Bioaugmentation Mitigates the Impact of Estrogen on Coliform-Grazing Protozoa in Slow Sand Filters

Sarah-Jane Haig,*,†,§ Caroline Gauchotte-Lindsay,† Gavin Collins,†,‡ and Christopher Quince†,∥

†School of Engineering, Rankine Building, University of Glasgow, Glasgow G12 8LT, U.K.
‡Microbial Ecophysiology Laboratory, School of Natural Sciences and Ryan Institute, National University of Ireland, Galway, Ireland

S *Supporting Information*

ABSTRACT: Exposure to endocrine-disrupting chemicals (EDCs), such as estrogens, is a growing issue for human and animal health as they have been shown to cause reproductive and developmental abnormalities in wildlife and plants and have been linked to male infertility disorders in humans. Intensive farming and weather events, such as storms, flash flooding, and landslides, contribute estrogen to waterways used to supply drinking water. This paper explores the impact of estrogen exposure on the performance of slow sand filters (SSFs) used for water treatment. The feasibility and efficacy of SSF bioaugmentation with estrogen-degrading bacteria was also investigated, to determine whether removal of natural estrogens (estrone, estradiol, and estriol) and overall SSF performance for drinking water treatment could be improved. Strains for SSF augmentation were isolated from full-scale, municipal SSFs so as to optimize survival in the laboratory-scale SSFs used. Concentrations of the natural estrogens, determined by gas chromatography coupled with mass spectrometry (GC-MS), revealed augmented SSFs reduced the overall estrogenic potency of the supplied water by 25% on average and removed significantly more estrone and estradiol than nonaugmented filters. A negative correlation was found between coliform removal and estrogen concentration in nonaugmented filters. This was due to the toxic inhibition of protozoa, indicating that high estrogen concentrations can have functional implications for SSFs (such as impairing coliform removal). Consequently, we suggest that high estrogen concentrations could impact significantly on water quality production and, in particular, on pathogen removal in biological water filters.
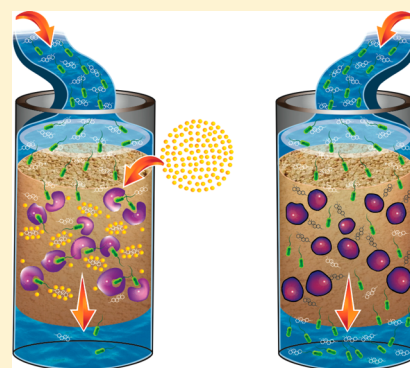
INTRODUCTION

The water industry faces a huge challenge in supplying a sustainable and safe supply of drinking water to a growing world population. Increasing demand has promoted the reuse of various water sources, including wastewater.[1] However, increasing urbanization and changes in agricultural practices are linked to anthropogenic contamination and reduced water quality. Common and emerging contaminants include the following: various metals; carcinogenic organic compounds; synthetic chemicals; pharmaceuticals; veterinary growth stimulators; ingredients in personal care products; and food supplements.[2−4] There is a growing body of scientific research indicating that these substances and in particular natural estrogens (estrone (E1), 17β-estradiol (E2), and estriol (E3)) may interfere with the normal function of the endocrine system of humans and wildlife by (i) mimicking and/or antagonizing the effect of endogenous hormones and (ii) disrupting the synthesis and metabolism of endogenous hormones and hormone receptors, resulting in various reproductive and developmental abnormalities and disorders.[3,5−8]

Since estrogens are excreted by all humans and animals, these compounds enter the environment via several routes, including from sewage treatment works discharge (in the case of incomplete removal) and agricultural runoff. It is, therefore, unsurprising that recent surveys revealed broad occurrences of E1, E2, and E3, of up to 85 ng/L, in surface waters in the U.S.A., Pan-European area, and Asia.[9−12] Due to increasing concerns about the adverse health effects posed by natural estrogens, the US EPA recently added E1, E2, and E3 onto its Contaminant Candidate List 3.[13] Likewise, the European Union Water Framework Directive added E2 as a "Hazardous" substance, meaning that EU countries must include removal measures for E2 from surface water and wastewater discharge by 2015 and meet the defined environmental quality standards by 2021.[14] Despite this, there has been little research into the impact of estrogens on the biological engineered systems used to remove them.

Drinking water treatment primarily relies upon adsorptive and oxidative processes to remove or transform organic materials; however, recent estrogen removal studies have shown that coagulation, sedimentation, filtration, and disinfection with chlorine achieve minimal removal.[15] Ozonation and granular activated carbon filters have been shown to be reasonably effective in removing EDC, but these methods are expensive and often difficult to incorporate into existing

drinking water treatment plants. As reclaimed wastewater and other surface waters will likely be required to supplement future drinking water supplies, information is required to determine how estrogen degradation can be improved in or introduced into biological water purification systems.

Numerous studies have focused on estrogen removal from wastewater using highly energy-intensive processes. However, the capacity of energy-passive, drinking water treatment technologies, such as slow sand filters (SSFs), to transform, or remove, natural estrogens has not yet been investigated – or is not recorded in the literature. Previous studies of wastewater treatment systems have shown that the removal of endocrine-disrupting chemicals (EDCs) can be enhanced by bioaugmentation with specific strains of degradative bacteria.[16] For example, Hashimoto et al. (2009)[17] and Roh and Chu (2011)[18] demonstrated that bioaugmentation of conventional activated sludge systems and sequencing batch reactors, respectively, with *Novosphingobium sp.* strain JEM-1 and *Sphingomonas* strain KC8, significantly enhanced estradiol removal. Furthermore, bioaugmentation was shown to be successful for the removal of various contaminants, including steroid hormones,[16] petroleum hydrocarbons,[19] and toluene[20] in various environments, including wastewater purification systems.[17,21−25] Unlike other water treatment methods, slow sand filtration (SSF) requires little energy input.[26] This, alongside the recent finding that SSFs can partially remove a range of pharmaceuticals, including E2,[27] underscores the catabolic potential of this technology. SSF may be an attractive candidate for bioaugmentation with EDC-degraders, potentially providing effective and economical EDC removal.

Previously, Haig et al. (2014a)[28] showed that it was possible to replicate the microbial community and water quality production of full-scale municipally operated SSFs and further revealed that the microbial communities underpinning SSFs are extremely complex and phylogenetically diverse.[29] Furthermore, recent work to explain pathogen removal in SSFs has shown that the filters host all kingdoms of life and that multitrophic interactions – particularly protozoan grazing – are required for optimal bacterial pathogen removal.[30] However, from a functional perspective, determining whether removal of emerging contaminants (e.g., estrogens) can be achieved or enhanced, by bioaugmenting SSF microbial communities, is also important. Furthermore, analysis of the functional impact of exposure to emerging contaminants on existing treatment systems is required, especially as the list of contaminants included in water quality guidelines expands. This study focused on understanding the functional effect estrogen exposure induced on SSF performance and determining whether bioaugmentation with estrogen-metabolizers can improve estrogen removal and overall filter performance.

