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Abstract

The biotransformation and bioconcentration of natural and synthetic steroid estrogens by *Chlorella vulgaris* were investigated using batch shaking experiments with incubation for 48 hours in the light or dark. Estradiol and estrone were inter-convertible in both light and dark conditions, however this biotransformation showed a preference to estrone. In the light, 50% of estradiol was further metabolized to an unknown product. Apart from biotransformation, estrone as well as hydroxyestrone, estriol and ethinylestradiol were relatively stable in the algal culture, while estradiol valerate was hydrolyzed to estradiol and then estrone within 3 hours of incubation. All the tested estrogens exhibited a degree of partitioning to *C. vulgaris*, however, the concentrations of estriol, hydroxyestrone, ethinylestradiol and estradiol valerate were always below the quantification limits. For estradiol and estrone, the partitioning of these estrogens in the algal extracts to the filtrates was below 6% of the total present. The average concentration factor for estrone was around 27, however the concentration factor for estradiol is not reported since no equilibrium was reached between aqueous solution and that within the cells due to continuing biotransformation.

Introduction

Natural phenolic steroid estrogens (estradiol and estrone) and the synthetic estrogen, ethinylestradiol, have been detected in aquatic systems and their occurrence is associated with vitellogenesis and reproductive abnormalities in wild fish (3,5,7,16,21). A more complete understanding of their behavior in the aquatic environment is required to enable accurate risk assessments to be undertaken (9). Algae, given their substantial biomass, extensive range of habitat and diversity play an important role in the fate of organic compounds in the aquatic ecosystem (14,15). They may degrade or take up contaminants thereby acting as a medium for bioconcentration and subsequent biomagnification in higher trophic levels (14,15,20). Biosorption of heavy metals and organic pollutants by algae has been frequently reported (12,14) and some macroalgae have been shown to have the same detoxification enzymes as those found in the mammalian liver, the so called “green liver” (15). It has been demonstrated that *Ochromonas danica* has the ability to break down phenolic compounds to pyruvate and carbon dioxide (19).

Ethinylestradiol and estradiol valerate are synthetic estrogens produced by modifying the structure of estradiol. Ethinylestradiol can be deethinylated to estradiol in the human liver, though a majority (up to 80%) of ethinylestradiol is excreted from humans unchanged in conjugated forms (4), while estradiol valerate is readily hydrolysed to estradiol in the liver to exhibit its estrogenic functions (18). In the environment, the persistence of ethinylestradiol has been reported in sewage treatment works and rivers (7,13,22). With respect to the natural estrogens, estradiol and estrone, they are readily transformed via oxidative and reductive pathways by 17β-hydroxysteroid dehydrogenase

(17β-HSDH) in a range of organisms (4). Microorganisms that do not produce estrogens, such as fungi and bacteria, also exhibit the ability to facilitate this biotransformation through such pathways when exposed to estradiol and estrone (4,11,13,23,27). These steroid estrogens may also be further metabolized to estriol and hydroxyestrone, which play an important role in a range of organisms (4).

The aim of this study was to investigate the interaction of a common and widely studied fresh water alga, *Chlorella vulgaris,* with different natural (estradiol, estrone, hydroxyestrone and estriol) and synthetic (ethinylestradiol and estradiol valerate) estrogens and establish whether they are biotransformed or bioconcentrated.

Materials and Methods

Chemicals

Individual working solutions of estradiol, estrone, estriol, hydroxyestrone, estradiol valerate, estradiol acetate and ethinylestradiol (Sigma, Poole, UK) were prepared in HPLC grade acetone (Rathburn, Walkerburn, UK) at 10 mg l^{-1}. *Chlorella vulgaris* was purchased from the Culture Collection of Algae and Protozoa (CCAP), Windermere, UK. The alga was grown in Jaworski's medium (JM), as recommended by CCAP, with continuous aeration and illumination (cool white fluorescent light at 300 lux was provided by Osram L58 W23 tubes) in a temperature controlled room set at 18±2^{o}C. The algal cultures were allowed to grow into the stationary phase (20 days) achieving approximately 2 g l^{-1} (dry weight). Stationary phase cultures maximised the amount of biomass available to allow for determination of trace levels of steroid estrogens sorbed to the solid phase.

