# *PRO 4000 X*

# Biotechnological Pond (and Hatchery) Water and Environment Quality Management

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# The Presentation

- Definitions
- Concepts
- Commentary
- PRO 4000 X Discussion
- Outlook and Conclusions

Ph.D in marine microbiology (vibrio). Developed vaccines for commercially reared fish and first commercial non specific immune stimulant for shrimp (PenStim, DS-1999, MBXC). Commercialized anti WSSV agent for feed. Microbial based products sold for digestion of sludge. Sold only patented products.

Auditor of Aquaculture Certification Council and Global Trust

# Definition

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

# Definitions

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) redefined a probiotic as

"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**."

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."

At this time there are no products that fall into this category for use in aquaculture (this definition is very broad).

Any products that would fall into this category are likely non specific immune stimulants.

# Definitions (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 not, by definition, 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 no easy matter and actually very unlikely to happen.

## ***Critical Elements of Definition***

Living microorganisms (can be more than one)

Affects animal health (increased survival by?)

Impacts flora in the digestive tract  which implies that they attach to the walls of the gut and impact gut flora (all products require repeat applications)

Delivery in the feed?  (heat kills bacteria)

# What is reality?

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.

# Extensive Literature Review

**Probiotics do not exist in aquaculture.** In fact the concept is contentious in human beings and in agriculture. Little doubt that some bacterial preparations do have an impact on animal although mechanism not proven. Nonspecific immune stimulation most probably the major affect. For fish this appears to be the case.

**Many poorly designed studies with inadequate controls, insufficient replicates and inappropriate conclusions.**

Shrimp molt to grow. Lose front and rear ends of intestinal tract with each molt. Claims about disease resistance are problematic. Can not eliminate non-specific immune stimulation as likely explanation. Does not require viable bacteria.

Studies of aquatic organisms (fish and shrimp) suggest that gut contents are related to what is in the water. No stable gut flora. Colonization problematic.

# What can we conclude?

- For 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 evidence to support this mode of action.
- Pseudo Scientific claims abound. It is often ignored that many pathogens enter immune suppressed shrimp through means other than the intestinal tract where the probiotics would act. Correlation does not equal causation.
- Likely non specific immune stimulation is the health affect

# What can we conclude (2)?

- 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.
- Farmers beware. "Caveat emptor"

# What is the difference between Probiotics and Prebiotics

- Probiotics-living organisms
- Prebiotics are non-digestible (for the host) 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. Also-not all pathogens enter through the gut.

# Probiotics in Aquaculture are really

# Microbial Ecology Management Tools or MEMs

Focus of this talk is  our tableted product PRO 4000 X. This is a tool that we developed to avoid some of the problems with traditional water applied based powdered materials.

Field proven tool for improving water quality with additional benefits that are a result of a cleaner and less stressful rearing environment.

What are the critical considerations that can affect effectiveness of bacterial based 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 ???

# THE CHALLENGE

Adding enough bacteria to cause a permanent change (or meaningful temporary 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.

