Investigating the Effects of Bis (2-ethylhexyl) Phthalate (DEHP) on the Growth of *Escherichia coli* strain BL21

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SUMMARY Phthalates (also known as phthalate esters) are organic chemicals used as plasticizers in a wide range of food packaging, containers, and other household plastic objects. Humans are exposed to phthalates through the air and by ingestion. Phthalate exposure has been shown to induce changes to the microbiome, causing shifts in the resident microbiota in a dose-dependent manner. One phthalate in particular, Bis(2-ethylhexyl) phthalate (DEHP), is known to be present at high concentrations in Canadian food packaging. Previous research has found that DEHP has biphasic effects on the growth of *Escherichia coli*. To further investigate the effect DEHP may have on the growth of *E. coli* relevant to the human gut, our research set out to determine the effects of dose-dependent exposure on *E. coli* BL21. To do this, minimum inhibitory concentration (MIC) and growth curve assays were conducted. In the MIC assay, growth was observed at all concentrations, with a limited inhibitory effect on *E. coli* growth. However, phase separation between the DEHP solution and *E. coli* culture media made it difficult to determine whether growth inhibition was due to the DEHP or to inadequate aeration of the growing culture. The addition of ethanol reduced the phase separation between DEHP and *E. coli* culture media. However, subsequent growth curves showed conflicting results suggesting that the addition of ethanol may be the reason for reduced growth rate.

INTRODUCTION

Phthalates (also known as phthalate esters) are organic chemicals used as plasticizers in a wide range of food packaging, containers, and other household plastic objects (1). Independent meta-analyses have detected phthalates in food packaging, citing Bis(2-ethylhexyl) phthalate (DEHP) as the most commonly used phthalate in commercial and food products (1, 9) in Canada. From such environments, phthalates commonly leak out into the environment and diffuse into food where they are capable of eliciting variable effects on bacterial growth; promoting it in some cases while eliciting cytotoxic effects in others (3).

In an environmental study using *Escherichia coli* and *Bacillus subtilis,* Sandy *et al.* (2010) observed growth promotion in both species at DEHP concentrations lower than 300 μg/mL. However, at concentrations higher than 300 μg/mL DEHP inhibited growth; causing cell elongation that was detectable by scanning electron microscopy (3).

Another important route of bacterial exposure to phthalates is through ingested food and drinks. Bioinformatic studies that utilize whole genome shotgun sequencing methods and environmental molecular genetics analyses were used to infer the effects of phthalates at the population-level of gut bacteria (2). Hu *et al.* (2016) used a murine model to detect changes in microbiome composition as a result of phthalate exposure (2), via whole genome shotgun sequencing, enrichment of gut microbiota, and performing PCR and sequence clustering of 16s rRNA isolated from mice stool. It was revealed that there was a significant microbiota shift in taxonomic composition when common phthalates were introduced into mice diets (2). The exposed mice exhibited lower body weight the authors partially attributed to a reduced *Firmicutes:Bacteroidetes* ratio. However, research into phthalates' ability to influence growth cycles of specific gut bacterial species has yet to be explored. Given that the constituents of the human gut microbiota play a vast role affecting everything from digestion to immune modulation (4), a deeper understanding of the impact phthalates have on commensal gut bacteria could provide insight into how chemicals we encounter every day in plastics and food consumption may be affecting our gut microbiota, and by extension, our health.

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In this investigation, the effects of varying concentrations of DEHP on the growth of *E. coli* BL21 are studied using optical density (OD) as a measure of cell culture density. Since phthalates have been shown to result in morphological effects in environmental studies (3), the morphology of cells was also examined to examine and confirm ways in which phthalates affect *E. coli* BL21. This organism is a non-pathogenic bacterium akin to *E. coli* B strains common in the guts of North Americans (6), and is implicated in producing proinflammatory secretions in the intestine (5). Due to the diverse characteristics and roles of *E. coli* in the gut, examining dose-dependent effects of phthalates could also provide insight into phthalate-related human disease manifestations associated with the digestive system.

It was hypothesized that patterns of biphasic growth could be observed in *E. coli* BL21 as a result of varying levels of phthalate exposure in growth media, similar to those previously observed in recent studies involving *E. coli* DH5α and *B. subtilis* (3). It was expected that a similar range of concentrations would either promote cell growth or toxicity – as determined by bacterial morphology and growth curves – depending on the concentration of DEHP in the growth media. Specifically, the growth and morphological effects on *E. coli* BL21 when grown in the presence of varying concentrations of DEHP was examined.

The study had two primary aims. First, to determine a minimum inhibitory concentration (MIC) of DEHP on *E. coli* BL21. Second, to generate growth curves for *E. coli* BL21 in growth media containing varying sub-lethal DEHP concentrations as determined from the minimum inhibitory concentrations.

METHODS AND MATERIALS

Bacteria Strains. *E. coli* strain BL21 was provided by the University of British Columbia Microbiology 421 teaching laboratory along with the Beckman spectrophotometer for optical density measurements. 98+% DEHP was acquired from Alfa Aesar (Catalog # CAAAA10415-36). For each 500mL of Lysogeny Broth (LB) growth media prepared; 2.5 g of yeast extract, 5 g of tryptone and 5g of sodium chloride were added to deionized water and autoclaved at 121°C. For all growth setups, *E. coli* BL21 were aerobically grown in slanted tubes at 37°C on a shaker at 150 rpm.

