Overall Research Proposal Goal: The goal of this research is to develop a biodegradation platform using division of labor in a single bacterial strain that dynamically adjusts its metabolism according to its environment. Preliminary modeling indicates that this strategy approaches the theoretical maximum efficiency in biodegradation of a compound. This research focuses on benzene biodegradation in contaminated groundwater as an initial application—a significant issue for multiple Department of Defense (DoD) agencies and specifically mentioned in ERDC BAA W912HZ-24-BAA-01. Successful implementation of this strategy has far-reaching implications for other biodegradation processes and bioproduction applications.

Introduction and significance of research: The deployment of living organisms for bioremediation is not a new concept. Many microorganisms possess biochemical pathways capable of breaking down harmful environmental contaminants into components the cells can use for growth. This approach can be deployed on a large scale due to microorganism's fast replication time and self-regulation. However, some contaminants require many chemical steps to process into non-harmful molecules, requiring many large enzymes to be expressed. This increases the burden on the cells, slowing their growth rate and therefore limiting biodegradation rates. Recent studies have attempted to overcome this barrier by using division of labor (DOL) strategies, dividing the proteins needing to be expressed between 2 or more strains of bacteria. Each strain can focus on creating proteins for specific steps, increasing their relative growth rate. However, balancing these strains in the environment poses a significant challenge. If one strain outcompetes others, essential steps in the biodegradation process are omitted, causing the process to halt. Further, interactions between different strains are difficult to quantify and can lead to more inefficiencies. While cells can inducibly express genes, this process is not self-regulated and requires constant monitoring and maintenance to achieve optimal biodegradation rates[1-4]. Recent studies have shown that when bacteria carry multiple distinct plasmids that have the same origin of replication, they maintain total copy number but adjust plasmid ratio to optimize growth[5]. By giving growth advantages to different plasmids that share an origin of replication at different steps of the biodegradation process, a singular bacterial strain can complete the biodegradation process efficiently. As a proof of concept, we will first apply this technology to the biodegradation of benzene in water, a common groundwater pollutant at military associated sites. On one plasmid, we will have the E1 gene cassette, encoding enzymes to process the benzene into catechol. The other plasmid will contain the E2 gene cassette, which encodes enzymes to process catechol into succinyl-CoA and acetyl-CoA, essential components of cellular respiration. The plasmids will confer growth advantages through inducible expression of a lysis protein. When benzene is present in significant concentrations, the E2 plasmid will start to produce a lysis protein that, in sufficient quantities, leads to cell death. The cells with lower amounts of the E2 plasmid will survive to process benzene and reproduce. When benzene concentrations are low and do not induce lysis protein expression, cells with higher amounts of the E2 plasmid gain a growth advantage due to the lower metabolic burden of expressing E2 enzymes and lower oxidative stress of processing steps. Preliminary modeling of this system shows that it approaches the theoretical limit of efficiency for these reactions, a revolution in biodegradation technology (**Figure 1B**). Additionally, this system is highly flexible and applicable to various biodegradation processes relevant to the DoD, including polycyclic aromatic hydrocarbons and other volatile organic compounds, as well as bioproduction applications such as pharmaceuticals and vitamins.

AIM 1: Construction of E1 and E2 plasmids and characterization. To build a dynamic DOL biodegradation system in a singular cell, the plasmids with E1 and E2 gene cassettes must first be constructed and characterized. The E1 plasmid will contain the E1 gene cassette, encoding benzene 1,2 dioxygenase and dihydrodiol dehydrogenase - enzymes that process benzene into cis-bezene dihydrodiol and then into catechol[6,7]. The E1 plasmid will also contain a type 1 toxin antitoxin system called Hok/Sok for plasmid upkeep. This system produces Hok, a toxic protein that causes cell death by depolarizing the cell membrane, and Sok, an antisense RNA that binds to Hok mRNA, preventing its transcription. This system ensures plasmid retention, as Hok mRNA is stable and constantly requires quickly degrading Sok RNA to prevent cell death[8]. The E2 plasmid will contain the E2 gene cassette, encoding enzymes of the β-ketoadipate pathway that convert catechol into succinyl-CoA and acetyl-CoA, essential intermediates in cellular respiration[9]. The E2 plasmid will contain a different toxin antitoxin system called ccdB/ccdA for plasmid upkeep. The enzyme ccdB binds to DNA gyrase, an enzyme critical for DNA replication, inhibiting its activity and killing the cell. The enzyme ccdA binds tightly to ccdB and neutralizes its toxic behavior. The toxic ccdB is very stable while the