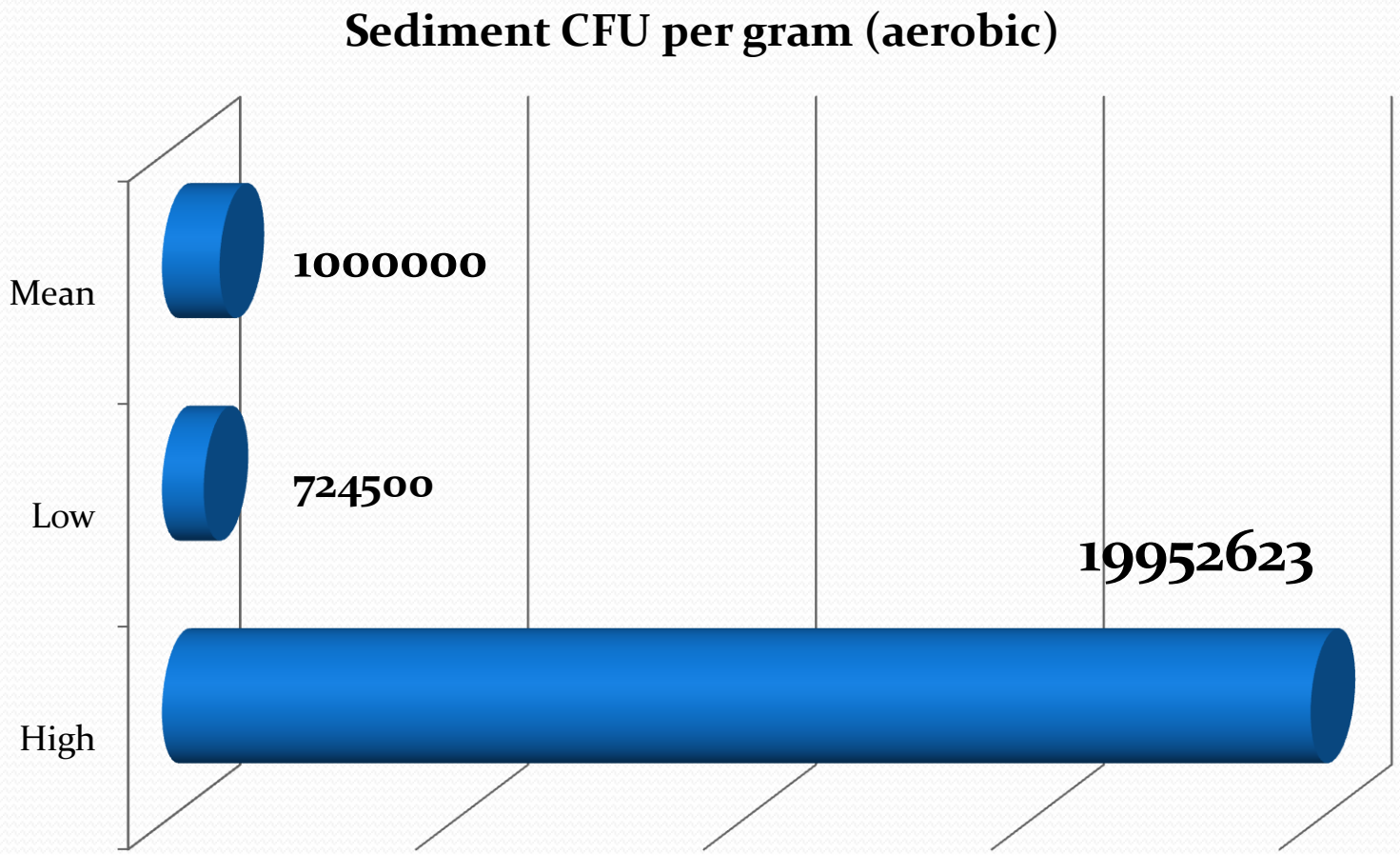
How can we maximize the chances that a product will have a cost effective benefit?

