

PRO 4000 X

Advanced Biotechnology for Pond Water Quality (Environment) Management

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The Presentation

- Definition of a Probiotic
 - Clearly defined. No such thing for aquaculture.
- Concepts
 - Makes no sense to add bacteria to the water column in a pond
- Commentary
- PRO 4000 X Discussion
- Outlook and Conclusions
- Testimonials

Definitions

Are we really talking about probiotics?

The classic definition refers to a change of the microbial flora in an animals intestinal tract with a subsequent benefit, usually some aspect of health or disease resistance based on inhibition of indigenous bacterial populations.

The definition that has been agreed upon by the “experts”

The term *probiotics* as defined by the Food and Agriculture Organization (FAO) and World Health Organization (WHO) as "*live microorganisms* which when administered in adequate amounts confer a health benefit on the host."

Early authors defined probiotics using a similar approach as described in the next slide.

Definition (continued)

- Fuller (1989) defined a probiotic as
“A live microbial feed supplement, which beneficially affects the host animal by **improving its intestinal microbial balance**”.
- Havenaar et al (1992) refined the definition:
“A mono- or mixed culture of live microorganisms that, applied to animal or man, affect beneficially the host by **improving the properties of the indigenous microflora.**”

Definition (continued)

- Moriarty (1998) extended the definition to include aquatic environments. Alteration of the bacterial flora in an aquatic environment could beneficially affect the animals growing in the aquatic environment. **This is in no way a probiotic.**
- He stated that it would take about 50,000 CFU/ml to effect meaningful change (10,000 to 100,000 CFU/ml). Later on we will talk about why this is not possible. No commercial products achieve these levels.

What is real?

Research has shown that while true probiotics might be possible in a laboratory environment there are, as of yet, no commercial products that conform with the properties of these laboratory tested products. Many claims are simply not substantiated by the facts.

What can we conclude?

- In aquaculture the term probiotic is being applied to both internal and external environments.
- These contrasting definitions have resulted in confusion and many products make claims and are sold based on changes in the intestinal tract flora of shrimp with no, little, or weak evidence to support this mode of action.
- **Pseudo science everywhere.** It is often ignored that many pathogens enter immune suppressed shrimp through means other than the intestinal tract where the probiotics would act.

Conclusions (continued)

- Term probiotic is misused in aquaculture.
- Products are in reality intended to alter environmental microbial composition with the idea that this will affect positive change on the growth of the organism being cultured (in this case shrimp).
- Addition to the feed would be more appropriate if we want to change flora in the gut (like yogurt). Not as easy as it sounds. Far from proven in humans and other animals.

PROBIOTICS DO NOT EXIST FOR AQUACULTURE

Probiotics or Prebiotics are not the same

- Probiotics are living organisms
- Prebiotics are non-digestible dietary supplements that can modify the balance of the intestinal micro flora, stimulating the growth and/or activity of beneficial organisms and suppressing potentially deleterious bacteria. No solid reproducible data that this happens in shrimp in the field. Cheap and harmless.

Probiotics in Aquaculture are really tools
for managing the microbial ecology in
the pond

So the reality is that the products that
work are tools for

Microbial Ecology Management
(MEM)

PRO 4000X is a tool that we developed to avoid some of the problems with traditional water applied based powdered materials.

PRO 4000X is a field proven tool for improving water quality with additional benefits that are a result of a cleaner and less stressful rearing environment.

THE CHALLENGE

Adding enough bacteria to cause a change in the composition of the bacterial population in any environment is very difficult and in fact is rarely if ever achieved by commercial products.

How can we maximize the chances that a product will have a cost effective benefit?

What are the critical considerations that can affect effectiveness of these products?

Environmental

Nutrient loads

High levels of bacteria naturally occurring in the environment

Water quality issues that can affect germination and subsequent growth

Metabolite loads

Biological

Shrimp biomass and health status of the population

Others we will only consider one for this presentation

Existing bacterial load in the environment

- There are bacteria in the water column
- There are bacteria in and on the shrimp
- There are bacteria in the sediments
- There are bacteria in the food that the shrimp eat (pellets and natural).

- Variable levels of bacteria depend upon species, environment (salinity, temperature, nutrient load and input, types of other microbes present, etc.), soil and water composition

