# Simple "Solutions" for Aquaculture

Susan E. Knudson Ph.D.

**WSR Pellets**

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# Finding Solutions for Aquaculture

Problem → Hypotheses → Research → Product

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# Waste and Sludge

- Feed applied to pond water results in the development of inorganic and organic matter, which contaminates the pond water and pond bottom soil, leading to poor health and productivity of the shrimp.
- Organic matter in the sludge can lead to high concentrations of ammonia, which helps create the perfect environment for pathogenic Vibrio and can cause early mortality syndrome and white feces disease. The combination of Ammonia, Vibrio, mineral deficiencies, and hydrogen sulfide can cause reduced growth  and increased mortality, ultimately resulting in less-than-optimal shrimp production.

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# Hypothesis:

# Probiotics Reduce Sludge in Aquaculture

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# What are Probiotics??

- Probiotics are a combination of beneficial bacteria, enzymes, minerals, and micronutrients on a carbon rich carrier. Probiotics are Important tools to manage the microbial ecology in the aquaculture ponds
- Probiotics are bacteria that have a beneficial effect in aquaculture through:
  - Competitive exclusion
  - Improved FCR/nutrient efficiency
  - Stimulate immunity to increase disease resistance
  - Improved water quality

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# Probiotics Effect Animal Health

- **Directly**
  - Ammonia
  - Nitrite
  - Nitrate
  - Pathogen Populations
  - Bottom Sludge
- **Indirectly**
  - Biological Oxygen demand
  - Hydrogen Sulfide
  - Animal Stress
  - Water Exchange
  - Use of antibiotics

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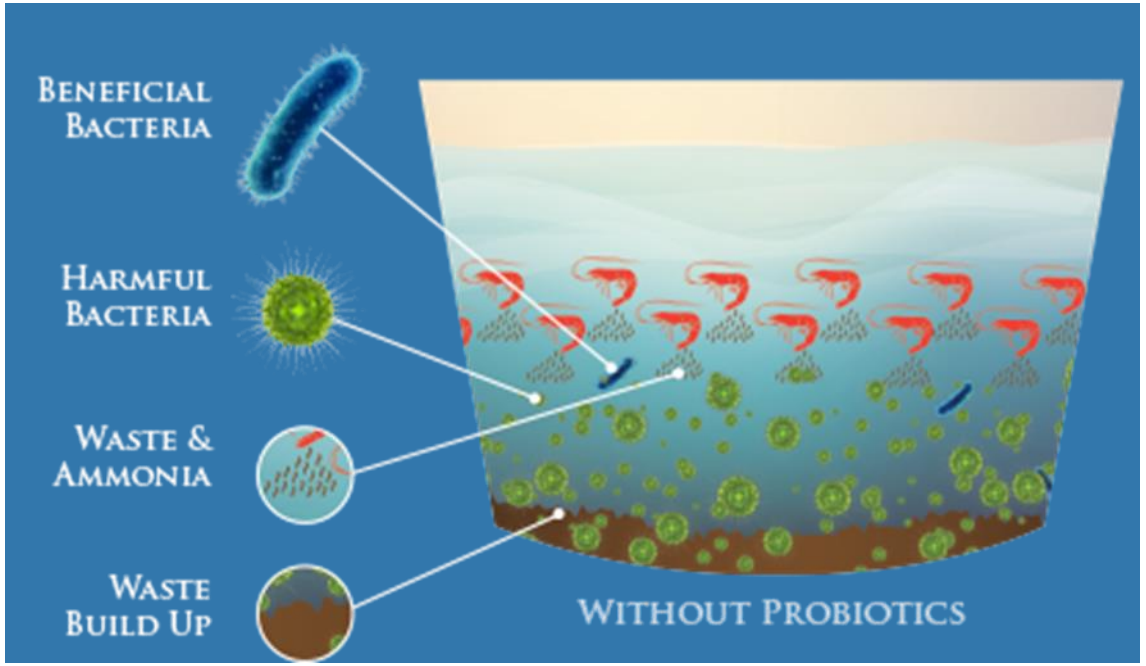
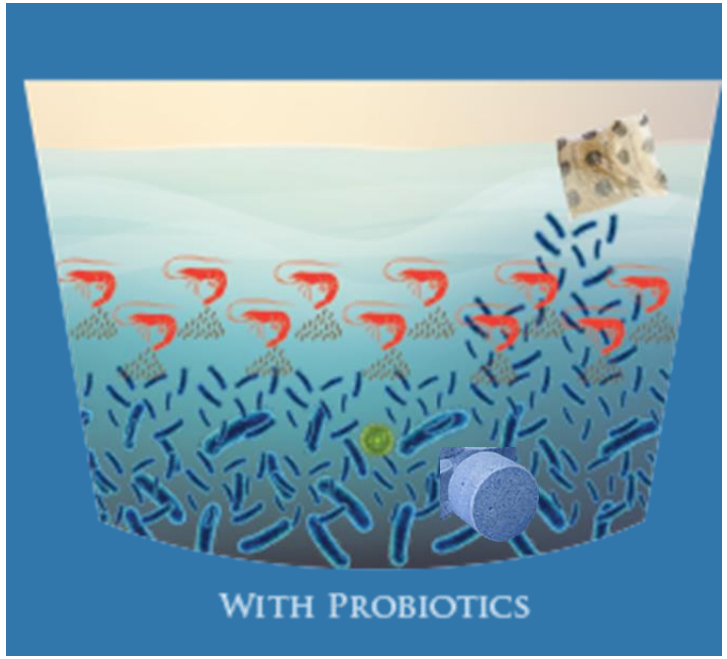
# Competitive Exclusion and the Microbiome




- Probiotic microbes outcompete *Vibrio*, *Pseudomonas*, *Aeromonas*, and other gram-negative microbes for resources and space.
- Enzyme production includes bactericidal and bacteriostatic compounds.
- They eliminate  the need for water exchange.

