The effect of Established Biofilms on the Survival of Environmental *E. coli, P. aeruginosa* and *E. faecalis* under Natural Swimming Pool Conditions

by Adam Gregg, 2017

Abstract/Summary

Biological filter systems are an established technology in many forms of water treatment, however, in recent years these water filtration systems have become increasingly popular for use within natural bathing waters. Because natural bathing water systems undergo no chemical disinfection, concerns have been raised about the microbiological safety of the systems for end users, especially when maintenance is neglected (e.g. Giampaoli, S et al. 2014). This paper addresses the question "how capable are biological filters at mitigating intestinal pathogen contamination?".

The primary health concern regarding biological filters is the potential for pathogenic strains to colonise and proliferate within the biofilm and persistently shed into the water body (Nocker (2014). If pathogens were able to be incorporated into the filter biota, this has the potential to periodically increase pathogen levels to infectious levels. The experiment intended to examine if a biological filter is indeed a source of such an 'infectious dose'.

The results from this experiment, obtained through membrane filtration, show that the three main intestinal pathogenic species (*E. coli*, P. aeruginosa and E. faecalis (EPA, 2009)) could not survive indefinitely within a natural pool system, and that the presence of a mature biofilm filter significantly decreases the residence time of pathogens within the system. Such information may be considered surprising, even to scientists, and if this information could be disseminated into the public sphere it would be a significant boost to the natural swimming pool industry.

As a means of showing this information in a digestible way, a real time bioluminescent assay was also carried out using bioluminescent *E. coli* Nissle 1917 pGlite. This video shows the same trend as the non-bioluminescent experiments. *E. coli* was not able to survive indefinitely within the system, and the mature biofilm filter has a much shorter residence time. This experiment not only showed that *E. coli* could not survive within the system but also serves as a very visual, digestible way (with a "wow" factor) to educate the public about the bioremediation capabilities of biological filters.

Method (4x Filter Pass) (Shown in Fig. 1)

Biofilm Preparation: Biofilms were matured in 1.5 L columns filled with ceramic substratum for 4 weeks prior to use. Biofilms were maintained in 25 L circulation tanks, supplemented daily with $3x10^{-3}$ g/day NH_4CL, $2.4x10^{-4}$ g/day $NH_4H_2PO_4$, and $6.5x10^{-2}$ g/day CH_3COONa. 175g of substratum was taken from the circulation tanks and placed in the experiment filters. Once transferred to the experiment filters, the system is dosed with $3.5x10^{-4}$ g/day NH_4CL, $2.8x10^{-5}$ g/day $NH_4H_2PO_4$, and $7.6x10^{-3}$ g/day CH_3COONa. The experimental filters were tested for nitrite, ammonia and ortho-phosphate daily to determine the stability of the filters. Once stability had been confirmed (the daily nutrient consumption equals the daily input), the biofilm is deemed ready for testing.

Preparation of Bottles, filters and Pipes: All bottles filters and pipes were washed and autoclaved (120°C, 15 min) prior to use.

Water Preparation: For each filter run, 1 L of natural pool water was taken from a mature 50,000 L onsite natural swimming pool (>2 years old). The Water was filtered through a 0.2 µm membrane filter and then supplemented with $3.5x10^{-4}$ g/L NH_4CL, $2.8x10^{-5}$ g/L $NH_4H_2PO_4$, and $7.6x10^{-3}$ g/L CH_3COONa.

Inoculum Preparation: The chosen species was grown for 18 h in nutrient broth. This was subcultured 1:1,000 into 20 ml nutrient broth and grown in a shaking incubator (37°C, 120 rpm) until an OD_{600} of 0.21 was reached (if overgrown diluting as appropriate). Cells were diluted 1:100,000 and 1 ml if this inoculum was added to 1 L prepared water giving an approximate CFU of 250 / 100 ml.

Sampling Processing (Membrane Filtration): *E. coli* was sampled as described by EPA (2009) documentation. Samples were membrane filtered (0.45 µm), membranes were placed on MLGA and grown at 37°C for 24 hours. For detailed EPA Membrane filtration method (2009) see Appendix 1.

Recovery of bacteria from filter substrate: (adaption of MSU Centre for biofilm Engineering, 2003)

1 g of substrate was placed in a 20 ml test tube with 10 ml ringer maximum recovery diluent. Sample tube placed in solicitor for 60 seconds. Tube removed, gently inverted three timed, and sonicated again for 60 seconds. Samples were then diluted, membrane filtered, placed on appropriate agar and incubated for the appropriate time

Experiment Procedure: ***Phase 1*** **(contamination/colonisation of pathogens - Fig. 1) –** The equipment was set up as shown in Figure 1. One litre of contaminated water was pumped through a mature biological filter four times. Water and substratum were sampled prior to filtration, and after each

filter pass. The number of pathogenic species in the water and on the substratum was tracked and the ultimate fate of the pathogens within the system was determined.

Experiment Procedure: *Phase 2* (Survival and fate of pathogens) - After the water has passed through the system four times. The water was disposed of and fresh water was circulated for 24-hours, before being sampled and replaced again. This was repeated every 24 hours until no more pathogenic species were detectable. Both the substratum and water were sampled every 24-hours. Enabled the tracking of pathogenic species and an assessment of; how readily pathogenic species attach/persists on the substratum, and at what rate it may sheer off into the circulating water body.