■ MATERIALS AND METHODS

Isolation of Estrogen Metabolizing Bacteria. Bold's basal medium[31] was prepared with the addition of E1, E2, or E3 (100 μg/L; Sigma-Aldrich, UK) as the sole carbon source and ammonium chloride (0.01 g/L) as the nitrogen source. Aliquots (9 mL) of medium were inoculated in 20-mL glass vials with 1 g of sand from municipally operated full-scale SSFs.[28] Enrichment cultures were incubated in complete darkness at room temperature (23 °C ± 2 °C). Every 3 weeks over one year, 1 mL of each culture was subcultured into fresh medium following the enrichment procedure described by ref 32. Then, 1 mL of each of the final cultures was streaked on

minimal media agar (1.5% w/v) plates comprising the same components as the enrichment cultures. Plates were incubated in the dark at room temperature for 7 days. One morphologically distinct colony was then selected from E1-, E2-, and E3-fed cultures, and the three isolated strains were named E1, E2, and E3.

Phylogenetic Identification of Enriched Isolates. Genomic DNA of E1, E2, and E3 cultures was retrieved using the FastDNA Spin Kit for soil (MP Biomedcal, Cambridge, UK) according to manufacturers' instructions. The 16S rRNA gene fragments were amplified using the following PCR reaction mixture: 200 ng of DNA, 25 Bioline PCR mix (Bioline, London), and 12.5 pmol of both the forward, 27F (5′-GAGTTTGATCCTGGCTCAG-3′), and reverse primer, 1392R (5′-ACGGGCGGTGTGTRC-3′), in a Gene Pro thermal cycler (Bioer Technology, UK). PCR amplification conditions were as described by McHugh et al. (2004),[33] and the resulting PCR products were Sanger sequenced (Source Bioscience, LifeSciences, Nottingham). After chimera checking using Bellerophon,[34] taxonomic classifications were assigned using the Ribosomal Database Project classifier[35] using an 80% confidence threshold.

GC/MS Quantification of Natural Estrogens. GC/MS analysis of E1, E2, and E3 followed a procedure adapted from Quintana et al. (2004).[36] Briefly, 1 L water samples were forced (at approximately 15−20 mL/min) through a 60-mg HLB Oasis SPE cartridge (Waters, UK), which had been sequentially preconditioned with 3 mL of ethyl acetate and 3 mL of Milli-Q water adjusted to the same pH as the sample. After the concentration step, cartridges were dried under a stream of nitrogen for 30 min and eluted with 1 mL of ethyl acetate. The resulting analytes were further concentrated by evaporating to dryness under a constant nitrogen stream and resuspended in 100 μL of ethyl acetate, which were derivatized with 200 μL of MSTFA (Fisher Scientific, UK) at 85 °C for 100 min. Derivatized samples were analyzed using a GC (Agilent 7890A) system equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) coupled to a single quadrupole (Agilent 3157 5975c Inert XL EI/CI MSD) run in splitless full scan mode (mass range 50−500 mz). Compounds were separated using the following oven program: 1 min at 50 °C, first ramp at 10 °C/min to 220 °C, second ramp at 5 °C/min to 280 °C (held for 10 min). The GC/MS interface temperature was set to 250 °C. Quantification of each of the estrogens was achieved using MSD ChemStation (Agilent, UK) using single ion extracts (estrone = 342 m/z, estradiol = 416 m/z, and estriol = 504 m/z). All GC/MS runs were done in triplicate alongside six standards, negative controls, and blanks. Calibration curves were built by plotting the ratio of analyte peak area versus the analyte concentration. SPE recoveries were determined for each estrogen at two concentrations (250 ng/L and 10 ng/L) in triplicate by adding known amounts of a mixed working solution to 1 L of Milli-Q water. The absolute recovery was calculated by comparing the analytical results of the samples through overall sample preparation with those of standard samples without SPE (Sup. 1).

Toxicological Assays. *Cell Cultures.* All toxicological assays used axenic cultures of *Dictyostelium discoideum* B10 strain DBS0304514 (DictyBase, Northwestern University, USA), *Tetrahymena pyriformis* strain CCAP 1630/1W, or *Euglena gracilis* strain CCAP 1224/5Z (Strains of Culture Collection of Algae and Protozoa, UK). Protozoan strains were chosen to represent members from the three protozoan

Table 1. Phylogenetic Classification Based on the 16S rRNA Gene of Estrogen Enrichment Cultures[a,b]

name	NCBI match	phylum	class	order	family	genus	species
E1-rm	CP003872.1	Proteobacteria (100)	Betaproteobacteria (99)	Burkholderiales (99)	Comamonadaceae (99)	Acidovorax (99)	Acidovorax sp. KKS102 (99)
E2-rm	CP003880.1	Proteobacteria (100)	Gammaproteobacteria (100)	Pseudomonadales (99)	Pseudomonadaceae (97)	Pseudomonas (95)	Pseudomonas sp. UW4 (95)
E3-rm	CP000094.2	Proteobacteria (100)	Gammaproteobacteria (100)	Pseudomonadales (99)	Pseudomonadaceae (97)	Pseudomonas (97)	Pseudomonas fluorescenes (97)

[a]Brackets designate the percentage match assigned by RDP. [b]NCBI results presented when match was >96%.

morphotypes, amoeba, ciliate, and flagellate, respectively, which were previously shown to be important in SSFs.[30]

The density of protozoa was adjusted to 10^4 cells/mL in fresh growth media (following the procedures of refs 37, 38 for *Dictyostelium discoideum, Tetrahymena pyriformis,* and *Euglena gracilis,* respectively).[39] Estrogen was added to the protozoan cultures at two different concentrations: a "high" concentration, of 101 ng estrogen/L, chosen to best represent and simulate agricultural or wastewater pollution events (based on averages from ref 41 and comprising 50 ng/L (estrone), 12 ng/L (estradiol), and 39 ng/L (estriol)) and a "low" concentration, which was one-tenth-strength (10.1 ng/L) and composed of E1, E2, and E3 in the same ratio as the high concentration. A culture without any estrogen and a culture exposed to only the solvent (ethyl acetate) used to dissolve the estrogen mixture were also set up. Assays were performed in triplicate in 25-mL, glass culture vessels. Growth was determined by cell counts using a hemocytometer under phase-contrast microscopy (Inverted Olympus IX71) using a 100× optic, and three separate samples were counted at each time point.

Determination of Growth Impairment. The effects of estrogen exposure on the generation time of the three protozoa were determined following the protocol outlined by ref 41. Three aliquots of 100 µL were immediately taken (T0) from the control assays and the estrogen-exposed cultures and subsequently after 4, 8, 12, 24, and 48 h. The samples were diluted in distilled water, and protozoa were enumerated as described earlier. The impact of estrogen exposure on protozoan growth was determined as a function of the generation time (g), which was calculated by the formulas in eqs 1 and 2

$$\text{Number of generations } (n) = \frac{\log N_1 - \log N_0}{\log 2} \quad (1)$$

where N_1 = number of cells at 24 h, and N_0 = number of cells at T0

$$\text{Generation time } (g) = \frac{\text{Time of growth}}{n} \quad (2)$$

where Time of growth = 24 h.

Filter Operation and Sampling. The same slow sand filter setup (eight filters; height, 2.5 m, ø, 54 mm) and operational procedures, as employed by refs 28 and 30, were used in this study. However, in this study, four of the filters were bioaugmented with the three estrogen-metabolizing isolates (E1, E2, and E3), collectively referred to from here as the isolates. As in Haig et al. (2014ab),[28,30] water quality analyses, including coliform measurements, were d1 weekly, along with analyses for E1, E2, and E3 to assess the removal capacities and overall performance of the SSFs.

