentration (EC_{50}) values were calculated for the parameters at different incubation times. It was found out that 17α-ethinylestradiol affects algal cell ability to grow, inhibits cell division and reduce photosynthetic processes in algal cells. Our research shows that inhibitory effect on growth of green algae D. communis start on concentration below 10 µg L-1 (4–8 µg L-1). Concentrations in the range of concentration 80–100 reduce growth by 50%, but concentrations 100–500 µg L-1 induce 100% reduction of growth rate and even calls initial algal cell destruction. Presence of EE2 in wastewater used for algal growth can affect productivity of a microalgae aquaculture.

Key words: microalgae, 17α-ethinylestradiol, EC_{50}, carbon, photosynthesis.

INTRODUCTION

In last 50 years the world's population has more than doubled, and due to great demand of energy, amount of fossil fuel is reducing. New sources of energy are being sought (Jones & Mayfield, 2012). Microalgae cultivation can be a significant source for energy production. Comparing to other sources like forestry, agriculture and macrophytes, microalgae has higher growth rate and productivity (Blumberga et al., 2011; Daroch et al., 2013). Comparing to other crops microalgae grow highly rapidly and usually double their biomass in 24 hours. These photosynthetic microorganisms convert sunlight, water and carbon dioxide to algal biomass (Chisti, 2007). Advantage is that they can be cultivated and harvested throughout the year (Chen et al., 2011). Besides that microalgae can be grown in wastewater to remove nutrients and toxic substances (Klein-Marcuschamer, 2013; Guiry, 2014; Zhang et al., 2014).

Using wastewater as microalgae nutrition is a way how to produce algal biomass with minimal cost and low environmental impact (Park et al., 2011). Wastewaters often have high concentrations of nutrients (nitrogen and phosphorus), and range of trace elements, which are necessary for the growth and metabolism of microalgae (Aravantinou et al., 2013). Microalgal growth can effectively remove phosphates and nitrates from wastewater, thus acting as treatment for the wastewater, and creating it as a suitable substrate for microalgal cultivation (Rawat et al., 2013). *Desmodesmus communis* is one of the most widely used microalgae species for wastewater treatment (Guiry, 2014; Zhang et al., 2014). Nutrients from wastewater are increasing algal biomass growth (Guo et al., 2013).

Wastewater contains not only essential nutrients, but also a lot of pollutants, that can harm aquatic organisms including microalgae. One of the most hazardous group of toxic substances in municipal wastewaters are pharmaceuticals. They can reach aquatic environment in different ways, of which the most common are the end products of human metabolism. Wastewater treating plants are not specifically designed to attenuate pharmaceuticals. Pharmaceuticals have been widely detected in the surface, ground and coastal waters and even in drinking water. Concerns have been raised about potential toxicity of pharmaceuticals and their adverse impacts on ecological safety (Sun et al., 2014).

Pharmaceutical pollution is growing as a result of increasing use of medicine. One of the most toxic substances is synthetic hormone 17α-ethinylestradiol (EE2), which is artificial estrogen derivate and is permanent in aquatic environment. After application in humans it is excreted with metabolic end products and it can reach wastewater treatment plants (Liebig et al., 2005). Even small quantities of this hormone may cause toxic effects on aquatic organisms. Although in natural habitat EE2 occurs just in small quantities it's one of the most dangerous water pollutants and it can affect not only animals, but also decrease photosynthetic processes in plants (Perron & Juneau, 2011).

Doubt exist that endocrine disruptor may leave negative impact on photosynthetic processes in microalgae. There are studies that endocrine disruptors have a negative effect on the level of non-photochemical quenching. This process occurs in almost all photosynthetic eukaryotes and it helps to regulate and protect photosynthesis in microalgae (Yi et al., 2014). Hormones change electron transport in algal cells reducing photosynthetic activity and inducing the same effects as low light conditions (Lohr et al., 2012). Loss of biomass production caused by reduced photosynthetic processes might make algae cultivation less profitable (George et al., 2014; Han et al., 2015). Damage to the photosynthetic apparatus is not permanent and a recovery occur when there is no more contact with the toxicant (Zhou et al., 2013).