Biotransformation and bioconcentration experiments

All the batch experiments were undertaken in triplicate. Individual estrogens (500ng) (estradiol, estrone, estriol, hydroxyestrone, estradiol valerate and ethinylestradiol) were spiked separately into 250 ml Teflon bottles (dark) or 500 ml conical flasks (light) and blown to dryness with a gentle nitrogen stream. Algal culture (200 ml) was transferred into the bottles or flasks so that the initial concentration was of 2.5 μg l^{-1} and placed on a rotary shaker in the dark or aerated in the light with triplicate samples taken at time intervals of 3, 6, 24 and 48 hours. The optical density of the algal culture was measured at 680 nm and converted into mass of algae (dry weight) for each sampling time. Further kinetic experiments on the biotransformation of estradiol were undertaken using different algal cell densities (0.8, 1.5 and 2.3 g l^{-1}, dry weight) shaking for 1, 18 and 48 hours and a range of estradiol concentrations per gram of algae (9.7, 97, 388 and 969 μl l^{-1} g^{-1}) in the dark for 6 hours.

Estrogen determination in filtrate and algal cells

Algal cells were filtered from the growth culture using a glass fibre filter (GF6) (Scheicher & Schuell, London, UK). Before extraction, 500 ng of estradiol acetate was spiked into the separated algal cells and filtrate for the determination of the final recovery in each sample. Estrogens in the algal cells were extracted overnight by soxhlet extraction using 150 ml dichloromethane (DCM) (Rathburn, Walkerburn, UK). Estrogens in the liquid phase were extracted by liquid-liquid extraction using 200 ml DCM. Extracted estrogens were evaporated to 1 ml using a rotary evaporator, transferred into a reaction vial and dried with a gentle nitrogen stream. The derivatization mixture (*N*-methyl-*N*-

(trimethylsilyl)-tri-fluoroacetamide: trimethylsilylimidazole: dithioerythritol; 1000:2:2; v/v/w) (Sigma, Poole, UK), 50 μl, was added, the reaction vial sealed, and placed in a heating block at 60 ^{o}C for 30 min. The solution was again evaporated to dryness and 0.2 ml of 2 mg l^{-1} mirex (internal standard) (Promochem, Welwyn, UK) in hexane was then added before gas chromatography-mass spectrometry (GCMS) analysis. The GCMS conditions, calibration, identification and quantification procedures have been described previously (8). The GCMS limit of quantification was 10 μg l^{-1} in final extracts, however, some samples exhibited responses of target ions at correct retention times below this level and such results have been reported as being less than the quantification level, but above the detection limit of 5 μg l^{-1}.

Quality Control

Before the experiments, quality controls were undertaken to ensure that the estrogens did not sorb to the laboratory equipment. The recovery efficiency of the soxhlet and liquid-liquid extraction for the algae and the filtrate spiked with 500 ng each of estrogen mixture was evaluated with respect to the recovery of estradiol acetate. The recovery for soxhlet extraction was 65% and for liquid-liquid extraction was above 85% for all the estrogens, except for estriol where recoveries were 50% in both extraction methods. Moreover, there was no estrogen peak detected from the blanks of the algae and filtrate extracts.

Results

Fate of estrogens in algal culture

The concentration of estradiol in the culture medium fell rapidly over the first 3 hours of incubation, and this decline was more pronounced in the light than the dark (Figure 1). However, subsequent change in the estradiol concentration was more rapid in the dark, and was accompanied by an increase in the concentration of estrone in the media solution. The stability of all estrogens was tested using crude and autoclaved algal filtrate (without algal cells) instead of the algal culture as controls. Experimental conditions were the same for all sample types. The estrogen concentrations in the crude and autoclaved (sterile) filtrates remained constant, which indicated that no biological, physical or chemical removal occurred in the algal-cell free or the sterile filtrates. Lack of change in the crude filtrates which may have contained bacteria not removed by the GF6 filter, would indicate that effects observed were due to the algae rather than other microorganisms. The most probable explanation for the source of estrone is that it is a result of biotransformation of estradiol by the algae, *C. vulgaris*. This is also supported by considering the mass balance of estradiol and estrone in the algal culture, which in the dark decreased by less than 20% in total (Table 1). However, in the light there was an overall decrease in concentration of both compounds, probably as a result of further metabolism (Table 1). These differences in final concentrations of estradiol and estrone in light and dark conditions suggest that biochemical processes in the algae have a different effect on the estrogens in light and dark conditions.