Estrogen removal efficiencies were calculated relative to the influent water supplying the filters, and total estrogenic potency

was determined using eq 3, where REA represents the relative estrogenic activity (values obtained from ref 5). Coliform removal was determined as a ratio (effluent coliform counts/influent coliform counts), where higher ratios correspond to lower removal.

$$
\begin{aligned}
\text{Estrogenic Potency} \\
= \frac{\text{E1 concentration}}{\sum \text{E1, E2, and E3 concentration}} \times \text{REA of E1 (0.2)} \\
+ \frac{\text{E2 concentration}}{\sum \text{E1, E2, and E3 concentration}} \times \text{REA of E2 (1.0)} \\
+ \frac{\text{E3 concentration}}{\sum \text{E1, E2, and E3 concentration}} \times \text{REA of E3 (0.01)}
\end{aligned}
$$
(3)

As in our previous studies,[28,30] sand samples were retrieved weekly from each sampling port to assess microbial community composition using qPCR (Sup. 2). Further, to monitor the abundance of the three isolates, custom qPCR primers and assays were designed (Sup. 2). To assess the effectiveness of SSFs for the removal of high concentrations of estrogens, filters were spiked with E1 (50 ng/L), E2 (12 ng/L), and E3 (39 ng/L) on Day 35, and again on Day 56, concentrations were chosen based on averages from refs 41 and 42 and simulate agricultural or wastewater pollution events. Effluent samples were collected from each filter 12 h after estrogen spiking to ensure complete passage of the water through the filters.

Bioaugmentation of SSFs with Estrogen Metabolizing Bacteria. Bioaugmentation of the SSFs was achieved following the method outlined by ref 19. At the beginning of the study 20 mL (1×10^8 cfu/mL [OD600 nm of 0.6]) of each of the three estrogen-metabolizing isolates (E1, E2, and E3) grown to exponential phase were added to the top of the filter-bed of four SSF every day for 4 days, equating to five hydraulic retention times. After which normal filter operation resumed.

Quantification of Estrogen-Metabolizing Organisms from SSFs. To accurately quantify the abundance of the three isolates specific qPCR primers, targeting their 16S rRNA gene sequences were designed (Sup. 2) using CODEHOP[43] and tested for their uniqueness using the Silva TestPrime database.[44] qPCR assays were conducted in triplicate as previously described[28,30] alongside two no-template controls, negative controls (*E. coli*), and six standards.

Statistics. Significant differences in the microbial composition between bioaugmented and nonaugmented filters, filter age, and depth were identified by permutation multivariate analysis of variance tests.[45] Significant differences in water quality production and estrogen removal efficiencies between bioaugmented and nonaugmented SSFs were tested using Wilcoxon tests.

In each toxicological assay, the experimental data represent the mean of three independent assays. Significant differences in protozoan generation time between the control and exper-

imental assays were determined by analysis of variance tests. All statistical analyses were done in the statistical software R[46] using the Vegan package, with statistical significance determined by P-values less than 0.05.

■ RESULTS

Characterization of Enrichment Cultures. Three estrogen-degrading bacteria, designated as E1, E2, and E3, were successfully isolated from the estrogen enrichment cultures. The analysis of the near full-length (1365bp) 16S rRNA genes of the isolates revealed they were phylogenetically distinct (Table 1), belonging to the *Proteobacteria* phyla. All strains were highly similar to three previously identified steroid-degrading bacterial species and represent the two most widely known steroid-degrading bacterial genera - *Acidovorax* and *Pseudomonas*.[47−49]

Protozoan Growth Impairment by Natural Estrogens. Under estrogen-free growth conditions, the generation times of *Dictyostelium discoideum*, *Euglena gracilis*, and *Tetrahymena pyriformis* were 10.6, 19.9, and 11.7 h, respectively. The addition of natural estrogens had little effect on *D. discoideum* growth (Table 4), and even the higher concentration (101 ng/L) of estrogens produced a close to significant effect on generation time (Anova, $p = 0.0508$, Figure 3A). A significant impact on growth (Anova, $p < 0.05$) was observed only after 48 h of exposure to the higher estrogen concentration. This is surprising as *Dictyostelium* is known to respond negatively to several other xenobiotics.

In contrast, exposure to natural estrogens, even at the lower concentrations, resulted in a significant increase in generation time for both *E. gracilis* and *T. pyriformis* (Table 4 and Figure 3B and C). Less than 50% of the original *E. gracilis* population was viable after 24 h in the higher estrogens concentration, which decreased further to 34% after 48 h. *T. pyriformis* was the most severely impacted by estrogen exposure and showed a clear concentration-dependent effect; the generation time doubled at the lower estrogens concentration, while no viable cells were detected after 24 h exposure to the higher concentration.

Effects of Bioaugmentation on SSF Functionality. Analysis of the water quality parameters and overall water quality performance showed that the augmented and nonaugmented SSFs produced water of a very similar quality to the full-scale municipally operated SSFs.[27] However, significant differences were found between augmented and nonaugmented SSFs (Wilcoxon, $p < 0.05$), which are solely attributed to differences in coliform removal capabilities (Sup. 3, 4, and 5). Interestingly, when data pertaining to the two spiking events are removed there were only marginal significant differences in filter performance. Additionally, the average total estrogen concentration of the raw, influent water (Sup. 3) supplying the filters was 23.86 ng/L, more than four times greater than the previously described highest concentration found in UK rivers.[50]

Estrogen degradation capacity results indicated that augmented filters removed significantly more estrone and estradiol than nonaugmented filters (Table 2). However, there was no significant difference in estriol removal. Although estrogen removal was lower than expected, bioaugmentation did significantly reduce the overall estrogenic potency (Wilcoxon, $p < 0.0001$) of the purified water by 25% on average, compared to 0% reduction in nonaugmented SSFs (Table 3).

Table 2. Average Estrogen Removal Efficiency over the 10-Week Study in Augmented and Nonaugmented Filters

	average percentage removal by filter type		
estrogen	augmented	nonaugmented	P-value
estrone	79.46	2.08	0.0007[a]
estradiol	34.58	−66.66	0.0146[a]
estriol	11.66	−11.60	0.2999

[a]Significant differences tested using Wilcoxon tests. Positive percentages correspond to removal. Negative percentages correspond to an increase in the concentration of estrogen i.e. no removal.

Irrespective of the low estrogen removal in SSFs, estrogen concentration was found to negatively correlate with coliform removal in nonaugmented filters (correlation = −0.34, $p < 0.05$) (Figure 1), which appeared to be due to inhibition of coliform-grazing protozoa (Figure 2). Referring to Figure 2, a reduction in the number of eukaryotes (i.e. protozoa) present during the two spiking periods of estrogen can be seen in both augmented and nonaugmented filters. However, intriguingly, the impact of estrogen on eukaryotic abundance appeared to be significantly lower in augmented filters (55% and 53% reduction at the two spiking events, respectively) compared to nonaugmented filters (93% and 85% reduction at the two spiking events, respectively). This, alongside the interesting finding that coliform removal in augmented filters was higher and appeared less affected by estrogen exposure than in nonaugmented filters, implies that augmentation reduces the toxic effect of estrogen on the coliform-grazing, protozoan community.