D. communis has been extensively used for evaluating the adverse effects of different toxic substances and environmental stressors. As stated before this microalgae is widely used for treating wastewater and biomass can be used as feedstock for biogas production. However, data regarding toxicity of hormonal pollution to microalgae growth ability produce biomass can hardly be found. The aim of this study was to evaluate hazard impact of this pollutant on microalgae *D. communis* growth. In order to have a picture of EE2 toxicity, growth, photosynthesis and total organic carbon were determined. Since the toxicity of chemicals vary with exposure time, the EC_{50} and Lowest Observed Effect Concentrations (LOEC) after 72 hours exposition were determined.

MATERIALS AND METHODS

Algal growth inhibition test

Algal growth inhibition test was carried out based on European standard (EN ISO 8692:2004).

Microscopic green algae *Desmodesmus communis* (DCGR-3) was used as a test organism. Culture was obtained from the Algal Culture Collection of Latvian Institute of Aquatic Ecology. Culture was pre-cultivated 3 days before test to reach exponential growth rate.

Algal growth medium BG-11 was prepared according Stanier et. al (1971). At the beginning of test, 1 mL suspended algae culture (2 10^5 cells mL^{-1}) was inoculated in 30 mL BG-11 medium and grown in Nalgene centrifuge tubes at 20 ± 2 °C, pH 8 ± 1 under the irradiance intensity of 60 µmol photon m^{-2} s^{-1}. *D. communis* was exposed to various concentrations of synthetic hormone under the defined conditions. The microalgal cultures were incubated for a period of 72 h and mixed manually once per day. Fluorometric determination was used to estimate algal biomass 0, 24, 48 and 72 h after the start of the test. Algal cell quantifying was used on the beginning of test and after 72 h.

Hormone 17α-ethinylestradiol (17α-ethinylestradiol ≥ 98%, Sigma-Aldrich, US) was initially dissolved in 96% ethanol to prepare the stock solution (100 mg EE2 in 100 ml ethanol) as EE2 in its original form is not soluble in water (Bell, 2001). In the test, the algal cultures were exposed to 10 different concentrations of the 17α-ethinylestradiol (10; 100; 500; 1,000; 1,500; 2,000; 2,500; 3,000; 4,000; 5,000 µg L^{-1}) and two different controls. First control was only algal culture with medium, second - was spiked with 96% ethanol in concentration 50 mL L^{-1} corresponding to the highest concentration of ethanol used for EE2 dissolution.

Growth measuring using algal cell counting

One mL of algal culture was aseptically sampled from each test tube and fixed with Lugol's solution. The cell number was counted with a Neubauer ruling under a light microscope (Leica ATC 2000) at magnification of 400×. Only healthy, pigmented, unbroken cells were counted.

Growth measuring using Fluorescence

Chlorophyll fluorescence was estimated using 10-AU Fluorometer (Turner Designs, Sunnyvale, CA, USA). When exposed to light, photosynthetic active organisms exhibits fluorescence radiation that originates from chlorophyll *a* (chl *a*) molecules of Photo System II (PSII). Increasing levels of fluorescence indicates for increased chl *a* concentration in algal cells. From this can be concluded algal growth rate and biomass yield. Samples were dark-acclimated for 30 min to reduce PSII to a constant fluorescence level.

Carbon quantifying

To calculate amount of carbon, fluorescence / cell number ratio was used. Ratio was established measuring fluorescence and algal cell number in different cell density levels. Total Organic Carbon (TOC) in the same cell density levels was calculated using amount of carbon in algal cell (Olguín & Sánchez-Galván, 2011). Fluorescence to TOC

ratio was established and used to calculate amount of Total Organic Carbon. Equation was derived (1), because it lets to quantify C in all cells including damaged cells.

$$TOC = (F = 390.89)/1.1588 \quad (1)$$

where *TOC* is the amount of total organic carbon in algal cells and *F* is fluorescence of chlorophyll a.

Data analysis

All the experiments were carried out in triplicates and data presented is mean values of these replicates. Data were further analysed using Microsoft Office Excel 2013 (Microsoft, USA). A statistical significance was considered at p = 0.05.

Data were processed by applying correlation and regression analysis.