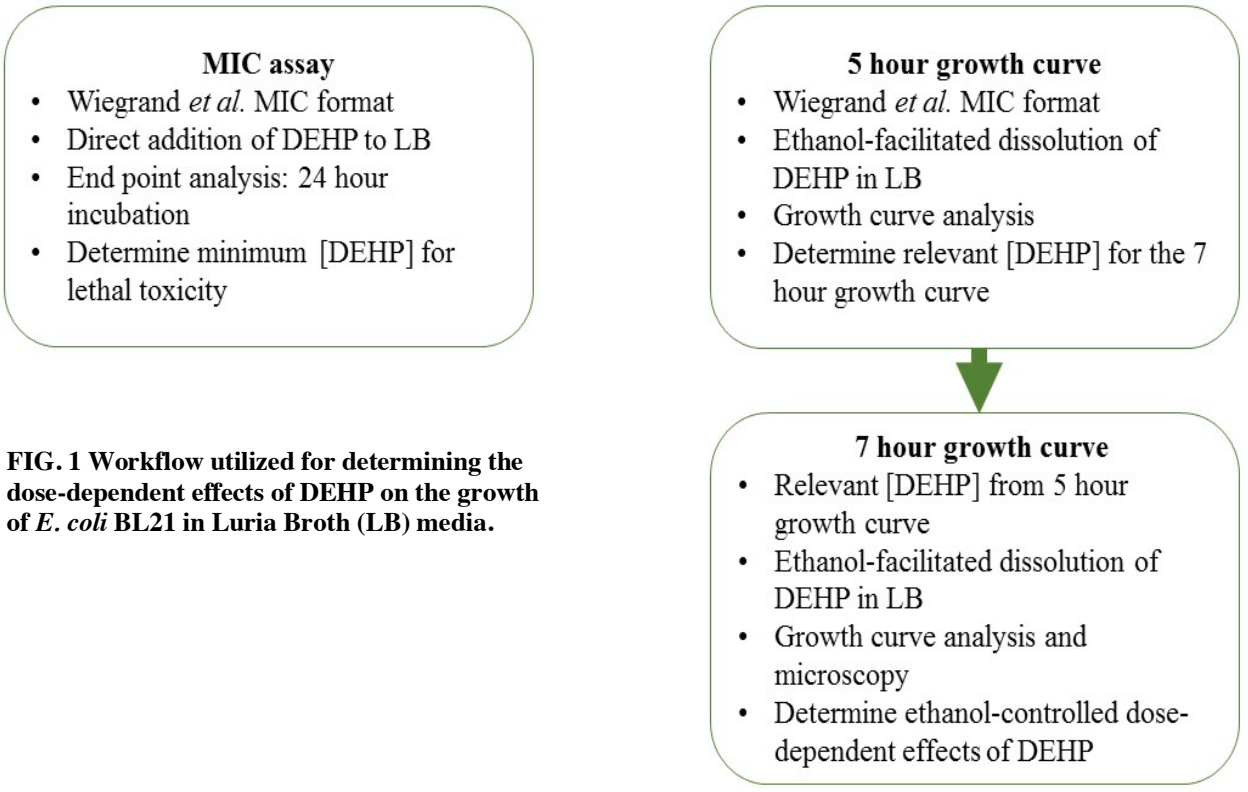

FIG. 1 Workflow utilized for determining the dose-dependent effects of DEHP on the growth of *E. coli* BL21 in Luria Broth (LB) media.

Minimum Inhibitory Concentration (MIC). To determine the MIC of DEHP for *E. coli* BL21 (Figure 2), DEHP was added directly to inoculated Luria Broth (LB) media to a total volume of 5mL. The double-dilution MIC protocol by Wiegrand *et al* (2008). was adapted to capture effects of DEHP concentrations between 1024 µg/mL - 4 µg/mL. After 24 hours, inoculated broth was vortexed, and samples taken for OD_{600} readings on a Beckman spectrophotometer.

Determining relevant concentrations for the growth curve. To determine the relevant DEHP concentrations for the growth curve, the MIC protocol above was repeated with a few modifications: *E. coli* were grown over a five-hour period, and OD readings were taken every hour for a growth curve. As well, the miscibility of DEHP in LB broth was enhanced by using ethanol as a solvent. This was done by dissolving DEHP in ethanol at a 1:1 ratio, and topping this up to a stock concentration of 10000 µg/mL with deionized water. Dissolved DEHP from this stock was then double-diluted.

Dose-dependent effects on the growth curve of *E. coli* BL21. To determine the dose-dependent effects of DEHP on the growth of *E. coli* BL21 over a seven-hour period (Figure 1), duplicate 1024 µg/mL, 128 µg/mL, 4 µg/mL, 0 µg/mL (growth control) DEHP conditions were set up in a total volume of 10 mL of inoculated LB broth. DEHP dissolution was facilitated by drawing from a 10000 µg/mL stock prepared by dissolving DEHP in ethanol at a 1:1 ratio, and topping this up to 10 mL with deionized water. To elucidate the effects of the solvent, an ethanol control was set up for the 1024 µg/mL DEHP condition. In this control, the same amount of ethanol used in the stock preparation was added to deionized water. The same volume drawn from this "ethanol control stock" as in the 1024 µg/mL DEHP condition. Growth was determined by hourly OD_{600} readings from a Beckman spectrophotometer. After 24 hours, 4uL samples were taken from the 1024 µg/mL and 0 µg/mL replicates to observe the morphological effects of DEHP on *E. coli* BL21 by light microscopy. The ZEISS™ microscope was used for imaging using the 20X and 63X objective lenses, and development was done using ImageJ software.