Effect of Bioaugmentation of Filter Community. Initial exploratory nonmetric multidimensional scaling (NMDS) and multivariate analysis of variance (MANOVA) analysis revealed that the microbial communities present in augmented and nonaugmented SSFs were significantly different ($p < 0.01$), with *Gammaproteobateria* and unclassified bacteria dominating augmented SSFs and *Bacteriodetes* and unclassified bacteria dominating nonaugmented SSFs (Sup. 6). The dominance of *Gammaproteobacteria* is unsurprising as two of the bacterial isolates used to augment the filters belong to this taxonomy. Additionally, significant differences in the concentration of 16S rRNA genes were found between augmented and nonaugmented filters ($p < 0.01$), with augmented filters possessing on average three times more copies than nonaugmented SSFs.

In order to determine which factors explain the differences between augmented and nonaugmented SSFs microbial communities, MANOVA and canonical correspondence analysis analyses were performed. This analysis revealed (Sup. 7) that the type of filter (augmented or nonaugmented) explained the biggest proportion (38%) of the difference in bacterial community composition, with age and the occurrence of estrogen spiking also being highly significant. Interestingly, individual filter identity within the bioaugmented and nonaugmented groups was not a significant variable in explaining differences between bacterial community compositions. This suggests as previously found that the communities within SSF are very reproducible[28]

In bioaugmented SSFs the initial number of E1, E2, and E3 degraders were 38, 25, and 16 times greater than that found in the nonaugmented SSFs. It is, however, important to note that nonaugmented filters did possess the three isolated estrogen degraders, which qPCR confirmed originated from the influent river water. Although bioaugmentation was shown to be

Table 3. Total Estrogenic Potency within the Influent and Effluent of Bioaugmented and Nonaugmented SSFs[a]

	total estrogenic potency				
week of expt	influent	augmented effluent	reduced	nonaugmented effluent	reduced
1	0.68	0.47	31%	0.72	0 (−6%)
2	0.73	0.64	12%	0.78	0 (−7%)
3	0.75	0.39	55%	0.46	39%
4	0.46	0.37	26%	0.33	28%
5[b]	0.30	0.22	27%	0.34	0 (−13%)
6	0.20	0.07	65%	0.07	65%
7	0.27	0.36	0 (−33%)	0.38	0 (−41%)
8[b]	0.26	0.35	0 (−35%)	0.54	0 (−108%)
9	0.66	0.35	53%	0.69	0 (−5%)
10	0.28	0.19	46%	0.29	0 (−4%)
av	0.46	0.33	25%	0.46	0 (−5%)

[a]Potency calculated using eq 3. [b]Designates time points when the SSFs were spiked with estrogen.
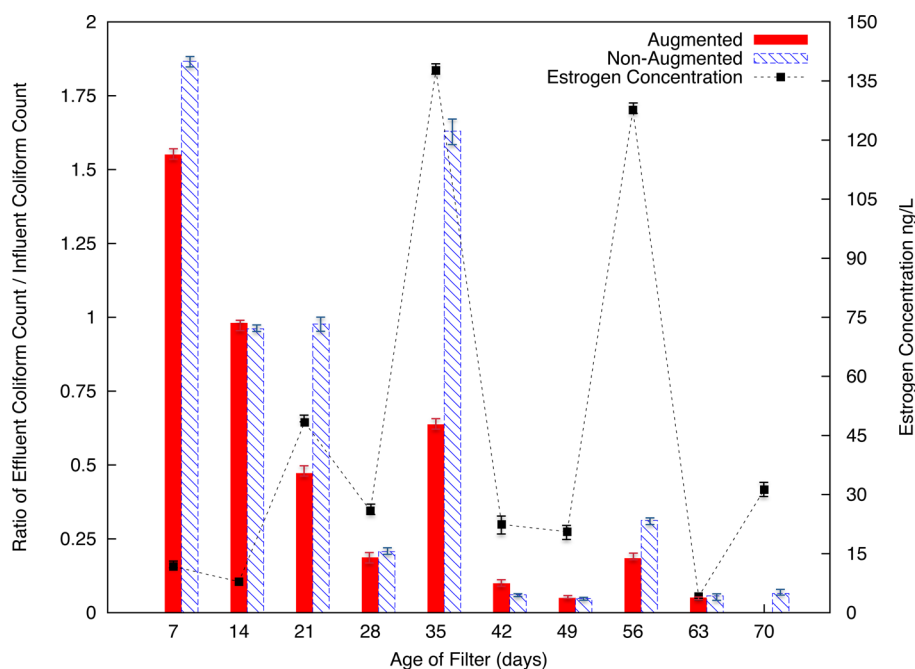

Figure 1. Average coliform removal in augmented and nonaugmented SSFs ($n = 4$), alongside the corresponding combined estrogen (estrone, estradiol, and estriol) concentration of the influent. Ratios below 1 correspond to coliform removal, and ratios above 1 correspond to an increase in coliforms relative to the influent concentration.

successful and sustainable, it is important to note that only 0.01%−1.3% of the initial inocula (1×10^8 cfu/mL) for each isolate remained at the top depth of the augmented SSFs by the end of the study, likely due to natural competition between other bacteria. Throughout the study the three isolates were present in greater abundance in the bioaugmented filters (Sup. 8), with the greatest abundance in both types of filters being seen during and after the two estrogen spiking periods.

■ DISCUSSION

Impact of Bioaugmentation on Estrogen Removal. The success of bioaugmentation is determined by two major principles: (1) the ability of the integrated bacteria to survive in the augmented environment and (2) the extent of degradation of the target pollutant.[51] The three isolated estrogen-metabolizing satisfied both criteria, supporting more estrone (E1) and estradiol (E2) removal in augmented than non-augmented SSFs (Table 2). Recently, several bioaugmentation studies have shown enhanced estradiol degradation in activated

sludge systems[52] and constructed wetlands,[16] but this is the first study, to the authors' knowledge, to show successful bioaugmentation of estrogen degradation in a drinking water system.

With respect to estrogen removal efficiencies, the SSFs achieved good removal of both E1 and E2, although the E2 removal efficiency was much lower than in a recent study.[27] Such differences may be due to the varying strategies and methodologies adopted to analyze and collect samples to determine estrogen removal, as well as to differences in SSF community composition. However, there is evidence that removal of the three natural estrogens is achieved by biodegradation into one of the other estrogens,[27] as demonstrated by the increased concentration of E1 and E3 (Sup. 3, 4, and 5). Similarly, poor E2 removal could be due to the recent discovery of a pathway which reduces the ketone group of E1 to form E2.[53] Indeed, an additional constraint on estrogen removal may be the possible top-down control of the bacterial community, including the estrogen-metabolizers, by
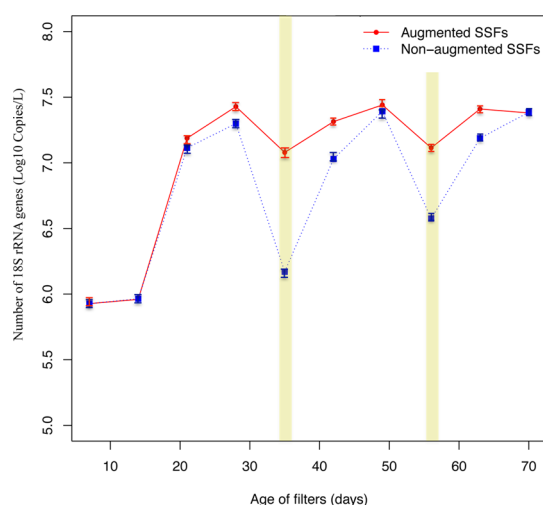
Figure 2. Concentration of 18S rRNA genes in sand samples from augmented and nonaugmented SSFs. Bars indicate the days at which influent was spiked with estrogen.

meiofauna (invertebrates smaller than 1000 μm, such as nematodes). Näslund et al. (2010)[54] found that naphthalene mineralization was significantly reduced by top-down control by meiofauna in marine sediments. Future work should aim to determine the effects of key eukaryotes, such as rotifers and nematodes − which are also abundant in SSFs − on estrogen degradation and on the success or sustainability of bioaugmentation.

