RECLAMATION

Managing Water in the West

Using Genetic Manipulation to Control Invasive Species

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Science and Technology Program
Final Report ST-2017-1746-01

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Mission Statements

Protecting America's Great Outdoors and Powering Our Future

The Department of the Interior protects and manages the Nation's natural resources and cultural heritage; provides scientific and other information about those resources; and honors its trust responsibilities or special commitments to American Indians, Alaska Natives, and affiliated island communities

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14. ABSTRACT *(Maximum 200 words)* With the discovery that CRISPR/Cas9 could be used to modify genomes and also be used to create gene drives, suddenly the idea of directly modifying the genome of an invasive species as a method of control or eradication could become a potential reality. From conception to actual use of CRISPR/Cas9 gene drive to control invasive species there are many issues that will have to be overcome. There is the potential that this technology could be used on invasive Dreissenid mussels. This scoping project had two goals. First, a summary what CRISPR/Cas9 and gene drives are, and some their applications. Second, to examine some of the steps involved in creating modified Dreissenid mussels. Some of these steps involve having a complete genome, determining the method for delivery of the gene modification into the mussel, and maintaining a mussel colony. It is not the intention of this scoping project to actually create modified mussels, but to instead understand the process. The main take home message from this project is that it is important to understand both the opportunities and limitations of these technologies so that informed choices can be made.

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Using Genetic Manipulation to Control Invasive Species

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Acronyms and Abbreviations

CGD- CRISPR/Cas9 gene drive

CRISPR- clustered regulatory interspaced short palindromic repeats

Cas9-CRISPR associated protein 9

PCR- polymerase chain reaction

RDLES- Reclamation Detection Laboratory for Exotic Species

TALENS-Transcription activator-like effector nucleases

QM- quagga mussel, *Dreissena bugensis*

ZM- zebra mussel, *Dreissena polymorpha*

Executive Summary

With the discovery that clustered regulatory interspace short palindromic repeats (CRISPR)/Cas9 could be used to modify genomes and also be used to create gene drives, suddenly the idea of directly modifying the genome of an invasive species as a method of control or eradication became a potential reality. Since its discovery, advances in CRISPR/Cas9 have been continually made. From conception to actual use of CRISPR/Cas9 gene drive (CGD) to control invasive species there are many steps and issues that will have to be overcome. There is the potential that this technology could be used to control or eliminate invasive Dreissenid mussels. This scoping project had two goals. First, a summary what CRISPR/Cas9 and gene drives are, and some of their applications. Second, to examine some of the steps involved in creating modified Dreissenid mussels. Some of these steps involve having a complete genome, determining the best method for delivery of the gene modification into the mussel, and maintaining a long term mussel colony. It is not the intention of this scoping project to actually create modified mussels, but to instead understand the process. The main take home message from this project is it is important to understand both the opportunities and limitations of these technologies so that the informed choices can be made.

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Introduction

It sounds like a straightforward solution, using genetic manipulation to control invasive species. The last few years have seen the emergence of the genome editing tool clustered regulatory interspace short palindromic repeats (CRISPR) Cas9 and gene drive as potential methods of controlling invasive species. The CRISPR/Cas9 system is allows for the rapid modification (both deletions and insertions) of an organism's genome. The complementary technique of gene drive enables researchers to spread the genome modification throughout a population. The pairing of these two technologies has been performed in fruit flies and mosquitoes [1]. Applying these methods to invasive organisms, such as Dreissenid mussels has been mentioned in publications [2]. If these ideas are going to be carried out there are many scientific, technical, and regulatory issues that will have to be overcome. The goal of this document is to start to consider some of these issues. In the coming years these technologies will continue to improve, and using genome editing to control invasive species could become a viable option. The advances are being made rapidly, and the research presented in this report, could become obsolete as new discoveries are made. Part I of this report will briefly go over genome editing, CRISPR and gene drives, and how these technologies could be applied. Part II will discuss the issues associated with using CRISPR and gene drive to modify Dreissenid mussels.