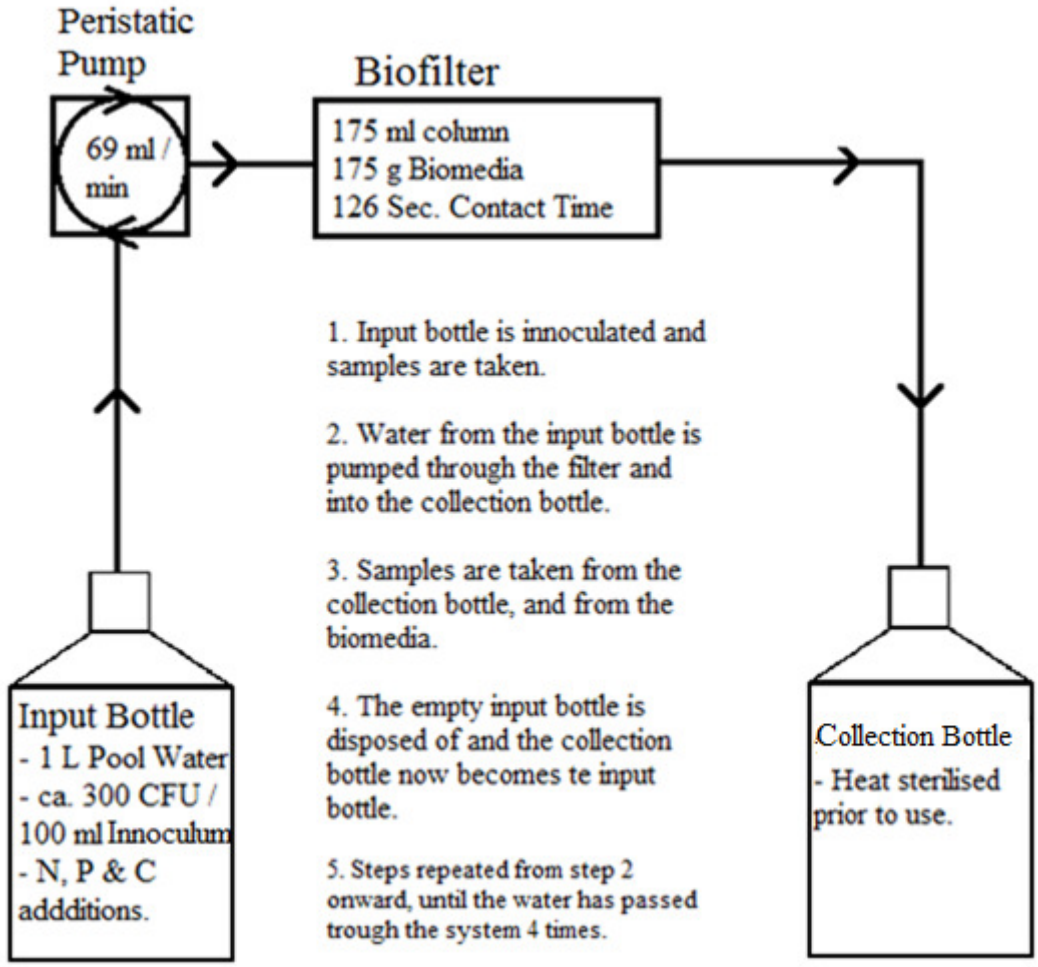


Figure 1. The experiment was set up as shown. The image in this figure details phase 1 of the experiment. This phase was followed by phase 2, where the contaminated water was replaced with uncontaminated water, which was circulated, before being sampled and replaced again - showing rate sheering from the substrate each subsequent 24 hours post-contamination.

Results

P. aeruginosa 4x Filter Pass (adherence) - Phase 1

Removal of *P. aeruginosa* from the Filtered Water: Figure 2 shows a stark difference between the mature biofilm and the no biofilm substratum. The mature biofilm removed, on average, 76% of *P. aeruginosa* over 4 passes (reduction of 797 CFU from 1050 CFU after 4x filter passes). Sterile substratum, with no biofilm, removed 24 % of *P. aeruginosa* from the water (192 CFU / 4x Filter passes).

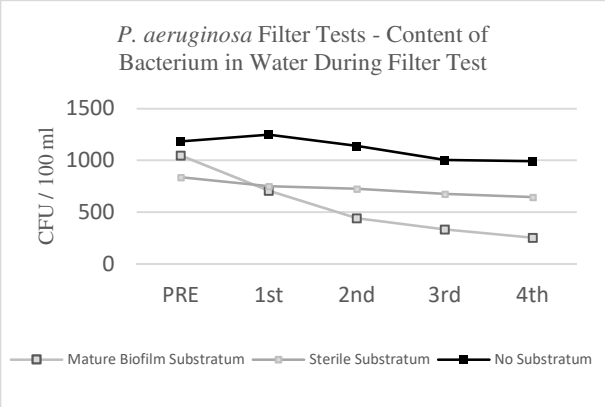


Figure 2. Inoculated water was filtered 4 times (n=9). Samples from the water were taken after each filter pass. Mature biofilm substratum consistently showed significant amounts of *P. aeruginosa* removed from the water. The mature substratum reduced *P. aeruginosa* water content by 76 % after 4 passes). While, the sterile substratum showed a reduction of 24 % after four passes (reduction of 192 CFU after 4 passes).

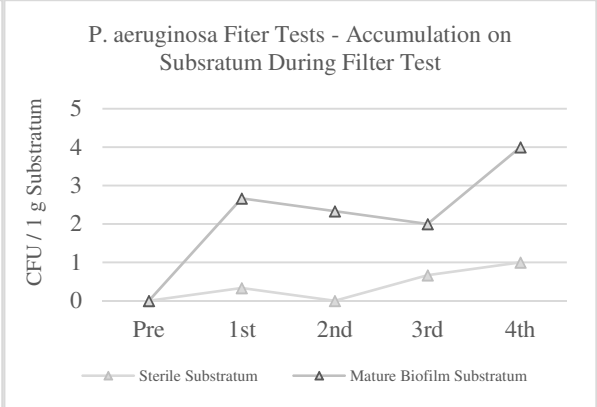


Figure 3. Water inoculated with *P. aeruginosa* was filtered 4 times (n=9). Samples were taken from the substratum after each complete filter pass. Mature biofilm substratum saw greater adherence (ca. 4 CFU / 1 g after 4 passes through the filter; 66 % removal). Sterile substratum, with no biofilm, saw little *P. aeruginosa* adherence compared to the mature biofilm substratum (ca. 1 CFU / 1 g), and little removal from the water (totalling 15 % removal over 4 passes).