# Existing bacterial load in the environment

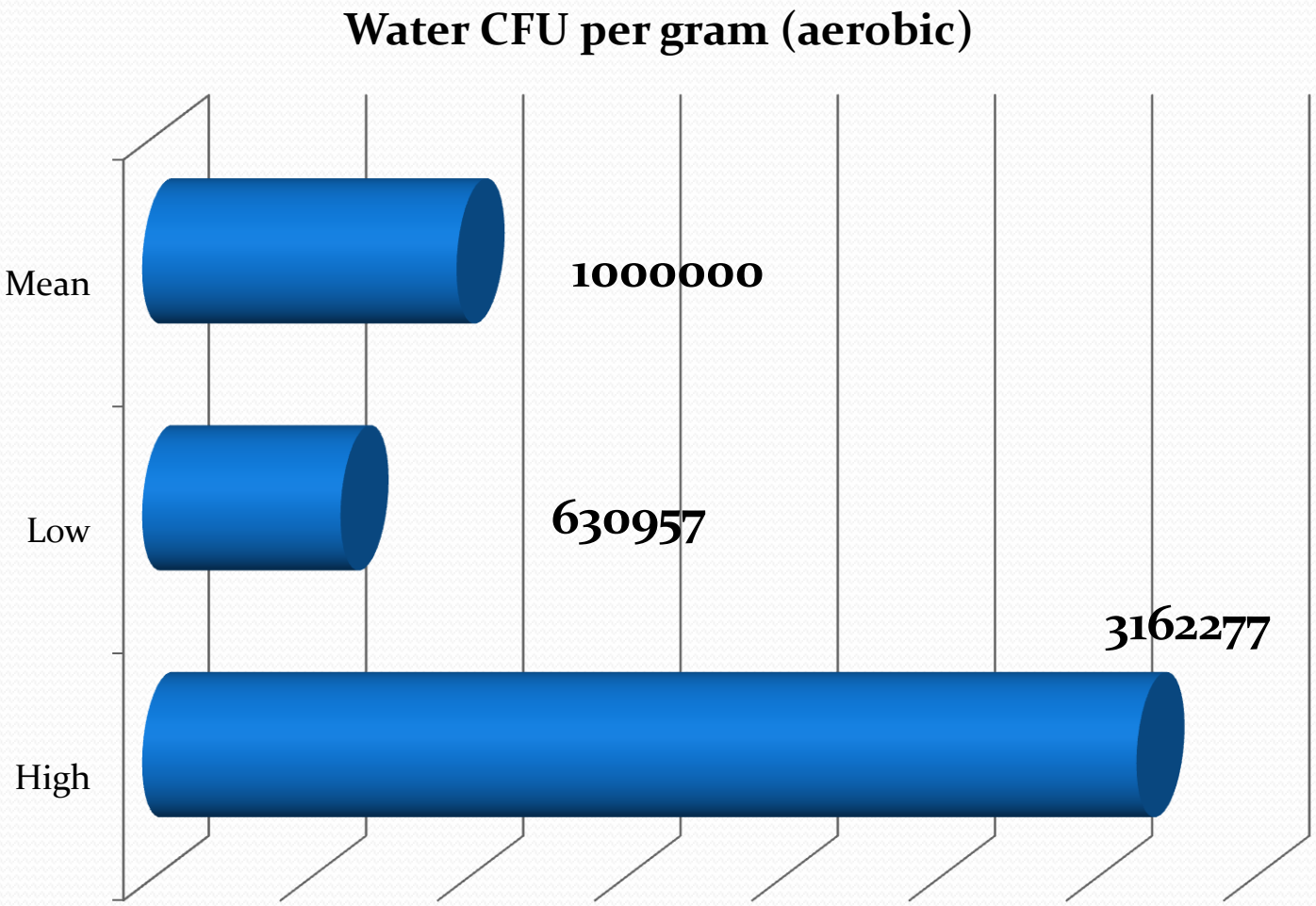
- There are bacteria in the water column
- There are bacteria in the shrimp
- There are bacteria in the sediments
- There are bacteria in the food that the shrimp eat (pellets and natural).

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

# High Natural Bacterial Counts in Soil



# High Natural Bacterial Counts in Water



- 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.

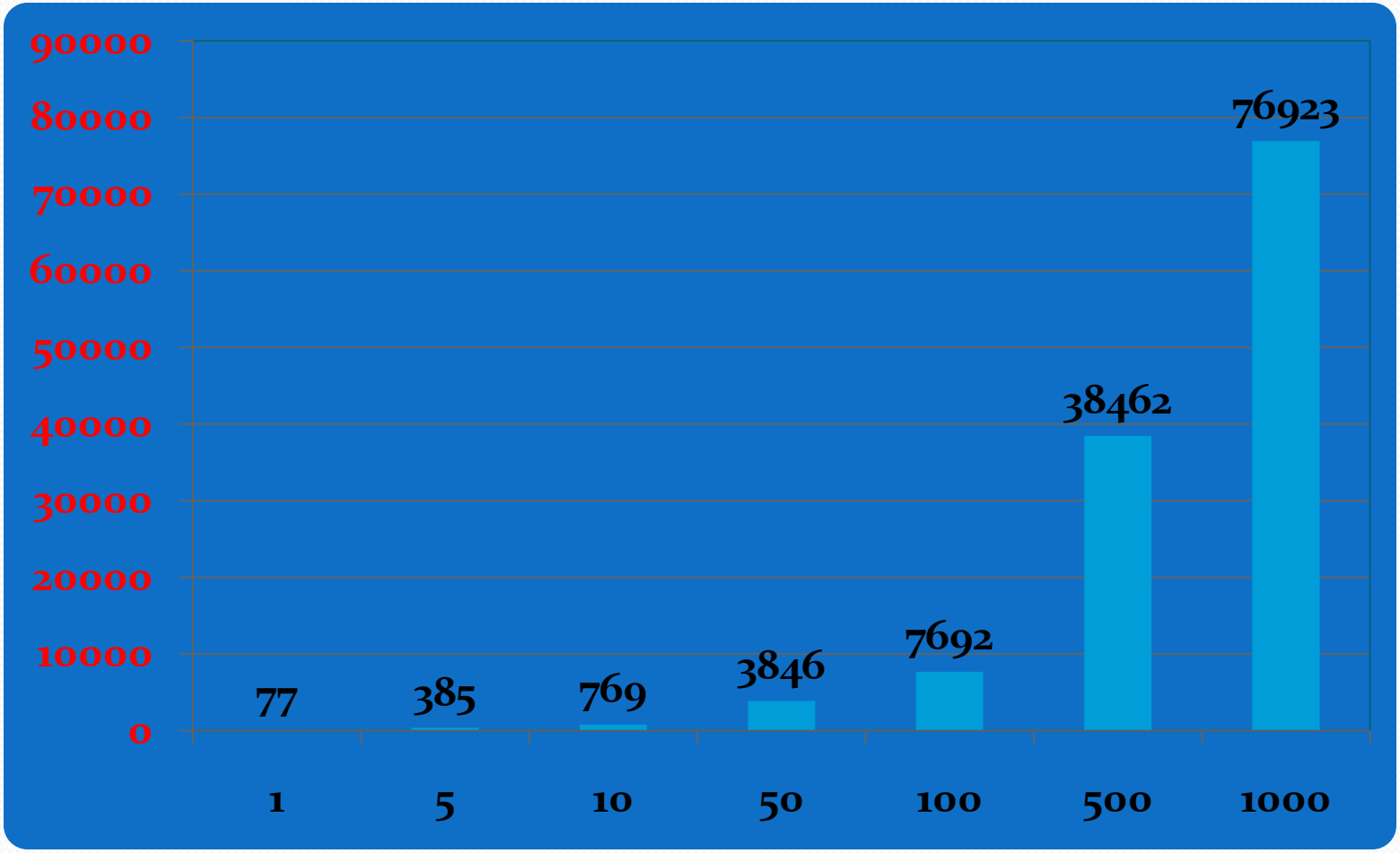
How many bacteria can be realistically added to these environments to try and affect change?