Part I: Background on CRISPR

The direct control of invasive organisms by genetic modification has been an ongoing topic of interest for many years [3]. With the advent of CRISPR/Cas9, and also gene drive technologies, using genome editing to control invasive species could because a reality. Genome editing is the when modifications, such as gene replacement, deletion, inversion, knockouts, and translocations, are made to the genome of an organism [4]. Genetic engineering can involve both the removal of a specific gene or the introduction of foreign DNA into the organism; such as the creation of genetically modified organisms (GMOs). This scoping proposal is not meant to be an exhaustive review of the current literature. There are many articles appearing in the popular press about CRISPR. Scholarly peer reviewed articles are also being published at a rapid pace. Keeping up the advances in knowledge and understanding of CRISPR/Cas9 can be a challenge.

Genome Editing

Genome editing involves the modification of an organism's genome, such as making a deletion. The molecular methods researchers use to make these genome modifications been developed over the last two decades. Zinc finger nucleases and TALENS (transcription activator-like effector nucleases) have been used with success for many years [5]. However, the development of the CRISPR/Cas9 system as a method for genome editing has in the last few years rapidly

become an very important tool for researchers [6]. The CRISPR/Cas9 system is easier, faster, and less expensive to use than other genome editing tools (Table 1). Researchers from a wide range of fields are using CRISPR every day to make modifications to organisms ranging from nematodes to mice [6]–[10].

Over the last year it has been interesting to track the advances being made in the understanding of both the advantages and disadvantages of the CRISPR/Cas9 system. Each week there seemed to be new articles about different advances or uses of this technology. The majority of the research has focused on human health [11], but at the same time there have been articles about using this genome editing tool to possibly control invasive species [2]. The development of CRISPR applications is still ongoing and its full potential is just starting to be understood.

Table 1: Table of gene editing tools, from [12]

Platform	Year Developed	First used in live animals	Time to do an experiment
Zinc finger nucleases	1996	2002	Month/year
TALENS	2010-2011	2011	Week(s)
CRISPR/Cas9	2012	2012-2013	Days

In prokaryotes, CRISPR/Cas9 system is used as a defense against bacteriophages and plasmids [13]. For two decades there has been research done on CRISPR genes in prokaryotes, it's only been in the last few years that there have been some major advances in the field. There are several key proof of concept experiments where using CRISPR/Cas9 as a genome editing tool was described. The first proof of concept publication was to show that CRISPR/Cas9 could edit the genomes of prokaryotes in 2012 [14]. The next step was to show that it could edit eukaryotic cells in 2014 [15]. CRISPR has now been used in many different organisms to perform genome editing.

CRISPR is able to edit specific strands of DNA in four steps. Figure 1 is a simplified version of these events. By using a "guide RNA" and the Cas9 protein, researchers are able to target a specific strand of DNA. Cas9 is an enzyme which cuts the target DNA, allowing another piece of DNA to be swapped into place of the old DNA. Additional enzymes in the cell then repair the cuts, securing the modification in the genome. An online search shows that there are many videos available to view that go into great detail on how the CRISRP/Cas9 system works.