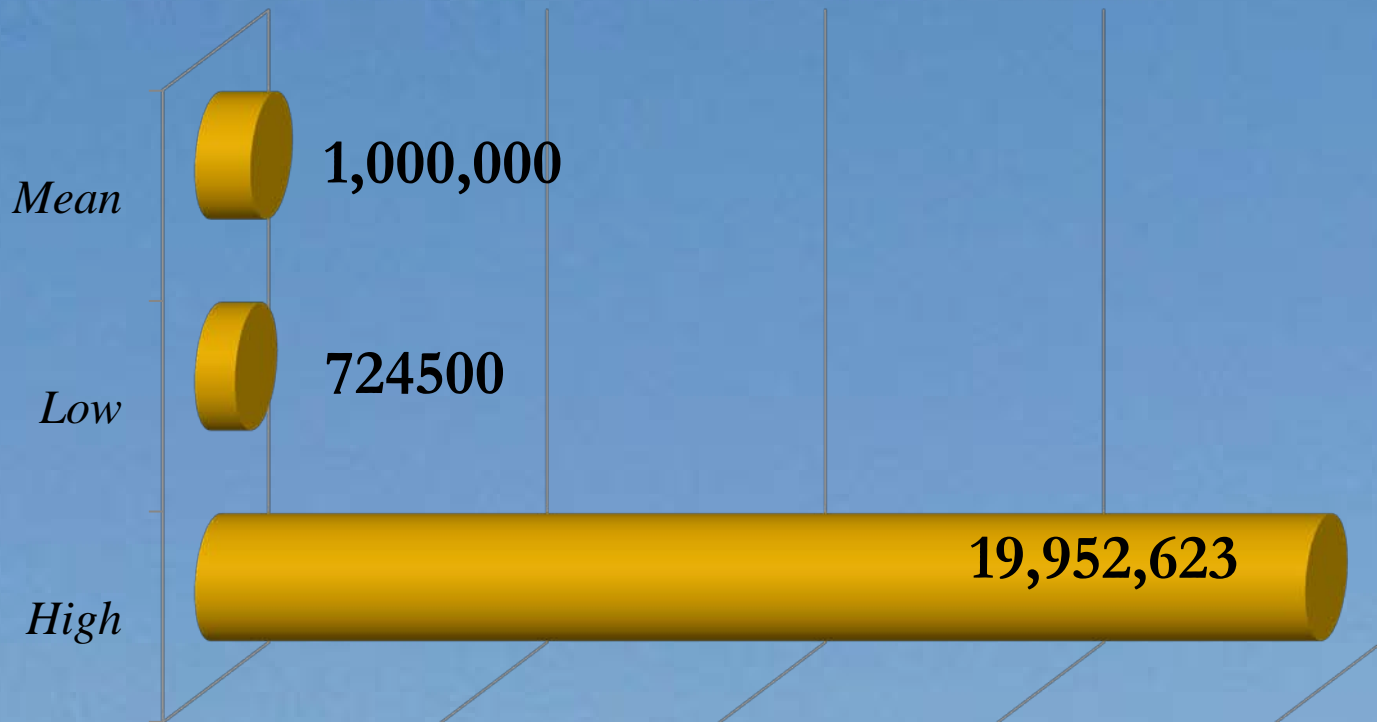
Bacteria are everywhere

Myth: When you count bacteria in an aquatic environment you can count them all. Many bacteria can not be cultured using traditional (agar plates) methods. Damaged bacteria can not be cultured.

Reality is that the bacterial counts in the following slides are actually low

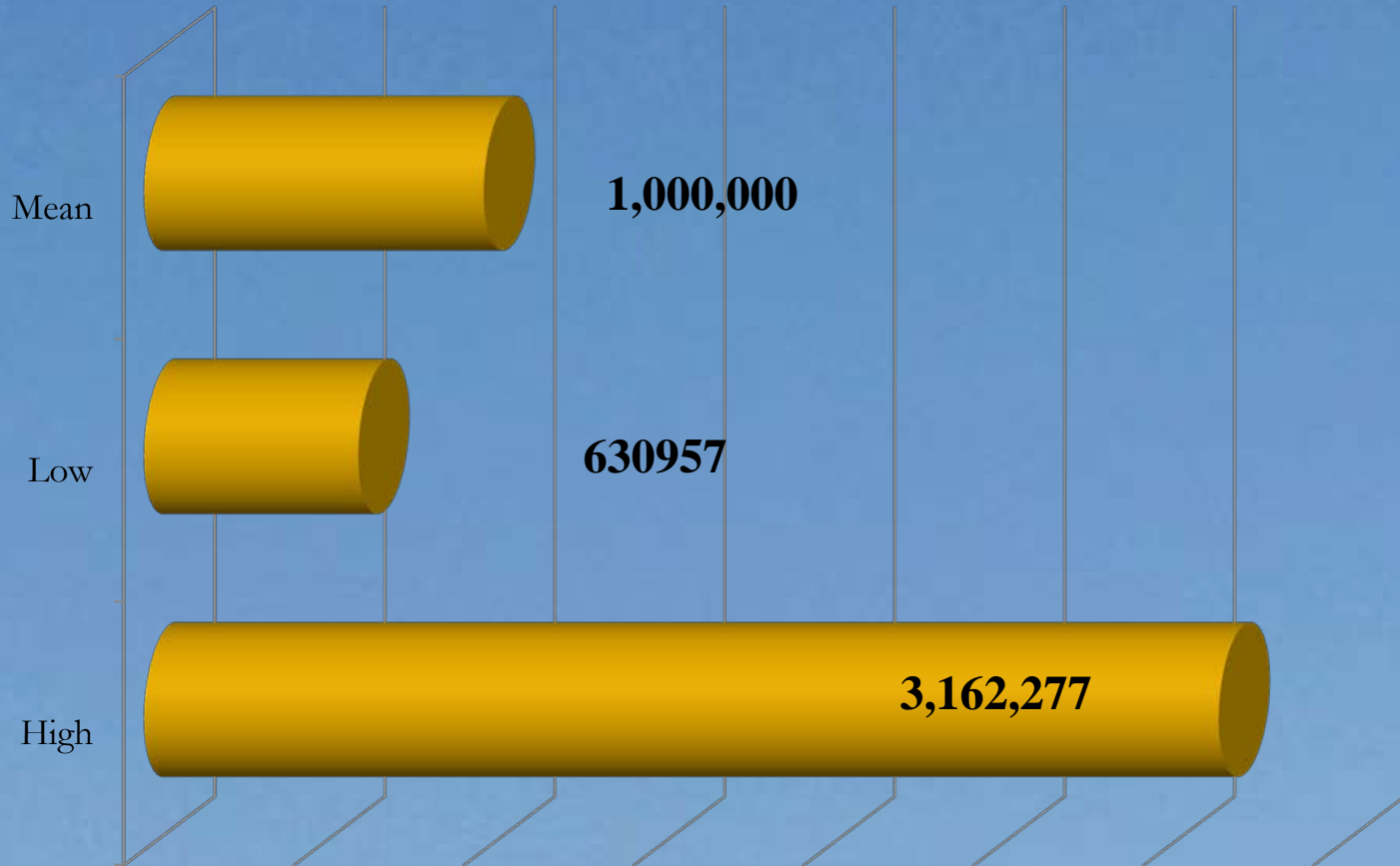
High Natural Bacterial Counts in pond sediments.
The mean of one million is low.