P. aeruginosa Survival post contamination - Phase 2

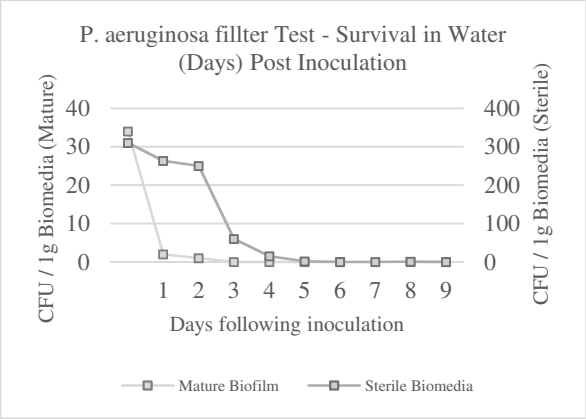


Figure 4. *P. aeruginosa* was recoverable from the sterile media for 3 days longer than on the mature media. It took 6 days for total removal compared to 8 days for the sterile media.

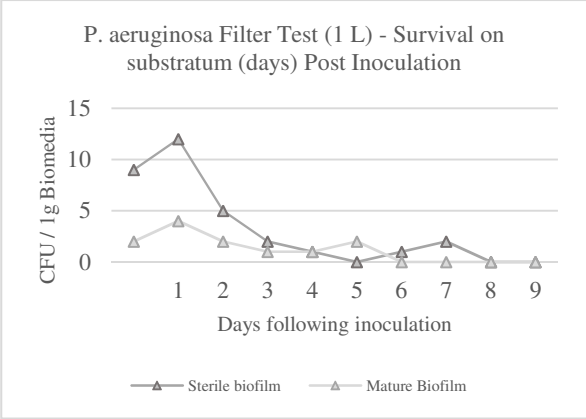
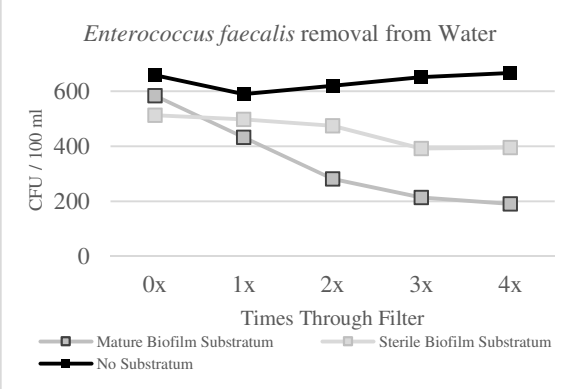


Figure 5. Huge reduction seen in the mature filter over the first 48 hours and by the 3rd day no *P. aeruginosa* was detectable. The sterile filter did see a significant reduction over the first 24 hours, but took 6 days until cells reached the limit of detection

E. faecalis 4x Filter Pass (adherence) - Phase 1



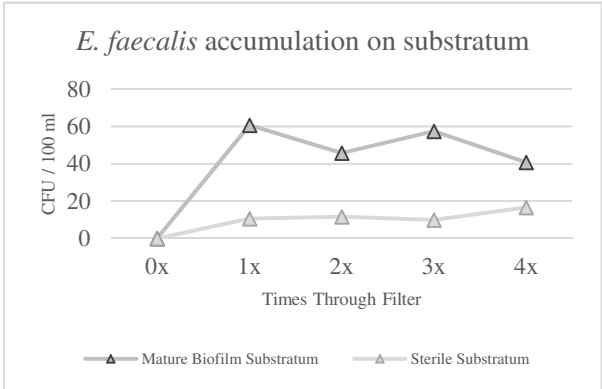


Figure 6: The mature biofilm removed *E. faecalis* from the water much quicker than the sterile and no biomedia filters. This is thought to be due to the extracellular polymeric substance produced by the mature biofilm. The Fresh (sterile) media shows little reduction from the water body, while the negative control (no media) show no reduction over the 4 passes.

***E. faecalis* Survival post contamination- Phase 2**

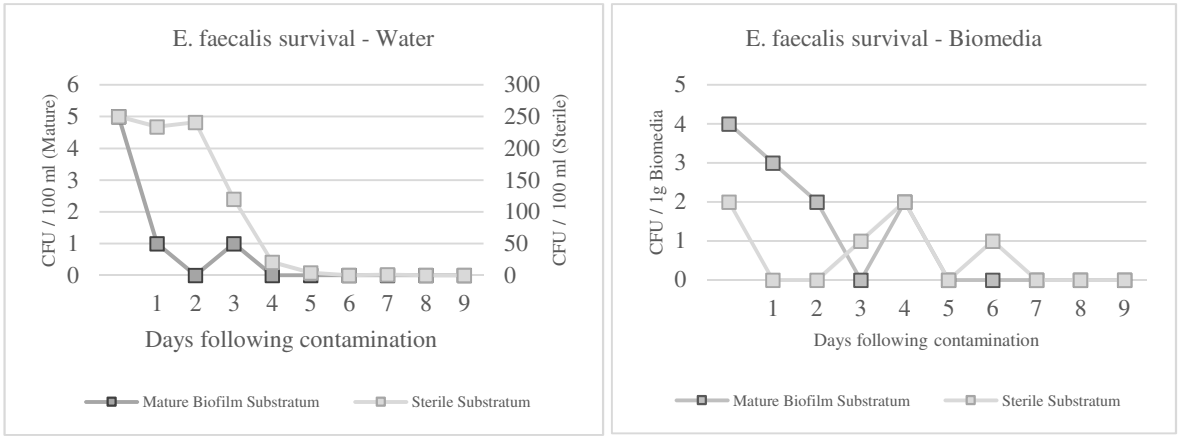


Figure 7: Greater accumulation can be seen on the mature biofilm compared to the sterile media. Showing the capacity of the mature media to remove *E. faecalis* cells from the water body.

