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Aquaculture

Science & Solutions

Probiotics

Farming shrimp intensively and successfully

Photo: Mati Nitibhon

Bacterial languages

Control pathogens by disrupting their communication

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Editorial

Growing sustainably

For several decades aquaculture production has been growing at rates unparalleled in any other animal production sector. Such growth, however, has come with problems of increased disease occurrences. This has been the case for the shrimp industry which, despite rapid growth, had experienced periods of production decline due to diseases.

Although the industry has improved its response to such challenges, the recent events in Southeast Asia, with the severe losses due to EMS/AHPND, show that there is still much to be done. Any solution needs a holistic approach based on the application of best management practices for disease prevention. Probiotics have been part of this solution and BIOMIN is proud to be contributing to sustainable solutions in this field.

BIOMIN has a long history in probiotic research. We are carrying out extensive research projects focused on the development and application of probiotics in aquaculture. These projects have shown that differences in strains within the same species can be quite important when searching for the most suitable strains for aquaculture. Moreover this also underlines the importance of a multi-strain product to take advantage of the strengths of each single strain.

Research on the topic of quorum quenching is also presented in this debut issue, along with an overview of the BIOMIN concept on multi-strain products combined with enzymes for bioremediation purposes, as well as an industry perspective using the example of the Thai shrimp sector.

Sound research is behind every BIOMIN solution and our activities in probiotic developments are a good example. This first issue of **Science & Solutions** Aquaculture is dedicated to the importance of these microorganisms that strongly influence developments in our industry.