Persistence of Estrogen-Metabolizing Isolates in Bioaugmented SSFs. Although bioaugmentation was achieved, only a small percentage of the original inoculum survived in the SSFs by the end of the study (Sup. 8). The prospect of scaling up the bioaugmentative approach to full-scale SSF does not seem imminently feasible − high volumes (e.g., several hundred liters of inoculum at the concentration used in this study) would be required over likely successive, periodically repeated applications to a full-scale SSF (e.g., surface area, >1,500 m^2). Nonetheless, recent studies have shown that bioaugmentation can be improved by augmentation at different times and through nutrient management. For example, Iasur-Kruh et al. [2011][16] successfully bioaugmented a mature wetland biofilm with E2-degrading bacteria, resulting in complete E2 removal; and Gallego et al. [2001][55] showed that a 50% increase in diesel oil degradation could be achieved by managing the carbon/nitrogen and carbon/phosphorus ratios in soil microcosms. Therefore, future work should also aim to enhance the survival of exogenous organisms in augmented SSFs by either augmentation at successive stages of the SSF lifecycle (e.g., after biofilm maturation or SSF ripening) or through biostimulation of the estrogen-degrading bacteria which may allow for realistic scaling up to full-scale SSFs. Furthermore, building on the findings from Ekelund et al. (2015)[56] who showed that the level of bacterial diversity in sand filters affected the degradation of BAM − a metabolite of the pesticide dichlobeni − future studies should explore the optimization of the bacterial diversity in augmented systems.

In addition, related studies may be pursued to optimize physical removal mechanisms in SSFs; for example, the use of anion exchange resin-coated sand could be used as part of bioaugmentation strategies for adsorption of the potentially toxic metabolites involved in inducing bacterial death.

Estrogen Exposure Affects Coliform Removal. Although estrogen removal was lower than expected, augmentation not only significantly improved estrogen removal (Figure 1 and Table 2) but also significantly increased the performance of augmented SSFs and improved water quality production. Enhanced performance was solely due to differences in the coliform removal capacity, with estrogen concentration negatively correlating with coliform removal in nonaugmented filters effluents (Sup. 7). The likely reason for the reduced coliform removal was the inhibition of coliform-grazing protozoa (Figures 2 and 3) and, in particular, *T. pyriformis,* which has been shown as integral for *E. coli* removal in SSFs.[30]

The toxicological assays indicated differential effects on growth depending upon the protozoan species and the estrogen concentration used (Figure 3; Table 4). Growth of both the flagellate (*Tetrahymena pyriformis*) and the ciliate (*Euglena gracilis*) was severely impaired. However, at lower concentrations the growth of only the ciliate was significantly affected. Although *D. discoideum* was unaffected by estrogens over the first 24 h of exposure, growth was significantly impaired after 48 h. This implies that although the amoeba is not as sensitive as either the ciliate or flagellate to initial estrogen exposure, extended exposure is detrimental. A possible reason for such a delayed effect may be the critical buildup of ROS or other damaging radicals (e.g., hydroxyl radicals), produced in the mitochondria when oxygen is reduced along the electron transport chain.[57]

Collectively, these observations help to clarify and provide explanations for the reduced coliform removal observed during the SSF study. Differential responses to EDCs are not surprising, as similar findings arose in human protozoan parasite studies[58] and in aquatic vertebrate studies.[59] Explanations for the impact of EDCs on growth can only be hypothesized as knowledge pertaining to protozoan endocrinology is sparse. However, in various other EDC studies involving invertebrates, estrogens have been found to alter the phosphorylation state of mitogen-activated protein kinases (MAPKs) and lysosomal membrane integrity[60] resulting in various detrimental effects. Similar processes might explain the effects observed in protozoa. Furthermore, and based on the reduced coliform removal performance observed during high estrogen exposure in the SSFs, it is possible that EDCs reduce the grazing capacity of protozoa; however, ingestion rate studies would be required to confirm this. This, alongside the interesting finding that coliform removal in augmented SSFs was less impaired by estrogen than in nonaugmented SSFs, implies that augmentation mitigated the toxic effect of estrogen on the coliform-grazing protozoan community. This is the first study to show that bioaugmentation not only improves the removal of the target chemical also but reduces the negative impact on treatment efficiency that such contaminants induce on water quality production.

Future Implications. The negative effects of estrogen exposure on coliform removal by SSFs pose questions for the operation of these and other biological water filters (for both waste and drinking water treatment); for example, how natural estrogens will impact the performance and micro- and macrocommunity of rapid sand filters, which are the most widely used drinking water purification systems and which are associated with similar microbial communities and fauna as SSFs.[61] This is important, especially since increasingly intensive agriculture and extreme weather phenomena result in elevated
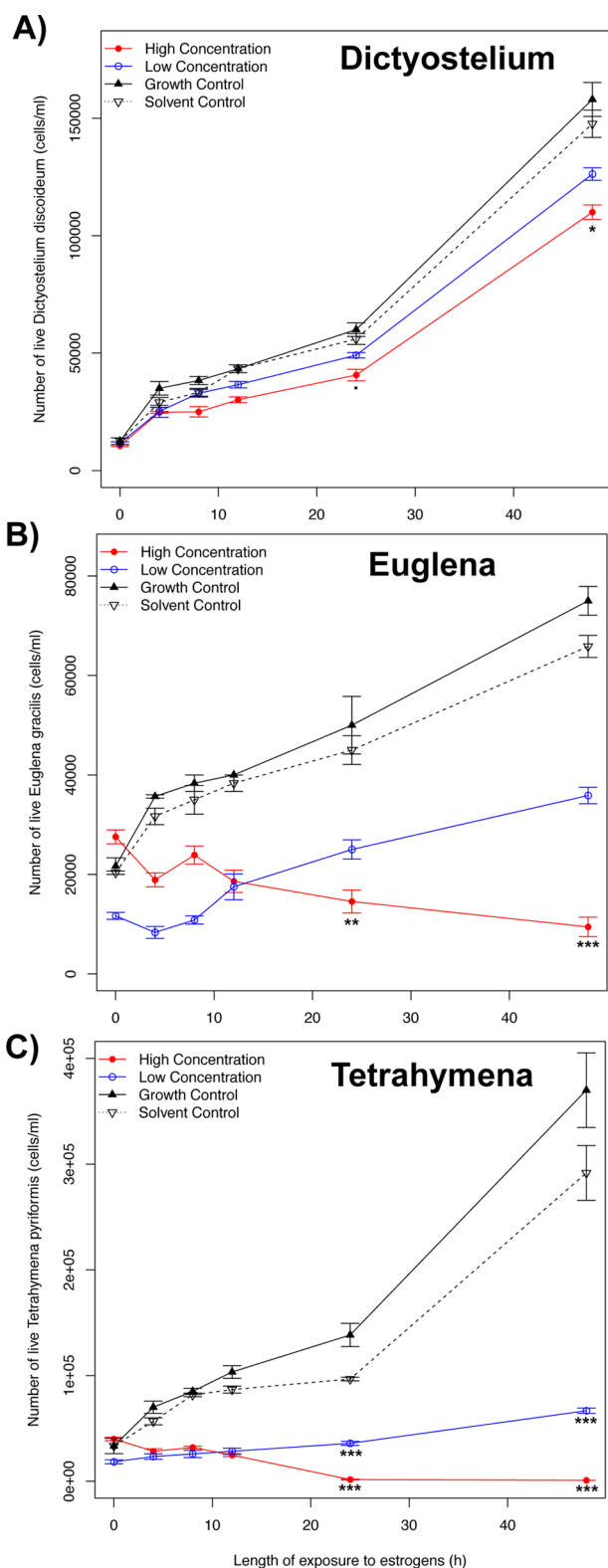
Figure 3. Effect of natural estrogens on A) *D. discoideum*, B) *E. gracilis*, and C) *T. pyriformis* population growth over 48 h. Concentrations of estrogens used were as follows: high (101 ng/L) and low (10.1 ng/L). Data points represent the mean of triplicate assays, and bars represent the standard error. ***Indicates significant differences (P-value: < 0.0005), **(P-value: 0.005) and (P-value: 0.03)) from the solvent control values.