***E. coli* 4x Filter Pass (adherence) - Phase 1**

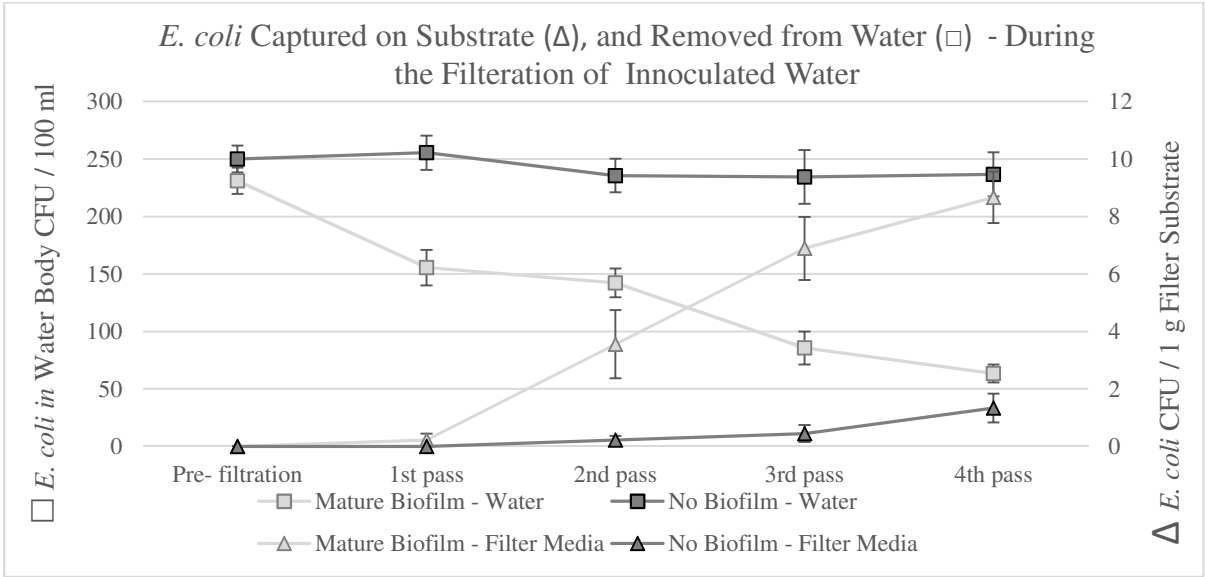


Figure 8. Contaminated water was filtered 4 times (n=9). Samples from the substratum and water were taken after each filter pass. Mature biofilm substratum consistently showed significant amounts of *E. coli* removed from the water after each filter pass (totalling 73% removal over 4 passes). Mature biofilm substratum also saw greater adherence (ca. 8 CFU / 1 g). Sterile substratum, with no biofilm, saw little *E. coli* adherence (ca. 1 CFU / 1 g), and little removal from the water (totalling 5% removal over 4 passes).

E. coli Survival post contamination- Phase 2

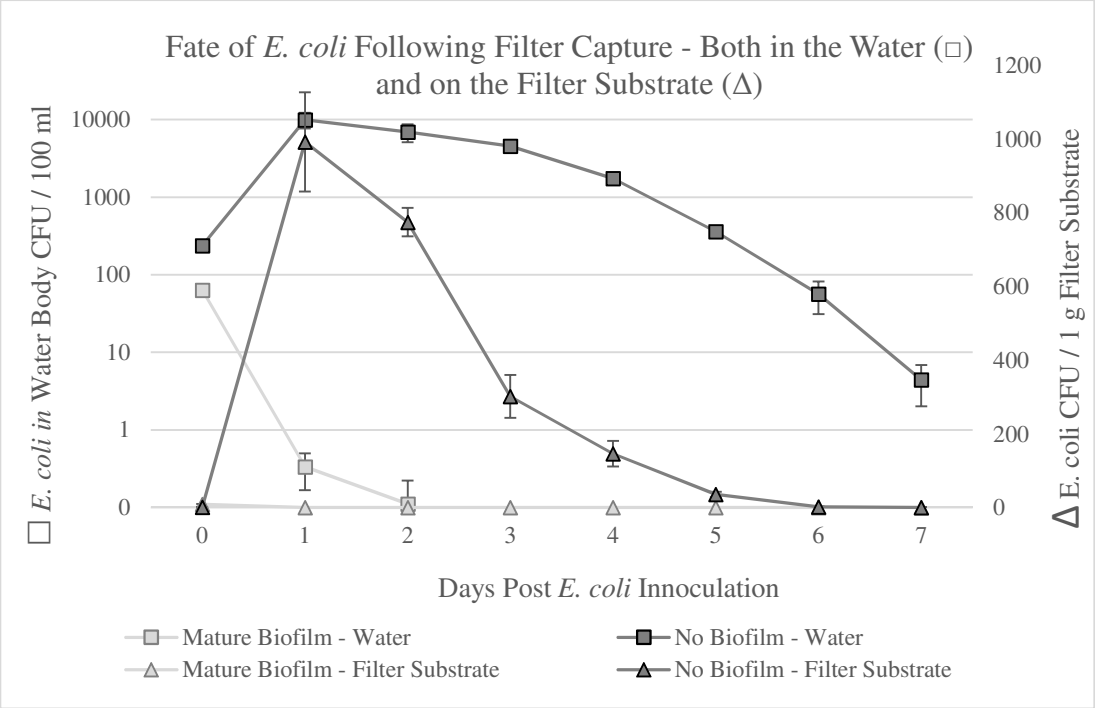


Figure 9. Substratum contaminated during phase 1 of the experiment was left to circulate, substratum and water samples taken every 24 hours. Mature biofilms saw a rapid reduction in *E. coli* over the first 24 hours, difficult to see on this graph due to the right hand scale. The sterile substratum, with no biofilm, saw an increase in *E. coli* over the first 24 hours, followed by a reduction, lasting 6 days until no more *E. coli* was recoverable.

Real-Time Bioluminescent *E. coli* Assay

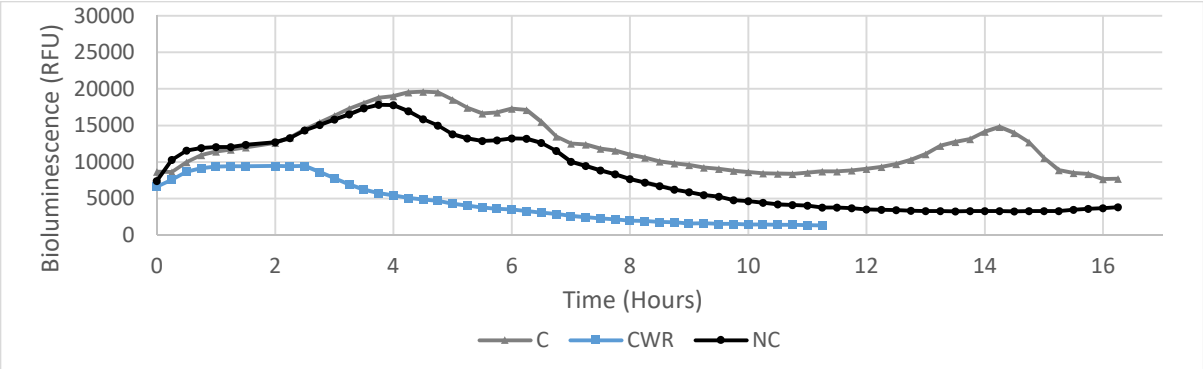
This experiment was conducted using a bioluminescent *E. coli* as a real-time observation of biofilter pathogen remediation. This was utilised for its ability to observe the bioremediation of a mature biofilm in real-time using a bacterial bioluminescent strain. In addition to this, the videos made using this method are visually impressive and could provide a useful tool for reassuring the public that biological filters are not havens for pathogens and that when properly maintained can remediate a contamination event in a relatively short amount of time.