Although further transformation products, hydroxyestrone and estriol were observed in media extracts, the concentrations were below quantification levels and these are therefore insignificant products or metabolic intermediates with short half-lives. It was also possible that the estrogens were removed from the media solution through bioconcentration within the algae. The amount of estrogens which partitioned to the algae did not exceed more than 9% of the total estrogens by mass (Table 1). Although the ultimate fate of estradiol is not clear, it is apparent that in the light, *C. vulgaris* reduces the concentration from 2.50 μg l^{-1} to 0.37 μg l^{-1}, although some 0.60 μg l^{-1} of the estradiol is converted to estrone, which is a potent steroid estrogen.

Media solutions were also spiked with estrone and again incubated in light and dark conditions. In this case, estradiol was observed in the solutions, which would indicate that the alga is able to interconvert estrone and estradiol. However, the amount of estrone transformed was less than 10% in comparison to the reverse reaction where over 70% and 22% of estradiol was transformed to estrone in the dark and light, respectively (Figure 2). Both estriol and hydroxyestrone were again observed in solution, yet still below the quantification limit. It was however, clear that mass balance equations (Table 2) indicate that overall reduction in concentration of estrone and estradiol was less than when solutions were spiked with estradiol. With respect to the partitioning of estrogens to the algae, the maximum percentage partitioning was 7%, with the majority of compounds remaining in solution (Table 2).

The algae also exhibited an ability to hydrolyze estradiol valerate to estradiol and estrone in both light and dark (Figure 3). The concentration of estradiol valerate had decreased after the first 3 hours of incubation to below the quantification level and this drop was associated with the occurence of both estradiol and estrone. The concentration of estradiol remained constant throughout the experimental period in both dark and light, however, the level of estrone increased continuously in the dark, but peaked at 24 hours in the light. Traces of both estriol and hydroxyestrone were also detected in the filtrate but their concentrations were below the quantification limits. This data may suggest that estradiol valerate was rapidly transformed to an intermediate before a slower hydrolysis to estradiol. Some uptake of estradiol valerate by the algae was observed, however, concentrations were below the limit of quantification.

For the samples spiked with estriol and ethinylestradiol, a slight drop in concentrations of both compounds were observed however the data points were not significantly different and thus evidence of removal of these compounds was not conclusive. Similarly, it was not possible to quantify any uptake of these estrogens by the algae since concentrations were generally below the quantification limits and in some cases below the detection levels.

The fate of hydroxyestrone in the algal culture was also tested and the result was compared to that in a culture filtrate (no algae present) as a control. A reduction in concentration of hydroxyestrone (<10% of initial spiked) was recorded in the culture with a detectable amount of estriol, while the concentration of hydroxyestrone was unchanged in the control. The relative stability of hydroxyestrone would indicate that it is not a rapidly metabolized intermediate in any transformation pathways.

Using the preceding observations, a possible pathway for transformation of the steroid estrogens by *C. vulgaris* is shown in Figure 4. Ethinylestradiol was persistent in the algal culture, while estradiol valerate was hydrolyzed to estradiol and estrone. Due to the interconversion preference for estrone, it is evident that estradiol valerate was hydrolyzed to estradiol and then transformed to estrone. In light conditions, approximately 50% of estradiol was metabolized into an unknown product and some of the estradiol was transformed to estrone, while in dark conditions, biotransformation to estrone was the major pathway. Although estrone, hydroxyestrone and estriol were detected in the algal culture under light conditions, the low concentration and the stability of these estrogens in the culture indicated that as a major metabolic pathway it is insignificant compared to the unknown pathway observed to occur in the light.