Gonçalo SANTOS
R&D Manager, Aquaculture

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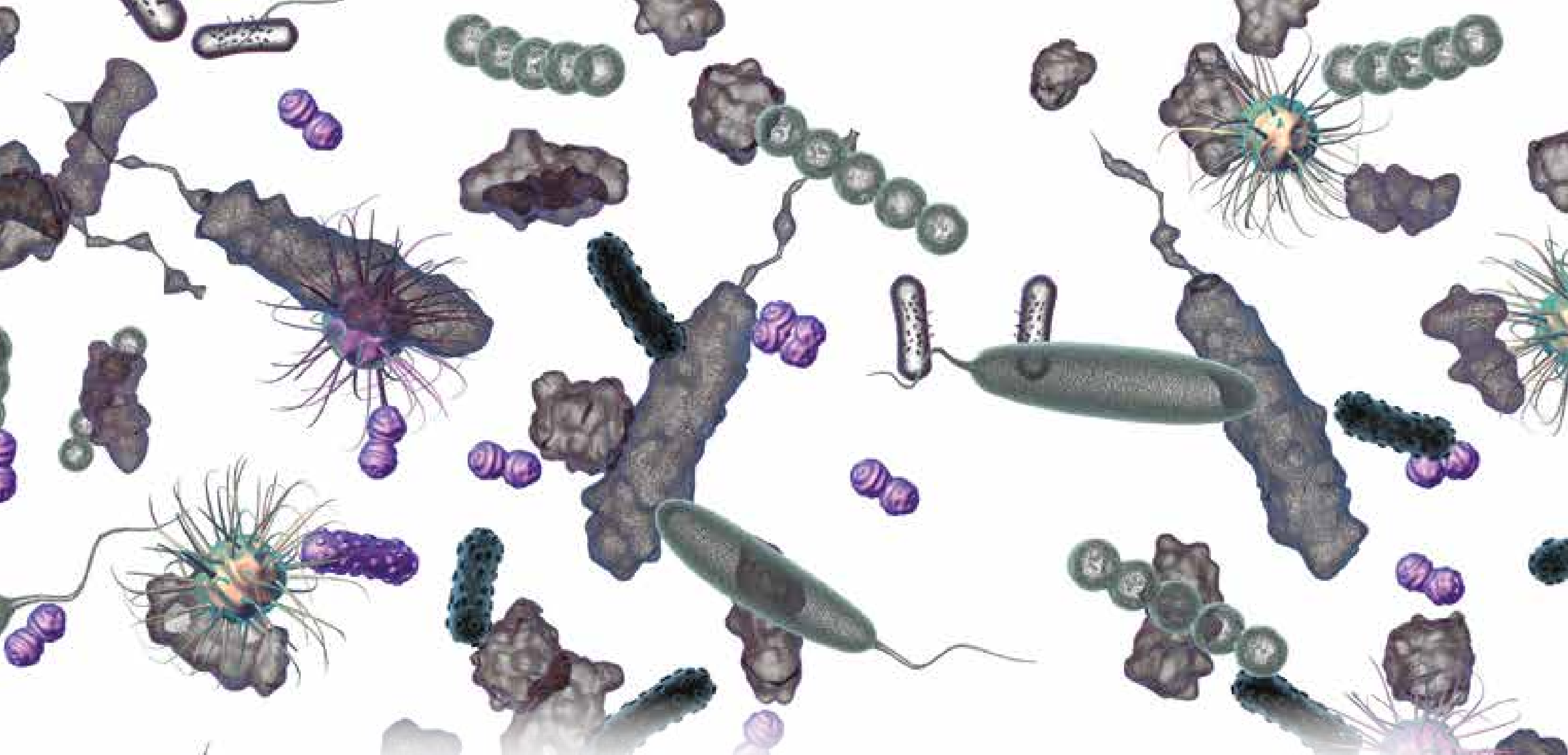
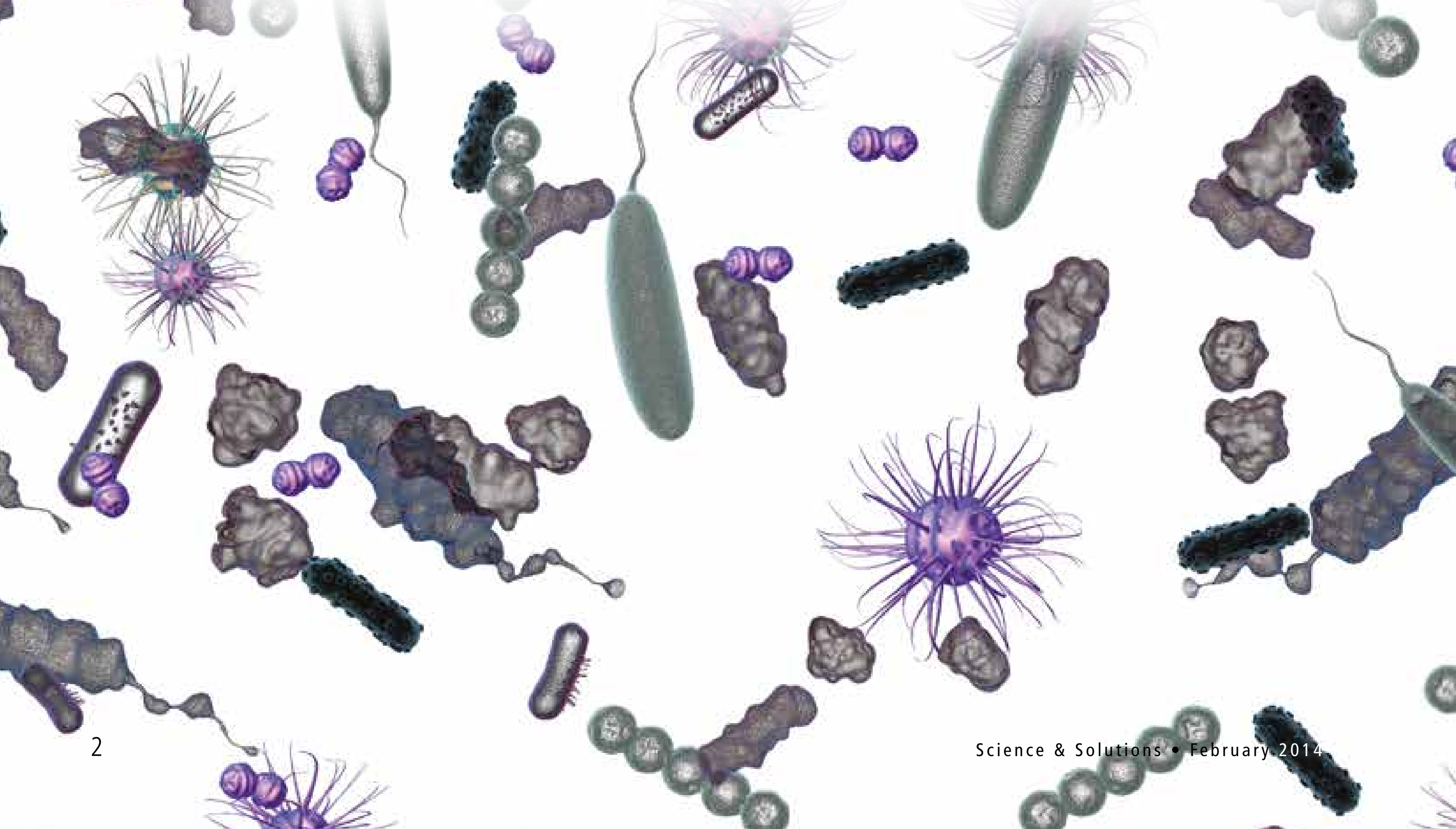