Is it a waste of time and money adding products that have low bacterial counts, typical of most liquid products.

Adding 10 kgs of a product with 1 trillion CFU/kg to one ha pond one meter deep, gives 769 bacterial cells per ml of water if all the bacteria germinate or survive.

**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.

# 500 kgs of $10^{12}$ CFU per kg product added per ha gives 38462 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 relatively 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.

Low levels have no scientific basis or logical basis for effectiveness.

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

Lactobacillus species
Photosynthetic bacteria such as purple sulfur
Nitrogen fixing bacteria
Nitrosomonas
Nitrobacter
Many other gram negative bacteria
Yeast-Torula, Candida and *Saccharomyces cerevisiae*

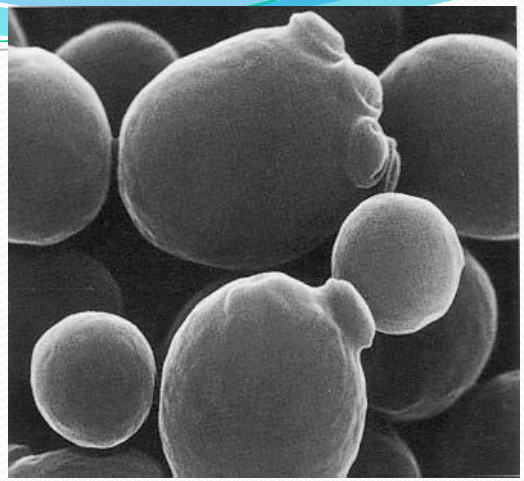
# Lactobacillus species

- 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-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". Beta glucan in cell wall.

Others

Nitrosomonas/Nitrobacter. Very difficult and expensive to grow. One product in market from legitimate vendor very expensive. Does nothing for sludge.

