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Evaluating Removal of Steroid Estrogens by a Model Alga as a Possible Sustainability Benefit of Hypothetical Integrated Algae Cultivation and Wastewater Treatment Systems

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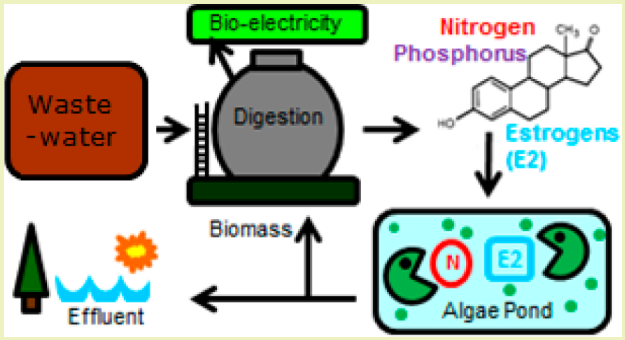
Supporting Information

ABSTRACT: The wastewater treatment field is increasing its emphasis on energy and resource recovery, while still prioritizing environmental protection. In this vein, there is growing interest in integration of algae cultivation and wastewater as a means to produce bioenergy while also removing dissolved nutrients. This paper assesses algae-mediated removal of estrogenic steroid hormones, which is an important but previously undocumented water quality benefit for municipal and livestock waste treatment facilities, because these entities discharge significant amounts of estrogens and nutrients. Bench-scale experiments were used to investigate apparent removal of four steroid hormones by a model alga, *Scenedesmus dimorphus*. Removal efficiencies were roughly 85% for 17α-estradiol and estrone and 95% for 17β-estradiol and estriol over eight days. Sorption, direct-photolysis, and algae-mediated biotransformation were evaluated as possible removal mechanisms. Removal was mainly achieved by algae-mediated biotransformation, and a partial mechanism has been proposed based on observed products. A bioassay indicates that removal of the parent estrogens does not always remove estrogenic activity, although estrogenicity associated with 17β-estradiol did decrease slightly. Overall, this study highlights a novel synergy between water and energy sustainability in integrated algae farming and wastewater treatment systems. These systems should be studied further to see if energy production could motivate voluntary removal of currently unregulated emerging contaminants.



KEYWORDS: *Environmental sustainability, Emerging contaminants, Estrogens removal, Algal bioenergy, Wastewater treatment*

INTRODUCTION

There is increasing expectation that wastewater treatment systems should deliver not only high-quality effluents but also energy and resource recovery, such that they can ultimately become net energy-producing entities without jeopardizing their capacity to remove harmful regulated and unregulated contaminants.[1,2] Integration of algaculture into wastewater treatment has been suggested as one means of creating bioenergy while also efficiently "recycling" aqueous nitrogen (N) and phosphorus (P) from treated effluents, thereby reducing downstream eutrophication.[3−6] Separately, discharge of steroid hormones and their structural analogs is a critical environmental concern because these "environmental estrogens" can impair the reproduction and development of aquatic wildlife even at very low concentrations (10−100 ng/L).[7,8] Steroid hormones are introduced into the environment primarily via human and livestock feces. The amount of estrogenicity discharged from livestock facilities is roughly 48.5 tonnes EEQ (17β-estradiol equivalents) per year in the United States, where 93% of this is from cattle installations including dairy farms.[9] Estradiol and estrone account for more than 95% of the total estrogenic potency of naturally excreted steroidal hormones by both humans and livestock; however, these chemicals (and also estriol) are present at much greater concentrations (hundreds of ug/L EEQ) in livestock manure waste compared to municipal wastewater.[9,10]

Currently, there are no relevant regulations pertaining to removal of steroid hormones and other so-called endocrine disrupting compounds. As a result, estrogen removal does not factor into the design of municipal and livestock wastewater treatment systems.[11] For example, a typical treatment strategy for dairy manure comprises use of covered or uncovered lagoons, in which estrogen removal may be as low as 15−23% over several months and the concentration of total estrogens in

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dairy lagoon wastewater varies from a few nanograms/liter to hundreds of micrograms/liter. The resulting material is often directly applied on land.[6,9,12] As a result, groundwater and runoff collected from research plots receiving dairy manure effluents may contain estrogens at concentrations ranging from 2.1−41 ng/L.[9,13] Similarly, conventional wastewater treatment plants (WWTPs) are not designed for these emerging contaminants, and the effluent of WWTPs contains estrogens at a level that may cause endocrine disruption (ng/L−ug/L).[9,11]

There have been a small number of previous studies reporting estrogen and/or other steroid removal by some microalgae species;[14−19] however, previous experimental and life cycle assessment (LCA)-based studies of integrated alga culture-based wastewater treatment systems have not accounted for this sustainability benefit.[2−6,20−24] Plugmacher et al.[14] reported that genera from several classes of marine macroalgae (*Chlorophyta*, *Phaeophyta*, and *Rhodophyta*) collected from the Arctic and Antarctic mediate transformation and conjugation of weakly estrogenic biphenyls, noting that these reactions proceed via pathways similar to those used by mammalian livers for detoxification of steroid hormones. Regarding water treatment applications, Lai et al.[15] observed photolysis, biosorption, and biotransformation of estradiol, estriol, and estrone in batch cultures of *Chlorella vulgaris*. Under light conditions, 50% of the initial estradiol was metabolized into unknown products within 48 h. Ge et al.[16] observed greatly accelerated photolysis of estradiol and ethynylestradiol in the presence of *Chlorella*, *Anabaena*, or *Microcystis*, attributing this to an increase in hydroxyl radical concentration arising from secretion of algal organic materials (AOM). Shi et al.[17] noted fair removal of estradiol (40% in 180 h) and ethynylestradiol (20% in 180 h) in a mixed algae culture, noting rapid sorption followed by biotransformation. Finally, Gatullo et al.[18] and Hirooka et al.[19] observed good removal of the estrogen analog bisphenol A (BPA), a key monomer most commonly used in production of polycarbonate plastic, by *Monoraphidium braunii* (40% over 4 d) and *Chlorella fusca* (80−90% over 7 d), respectively. Hirooka[19] also reported "complete disappearance" of BPA's estrogenic toxicity, as measured using a yeast two hybrid assay.