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# How do Probiotics Work?

- Control of Microbiome
- Improvement of water quality
- Improvement of pond bottoms

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# Probiotics that Reduce Sludge

- *Bacillus* species are known to enhance the natural sludge digestion process by breaking down complex organic matter such as ammonia, carbohydrates, or fatty acids.

- Biodegradation of sludge is a multi-step process involving the whole bacterial community with a variety of organisms that produce proteases, cellulase, lipase and amylase.

- Each *Bacillus* strain is unique, so a product designed to incorporate several complimentary strains is an effective approach to wastewater sludge treatment

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# Research and Development Program

- Keeton is dedicated to making the best possible products.  Our mission is to identify microbes with improved efficacy at different salinities, temperatures, pH and against known pathogens.
- Our research and development program goal is to find novel bacteria that are more reactive against emerging strains of aquaculture related pathogens.

- Dr. Susan Knudson, MPH, PHD
- Senior Research Scientist

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# Mining for New Microbes

Mining for microbes where they will be used provides isolates that will grow well where needed.

Samples were collected from the ocean, estuaries, streams, lakes and animals around the world, including the United States, India, Malaysia, Vietnam, Ecuador, Brazil, the Caribbean, Canada, and Portugal


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# Microbial Selection Strategies

We look for microbes that create a natural, beneficial Bio-Floc, that can reduce ammonia, nitrite and nitrate. We screen for microbes that produce enzymes that are capable of digesting organic solids and bottom sludge.

These microbes then can help provide a natural healthy environment that is safe and less stressful for the animals.

In addition, we screen for microbes that are antagonistic against known animal pathogens which would further improve animal health and enhance our products.

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# *In Vitro* Screening

- Gram Stain
- Inhibitory Characteristics
- Growth Characteristics
- Salt Tolerance
- Enzyme profiles
- Water management capabilities
- Identification

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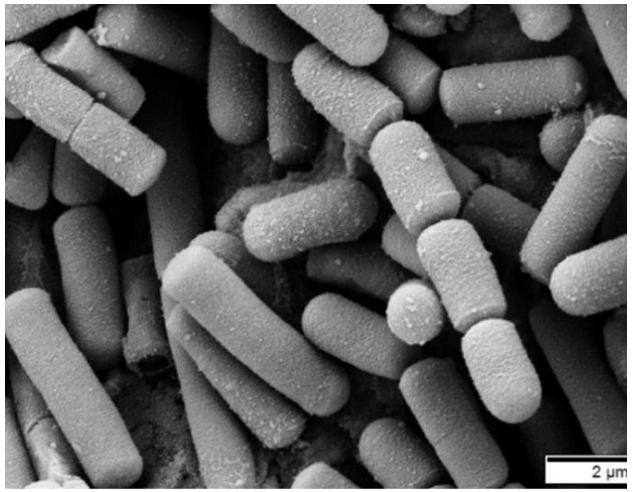
# Natural Occurring Bacillus

Non-GMO, **Gram +,** Spore formers

- *Bacillus amyloliquefaciens.*
- *Bacillus licheniformis.*
- *Bacillus pumilus*
- *Bacillus subtilis.*
- *Bacillus megaterium.*

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# Inhibition Studies with Aquaculture Pathogens

| | | | *V. harveyii* | *V. parahaemolyticus* | | | | *V. vulnificus* | *F. psychrophilum* | *E. ictaluri* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | A3 | O3K6 | VNM | ECU | | | |
| *subtilis* | RA24 | oyster, HI | *** | | | ### | # | * | # | |
| | RA26 | oyster, HI | | | | **** | | | # | |
| | RA47 | oyster, WA | # | | # | ## | # | | | ***** |
| | RA54 | oyster, WA | * | * | | * | # | # | | * |
| | RA165 | oyster, WA | ### | | | ** | | # | ## | |
| *pumilus* | 6G11 | water,ECU | ##### | | *** | *** | **** | * | ##### | |
| | RA158 | oyster, VA | | ## | | | ## | | # | * |
| amyloliqu | RA203 | ocean, MYS | ## | * | | # | *** | * | # | *** |
| | RA21 | oyster, HI | ## | * | * | | | ** | ## | |
| | RA259.2 | ocean, CT | | * | | * | * | * | | * |
| | RA19 | oyster, HI | ## | * | * | | # | * | | |