Table 4. Effect of Natural Estrogens on the Generation Time of *D. discoideum*, *T. pyriformis*, and *E. gracilis* after 24 h of Growth[a]

protozoa	estrogen concn (ng/L)	generation time (h)	increase in generation time (%)
T. pyriformis	growth control	11.67 ± 1.08	
T. pyriformis	solvent control	12.77 ± 1.51	9.43
T. pyriformis	101	b	
T. pyriformis	10.1	22.74 ± 1.49[b]	94.86
D. discoideum	growth control	10.61 ± 0.88	
D. discoideum	solvent control	11.09 ± 0.76	4.52
D. discoideum	101	12.44 ± 0.82	17.25
D. discoideum	10.1	11.57 ± 0.69	9.05
E. gracilis	growth control	19.89 ± 0.94	
E. gracilis	solvent control	20.94 ± 1.36	5.28
E. gracilis	101	b	
E. gracilis	10.1	23.86 ± 2.39[c]	19.96

[a]Increase in generation time is with respect to the solvent control. [b]Indicates significant differences (P-value: < 0.0005). [c](P-value: 0.005) and (P-value: 0.03).

estrogen levels reaching various water sources. Future work should aim to determine the level and mode of toxicity induced by estrogen exposure to functionally important protozoa. Further studies in SSFs; river microcosms; soils and sediments; and, indeed, SSFs with varying concentrations, periods of exposure, and estrogenic potencies will all allow more accurate conclusions to be drawn about the potential deleterious effects of estrogen exposure on SSFs. Finally, a wider focus is justified on the impact of estrogens on complex ecosystems that are based on interactions between microorganisms and various meiofauna, such as rotifers and nematodes. In addition to "bottom-up" effects of bioaugmentative bacteria, such as in this SSF study, positive − and negative − "top-down" feedback mechanisms from a variety of meiofauna have been reported in the literature in the presence of xenobiotic chemicals.[54,56,61]

To conclude, this study is, to the authors' knowledge, the first example in any system to examine the effect of natural estrogens (E1, E2, and E3) on the growth of protozoa and to link the findings to compromised pathogen removal capacity in a complex ecosystem. Most importantly, this study highlights the potentially serious consequences of unmonitored estrogen exposure, not only for water quality production but also for the health of aquatic ecosystems in water sources, such as reservoirs and rivers used as water sources for SSFs and other biological water systems.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b05027.

Sup. 1 Linearity, recovery, and limit of quantification of estrogenic species spiked in Milli-Q water (PDF)
Sup. 2. qPCR primers used in the study (PDF)
Sup. 3 Summary of the physical and chemical characteristics of the influent water (River Kelvin) supplying bioaugmented and nonaugmented SSFs. The spiking events occurred 14 h prior to sampling to ensure filtration through the SSFs. Age: number of days since

filters were scraped. Units of measurement are TEMP: °C, PH: pH, NTU: turbidity, DO (Dissolved Oxygen): mg/L, COD (Chemical Oxygen Demand): mg/L, PO4 (Phosphate): mg/L, NO3 (Nitrate): mg/L, NO2 (Nitrite): mg/L, NH4 (Ammonium): mg/L, Total Viable Bacteria grown at 30 °C (TVB) and 13 °C (TVB13), coliforms: cfu/mL, and estrogens: ng/L (PDF)

Sup. 4 Summary of the physical and chemical characteristics of the effluent from the augmented SSFs (see Sup. 3 for additional information) (PDF)

Sup. 5 Summary of the physical and chemical characteristics of the effluent from the nonaugmented SSFs (see Sup. 3 for additional information) (PDF)

Sup. 6 Stacked barplots of the relative abundance of each of the phyla quantified by qPCR at the different depths sampled from A-D (augmented filters) and E-H (nonaugmented filters). From left to right, depths are 0, 5−15, 20−45, and 75 cm (PDF)

Sup. 7 Canonical-correspondence analysis of the relative abundance of the bacterial phyla quantified, and class abundances, against various parameters from augmented and nonaugmented SSFs (PDF)

Sup. 8 Abundance of the protozoa and the estrogen-degrading bacteria at the uppermost depth from A) augmented and B) nonaugmented SSFs (PDF)

■ AUTHOR INFORMATION

Corresponding Author
*Phone: 734-764-6350. E-mail: sarahjane.haig@googlemail.com.

Present Addresses
§Department of Civil & Environmental Engineering, University of Michigan, Michigan, USA.
∥Warwick Medical School, University of Warwick, Warwick, UK.

Notes
The authors declare no competing financial interest.

■ REFERENCES

(1) Falconer, I. R.; Chapman, H. F.; Moore, M. R.; Ranmuthugala, G. Endocrine-disrupting compounds: A review of their challenge to sustainable and safe water supply and water reuse. *Environ. Toxicol.* **2006**, 21 (2), 181−191.

(2) Kolpin, D. W.; Furlong, E. T.; Meyer, M. T.; Thurman, E. M.; Zaugg, S. D.; Barber, L.; et al. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999−2000: A national reconnaissance. *Environ. Sci. Technol.* **2002**, 36 (6), 1202−1211.

(3) Caliman, F. A.; Gavrilescu, M. Pharmaceuticals, personal care products and endocrine disrupting agents in the environment—a review. *Clean: Soil, Air, Water* **2009**, 37 (4−5), 277−303.

(4) Goldman, J. M.; Laws, S. C.; Balchak, S. K.; Cooper, R. L.; Kavlock, R. J. Endocrine-disrupting chemicals: prepubertal exposures and effects on sexual maturation and thyroid activity in the female rat. A focus on the EDSTAC recommendations. *Crit. Rev. Toxicol.* **2000**, 30 (2), 135−196.

