Design Brief

Utilizing the CRISPR–Cas9 system to create hypervirulent cyanophages as a remedy for harmful algal blooms in saltwater ecosystems

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Overgrowth of cyanobacteria results in harmful algal blooms (HABs), or 'red tides,' which have many detrimental impacts on the aquatic ecosystem, human health, and the economy. More specifically, HABs release toxins, block sunlight, and consume oxygen, creating 'dead zones' and making water unsafe for fish, shellfish, humans, and other mammals. They not only harm the marine environment, but also pose risks to fishing and tourism, resulting in the loss of jobs and closure of tourist attractions with significant economic impacts that can run into millions of dollars. Finding ways to combat HABs will therefore not only preserve marine life, but will also benefit the economy. We propose to create a spray solution of virulent, host-specific cyanophages that will infect and prevent the harmful overgrowth of *Karenia brevis*, a cyanobacterium notorious for causing red tides, in which the water acquires a red hue and becomes toxic. To increase the effectiveness of the spray solution, we intend to edit the genome of the cyanophage by knocking out the integrase gene using the CRISPR–Cas9 system. By inactivating this gene, onset of the lysogenic cycle will be prevented and the lytic cycle will be induced instead, resulting in increased virulence.

Keywords: Harmful algal bloom, cyanophage, cyanobacteria

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Background

One of the most well-known types of harmful algal bloom (HAB) is the 'red tide,' which is widespread along the Atlantic and Pacific coasts, with a higher frequency in the Gulf of Mexico. Red tide results from overgrowth of the cyanobacterium *Karenia brevis* (Kirpatrick et al., 2004; Anderson et al., 2012), which harms aquatic life in several ways. When an algal bloom develops, it blocks sunlight from reaching plants that provide the food and habitats necessary for fish and other animals to thrive. Furthermore, when *K. brevis* dies, it is decomposed by bacteria that often deplete the surrounding oxygen, creating 'dead zones' that kill fish and also make the water unfit for recreational use. Thus, HABs result in dramatic changes in water chemistry (pH, temperature, depleted nutrients, toxicity), causing many organisms to suffocate and die, or forcing them to find an alternative habitat in order to survive. In addition, *K. brevis* produces a potent suite of neurotoxins (Kirkpatrick et al, 2004), known as brevetoxins, which bind to sodium/potassium channels and promote unrestricted flow of sodium ions into cells. This dampens electrical impulse control and causes ataxia, or loss of limb control, as well as bronchospasms if brevetoxins are inhaled (Anderson et al., 2012; Stauffer et al., 2019; Watkins et al., 2008).