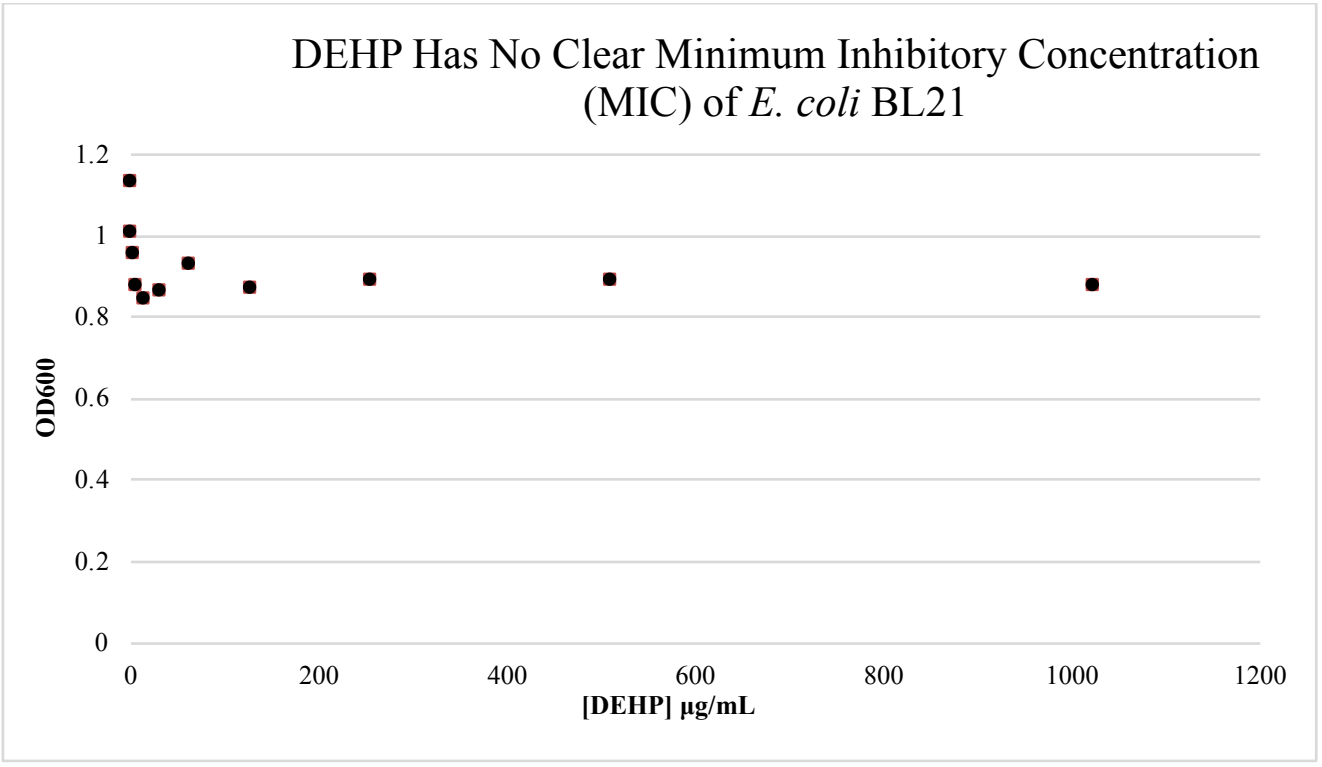

FIG. 2 *E. coli* BL21 culture density varied after incubation for 24 hours at 37 °C in concentrations of DEHP ranging from 0 - 1024 µg/mL. The growth control (GC) without DEHP resulted in OD_{600} reading of 1.136. n = 1 replicates of 11 concentration points.

RESULTS

DEHP had no minimum inhibitory concentration for *E. coli* BL21 within the tested range. The concentration at which there was no growth was to be recorded as the MIC. However, because there was growth at all concentrations of DEHP, no minimum inhibitory concentration was determined by the MIC assay. Furthermore, altering DEHP concentrations did not markedly influence the end-point growth of *E. coli* BL21 (Figure 2). Increasing concentration of DEHP in the *E. coli* culture media did not consistently decrease OD_{600} readings. Both low and high DEHP concentrations decreased OD_{600} relative to that of the growth control (0 µg/mL DEHP), however, concentrations in the middle of the tested range showed higher OD_{600} readings than the low or high concentrations. Hence, no MIC was determined and increasing DEHP concentration had no conclusive impact on growth.

It was observed that the *E. coli* culture media and the DEHP liquid layers were physically separated after incubation. Distinct phase-separation was especially observed for the higher concentrations of DEHP. Insufficient mixing of the two layers may have affected the ability to produce an MIC result and inhibit *E. coli* growth.

Initial growth curve suggests biphasic growth properties of DEHP. To determine whether DEHP had other effects during growth of *E. coli* BL21, a growth curve was performed over a five hour time period. Effects of DEHP were investigated over the initial growth of *E. coli* BL21 as opposed to solely the end-point culture density after 24 hours of incubation. In order to facilitate dissolution of the DEHP in the *E. coli* BL21 culture media, DEHP was mixed with ethanol and deionized water, which prevented phase separation between the DEHP and culture media in the test tube as had previously occurred in the MIC assay (Figure 2).