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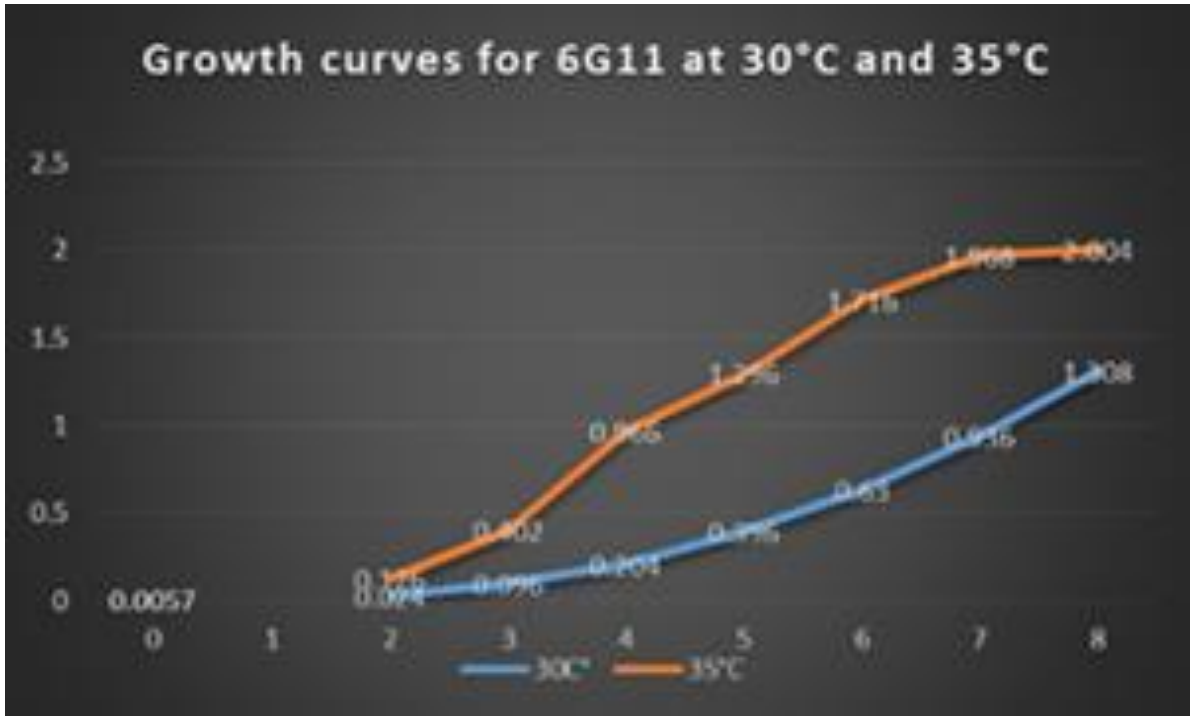
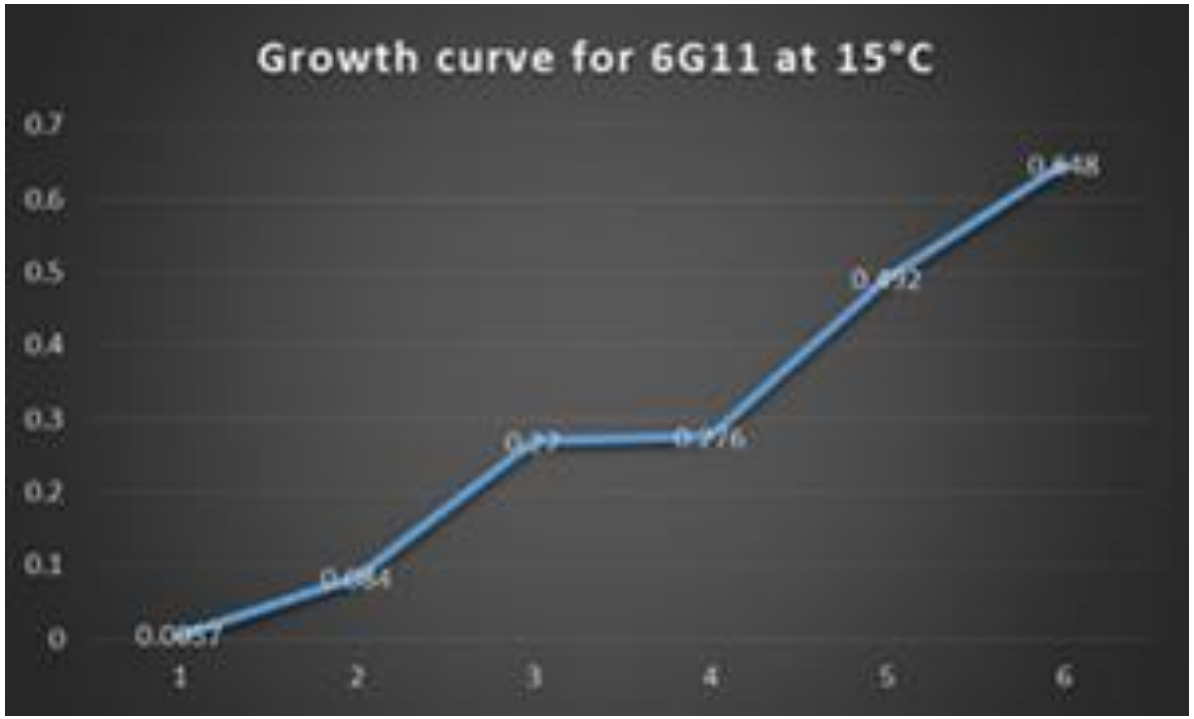
# Growth Curves

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# Enzyme Production

| | | cellulase | amylase | caseinase | urease | lipase | Phosphate Solubilization |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| | | | | | | | |
| *subtilis* | BS | 5 | 5.3 | 1 | 0 | 10 | POS |
| | RA165 | 5 | 4 | 1 | 0 | 10 | POS |
| | RA24 | 5 | 5 | 1 | 0 | 10 | NEG |
| | RA47 | 5.5 | 5 | 1 | 0 | 6 | POS |
| *amyloliquefaciens* | BA | 7 | 5 | 11 | 0 | 7 | POS |
| | RA203 | 7 | 5.5 | 11 | 0 | 6 | ? |
| | 9H6 | 7 | 5 | 11 | 0 | 5.5 | POS |
| *pummilus* | BP | 0 | 0 | 0 | 0 | 7 | POS |
| | 6G11 | 0 | 0 | 0 | 0 | 6 | POS |
| *licheniformis* | BL | 4 | 0 | <1 | 2mm | 5 | NEG |

Cellulase, catalyzes the decomposition of cellulose and of some related polysaccharides.
Amylase, hydrolyzes the glycosidic bonds in starch molecules, converting complex carbohydrates to simple sugars.
Caseinase, hydrolyzes casein into smaller soluble amino acids.
Urease, catalyzes the hydrolysis of urea to ammonia and carbon dioxide.
Lipase, break down triglycerides into free fatty acids and glycerol.
Phosphate solubilizing inorganic phosphorus from insoluble phosphate.