Bioconcentration of estrogens in the algae

On exposure of *C. vulgaris* to estradiol and estrone, a degree of partitioning of both estrogens was observed to occur between the algae and aqueous phase. However, a true "bioconcentration factor" cannot be reported as equilibrium was not been reached, and transformation continued. For the samples spiked with estradiol, the concentration of estradiol decreased significantly in the filtrate with time, and the occurence of estrone in the algal extract was most probably a result of intracellular biotransformation (rather than uptake from the filtrate), thus no bioconcentration factor can be reported (Table 1). For the samples spiked with estrone, although biotransformation did occur, the level of estrone in the filtrate remained relatively constant and the concentration of estradiol was also consistently low. It was therefore likely that estrone extracted from the algae originated from the filtrate solution and thus a concentration factor for estrone between the algae and filtrate was calculated to give an insight into the probable bioconcentration factor for estrone. The average concentration factors for estrone in both light and dark conditions was approximately 27 (Table 2).

Effect of cell density on transformation of estradiol

The kinetics of the biotransformation process from estradiol to estrone were determined over a range of cell densities in the dark only as in light the end products of transformation were unknown. The rate of biotransformation of estradiol to estrone declined with an increase in cell density and shaking time (Figure 5), which indicated that substrate levels were limiting under these experimental conditions.

Effect of substrate level on the rate of transformation

In order to investigate the effect of substrate level on biotransformation, different concentrations of estradiol were spiked into the culture. The final concentration of estrone produced increased with initial estradiol concentration up to 400 μg l^{-1} g^{-1} of algae, however at 969 μg l^{-1} g^{-1} there was no significant increase in the final estrone concentration. The regression line produced using the first 3 data points indicated that within experimental error, the concentration of estradiol became limiting below 400 μg l^{-1} g^{-1} (Figure 6) which is equivalent to 2.58 μg of estrone produced per gram of algae per hour and is likely to indicate the maximum capacity of *C. vulgaris* to transform estradiol to estrone under experimental conditions.

Discussion

In the present study, the hydrolysis of estradiol valerate to estradiol by *C. vulgaris* has been demonstrated, however, under the conditions used in this study, transformation of ethinylestradiol, through deethinylation or other pathways, was not observed. With respect to estradiol and estrone, *C. vulgaris* exhibited a preference toward estrone in both light and dark. This biotransformation may be significant to the alga by reducing the estrogenic potency and increasing the polarity of estrogens for excretion or further metabolism (4,25). The occurrence of estriol and hydroxyestrone is an indicator of further hydroxylation of estrone to more polar metabolites (4,25). However, such a pathway accounts for a small proportion, < 4% of total loss of estrone/estradiol in the light and another, unidentified pathway must therefore be responsible for 50% loss of the estrogens. It is possible that conjugation of the estradiol may occur in the light. Conjugation increases polarity and is the method by which the steroid estrogens are excreted in urine in a range of animal species (4). However, this process also occurs in plants and partially or completely deactivates plant hormones and phytoestrogens (2). This process has also been observed to occur in plants to detoxify xenobiotics such as DDT and chlorinated phenols (17,24). It is evident that conjugation is a rapid detoxification mechanism and an absence of a lag phase for conjugation has been demonstrated for plant hormones (2). Such transformation would explain the rapid decline in estradiol concentration after the first 3 hours of shaking. Moreover, in the same review (2) the effect of light on the stimulation and regulation of conjugation has been exemplified in different plant hormones.

Estradiol/estrone biotranformation by the algae was observed to be dependent on cell density and substrate concentration. In this study a relatively high concentration of estrogens in comparison to environmental levels, of around 0.1 – 2 ng l^{-1} (9), and algal cells, were used to allow for monitoring of changes and uptake. The results of the kinetic study demonstrated that the biotransformation process operated under conditions of substrate limitation, however if the same pattern of substrate limitation occurs in the natural environment, it would be dependent on the ratio of estrogen concentrations present, the amount of algae and a range of other factors not included in this study. However, our results suggest that, it is likely that biotransformation would reach a saturation when the concentration of estradiol in aqueous solution per gram of algae is greater than 400 $\mu g\ l^{-1}\ g^{-1}$. This kinetic study may be used in estimating the probable rate of biotransformation of estrogens by algae in different environmental conditions.

