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Escherichia coli O157:H7 attachment and persistence within root biofilm of common treatment wetlands plants

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ABSTRACT

Pathogen retention and subsequent release within the rhizosphere of wastewater treatment wetlands may be a concern for human health. To address this concern, the enteric pathogen *Escherichia coli* O157:H7 with a DsRed plasmid insertion was used as a model pathogenic organism in an open-air chemostat reactor with constant flow of simulated wastewater. Colonization and persistence of the organism was tracked on roots of two obligate wetland plant species, *Carex utriculata* and *Schoenoplectus acutus*, originally grown in pilot scale wetland reactors. Teflon nylon string, clean and with existing indigenous biofilm, was used as an inert surface control. Epifluorescence microscopy and qPCR were used to verify *E. coli* O157:H7 abundance for up to 1 week. Initial attachment was seen on all surfaces, with colonization decreasing through 1 week. qPCR showed preferential association of the pathogen with roots over the nylon. There was a significant difference between plant type; *S. acutus* showed significantly higher numbers compared to *C. utriculata*. *E. coli* O157:H7 binding and persistence on root surfaces may be a means of survival in treatment wetlands.

INTRODUCTION

Wastewater treatment is important for the environment and human health. Many alternative methods to conventional treatment have been researched (Tonderski, 2009), including using treatment wetlands (TW). TW are the adoption and adaptation of a wetland's ability to remove common contaminants in water. In general, TW have a nonpermeable layer to prevent leaching and are planted with single or mixed species plants in a medium of gravel or soil (Akratos and Tsihrintzis 2007; Baskar et al., 2014). TW can vary in terms of wastewater loading and retention time (Kotti et al., 2010; Austin 2014).

For wastewater treatment, TWs provide both aerobic and anaerobic microniches (Faulwetter et al., 2009). Plant roots release oxygen as well as other exudates into the subsurface, providing an environment for aerobic bacteria to survive at depths below the dissolved oxygen level (Carballeira et al., 2016). This allows aerobic processes such as nitrification and phosphorus removal, leaving

the anaerobic matrix to continue other anaerobic reactions such as denitrification (Hench et al., 2003; Taylor et al., 2011).

The root biofilm of TW may also reduce pathogenic bacteria present in the wastewater (Licence et al., 2001; Boutilier et al., 2009; Zhang et al., 2016). Existing biofilm competition and predation in the rhizosphere limits attachment and settling (Boutilier et al., 2009; Jasper et al., 2013). However, there is extensive research revealing that pathogens may survive and be released in effluent water (Barcina and Arana 2009; Liu et al., 2009; Paruch, 2011). Even a low count release, such as 100 cells for *Escherichia coli* O157:H7, is enough to cause human infection and disease (Bitton, 2005; USEPA, 2013).

E. coli O157:H7 is a food- and waterborne pathogen transmitted via the fecal-oral route, usually through contaminated water or vegetables (Licence et al., 2001; Dinu and Bach 2011; Ongeng et al., 2011). Previous research has shown a decrease of *E. coli* O157:H7 through TWs, however these studies are solely based on plate counts (Liu et al., 2009; Ongeng et al., 2011; García et al., 2013). Direct microscopy or molecular techniques for enumeration would likely reveal much higher survival, as well as the ability for later release. This is due to a phenomenon known as the viable but non-culturable (VBNC) state in which bacteria are no longer culturable on a plate but can be resuscitated from the environment when returned to growth-promoting conditions (Barcina and Arana 2009; Dinu and Bach 2011; Paruch, 2011).

Fig. 1. Representative images of the four experimental surface types taken at 2, 24 and 168 h. Fluorescence was observed under 558 nm wavelength. Due to natural fluorescence of photosystem II chlorophyll in the plant tissue, plant surface is shown in *C. utriculata* and *S. acutus* images. No stains or dyes were used in Teflon nylon imaging under the assumption stationary cells were attached to a surface. Scale bar represents 50 μm .

Another serious limitation is the focus on influent to effluent measurements, assuming that *E. coli* O157:H7 is not surviving within the TW. Long-term lab studies in river and reservoir water found *E. coli* O157:H7 can persist up to 6 months (Liu et al., 2009). *E. coli* O157:H7 may also be surviving within the biofilm matrix or incorporated into the plants, as seen in vegetable production (Fu et al., 2005; Dinu and Bach 2011) *E. coli* O157:H7 can invade plant tissues of leafy greens and stalks (Deering et al., 2012). Although this is not a health concern in TW as with edible plants, *E. coli* O157:H7 within vascular openings of plants could provide means of survival and potential release.