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# Salt Tolerance of Beneficial Microbes

| *subtilis* | TSB/Salt % | TSB/Salt 2.5% | TSB/Salt 5% | TSB/Salt 7.5% | TSB/Salt 10% | Source |
|---|---|---|---|---|---|---|
| RA47 | + | + | + | + | - | oyster |
| RA54.1 | + | + | ? | + | - | oyster |
| RA165 | + | + | + | + | - | oyster |
| 11H9 | NG | + | + | + | - | estuary |
| 11H5.2 | + | + | + | + | - | estuary |
| RA768 | + | + | + | - | - | beach |
| RA804 | + | + | + | + | - | ocean |
| K145 | + | + | + | -/+ | - | shrimp farm |
| RA864 | + | + | + | -/+ | - | Feather |
| RA866 | + | + | + | + | - | Feather |
| RA889 | + | + | + | -/+ | - | Honey |
| *amyloliquefaciens* | TSB/Salt 0% | TSB/Salt 2.5% | TSB/Salt 5% | TSB/Salt 7.5% | TSB/Salt 10% | Source |
| RA383 | + | + | + | + | - | Trout Farm |
| RA388 | + | + | + | + | - | Trout Farm |
| RA21 | + | + | + | + | - | Oyster |
| RA259.2 | + | + | + | + | - | Ocean |
| RA203 | + | + | + | + | - | Oyster |
| RA810 | + | + | + | -/+ | - | Kelp |
| K176 | + | + | + | + | - | Worm Casting |
| RA806 | + | + | + | + | - | Ocean |

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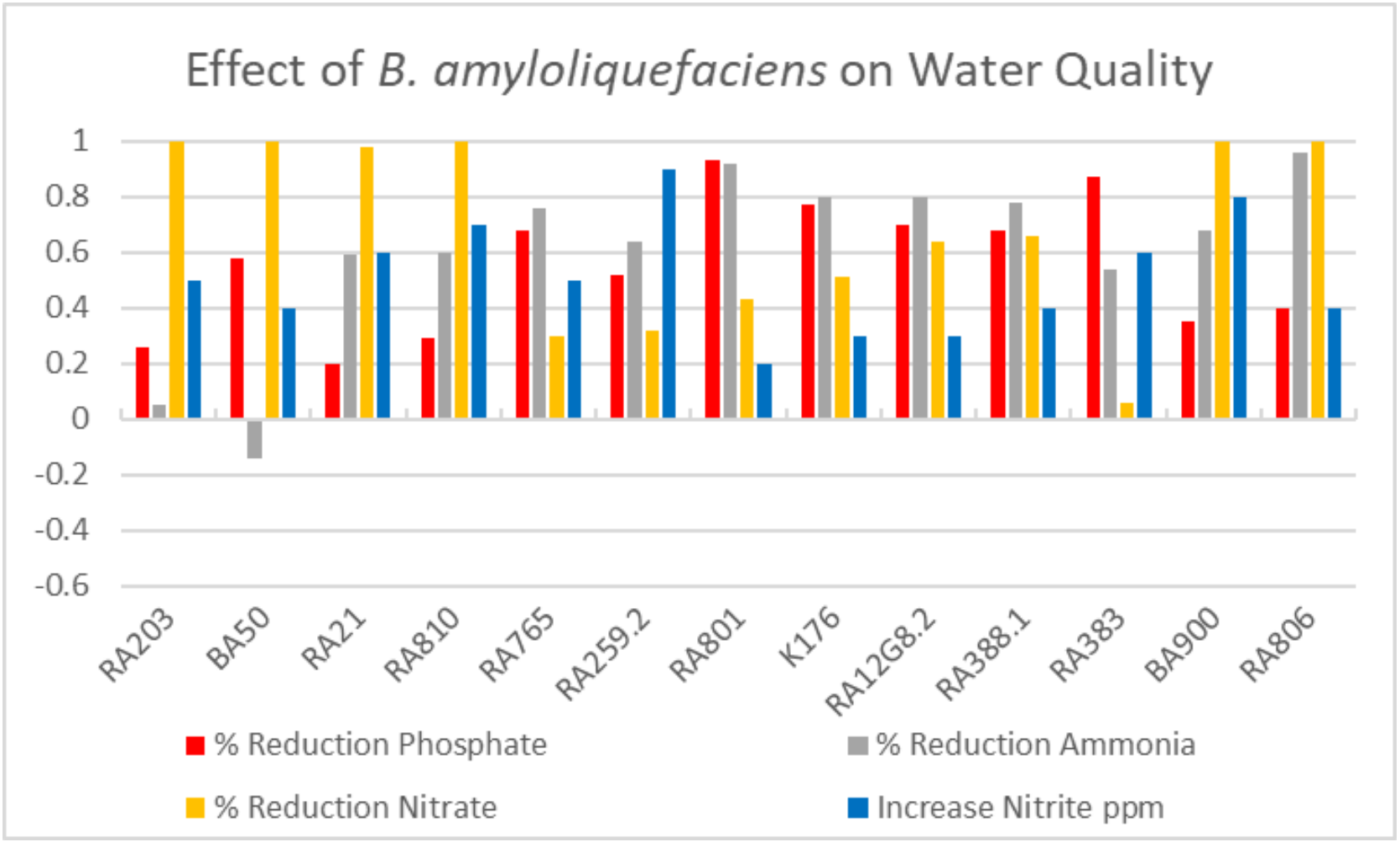
Sea water is equal to 3.5% salt