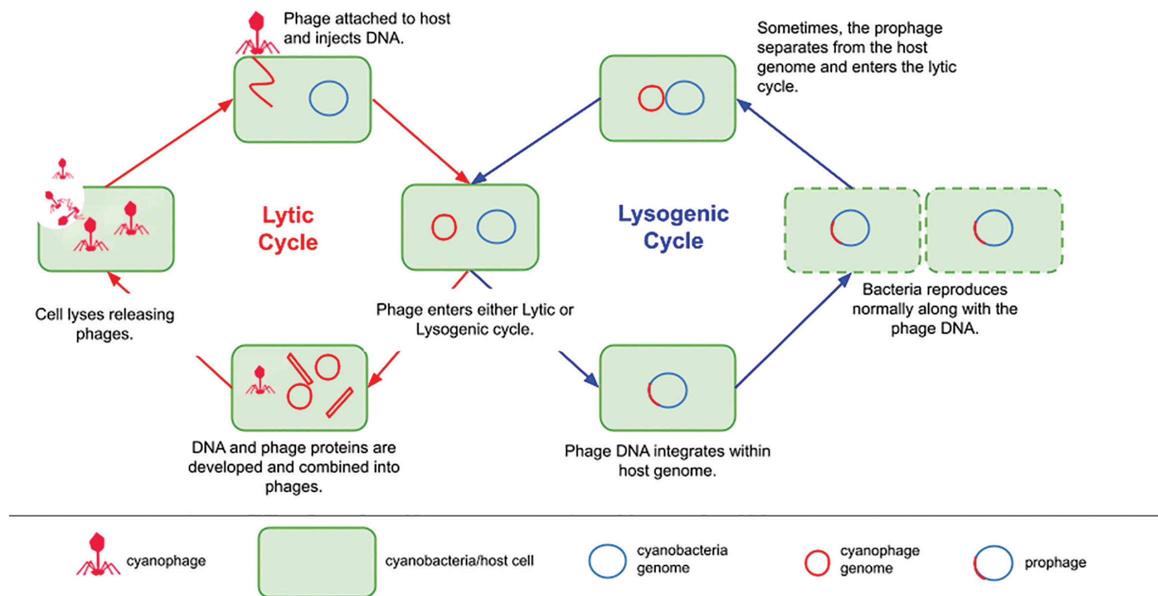

Figure 1. *Schematic of the lytic and lysogenic cycles of phages.*

These compounds are particularly harmful to fish, and can contaminate shellfish. Animals that then eat these shellfish often display symptoms of neurotoxicity, which may even include death. Additionally, brevetoxins can accumulate in the air and affect nearby animals, causing respiratory illnesses (Anderson et al., 2012; Stauffer et al., 2019; Watkins et al., 2008). Symptoms of neurotoxic poisoning by these agents include tingling and numbness of extremities, muscular aches, ataxia, dizziness, diarrhea, and vomiting. In some cases, the symptoms can progress to partial paralysis or severe respiratory distress, potentially leading to the death of fish, mammals, and humans.

Thus, red tide leads to severe loss of marine life and has negative impacts on local economies (Kouakou & Poder, 2019; Northwest Fisheries Science Center, 2021). In 2013, 277 manatees—a species already classified as threatened under the Endangered Species Act—died due to *K. brevis* poisoning, placing a significant financial strain on local wildlife preservation groups in Florida (Northwest Fisheries Science Center, 2021). In addition, 179 loggerhead sea turtles died from *K. brevis* poisoning on Florida's Gulf Coast between 2005 and 2006. These impacts are not confined to the natural environment, but also affect the local economy and human health. In 2011, the oyster industry in Texas suffered a $10.3 million loss due to a ban on oyster harvesting for three months out of the six-month season, caused by unrelenting overgrowth of *K. brevis*. In 2000, Galveston County collectively suffered an estimated economic fallout of $22–25.4 million due to *K. brevis*. At the same time, hospital visits for respiratory illnesses increased by 54% in Saratoga County, resulting in considerable financial and personal strain on healthcare systems.

It is clear that the impacts of red tide are devastating, and require an urgent solution. Of the naturally occurring forces that combat algae, cyanophages are among the most potent (Clokie et al, 2011; Chen & Lu, 2002). Phages are host-specific viruses that are found naturally near their hosts and are often integrated within the host genome (then known as prophages), where they lie dormant. When activated, they infect the cell and hijack its cellular machinery to produce viral components and propagate the viral progeny. Eventually, the phage lyses the host, killing it by rupturing the cell membrane and releasing its progeny to infect neighboring host cells. The major advantage of using phages as a potential solution for HABs is their specificity to one particular species. Thus, they can be employed to discriminately target only a single particular host species, without causing harm to other organisms and the surrounding environment (Clokie et al., 2011; Chen & Lu, 2002).

Once a phage infects a susceptible host, it may pursue one of two cycles of replication: lysogenic or lytic (Figure 1). In the lysogenic cycle, also referred to as non-virulent infection, the phage incorporates its DNA within the genome of the host and stays dormant. The phage genome is replicated passively along with that of the host, until external factors—such as starvation or exposure to poisonous chemicals—cause the prophage to excise from the host genome and trigger the lytic cycle. In the lytic cycle, also referred to as virulent infection, the phage enters the host, starts replicating, and ultimately kills the host through lysis. Upon