At the two opposite ends of the spectrum, DEHP appeared to exhibit a somewhat biphasic effect on the growth of *E. coli* BL21 (Figures 3, 4). That is, growth appeared to decrease at high concentrations between 32 and 1024 µg/mL DEHP but increase at low concentrations between 4 and 16 µg/mL DEHP (Figure 3). However, the growth inhibition was most pronounced at 1024 µg/mL DEHP and, conversely, growth inhibition at 4 µg/mL DEHP (Figure 3). This trend were especially clear in the two to five hour time period in the growth curve analysis (Figure 3).

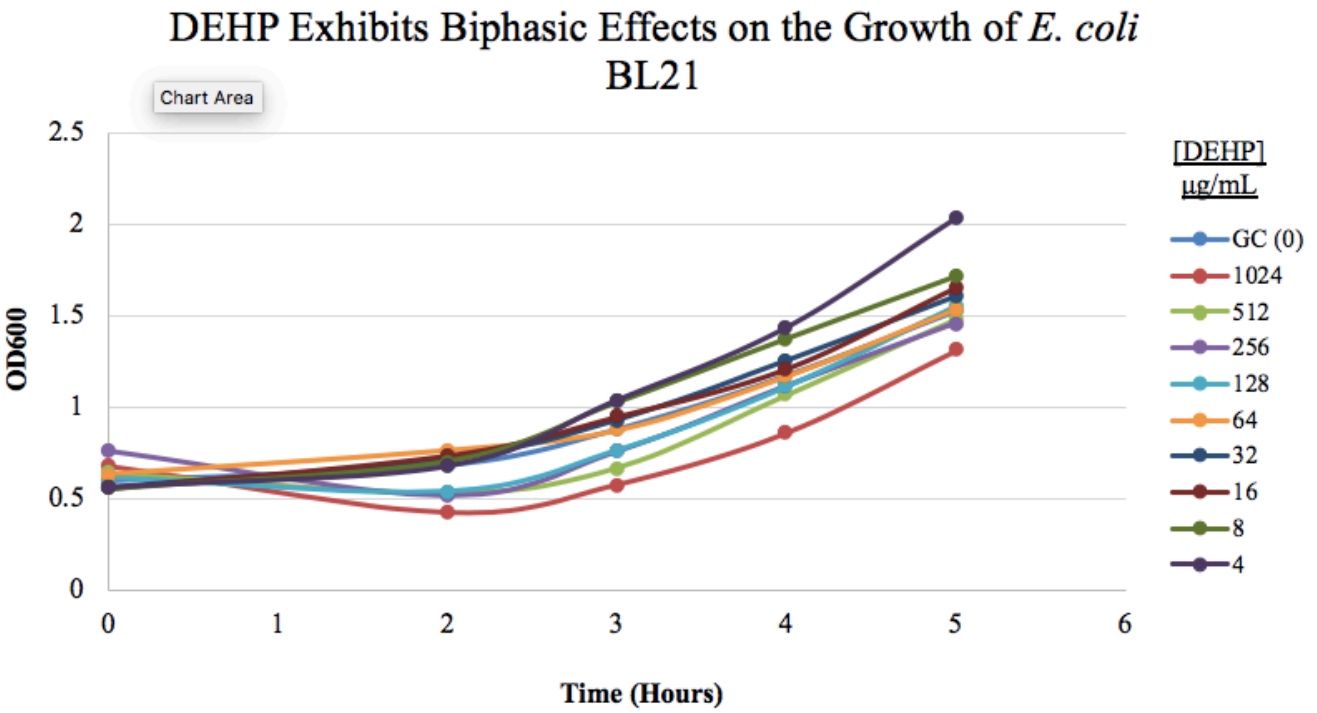

FIG. 3 DEHP exhibits modest biphasic effects on *E. coli* BL21 growth. Low concentrations (i.e. 4 - 16 µg/mL) of DEHP modestly promote growth over the growth control (0 µg/mL DEHP). Higher concentrations (i.e. 32-1024 µg/mL) decreased growth over time relative to the growth control. n = 1 replicates of five time-points.