Method

This experiment was set up in the same way as the first experimental configuration (four filter pass), except phase 1 was replaced with a real-time bioluminescent assay. This was done using a bioluminescent strain of *E. coli* (Nissle 1917 pGlite) and measured using a CCD camera. This allowed for real time tracking of *E.coli* within the system. However, due to the biofilter being polymicrobial, the antibiotic that selects for this gene could not be used within this system. Therefore, this real-time tracking can only maintained for as long as the plasmid is retained. Once *E. coli* completely loses the plasmid in all three configurations, phase 2 is implemented in the same way as the first experiment.

Results

a)



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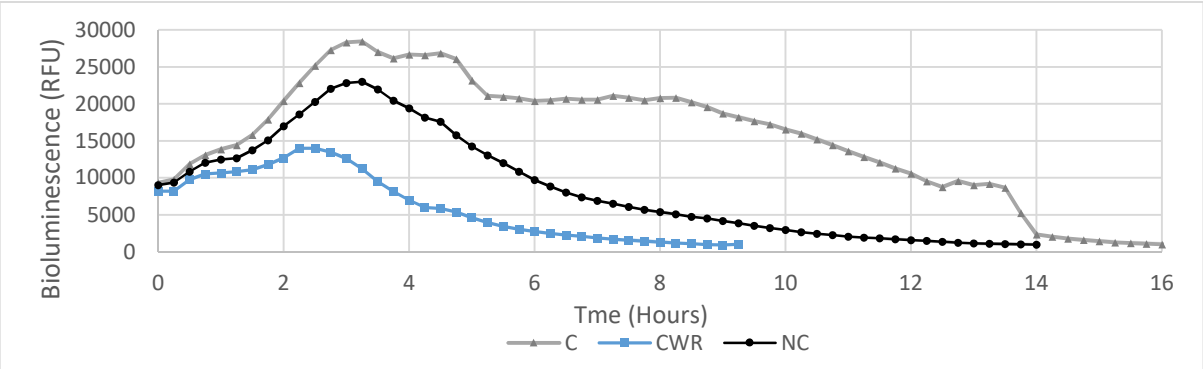


c)