# Effect Probiotics Have on Water Quality

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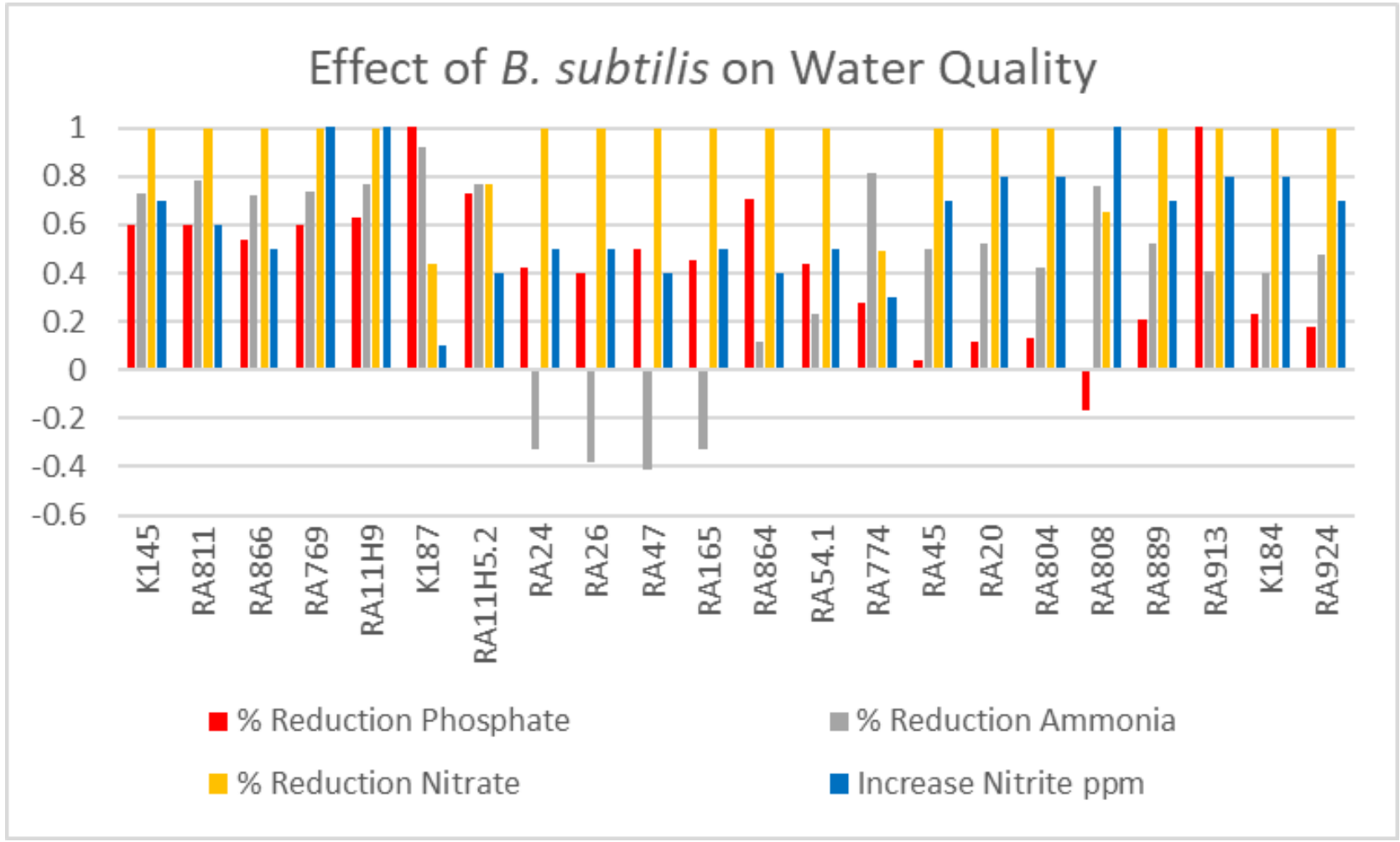