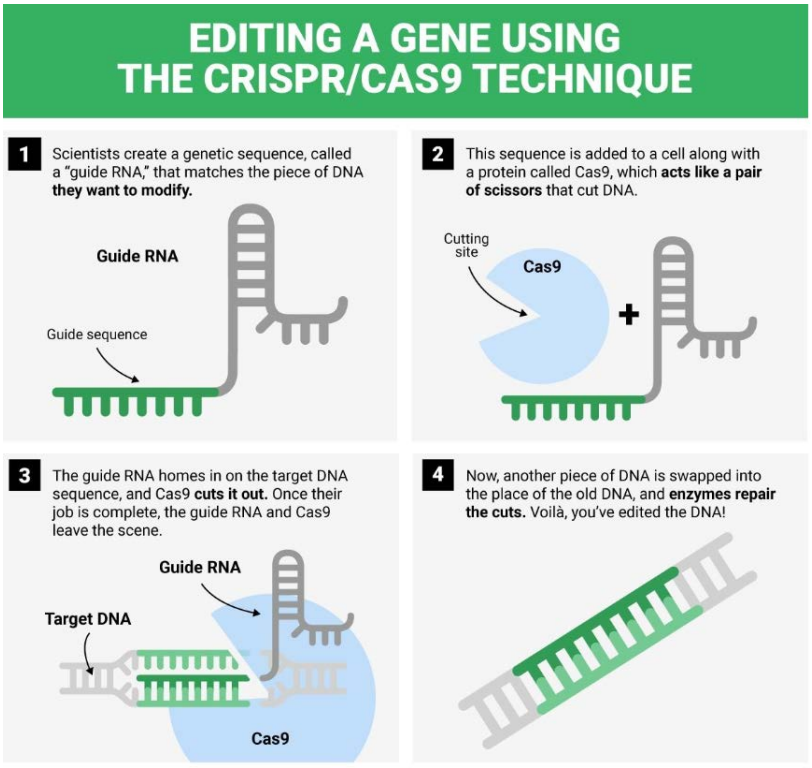

Figure 1: Editing of DNA with CRISPR/Cas9, Source Nature News; Carl Zimmer [16].

There is currently research going on to expand our understanding of CRISPR/Cas9. Additional enzymes (other than Cas9) that can cleave DNA have been found, making the system more specific, and improving the delivery of the construct into cells are just a few of the areas of research [17]. There is still a great deal of basic research to be done.

CRISPR and Gene Drive

If the goal is to control an invasive species, once genome editing has been performed it is necessary to have that genetic manipulation spread efficiently throughout the targeted population. Mendelian inheritance means that only 50% of the offspring will receive the genome edit. If a gene drive is used instead of only 50% of the offspring inheriting the trait, the majority of offspring will inherit the trait (Figure 2).

In an effort to overcome Mendelian inheritance, Austin Burt, in 2002, proposed using site specific selfish genes or natural gene drives to manipulate populations [18]. In his publication, he suggested if the gene that is selected is essential to the host that it would be possible eradiate a population in as few as 20 generations. His paper is the first to suggest using synthetic gene drives. There are three goals for gene drives: evolutionary stability, reversibility, and wide applicability [18]. The discovery of CRISPR/Cas9 as a genome editing tool has allowed researchers to construct and study gene drives in the laboratory [19].

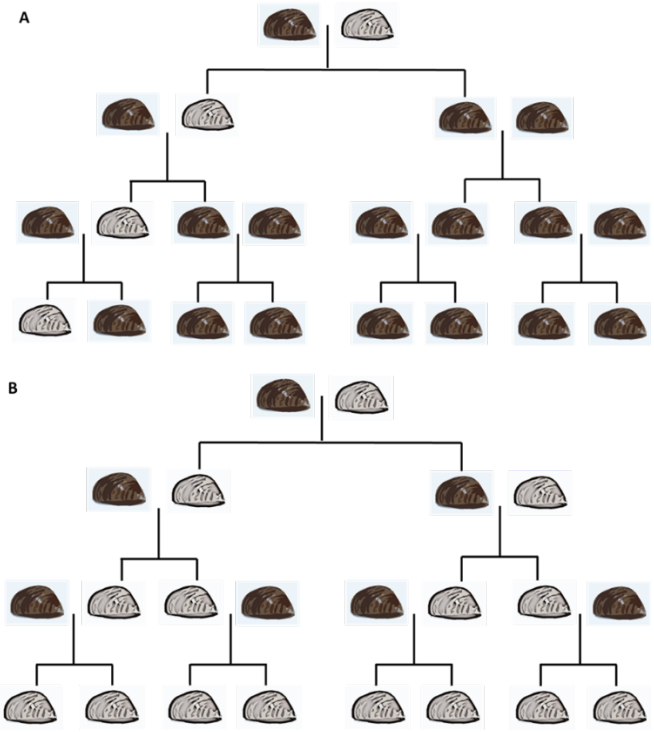

Figure 2: Gene drive and inheritance. A. Under normal inheritance an organism has a 50 % chance of inheriting a particular gene. B. When a gene is tied to a gene drive, all of the offspring will inherit the altered gene.