The mean specific growth rate (μ) for exponentially growing cultures was calculated by using equation (2)

$$\mu = (\ln N_2 - \ln N_1)/t_2 - t_1 \quad (2)$$

where: t_1 is the time (h) of the first measurement at the start; t_2 the time of the final measurement at the end of the test; N_2 the number of cells per mL at time t_2 and N_1 the number of cells per mL at t_1. The inhibition of the growth rate was determined in the relation to the control (culture with ethanol). The same calculations were made with fluorescence measurements, replacing number of cells with fluorescence values.

The percent inhibition of growth rate for each treatment was calculated by using formula (3)

$$\%I_{\mu_i} = \frac{\mu_c - \mu_i}{\mu_c} \times 100 \quad (3)$$

where: I_{μ_i} is percent inhibition in mean specific growth rate; μ_c is value for mean specific growth rate in the control; μ_i is mean specific growth rate in concentration *i*.

Values for the mean growth inhibition were calculated for each test substance concentration (in logarithmic scale) to establish a dose-response curve.

EC_{50} and Lowest Observed Effective Concentration (LOEC) were calculated using dose-response curves for this biotest. The parameter EC_{50} expresses the effective concentration of EE2 that results in a 50% growth rate inhibition comparing to the control. LOEC is a lowest concentration, which has an impact to algal growth and reduce algal growth by 10%. EC_{50} and LOEC were determined using dose-response curves.

RESULTS AND DISCUSSION

Algal cell counting

Algal growth inhibition was determined from results of algal cell counting. The dose–response curve was designed to display inhibition in a range 10–1,000 μg L^{-1} for used concentration (Fig. 1).

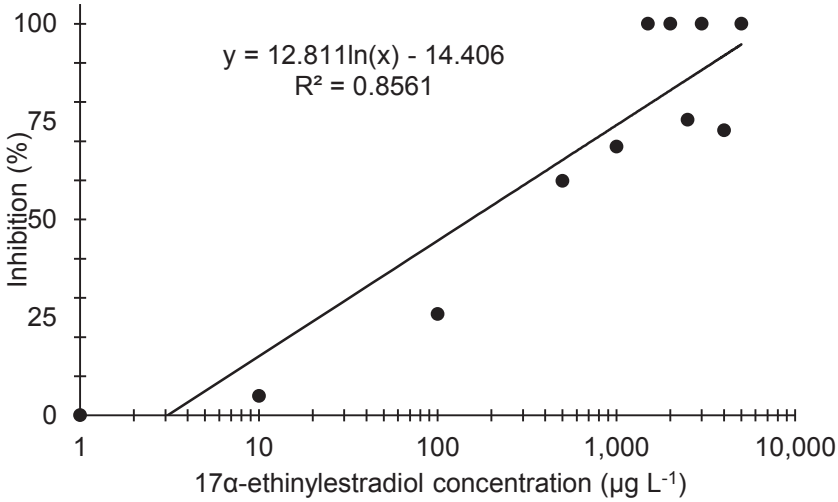

Figure 1. Dose–response curve (concentration vs. inhibition of growth rate) for 17α-ethynilestradiol, quantified with cell counting.

Strong correlation between the inhibition of growth rate of *D. communis* detected by cell counting and EE2 concentrations was detected ($R^2 = 0.8561$). Growing hormone concentrations cause increase of algal growth inhibition. Already 8μg L^{-1} of EE2 is able to reduce algal growth by 10%, but concentrations around 80 μg L^{-1} can evoke a 50% algal growth inhibition. Concentrations exceeding 1,300 μg L^{-1} are able not only completely stop cell division, but can even cause damage of initial cells. In the range of concentrations of EE2 (1,000–5,000 μg L^{-1}) there are more damaged and deformed cells observed.

Fluorescence measurements

Measurements of chlorophyll fluorescence were made to the same samples as cell counting. From measurements it is possible to assess changes of photosynthetic processes in algal cells in relation to EE2 concentrations (Fig. 2).

Statistically significant correlation between fluorescence measurements was detected ($R^2 = 0.8642$). Growing hormone concentrations decrease photosynthetic activity in algal cells. Results show that 4 μg L^{-1} of EE2 is able to reduce fluorescence of chlorophyll a by 10%, but concentrations around 100 μg L^{-1} evoke a 50% inhibition of photosynthesis. Concentrations in the range 1,000–5,000 μg L^{-1} can cause 75–100% inhibition of photosynthetic activity.

