ZEQUANOX®
Invasive Mussel Control

THE ZEQUANOX® STORY

An in-depth review of the discovery and commercialization of a non-chemical, natural alternative for invasive mussel control

The naturally occurring microorganism in Zequanox (a bacterium that kills invasive zebra and quagga mussels but leaves other mollusks unharmed) was discovered by the New York State Museum.

Marrone Bio Innovations commercialized the product Zequanox. More recently, Invasive Species Corporation, co-founded by the founder and former CEO and former president/CFO of Marrone Bio, has taken over Zequanox to continue to expand its use and develop an improved, lower cost product for larger facilities and water bodies.

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They are tiny—the size of a person's fingernail—yet they are having a billion-dollar impact on the North American economy and causing great harm to countless freshwater ecosystems. Zebra mussels (Dreissena polymorpha) and quagga mussels (Dreissena rostriformis bugensis) are highly invasive bivalves native to Eastern Europe. These tiny, freshwater species originated in the Caspian and Black Seas and were transported to North America in ballast water from a cargo ship. First discovered in the Great Lakes in the late 1980s, these mussels have now invaded waterways across the United States, causing numerous ecological impacts and creating operations and maintenance challenges for commercial facilities that draw water from infested lakes and rivers. By the early 1990s facility operators were employing a variety of methods to try and stave off impending damage that could be caused by the ever-expanding mussel colonies. Some methods were labor intensive, some turned out to be completely ineffective and others posed a tremendous risk to the facilities' employees and the surrounding ecosystem. Without a better alternative, operators had to tolerate these shortcomings. In 2007, however, an exciting scientific breakthrough led to the development of Zequanox®, a naturally derived molluscicide that offered a highly effective AND environmentally compatible control method for these invasive mussels. Presented herein is the story of Zequanox: how it was discovered, how the product was developed, and an overview of the science behind this innovation. This document also summarizes results that demonstrate the efficacy and safety of Zequanox and highlights the product's many advantages over traditional control options.

A MULTIBILLION-DOLLAR PROBLEM

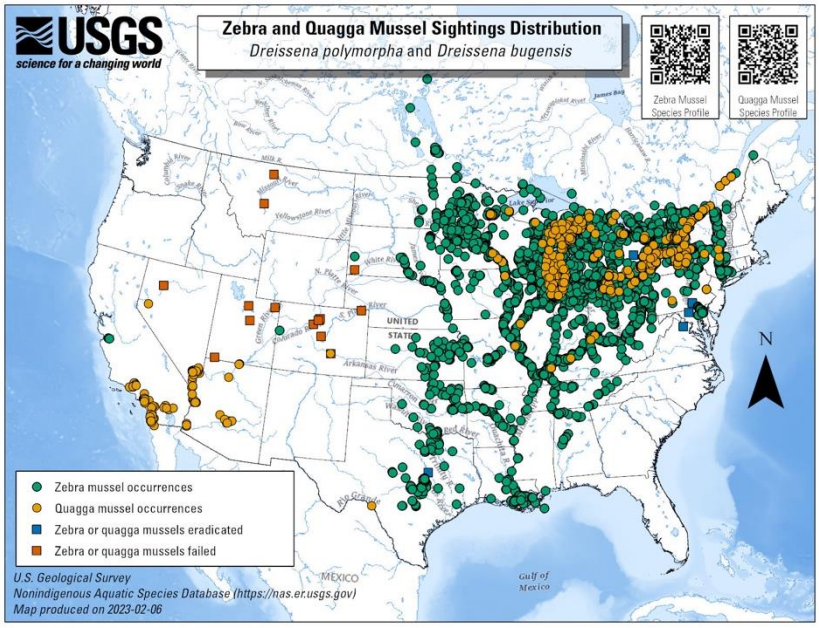
Zebra and quagga mussels can attach by the millions to one another, forming dense colonies in heavy masses up to a foot thick (U.S. Department of the Interior, U.S. Geological Survey [USGS] 2008). These colonies can clog piping, filters and screens, impeding or preventing the flow of critical cooling or process water. The mussels can also coversystem components and mechanical parts, causing system damage and weighing down equipment and infrastructure. These effects can hinder or in some cases shut down operations. Continual attachment of *Dreissena* can also significantly increase corrosion rates of steel and concrete (Benson and Raikow 2011), leaving equipment and infrastructure vulnerable to failure. Many of the traditional chemical control options, chlorine in particular, exacerbate the increased corrosion and pitting that invasive mussels initiate. Once introduced into a new water body, the population growth of these mussels can be explosive.