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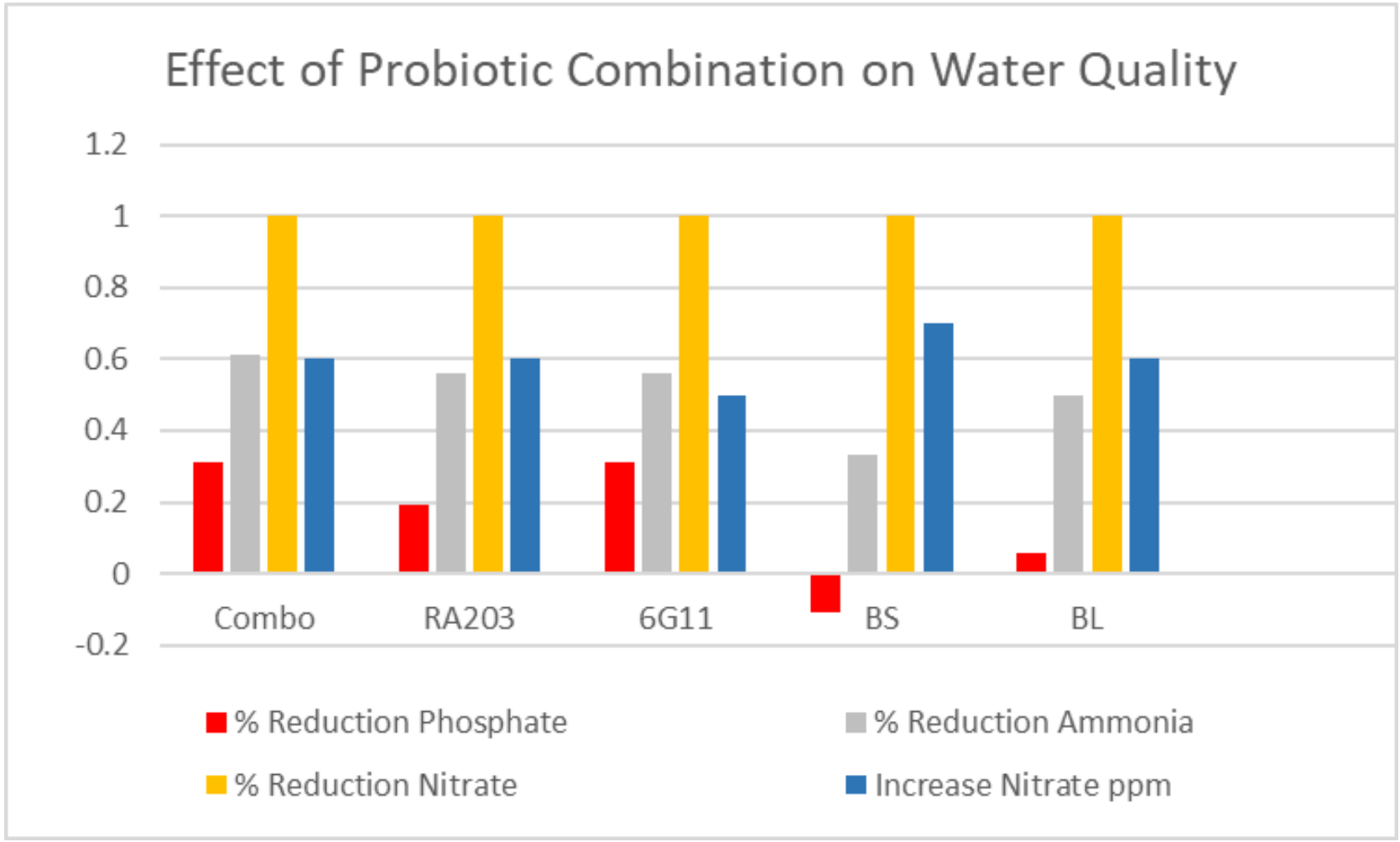


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# Identification

**MALDI-TOF Microbial ID Services Using Accugenix**

- Higher confidence for species-level identifications when applying MALDI-TOF for microbiology lab applications.
- AccuPRO-ID MALDI-TOF is cGMP-compliant and ISO 17025-accredited.
- Accugenix proprietary MALDI-TOF microbial database provides more extensive coverage for environmental monitoring isolates than any other commercially available MALDI-TOF system for microbiology labs.

**Accugenix ProSeq**

- Highly discriminatory and accurate sequence-based method that can be used to distinguish between very closely related microorganisms to the species level.
- Resolution is achieved by analyzing protein-coding genes.

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# A Probiotic Profile

**6G11 *Bacillus pumilus* BOX 6 4: 4-6**

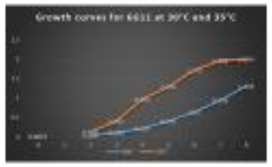
**Isolate History:** October 14, 2017 6G11 was isolated from a water sample collected from an Estuary in Ecuador. Identified by Charles River Accugenix® Services

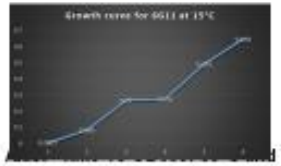
**General Characteristics of *B. pumilus* species:**

- Chromosome circular
- Size approximately 3.68 Mbp
- Predicted proteins approx. 3928
- GC content approx. 41%
- Aerobic chemoheterotrophic
- Mesophilic (grows 20°C-45°C)
- Salt Tolerant 0-10%
- Optimal Growth 30-37°C
- Motility flagellated
- Endospores

**Specific Characteristics 6G11**

**Temperature Growth Curves:**





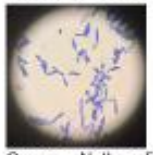
**35°C hours, 15°C days**

**Phenotype:**

Colony Morphology: small circular colony

30°C

Gram Positive: rod shaped



Genome NotI profile

**Salt Tolerance:** **6G11** grows well in TSA with 2.5%, 5% 7.5% or 10% salt.

**pH tolerance:**

**6G11 *Bacillus pumilus.***

**Inhibition studies:**

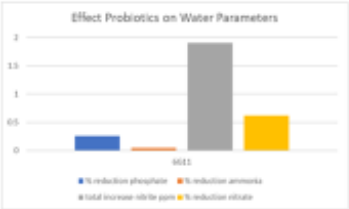
*Bacteriostatic* = ***BS+***, *Bactericidal* = ***BC+***