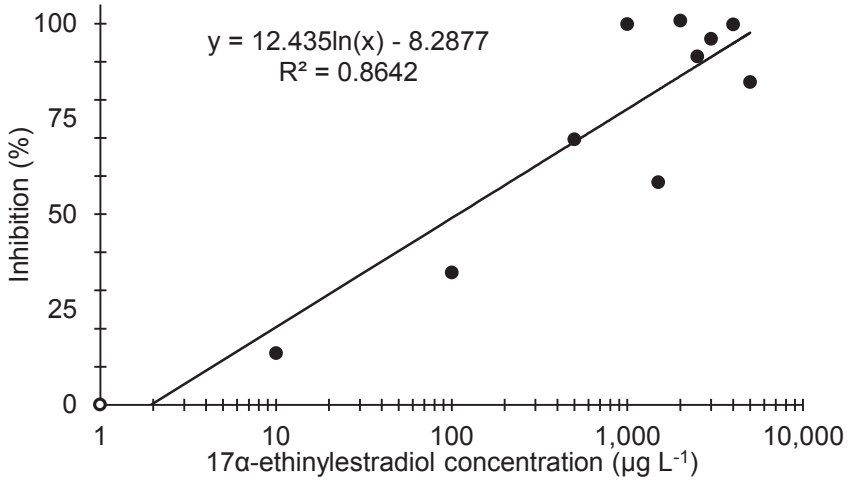

Figure 2. Dose–response curve (concentration vs. inhibition of growth rate) for 17α-ethynilestradiol, quantified with fluorescence.

Pearson's correlation was used to estimate correlation between fluorescence and cell counting. Obtained results revealed about correlation between fluorescence and cell counting results ($r^2 = 0.96$, $p = 0.019$).

Carbon quantifying

Photosynthesis fixed carbon dioxide is the main source of carbohydrates and other organic compounds containing carbon in algal cells. Quantity of carbon predicts potential amount of CH_4 and CO_2 produced by algae. Amount of Total Organic Carbon (TOC) in samples were calculated using data of fluorescence to see how algal biomass change in a time depend from concentrations. Data of fluorescence was used instead of cell counting data because fluorescence measurement show amount of carbon in all cells including damaged cells. Fig. 3 shows the results obtained for a daily changes of an amount of carbon in a range of EE2 concentration.

Graph shows that increased level of EE2 reduce production of TOC 100 µg L^{-1} of EE2 decreased production of TOC by 25%. Concentrations exceeding 500 µg L^{-1} can evoke 50% inhibition of biomass production, but over 1,500 µg L^{-1} it can stop biomass production. The most significant impact occurred in the first 24 hours. On EE2 concentration 100 µg L^{-1} small decrease of TOC was observed in 48 h, but in 72 h exposition, recovery was noticed. Higher EE2 concentrations stops carbon production. Recovery could be explained as algal ability to accumulate pollutants, and maybe some cells was not harmed and continued to divide. It is possible that recovery can happen also in higher concentrations, that 100 µg L^{-1} but it was not observed in this experiment.

Levels of pharmaceuticals in wastewaters used as nutrient source for microalgae cultivation may decrease and even stop producing biomass, thus making cultivation process inefficient.

Although EE2 is prepared to interact with human endocrine system, it is also presented in aquatic ecosystems and can reduce the development of phytoplankton (Hense et al., 2008). Even a small amount of endocrine disrupters can significantly

reduce the photosynthetic activity in algae cells (Perron & Juneau, 2011). Endocrine disruptors make damage to the algal cells leading to growth inhibition (Liu et al., 2010). Although EE2 is not soluble in water, after exposure to human body it passes in water in dissolved form and is free ingestible aquatic organisms (Feng et al., 2010). Our research shows that inhibitory effect on growth of green algae *D. communis* start on concentration below 10 µg L^{-1} (4–8 µg L^{-1}). Concentrations in the range of concentration 80-100 reduce growth by 50%, but concentrations 100–500 µg L^{-1} can reduce algal growth to minimum and even cause destruction of algal cells.