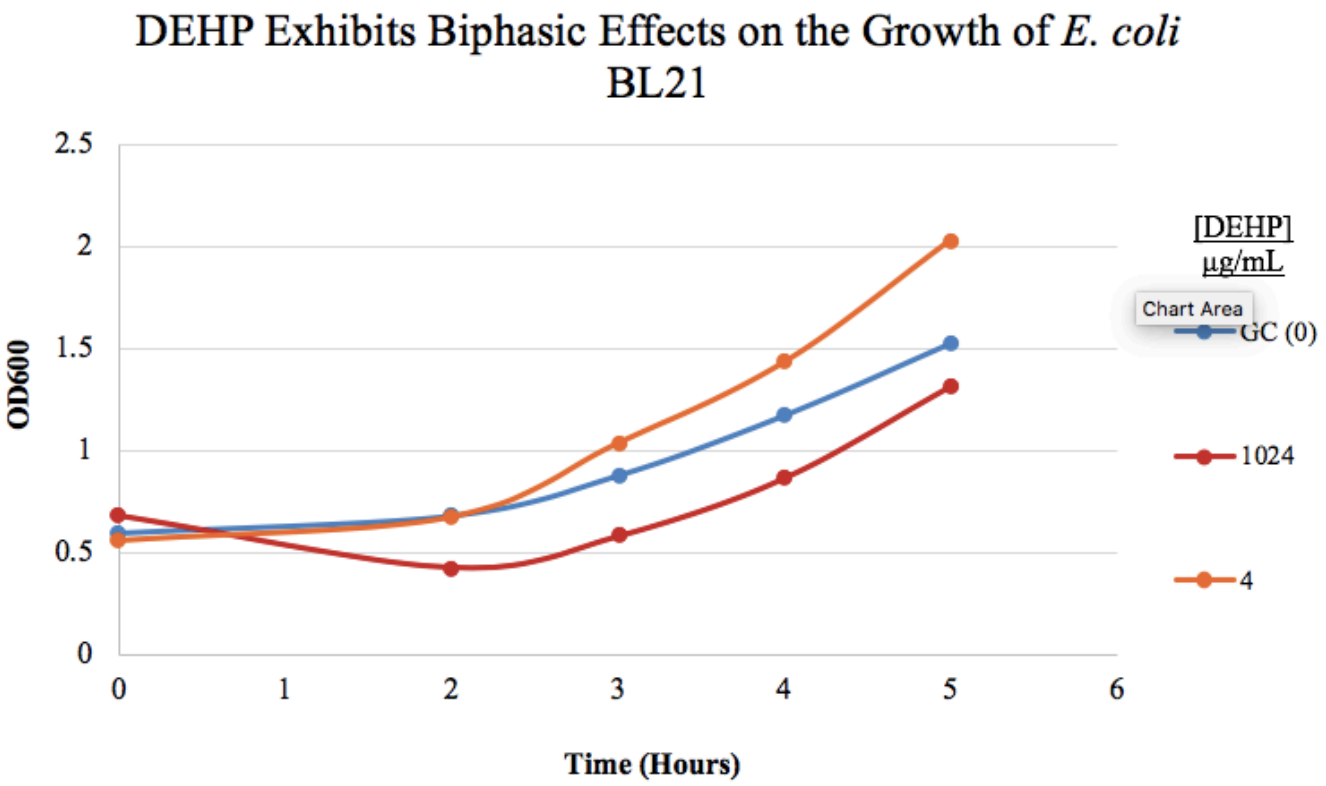

FIG. 4 DEHP exhibits modest biphasic effects on *E. coli* BL21 growth. The low DEHP concentration of 4 µg/mL promoted *E. coli* BL21 growth to a higher culture density than the growth control (GC) containing 0 µg/mL DEHP, as determined by OD_{600} readings. The higher concentration of 1024 µg/mL appeared inhibited growth relative to the GC. n = 1 replicates of five time points.

Figure 4 shows that the lowe3st DEHP concentration tested, 4 µg/mL, promoted *E. coli* BL21 growth with OD_{600} readings that surpassed the growth control without DEHP. Conversely, the highest concentration of DEHP tested, 1024 µg/mL, reduced *E. coli* BL21 growth to readings below the growth control, as measured by OD_{600}. Based on these results (Figure 4), 1024 µg/mL, 128 µg/mL and 4 µg/mL concentrations appeared to be suitable concentrations to capture the effects of low, medium and high DEHP concentrations on *E. coli* BL21 growth.

Ethanol-controlled growth curve indicates solvent-confounding effects. Unlike the 7-hour growth curve, the ethanol-controlled 7-hour growth curve showed no growth enhancement at low (4 µg/mL) and medium (128 µg/mL) DEHP concentrations when compared to the growth control (Figure 5). Specifically, *E. coli* BL21 culture with DEHP at concentrations of 4 to 128 µg/mL grew to approximately equivalent OD_{600} by six hours relative to growth control (0 µg/mL DEHP). As well, there was convergence in their growth trends at seven hours. The high (1024 µg/mL) DEHP dose and ethanol control replicates showed decreased growth, and had similar and convergent trends (Figure 5). That is, the culture containing 1024 µg/mL DEHP grew to the same OD_{600} as the ethanol condition at five and six hours incubation (Figure 5). It is possible that the ethanol used to facilitate dissolution of the DEHP in the *E. coli* BL21 culture media could have confounded the previously observed biphasic effect of DEHP (Figure 3, 4). However, more precise experimentation into such confounding solvent effects will be needed to fully elucidate this possibility.

Microscopy reveals no distinct morphological effects of DEHP. While the spectrophotometry results indicated that the high DEHP concentration of 1024 µg/mL inhibited growth relative to the growth control, this effect was not observably reflected in the morphology of *E. coli* BL21 cells after 24 hours incubation. As seen at 63X magnification in

Figure 6, rods were observed in both conditions, with a similar occurrence of cell and replicating-chain lengths. Because the morphology of the cells was relatively similar, our results suggest that DEHP had no evident effect on the *E. coli* BL21 at the tested conditions and magnification.

DISCUSSION

In the MIC assay, the growth of *E. coli* BL21 showed only minimal response to varying DEHP concentrations (Figure 2); which most likely occurred as a result of the phase separation that formed between DEHP and the growth medium. Initially, methanol was chosen as the first solvent to test for confounding effects (data not shown). The tested concentrations of methanol did not increase the solubility of the DEHP in the *E. coli* BL21 culture media. DEHP demonstrated near complete phase separation from the growth media and was speculated to be unable to influence the growth of *E. coli* BL21 (Figure 2). However, this problem was later ameliorated in the five- and seven-hour growth curves (Figures 4 and 5, respectively) wherein ethanol was used as solvent that successfully dissolved DEHP.