Very successful invaders, zebra and quagga mussels thrive in a variety of temperatures, readily find food, reproduce prolifically and rapidly, and lack natural predators. The average female zebra mussel, which is ready to reproduce in its first year of life, can release 30,000 to 40,000 eggs per year. After hatching, the planktonic larvae can not only move great distances in flowing water, but can also easily invade small places in both natural systems and industrial systems that draw from infested waters. Mussel colonies can build up very quickly. It has been reported that these mussels have been able to clog a three-foot-diameter pipe in less than three months (U.S. Department of Energy, National Energy Technology Laboratory [NETL] 2006

Six-inch process water intake pipe clogged with zebra mussels. Photo taken at an industrial facility in Michigan by Marrone Bio Innovations

An added challenge is that these mussels can rapidly disperse to other water bodies, primarily by the larval

movement and their inadvertent transport by barge and boat traffic, and can survive for many days out of water, factors that have caused the zebra and quagga mussel invasion to spread to many previously uninfested waters throughout the United States. They arrived in Lake Erie in the late 1980s, likely in the ballast water of transoceanic vessels (McMahon, 1996). Dreissenids can survive dry conditions for several days on or in boats, motors, and trailers. They also hitchhike on aquarium plants, such as moss balls available at petand aquarium stores (U.S. Geological Survey, 2021). Zebra mussels were the first to arrive and establish. Where both species exist, quagga mussels frequently replace zebra mussels because they are larger. Since their invasion, zebra mussels have spread to 31 states and quagga mussels to 18 states (U.S. Geological Survey, 2023). Bilge and livewell water of recreational vessels and ballast water of shipping vessels have been the primary vectors of transmission.



USGS map of zebra and quagga mussel spread June 2023

There is widespread agreement that zebra and quagga mussels annually cause millions of dollars in additional maintenance expenses in North America. United States Congressional researchers estimated that zebra mussels alone cost the power industry $3.1 billion during 1993–1999. The U.S. Fish and Wildlife Service estimated the economic impact during 2000–2010 at $5 billion. The mounting costs, combined with an ever-expanding geographical area of impact, have increased the need for reliable control methods that are suitable for a variety of industrial and civil applications.

THE NEED FOR A NEW APPROACH

Enclosed Systems

Commercial and public entities facing zebra and quagga mussel infestations have applied a variety of methods when seeking to control mussel populations, including aqueous controls, antifouling coatings, physical removal and mechanical controls. Each of these methods has significant drawbacks.

A common approach to mussel control is aqueous applications of chemicals such as chlorine for enclosed systems such as pipes and bays. Chlorine-based methods using hypochlorite, chlorine gas and chlorine dioxide necessarily involve careful practices to ensure that the chemicals are safely stored, and that employees handling the chemicals are not exposed to hazards and unnecessary risk. In addition, chlorine and other oxidizing chemicals are corrosive to

equipment. Chemical treatments are toxic to other aquatic organisms and because of this non-targeted toxicity, facilities using chlorine and other chemical- based molluscicides may be required to deactivate or detoxify the treated water before discharge to meet environmental requirements (NETL 2006). Bisulfate or similar salts are used to help prevent the release of chlorine into the environment and reduce the impact on other aquatic organisms, contributing to salt loading in water bodies. Many molluscicides require the addition of clay to a treated water system to quench or deactivate the chemicals' toxicity before discharge into the environment. The ultimate fate and transport of the clay-bound molluscicides once discharged is unknown; many of these substances are nonbiodegradable and stay in the ecosystem long after discharge.