Sediment CFU per gram (aerobic)



High Natural Bacterial Counts in Water

Water CFU per gram (aerobic)



This study showed an average of 1,000,000 CFU per ml (with high degree of variability) of viable aerobic bacteria already established in pond water and soils. In fact this is a low number but useful for understanding why dried and liquid products probably cannot work.

How many bacteria can be realistically added to these environments to try and affect change?

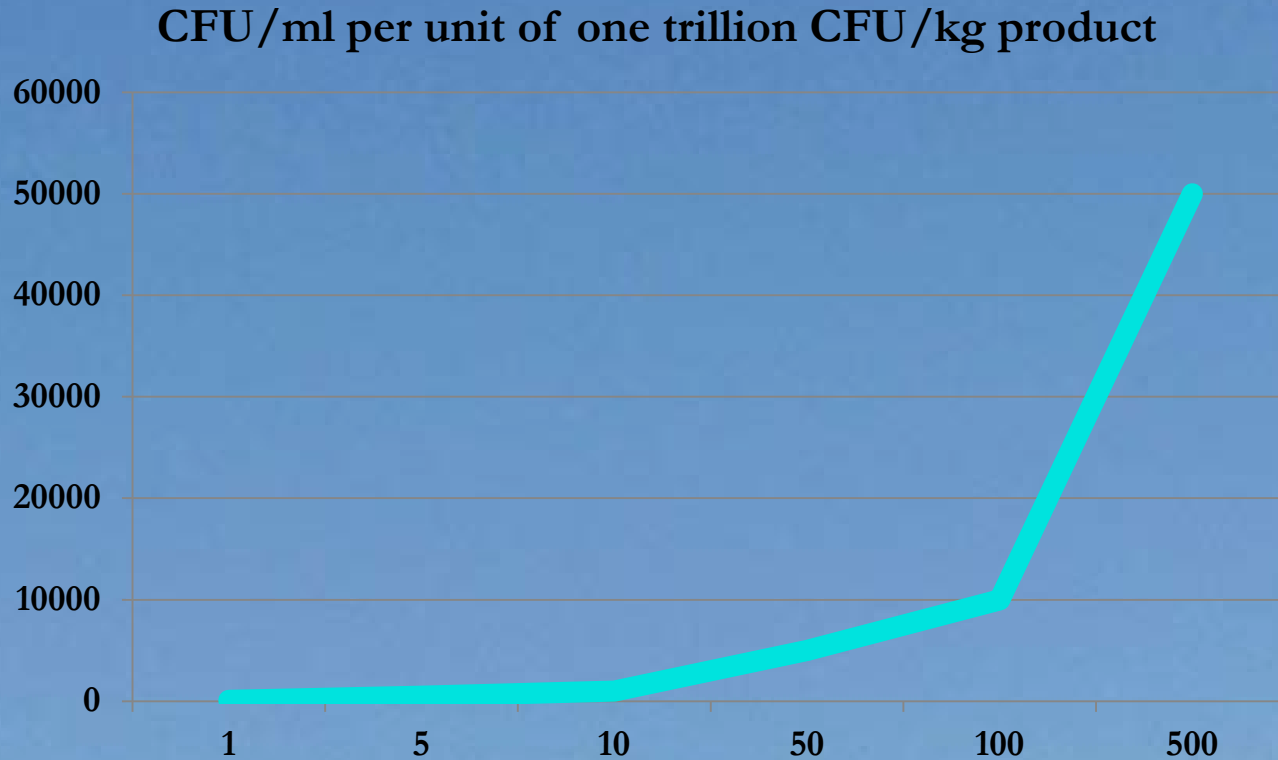
Reiterates the futility of adding products that have low bacterial counts. This is typical of most liquid products.

Based on this it is not going to be cost effective to add biologically meaningful levels of product

Consider: adding 10 kgs of a product with 1 trillion CFU/kg to one ha pond one meter deep, only gives 1000 bacterial cells per ml of water if all the bacteria germinate.

Can this be cost effective? This is 1 billion CFU/gram. Many commercial liquid and powdered products contain as little as 1 million CFU/gram. Adding 10 kgs of a product every other week for a 150 day cycle will require more than 200 kgs of product. Even at \$5.00 per kg this is a \$1000 per ha per cycle.

500 kgs of 10^{12} CFU per kg product added per ha gives 50000 CFU if 100% germinate (this is not likely)



Conclusion

Adding low levels of bacteria to ponds makes no sense from a strictly microbiological perspective. Natural bacterial loads are very high and typical products have low levels of bacteria. They must out compete existing bacteria in order to grow. Products would have to be used at very high rates of application to be effective. This is not cost effective nor does it make sense biologically.

