

NMSBA: Aken Technologies

Final Report: Toxicity Testing of Liquidoff

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Bacteria Toxicity Testing

To determine the effect of Liquidoff on bacteria, three bacterial strains were tested: *Escherichia coli* DH5 α , *Synechococcus* sp. PCC 7002, and *Synechococcus elongatus* PCC 7942. *E. coli* DH5 α is a Gram-negative, aerobic bacterium that is often found in normal gut flora and is commonly used in the laboratory due to its fast growth rate. *Synechococcus* sp. PCC 7002 and *S. elongatus* PCC 7942 are Gram-negative, aquatic, autophototrophic cyanobacteria. *Synechococcus* sp. PCC 7002 is a marine cyanobacterium isolated from 'fish pens' on Magueyes Island, Puerto Rico in 1962, while *S. elongatus* PCC 7942 is a freshwater cyanobacterium. It should be noted that no Gram-positive bacterium was tested in this study.

To test the toxicity of Liquidoff for each bacterium, a range of Liquidoff concentrations were added to test tubes containing 3 mL of growth medium. Sterilized water was added to the cultures so that the final volume in each test tube was 4 mL. Luria Broth, Medium A+, and BG-11 Medium were used as the growth media for *E. coli* DH5 α , *Synechococcus* sp. PCC 7002, and *S. elongatus* PCC 7942, respectively. Approximately 20 μ L of bacterial culture was added to each test tube, and the test tubes were placed under appropriate growth conditions for each bacterium (37°C, 200 rpm for *E. coli* DH5 α and 30°C, 150 rpm, and 50 μ mol photons $m^{-2}s^{-1}$ for both *Synechococcus* sp. PCC 7002 and *S. elongatus* PCC 7942). A control, containing the highest percentage of Liquidoff in media and no bacterial culture, was added to determine if any bacterial growth is resulting from the unsterilized Liquidoff solution. The *E. coli* DH5 α cultures were grown for approximately 16 hours, while *Synechococcus* sp. PCC 7002 and *S. elongatus* PCC 7942 were cultivated for approximately 5 days. As Liquidoff is not soluble in aqueous solution, the Liquidoff phase separated from the growth medium (Figure 1). Therefore, we used multiple methods to analyze the cultures. First, images were taken to show the distribution of the bacterial culture in the two phases (Liquidoff – top phase, growth medium – bottom phase). Then, culture was extracted from the growth medium phase, and the optical density of this phase was measured to determine the cell density. The optical density for *E. coli* DH5 α was read at 600 nm while the optical densities for *Synechococcus* sp. PCC 7002 and *S. elongatus* PCC 7942 were measured at 730 nm, standard practice for these organisms. The optical density reading for the culture with no Liquidoff addition was set to be 100% growth, and the remaining cultures were normalized to this control (Figure 2). Triplicate biological replicate experiments were performed to assess reproducibility of the results.