There are several requirements for establishment of synthetic gene drive: the organism has to have sexual reproduction, reproduce rapidly, and the organism must be able to be transformed (have exogenous DNA introduced to its genome) [20]. If an invasive pest meets these requirements then using a gene drive to control it could be an option. In 2007, there was a publication that discussed genetic options for the control of invasive vertebrate pests, where five different 'autocidal' strategies for control were discussed [3]. Two strategies of particular interest are sex or stage specific lethality/sterility and gender distortion (daughterless or sonless) [3]. By distorting the gender and making a population without females it is possible over time to drive the targeted population to extinction. Using CRISPR/Cas9 to create a synthetic gene drive, researchers were able to modify genes in fruit flies and mosquitoes and show that it was possible to spread a specific genetic change throughout a population [1], [19]. To date no one has released a synthetic gene drive into the wild to control a pest.

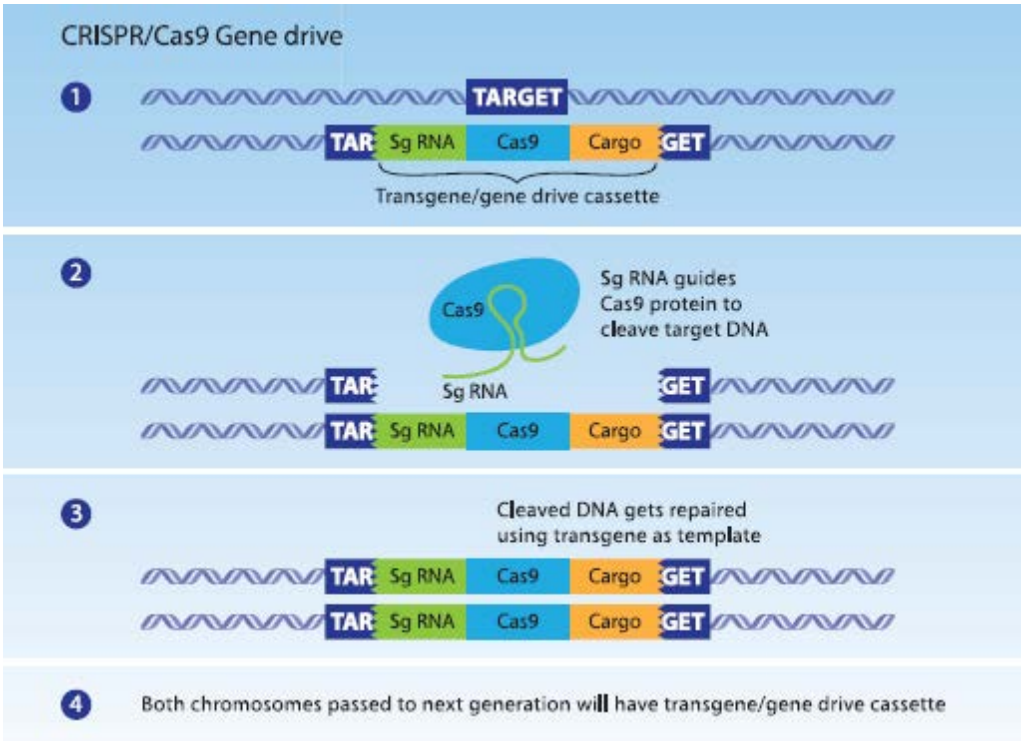

Figure 3: "A synthetic CRISPR/Cas9 gene drive. Sg RNA is the guide RNA, Cas9 is an endonuclease which cuts the DNA and cargo is the desired genetic material added. When all three elements are present in a gene drive cassette this ensures that each chromosome will have the desired cargo and will be inherited by the next generation thereby spreading the gene drive", from [20]**.**

Figure 3 shows how a gene drive cassette that contains the guide RNA, Cas9, and genetic cargo that is to be added can be inserted into a chromosome [20]. The target DNA is cleaved by the Cas9 enzyme, and the cassette is inserted. The transgene is then used as a template to ensure that the organism has two copies of the cassette. There are online sources and videos available that go over this process in great detail.