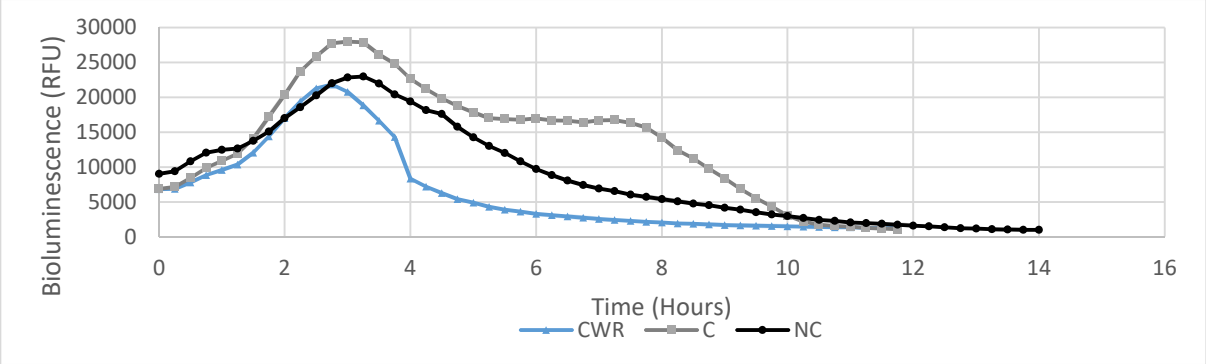
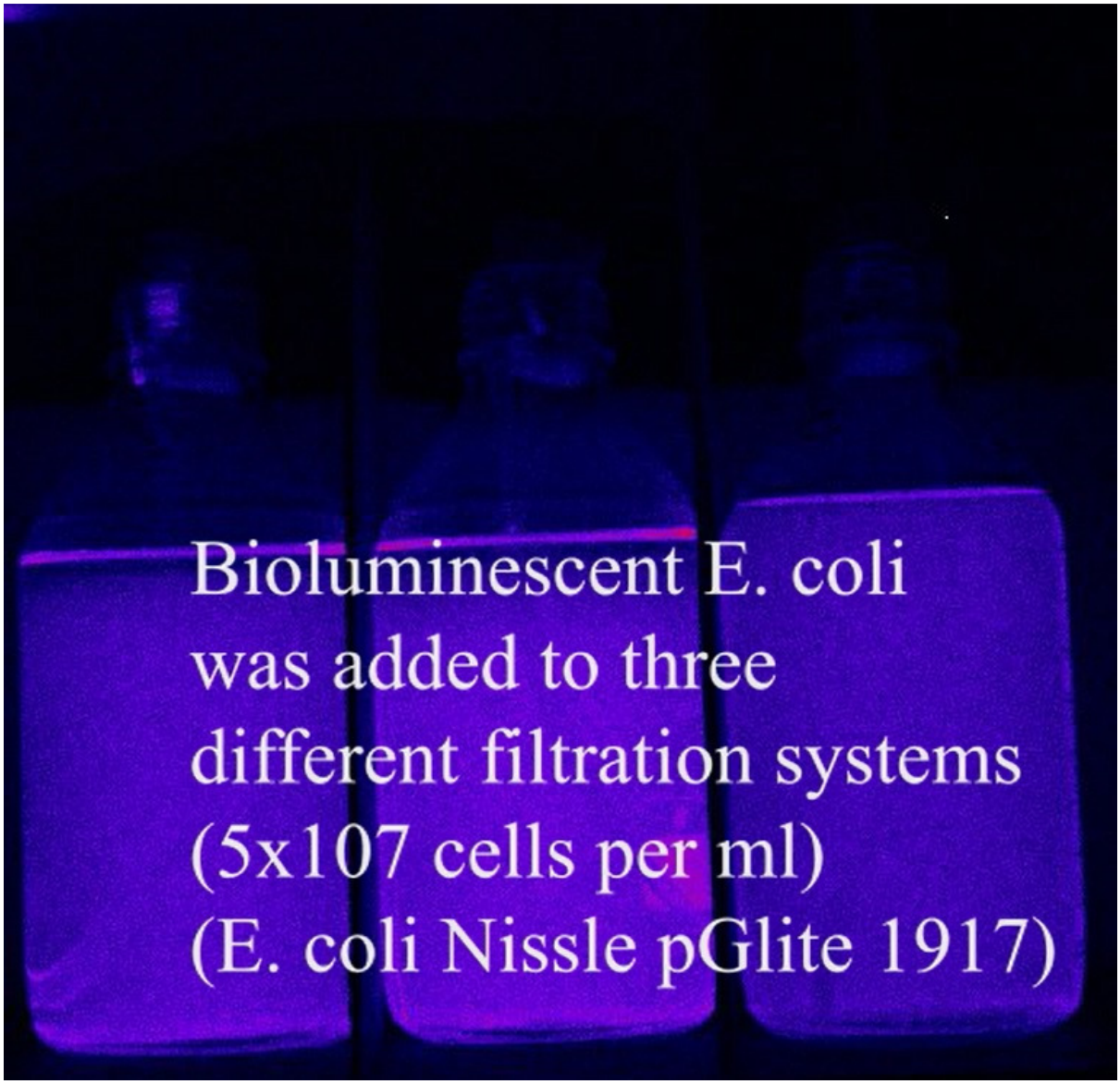
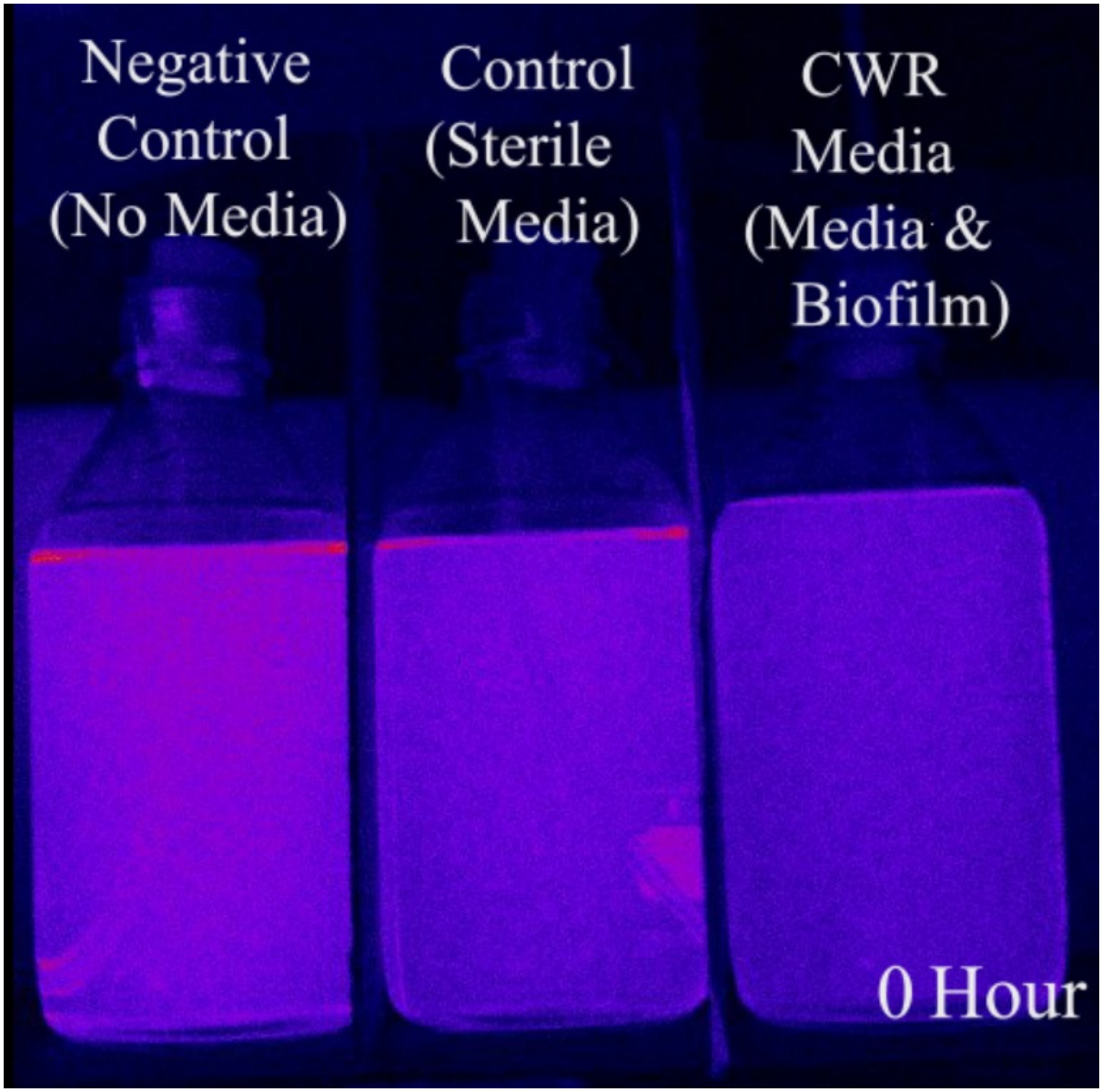
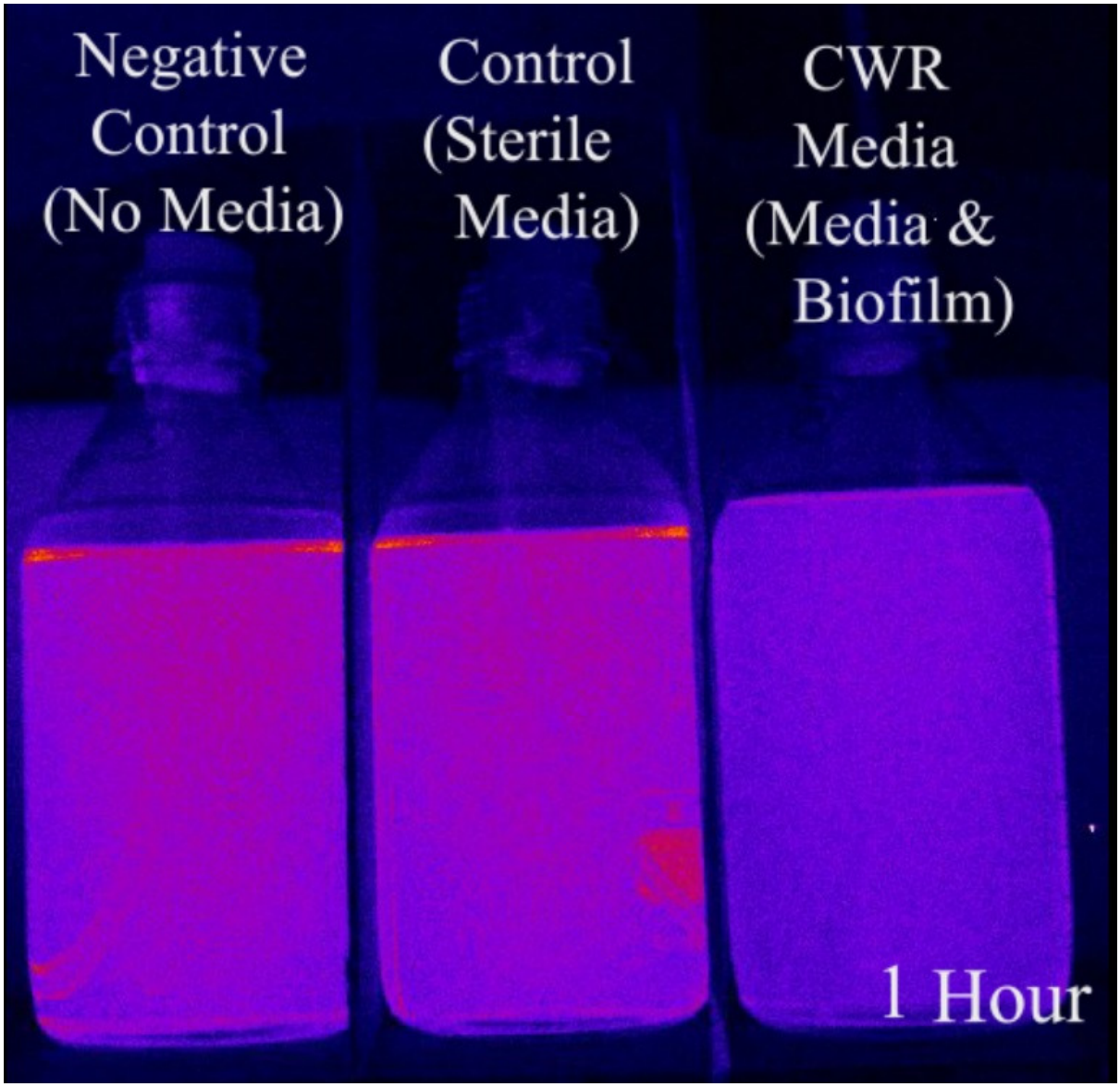