(5) Conroy, O.; Saez, A. E.; Quanrud, D.; Ela, W.; Arnold, R. G. Changes in estrogen/anti-estrogen activities in ponded secondary effluent. *Sci. Total Environ.* **2007**, 382 (2-3), 311−323.

(6) Caserta, D.; Maranghi, L.; Mantovani, A.; Marci, R.; Maranghi, F.; Moscarini, M. Impact of endocrine disruptor chemicals in gynaecology. *Hum. Reprod. Update* **2008**, 14 (1), 59−72.

(7) Alvarez, D. A.; Shappell, N. W.; Billey, L. O.; Bermudez, D. S.; Wilson, V. S.; Kolpin, D. W.; et al. Bioassay of estrogenicity and chemical analyses of estrogens in streams across the United States associated with livestock operations. *Water Res.* **2013**, 47 (10), 3347−3363.

(8) Ternes, T. A.; Stumpf, M.; Mueller, J.; Haberer, K.; Wilken, R. D.; Servos, M. Behavior and occurrence of estrogens in municipal sewage treatment plants. (i) Investigations in Germany, Canada and Brazil. *Sci. Total Environ.* **1999**, 225 (1), 81−90.

(9) Kuch, H. M.; Ballschmiter, K. Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range. *Environ. Sci. Technol.* **2001**, 35 (15), 3201−3206.

(10) Matthiessen, P.; Arnold, D.; Johnson, A. C.; Pepper, T. J.; Pottinger, T. G.; Pulman, K. G. T. Contamination of headwater streams in the United Kingdom by oestrogenic hormones from livestock farms. *Sci. Total Environ.* **2006**, 367 (2), 616−630.

(11) Zhao, J. L.; Ying, G. G.; Wang, L.; Yang, J. F.; Yang, X. B.; Yang, L. H.; et al. Determination of phenolic endocrine disrupting chemicals and acidic pharmaceuticals in surface water of the Pearl Rivers in South China by gas chromatography, negative chemical ionization and mass spectrometry. *Sci. Total Environ.* **2009**, 407 (2), 962−974.

(12) Wang, L.; Ying, G.-G.; Zhao, J.-L.; Liu, S. B.; Yang, B.; Zhou, L.-J.; et al. Assessing estrogenic activity in surface water and sediment of the Liao River system in northeast China using combined chemical and biological tools. *Environ. Pollut.* **2011**, 159 (1), 148−156.

(13) U.S. Environmental Protection Agency. Drinking Water Contaminant Candidate List 3-Final: E9-24287. Federal Register: U.S. Environmental Protection Agency, *Code of Federal Regulations*, **October 2009b**, 74 (194), 51850−51862.

(14) European Commission Scientific Committee on Health and Environmental Risks *Opinion on: Chemicals and the water framework directive: environmental quality standards: ethinylestradiol (EE2)*; 2011.

(15) Ternes, T. A.; Meisenheimer, M.; McDowell, D.; Sacher, F.; Brauch, H. J.; Gulde, B. H.; et al. Removal of pharmaceuticals during drinking water treatment. *Environ. Sci. Technol.* **2002**, 36, 3855−3863.

(16) Iasur-Kruh, L.; Hadar, Y.; Minz, D. Isolation and bioaugmentation of an estradiol-degrading bacterium and its integration into a mature biofilm. *Appl. Environ, Microb* **2011**, 77 (11), 3734−3740.

(17) Hashimoto, T.; Murakami, T. Removal and degradation characteristics of natural and synthetic estrogens by activated sludge in batch experiments. *Water Res.* **2009**, 43 (3), 573−582.

(18) Roh, H.; Chu, K.-H. Effects of solids retention time on the performance of bioreactors bioaugmented with a 17β-estradiol-utilizing bacterium, *Sphingomonas* strain KC8. *Chemosphere* **2011**, 84 (2), 227−233.

(19) Mishra, S.; Jyot, J.; Kuhad, R. C.; Lal, B. Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. *Appl. Environ, Microb* **2001**, 67 (4), 1675−1681.

(20) Kim, H.; Jaffe, P. R.; Young, L. Y. Simulating biodegradation of toluene in sand column experiments at the macroscopic and pore-level scale for aerobic and denitrifying conditions. *Adv. Water Resour.* **2004**, 27 (4), 335−348.

(21) Runes, H.; Jenkins, J.; Bottomley, P. Atrazine degradation by bioaugmented sediment from constructed wetlands. *Appl. Microbiol. Biotechnol.* **2001**, *57* (3), 427−432.

(22) Lorah, M. M.; Voytek, M. A. Degradation of 1, 1, 2, 2-tetrachloroethane and accumulation of vinyl chloride in wetland sediment microcosms and *in situ* porewater: biogeochemical controls and associations with microbial communities. *J. Contam. Hydrol.* **2004**, *70* (1), 117−145.

(23) Ren, Y. X.; Nakano, K.; Nomura, M.; Chiba, N.; Nishimura, O. Effects of bacterial activity on estrogen removal in nitrifying activated sludge. *Water Res.* **2007**, *41* (14), 3089−3096.

(24) Silva, C. P.; Otero, M.; Esteves, V. Processes for the elimination of estrogenic steroid hormones from water: A review. *Environ. Pollut.* **2012**, *165*, 38−58.

(25) Yu, C.-P.; Deeb, R. A.; Chu, K.-H. Microbial degradation of steroidal estrogens. *Chemosphere* **2013**, *91*, 1225−1235.

(26) Haig, S. J.; Collins, G.; Davies, R. L.; Dorea, C. C.; Quince, C. Biological aspects of slow sand filtration: past, present and future. *Water Sci. Technol.: Water Supply* **2011**, *11* (4), 468−472.

(27) D'Alessio, M.; Yoneyama, B.; Kirs, M.; Kisand, V.; Ray, C. Pharmaceutically active compounds: Their removal during slow sand filtration and their impact on slow sand filtration bacterial removal. *Sci. Total Environ.* **2015**, *524-525*, 124−135.

(28) Haig, S. J.; Quince, C.; Davies, R. L.; Dorea, C. C.; Collins, G. Validating the Performance and Microbial Community of Laboratory-Scale Slow Sand Filters with respect to Full-Scale Industrial Filters. *Water Res.* **2014**, *61*, 141−151.

(29) Haig, S. J.; Quince, C.; Davies, R. L.; Dorea, C. C.; Collins. The Relationship Between Microbial Community Evenness and Function in Slow Sand Filters. *mBio* **2015**, *6* (5), e00729-15.

(30) Haig, S. J.; Schirmer, M.; D'Amore, R.; Gibbs, J.; Davies, R. L.; Collins, G.; Quince, C. Stable-isotope probing and metagenomics reveal predation by protozoa drives *E.coli* removal in slow sand filters. *ISME J.* **2015**, *9*, 797−808.

(31) Shelton, D. R.; Tiedje, J. M. General method for determining anaerobic biodegradation potential. *Appl. Environ. Microb* **1984**, *47* (4), 850−857.

(32) Madigan, M. T.; Martinko, J. M.; Stahl, D. D. P.; Clark, D. P. *Brock Biology of Microorganisms*, 13th ed.; Pearson Education: 2011; Vol. *13*.