In Figure 3, we observed different growth rates in the different DEHP concentrations. High concentrations were observed to inhibit growth, while low concentrations led to higher growth compared to the growth control without DEHP. It was observed that low DEHP concentrations (4 - 16 µg/mL) exhibited growth enhancement; whereas high concentrations of DEHP of 32 - 1024 µg/mL, exhibited modest growth inhibition. This trend can be more clearly seen in Figure 4 showing only the growth of *E. coli* BL21 in the lowest and highest concentrations of DEHP relative to that of the growth control (Figure 4). The *E. coli* culture containing 4 µg/mL DEHP grew to a culture density that contained approximately 5 x 10^8 cells more than the growth control. Conversely, 1024 µg/mL DEHP resulted in an *E. coli* culture density of 0.2 OD_{600} units less than the growth control, or the equivalent of approximately 2 x 10^8 cells less than the 0 µg/mL DEHP condition (Figure 4). Hence, although the intermediate DEHP concentrations between these two extreme values appear to have more a more limited ability to either promote or inhibit *E. coli* growth, culture media containing 4 and 1024 µg/mL DEHP did show a trend of varied growth relative to the control.

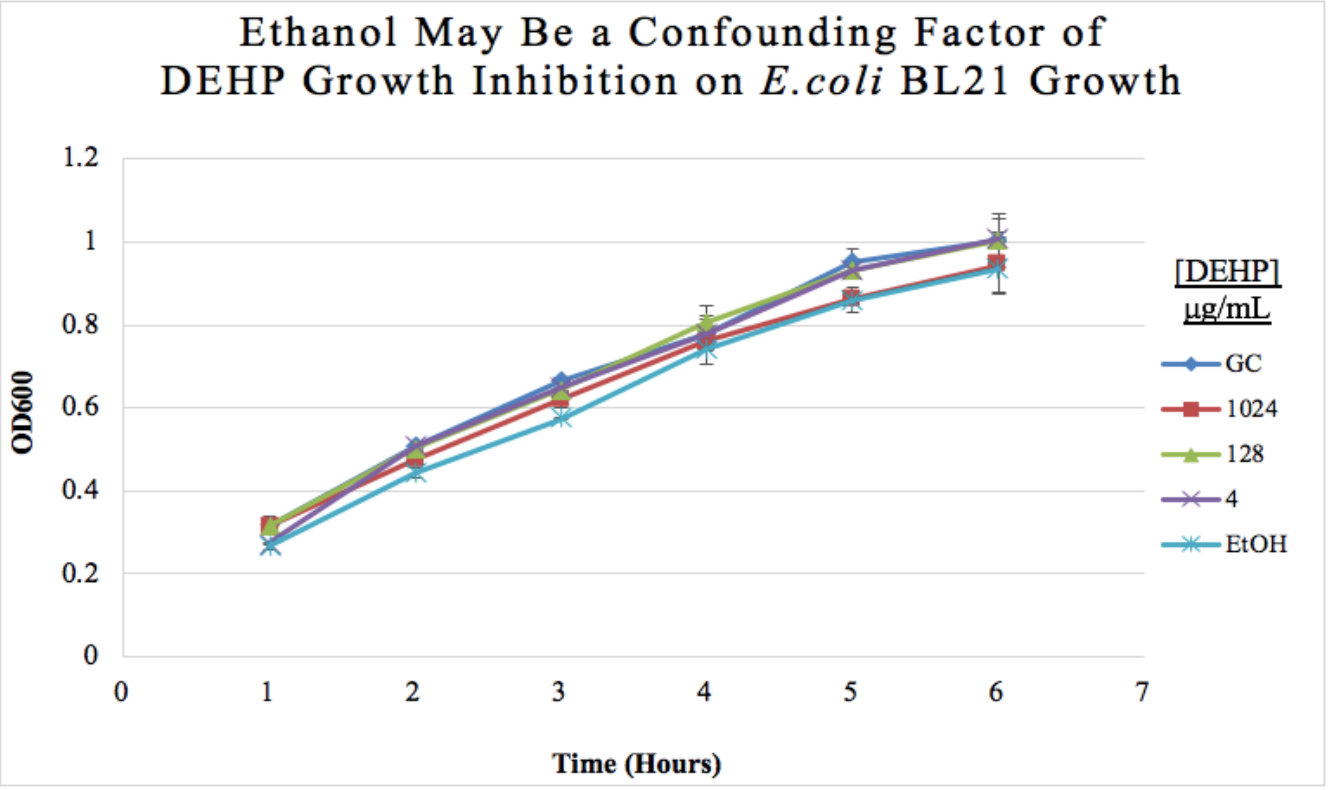

FIG. 5 The growth-inhibitory effect of high DEHP concentrations may be confounded by ethanol used to facilitate dissolution of DEHP in *E. coli* BL21 culture media. n = 2 replicates at each time point.

Yet, the five hour growth curve assay (Figure 3 and 4) lacked an ethanol control needed to indicate that the growth inhibiting effect at high DEHP concentrations could be confounded by the increased ethanol concentration respectively. The following growth curve analysis (Figure 5) was performed with the inclusion of an ethanol control. In this experiment, the *E. coli* BL21 grown in the ethanol control showed very similar growth to the ones in the medium containing the highest concentration of DEHP at 1024 µg/mL. Both this DEHP concentration and the ethanol control contained the same concentrations of ethanol. The fact that the same growth effect was observed between the two conditions indicates that the growth inhibition that supposedly occurred at high concentrations of DEHP observed in Figures 3 and 4, may have in fact been due to ethanol-mediated inhibition of *E. coli* BL21 cell growth, as opposed to a growth-inhibitory effect of DEHP. More experimentation will need to be performed on using ethanol as a solvent for DEHP to see if the growth inhibitory effect observed at high concentrations was actually due to the DEHP, or rather from the ethanol solvent.