Although the previous research suggests that algae-mediated photolysis, biosorption, and biotransformation could contribute to good removal of estrogenic steroid hormones in integrated algaculture-based wastewater treatment systems, there is need for additional mechanistic information related to commercially relevant algae strains. Additionally, none of the previous studies have tested algae-mediated removal of 17α-estradiol, even though this is a significant contribution to estrogenicity in dairy cattle manure.[9]

This study investigates algae-mediated estrogen removal in hypothetical algae-to-energy systems with three key objectives: (1) measure algae-mediated removal of steroid estrogens, and assess the contributions of three hypothesized removal mechanisms, for example, sorption, photodegradation, and/or biotransformation, (2) elucidate a pathway for algae-mediated estrogen transformation, and (3) assess changes in associated estrogenic activity during algae-mediated estrogen removal. *Scenedesmus dimorphus* was selected as the model alga for this study because it is abundant in temperate freshwater ecosystems.[25] Additionally, several *Scenedesmus* species have been included in various (LCA)-based evaluations of algae-to-energy systems[26,27] but have not been included in previous examinations of algae-mediated steroid hormone removal.[14−19] The resulting information is valuable for understanding the full benefits of integrated algae systems and, more broadly, for improving our understanding of how energy and water sustainability priorities related to net-energy production during wastewater treatment can complement one another.

MATERIALS AND METHODS

Chemicals and Medium. 17α-Estradiol, 17β-estradiol, estrone, and estriol were chosen for this study in order to represent the steroid estrogens typically found in municipal and livestock wastewaters. Analytical standards for these four estrogens were from Sigma-Aldrich Chemicals (St. Louis, MO). All other reagents were from Fisher Scientific (Waltham, MA). Individual stock solutions were prepared in HPLC-grade methanol at 100 mg/L and stored at −20 °C for up to one month.

Protease−peptone medium (PPM) and modified Bold 3N medium (MB3N) were used for aseptic algae cultivation of *S. dimorphus*. These media were selected, despite key differences between their makeups and the composition of municipal wastewater effluents, based on the recommendation of the UTEX Culture Collection (Austin, TX) who provided the original algae cultures. A three-step cultivation process was used. First, algae were inoculated into 15 mL capped tubes containing 10 mL of PPM medium. These were incubated for 4 d in a rotator subjected to 12 h of illumination (cool white fluorescent with light flux of 100 μE/m^2 sec) and 12 h of darkness per day. Cultures were then transferred to 250 mL flasks containing 50 mL of PPM medium and incubated for another 4 d in a shaker subjected to the same light−dark sequencing until they achieved stationary growth phase based on measurement of optical density at 662 nm (corresponding to chlorophyll *a*) to quantify algae concentrations over time (section 1.3, Supporting Information). These preliminary cultures were then diluted to a concentration of 40 mg/L, and 500 mL aliquots of the diluted algae culture were transferred to 1000 mL flasks. The resulting cultures were used for subsequent experiments. Under the same light−dark sequencing as in previous steps, the flasks were stirred by magnet (250 rpm) and continuously aerated using filtered air at 7 scfm (standard cubic feet per minute). Detailed algae cultivation information is summarized in section 1 of the Supporting Information.

Experimental Reactions. Two wastewater types have been most frequently proposed for algae cultivation in previous studies: (1) secondary effluent of WWTPs, whereby algae cultivation serves as a tertiary treatment to remove N and P, and (2) fresh or anaerobically digested manure waste from lagoons at livestock facilities.[2,4−6,20−24] Concentrations of estrogens in these wastewaters typically vary from a few nanograms/liter to several micrograms/liter, although fresh manure waste can occasionally contain concentrations up to hundreds of micrograms/liter.[7,28] Therefore, an initial estrogen concentration of 5 ug/L was used in this study. A preliminary experiment was also performed to see if high estrogen concentrations impact algae growth. 17β-Estradiol was spiked into 500 mL samples of algae culture obtained from the third stage of algae cultivation at 100 ug/L, which was used to represent a likely upper limit for estrogen concentrations in fresh manure waste. In all experiments, algae were cultivated over 8 days.[20,23]

Subsequent experiments were performed to measure the removal efficiencies for four selected estrogens and also characterize the contributions of hypothesized removal reactions. For each of these experiments, four types of reactors were used in duplicate: (1) algae (A) reactors containing 500 mL of algae culture from the third stage of algae cultivation (see Chemicals and Medium section) spiked with 5 ug/L of one estrogen, (2) dark control (DC) reactors comprising 500 mL of MB3N medium spiked with 5 ug/L of one estrogen without algae and wrapped in foil to prevent light penetration, (3) light control (LC) reactors containing 500 mL of MB3N medium spiked with 5 ug/L of one estrogen without algae but exposed to the same light conditions, and (4) autoclaved algae sorption control (AASC) reactors, which contained 500 mL of autoclaved algae culture spiked

with 5 ug/L of one estrogen and wrapped in foil to prevent light penetration. A batch of algae in culture medium without any estrogen was used as negative control for subsequent analyses.