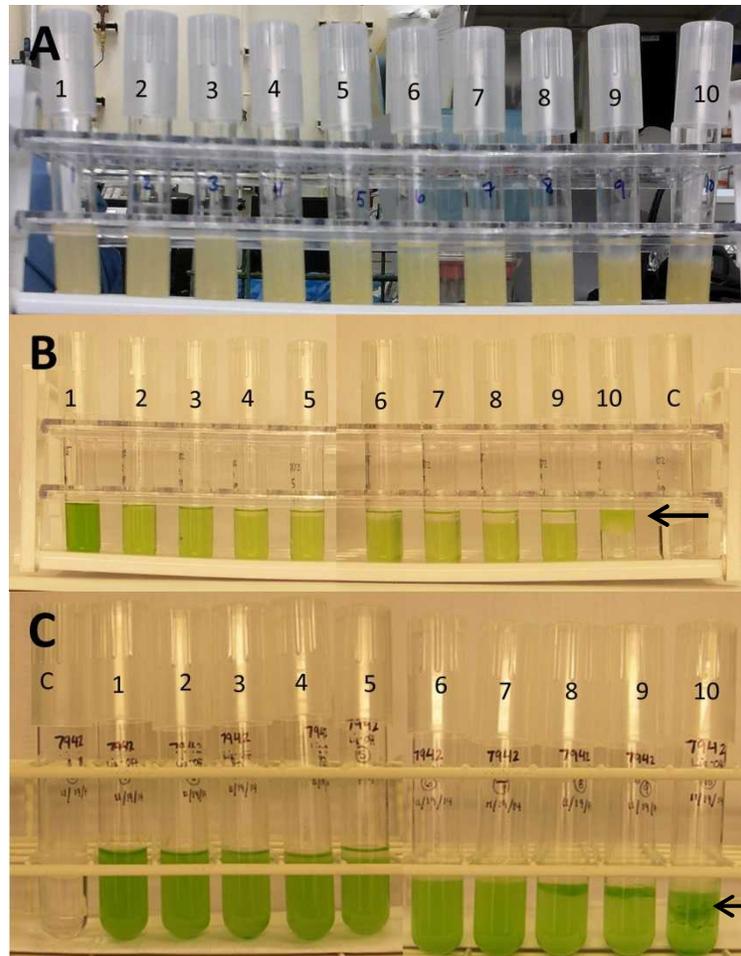


Figure 1. Images of *E. coli* DH5 α (A), *Synechococcus* sp. PCC 7002 (B), and *S. elongatus* PCC 7942 (C) cultures as a function of Liquidoff concentration after 16 h or 5 days of growth. C = no bacteria, Liquidoff concentrations: 1 = 0%, 2 = 1.25%, 3 = 2.5%, 4 = 5%, 5 = 7.5%, 6 = 10%, 7 = 12.5%, 8 = 15%, 9 = 20%, 10 = 25%, C = 25%. Arrows point to bacterial growth in the Liquidoff phase.

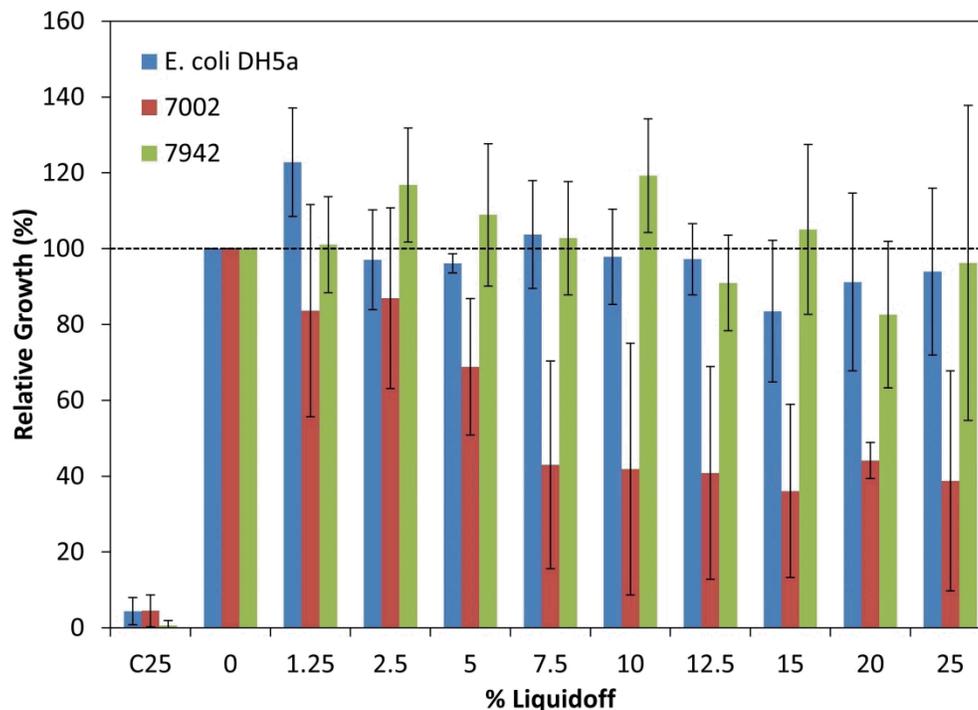


Figure 2. Growth inhibition of *E. coli* DH5 α , *Synechococcus* sp. PCC 7002, and *S. elongatus* PCC 7942 as a function of Liquidoff concentration after 16 h or 5 days of growth. Growth is relative to the culture without Liquidoff. Data is the average of three biological replicate experiments. Error bars indicate the standard deviation of the three measurements. The dashed line indicates 100% growth or no growth inhibition.

From the images and optical density measurements (Figures 1 and 2), it appears that Liquidoff does not inhibit the growth of *E. coli* DH5 α and *S. elongatus* PCC 7942. However, there is measurable growth inhibition of *Synechococcus* sp. PCC 7002 at Liquidoff concentrations greater than 2.5%. Interestingly, cyanobacterial growth was observed in the non-aqueous, Liquidoff phase (Figure 1, arrows). Therefore, while there appears to be some growth inhibition for *Synechococcus* sp. PCC 7002, the bacterial cells associated with the non-aqueous phase are not accounted for in the optical density measurements of growth in Figure 2. Overall, the results show low toxicity for most Gram-negative bacteria; however, these results appear to be strain dependent. Additional testing of other Gram-negative bacteria as well as Gram-positive bacteria is required to provide an accurate assessment of toxicity for bacteria.