Furthermore, the microscopy performed did not show any alteration in morphology of cells while grown in high DEHP concentrations compared to *E. coli* BL21 culture media grown in 0 µg/mL. If DEHP was indeed inhibiting the growth of these bacteria, the cells were expected to show altered different morphology compared to the control cells grown in the presence of no DEHP. It is generally observed that when cells undergo growth-inhibitory environmental stress, they exhibit elongated morphology as a representation of some form of cell cycle disruption at the physiological level (11). Therefore, the lack of altered morphology lends support to the idea that there was no clear bacteriostatic effect of DEHP observed in the growth curves in Figures 3 and 4.

Another observation in Figure 5 was that *E. coli* BL21 grown in low DEHP concentrations did not grow to higher densities than the growth control. In past research by Sandy *et al.*, the concentrations of DEHP used in their respective growth studies (from 150 to 600 µg/mL, in increments of 150 µg/mL) were substantially lower than the concentration that was used in Figures 2 - 5. It is unclear what methodological differences in facilitating dissolution of DEHP in media are between our research and Sandy *et al.*, since limited information on this topic was provided by past researchers. However, what is clear from their research is that that no respective solvent control was performed or discussed while conducting the growth curves. In contrast, our studies growth curves in Figures 3, 4, and 5, ethanol was used as a solubilizing agent for DEHP and a respective ethanol control was introduced in Figure 5 to account for such confounding solvent effects. Examination of Figures 3 and 4 yields a similar conclusion to Sandy *et al.* that DEHP influenced the growth of *E. coli* in a biphasic manner, but once

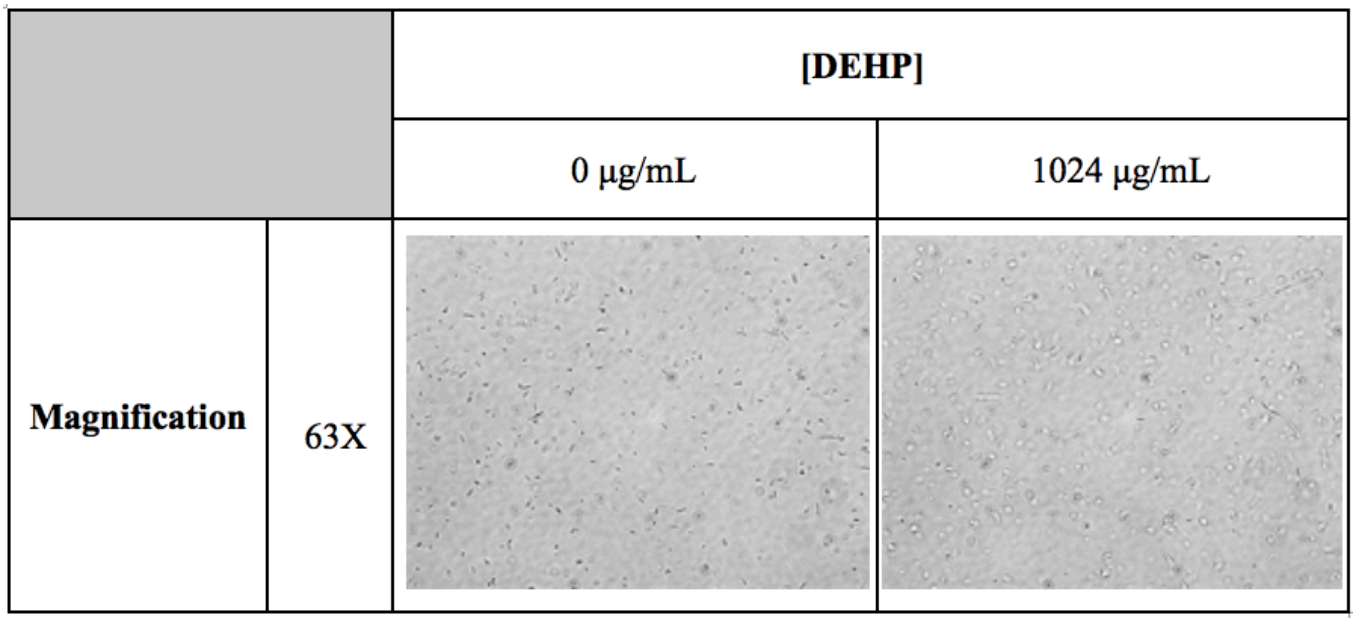

FIG. 6 DEHP did not produce distinct visible effects on the morphology of individual *E. coli* BL21 cells after incubation. Light microscopy demonstrates that the morphology of individual cells grown in LB media for 24 hours at 37 °C is similar at 63X magnification, between the media containing either 0 or 1024 µg/mL DEHP.

ethanol controls were factored, the growth effects became confounded (Figure 5). Furthermore, Sandy *et al.* inferred metabolic inhibition from the dramatically reduced growth rate as observed in high DEHP concentrations but compared with the observations made in Figures 2 and 6, no metabolic effects could be inferred from any end-point minimum inhibitory concentrations and physiological states of *E. coli* BL21 cells grown in the presence of high concentrations of DEHP. Moreover, the similarity in population size observed between the cells grown at a high DEHP concentration and a condition with no DEHP runs counter to a primary observation in Hu *et al.* (2016), where population enrichment for specific microbial taxonomic groups were attributed to the consumption of DEHP. Future research could thus look into the ability of DEHP to alter population size on a macroscopic level, focussing on microscopic and cell counting methods.