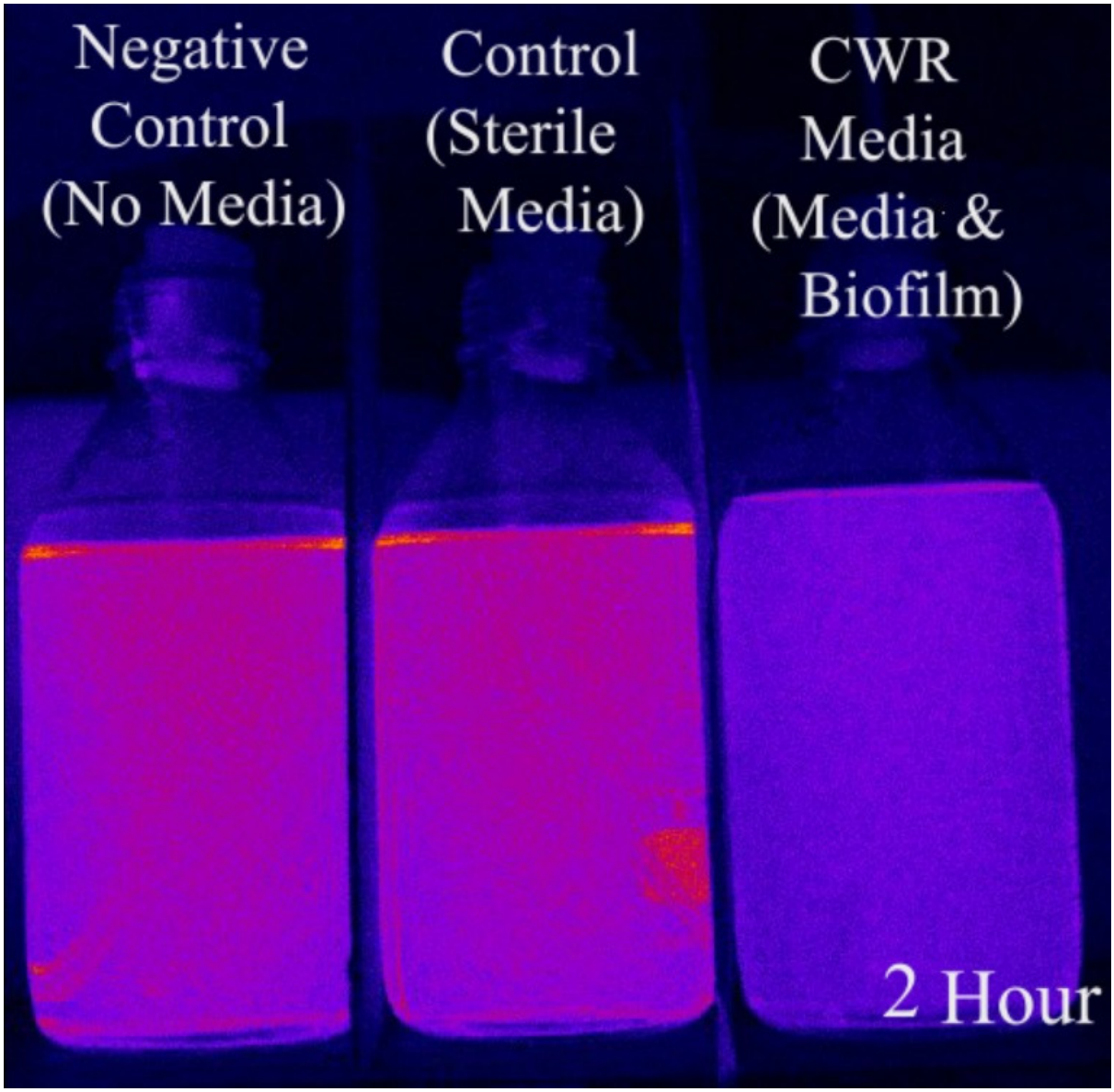
Figure 10a, 10b and 10c. Filtration systems were inoculated with bioluminescent *E. coli*, water was circulated and images were taken using a CCD every 30 seconds (n=1 per graph, n=3 repeat). A similar trend can be seen across all three repeats. The mature biofilm substratum consistently showed *E. coli* decline hours before the natural plasmid loss. This confirms the theory that predation, competition and nutrient availability play a significant role in controlling non-indigenous micro biota.