The hypothesis was that indigenous biofilm and living plant tissue (with an established biofilm) impact the persistence of *E. coli* O157:H7 in a simulated TW. Two plant species, *C. utriculata* and *S. acutus*, were used in hydroponic reactors to determine interactions with the roots. In previous research these two obligate wetland species were the top performers for removal efficiency of carbon, nitrogen and sulfate in pilot-scale wetland systems (Taylor et al., 2011). Inert Teflon nylon material was used as a control both with and without a pre-established biofilm. Pathogen persistence on these surfaces was assessed using qPCR and microscopy.

2. Materials and methods

2.1. Growth of *E. coli* O157:H7

The *E. coli* O157:H7 was isolated from an outbreak in 1993 (Pyle et al., 1995). A constitutively expressed DsRed plasmid with a carbenicillin resistance selective gene (Clonetechn) was incorporated (Klayman et al., 2009) for detection by fluorescent microscopy. Tryptic Soy Broth (TSB) was made using non-casein enzymatic digest of protein (Klayman et al., 2009) for plasmid retention and expression. *E. coli* O157:H7 was grown overnight to approximately 10^8 cells mL^{-1} as determined by plate count. Survival and plasmid stability in simulated wastewater (SW; Supplemental Table 1)

was verified by plate counts, qPCR, and epifluorescence microscopy (VanKempen-Fryling et al., 2015).

2.2. Plant growth and strain collection

Shoots with substantial root mass of *C. utriculata* and *S. acutus* grown in a controlled greenhouse environment were harvested and washed with nonsterile H_2O to remove excess planting medium and loosely associated bacteria while keeping the attached biofilm. The rinsed plants ($49.3 \text{ mL} \pm 26.4 \text{ mL}$, determined by the mass displacement) were transferred to 300 mL hydroponic chemostat reactors under 24 h light at 25°C and grown for 3–7 days in SW.

Comparisons were made between living roots to an inert surface of Teflon nylon strings (D'Addario; GHS strings) similar in dimensions to the roots (0.79, 0.81 and 1.01 mm diameter). Clean nylon strings were added to chemostats with SW immediately prior to *E. coli* O157:H7 inoculation. Alternatively, strings were added to effluent of the established plant mesocosms for 24 h in batch, followed by the addition of SW (flow conditions) for 1 week to encourage biofilm growth prior to pathogen inoculation. Total weight of the nylon strings, cut into 17.5 cm lengths (half immersed), was 2.94 g.

2.3. Determination of surface area

To estimate the *E. coli* O157:H7 surface area attachment, standard curves were generated relating wet mass to root surface area. Representative roots were excised, imaged, and analyzed with the Rootscan[®] program to determine the surface area. The wet mass was plotted against surface area to generate standard curves (Supplemental Fig. 1). *C. utriculata*'s two distinct root types (taproot and branched) formed unique standard curves; DNA extraction for *C. utriculata* samples were separated by root type. Teflon surface area was estimated by treating the strings as open ended cylinders. The

Fig. 2. Distribution of cell clumping over total counts per time point for each surface: (A) *C. utriculata*, (B) *S. acutus*, (C) clean Teflon nylon, and (D) biofilm Teflon nylon. Root locations were combined for a total mean. Bars represent standard deviation from all experimental replicates.

total surface area of the 24 sample strings, 8 of each diameter was 53.32 cm².

2.4. Reactor series

20 mL of overnight *E. coli* O157:H7 culture was spun at 4816 x g in triplicate, washed in nanopure water, and concentrated into 1 mL. For each triplicate experimental condition (*C. utriculata*, *S. acutus*, clean nylon, and established biofilm nylon), 1 mL of inoculum was added and mixed via pipetting and rotating the plant or nylon. One additional non-inoculated reactor served as a control. Flow was at 3 mL min⁻¹ (100 min hydraulic residence times (HRT)) for one week. A time zero sample was taken from each reactor immediately after inoculation. Effluent samples were collected at 45-min intervals for 6 h. One replicate reactor was destructively sampled at 135 min, approximately 1 HRT (initial attachment). Another effluent sample was taken at 24 h and the second replicate reactor destructively sampled. The third replicate was destructively sampled at one week. The uninoculated control reactor effluent was

sampled at 0 and 6 h and destructively sampled at one week. All water samples were frozen for further analysis.