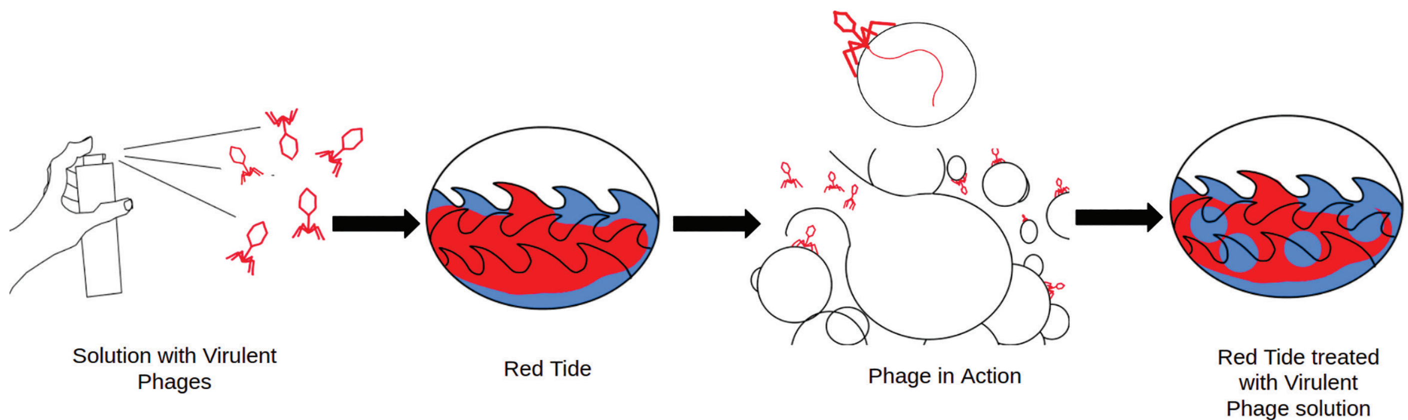

Figure 2. *Proposed mechanism of action for combating HABs.*

injection into the host, a lytic phage therefore takes immediate control of the host's molecular machinery and synthesizes proteins to assemble more phages. Following this replication cycle, phage progeny lyse the cell, burst out, and infect surrounding susceptible hosts. We plan to utilize this lytic cycle as the main mechanism of action to combat red tide.

Cyanophages are typically of either the T4 or T7 phage family, tailed, and double-stranded (Suttle & Chan, 1993). They share several sets of core genes with *Escherichia coli* T4 and T7 phages, including those for virion formation, DNA replication and packaging, transcription, and other fundamental processes of the lytic and lysogenic cycles (Huang, 2015). Of particular interest are the genes associated with lysogeny. Integrated phages have been found in many bacterial genomes, and integrase genes are common in viromes and cellular metagenomes (Knowles et al., 2006; Roux et al., 2015). Integrases provide phages with the functional ability to initiate the lysogenic cycle by incorporation into the host genome. Many members of the T7 class of cyanophages have been shown to carry an integrase gene. In some cases, this is flanked by putative attachment sites (attP) that facilitate site-specific integration of the lysogenic phage into the host genome (Huang, 2011). Deleting the integrase gene in a wild type (wt) cyanophage will prevent its incorporation into the host genome. The resultant mutant (m) cyanophage would then propagate the lytic cycle, thereby ensuring the host cell will be lysed.

Systems level

To prevent HABs, excessive growth of the pathogenic cyanobacterium, *K. brevis*, must be managed and controlled. A non-toxic solution that will clear *K. brevis* from the waters may work as a potential application to avoid or combat HABs.

Device level

We plan to develop a non-invasive and non-chemical-based, liquid-form product containing mutant cyanophages to control the growth of *K. brevis*. First, we intend to identify and isolate cyanophage variants that specifically target this organism. Once identified, we will use a DNA editing technology, the CRISPR–Cas9 system, to delete the integrase gene in order to increase virulence. The modified phage will be mass produced and used as the active ingredient in a spray to stop red tide (Figure 2).

The Culture Collection of Algae at the University of Texas offers several strains of *K. brevis* that may be used for research in academic labs; for example, strain UTEX LB 2929. Alternatively, we may be able to obtain *K. brevis* directly from local beaches (Niederhelman, 2021). Once we successfully obtain a sample and culture the organism to safe and sufficient levels, we plan to artificially expose the algae to environmental stressors in order to induce the lytic cycle of the already integrated phages. This can be done by culturing the algae in petri dishes on an agar-based solid growth medium, then exposing them to UV light. This will stress the cells and disrupt phage latency, inducing the lytic cycle. The areas on the plate where the phage has lysed cells will be cleared. These clear spots are called plaques, and a stabilizing solution will be dispensed over them to wash off and extract phages. The extracted phages will be edited via the CRISPR–Cas9 system to delete the integrase gene to increase virulence, then they will be tested for lytic effectiveness against *K. brevis*.