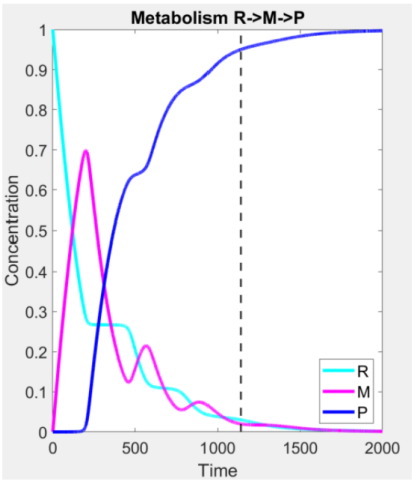
neutralizing ccdA is unstable, leading to cell death if the plasmid is lost[10]. Additionally, the E2 plasmid will incorporate an inducible TodS/TodT system to control the expression of a lysis protein in the presence of benzene. TodS is a sensor kinase that recognizes benzene and autophosphorylates upon binding. It then transfers the phosphate group to TodT, which activates lysis protein transcription by binding to the P_{Tod} promoter[11]. At high levels of expression, this lysis protein kills the cell, conferring a growth disadvantage. When benzene is present in significant amounts, the E1 plasmid will have a higher copy number than the E2 plasmid due to its growth advantage over the lysis protein producing E2 plasmid. Once benzene is processed into catechol and its concentration decreases, catechol's hydrophilic groups prevent it from binding to the hydrophobic pocket of TodS, halting lysis protein production. Since expressing the E2 enzymes imposes a lower metabolic burden, the E2 plasmid will become predominant. Both plasmids will use the ColE1 origin of replication due to its high copy number, allowing large changes in expression at different benzene concentrations. For the purposes of characterization, the E1 gene cassette will include green fluorescent protein (GFP) and the E2 gene cassette will include red fluorescent protein (RFP). The E1 plasmid and E2 plasmid will then be transformed into separate E. coli. They will then be loaded onto a microfluidics gradient chip and subjected to 0-0.3 mg/L benzene and then 0-0.3 mg/L catechol. Microfluidic devices enable observation of microorganisms in continuous culture, allowing us to monitor long-term behavior under conditions akin to a water treatment plant. In the E1 strain, we expect to see high GFP expression both in the presence of benzene and catechol. In the E2 strain, we expect to see cell lysis in benzene and high RFP expression in catechol. The growth of the two strains will be compared to confirm that the E1 strain has slower growth. Growth data from the various environmental conditions will be gathered and used to fit model parameters.

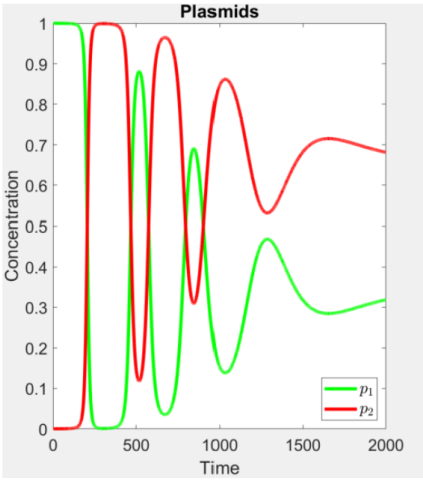
Equations

$$\dot{R} = -\frac{k_A E_1 R}{K_A + R} + D(R_f - R)$$
$$\dot{M} = \frac{k_A E_1 R}{K_A + R} - \frac{k_B E_2 M}{K_B + M} - DM.$$
$$\dot{P} = \frac{k_B E_2 M}{K_B + M} - DP$$

A.

Results





B.