Low levels have no scientific or logical basis for effectiveness.

So we can conclude that it would take very high levels of viable bacteria to impact the existing bacterial composition.

This is compounded by the inclusion of bacterial species that also make no sense scientifically for a variety of reasons.

What types of bacteria are often included in these type of products?

Lactobacillus species

Photosynthetic bacteria (EM)

Nitrogen fixing bacteria

Nitrosomonas

Nitrobacter

Many other gram negative bacteria

Yeast-Torula, Candida and *Saccharomyces cerevisiae*

Various bacteria including *Vibrios*, *Pseudomonas* and *Aeromonas species, etc.*

Only a few are considered in this analysis, starting with a bacterium that most people are familiar with because of its presence in yogurt, *Lactobacillus* species.

Lactobacillus species

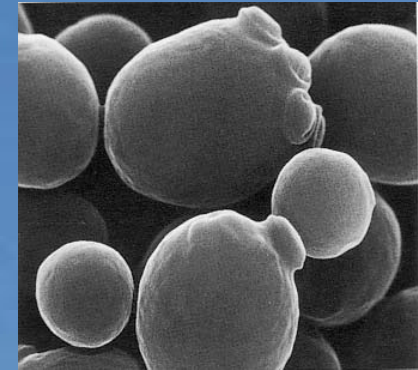


- Most widely used human probiotic
- No means to stabilize without refrigeration or freezing
- Might work in the feed if the heat did not kill them. Top dressing possible but material must be kept cold.
- Dead in dried products held at room temperatures. Usually die off quickly (weeks to months).
- Can not possibly work in off the shelf dried products sold for application in the water or for use in or on the feed, although there are some “stabilized” products available that are not being used in aquaculture. (cost considerations-expensive with poor cost benefit)

Lactobacillus (continued)

- Most are not spore formers (except for *L. sporogenes* which is really a *Bacillus* species)
- Less common in aquatic environments than *Bacillus* species
- Colonize gut perhaps but no significant effects on water quality parameters???
- Good candidate for oral application if there is a willingness to keep material frozen before use and top dress

Yeast



- Saccharomyces or Torula species
 - Known as brewers or bakers yeast
 - Poor survival through feed manufacturing process
 - Top dressing might work
 - Might work by direct addition to the water of high numbers.

Benefits might be as a non specific immune stimulant rather than a “probiotic”

Others

Nitrosomonas/Nitrobacter Difficult and expensive to grow. One product in market from legitimate vendor. Big item for scammers. Typically not needed in ponds.

Photosynthetic bacteria-no third party verification of efficacy-difficult to grow. Obligate anaerobes die in the presence of oxygen.

Conclusions:

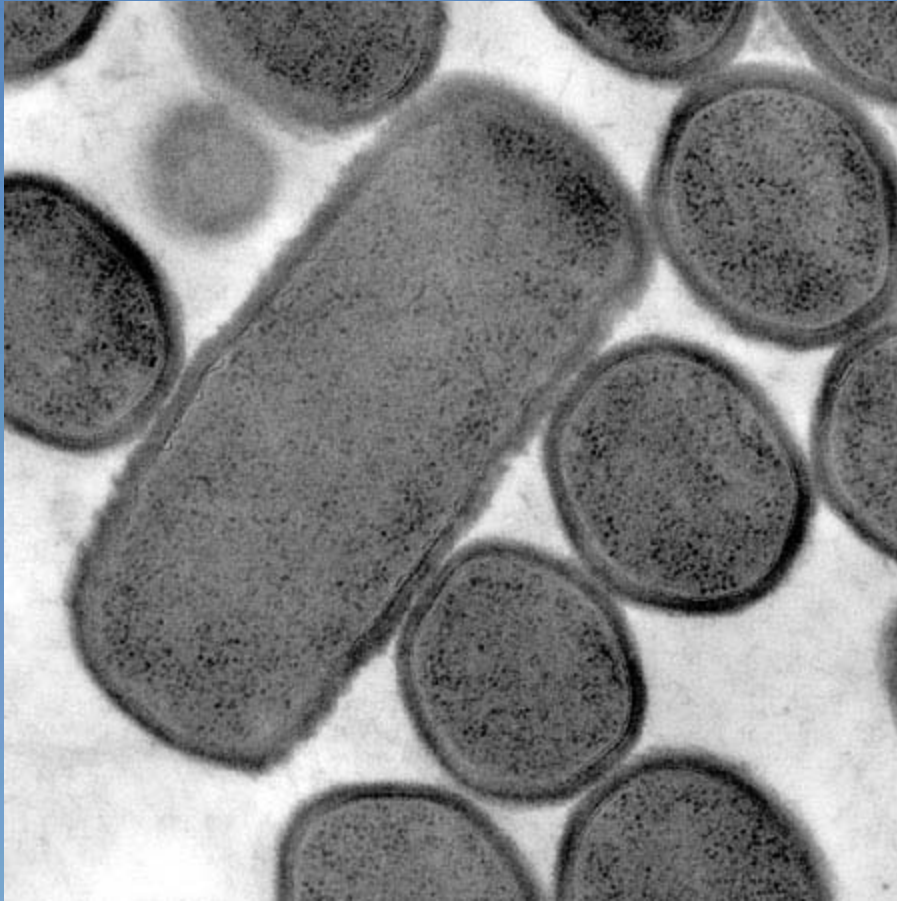
Most bacteria that are in these products are for the farmer not for the animals in the ponds.