Probiotics

An essential tool in intensive shrimp aquaculture

As aquaculture develops, the industry faces several challenges related to its environmental impact. The application of beneficial bacteria (probiotics) is not only associated with gut health (feed probiotics), but also with bioremediation that improves the environment (water and soil) in which the animals are reared.



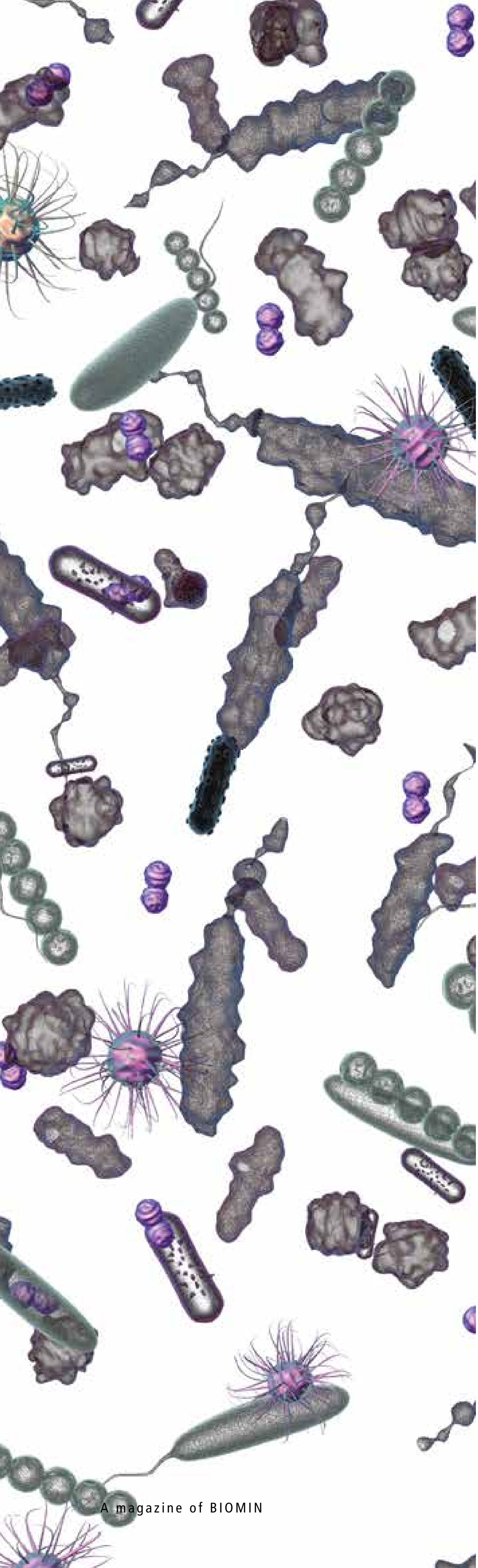
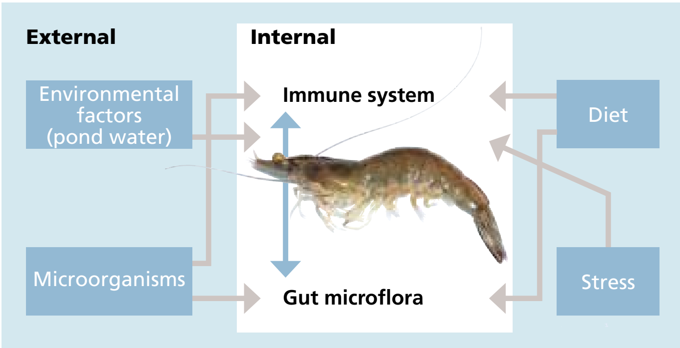