*Figure 1: **A.** Equations modeling the Division of Labor (DoL) system, where R represents benzene concentration, M denotes catechol concentration, and P signifies the combined concentrations of acetyl-CoA and succinyl-CoA. **B.** (Left) Metabolite concentrations over time show a decrease in benzene (R) and catechol (M) concentrations over time, while the concentration of acetyl-CoA and succinyl-CoA (P) increases. The dotted horizontal line indicates the theoretical maximum efficiency of reactions based on Michaelis-Menten kinetics. (Right) Plasmid ratio dynamics over time corresponding to the concentrations of R and M, where plasmid E1 is denoted as p_1 and plasmid E2 as p_2.*

AIM 2: Studying plasmid regulation dynamics in continuous culture and chemostat. After characterizing each plasmid individually, we must then characterize their dynamics when co-transformed into the same E. coli strain. The co-transformed cells will be characterized on a gradient microfluidics chip. In the absence of both benzene and catechol, we expect high RFP expression, indicating a higher copy number of the E2 plasmid. If GFP expression is detected, the expression of the enzymes in the E1 gene cassette will be increased using directed mutagenesis on the ribosome binding site of each enzyme, increasing E1 plasmid burden. In the presence of 0-0.3 mg/L benzene, we expect the bacteria will have high GFP expression and low RFP expression, indicating high copy number of E1 plasmid. If this is not observed, directed mutagenesis will be used on the P_{Tod} promoter to strengthen lysis protein expression, enhancing the growth disadvantage of the E2 plasmid under benzene conditions. In the presence of 0-0.3 mg/L catechol, we expect that the bacteria will have

low GFP expression and high RFP expression, indicating high copy number of E2 plasmid. If this is not observed, the TodS/TodT gene sequences will be checked to ensure binding pocket fidelity. We will monitor the bacteria as the media transitions from benzene to catechol, observing the shift from GFP to RFP expression. This will be compared to a negative control where bacteria have the E1 and E2 plasmids with separate origins of replication. In this control, GFP and RFP levels would remain constant in catechol, and cells would lyse in benzene due to unregulated plasmid copy numbers.

To characterize the benzene and catechol degradation rate, the co-transformed cells in a chemostat will maintain a steady growth state by filtering effluent media to remove cells and recycling it as input media. Every 2 hours, samples will be collected for high-performance liquid chromatography (HPLC) analysis of chemical composition, and GFP and RFP fluorescence will be measured. When the chemostat is supplied with benzene containing media, we expect catechol to appear in HPLC analysis after some time, followed by the disappearance of catechol and benzene as they are degraded. The dynamics of the fluorescent proteins should coincide with the dynamics of the chemical species. The rate at which catechol appears and both catechol and benzene disappear will inform reaction rates and other parameters in the model. This data will also be used to find rate degradation bottlenecks, guiding optimal expression rates of the E1 and E2 gene cassettes.

AIM 3: Evolution of chassis strain and recharacterization of plasmid dynamics. E. coli typically grow slower in media containing benzene than in standard media. This is due to the lipophilic nature of benzene, allowing it to partition into cell membranes and disrupt their structure and function. However, some species of gram-negative bacteria have evolved to thrive in high concentrations of benzene, suggesting mechanisms for bacterial adaptation. To adapt E. coli to benzene rich environments, adaptive laboratory evolution (ALE) will be used. In this strategy, consecutive generations of bacteria are grown in a benzene rich environment. Each generation accumulates strategies for thriving in the stressful environment via beneficial mutations[12]. After a sufficient number of generations, E. coli will exhibit increased growth rates in benzene rich environments. The evolved E. coli will be transformed with the E1 and E2 plasmids from AIM 1 and recharacterized in benzene and catechol containing media. We will then co-transform the plasmids into the same strain and recharacterize as in AIM 2, updating parameter values in the model. We anticipate faster growth rates and increased degradation rate of benzene to the non-evolved strain.

Benefit to society: While this technology has immediate implications for the bioprocessing of benzene-contaminated water, its long-term implications are much greater. This revolutionary technology can be extended to virtually any biodegradation process, adding more plasmids for more complex processes. In addition, this technology can also be applied to multistep bioproduction processes with significant societal impact such as biofuel production, pharmaceutical synthesis, and industrial enzyme manufacturing. This research will aid in remediating contaminant spills associated with United States service activities and help maintain the nation's leadership in biological production of industrially relevant molecules.