It is widely agreed upon that *Bacillus* species make the most sense for use in bioremediation of aquatic ecosystems.

Bacillus Species

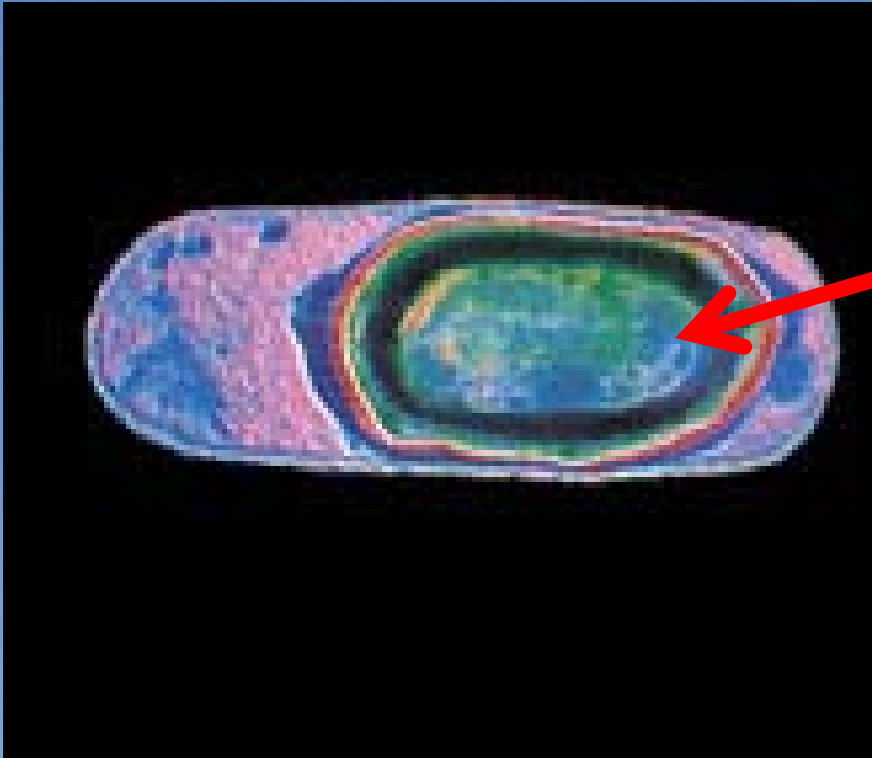
- Ubiquitous in marine environments
- Make up an important part of the natural bacterial flora of shrimp
- Naturally ingested
- Inhibit other bacteria by competition
- Heat resistant spores-stable storage

Bacillus Cells SEM



Typical rod shaped
bacteria; round forms
are cross sectioned
bacteria

Bacillus Spores-allow a shelf stable and feed stable product



This is a picture of a Bacillus cell containing a spore

AquaInTech's products for targeted delivery to the pond bottom

Our products contain Bacillus species selected for ability to digest organic material based on 30 plus years of use in waste management.

PRO4000X tablets contain only bacteria

AquaPro EZ contain bacteria and nutrients

PRO 4000 X Tablets



Bulk Tablets

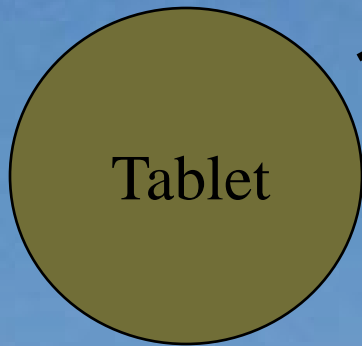


Tablets weigh 13 grams each
Contain 52 billion CFU each min

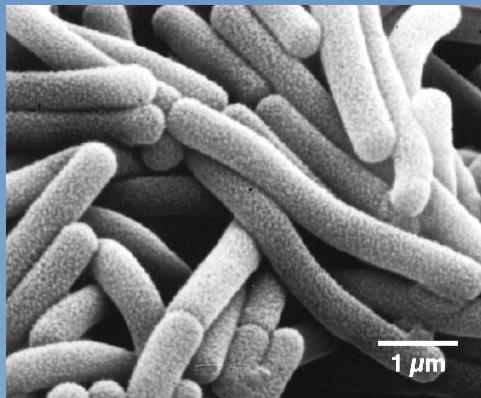
AquaPro EZ Biodegradable bags that allow targeted delivery of bacteria and nutrients



Spore forming Bacillus



Germination



Degradation of organic material
Nitrogen fixation
Water quality improvement

Advantages of using tablets

- Delivery directly to pond bottom where organic material is accumulated and where shrimp are most active.
- Highly concentrated; more than 50 billion CFU per tablet.
- Bacteria germinate at the sediment/water interface where the shrimp are and where the organic matter accumulates.
- Very high levels of bacteria will be present for a short period of time digesting organics

PRO 4000X is an inexpensive tool for delivering high levels of bacteria directly to where they are needed.



Before



24 hours post



After

Why the use of liquids (or activated liquid suspensions) of bacteria addition to ponds makes little if any sense?

A one ha pond one meter deep contains 10 million liters of water or 10 billion mls of water.

Upon addition of a liquid or powder there is a very high dilution.

Assumption is that the bacteria in the suspensions continue to grow after they are added. No data to show that this actually occurs. Frequent addition suggests otherwise.

Bacteria are diluted and move by flagella where currents allow. Levels of bacteria at the sediment/water interface would be very low.