| Pathogen | Isolate | 30°C | 15° C | RT |
|---|---|---|---|---|
| *Vibrio harveyi* | RV11 | | | |
| *V .parahaemolyticus* | O3K6 AHPND- | **BC+** | | |
| | K144 AHPND+ | **BC+** | | |
| | ECA1 AHPND- | **BC+** | | |
| | ECA2 AHPND+ | **BS+** | | |
| | A3 AHPND+ | | | |
| | K143 | | | |
| *V. vulnificus* | RV9 | **BC+** | | |
| *V. alginolyticus* | RV13 | | | |
| | RV61 | | | |
| | RV192 | | | |
| *V. angularum* | RV50 | | | |
| | RV51 | | | |
| *V. proteolyticus* | RV58 | | | |
| *V. ponticus* | RV12 | | | |
| *V. corallyticus* | | | | |
| *V. tubiashii* | | | | |
| *v. owensii* | RV7 | | | |
| *Flavobacterium psychrophilum* | | | + | |
| *F. Limicola* | RV601 | | + | |
| | RV602 | | + | |
| *Aeromonas salmonicida* | RV417 | + | | |
| | RV600 | | | |
| *Flavobacterium spp.* | RV599 | | | |
| *Edwardsiella ictaluri* | | | | **neg** |

**Enzyme Activity**

| Enzyme | 30°C | 35°C |
|---|---|---|
| Lipase | + | |
| Amylase | | |
| Cellulase | | |
| Protease | | |
| Phosphate solubilizing | + | |
| Catalase | | |
| Citrase | | |
| Oxidase | | |
| Urease | | |
| Gelitinase | | |

**Effect of 6G11 on Phosphorus, Ammonia, Nitrate and Nitrite in water**

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# Product Formulation

Choose a set of microbes that demonstrate  properties that digest sludge and enhance the animal environment.

- They need to work together and not be competitive.
- The right proportions for each microbe needs to be established.
- The application method that best suits the target needs to be determined.
  - Granular for water column.
  - Pellets sink to sludge level.
- The product needs to be tested *in vivo.*
- Application options tested.


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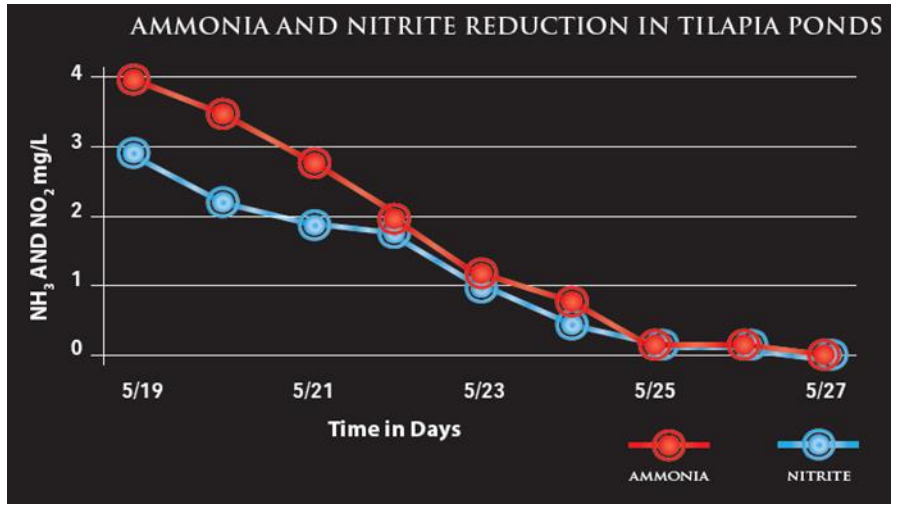
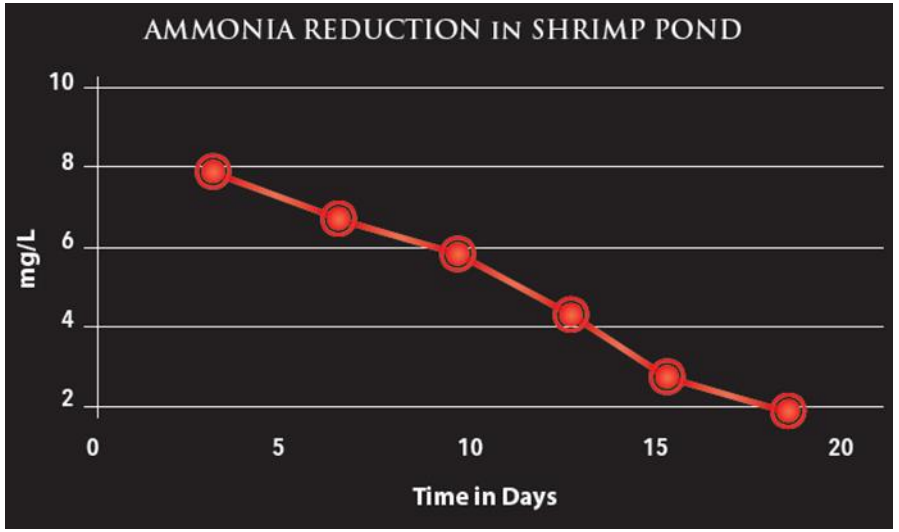
# *In vivo* Studies with WSR on Water Quality




Eliminate the need for water exchange & increase biosecurity

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# Waste and Sludge vs Microbes


Before Treatment

After Treatment

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# Assessing Beneficial Microbial Interactions