Fifty milliliter samples were collected from LC, DC, and A reactors at 0, 6, 24, 48, 96, 144, and 192 h. AASC reactors were sampled at 0 and 192 h. A and AASC samples were filtered through 0.7 μm pore size glass microfiber filters to remove algae cells. Estrogens in the medium were extracted and concentrated using solid phase extraction (SPE) on Oasis HLB SPE 3 cm^3 (160 mg) cartridges (section 2, Supporting Information). Post-extraction SPE cartridges were stored at −20 °C until analysis. Before analysis, SPE cartridges were eluted with 2 mL of HPLC-grade methanol, evaporated to dryness, and then reconstituted in 400 uL of methanol.

Analytical Methods. *High-Performance Liquid Chromatography (HPLC).* HPLC was used to measure the concentrations of estrogens in extracted samples. The instrument was a Shimadzu 2010-AB HPLC with UV and fluorescence detectors. 17α-Estradiol, 17β-estradiol, and estriol were measured on the fluorescence detector with excitation at 280 nm and emission at 310 nm. Estrone was quantified using the UV detector at 280 nm. Chromatographic separation was achieved using a 250 mm × 2.1 mm C18 column (Thermo Scientific). Mobile phase was a mixture of HPLC-grade water (A) and acetonitrile (B) pumped at 0.5 mL/min per the following gradient program: 0−8 min, 20% B; 8−18 min, 20% → 35% of B; 18−30 min, 35% → 50% of B; 30−32 min, 50% → 20% of B; and 32−35 min, 20% of B. Injection volume was 20 μL. Retention times were 11.9 min for estriol, 23.4 min for 17β-estradiol, 24.5 min for 17α-estradiol, and 26.2 min for estrone. Additional HPLC details are summarized in section 3 of the Supporting Information.

Estrogenicity Assay. The E-SCREEN test was used to measure estrogenicity of A and LC samples at selected times, according to a modified protocol described by Mao et al.[29] The SPE eluate was diluted with Dulbecco's Modification of Eagle's Medium (DMEM), so that the final methanol concentration was 0.01% to minimize its impact on the MCF-7 cells.[30] The test was carried out with 100 uL of each diluted sample with three analytical replicates in a 96 well plate containing MCF-7 cells and 900 ul of DMEM. The relative estrogenic effect (REE) for each sample was calculated as described in Olsen et al.[31]

Statistical Analysis. All statistical analyses were performed using Microsoft Excel 2010. One-way ANOVA was used to analyze differences among estrogen concentrations at each sampling time in A, DC, LC, and AASC reactors. Two-way ANOVA was used to assess differences in estrogen removal for DC versus LC, AASC, and A reactors and to evaluate differences among direct photolysis, sorption, and algae-mediated biotransformation reactions. A two-tailed student's t test was used to compare the estrogenicity at 0 and 192 h; p-values < 0.01 were considered significant.

RESULTS AND DISCUSSION

Evaluating the Impacts of Estrogens on Algae Growth. It has been previously documented that total estrogenicity, as measured using combined estrogen concentrations, can be as high as 100 ug/L in manure waste.[7,32] Therefore, an important first step was to evaluate whether algae growth is affected by estrogens at such high concentrations. Our result indicate that there is no statistically significant difference (Student's t test, $p = 0.24$) in algae growth over 8 d for reactors with and without 17β-estradiol. Final biomass concentrations were 820 ± 150 mg/L for 0 ug/L 17β-estradiol and 770 ± 130 mg/L for 100 ug/L (ranges denote standard deviations for $n = 2$). This is consistent with other reports that even relatively high estrogen concentrations do not significantly affect algae growth.[33,34]

Assessing Algae-Mediated Estrogen Removal. Having demonstrated that typical estrogen concentrations should have no significant impact on algae growth, it was desirable to assess algae-mediated estrogen removal. Figure 1 depicts estrogens

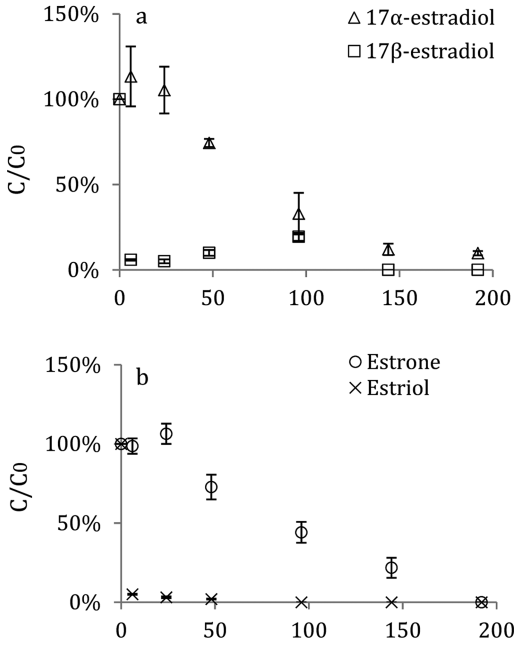

Figure 1. Removal efficiency of four selected estrogens mediated by algae culture *Scenedesmus dimorphus*. X axis presents incubation time (h); Y axis presents estrogen removal rate (C/C_0, %). C is the concentration of individual estrogen at the sampling time, and C_0 is the initial concentration of individual estrogen spiked into the algae culture at 5 ug/L. Error bars are standard deviations for two replicates.

concentrations over time for algae reactors spiked with 5 ug/L of one individual steroid hormone. 17β-Estradiol (Figure 1a) and estriol (Figure 1b) are removed quickly, up to 90% within the first 24 h. In contrast, 17α-estradiol (Figure 1a) and estrone (Figure 1b) require 6−8 d to achieve this same removal. The data in Figure 1 were fit to the pseudo-first-order kinetic model for all estrogens except estriol (section 4, Supporting Information).