Parts level

Cyanophage genomes have been sequenced, and some understanding of lytic and lysogenic pathways has been gathered. As stated above, we propose to edit the phage genome by targeting the integrase gene that

is essential for the lysogenic life cycle. By knocking out this gene, we will ensure that the phage cannot integrate into the host genome and will instead initiate a lytic cycle. Sequence information for an example phage integrase is shown in Figure 3.

There are several ways to edit a phage genome. The most desirable would be to use the clustered regularly interspaced short palindromic repeat/CRISPR-associated gene (CRISPR–Cas9) system. This is a highly accurate gene editing tool capable of targeting and altering specific genes and DNA sequences. As its name suggests, the system contains two main components: Cas9 protein and CRISPR RNA (crRNA) strands, also referred to as guide RNA (gRNA). The crRNA sequences define the target gene or region of DNA to be edited. They direct the Cas9 enzyme to locate and cleave the DNA at this specific target site, by creating double-strand

a. Nucleic acid sequence

1 atggccactg ggaacgtcaa aaggcagtcg gtttgggatc gatgtgttga ggatctgcag

61 aagcggggga cgaatttcag gatggtccaa cgtggcaagt cagcaatcat ccagattcgc

121 cattaccagg acggagagct ggtgagagcg ttcagctctc agggttatcg atggaggaac

181 gcccaaggcc tgaccaccgc caaggaagag aaagagatcg agagctgcta caagctctgc

241 gtcgatgccc acgagcaagg gtcttgggtg gcagccggtg gagtcctggc agttgaggag

301 atcaaagact ggccaacctt tgctcagaga gtcacgcagg acctggaatc ccgcaccctg

361 atcaagggct ccaagaagaa ccagatcggc cacctcaagg aattagctct gcttaacggt

421 cccgtctgtg caggtgctct ggagaagtgg gccttagaga agagtcctgt tgaagaaccc

481 ggcgccttca ggaacagaag agaaacaatc agcgtgatca atcgggtcgg catcatcgac

541 ctaactgatg tgatcaagag attaaagagc aaggtcgtta ataagaaatc agccaggggc

601 aaggagctgg ccagccagca cgaaaagcct cgagcaattc ctacagatga cgagctattt

661 aattggctca aatcaatcga tgatccattg atccaatggg cctttgcaat gcaggccacc

721 tatggactga gaacctcaga ggtttggcac gttcttgaca tcgatagaga aggttgggca

781 cacattggcc ccatgaccaa aacaggcgaa cgacttgctt acccctgtcc aatggcatgg

841 gttgaagagt tcggattacg caccaacctg aaacgattct ccaaacaact ggatagcgaa

901 gaccatcagc ggcagatcgt tcggcgcgga cgacttagta aatgcatcaa taacgacgac

961 ctagggaatt ggttatggcg tcgcattgac ggtcagatca tccccaggct ctgggctgct

1021 gcagaagaca gcgagagcaa gacggcaaga gatacaggat taggcagggt tgaggattac

1081 tgccggccct acgacttcag gcatgccttt gcaatcagat gcttcactca tcctgaagtg

1141 ttctctgaat cagatgaaga gcacgcgaga tggatgggtc acggcgtaca cgtccacacg

1201 cggatctatc gcaaatggat gccaatggaa cgtcagaagg aagctgtcag gtctcgacgt

1261 caacagcgca tgaacgagga gccacaagaa aggcccgcct tagcgagcct cccagatgat

1321 gtgatggaaa agttagccaa gttagccaag ctcgaaaagc tcatggcttc atag

b. Amino acid sequence

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MATGNVKRQSVWDRCVEDLQKRGTNFRMVQRGKSAIIQIRHYQDGELVRAFSSQGYRWRNAQGLTTAKEEKEIESCYKLC
VDAHEQGSWVAAGGVLAVEEIKDWPTFAQRVTQDLESRTLIKGSKKNQIGHLKELALLNGPVCAGALEKWALEKSPVEEP
GAFRNRRETISVINRVGIIDLTDVIKRLKSKVVNKKSARGKELASQHEKPRAIPTDDELFNWLKSIDDPLIQWAFAMQATY
GLRTSEVWHVLDIDREGWAHIGPMTKTGERLAYPCPMAWVEEFGLRTNLKRFSKQLDSEDHQRQIVRRGRLSKCINNDDL
GNWLWRRIDGQIIPRLWAAAEDSESKTARDTGLGRVEDYCRPYDFRHAFAIRCFTHPEVFSESDEEHARWMGHGVHVHTRI
YRKWMPMERQKEAVRSRRQQRMNEEPQERPALASLPDDVMEKLAKLAKLEKLMAS
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Figure 3. *Nucleotide (a) and protein (b) sequences for the integrase gene of cyanophage PSS2 (Gene ID: 8207508; Symbol: PSS2_gp101; Sullivan, 2009).*