Photo: EKS

Gonçalo Santos
R&D Manager, Aquaculture

A key factor for successful aquaculture is to understand the interactions between the microbial environment, gut flora and immune system of the shrimp, as well as factors that determine the persistence of microbial species in the internal and external microbial ecosystems (*Figure 1*). While natural eco-systems are balanced, the farming environment favours the growth of microorganisms as it is rich in nutrients and feed waste.

Figure 1. Interactions within the microbial environments.



This environmental challenge is influenced by different factors, including farm management and rearing methods. Aquaculture operations generally involve the stocking and feeding of shrimp in open or semi-closed water systems. Semi-closed pond systems have a low water turnover and can accumulate nutrients, metabolites and waste, which deteriorate water quality and create anoxic conditions in the soil.

Pond management

The accumulation of waste compounds favours the growth of potentially harmful microorganisms while impacting the performance of farmed species. Good pond management is crucial for high production and a healthy crop. As water quality plays an important role, it is of great value to understand the various interactions taking place within the ponds. These are quite complex and

Gonçalo Santos
R&D Manager, Aquaculture

Enzymes at work

Enzymes have the capacity to stabilise the soil organic matter and can be used effectively to manage soil quality and rearing conditions for aquatic species under intensive conditions. There is no one specific enzyme that works best in all cases. A blend containing a variety of enzymes may be the most effective means for bioremediation in aquaculture.

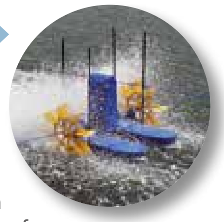


Table 1. Summary of pond interactions with and without the addition of beneficial bacteria.

Effect on	Without beneficial bacteria	With beneficial bacteria
Feed waste, faeces and organic matter	Accumulation	Reduction
Ammonia (NH_3), Nitrogen dioxide (NO_2), Hydrogen sulphide (H_2S)	▲ Increase	▼ Decrease
Oxygen (O_2)	▼ Decrease	▲ Increase
Eutrophication	▲ Increase Contaminated pond, water and bottom	▼ Decrease Improved pond, water and bottom
Pathogen and disease	▲ Increase	▼ Decrease
Shrimp growth and crop	▼ Decrease	▲ Increase

depend directly on the pond environment, stocked biomass, input of nutrients and pond management.

The accumulation and degradation of organic waste in the pond increases the consumption of oxygen (O_2) and production of waste compounds such as ammonia (NH_3), nitrites (NO_2-) and hydrogen sulphide (H_2S), which can lead to a phytoplankton bloom. The massive growth of phytoplankton can further deplete oxygen during the night and contribute to a phytoplankton bloom crash.