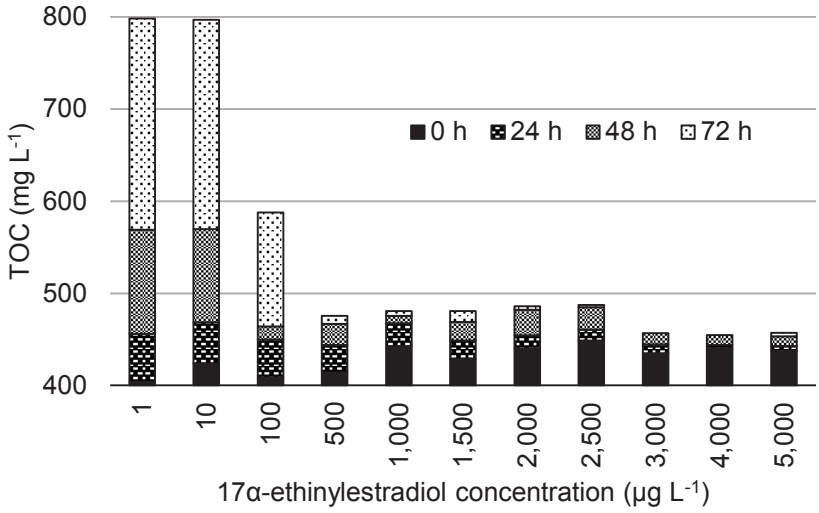

Figure 3. Effect of concentration on production of total organic carbon.

This was also demonstrated in a study, where several green algae and blue-green algae species were affected by different endocrine disruptors. Study represents reduced activity of chlorophyll in affected algal cells, showing that photosynthetic activity is reduced (Perron & Juneau, 2011). Interesting fact is that EC_{50} obtained from fluorescence is higher than EC_{50} obtained from cell counting, which can be caused by cell damage and deformation observed during cell counting.

Environmental concentrations of EE2 are 0.002 µg L^{-1} in Germany to 0.273 µg L^{-1} in North America and concentrations tend to increase (Adler et al., 2001; Daughton, 2013; Kolpin et al., 2002). Concentrations used in our experiments are much higher to predict toxic effect that may happen if there will be no improvements in wastewater treatment system. Already the smallest of the test concentrations (10 µg L^{-1}) caused above 10% algal growth inhibition as well as inhibited algal fluorescence. Consumption of endocrine disruptors is increasing due to raising living standard (Daughton, 2013). Taking into account the inflation of hormonal preparations environment and insufficient treatment of wastewater, presence of pharmaceuticals can cause significant changes on hydroecosystems.

Unfortunately EE2 is not only one pharmaceutical found in environment. There are several studies, which shows effect of pollutant mixtures on different water organisms (Silva & Kortenkamp, 2002). Different pharmaceuticals are found in environment in low

level, that are not affecting aquatic organisms, but studies show that mixture of these pharmaceuticals is affecting water organisms. Wastewaters usually contain high levels of various pharmaceuticals and other pollutants which may affect growth of microalgae and inhibit the increase of the biomass.

As current level of EE2 in nature is not affecting the algal biomass and the growth rate of cultivated microalgae, using wastewater as nutrient source is a way how to remove pharmaceuticals from water. Under natural conditions, endocrine disruptors existing in environment hinder the normal development of the phytoplankton that cause changes in phytocenosis structure, thus posing a threat to the entire hydroecosystem. In aquaculture (artificial environment) increased concentrations of hormones can reduce the practical application not only in cosmetics and pharmaceuticals, but also in energy production.

CONCLUSIONS

Endocrine disruptors have an effect on microalgae photosystem. Increased concentrations of synthetic hormone 17α-ethinylestradiol can damage phytoplankton cells and can stop algal cell division. It was observed that synthetic hormone has a negative influence on carbon production. Decreased biomass leads to smaller carbon production in this way making bioenergy production from microalgae less effective. It is suggested not to use wastewater with pharmaceutical pollution for microalgae cultivation. In further studies it is suggested to analyse carbon content changes in algal cells. Concentrations of EE2 used in experiment are above average concentrations abundant in environment, but increasing pharmaceutical pollution in long term may cause significant loss of microalgal biomass production and impede sustainable development of aquatic ecosystems.

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