Due to the difficulty in testing Liquidoff toxicity with aqueous solutions, we also tested for toxicity by spreading a culture on an agar plate of media and placing a drop of Liquidoff in the center of the plate. The plate was then incubated under growth conditions. If Liquidoff inhibits bacterial growth, there will be a spot in the center of the plate where no bacteria grow; if there is no growth inhibition with Liquidoff, bacteria will grow uniformly across the plate in what is known as a bacterial ‘lawn’. Drops of water and antibiotic (kanamycin) were also tested as positive and negative controls (i.e. there should be no growth inhibition from water and complete growth inhibition with the antibiotic). Only the two

cyanobacterial strains, *Synechococcus* sp. PCC 7002 and *S. elongatus* PCC 7942, were tested in this manner. The results are shown in Figure 3.

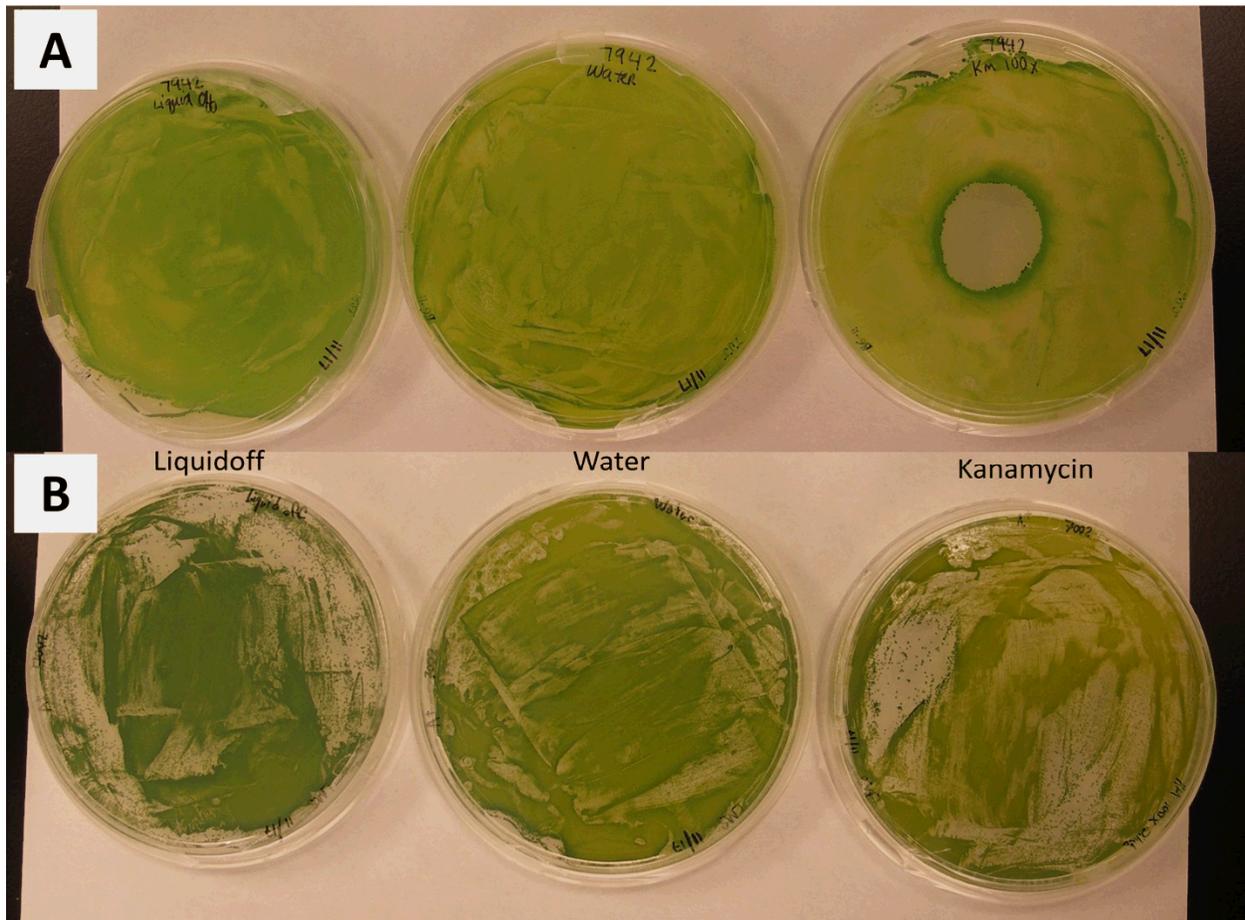


Figure 3. Solid medium toxicity testing for *S. elongatus* PCC 7942 (A) and *Synechococcus* sp. PCC 7002 (B). After spreading culture on the plates, a drop was placed in the center: Liquidoff (left plate), water (middle plate), and kanamycin at 500 µg/mL (right plate).