Submerged roots from destructively sampled reactors were divided in half by length for differentiation of basal and root tip, rinsed with nanopure water to remove unattached cells, placed into 2 mL microcentrifuge tubes and frozen for DNA extraction. The nylon samples were taken in entirety, placed into 50 mL Falcon tubes, and frozen. A representative root or nylon sample was pulled through a 2 mm square glass capillary approximately 90–110 mm in length (Wale Apparatus, PA) using a wire hook (Aircraft Spruce), the interior of the capillary rinsed with nanopure water to slough off unattached cells, and attachment observed by epifluorescence microscopy.

2.5. Epifluorescence microscopy

The glass capillaries were viewed under 600× magnification (Nikon; Melville, NY) and 558 nm wavelength for DsRed plasmid excitation on a Nikon Eclipse E-800 microscope with a cooled CCD camera (Photometrics). The root tip, mid-root, and top root were

Fig. 3. Relative abundance of *E. coli* O157:H7 per surface area normalized to background fluorescence. The data is differentiated over time and by root type or nylon condition for each respective surface.

Table 1
DNA extraction quantification.

Sample Type	Concentration (ng/μL)	A ^{260/230}
<i>C. utriculata</i> water	80.08 ± 40.56	2.01 ± 0.21
<i>C. utriculata</i> root	96.38 ± 30.02	2.02 ± 0.15
<i>S. acutus</i> water	57.34 ± 51.16	1.49 ± 0.61
<i>S. acutus</i> root	72.09 ± 37.17	1.88 ± 0.25
Nylon clean water	59.81 ± 29.91	2.04 ± 0.21
Nylon clean surface	48.37 ± 36.63	1.94 ± 0.70
Nylon biofilm water	72.22 ± 45.80	1.95 ± 0.48
Nylon biofilm surface	40.03 ± 24.86	1.99 ± 0.34

scanned and 20 images taken in each area for samples collected at 2, 24 and 168 h. Natural photosystem II chlorophyll fluorescence allowed the plant surface to be viewed without stain. The images were semi quantitatively analyzed (MetaMorph® Image Analysis Software) to determine number of cells and colony clump size using the average pixel area for *E. coli* O157:H7 single cell fluorescence (Wilson et al., 2004; Behnke et al., 2011). Thresholding was used to subtract background autofluorescence and remaining objects were counted.

2.6. DNA extraction and qPCR analysis

Thawed water samples were spun down at 4816 × g for 20 min at 4°C. The pelleted sample was resuspended and transferred to a PowerBead tube from the PowerSoil® DNA Isolation Kit (MoBio Laboratories; Carlsbad, CA, USA). Mass of the roots was determined prior to transfer to a PowerBead tube. DNA extraction followed the kit protocol except a Fastprep®-24 bead beater (M.P. Biomedicals; Santa Ana, CA, USA) was used at 5.5 msec⁻¹ for 45 s. Cell material on the nylon strings was removed by adding glass beads to the 5 mL mark of the 50 mL conical Falcon tube with 10 mL autoclaved nanopure H₂O, and vortexed for 60 s. The liquid fraction was removed and centrifuged at 4816 × g for 5 min. The supernatant was removed and the pellet resuspended and added to a PowerBead tube for extraction as previously described. DNA was isolated in 100 μL of DEPC water (Ambion®; Grand Island, NY, USA) and quantified using a Nanodrop® ND-1000 spectrophotometer (Thermo Scientific; Wilmington, DE, USA). Average quantification and purity per sample type is listed in Table 1.

qPCR primers targeted the Shiga toxin 2 gene unique to the O157:H7 serotype (Jothikumar and Griffiths, 2002). Control experiments using plate counts verified that the cells had one copy of the *fStx1* and *Stx2* genes (Shaikh and Tarr, 2003). A 25 μL reaction

mixture was prepared using SYBR Green Master Mix (Kapa Biosystems; Wilmington, MA, USA), 0.5 mM primer, and 3.1 × 10⁻³ mM DNA diluted from the extraction. Programs for qPCR can be found VanKempen-Fryling et al., 2015.

2.7. Data analysis

Multifactor analysis of variance (ANOVA) was performed to determine statistical significance between surface types and time. Comparisons were analyzed between plants and Teflon nylon strings, the root tip or basal root, established biofilm compared to clean nylon, and time. General linear models (GLM) were used to compare interactions using Minitab 17®. Statistical significance between specific factors for each variable were identified by pairwise Tukey's *post hoc* multiple comparison test using a 0.05 *p*-value cut-off. The experimental replicates were input as random variables nested within time.

3. Results

Initial washout of *E. coli* O157:H7 during the first four HRT was similar for all systems (Supplemental Fig. 2). A twenty-four hour sample showed the continued release of *E. coli* O157:H7 from the inoculated reactors, which would result from detachment of previously attached cells.