The log K_{ow} values for the steroid estrogens indicate that ethinylestradiol should partition to the organic cellular material to a greater extent than other estrogens studied, assuming that simple diffusion controls the partitioning process (10,23). However, this was not the case and the apparently variable uptake of the compounds suggests that an active transport (or exclusion) or a selective binding mechanism may be operating. Since it is generally agreed that estrogens pass across membranes via a simple diffusion pathway (25), it is likely that bioconcentration of estrogens in the algae is due to an active binding mechanism such as binding to receptors or enzymes for biotransformation.

It has been suggested that accumulation of persistent organic compounds in phytoplankton is the first step of biomagnification in the food chain (12,20). However, the concentration factors of estrogens in the algae are significantly lower than reported for other endocrine disrupting substances such as DDT (3×10^4) (10). It is likely that biomagnification of estrone is of less significant than for other endocrine disrupting substances in ecosystems.

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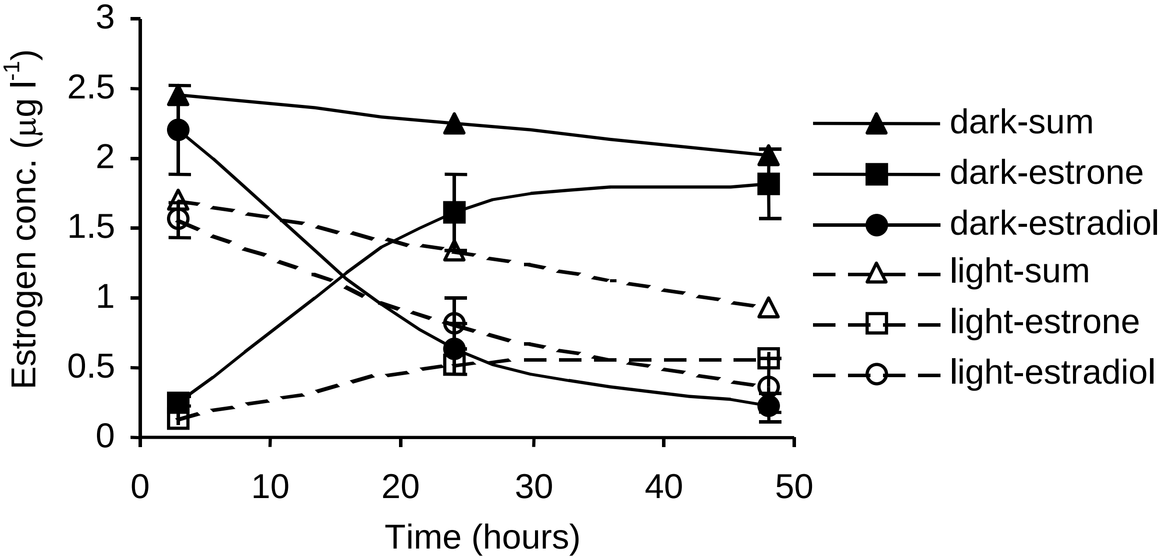

Figure 1. Changes with time (in light and dark conditions) observed after spiking the culture with estradiol