17β-Estradiol and estriol are the most rapidly removed estrogens during reactions mediated by *S. dimorphus,* followed by 17α-estradiol and then estrone (Table S6, Supporting Information). This ranking is qualitatively consistent with previous studies evaluating estrogen removal from anaerobically digested dairy lagoon water and conventional WWTPs;[9,12] however, the 17β-estradiol and estrone removal rates measured in this study are two and ten times faster, respectively, than in an anaerobic dairy lagoon.[12] The rate of 17α-estradiol removal by *S. dimorphus* is similar to what occurs in an anaerobic diary lagoon, but it cannot be compared to a municipal WWTPs because this compound is only found in dairy wastes. In contrast, the 17β-estradiol and estrone removal observed in this study can be compared with WWTP removal rates. Both compounds are widely present in municipal wastewaters, and both are removed to varying levels via biodegradation during activated sludge treatment. 17β-Estradiol removal under typical conditions is 85−99%, whereas estrone removal is only 25−80%.[9,35] Increased solids retention times (SRTs), of at least 10−13 d, are associated with more effective removal of both estrogens.[35]

The results of this study indicate that algae-mediated removal of 17β-estradiol and estrone (90% in 1 and 8 d, respectively) is quite effective and algae-mediated polishing could be a potential strategy for removing these emerging contaminants in a WWTP; although it should be noted that the algae

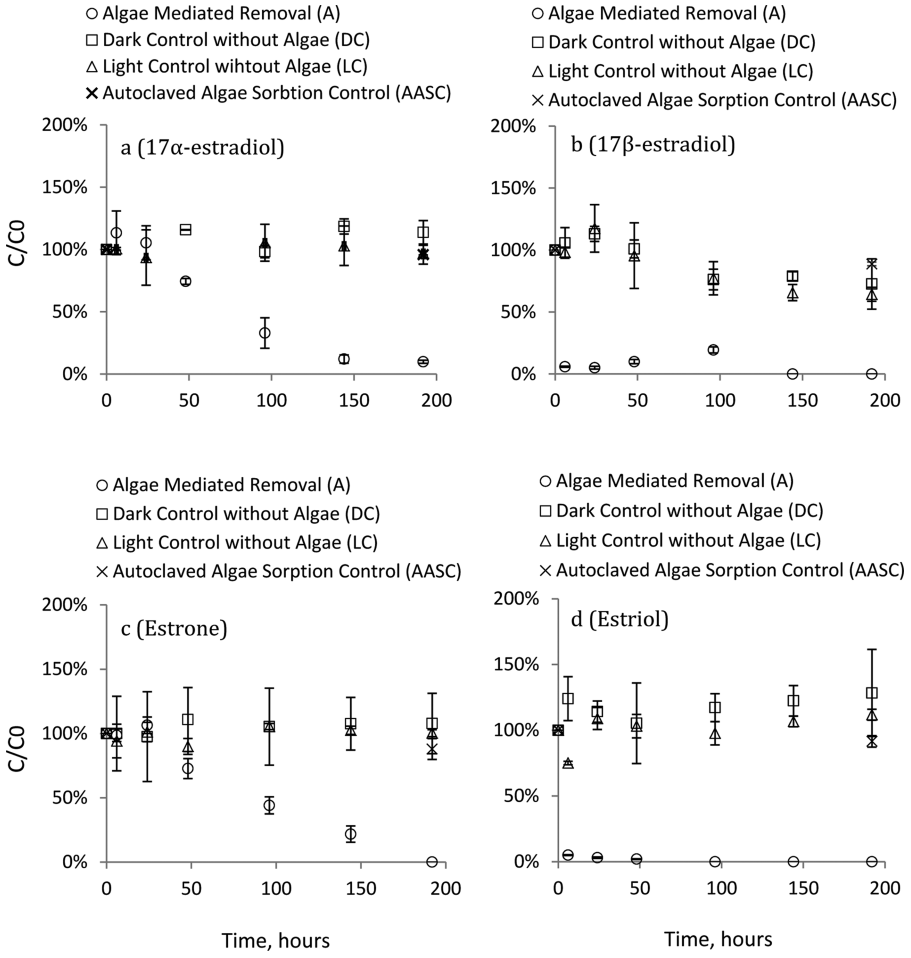

Figure 2. Fates of four tested estrogens under various experimental conditions. *X* axis presents incubation time (h); *Y* axis presents estrogen removal rate (C/C_0, %). *C* is the concentration of individual estrogen at the sampling time, and C_0 is the initial concentration of individual estrogen spiked into the algae culture at 5 ug/L. Error bars are standard deviations for two replicates.

cultivation conditions in this study were highly controlled at bench-scale (e.g., pure algal culture grown in autoclaved standard medium) and therefore quite different from what would be expected in real wastewater treatment systems. As such, future work will be needed to evaluate the removal of these chemicals in real integrated algae cultivation and wastewater treatment systems.

Elucidating the Mechanisms of Algae-Mediated Estrogen Removal. Another objective of this research was to apportion estrogen removal among three mechanisms that had been referenced in previous studies: biotransformation, biosorption, and photolysis. Different removal mechanisms give rise to different intermediates and byproducts, which could affect the suitability of the harvested algae biomass for downstream processing into useful energy carriers (e.g., via anaerobic digestion, transesterification, etc.). Figure 2 summarizes concentrations of each estrogen over time in dark control (DC) reactors, light control reactors (LC), autoclaved algae sorption controls (AASC), and live algae cultures (A). Comparison among these four sets of reactors enables evaluation of the contribution of each removal mechanism.