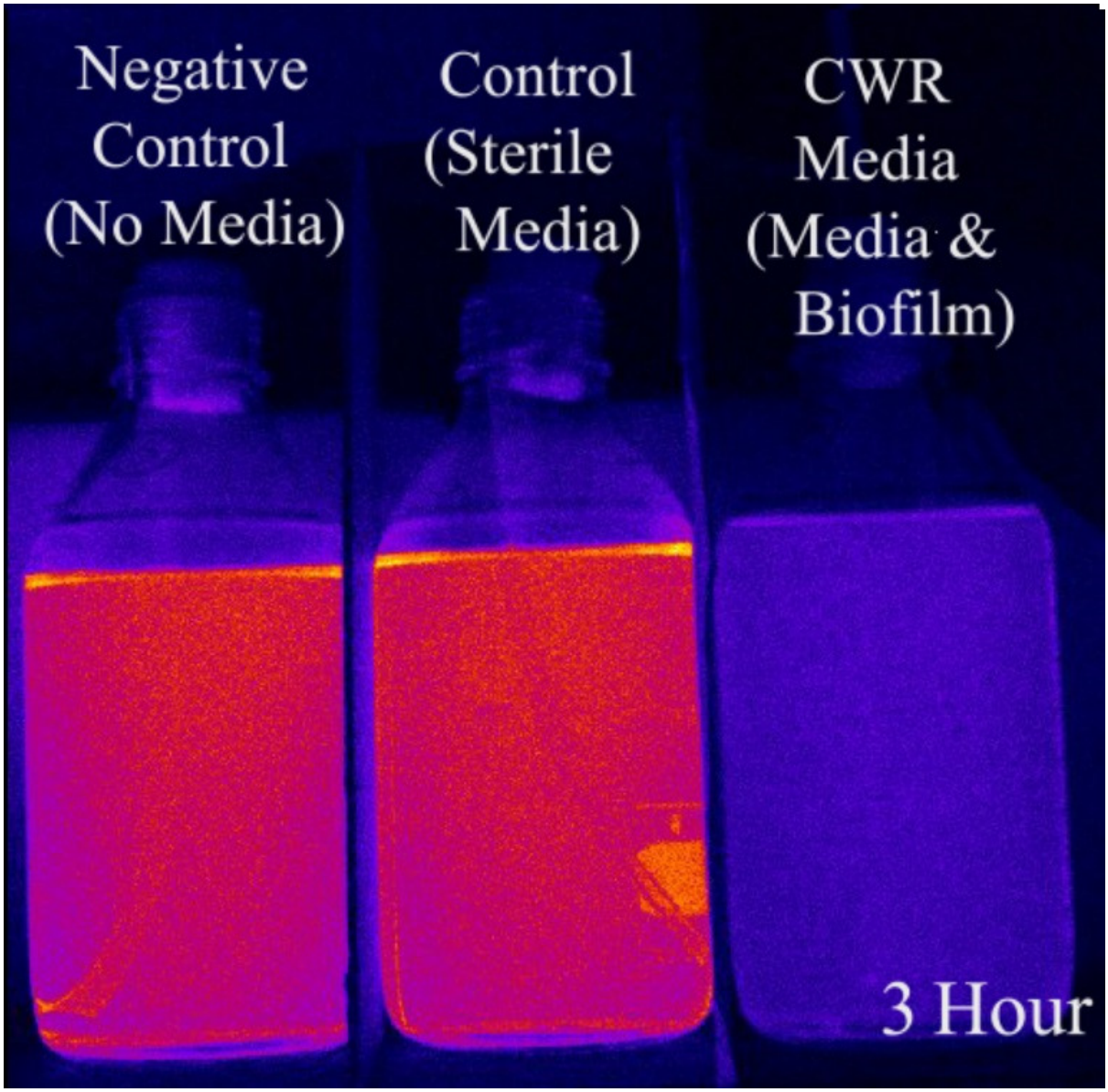
STILLS TAKEN FROM BIOLUMINESCENCE EXPERIMENT




Negative Control (No Media)
Control (Sterile Media)
CWR Media (Media & Biofilm)
0 Hour


Negative Control (No Media)
Control (Sterile Media)
CWR Media (Media & Biofilm)
1 Hour


Negative Control (No Media)
Control (Sterile Media)
CWR Media (Media & Biofilm)
2 Hour



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