breaks (DSBs). The model integrase DNA sequence (Figure 3) will be used to design integrase-specific crRNA fragments. Multiple crRNAs will be tested to determine which is most effective at knocking out the integrase gene. The CRISPR plasmid will be designed to include the optimal crRNA sequence along with a fluorescent reporter gene, *RFP*, encoding red fluorescent protein and flanked by regions of homology (ROH) to the integrase gene. Upon successful Cas-9 mediated cleavage at the target site, the integrase gene will be fully or partially removed and swapped with the fluorescent reporter gene. The *RFP* gene will be used as a marker to screen for and identify the modified phages (integrase-KO mutant phages) in a fluorescent assay (Nano-Glo). A schematic representation of how the CRISPR–Cas9 system will be applied is shown in Figure 4. We have based our phage engineering workflow on the research of Chen et al. (2019) and Duong et al. (2020).

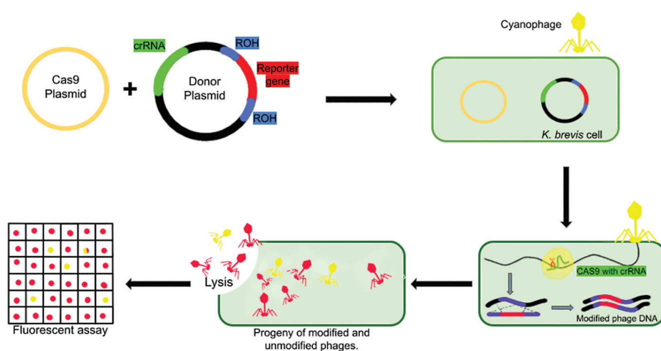

Figure 4. *Genome editing of cyanophages utilizing the CRISPR–Cas9 system. The Cas9 and donor plasmids are co-transfected into K. brevis. The latter plasmid contains a reporter gene (RFP) flanked by ROH to the cyanophage integrase gene, as well as the CRISPR recognition sequence (crRNA) that facilitates targeting of this gene. Upon phage infection, the Cas9 nuclease will cut the integrase gene and, as defined by the ROH, it will be replaced with the reporter gene. The modified (integrase-KO) phages will enter the lytic cycle, proliferate, and be released during lysis. A Nano-Glo fluorescent screening assay will be used to distinguish between wild type and mutant phages by detecting the reporter gene product, RFP protein.*

Depending on the cost and availability of reagents, as well as instruments and laboratory space, we may or may not be able to use the CRISPR–Cas9 system. If this is not possible, an alternative method involves exposing phages to UV light to generate random mutations (Figure 5). Multiple phage batches will be exposed to UV light for different durations. Consecutively, the exposed (mutated) phage samples will be tested against *K. brevis* to identify which conditions resulted in the highest virulence efficiency based on the presence, frequency, and size of plaque formation. Through multiple iterations, it may be possible to identify the desired phage phenotype. This method is not as precise or as targeted as the gene editing approach, but it is comparatively inexpensive and straightforward.

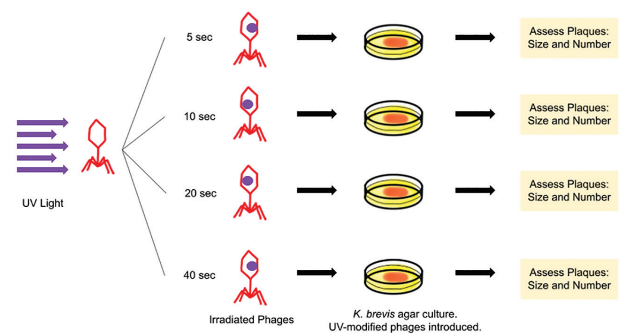

Figure 5. *Using UV exposure to introduce mutations, followed by screening of phages for virulence. In this approach, UV light is used to irradiate phages for different time intervals to create mutations. Mutated populations are screened for lytic efficiency by infection of K. brevis cultured on agar plates.*