An additional disadvantage of using chlorine is that the mussels perceive the chlorinated water as a threat, causing them to shut their valves for so long that very long application times are necessary to achieve results. The formation of harmful by-products is yet another area of concern; when chlorine combines with organic compounds in water, potentially carcinogenic substances such as trihalomethanes, haloacetic acids and dioxins are formed (U.S. Environmental Protection Agency [EPA] 1999; Thornton 2000).

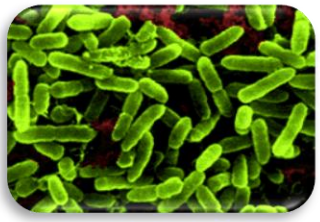
Open Water Treatment

Copper-based pesticides have a long history of use in aquatic ecosystem management and were used in open water to control dreissenid mussels as early as 2004 (reviewed by Dahlberg et al, 2023; doi: 10.1038/s41598-023-36522-5). Several forms of copper-based pesticides have been used in these efforts, including copper sulfate ($CuSO_4$), Cutrine-Ultra (Applied Biochemists, Alpharetta, Georgia), Natrix (SePro Corporation, Carmel, Indiana), and EarthTec QZ (Earth Science Laboratories, Rogers, Arkansas). Currently, EarthTec QZ and Natrix are the only copper-based products registered by the U.S. EPA as molluscicides for dreissenid mussel control, although Cutrine-Ultra is registered for use as an algaecide, herbicide, and cyanobactericide.

Potassium chloride (KCl), or potash in its unrefined form, has also been used to control dreissenid mussels. KCl is not registered by the U.S. EPA as a molluscicide, but through site-specific regulatory exemption processes (i.e., Section 24 (c) Special Local Needs exemption[31], Section 18 Emergency Exemption[32]) has been used in open water to control zebra mussels[33]. Section 24(c) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) allows states to register a pesticide for a Special Local Need. Special Local Needs are defined as existing or imminent pest problems for which there is no appropriate federally registered pesticide available[31]. Alternatively, Section 18 of FIFRA authorizes the U.S. EPA to allow unregistered uses of pesticides to address emergency conditions[32].

Although the currently available chemical products can effectively kill dreissenid mussels, they can have unintended negative effects on non-target organisms. For example, high concentrations of dissolved copper can be toxic to aquatic plants, algae, fish, snails, and other invertebrates. KCl, at the concentrations lethal to dreissenids, has minimal effects on fish populations, but can be lethal to shelled organisms, including native mollusks, crayfish, and zooplankton. In fact, a recent treatment to kill quagga mussels in the Snake River in Idaho killed the native mollusks and thousands of fish at the doses used.