E. coli O157:H7 could be imaged on the surfaces throughout the time course (Fig. 1). Initial attachment was mainly single cells and there was no observable difference in abundance by location or surface type. At 24 h, microcolony formation was seen. The final time point at 168 h showed a lower, sustained *E. coli* O157:H7 cell presence and more variable microcolony formation. These observations were substantiated with the MetaMorph® Image Analysis software (Fig. 2) and reported as a percentage of the total events for all size ranges from small and medium clusters to large, >100 cell formations. The number of clumps greater than single cells increased on all surfaces except the biofilm-colonized nylon, where increases were limited to clumps >100 cells. This is more dramatic in *S. acutus* (23 to 36 events) and clean nylon experiments (96 to 109 events). Single cells were more varied between replicates, as seen through the large error bars. The total clump counts decreased from 2 to 168 h (22.6 average events to 13.3 average events), however the largest formations increased (average increase of 1 event for >100 cell clump size).

qPCR determined relative target cell abundance on inoculated vs non-inoculated surfaces over time. For the root experiments, location (root tip or basal root) and condition (branched or thick for *C. utriculata*; red/live or black/dead coloration for *S. acutus*) of the root were analyzed. For nylon experiments, the biofilm presence/absence was compared. For all experimental surfaces (Fig. 3), the population decreased during the period between 2 and 168 h, averaging a reduction from 704 gene copies cm⁻² to 55 gene copies cm⁻² (*P* < 0.001). Attachment on *S. acutus* was significantly higher (average 493 gene copies cm⁻²) than *C. utriculata* by 209 gene copies cm⁻² (*P* = 0.002), as well as both nylon replicates (*P* = 0.021) in all time points. Nylon replicates and *C. utriculata* were statistically equivalent with the lowest *E. coli* O157:H7 attachment across all time points. For *C. utriculata*, *E. coli* O157:H7 preferred branched roots to uniform taproots with an average difference of 82.1 gene copies cm⁻² to 281 gene copies cm⁻² (*P* < 0.001) across all time points. In *S. acutus*, no meaningful effect of live roots was observed. Localization was taken into account between root tip and the basal roots, but there was no preference.

The two abiotic surfaces were also compared to identify any difference with respect to preexisting biofilm alone. There was no significant difference in *E. coli* O157:H7 attachment. A slight inter-

action between surface and time was seen from initial attachment to 24 h in clean nylon over biofilm-colonized nylon (Supplemental Fig. 3).

4. Discussion

Epifluorescence microscopy and qPCR showed that *E. coli* O157:H7 attached and persisted on all surfaces under flow conditions for the duration of the experiment. Attachment was significantly lower on abiotic nylon controls compared to *S. acutus* roots. This supports the hypothesis that there is a benefit for survival or potential growth for bacterial pathogens within an established root biofilm (Tyler and Triplett 2008). Many plants have been shown to foster biofilm growth along the root structures for reasons ranging from protection against plant pathogens to nitrogen fixation (Gruyer et al., 2013). Wetland plants provide labile organic carbon exudates during active periods and by slow release during decay (García et al., 2010; Iannelli et al., 2011), and also contribute ions and oxygen (Hawkes et al., 2007). Given previous extensive research on interactions between plants and bacteria (Danhorn and Fuqua, 2007) it is possible that *E. coli* O157:H7 attachment and persistence may benefit from these interactions (Deering et al., 2012). Less attachment to *C. utriculata* roots suggests that this plant may have a lower potential for pathogen retention in TW.

Interestingly, there was no statistical difference in pathogen attachment and persistence between the clean nylon surface and nylon with an established biofilm. It was hypothesized that competition with the natural consortia of bacteria in an environment would protect against invading *E. coli* O157:H7 cells since this is common with other organisms (Hibbing et al., 2010) and pathogens in aquatic biofilms (Nocker et al., 2013). The mechanisms in this system remain unknown and will warrant further research.

The fate of the undetected *E. coli* O157:H7 cells and whether they were sloughed into the effluent feed and still viable or inactivated by predation or competition was not determined. Other studies have indicated these factors play a large role in lowering human pathogen retention in the environment (Boutillier et al., 2009; Jasper et al., 2013; Li et al., 2014), however for strains with a low infection rate such as *E. coli* O157:H7, these may not be enough to completely eradicate subsequent release and the potential infection. If *E. coli* O157:H7 is able to attach to roots, it may have prolonged ability to survive in a TW. Further research is needed for optimizing pathogen removal in TW.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ecoleng.2016.10.018>.

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