Safety

The alga *K. brevis* is a toxin-producing organism and is harmful when ingested. It should therefore be handled with proper laboratory care, and requires the use of personal protective equipment that includes chemical-resistant gloves, chemical safety goggles, and sufficient clothing to prevent skin exposure. We will need to conduct experiments in biosafety cabinets and have

dedicated incubators for culturing *K. brevis*. Culturing the algae on agar plates is safe as it will not result in sufficient biomass to secrete neurotoxins at levels that will pose a risk to laboratory personnel. The cultures will be disposed of as biohazardous waste and eventually incinerated.

Cyanophages isolated from *K. brevis*, and those edited by the CRISPR–Cas9 system, are not known to be hazardous or toxic to humans. However, care still must be taken when handling the reagents.

Discussions

As described above, HABs are devastating to the environment, and ways to prevent them must be developed and implemented. Combating HABs is critical to preserve aquatic ecosystems, and protect human health and affected animals. It should be noted that aquatic ecosystems are delicate and sensitive, and thus, care must be used when designing potential solutions. Chemicals and other toxic substances cannot be administered since they would likely destroy both the harmful algae and the life we are trying to protect. Our proposal of utilizing virulent cyanophages prevents such indiscriminate killing, and does not introduce toxic chemicals into the ecosystem. Cyanophages are also self-limiting. If the host is no longer present, or only exists in small numbers, the phages will stop propagating.

Moreover, at low levels, cyanobacteria are beneficial as they produce oxygen via photosynthesis. It is specifically overgrowth, as occurs in HABs, which presents a problem. To improve upon our idea, it would therefore be optimal to control the lytic cycle by including inducible elements; for example, engineering the phage to initiate a killing cycle only if the cyanobacteria reach a certain level of growth. More research is required to understand what environmental factors control the onset of lytic or lysogenic cycles, and which genes regulate the fate of phages.

Another challenge that we must address is the potential immunity associated with hosts that contain a latent phage. This means that cells of *K. brevis* that already possess prophage DNA may be resistant to superinfection (Bondy-Denomy et al., 2016). The mechanisms involved are not well understood, so it is unclear how this may impact our proposal, and what we may need to implement to resolve it.

Nevertheless, we hope that our experiments will spark further research in administering natural and non-intrusive ways to help the environment. Employing cyanophages in saltwater conditions may inspire related research to combat HABs in freshwater, also using phages. Perhaps our ideas could be extended to other applications. For example, phages could be integrated within the water purification system to remove deadly pathogens from drinking water. Utilizing phages in this way may be more effective than relying on large water treatment plants, which often employ environmentally harmful chemicals. Phages may also offer a low-cost solution that can be implemented in developing countries to improve sanitary conditions.

Next steps

Once we successfully knock out the integrase gene, or create mutations that render the integrase gene inactive and identify desirable mutant phage(s), we plan to increase virulence even further by overexpressing the lytic promoters. The P3 promoter has been isolated from the GIL01 bacteriophage, where it controls the expression of lytic genes (Fornelos et al., 2018). The P3 promoter will serve as a substrate for future experiments. For example, using CRISPR–Cas9 to introduce P3 into the genome of integrase-KO cyanophages may enhance their lytic attributes considerably.

In parallel, we intend to test the solution of virulent phages on a pilot scale by building a small saltwater aquarium. We will set the temperature between 22 °C and 28 °C, and the salinity level between 30 and 35 g/kg, to mimic the conditions in which *K. brevis* flourishes (Tominack et al., 2020). Once the alga blooms, we will apply a cocktail of selected phages to determine the efficiency and rate at which they lyse the host. We will also observe what happens to the aquatic environment once the algae are destroyed, and measure how fast the organism recovers, if at all. Lastly, we will determine the rate at which the modified phages turn lysogenic.

Author contributions

All authors participated in the development of various ideas aimed at combating HABs, and contributed to the necessary research. A.E.L. initiated the concept of using phages and CRISPR-Cas9 genome editing. V.R., A.E.L., M.V., B.B., and A.C. were instrumental in authoring the manuscript. A.E.L. created the Figures. A.E.L. and V.R. addressed editors' comments and suggestions. All authors were involved in the revision and citation processes, as well as proposing potential experiments.

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