Estrogen Removal by Sorption. The AASC controls are useful for assessing the contribution of sorption to overall apparent removal of each estrogen. From Figure 2, there is no significant difference between estrogen concentrations in the DC versus the foil-wrapped AASC reactors (two-way ANOVA, $p > 0.01$). Beyond this, the total change in concentration for each estrogen was no more than ∼10% after 8 d. This suggests that sorption constitutes a minor contribution to overall apparent removal. Other studies have reported similar findings, whereby sorption comprises comparably small (4−9%) contributions of overall algae-mediated estrogen removal.[15,17] It should be noted that these previous studies used live algae biomass for their sorption controls. This suggests that our use of autoclaved algae did not interfere with our ability to assess sorption-based estrogen removal.

Estrogen Removal by Direct Photolysis. In all panels of Figure 2, there is no statistically significant (one-way ANOVA, $p > 0.01$) change in estrogen concentration for any of the DC reactors. This confirms that there is no significant estrogen removal occurring by any pathway other than the three hypothesized mechanisms of interest. Comparison of DC and LC concentrations (specifically DC minus LC) reveals that the differences between these data are quite small and not significant (one-way ANOVA, $p > 0.01$). This indicates that direct photolysis does not constitute an appreciable contribution to overall estrogen removal. This is consistent with previous studies in which removal of free estrogens is achieved mainly through biotic routes.[9,36,37] In particular, Jürgens et al.[37] reported that the half-life of estradiol, assuming first-order decay via biotic routes, could be as low as 0.2 days; whereas, the

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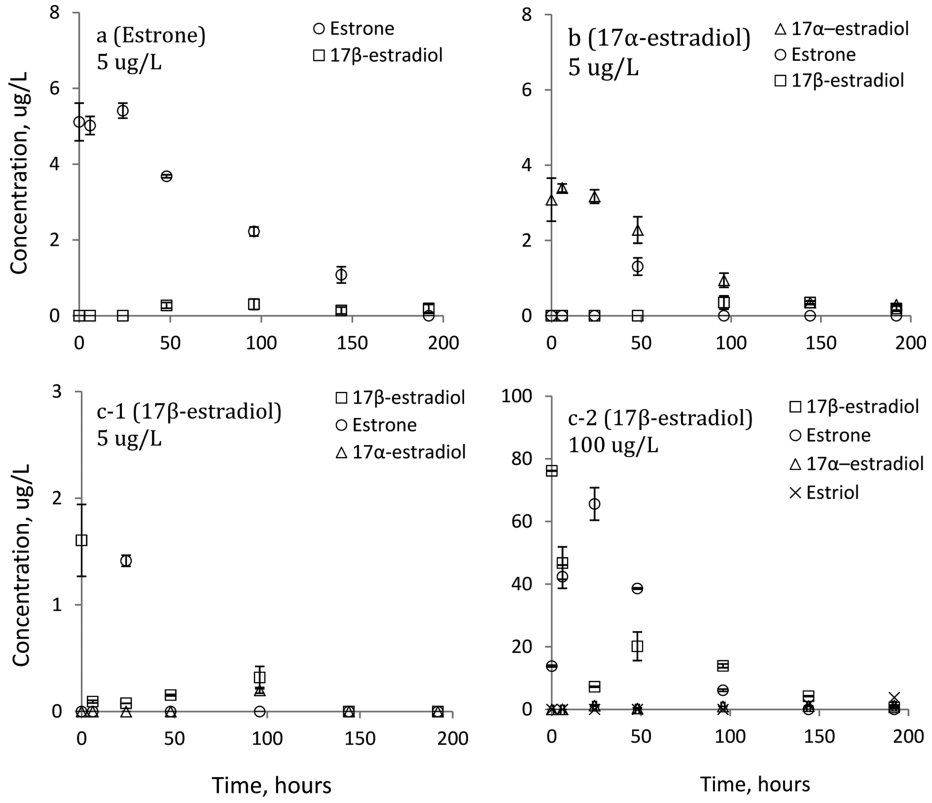

Figure 3. Biotransformation of four estrogens and formation of their transformed products mediated by algae *Scenedesmus dimorphus*. (a) Estrone with initial concentration of 5 ug/L. (b) 17α-Estradiol with initial concentration of 5 ug/L. (c-1) 17β-Estradiol with initial concentration of 5 ug/L. (c-2) 17β-Estradiol with initial concentration of 100 ug/L. Error bars are standard deviations for two replicates.

half-life of direct photolysis under ideal conditions was at least 10 days.

It should be emphasized that our experimental setup only accounts for direct photolysis, which occurs when the compound itself absorbs light energy and, as a result, is transformed into another product. The use of LC reactors containing only growth medium and estrogen captures this type of removal. In contrast, indirect photolysis occurs when another constituent in the water (e.g., natural organic matter, NOM) absorbs light energy and initiates a reaction that creates a radical or other reactive intermediate. This intermediate then mediates transformation of the target chemical (e.g., estrogen). The reaction setup in our study does not capture indirect photolysis, which may occur in the presence of live algae, especially if the algae are excreting appreciable quantities of AOM as suggested by Ge et al.[16] This should be assessed more mechanistically in future studies.

Estrogen Degradation by Algae-Mediated Biotransformation. The last remaining removal mechanism is biotransformation, which can be assessed by comparing the concentrations in A, LC, and AASC reactors (Figure 2). This comparison reveals that algae-mediated biotransformation offers significant estrogen removal compared to direct photolysis and sorption controls (two-way ANOVA, $p < 0.01$). The concentrations of all four estrogens in the A reactors decreased by more than 90% over 8 d. At the end of this period, only 10% of the initial 17α-estradiol remained, and the residual concentrations of the other estrogens were below their detection limits. This suggests that live algae cells are very effective at removing estrogens, which is somewhat consistent with limited data from previous studies. Lai et al.[15] observed that the half-life of 17β-estradiol decreased from 48 to 3 h in the presence of live *Chlorella*, similar to the rapid transformation depicted in Figure 2B; however, they also reported a much slower rate of initial degradation for estrone compared to what we observed. Shi et al.[17] observed slower removal rates for 17β-estradiol and estrone in an algae-based system, roughly 70% for 17β-estradiol and 40% for estrone, over 48 h. These differences could be attributed to different algae species, light conditions, or cultivation times. Regardless, there is good evidence to suggest that algae can mediate efficient removal of steroid hormones.