(33) McHugh, S.; Carton, M.; Collins, G.; O'Flaherty, V. Reactor performance and microbial community dynamics during anaerobic biological treatment of wastewaters at 16−37 degrees C. *FEMS Microbiol. Ecol.* **2004**, *48* (3), 369−378.

(34) Huber, T.; Faulkner, G.; Hugenholtz, P. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **2004**, *20* (14), 2317−2319.

(35) Cole, J. R.; Wang, Q.; Cardenas, E.; Fish, J. B.; Chai, B.; Farris, R. J.; et al. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* **2009**, *37* (S1), D141−D145.

(36) Quintana, J. B.; Carpinteiro, J.; Rodrıguez, I.; Lorenzo, R. A.; Carro, A. M.; Cela, R. Determination of natural and synthetic estrogens in water by gas chromatography with mass spectrometric detection. *J. Chromatogr A* **2004**, *1024* (1), 177−185.

(37) Fey, P.; Kowal, A. S.; Gaudet, P.; Pilcher, K. E.; Chisholm, R. L. Protocols for growth and development of *Dictyostelium discoideum*. *Nat. Protoc.* **2007**, *2* (6), 1307−1316.

(38) Cassidy-Hanley, D. M. *Tetrahymena* in the Laboratory: Strain Resources, Methods for Culture, Maintenance, and Storage. *Methods Cell Biol.* **2012**, *109*, 237−276.

(39) Fleck, R. A.; Benson, E. E.; Bremner, D. H.; Day, J. G. Studies of free radical-mediated cryoinjury in the unicellular green alga *Euglena gracilis* using a non-destructive hydroxyl radical assay: a novel approach for developing protistan cryopreservation strategies. *Free Radical Res.* **2000**, *32* (2), 157−170.

(40) Chen, T.-S.; Chen, T.-C.; Yeh, K-J.C; Chao, H.-R.; Liaw, E.-T.; Hsieh, C.-Y.; et al. High estrogen concentrations in receiving river discharge from a concentrated livestock feedlot. *Sci. Total Environ.* **2010**, *408* (16), 3223−3230.

(41) Dias, N.; Mortara, R. A.; Lima, N. Morphological and physiological changes in *Tetrahymena pyriformis* for the *in vitro* cytotoxicity assessment of Triton X-100. *Toxicol. In Vitro* **2003**, *17* (3), 357−366.

(42) Fine, D. D.; Breidenbach, G. P.; Price, T. L.; Hutchins, S. R. Quantitation of estrogens in ground water and swine lagoon samples using solid-phase extraction, pentafluorobenzyl/trimethylsilyl derivatizations and gas chromatography−negative ion chemical ionization tandem mass spectrometry. *J. Chromatogr A* **2003**, *1017* (1), 167−185.

(43) Rose, T. M.; Henikoff, J. G.; Henikoff, S. CODEHOP (COnsensus-DEgenerate hybrid oligonucleotide primer) PCR primer design. *Nucleic Acids Res.* **2003**, *31* (13), 3763−3766.

(44) Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **2013**, *41* (1), e1.

(45) Anderson, M. J. A new method for non-parametric multivariate analysis of variance. *Austral Ecol* **2001**, *26* (1), 32−46.

(46) R Development Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2011. URL http://www.R-project.org/ (accessed Feb 1, 2016).

(47) Silby, M. W.; Cerdeno-Tarraga, A. M.; Vernikos, G. S.; Giddens, S. R.; Jackson, R. W.; Preston, G. M.; et al. Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*. *Genome Biol.* **2009**, *10* (5), R51.

(48) Ohtsubo, Y.; Maruyama, F.; Mitsui, H.; Nagata, Y.; Tsuda, M. Complete genome sequence of *Acidovorax sp.* strain KKS102, a polychlorinated-biphenyl degrader. *J. Bacteriol.* **2012**, *194* (24), 6970−6971.

(49) Duan, J.; Jiang, W.; Cheng, Z.; Heikkila, J. J.; Glick, B. R. The complete genome sequence of the plant growth-promoting bacterium *Pseudomonas sp.* UW4. *PLoS One* **2013**, *8* (3), e58640.

(50) Xiao, X.-Y.; McCalley, D. V.; McEvoy, J. Analysis of estrogens in river water and effluents using solid-phase extraction and gas chromatography−negative chemical ionisation mass spectrometry of the pentafluorobenzoyl derivatives. *J. Chromatogr A* **2001**, *923* (1), 195−204.

(51) El Fantroussi, S.; Agathos, S. N. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? *Curr. Opin. Microbiol.* **2005**, *8* (3), 268−275.

(52) Yu, C.-P.; Roh, H.; Chu, K.-H. 17β-Estradiol-degrading bacteria isolated from activated sludge. *Environ. Sci. Technol.* **2007**, *41* (2), 486−492.

(53) Isabelle, M.; Villemur, R.; Juteau, P.; Lepine, F. Isolation of estrogen-degrading bacteria from an activated sludge bioreactor treating swine waste, including a strain that converts estrone to β-estradiol. *Can. J. Microbiol.* **2011**, *57* (7), 559−568.

(54) Näslund, J.; Nascimento, F. J.; Gunnarsson, J. S. Meiofauna reduces bacterial mineralization of naphthalene in marine sediment. *ISME J.* **2010**, *4* (11), 1421−1430.

(55) Gallego, J. L.; R Loredo, J.; Llamas, J. F.; Vazquez, F.; Sanchez, J. Bioremediation of diesel-contaminated soils: Evaluation of potential *in situ* techniques by study of bacterial degradation. *Biodegradation* **2001**, *12* (5), 325−335.

(56) Ekelund, F.; Harder, C. B.; Knudsen, B. E.; Aamand, J. *Aminobacter* MSH1-mineralisation of BAM in Sand-Filters Depends on Biological Diversity. *PLoS One* **2015**, *10* (6), e0128838..

(57) Jafar, G.; Hamzeh, G. Ecotoxicity of Nanomaterials in Soil. *Ann. Biol. Res.* **2013**, *4* (1), 86−92.

(58) Nava-Castro, K.; Hernandez-Bello, R.; Muniz-Hernandez, S.; Camacho-Arroyo, I.; Morales-Montor, J. Sex steroids, immune system, and parasitic infections: facts and hypotheses. *Ann. N. Y. Acad. Sci.* **2012**, *1262* (1), 16−26.

(59) Segner, H.; Caroll, K.; Fenske, M.; Janssen, C. R.; Maack, G.; Pascoe, D.; et al. Identification of endocrine-disrupting effects in

aquatic vertebrates and invertebrates: report from the European IDEA project. *Ecotoxicol. Environ. Saf.* **2003**, *54* (3), 302−314.

(60) Canesi, L.; Lorusso, L. C.; Ciacci, C.; Betti, M.; Zampini, M.; Gallo, G. Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. *Gen. Comp. Endocrinol.* **2004**, *138* (1), 58−69.

(61) Albers, C. N.; Ellegaard-Jensen, L.; Harder, C. B.; Rosendahl, S.; Knudsen, B. E.; Ekelund, F.; Aamand, J. Groundwater Chemistry Determines the Prokaryotic Community Structure of Waterworks Sand Filters. *Environ. Sci. Technol.* **2015**, *49* (2), 839−846.