From Figure 3, there appears to be no growth inhibition observed with Liquidoff for either *Synechococcus* sp. PCC 7002 or *S. elongatus* PCC 7942. Unexpectedly, the antibiotic control for *Synechococcus* sp. PCC 7002 did not inhibit growth. Despite this anomaly, these results suggest that Liquidoff is not toxic to *Synechococcus* sp. PCC 7002, indicating that the growth inhibition observed in Figure 2 for *Synechococcus* sp. PCC 7002 may be due to partitioning of the cells in the nonaqueous Liquidoff phase. Overall, this indicates little to no toxicity of Liquidoff for Gram-negative bacteria.

Human/Mammalian Tissue Culture Toxicity Testing

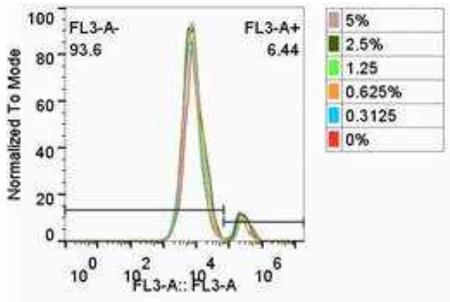
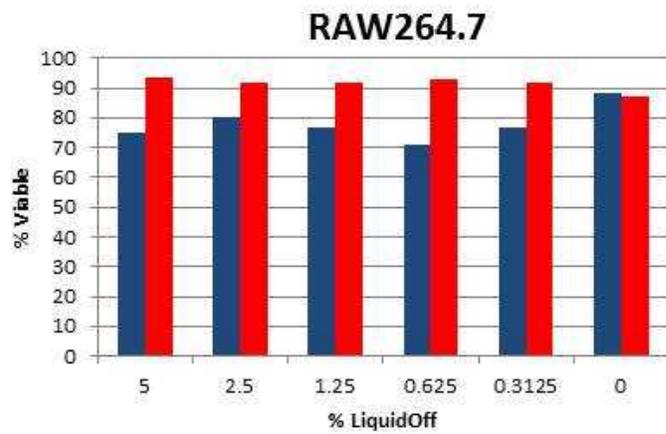
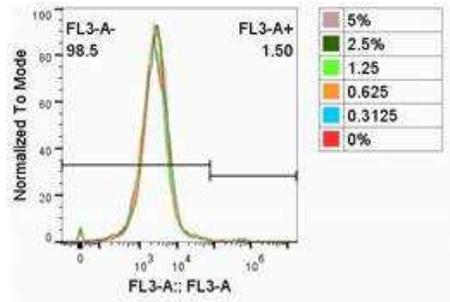
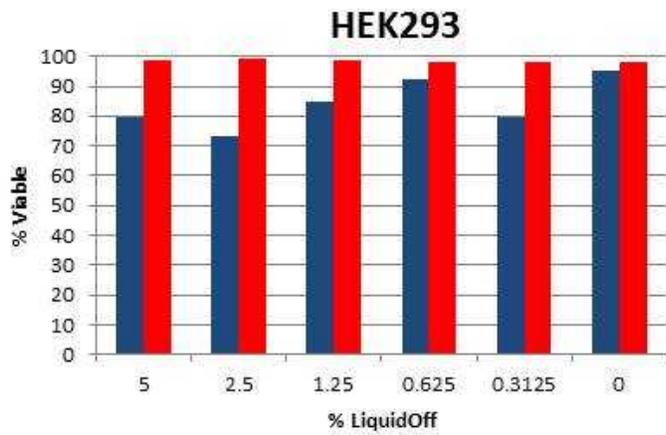
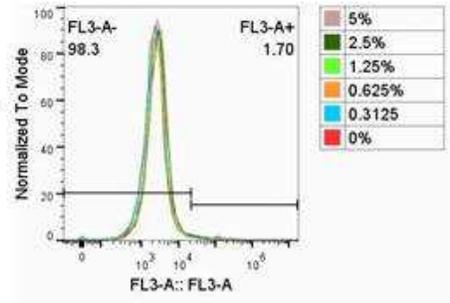
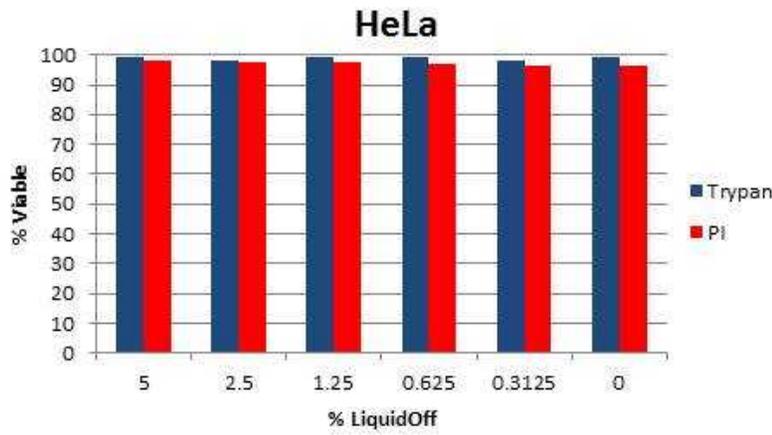
To perform a preliminary determination of potential human toxicity (e.g. skin, mucous membranes) of Liquidoff, three adherent mammalian cell lines were cultured in the presence or absence