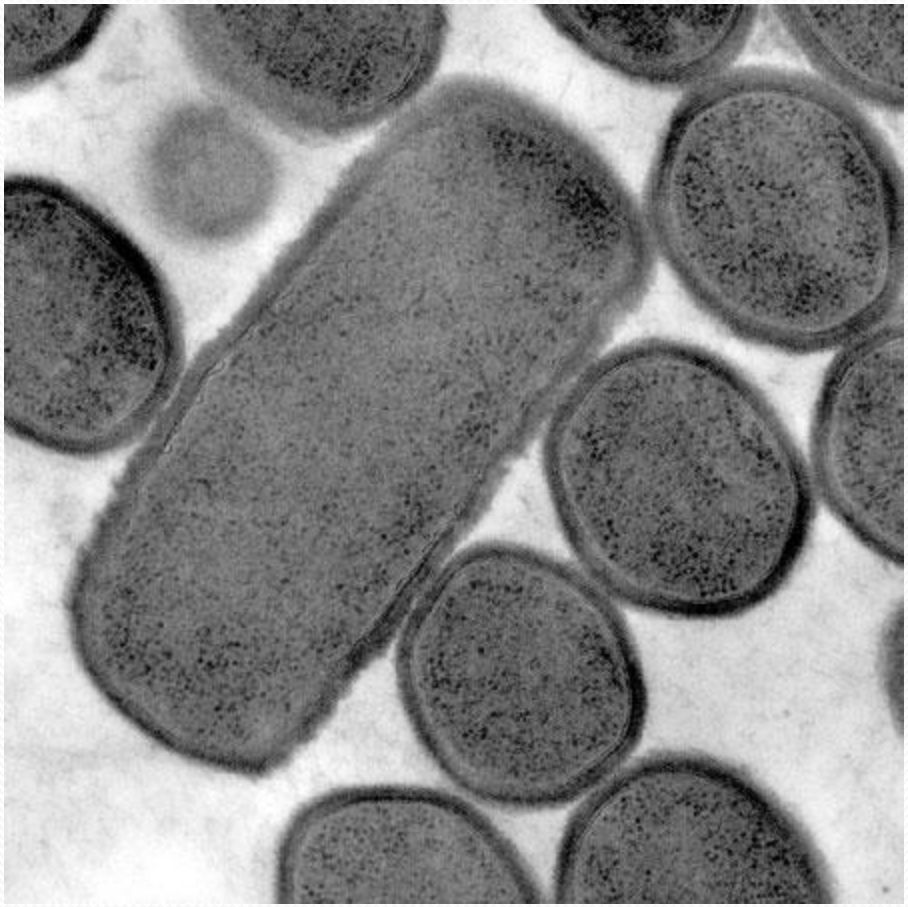
Photosynthetic bacteria-no third party verification of efficacy. On pond side production is dangerous and seriously flawed. Obligate anaerobes die in the presence of oxygen. Even facultative anaerobes that are cultured in the absence of oxygen die in the presence of oxygen.

Bacillus species-most promising

# Bacillus Species

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

# Bacillus Cells SEM



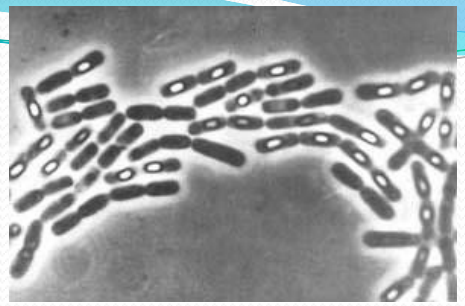
Bacillus Spores-allow a shelf stable and feed stable product.  Spores can remain viable for centuries.

# Tablets

Each tablet contains at least 2 billion spores of each of two species, Bacillus subtilis and B. licheniformis, for a minimum of 4 billion spores per gram of tablet.

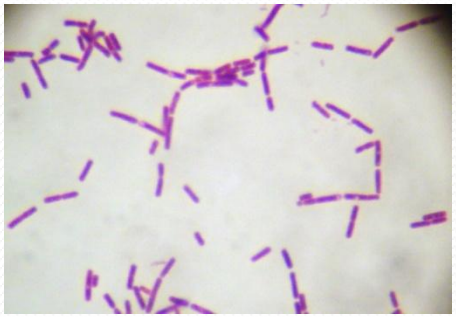
Quality control is done by Auburn University environmental lab that knows how to do tests of Bacillus spore containing products that agglomerate.

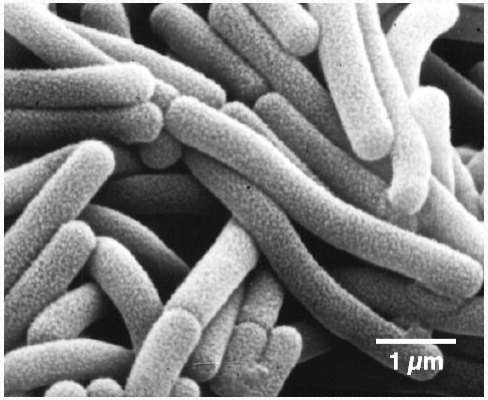
# Spore forming Bacillus



Tablet

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. Thirteen gram tablets contain at least 52 billion CFU. Shelf stable-no shelf life for all practical purposes.