Elucidating the Pathway of Estrogen Biotransformation by *S. dimorphus*. Having demonstrated that algae-mediated biotransformation significantly contributed to the removal of steroid hormones by the model algae species, it was desirable to also determine the pathway by which transformation(s) occurs for each of the selected estrogens. These results are depicted in Figure 3. The measured initial concentrations of each parent estrogen in these experiments are somewhat different from the intended spiked concentration (5 ug/L): 5.1 ug/L for estrone, 3.4 ug/L for 17α-estradiol and 1.7 ug/L for 17β-estradiol. This likely happens for two reasons: (1) Different estrogens have different SPE recovery efficiencies (estrone >17α-estradiol >17β-estradiol, data not shown). (2) Estrogens with very short half-lives could be degraded during the extraction process (e.g., 17β-estradiol). Despite these differences in initial measured concentrations, the experimental results are useful for evaluating the efficiency and pathway of removal for each estrogen.

Biotransformation of Estrone. Figure 3a shows estrogen concentrations over time arising from biotransformation of estrone. From these measurements, estrone concentration

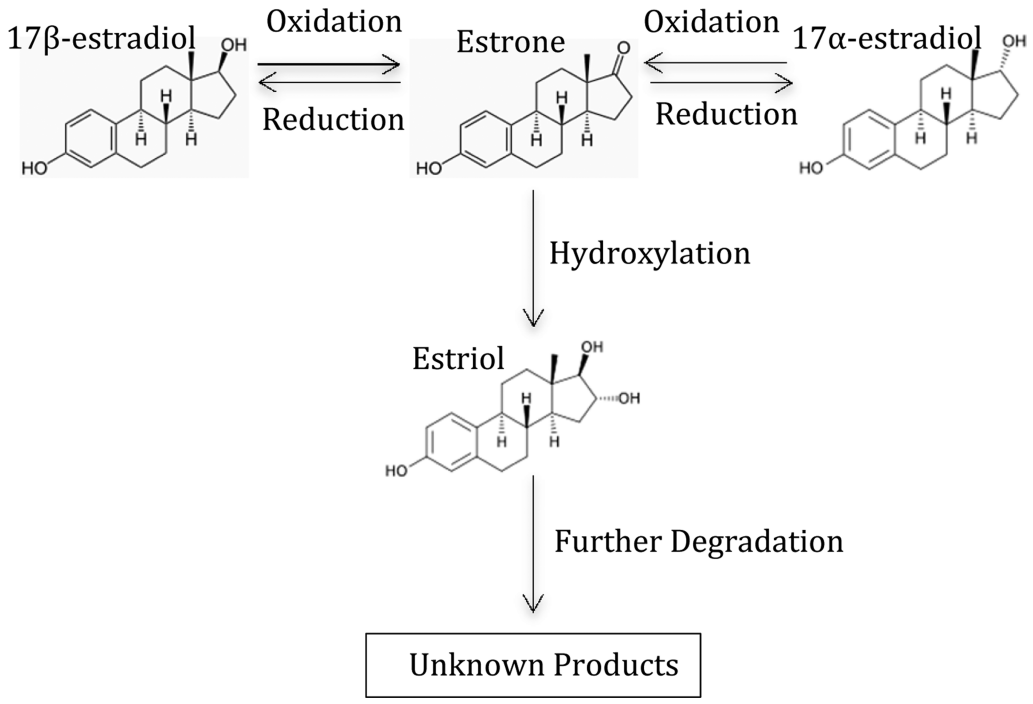

Figure 4. Possible biotransformation pathways of 17β-estradiol, 17α-estradiol, estrone, and estriol mediated by the algae *Scenedesmus dimorphus*.

decreases as 17β-estradiol concentration increases starting at around 24−50 h. This suggests that estrone may be biotransformed into 17β-estradiol via a reduction reaction, whereby two hydrogen atoms are added to each estrone molecule to produce one molecule of 17β-estradiol. Estriol and 17α-estradiol were also observed in the reaction mixture toward the end of the incubation period (~192 h), but their concentrations were below the quantification level. Their low levels may reflect the low initial concentration of the parent compound, or it may indicate that these compounds are short-lived intermediate metabolites in this reaction mixture.

Biotransformation of 17α-Estradiol. Figure 3b shows estrogen concentrations over time arising from biotransformation of 17α-estradiol. From this data, 17α-estradiol concentration decreases as estrone concentration increases starting at around 24−50 h. The amount of 17α-estradiol removed from solution (0.0029 uM) is roughly equal to the amount of estrone produced (0.0031 uM). This suggests that estrone is a biotransformation product of 17α-estradiol in the presence of active *S. dimorphus*. This conversion comprises an oxidation reaction, whereby two hydrogen atoms are removed from one molecule of 17α-estradiol to produce one molecule of estrone.

Later in this experiment reaction (96−192 h), the estrone concentration begins to decrease. At around the same time, a small amount of 17β-estradiol (0.2- 0.4 ug/L) begins to appear. This is further indication that estrone is converted to 17β-estradiol by *S. dimorphus* (Figure 3a). It is interesting that this conversion occurs roughly 24 h after the estrone is introduced into each reactor. Finally, a small quantity of estriol was observed toward the end of the incubation, but its concentration could not be quantified.