A BIOLOGICAL BREAKTHROUGH

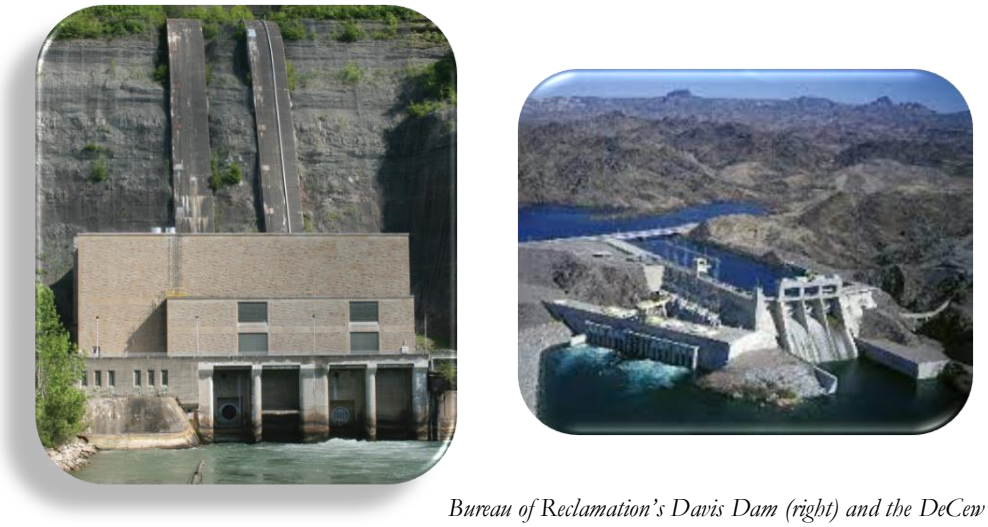


The need for a new control method drove extensive research that led to an industry-changing discovery. Faced with the threat of zebra mussels fouling electric power facilities within New York State, a research consortium of New York State's electric power generation companies contracted with New York State Museum Field Research Laboratory in 1991 for the screening of bacteria as potential biological control agents. The use of microbial, natural product compounds already had a clear record of commercial success and environmental safety in the control of invertebrate pests in North America, as well as globally (Rodgers 1993). Extensive laboratory screening trials of more than 700 bacterial strains identified a North American isolate, strain CL145A of *Pseudomonas fluorescens*, to be lethal

to zebra and quagga mussels (Molloy 2002). *Pseudomonas fluorescens* is worldwide in distribution and is present in all North American water bodies. In nature, it is a harmless bacterial species that is found protecting the roots of plants from diseases.

BRINGING THE SOLUTION TO MARKET

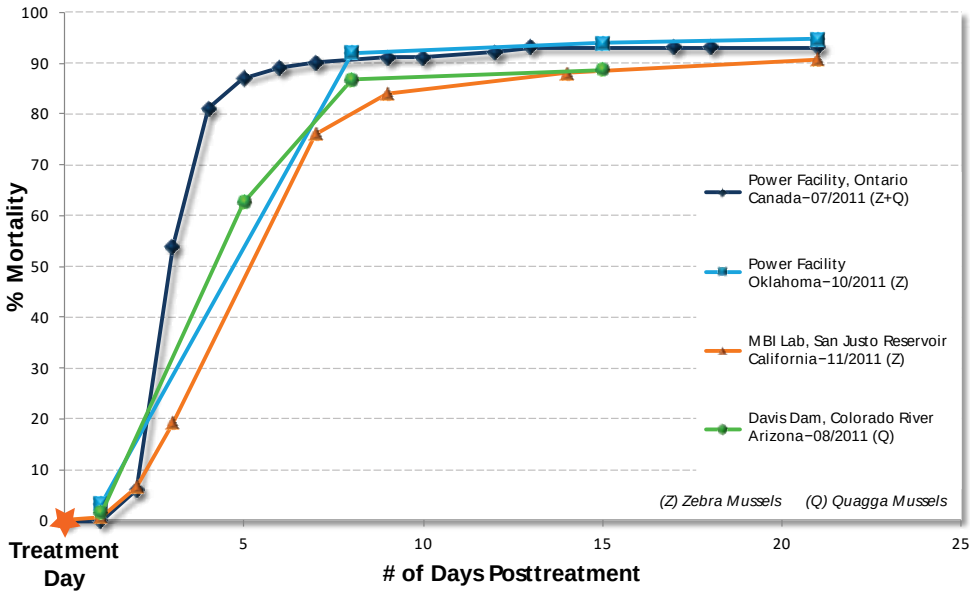
In 2007 Marrone Bio Innovations (MBI) entered into a commercial partnership with the New York State Museum to bring this naturally occurring soil microorganism to market for the control of zebra and quagga mussels. The result was Zequanox—**the industry's first environmentally compatible molluscicide.** The EPA registered Zequanox on July 29, 2011 and it is now registered in all states except Hawaii and In Canada for pipe use. Beginning in 2009, MBI in cooperation with the U.S. Bureau of Reclamation (Reclamation) conducted field trials of Zequanox under a Cooperative Research and Development Agreement (CRADA). The product was tested at Reclamation's Davis Dam on the lower Colorado River, where supply lines were heavily infested. MBI also teamed with Ontario Power Generation of Ontario, Canada, to perform testing at the DeCew II Generating Station Facility. Ontario Power Generation, which had a 20-year history of chlorine control that had reached its maximum optimization potential and wanted to help bring a more sustainable mussel control solution to the market, assisted MBI in its commercial development of Zequanox (Van Oostrom, Peterson-Murray and Dow 2010).