•Stable spores; heat resistant, germinate in presence of nutrients

•Wide range of abilities to consume types of nutrients (waste products that are a by product of shrimp farming)

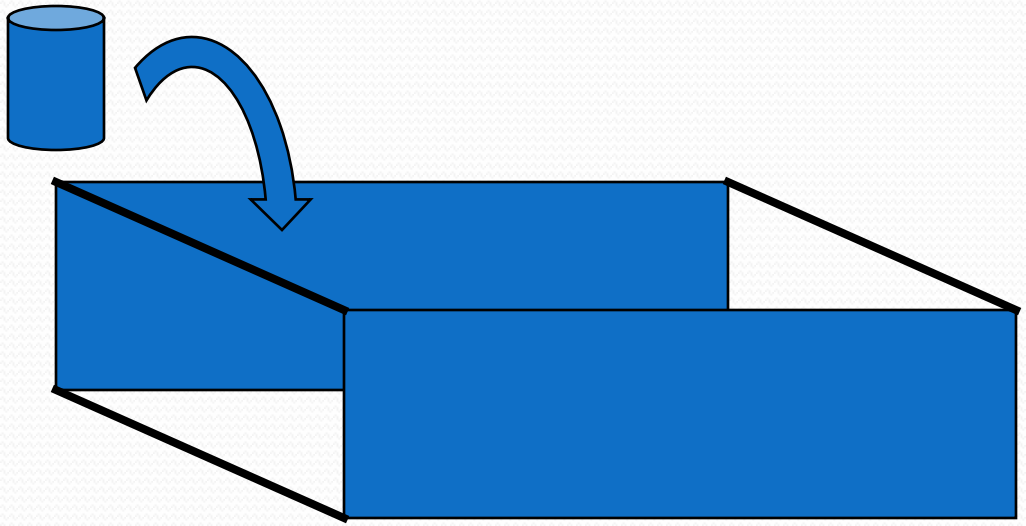
Principle: Bacteria germinate and colonize area around where tablets have dissolved. Spread into water column

Inexpensive tool for delivering high levels of bacteria directly to where they are needed.

- Problem areas of pond bottom
- In accumulated sludge
- Excess feed accumulation
- Areas of dead algae
- Around feeding trays
- Hatchery Tanks (1 tablet per 5 to 10 MT of water)

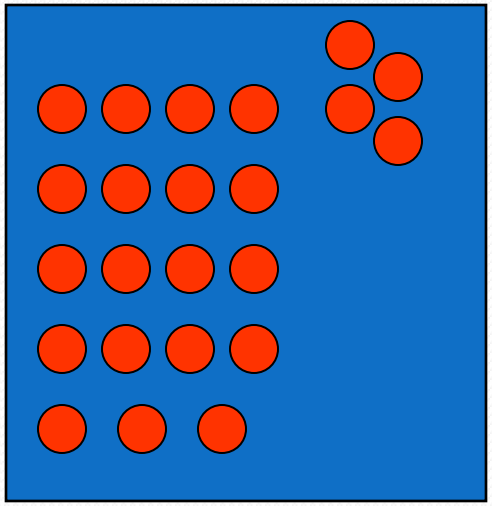
# Comparing direct (liquids or powdered products) with targeted addition (PRO4000X)

- Adding bacteria directly to water
  - Bacteria are diluted
  - Bacteria must spread through water column. Result is low levels of bacteria at bottom.



# The advantage of tablets

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



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

Tablets go where you want them and the bacteria go where you want them. More 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

Hatchery Tanks  Improved water quality and survival

# Benefits Noted by our Clients

- Sludge Reduction-cleaner water, Cleaner pond bottoms
- Decreased water exchange-*reduced cost of production*
- Increased survivals *cleaner water=less stress*
- Eliminated antibiotics *less disease*
- Less blue green algae *competitive inhibition*
- Lower cost of production
- Reduced hydrogen sulfide levels in sludge
- Cleaner hatchery tanks with higher survivals

- Low Cost (tablet costs around 22 cents US each 2050 RP)

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

Relatively small farm in Belize, Central America

13 ponds, 1.6 to 10.3 ha in size

Max 70 animals per square meter

# 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

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

Less dependence on variable water quality in your 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 <u>no differences</u> in overall survival