Biotransformation of 17β-Estradiol. Figure 3c-1 presents estrogen concentrations over time arising from biotransformation of 17β-estradiol. Estrone appears at around 24 h as 17β-estradiol is initially removed. 17α-Estradiol appears at around 96 h, following a significant decrease in estrone concentration during the period of 24−96 h. Estriol was also observed toward the end of the reaction (192 h), but it could not be quantified. To overcome analytical difficulties associated with quantification of all intermediates and products, the 17β-estradiol degradation reaction was repeated at an initial concentration of 100 ug/L. These results are depicted in Figure 3c-2.

At higher initial concentration, it is apparent that all four target estrogens arise in turn from biotransformation of 17β-estradiol. As in Figure 3c-1, the concentration of 17β-estradiol rapidly declines within 24 h, while the concentration of estrone rapidly increases. From Figure 3c-2, more than 80% of the 17β-estradiol is converted into estrone. This is much more significant than the 6% of estrone that is converted into estradiol for the reverse reaction (Figure 3a). This is also more significant than the transformation of 17α-estradiol into estrone (42% from Figure 3b), which might partly explain 17α-estradiol's longer half-life compared to 17β-estradiol in initial experiments. After 24 h, the estrone concentration decreases rapidly to a level below the quantification limit by the end of the experiment. As the estrone decreases, small quantities of estriol and 17α-estradiol appear. There is a substantial difference between the amount of 17β-estradiol removed (~0.27 uM) and the combined amounts of estrone, estriol, and 17α-estradiol observed (0.02 uM) at the end of cultivation. One explanation for this discrepancy may be that 17β-estradiol is converted primarily through estrone into estriol, which is then rapidly converted to unknown metabolites, consistent with Figure 2. Another possible explanation could be that estradiol was transformed into intermediates that cannot be identified using the analytical techniques of this study. For example, conjugated species are a known detoxification product of steroid hormones in algae and plant species.[14,15]

Biotransformation of Estriol. From Figure 2d, there was rapid and nearly complete removal of estriol within 24 h. Samples were analyzed for all four estrogens of interest, but there was no formation of either estradiol isomer or estrone

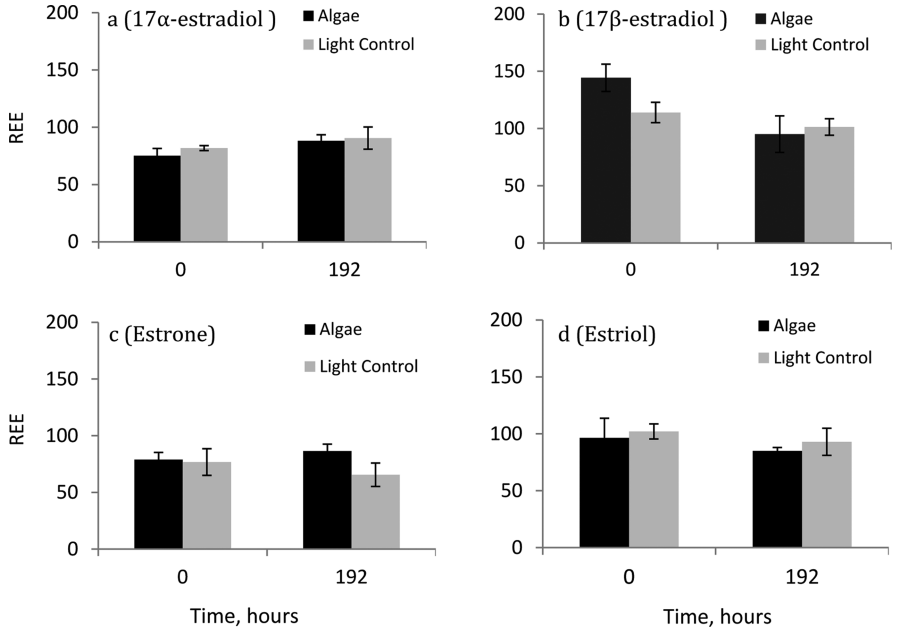

Figure 5. Measurements of total estrogenic activity at the beginning and end time over 192 h (8 days), during reaction with light control and algae *Scenedesmus dimorphus*. Starting concentration of each estrogen was 5 ug/L. *X* axis is the incubation time (hours). *Y* axis is relative estrogenic effect (REE), which is the estrogenic effect of the sample of interest as a percent of the estrogenic effect of 10^{-9} M 17β-estradiol solution (REE of 10^{-9} M 17β-estradiol solution is 100). Error bars are standard deviations for six replicates (duplicates at each sampling time × three replicate wells per sample during E-SCREEN = 6).

over 8 d. This suggests that estriol was converted to some product(s) very rapidly, in marked contrast to previous research in which there was no conclusive evidence for biotransformation (or even removal) of estriol by *Chlorella vulgaris* within 48 h.[15]

Summary of Estrogen Biotransformation Reactions. The results in Figure 3 demonstrate that the four target estrogens are interconverted among one another during reactions mediated by *S. dimorphus*. Figure 4 presents a possible pathway based on these observations, whereby both 17β-estradiol and 17α-estradiol are first biotransformed into estrone, which is then either transformed into estriol or converted back to 17β-estradiol or 17α-estradiol. Thus, all of the estrogens with two oxygen molecules (17β-estradiol, 17α-estradiol, and estrone) are interconvertible during interactions with *S. dimorphus*, and the sole three-oxygen form (estrone) is an important intermediate for further biotransformation and degradation. The reactions corresponding to the conversions among these four estrogens (e.g., hydroxylation, reduction, and oxidation) have been previously reported in published studies of algae-mediated estrogen biotransformation.[14,15] Lai et al.[15] also reported 17β-estradiol and estrone to be interconvertible during reactions mediated by *Chlorella*, and similar to Figure 4, that estrone is then converted into hydroxyestrone and estriol. They did not observe subsequent removal of the estriol; however, this could be because their reaction time was only 48 h. In the present study, estriol is further degraded to unknown product(s) and is likely not converted back into estrone or estradiol. Unfortunately, these products(s) could not be identified by the techniques used in this study, and no previously published studies have put forth a possible biotransformation pathway for estriol removal by algae.