All these factors contribute to the contamination of water and soil, creating favourable conditions for pathogens to grow and affecting the condition of shrimp. Under such poor conditions, shrimp face higher levels of stress and are more susceptible to disease, which could result in poor growth or a failed crop through disease outbreaks (*Table 1*).

With the inclusion of beneficial bacteria, organic matter is utilised as a source of nutrients by the bioremediation bacteria. This reduces the amount of waste accumulating in the pond. Specific nitrifying and denitrifying bacteria will convert NH_3 and NO_2- into nitrogen gas, reducing the level of such toxic compounds. Some beneficial bacteria can also degrade toxic H2S, improving water quality and odour. The combination of all these factors will improve water quality and the condition of the pond soil, resulting in a better environment for shrimp with better growth and health status.

Table 2. A diverse range of enzymes used as bioremediation agents in aquaculture.

Enzyme	Substrate
Amylase	Starch
β-Glucosidase	β-Glucoside
Cellulase	Cellulose
Lipase	Lipids and fat
Protease	Protein
Xylanase	Xylan, Hemicellulose
Pectinase	Pectin

Enzymes

In the bioremediation process, enzymes play the role of catalysts that accelerate biochemical reactions in pond soil and water. When added to culture water or spread on top of pond soil, enzymes are able to degrade the major organic constituents normally found in shrimp and fish ponds. Each enzyme has its mode of action and is very specific in the chemical reaction it catalyses (*Table 2*).

Enzymes are also naturally produced and excreted by some microbes. These extracellular enzymes, such as cellulase, protease and amylase, are produced during the aerobic fermentation of organic matter by micro-organisms, for example by some *Bacillus* species.

Bacilli are commonly found in pond sediments and can also be added to the pond water for bioremediation purposes. Some *Bacillus* sp. are also able to degrade nitrogenous compounds and their large variety of excreted (extracellular) enzymes also helps to speed up the degradation of organic matter and toxic compounds such as ammonia. Efficient removal of nitrogenous compounds can also be carried out by nitrifying and denitrifying bacteria such as *Thiobacillus* and *Paracoccus*.

Enzymes reduce sludge accumulation and anaerobic conditions in pond bottoms. They promote a faster degradation of the organic matter that accumulates in ponds, especially under intensive production conditions. This organic matter comprises uneaten feed, dead plankton, mineral soils, faeces and pathogenic micro-organisms in the soil where conditions are often anaerobic.

For all these bioremediation processes catalysed by enzymes, the presence of beneficial bacteria is important as well. Enzymes accelerate microbial processes by breaking apart large sludge particles, thus creating more surface areas which can then be attacked and fermented by microbes. This reduction of sludge and dead organic matter can be seen visually not only with better water quality, but also better soil quality.

Field study

In a field study in China, the combined application of the bioremediation products AquaStar® Pond (multi-strain beneficial bacteria) and AquaStar® PondZyme (beneficial bacteria and an enzyme blend) were added to the water. It was observed that water quality improved, as did soil condition and ultimately, shrimp performance.

Four earth shrimp ponds (0.7-0.8 ha/pond) with a depth of 1-1.2 m were stocked with juvenile shrimp (approximately 1.4 g/shrimp) at a density of 50 shrimp/m². The trial was carried out for a period of 57 days with an application of 1 kg/ha AquaStar® Pond weekly and 500 g/ha of AquaStar® PondZyme applied once a month to the treatment group (two ponds). The control ponds consisted of two ponds with normal production operations.

The soil of the AquaStar® ponds in *Figure 2 (A)* was yellow, the colour which represents the best bottom type. The soil of the control ponds in *Figure 2 (B)* however, exhibited a dark black color, indicating the accumulation of dead organic matter.

Results suggested that with the combined use of beneficial bacteria and enzymes, pond soils containing black and glutinous organic sludge turned into a yellowish soil.

Figure 2. Pond bottom soil from ponds with the AquaStar® programme (A) and from the control ponds (B).

In terms of performance, the average daily weight gain of shrimp in the AquaStar® group was higher by 36% and feed conversion ratio was lower by 9% compared to the control ponds. The results are shown in *Figure 3* and *4*.