# Cleaner Pond bottoms

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

Sludge Reduction

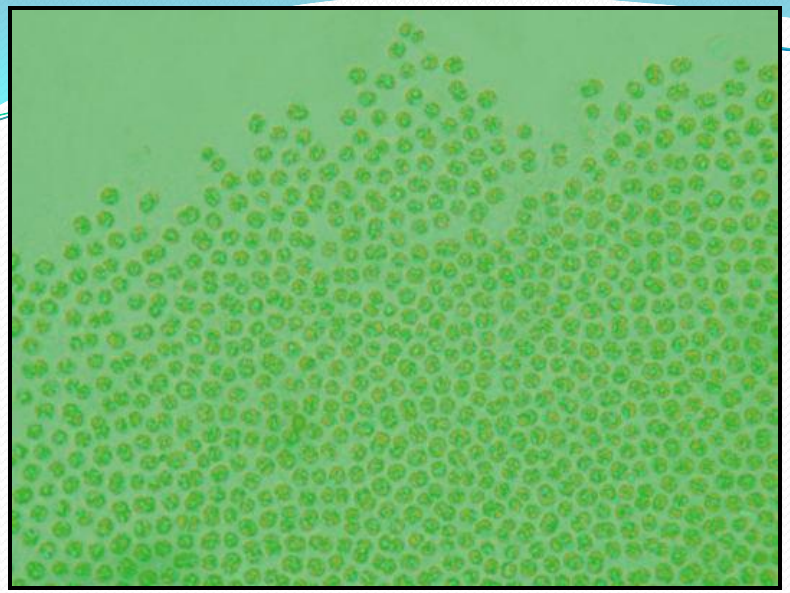
Primary benefit of use of the product. Bacteria germinate, digest organic material converting into bacterial biomass

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.

High Density farming environments

Unique challenges because of extremely high density of shrimp. Clients in Guatemala have seen changes in the color of sludge from black to brown. Clear cut liquefaction of sludge due to bacterial degradation. Significant drops in hydrogen sulfide production in ponds.