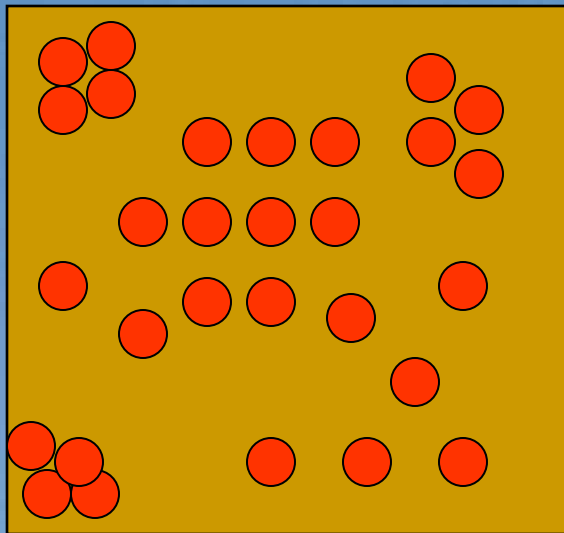
Adding bacteria directly to water

Bacteria must spread through water column. Result is low or no bacteria at bottom. Not possible to measure.



The advantage of tablets

- Tablets go to the bottom and germinate in the pond bottom



You decide where you want the bacteria to go and how much bacteria to put where.

Tablets go where you put them and the bacteria go where you want them. Very effective tool for getting large numbers of bacteria into areas where needed.

What Benefits have our clients reported?

Benefits will vary based on your farm

Density

Water Quality (reduced Hydrogen sulfide levels)

Use of antibiotics

Water exchange philosophy

Aeration

Feeding philosophy

Nutrients present in ponds

Lined versus soil bottoms

Benefits Noted by our Clients

- Sludge Reduction-cleaner water, elimination of hydrogen sulfide, restoration of anaerobic pond bottoms to aerobic
- Decreased water exchange
- Increased survivals
- No need to use antibiotics
- Cleaner pond bottoms
- Less blue green algae shift to algae not present in eutrophied pond systems
- Lower cost of production

Results discussed here are from a farm in Belize Royal Mayan Shrimp Farms



Decreased Water Exchange

Why?

Better Water Quality, less organic material to cause problems

Real Time Observations in Belize:

200 to 300 % water exchange per pond per cycle-once every 7 to ten days before tablet and molasses applications.

Cleaner pond bottoms equals less need for flushing out accumulated organic detritus

Decreased Water Exchange (continued)

Reduced use of pumps, reducing overhead and repair costs and down time.

Less dependence on variable water quality in incoming water

You decide when you exchange.

Increased Survivals

Why?

Subjective as many things can impact survival.

Better water quality equals less stress equates with stronger shrimp.

Decreased Antibiotic Usage

- Typical use in aquaculture is not responsible
- Responsible use entails:
 - Identification of cause of problem
 - Isolate organisms causing problem
 - Determine antibiotic sensitivity pattern
 - Treat with the appropriate antibiotic
- Selective use of tablets substituted 100% for the use of antibiotics

Antibiotics can be done away with

Successful control of common vibrio infections by the use of the tablets in conjunction with molasses and selective water exchange.



RMSF reported a 100% reduction
in use of antibiotics with no difference in overall survival

Cleaner Pond bottoms



Sludge Reduction

Fish Farmer in the Philippines reports substantial reduction in organic material in ponds and much improved water quality

Bacteria digest organic material and compete with other bacteria and algae for food.

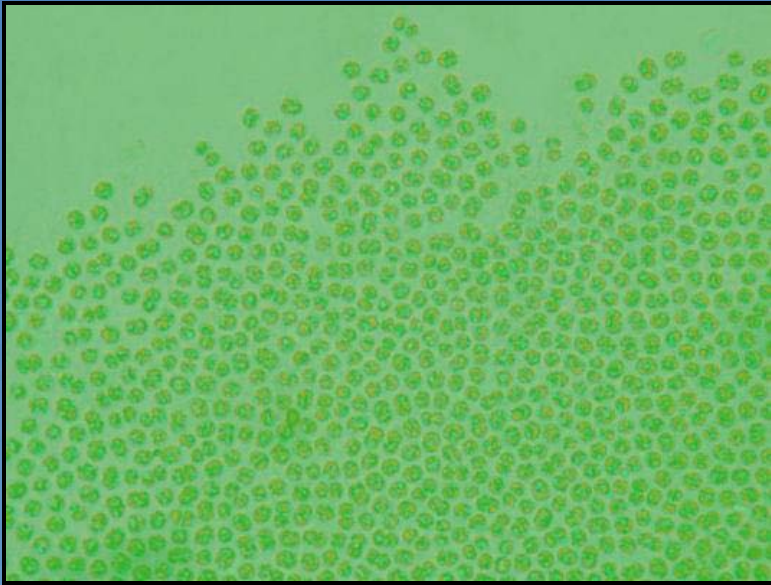
Tablets cleaned up a pond that used to require extensive flushing prior to restocking

Shrimp farmer in Venezuela reported complete reduction of problem areas on pond bottoms

See testimonials at end of the presentation.

Cleaner Pond Bottoms

Less time between cycles because of lower organic material accumulated on pond bottoms.



Microcystis

Less Blue Green Algae

Schizothrix calcicola



Managers noted that when they added tablets along with molasses applications usually within 12 hours of tablet application pond population shifted from blue green to green and brown. They reported a noticeable difference in coloration and composition occurring within 24 hours of application. (More so than with molasses alone.)