Relevant qualifications and support: The lab I work in has extensive experience with microfluidics devices, synthetic gene circuits, and working with dynamic bacterial populations. It is at a large research university which has ample access to microscopes and other resources, ensuring no materials or equipment bottlenecks for this research. I have experience with the construction of gene circuits, characterizing them in microfluidics devices, and performing image analysis for data quantification. Taken together, the likelihood of success for this project is high. Receiving the NDSEG fellowship would provide crucial funding and support to continue my research into transformative biotechnologies in microorganisms.

References:

[1]: Rafieenia, R., Atkinson, E., & Ledesma-Amaro, R. (2022). Division of labor for substrate utilization in natural and synthetic microbial communities. Current Opinion in Biotechnology, 75, 102706. https://doi.org/10.1016/j.copbio.2022.102706

[2]: West, S. A., & Cooper, G. A. (2016). Division of labour in microorganisms: an evolutionary perspective. Nature Reviews Microbiology, 14(11), 716–723. https://doi.org/10.1038/nrmicro.2016.111

[3]: Tsoi, R., Wu, F., Zhang, C., Bewick, S., Karig, D., & You, L. (2018). Metabolic division of labor in microbial systems. Proceedings of the National Academy of Sciences, 115(10), 2526–2531. https://doi.org/10.1073/pnas.1716888115

[4]: Lilja, E. E., & Johnson, D. R. (2016). Segregating metabolic processes into different microbial cells accelerates the consumption of inhibitory substrates. The ISME Journal, 10(7), 1568–1578. https://doi.org/10.1038/ismej.2015.243

[5]: Kumar, S., Lezia, A., & Hasty, J. (2024). Engineering plasmid copy number heterogeneity for dynamic microbial adaptation. Nature Microbiology, 9(8), 2173–2184. https://doi.org/10.1038/s41564-024-01706-w

[6]: Zamanian, M., & Mason, J. R. (1987). Benzene dioxygenase in Pseudomonas putida. Subunit composition and immuno-cross-reactivity with other aromatic dioxygenases. Biochemical Journal, 244(3), 611–616. https://doi.org/10.1042/bj2440611

[7]: Fong, K. P., & Tan, H. (1999). Characterization of a novel cis-benzene dihydrodiol dehydrogenase from Pseudomonas putida ML2. FEBS Letters, 451(1), 5–9. https://doi.org/10.1016/s0014-5793(99)00520-7

[8]: Rhun, A. L., Tourasse, N. J., Bonabal, S., Iost, I., Boissier, F., & Darfeuille, F. (2022). Profiling the intragenic toxicity determinants of toxin–antitoxin systems: revisiting hok/Sok regulation. Nucleic Acids Research, 51(1), e4. https://doi.org/10.1093/nar/gkac940

[9]: Stainer, R., & Ornston, L. (1973). The β-Ketoadipate Pathway. Advances in Microbial Physiology/Advances in Microbial Physiology, 89–151. https://doi.org/10.1016/s0065-2911(08)60377-x

[10]: Zhang, H., Tao, S., Chen, H., Fang, Y., Xu, Y., Chen, L., Ma, F., & Liang, W. (2024). The biological function of the type II toxin-antitoxin system ccdAB in recurrent urinary tract infections. Frontiers in Microbiology, 15. https://doi.org/10.3389/fmicb.2024.1379625

[11]: Lacal, J., Busch, A., Guazzaroni, M., Krell, T., & Ramos, J. L. (2006). The TodS–TodT two-component regulatory system recognizes a wide range of effectors and works with DNA-bending proteins. Proceedings of the National Academy of Sciences, 103(21), 8191–8196. https://doi.org/10.1073/pnas.0602902103

[12]: Dragosits, M., & Mattanovich, D. (2013). Adaptive laboratory evolution – principles and applications for biotechnology. Microbial Cell Factories, 12(1), 64. https://doi.org/10.1186/1475-2859-12-64