There are some issues that are emerging with the use of gene drive technology. First, once the gene drive is released into wild populations there is no control on its spread. There are already published recommendations regarding the best ways to study and control gene drives in the laboratory setting [21]. The National Academies of Sciences has also issued recommendations on best practices [22]. Once a gene drive is released into the world, there are no controls on it. That means that the potential is there for unintended consequences. To combat this, researchers are looking at designing reverse drives to stop the spread of a gene drive in a wild population. Another issue that is starting to emerge is that resistance to gene drive can develop in a population over time [23]. Both of these issues are ones that are starting to receive attention by researchers and would have to be addressed in any project that wants to use this technology to control invasive species.

Applications

Over the last year there have been regular reports of new applications for the CRISPR/Cas9. Listed below are just some of the applications of the CRISPR/Cas9 system.

Mosquitoes

Controlling mosquitoes through CRISPR/Cas9 and gene drive could potentially save many lives. Because mosquitos are carriers of several diseases (malaria and Zika virus) they are an ideal candidate for genome editing [1]. There are two different approaches being done with these organisms. First, alter the mosquitoes so that they cannot carry the disease by giving them genetic immunization to the disease. Second, eradicate mosquitoes by targeting genes to make a daughterless society. The males will be able to still reproduce and pass the genetic modification on to their offspring. All of the female offspring will be infertile. Research into both of these methods is ongoing and being looked at by the World Health Organization and other organizations as a way to control the spread of mosquito borne disease.

Agricultural

A second area where there are rapid advancements being made is in the creation of transgenic crops. Already mushrooms that do not turn brown have been created using CRISPR technology [24]. Using CRISPR to create plants that are disease or drought resistant, or able to withstand higher salinity, are all areas where research is ongoing [25]-[26].

Human Health

The use of CRISPR to treat human diseases is an area where major research is ongoing. Cell lines and mouse models are being made, and there are even clinical trials planned for the treatment of a certain types of cancer [27]. From AIDS [28] to cancer, researchers are applying CRISPR technology. Inherited diseases where a single mutation is responsible, such as sickle cell anemia, are of particular interest for researchers because with CRISPR/Cas9 the hope is to use this technology to delete and/or replace the gene responsible for the disease. New discoveries are being made at a rapid pace through the use of CRISPR.

Vertebrates

Using CRISPR to made genetic modifications on vertebrates is ongoing in a wide range of organisms for both improving human health and also pest control. First, one of the goals has been to create lab animals, such as mice and pigs, which are more human like. With pigs, the goal is to use CRISPR to give them a humanized immune system and remove any risky viruses that they carry [29]. Ultimately, using pigs as a source of organs for transplants is one of the goals with this research.

The removal of vertebrate pests is being looked at by several different groups. New Zealand has set the goal of removing damaging predators, such as mice and rats, from their island by 2050

[30]. This project, Predator Free 2050, could make use of CRISPR/Cas9 and gene drives to remove the predators on the island. Island conversation groups are also looking at this method for the removal of mice and rats from Pacific islands to protect native bird populations. For vertebrates, there is a great deal of research being done both to create research models and also to control pests.

Conclusion

Research into using CRISPR to edit genomes is being done in a wide range of fields, from agriculture to human diseases. Using CRISPR to insert a gene drive that will could eradicate or control an invasive species is research that is starting to be undertaken by researchers around the world. The number of publications related to CRISPR and also gene drive continue to grow each year with over a thousand in 2015 [22]. Being aware of both the benefits and limitations of these methods is important. This short review was only meant to give an overview of the field. Additional research will be needed for each organism of concern. Input from researchers, policy makers, and the public will be needed for any project that hopes to release a genetically manipulated organism that has a synthetic gene drive into the wild.