Figure 3. Growth rate of shrimp in the control and AquaStar® groups.

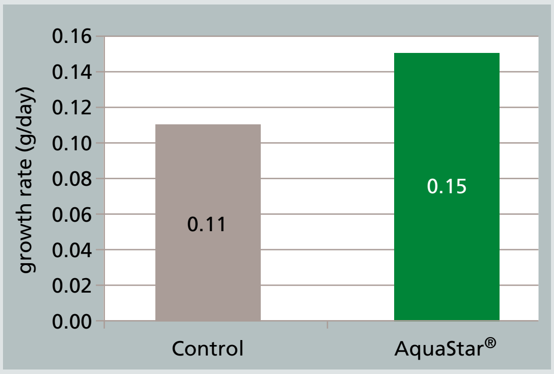
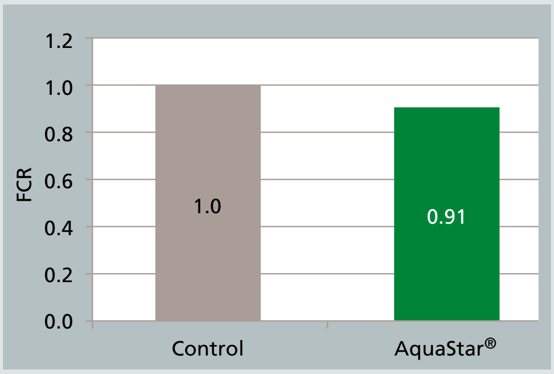


Figure 4. Feed conversion ratio of shrimp in the control and AquaStar® groups.



Based on these results, it was concluded that in the search for more effective and environmentally-friendly treatments, beneficial bacteria have emerged as a viable alternative. Enzymes can play a complimentary role in bioremediation solutions for aquaculture, especially in intensive productions.

Barbara Weber
Scientist, R&D

Bacterial languages
Communication and Coordination?

Several decades ago bacteria were primarily considered as separate entities of unicellular organisms that do not interact with each other. However, this view has changed and many bacterial species are now known to coordinate behaviour at a multicellular level and not as single cells. This phenomenon is called quorum sensing.

How does bacterial chatter work?

Quorum sensing is mainly mediated by small, diffusible signal molecules. Cells produce these molecules continuously and as the population grows, these molecules accumulate. When molecules reach a certain threshold, concentration changes in gene expression are triggered and lead to changes in population behaviour.

Signal molecules can be viewed as languages. Many species have their own specific language, but they can also communicate with other species. For Gram-negative bacteria the language is generally based on acylated homoserine lactones (AHLs), whereas for Gram-positive bacteria the language is based on small peptides.

Variations and modifications of these structures confer species-specificity. Both types of bacteria produce and respond to a molecule called AI-2. AI-2 represents a universal language comparable to English that allows the transfer of information within a multispecies community. Different groups of bacteria, e.g. *Vibrios* have their own language that is not specific for a distinct strain, but a group of related species.

Why do bacteria need to communicate?

Communication helps bacteria to organise themselves and to decide whether it is worthwhile to invest energy in the expression of virulence genes, biofilm formation, light emission, DNA uptake, production of antimicrobials and exoenzymes and many other traits. Many of these process-

Table 1. Various tools to measure the production of signal molecules and quorum quenching.

Biosensor	Read out	Signal molecule
Chromobacterium violaceum — McClean, 1977	Purple pigment	AHL
Escherichia coli — Andersen, 2001; Winston, 1998	Green fluorescent Light production	AHL
Agrobacterium tumefaciens — Luo, 2001	Blue color reaction	AHL
Vibrio harveyi MM32 mutant *— Miller, 2004*	Light production	AI-2
Vibrio harveyi JMH626 mutant *— Henke, 2004*	Light production	CAI-1
Vibrio harveyi BB120 wild type *— Bassler, 1997*	Light Production	AHL,AI-2,CAI-1

Figure 1. Different concentrations of cinnamaldehyde were tested for the inhibition of light production.
First, light production was induced by a signal molecule.
Second, cinnamaldehyde was added.
Third, light production and growth were recorded after five hours.