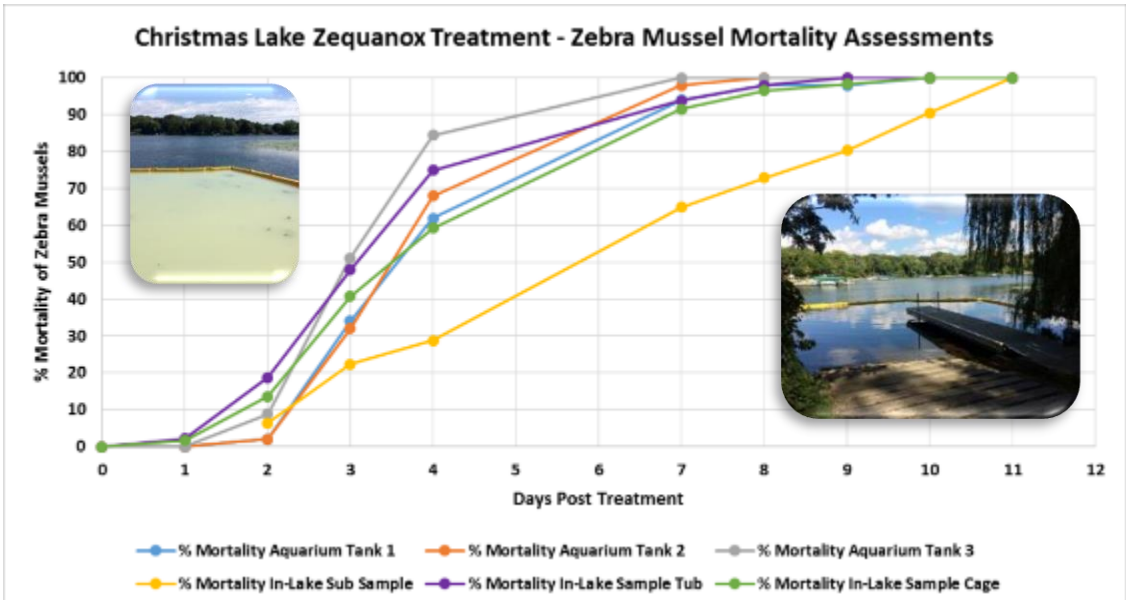


Bureau of Reclamation's Davis Dam (right) and the DeCew II Generating Station operated by Ontario Power Generation (left).

In 2011, MBI conducted a number of demonstration and full-scale Zequanox treatments throughout North America. The chart below summarizes test results of adult mussel treatments—conducted in different locations with varying water qualities—on both zebra and quagga mussels. Typical treatments ranged from six to eight hours and mortality was scored from two weeks up to one month posttreatment. The following chart summarizes the early work in power and San Justo reservoir. Since these initial studies, several more facilities have been successfully treated such as Hoover Dam, First Light & Power, NRG, OGE, two drinking water plants (one in Mexico and one in Ireland), and others.

In addition, several lake treatments have been conducted including an eradication in Christmas Lake, MN.