Part II: Genome Editing of Dreissenid Species

Using genetic control methods to stop the spread or control a population of invasive organisms is being studied by groups around the world from the United States to New Zealand. Using CRISPR and a gene drive to modify invasive organisms has been suggested in the literature [19]. To be an ideal candidate for modification there are several factors that need to be considered: lifespan, number of offspring, reproduction mechanisms, gestation period, and availability of genome information [20]. Dreissenid mussels appear to be ideal candidates for genetic engineering, based on their short lifespan, sexual reproduction, and large number of offspring. Creating transgenic quagga and zebra mussels will be a major undertaking because there are many issues that will have to be resolved before it is even possible to begin to modify these organisms.

Based on recommendations from the National Academies of Sciences, there are five phases to the process of creating and then the eventual release of an organism genetically modified to contain a gene drive (Figure 4). These recommendations are for any organism that is being considered for release into wild populations. Each step has several parts that would have to be considered. This list incorporates both the laboratory research and also the regulatory needs. For Dreissenid mussels there are additional issues that will have to be addressed as researchers move through each of these phases.

Phase 0: Research Preparation

- Develop a Target Product Profile
- Identify containment and confinement strategies
- Develop mitigation strategies
- Identify and plan for regulatory requirements
- Use models to inform standards, thresholds of acceptance, and study design
- Establish site-selection criteria (if research includes phase 2-4 trials)
- Identify risk assessment needs

Phase 1: Laboratory-Based Research

- Acquire required laboratory regulatory approvals
- Develop containment and confinement strategies
- Detect and measure off-target effects
- Optimize design of guide RNAs (when using CRISPR/Cas9-based gene drives)
- Utilize an optimized endonuclease with high cutting efficiency and accuracy
- Optimize for the use of homology-directed repair versus non-homologous end joining in order to maximize precision of editing
- Evaluate effects on organismal fitness in the presence of the gene drive
- Evaluate gene drive stability over multiple generations
- Mark gene-drive modified organisms
- Use quantitative and computational methods
- Set baseline population-level effects

Phase 2: Field-Based Research

- Acquire site-specific regulatory approvals
- Validate efficacy
- Validate population-level effects
- Estimate impact on selected non-targets

Phase 3: Staged Environmental Release

- Acquire site-specific regulatory approvals
- Conduct monitoring and surveillance for efficacy
- Conduct monitoring and surveillance for harms

Phase 4: Post-Release Surveillance

- Acquire regulatory approvals
- Conduct monitoring and surveillance
- Measure impact

Figure 4: National Academies of Sciences recommendations for gene drive [30].

Laboratory Issues

There are several major decisions that will have to be made before beginning to attempt to genetically manipulate quagga and zebra mussels. Three big questions that have to be addressed in the laboratory setting are: Which autocidal strategy for the control should be used? Which gene or genes to target? How will the CRISPR element be introduced into the mussels? Many additional questions will likely arise as each of these decisions are made.

The first step should be to model the different autocidal control strategies to determine which one could best be applied to the mussels. For example, would it be best to use a sex or stage-specific lethality/sterility or a gender distortion approach. Modeling these different approaches will be important because it will help to predict how long it would take the modified gene to

spread throughout the population, how long it might take to decrease the population, and how many of the modified organisms need to be released to have an impact on the wild population. Modeling will enable researchers to have a better idea of the type of gene or genes that should be targeted. Modeling will also allow researchers to begin to study the risks involved with releasing modified mussels into a wild population. Determining by modeling the best strategy for the control will help to guide the selection of the types of genes that should be targeted.

This leads to the second decision: Which gene or genes to target? The first step will be to obtain more genetic information about the invasive mussels. Currently, for both species of Dreissenid mussels there are only a few genes that have been sequenced and are available publically. Public databases mainly has the genes available that are used for the detection of the mussels (cytochrome oxidase I), and also the microsatellite genes that are used to track their population dynamics. It will be important to have the whole genome sequenced so that it will be possible to select genes for modification. Ideally, a whole genome for both organisms would be sequenced, and annotated. Once a gene is selected it will be critical to determine how many copies of it are in the genome. Ideally, a gene that has only one copy in the genome will be selected. Having a genome is a critical part of creating genetically modified mussels.