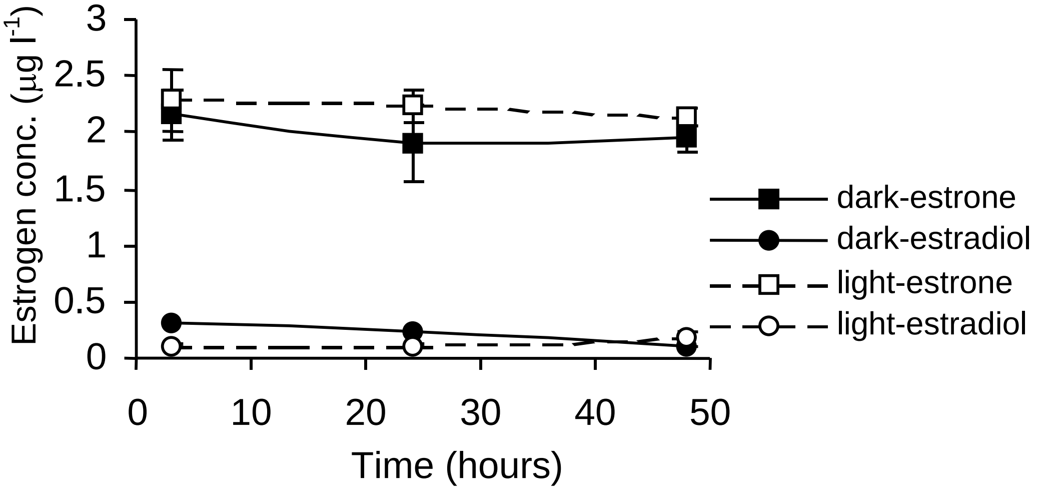

Figure 2. Changes with time (in light and dark conditions) observed after spiking the culture with estrone

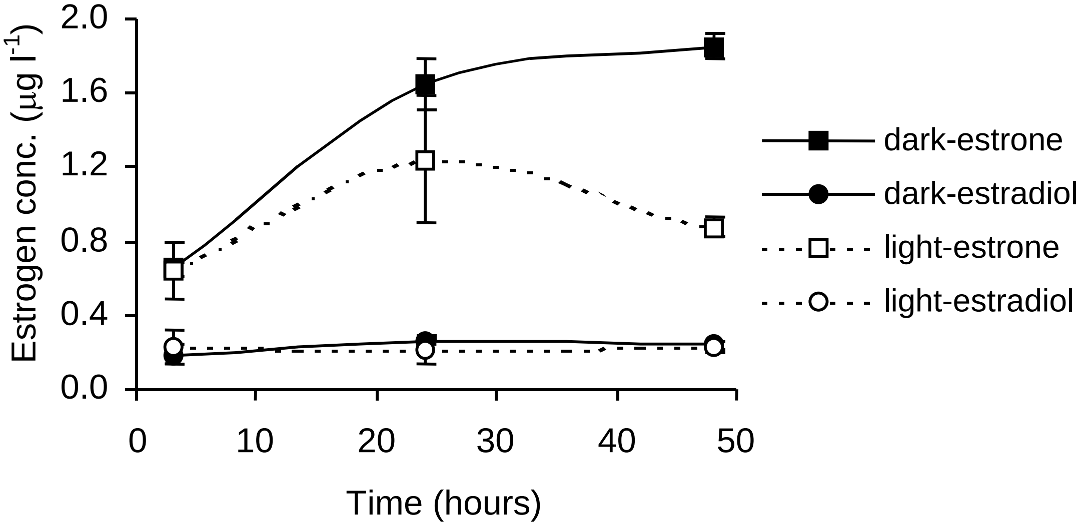

Figure 3. Changes with time (in light and dark conditions) observed after spiking the culture with estradiol valerate