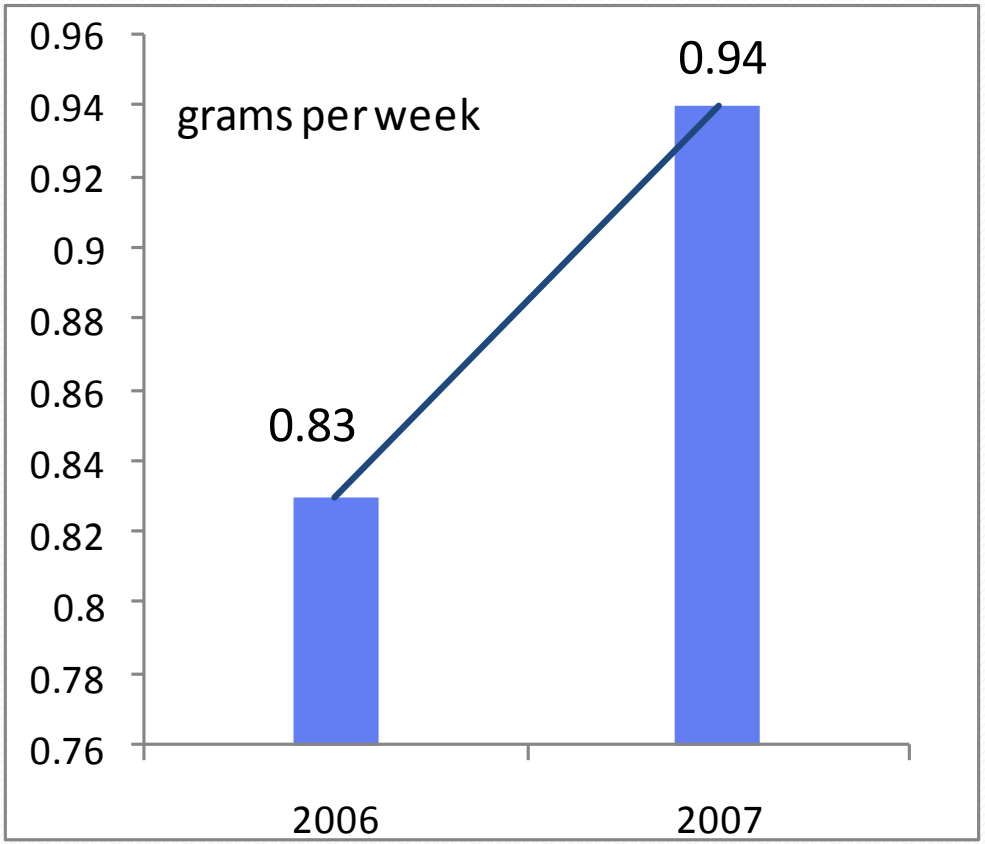
*Microcystis*

Less Blue Green Algae

*Schizothrix calcicola*

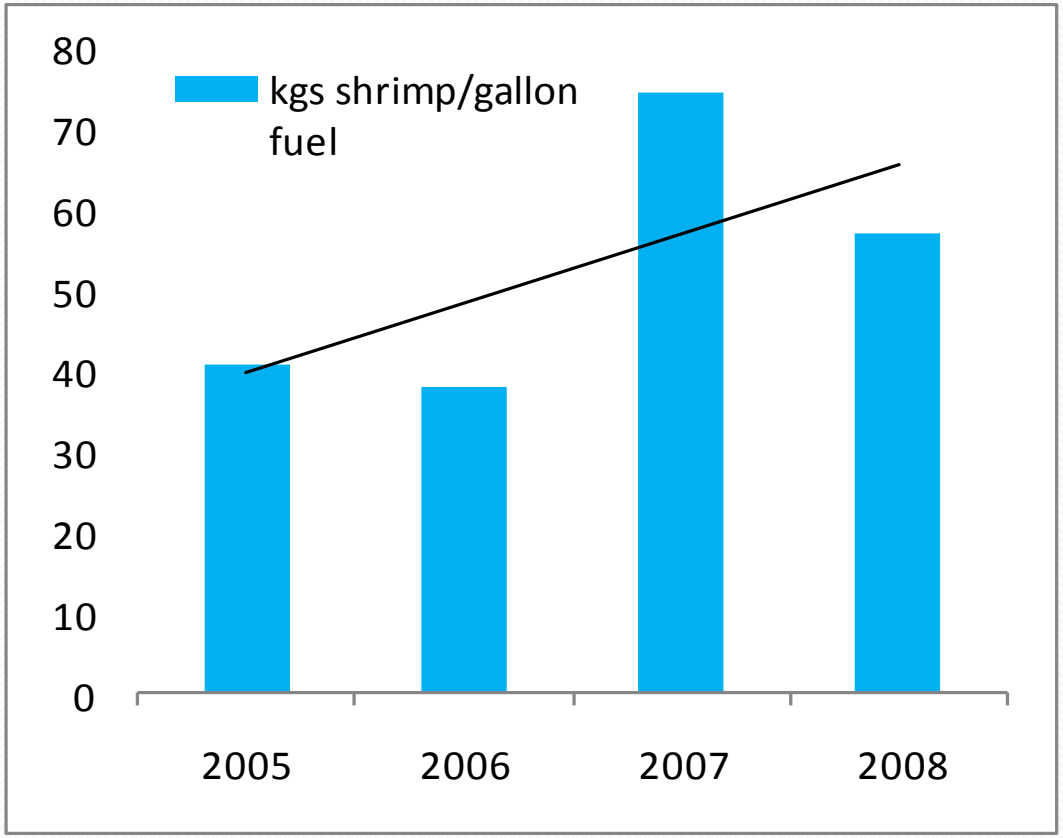
Managers noted that when they added tablets along with molasses applications usually within 12 hours of tablet application pond population shifted from blue green to green and brown. They reported a noticeable difference in coloration and composition occurring within 24 hours of application. (More so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

# 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

What are suggested application rates?

If I tell you that there is one best way, I am not being honest. The best application rate <u>will depend on your farm and your ponds</u>. There is simply no way that a given product can be used at the same application rate in every pond and every farm.

We suggest 600 to 800 tablets per ha per cycle as a baseline to work from. Frequency of application is every other week.

You need to determine what the rates are that will work best for your farm.

Number of tablets per application
Location of tablet addition
Frequency of application

As cycle progresses higher levels of organic material accumulate and more tablets should be used.

**DON'T BE AFRAID TO EXPERIMENT TO OPTIMIZE PARAMETERS.** The bacteria will digest the sludge.

## Conclusions

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

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

Clients have seen **many cost beneficial impacts**

Reduced water exchange
Reduced disease and use of antibiotics
Better water quality
Cleaner pond bottoms and hatchery tanks

Thank you for your time.

QUESTIONS?