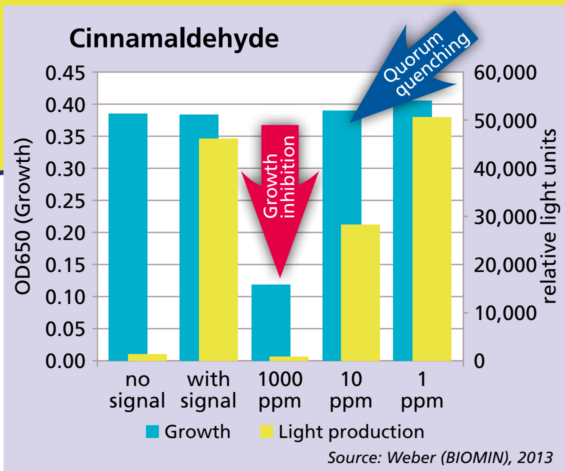
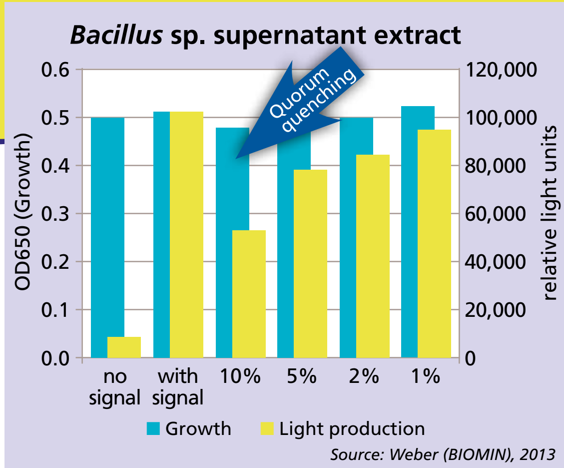


Figure 2. Supernatant of a probiotic *Bacillus* was tested for quorum quenching and potential production of AHL lactonase.
First, light production was induced by a signal molecule.
Second, probiotic supernatant was added at different concentrations.
Third, light production and growth were recorded five hours later.



es, such as virulence, are costly and might only be successful, for instance if the population is large enough to buffer losses due to the hosts´ defense system. Quorum sensing also provides information about the metabolic state of a community, species composition, and competitors for nutrients in a certain niche.

Quorum sensing is important for virulence, especially for pathogens that are relevant for aquaculture, such as *Aeromonas*, *Vibrio*, or *Yersinia*. *Vibrio harveyi*, the causative agent of luminescent Vibriosis in shrimp, is known to use quorum sensing mechanisms to regulate its virulence.

Can communication be disturbed?

The inhibition of communication is called quorum quenching. Several quorum sensing inhibitors that interfere with communication lines have already evolved in nature. Algae, sponges, plants and many bacteria produce different kinds of inhibitors that either destroy signal molecules, block signal molecule production or sabotage signal molecule detection.

Test systems

To test quorum quenching, several biosensor strains are available. In the presence of signal molecules, these strains induce light production, purple pigmentation, fluorescence, or other easily detectable traits. If at the same time an inhibitory substance or enzymes that destroy the molecules are present, these traits are quenched (*Table 1*).

Quorum quenching—practical examples

Algae and plants (e.g., red macroalgae, cinnamon, garlic, ginger) produce compounds that mimic AHLs or destabilise the detectors and thereby block the information flow. A famous quorum quenching phytogenic substance is cinnamaldehyde (*Figure 1*). We have performed several tests in our laboratories and could confirm its quorum quenching activity.