of varying Liquidoff concentrations. Following 24hr of exposure, viability was measured by both propidium iodide (flow cytometry) and Trypan blue (cell counter) exclusion.

Cell lines tested were HeLa (human cervical tumor origin), HEK293 (human embryonic kidney origin), and RAW264.7 (mouse macrophage-like cell line). Each cell line was cultured separately in DMEM-10 medium for 24hrs in 6-well tissue culture plates such that each reached 25-50% confluence. At that time, Liquidoff was added at 5.0%, 2.5%, 1.25%, 0.63%, 0.32%, or 0% to separate wells then plates were swirled to mix for 10 seconds. After 24hrs of incubation with Liquidoff, HeLa and HEK293 cells were harvested by trypsinization and RAW264.7 cells were harvested using non-enzymatic cell dissociation solution (Cell Stripper, Mediatech). Cell suspensions were then centrifuged (200xg, 7min) and resuspended in 1ml PBS. Aliquots of the cell suspension were then combined with either trypan blue (0.2% final) or propidium iodide (100ug/ml final) and incubated for 5min (Trypan) or 15min (propidium iodide). Trypan-incubated cells were counted using a Bio-Rad TC-10 automated cell counter and % viable cells values were recorded. Propidium iodide-stained cells were quantified by flow cytometry using an Accuri C6 instrument and FlowJo software.

Because Liquidoff is a hydrophobic fluid less dense than water, the substance beaded and remained on the surface of the cell culture media in the plate wells. However, the plates were mixed orbitally in an attempt to release any water-soluble components into the culture medium for exposure to the cells adhered to the bottom of each well. When the culture medium was aspirated prior to cell harvest, care was taken to avoid direct contact between the superficial (floating) Liquidoff layer and the cell lawn. However, this appeared to be inevitable so the Liquidoff most likely came into direct contact with the cells during harvest prior to washing with PBS. Nevertheless, viability did not appear significantly affected by Liquidoff at any concentration between 0-5%. Positive cell death controls were omitted due to lack of time and funding, but it should be noted that there was little difference between viability of Liquidoff-treated cells and medium alone control cells.

Figure 4. Viability of three mammalian cell lines following 24hrs of culture in the presence of 0-5% Liquidoff. Bar charts on left show comparative Trypan blue and propidium iodide exclusion viability data. Histogram overlays on right show propidium iodide viability comparison and gating (plots pre-gated on non-debris events).



Conclusions

- Minimal growth inhibition was observed for three species of Gram-negative bacteria, suggesting low toxicity.
- Slight growth inhibition was observed for *Synechococcus* sp. PCC 7002 at high Liquidoff concentrations. However, it is likely that this perceived inhibition is due to partitioning of the cells in the non-aqueous phase, as no significant inhibition was detected using solid medium.
- Three distinct lineage mammalian cell lines showed no obvious loss in viability after 24hrs of culture in the presence of Liquidoff, suggesting the substance has low or no human toxicity related to water-soluble components. Future tests should include extended incubation times and perhaps pre-extraction of the Liquidoff with cell culture media to better represent exposure to water-soluble components. In addition, a means of direct exposure (rather than through aqueous media) could be used, e.g. using supported dermal and epithelial cell cultures.



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