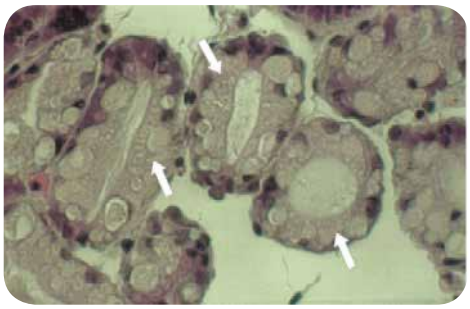




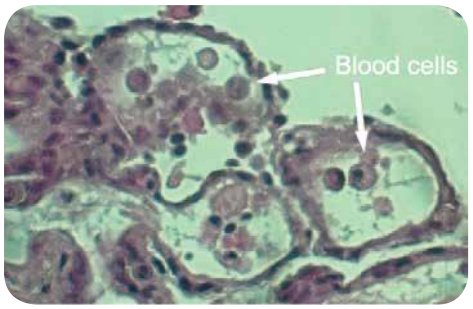
Keegan Luna, Kyle Bloodsworth Cattoor, Eric Preisler, Jill Sweet & Michael A. McCartney (2018) Zebra mussel (Dreissena polymorpha) eradication efforts in Christmas Lake, Minnesota, Lake and Reservoir Management, 34:1, 7-20. https://doi.org/10.1080/10402381.2017.1360417

HOW DOES ZEQUANOX WORK?

Zequanox is composed of **dead cells** of the *Pseudomonas fluorescens* microorganism. The cells contain natural compounds that, when ingested, are lethal to zebra and quagga mussels during all life stages (veliger to adult). The primary natural product compound identified as causing the efficacy is a protein called FitD produced in the bacterial cells. It is patented by MBI and exclusively licensed to ISC. ISC has cloned this protein and developed a method to determine the protein levels in manufactured cells so we can ensure the product is efficacious from the fermentation factory. In the past, the only method to determine efficacy was a live mussel assay in the laboratory, which is very cumbersome. Now we can make product improvements more quickly using a gel method to detect the protein rather than using the mussel bioassay. The protein binds to the mussels' gut cells and causes hemorrhaging. The mussels stop feeding and die.



In healthy mussels, epithelial cells (arrows) appear as a thick layer lining the tubules of the digestive gland.



Following bacterial treatment, epithelial cells are destroyed. Blood cells are abundant as the digestive gland hemorrhages.

The mussels perceive Zequanox as a nonthreatening food source and readily consume the product along with their normal phytoplankton diet. As mentioned previously, this feeding mechanism contrasts with biocides such as chlorine, which mussels sense as threatening, causing them to quickly shut their valves to guard themselves against the chemical. While susceptibility increases with water temperature, with more than 90% mussel mortality when Zequanox is used in water temperatures greater than 10°C, high mortality is achievable even in very cold waters. The higher the temperature the faster the kill (hours in warm water and days to weeks in cold water).

EXTENSIVE TOXICOLOGY STUDIES DEMONSTRATE SELECTIVITY

As previously noted, *P. fluorescens* is worldwide in distribution and is present in all North American water bodies. In nature it is a harmless bacterial species. Extensive toxicology studies have been conducted with Zequanox and the findings demonstrate that, unlike chemical molluscicides, Zequanox is highly selective toward zebra and quagga mussels and is of inherently low toxicity to non-target organisms (including other mussel species).