Once the gene or genes have been identified, the CRISPR/Cas9 gene drive construct can be made. This construct will then have to be used to transform the mussels. Which leads to decision three: How to get this construct into the mussels and which life stage is the best one for transformation? Transfection is the introduction of DNA into cells. This can be done either physically (microinjection or electroporation), chemically, or by biological (viral) means. There is a whole literature on the different methods that can be used to transform a wide range of organisms. Determining which method works best for Dreissenid mussels will be a major undertaking. This step represents a central hurtle that will have to be overcome. Once the mussels have been transformed it will be necessary to monitor them over time to ensure that the CRISPR/Cas9 gene drive is active.

For all these steps identifying researchers that are well versed in modeling, genome analysis, creating CRISPR/Cas9 constructs, and transformation methods would facilitate the research. These laboratory research steps could take several years to perform. And like any biological system there is no guarantee of a successful outcome. But the knowledge gained by performing this initial research would be valuable and increase our knowledge of Dreissenid mussel biology and genetics.

Working with live mussels

To have mussels for transformation, it will be important to have a long-term captive quagga or zebra mussel populations to perform experiments on. This limits the research to locations that already have mussel populations. A secure facility where these mussels can be maintained will have to be created. It will be necessary to have both a control population and experimental groups. Once the transformation has taken place it will be important to monitor the mussels for any unintended side effects. Modified mussels will have to be maintained and induced to breed. Their offspring will have to be monitored to determine if the genome modification is still present

over several generations. Crossing the engineered mussels with control mussels will have to be done to determine how the genome editing behaves when crossed into an unmodified population.

Keeping a Dreissenid mussel population in the laboratory over several years is in itself a large challenge. One publication about keeping Dreissenid mussel in the laboratory maintained them for one year [31]. Having the conditions (water, temperature, food) that will enable the mussel colony to thrive could be a challenge to maintain. Separating the effects of the genome modification from the conditions that the colony is kept is could be an issue. For example, if the offspring are not surviving to adulthood what is the cause? Is it due to the genome modification or the colony conditions? These technical issues will have to be addressed.

Field Studies and Releasing into the Wild

The final step will be to take mussels from the laboratory and place them in to the environment. A major issue will be to determine the site or sites that the testing can happen. It will have to be at a site where there is already a mussel population. Ideally it would be an isolated small water body with no recreation. A through survey of the wild population should be made prior to the introduction of the modified mussels. How many modified mussels should be released? How many releases should be made? How soon can an impact on the wild population be seen? Modeling will help to answer these questions. Setting up a monitoring program to determine how the modified gene(s) are spreading through the population will be an important step.

At the same time, it will be critical to work with the state and local government to get their input on performing these trials. Making sure that the public is informed and has input on this work will also be vital. Based on how the public has received other transgenic organisms having a public education component will be fundamental for a successful release of the modified mussels.

It is one thing to create a CRISPR gene drive mussel and have it in the laboratory. The ultimate goal of using this organism as a control method will ultimately depend on the regulatory rules. Regulatory issues will have to be addressed. Determining who has the authority to release these organisms into the wild and the regulations associated will be a key hurtle to overcome. Determining which agency or agencies that need to be consulted will be a first step. Then working closely with the regulators throughout the whole process from laboratory experiments to working with live mussels, and finally the release of these mussels into the environment.

Conclusions

The field of genome editing has been changed with the advent of CRISPR/Cas9 with research rapidly advancing. The idea of releasing transgenic invasive mussels into the environment as a control measure is very seductive. Could this be a magic bullet that causes a decrease or eradication in quagga and zebra mussel populations? While it seems like a straightforward concept, like any biological system there could be unknown or unplanned issues or risks that

arise. Also, in reality there are several major issues that would have to be overcome. Having a genome, determining the best transformation methods, and maintaining a colony of mussels for years are just three issues that would have to be addressed. It is important to consider all of these issues prior to undertaking a project such as this.

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