Algae control is a result of competition for nutrients. By lowering the amount of suspended organic material in the ponds, there was less food for blue green algae

Broad reaching ramifications

As per slide before this, dramatic change in water color typically within 24 hours of addition of tablets



Before

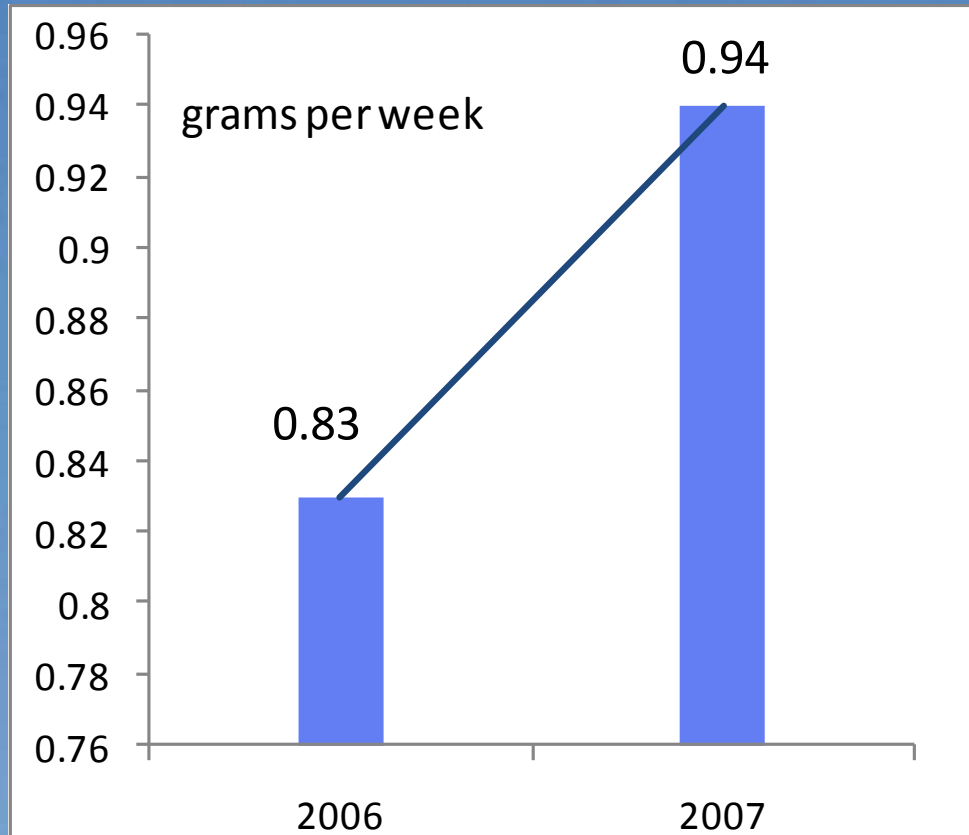


24 hours post



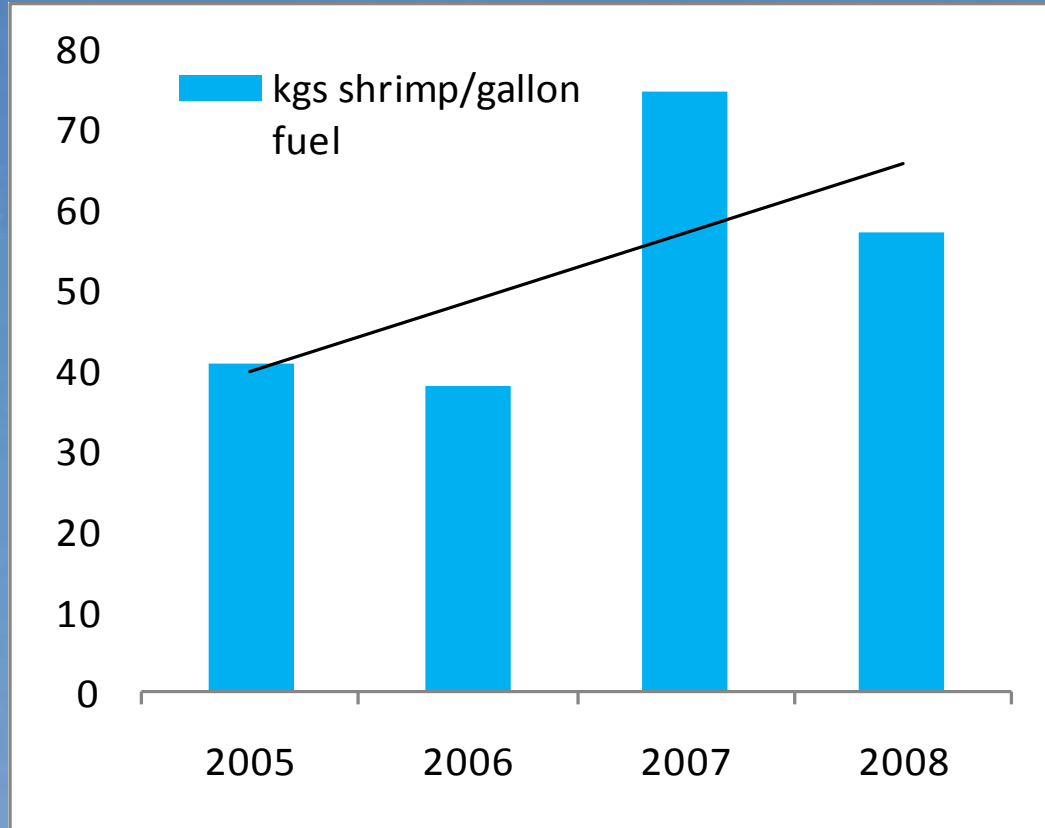
After (at harvest)

Better Growth



Growth depends on many factors but in general better environment equals healthier shrimp equals better shrimp growth.