Overall, nearly 95% of the total estrogens were removed during individual reactions with 17β-estradiol, estrone, and estriol over 8 d, and roughly 85% of total estrogens were removed during individual reaction with 17α-estradiol. These are surprisingly high removal efficiencies, given that many removal reactions are initiated by conversion of one estrogen into another. Comparable removals of 17α-estradiol, 17β-estradiol, and estrone in other treatment systems are much lower or on the same magnitude: 15−23% after 52 d in an anaerobic lagoon[12] and 85%−99% (for 17β-estradiol), 25%−80% (for estrone), or 0%−89% (for estriol) in conventional WWTPs.[9] The results of this study indicate that algae-mediated treatment is very competitive for estrogen removal compared to these conventional engineered systems. In addition, there has been no previously published study of algae-mediated 17α-estradiol biotransformation. Results from the present study indicate that this estrogen could share the same transformation pathway as 17β-estradiol under algal culture conditions but at a much longer half-life. Considering the large amount of 17α-estradiol present in dairy manure, this information is important for understanding the benefits of algae-mediated livestock manure waste treatment.

Evaluating Estrogenic Activity Removal Mediated by *S. dimorphus*. Because many of the estrogen removal reactions observed in this study and previous studies comprise interconversions, tracking of the parent compound concentrations is not adequate to assess whether algae-mediated reactions also reduce estrogenic activity. For this reason, we used a standard *in vitro* assay (E-SCREEN) to measure estrogenic activity over time during algae-mediated reactions. This assay uses units of "relative estrogenic effect (REE)", which describe the estrogenic effect of a water sample of interest relative to 10^{-9} M 17β-estradiol solution.[31] Figure 5 summarizes results from the E-SCREEN test for the different treatments.

From Figure 5, there are some slight differences in initial REE between the light control (LC) samples and corresponding algae treatments for the four selected estrogens, but these

differences are not statistically significant based on two-tailed student's t test. Likewise, there is no statistically significant difference between starting and ending REE for any of the LC samples. This observation is consistent with the observation of little to no change in estrogen concentration for the LC samples in Figure 2, and it rules out changes in estrogenicity due to pathways other than algae-mediated processes. For algae culture reactors (A), only the 17β-estradiol experiment (Figure 5B) exhibits a statistically significant difference in estrogenicity (student's t test, $p = 0.002$). Estrogenic activity is reduced by roughly 30%, mostly during the first 24 h (data not shown). This drop in estrogenicity is consistent with the hypothesized conversion of 17β-estradiol into estrone and estriol, which are only 10−20% as estrogenic as 17β-estradiol.[38,39] The other three evaluated estrogens show little change of REE over 8 d. This is a significant mismatch when compared to removal of the individual parent compounds (Figure 2). One possible explanation could be that the degradation products are as strongly or more strongly estrogenic than the parent estrogen, such that reduction in estrogenicity is not as significant as reduction in concentration of the initial estrogen. Taking estrone as an example, conversion of estrone into estriol followed by transformation could result in decreasing of estrogenic toxicity, but transformation back to either 17β-estradiol or 17α-estradiol even at a very low level could increase the estrogenicity back to its initial level because these two estrogens are much more potent.

There have been very few studies assessing algae-mediated estrogenicity removal, especially in comparison to the large volume of work on estrogenicity removal in conventional WWTPs. The total estrogenicity reduction in municipal WWTPs varies dramatically, from nearly no change to 99% removal, depending on operational variables such as solids retention time.[40−43] This removal occurs primarily via bacteria-mediated transformation reactions during activated sludge treatment, and these reactions show some interesting similarity to the algae-mediated estrogen removal pathway presented in this study, namely, initial removal of 17β-estradiol via conversion into estrone.[9,10] Anaerobic treatment is another biological system that is frequently used for wastewater treatment, especially for livestock applications, but it is much less effective for removal of estrogens and estrogenicity. Zhao et al.[44] reported that there was no or little REE reduction during anaerobic treatment. Other studies have even reported increase in total estrogenic activity (REE) during anaerobic sludge digestion.[45] Due to dramatic variability in estrogenicity change during WWTPs and anaerobic treatment facilities, it is hard to compare the efficacy of algae-mediated estrogenicity removal to that of conventional treatments. However, the results from the current study give the first insight of estrogenicity change in algae cultivation. They also emphasize the need for further research, especially for investigating algae-mediated removal of estrogens from real wastewaters sources and assessing possible ecological or human health impacts of the biotransformation products. This information is critical for developing a better understanding of the environmental services and the overall sustainability afforded by the proposed integrated algae-to-energy systems. Under the best case scenario, integration of algaculture into existing WWTPs with anaerobic digestion could yield a much less energy-intensive (or even net energy-producing) means of removing currently unregulated estrogen compounds from secondary effluents.

Future work should focus on evaluating removal of the steroid hormones in actual wastewater effluents using more realistic assemblages of WWTP-grown algae strains, more detailed characterization of the contributions of each removal mechanism of interest, and further evaluation of the degradation pathway to identify currently unknown intermediates and products. Future work with real wastewaters should especially focus on understanding the impact of other constituents (e.g., chemical oxygen demand, inorganic materials, other emerging contaminants, etc.) on the observed algae-mediated estrogen reactions and evaluating the possible formation of algal toxins. This information will be critical to assess, in a holistic manner, the practical tenability of integrating algae-mediated estrogen removal into municipal WWTPs.

■ ASSOCIATED CONTENT

Supporting Information

Detailed documentation of algae procedures, SPE protocols, and HPLC analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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