A number of fish species from representative taxonomic groups have been tested. Several high-dose, multi-day toxicity studies on either Brown or Rainbow trout, which are often noted to be the most sensitive species in ecotoxicological studies, indicate minimal toxicity to trout. The results of studies on more hardy species—the fathead minnow of the cyprinid family, striped bass, and suckers—also show that the fish would be safe at the concentrations and exposure durations that they would experience in water bodies near facilities undergoing Zequanox treatments. Considering the relatively short treatment times using Zequanox, the immediate dilution in receiving waters and the rapid environmental breakdown of the product, no toxic effects to non-target fish species are expected or likely. This has been confirmed in open water treatments such as the Sleeping Bear Lake Michigan demo. https://www.mlive.com/news/2020/12/experimental-project-successfully-removes-invasive-mussels-near-sleeping-bear-dunes.html

In addition to fish species, a broad range of invertebrate taxa has also been tested. No eff were noted on *Daphnia* (a common, small, free-swimming crustacean) or the sediment-dwelling amphipod crustacean *Hyalella azteca*. Based on the results of studies conducted on two benthic insects—mayfly nymphs and chironomids—no effects would be expected on free-swimming and benthic invertebrates. A 14-day study conducted with the mallard duck (a common, representative aquatic avian species) showed no mortality, no clinical signs of toxicity, no effect on body weight or feed consumption, and no pathological findings in all cases and at all concentrations tested. It is expected that exposure to water containing maximum treatment concentrations would not pose a threat to aquatic birds. Six common species of aquatic plants (common water plantain, small-flower umbrella sedge, nightshade, bindweed, mallow and curly dock) were immersed in water containing Zequanox at a concentration higher than that typically used for treatments. After six days of immersion, no signs of phytotoxicity were observed in any of the plants.

A range of freshwater mussel species (in the unionid family) was exposed to the maximum Zequanox concentrations. There was a complete absence of mortality in all cases, while in the same studies mortality in the zebra and quagga mussels consistently approached 100%. In addition, no mortality was observed in the native freshwater anadonta mussel. Based on these studies, no risks to native mussels are expected.

HOW DOES ZEQUANOX COMPARE WITH ALTERNATIVE SOLUTIONS?

Zequanox offers several advantages over chlorine, copper and other chemical pesticides, including safety, flexibility and ease of use. First and foremost, Zequanox poses very limited to no risk to workers, non-target species and the environment. As a reduced-risk pesticide, Zequanox is safe to store, handle and apply; only minimal personal protective equipment is needed. In contrast, chlorine, copper and other chemical pesticides are toxic to aquatic life and the environment (i.e., they often fall into the level 1 pesticide, or other high-risk category). These products require special handling, safety warning placards, sophisticated permitting, tracking and monitoring. If not properly managed, chlorine and other hazardous chemicals can cause serious (even fatal) harm to humans, and can cause irreparable harm to the environment. And as mentioned previously, the corrosive nature of oxidizing chemicals can limit the life span of valuable equipment or create the need for additional maintenance. To comply with the National Pollutant Discharge Elimination System, chlorine and harmful chemicals require special permitting, tracking, monitoring and detoxifying before discharge. The use of Zequanox carries none of these requirements, and detoxification is not required before discharge of the treated water. Applications of Zequanox are less labor intensive and less operationally disruptive than chemical methods. Zequanox treatments can be done during normal facility operations and typically occur within a six- to eight-hour period. This timeframe is in contrast to chlorine treatments, which can require several weeks of around-the-clock treatment, and often require special procedures to ensure worker safety during the treatments. Zequanox offers additional flexibility in that it is proven effective in a broader range of water conditions and temperatures than chlorine, thus expanding the "treatment season" during which Zequanox treatments can be effective. Zequanox also offers a number of advantages when compared with UV and microfiltration solutions. First, Zequanox can be applied using standard injection equipment, so facility operators can implement a Zequanox control program quickly and easily. Zequanox can be employed without having to undergo an arduous capital budgeting process and equipment installation, and without incurring the additional overhead of ongoing equipment maintenance. The aqueous formulation of Zequanox provides the added benefit of being able to reach and treat even the smallest of crevices in the water system, whereas mechanical solutions offer control only at a fixed location. Zequanox also offers the unmatched ability to tailor the treatment regimen to achieve the desired balance of mussel control, application frequency and shell debris management.