Lower costs of production



Based on pumping costs associated with water exchange

Conclusions

PRO4000X is a tableted formulation of several proprietary Bacillus species that have been the subject of a US patent.

A convenient tool for managing water quality. Not a solution but a management tool.

Clients have seen **many cost beneficial impacts**

Reduced water exchange

Reduced disease and use of antibiotics

Better water quality

Cleaner pond bottoms

Conclusions (continued)

A very cost effective tool for managing water quality problems in shrimp and fish ponds as well hatcheries and for that matter any body of water.

Clients in Venezuela, Belize, Mexico (trials in Tilapia ponds underway with early indications of success), Vietnam, Taiwan, India, Bangladesh and Indonesia.

Tablets cost around 20 cents US each (volume dependent).

Hatchery -1-2 tablets per 10-20 MT water/day (20 to 40 cents)

Farm-depends on amount of organic matter present

Average 600 to 800 tablets per ha per cycle. (\$120-\$160)

The following slides contain testimonials from clients in three different countries. It is clear that the product works and provides a consistent cost effective benefit.

It is much less costly than other competitive products and is designed by experienced professionals with more than 30 years of experience in the science of aquaculture.

While we can guarantee what results you will see as we have no control over how you use the product, we can guarantee you that the bacteria digest sludge.

Testimonials from the Philippines

“I am now in the Philippines and have been in the fish farm quite a bit. We have just completed a round of initial treatment and the water quality seem to stay good, oxygen content is satisfactory. The weather is not yet that hot, water temperature is actually abnormally low this time of the year. The real test of the waste clean up would be the hot temps in the coming months.

By the way, should we expect the sludge on the ponds to decrease with the use of the bacteria? Our people seem to think so! Before I arrived, one pond badly deteriorated to a point where the water really smelled bad and discolored that they had to take the fishes out of it for fear of a fish kill. Our caretaker decided not to pump out the water as they normally do (in fact, several times before the water improves) but instead applied the tablets. IT WORKED! The water cleared and the odor disappeared allowing them to re-seed the pond after a week.

Yesterday, the provincial agricultural office personnel visited the farm and wanted to enter it into a regional agricultural contest but I declined. They said we are the only ones they know of utilizing probiotics in the province. In fact, they asked for samples of the tablets to do some experimentation. I was told that their boss is interested on the waste clean up capabilities because there is a huge lake here that is polluted. I know that lake, it is beside a golf course that we play on. The fish they sell in the market coming from that lake is foul tasting!”

Testimonial from Venezuela

Estimado Stephen:

Gracias por los comentarios hechos en su visita por estas tierras. Quiero que sepa que las pastillas de bacilos que gentilmente José Enrique Rincón me facilitó, las hemos utilizado en nuestro sistema semi-cerrado con gran éxito. Definitivamente los canales de drenaje o de alimentación que no se les puede mover el agua y crean puntos muertos son los que más necesitan de la aplicación de algún probiótico. Estos puntos muertos tienden a presentar fuertes olores, típicos de la presencia de azufre, los cuales fueron tratados con las pastillas por una semana. La dosis fue de una libra por esquina quieta. A la se-mana, el olor desaparece totalmente y suponemos que mejora la calidad del agua. No tenemos como medir los metabolitos.

Como pudo ver, nuestras aguas en un sistema tan eutroficado como el del Lago de Maracaibo, son de buena calidad y son producto de trabajar conjuntamente con la melaza y los bacilos.

Rough Translation

Dear Stephen:

Thanks for the comments made during your visit in these parts. Thank you for providing us with the Bacillus tablets (PRO4000X) . We have used them in our semi-closed system with great success. Our production system results in dead spots that are in need of the application of your product. These dead spots tend to have strong odors, typical of the presence of sulfur, which we treated with pills for a week. The dose was about 25 tablets per area. After a week the smell disappeared altogether and the water quality improved.

As you saw, our water in a system as eutrophic as Lake Maracaibo, are now of good quality and are the product of working with your product.

Testimonial from Belize

To Whom It May Concern:

In early 2007, we began using AquaInTech's tableted probiotics, PRO4000X, on our shrimp farm in the Southern part of Belize. We had worked with their powdered product in the past cycles and felt that the tablets offered an innovative approach to targeted pond bottom delivery and elected to use the tablets on all of our 23 ponds. We stocked our farm at the usual densities using the same seed sources that we have in prior years. Feed came from the same suppliers as well.

We are pleased with the results that we observed. Using these tablets on a consistent basis allowed us to dramatically cut back on our water exchange. The savings in fuel consumption have been significant, greatly offsetting the cost of the product. We also saw a dramatic increase in the average growth rate of the animals from previous cycles, with almost a 15% increase in weekly growth rates. Other benefits included a reduction in the incidence and severity of disease due to bacteria. We also have noted changes in the overall composition of the algae in the ponds as well and cleaner pond bottoms. Second cycle results are confirming these first cycle observations.

We whole heartedly endorse the use of this product and have found it to be a cost effective tool that has had a significant impact on our productivity and profitability.