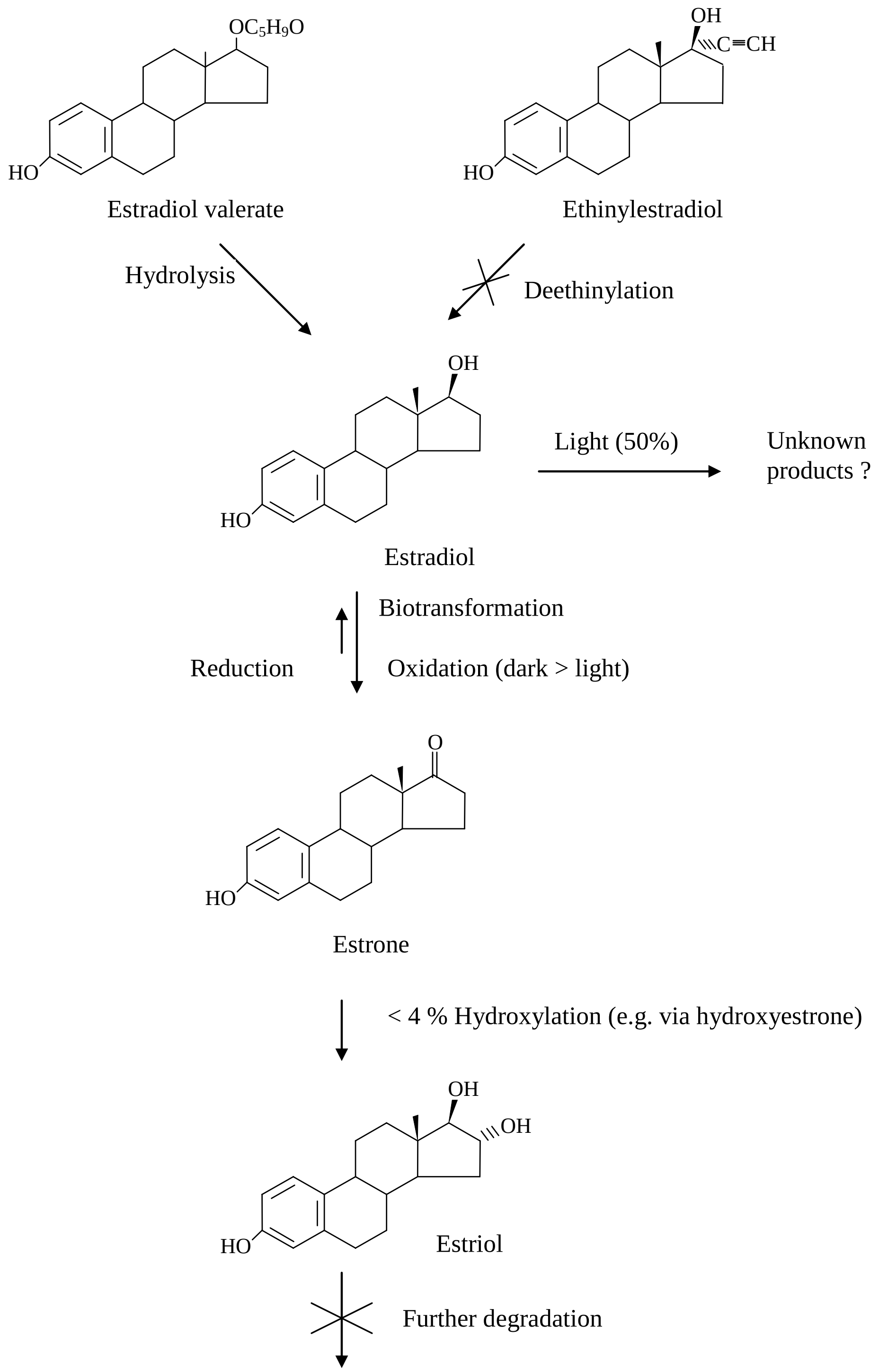

Figure 4. Proposed biotransformation pathway of steroid estrogens by *C. vulgaris*