The immiscibility of DEHP in LB broth presents important implications for DEHP toxicity in the gut and environment. First, culture media experiments using gut bacteria are poor models for the bactericidal effects of phthalates in the human gut. Because the gut contains digestive factors that emulsify large organic compounds and catalyze their breakdown, the extent of dissolution and subsequent bacterial exposure to phthalates is difficult to reproduce in a standard bacterial growth model. Experiments using exposure in water-based growth media will always be fundamentally limited in their applications to complex biological systems. Second, the distinct phase separation of DEHP and LB broth in our experiment provides insight into the perceived threat of leaching plastic pollutants in drinks. Our observations show that leaching of plastics into water is limited and might not be uniform due the density differences between large-molecule phthalates and water. This also means that large-molecule phthalates, despite their higher perceived toxicity, might not be as important because of their limited dissolution into water-based drinks. This toxicity is likely to increase depending on the extent to which the drink can mix with phthalates. For example, ethanol, the solvent used for our exposures, might increase phthalate exposure if consumed from plastic containers.

Our investigations did not lead to determination of a conclusive MIC for DEHP on growth of *E. coli* BL21, as growth was observed even at the highest tested concentration of 1024 μg/mL. Phase separation between DEHP and growth media complicated the investigation of growth effects due to DEHP. Addition of ethanol reduced phase separation but led to confounding effects on growth of *E. coli* BL21. The growth limiting effects observed in following growth curves could not conclusively be attributed to high concentrations of DEHP, because ethanol controls containing no DEHP showed similar effects.

In all, this research demonstrated that bis(2-ethylhexyl) phthalate (DEHP) had mixed effects on the growth of *E. coli* BL21 organisms; and while it appeared that low concentrations of DEHP promoted *E. coli* growth and high concentrations inhibited it, the fact that *E. coli* culture media containing only ethanol at the same concentration as in the highest DEHP concentration tested exhibited similar growth inhibition, illustrates the need for more research to elucidate the exact effects of DEHP, as well as the solvent it is in, on the growth of bacteria such as *E. coli* and other model organisms.

Future Directions Primary difficulties in conducting growth assays in phthalates pointed to poor miscibility and solubility in polar solvents. To conduct growth assays, partial chaotropic interaction with liquid media was found to be required by the phthalate and its respective solvent for growth-related effects to be observed. Despite the ability of ethanol enhance the miscibility between the *E. coli* BL21 culture media and liquid DEHP, at the concentrations required to demonstrate any growth effects, ethanol exhibited negative growth modulation. That is, due to the higher amount of ethanol needed to dissolve the higher concentrations of DEHP, the growth of *E. coli* BL21 bacteria was inhibited not by the increasing concentrations of DEHP, but rather by the ethanol itself.

Further study into this matter may assay multiple organic and inorganic chemical solvents for DEHP solubility, as well as solvent-dependent growth modulating effects. The potency of other phthalates may also provide opportunity to overcome solubility-related confounding factors. Other commercially available phthalates possessing varying chemical structures and chaotropic properties that may be more favourable to enhance miscibility between it and water-based growth media. Moreover, subsequent research may explore the use of

lipophilized DEHP dissolved in newfound solvents of choice to enhance the ability to test for the ability of DEHP to affect bacterial growth.

S. thermophilus is a known active member of the gut microbiota and expected to grow better in the presence of high phthalate concentrations than *E. coli* BL21. The former bacteria taxa has esterases that have been suggested to expedite phthalate catabolism (7, 8). It is thus possible that *S. thermophilus* will be able to metabolically accelerate DEHP degradation relative to *E. coli* BL21. At low concentrations, this catalytic potential may facilitate the metabolic degradation of phthalate groups from growth inhibitory compounds, to the biosynthesis of growth-promoting biomolecules from breakdown of phthalate components. Furthermore, the keystone biological significance of *S. thermophilus* organisms in the gut microbiome in conjunction with other taxa, allows future studies to investigate the taxonomic distribution and community-level biochemistry involved in nutrient cycling, as affected by overconsumption of commercially available phthalates

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CONTRIBUTIONS

JB: Wrote the abstract, introduction, results, and discussion. Contributed to the future directions and performed microscopy. Revised paper for submission. Performed lab work in all experiments, carrying out the minimum inhibitory concentration analyses, the growth curves, and microscopy. **JO:** Wrote the methods section. Contributed to the introduction, results and discussion sections. Acquired bacterial strains. Performed general lab prep work, set up one of the minimum inhibitory concentration and growth curve analyses, developed ways to enhance DEHP dissolution, and performed microscopy. **LS:** Performed lab work, carried out the minimum inhibitory concentration analyses and the growth curves. Assayed DEHP dissolution. Wrote the discussion section and contributed to the results and methods. **AY:** Set up and carried out minimum inhibitory concentration analysis and growth curves. Assayed DEHP dissolution. Collected and analyzed data. Contributed to discussion and future directions sections.

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