Since Zequanox was developed, copper has gained usage for controlling zebra and quagga mussels in open waters, particularly Earthtec QZ. It is an effective product, however it is less environmentally friendly and

more toxic to non-target organisms than Zequanox. For humans, Zequanox has a CAUTION label – the safest category, whereas copper is WARNING, a higher risk category. Also, there are warnings about non-target organisms on the Earthtec QZ EPA label. "This pesticide is toxic to fish and aquatic invertebrates." "Waters treated with this product may be hazardous to aquatic organisms." This is not the case with Zequanox, which has similarly high efficacy as copper.

In addition, Zequanox can mesh with sustainability initiatives. Because Zequanox is fermented in large stainless steel vessels instead of made synthetically or mined, it has a lower carbon footprint and is less fossil fuel intensive than competing products. Including Zequanox's high safety to humans and nontarget organisms, it makes it the product of choice for increasing sustainability and environmental compliance initiatives.

A Comparison of Zebra and Quagga Mussel Control Methods

	Chlorine & Other Chemical Pesticides	**Microfiltration/UV**	**Copper**	**Zequanox**
Application Time	Days to weeks	Continuous	24 hours to several days	6 hours
Startup Investment	Medium	High	Medium	Limited
Worker Safety Requirements	High	Minimal	Medium	Minimal
PPE Requirements	High	Minimal	High	Minimal
Discharge Requirements	Detoxification may be required	None	Some restrictions for potable water	None
Environmental & Nontarget Risk	Highly toxic to most organisms; Forms toxic byproducts	None	EPA classifies copper as an environmental pollutant; Low doses may have lower effect but cannot be used at pHs below 5.5 & low DO; Specific fish restrictions for lake treatments	None
Equipment Corrosion Risk	High	None	Cannot use steel, nylon, brass or copper containers/pipes	None
Water Temp & Water Quality Effects on Control	Limited efficacy below 8C; lower efficacy when organic matter & algae are present	Efficacy comprised in cloudy waters with organic matter, & algae infested waters	Minimal; Lower temps may require longer treatment times	Minimal water quality effects. Effective down to 8C
Regulatory Restrictions	High. May require state permits	NA	Some. May require state permits	Low

ZEQUANOX SECOND GENERATION PRODUCT UNDER DEVELOPMENT

Because Zequanox is fermented in large stainless steel vessels instead of made synthetically or mined, it has a lower carbon footprint and less fossil fuel intensive than competing products. However, fermentation is generally more expensive than synthesis. As such, it has been more expensive than chemicals on a line item basis (not including all the permitting, safety requirements, corrosion, etc. for chemicals). ISC is working on enhancing the fermentation manufacturing process and formulation to drive down the cost to cement it as the product of choice. ISC has a CRADA with the USGS to develop and test a new formulation that sinks to the bottom of a water body where the mussels live. This formulation will eliminate the need for barriers and curtains required with the current wettable powder product for large bodies of water.

CONCLUSION

Throughout North America and Europe, zebra and quagga mussels have seriously affected industrial and commercial operations by restricting water flow in heat exchangers, condensers, fire suppression systems, and service and cooling water systems, as well as by damaging other infrastructure and equipment. In addition, they continue to spread into lakes and rivers, including tributaries to sensitive salmon habitats. Just recently (July 2024), the mussels were reported in new areas of the Colorado River, a lake in Minnesota and Manitoba Canada.

The first and only biological mussel control solution, Zequanox offers what no other mussel control solution does—a highly effective, flexible method that requires little or no capital investment and that can be used without putting employees or the environment at risk from harsh chemicals. Using Zequanox for invasive mussel control allows facility owners to support environmental stewardship while protecting operations and assets. In addition, Zequanox offers water resource managers a very effective but safer and more environmentally friendly solution **for rapid response to eradicate new infestations as well as treatment of established infestations.**

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