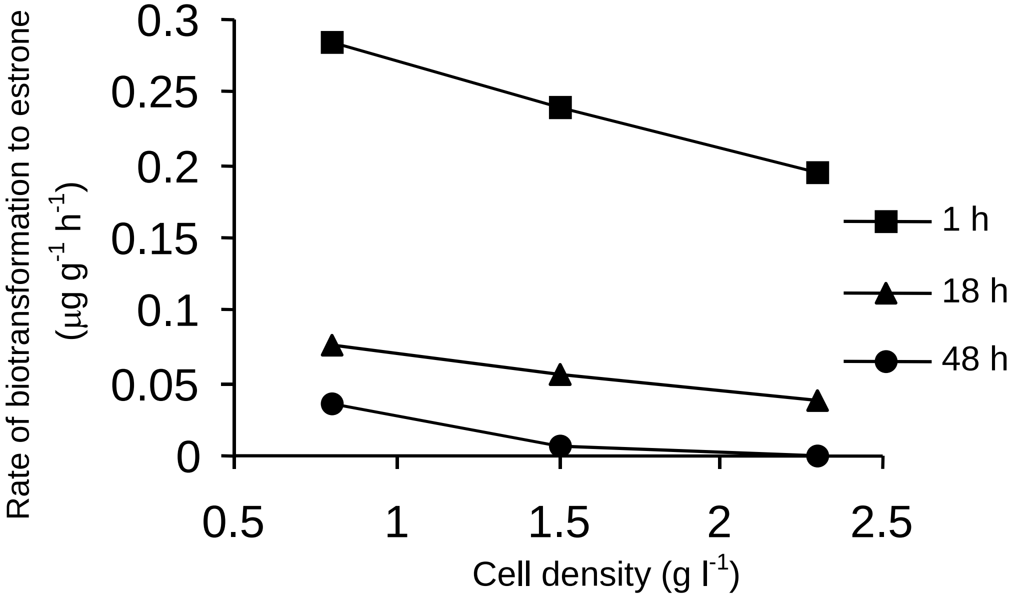

Figure 5. Effect of cell density on the formation of estrone from estradiol

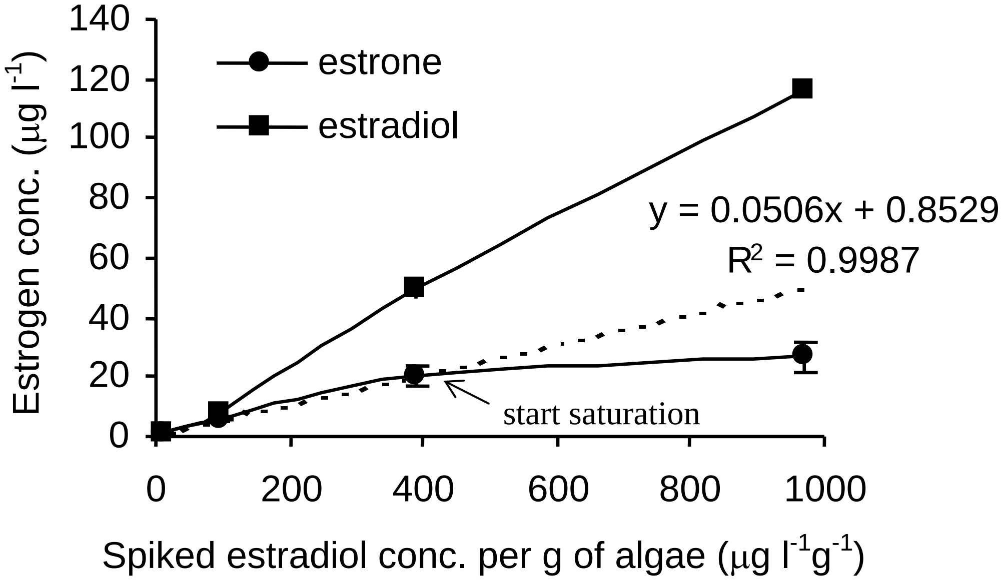

Figure 6. Effect of estradiol concentration on the biontransformation

Table 1. Mass balance and distribution of estrogens in algae and filtrate in samples spiked with estradiol

DARK Hours	Mass balance (ng)	Algae (ng g^{-1}) Estradiol	 Estrone	Filtrate (μg l^{-1}) Estradiol	 Estrone	Partitioning to algae* (%)
Initial	500	0	0	2.5	0	0
3	506	98	66	2.2	0.26	3
24	485	201	175	0.63	1.62	8
48	426	137	72	0.22	1.81	5
LIGHT						
Initial	500	0	0	2.5	0	0
3	362	206	17	1.57	0.13	6
24	298	59	269	0.82	0.52	12
48	252	30	97	0.38	0.57	9

* Sum of estrogens in algae/ in filtrates

Table 2. Mass balance, distribution and concentration factor of estrogens in algae and filtrate in samples spiked with estrone

DARK	Mass	Algae (ng g^{-1})		Filtrate (µg l^{-1})		Partitioning to	Conc. factor[#]
Hours	balance (ng)	Estradiol	Estrone	Estradiol	Estrone	algae* (%)	
Initial	500	0	0	2.5	0	0	0
3	509	103	47	0.31	2.16	3	22
24	445	83	98	0.24	1.90	4	52
48	424	109	41	0.10	1.95	4	21
LIGHT							
Initial	500	0	0	2.5	0	0	0
3	510	240	70	0.11	2.29	6	31
24	481	72	26	0.12	2.24	2	12
48	487	157	47	0.17	2.14	7	22

* Sum of estrogens in algae/ in filtrates; [#] Concentration factor of estrone in algae/ in filtrate