- Four microbes commonly used in aquaculture

| Culture Filtrate | Bacterial lawn on agar plate surface | | | | |
|---|---|---|---|---|---|
| | ***Bacillus licheniformis*** | ***Bacillus pumilus*** | ***Bacillus subtilis* (A)** | ***Bacillus amylolique-faciens*** | **Bacillus methylo-trophicus** |
| ***B. subtilis* A** | **Diffuse** | **14 mm** | **-** | **16 mm** | **17 mm** |
| ***B. subtilis* B** | **-** | **-** | **11 mm** | **12 mm** | **10 mm** |
| ***B. subtilis* C** | **-** | **-** | **-** | - | **-** |
| ***B. subtilis* D** | **-** | **-** | **-** | **-** | **-** |

Summary:

- Inhibition was prevalent between Bacillus species, but varied from no inhibition to largely inhibited.

# Testing Application Methods

Pond side grow out of multispecies probiotics changes the composition and the efficacy compared to direct application

Simulate pond conditions *in vitro* by  growing microbes in TSB media with 2% salt.

Total Microbe Concentration based on field application rates. Standardized for all tests.

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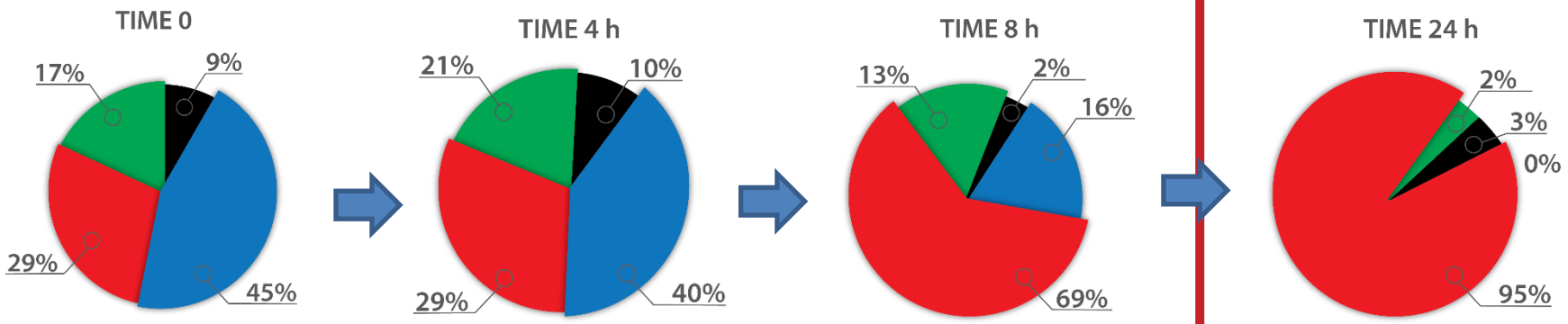
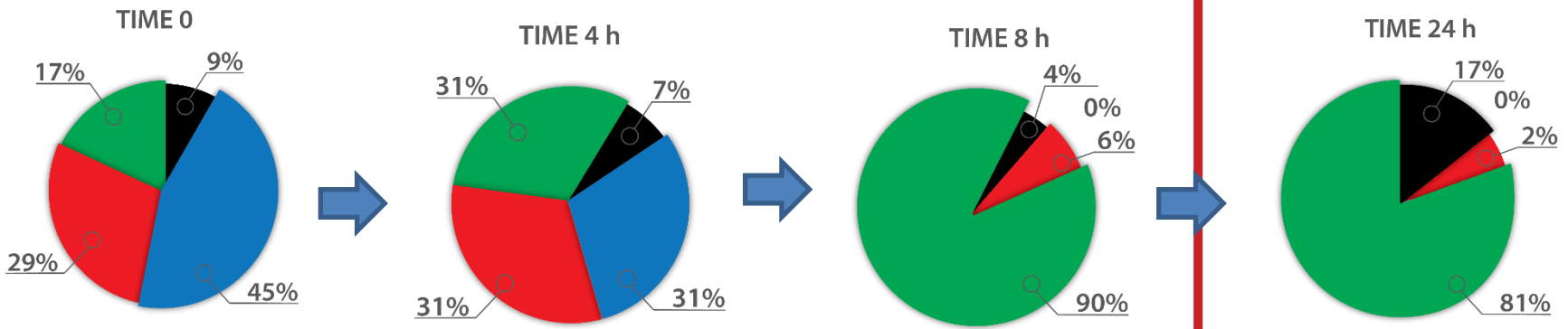
# Comparing Direct Pond Application vs. Pond Side Grow Out at 28°C Over time

## Direct Pond Application




## Pond Side Grow Out



# Pond Side Grow Out vs Direct Application

- The loss of microbial diversity in Pond Side Grow Out products is significant
- Pond side grow out products are not cost effective if you lose microbe diversity important to pond health.
- Direct Application of probiotics is better when more than one microbe is present

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# Recommendations for Direct Application

Direct application of beneficial bacteria products should be performed every 5-7 days to ensure high beneficial microbe concentrations

WSR tablets fall to the pond bottom to directly work where they are needed most

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# Conclusions

Collecting microbes from various environments have enhanced our collection and provided an important resource to produce a myriad of products to help the future of sustainable aquaculture. The profiles we develop for our microbes allows us to determine which probiotics are appropriate for products to reduce pond waste and sludge and which products would be appropriate to use for animal health. The **WSR** product was tested in the field and was found to be highly effective in reducing waste and sludge.


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# Questions on Products and Applications

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# Acknowledgements


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