Many Gram-positive bacteria, including probiotic strains such as *Bacillus* sp. produce enzymes such as AHL-lactonase or AHL-acylase that deactivate the communication of Gram-negative bacteria by degrading the AHL signal molecule. Analysis of the probiotic *Bacillus* present in AquaStar® revealed that this strain is able to interfere with bacterial communication (*Figure 2*). Thus, quorum quenching is part of the mode of action of AquaStar® probiotics.

Information is critical

Disrupting communication lines will interfere with information transfer and lead to uncoordinated behaviour. In the case of pathogens, this interference could prevent the outbreak of infectious diseases, because the pathogens cannot arm themselves properly and are thus more vulnerable and easier to kill. In the light of increasing microbial resistances to antibiotics, targeting a mechanism not related to growth provides a means for controlling pathogens.

Pitfalls of quorum quenching test systems

Because cell-free probiotic supernatants, plant- and algae extracts contain antimicrobial substances that repress bacterial growth, analysis for quorum quenching needs to be done with care. When growth of a strain is repressed, it will by default not produce the quorum-sensing related trait. Thus, it is important to test a range of concentrations to find a concentration where bacteria are growing but are not able to communicate.

Pedro Encarnação
Director Business Development

Since the last decade Thailand has been the world's leading shrimp exporter with annual foreign exchange earnings of more than US$3 billion. In 2010, Thailand produced 600,000 tonnes of marine shrimp. The strong growth observed in the industry in the first decade of the century was mainly achieved by increasing the density of the farming process, resulting in higher yields.

Supporting the Thai shrimp industry

However Thai shrimp production has been on the decline in recent years. If most of the decline (10%) in 2011 was related to natural disasters like floods, the main culprit in 2012 has been the EMS/AHPND disease outbreak. This new disease resulted in massive mortalities at the early farming stage (15-30 days) and caused devastating economic damage to the sector. Production in 2013 plunged 54% from 2012 to just 250,000 tonnes.

EMS/AHPND caused widespread panic among Thai shrimp farmers and many did not even stock their ponds as they were afraid of losing their investment. Thai farmers quickly recognised that shrimp health management is strongly linked to other aspects of shrimp farming which should be addressed in order to achieve effective shrimp health management. As such, farmers started focusing on maintaining a healthy environment in the ponds at all phases of the culture cycle so as to prevent problems in the ponds before they occur and reduce the likelihood of disease transmission outside the farms.

Emphasis has been placed on farming practices like the quality of post larvae, pond preparation and the improvement of water quality through disinfection, aeration, temperature control, pH, alkalinity and salinity. Farmers are also paying more attention to sludge reduction, reducing water exchange, removal of nitrogenous compounds, and the stability of the algae bloom. Feeding issues have also been managed in order to restrain the use of antibiotics through the application of probiotics.

Farmers are focusing on maintaining ***a healthy pond environment*** *to prevent pond-related problems and reduce disease transmissions.*

Working together

The use of probiotics became a successful tool to manage pond environments and the prophylactic control of pathogenic bacteria. The success of the aquaculture team in BIOMIN Thailand is based on improving shrimp farming practices.

Starting with only one sales person in 2010, the success of the products in the field led to a rapid expansion of the sales team that now accounts for four sales people and one technical person covering the major shrimp farming areas of the country.

Technical support

The BIOMIN Thailand sales team works actively with the farmers to improve pond environment and shrimp health with the application of probiotics (AquaStar®) and liver protection products (HEPAProtect-Aqua). Water quality parameters (pH, salinity, NH_3, NO_2) are analysed to assess the efficiency of product applications (AquaStar® Pond and PondZyme®) on improving pond conditions.

With regard to EMS/AHPND, BIOMIN has been developing and testing integrated strategies that minimise the presence of the vibrio in water (AquaStar®–probiotics) and feed (organic acids–Biotronic® Top3 and phytogenics–Biomin® P.E.P. MGE). Results so far have been promising with many customers reporting improvements in survival and good harvests (10-15 tonnes/ha) in areas affected by EMS/AHPND.

R&D

The BIOMIN R&D team supports the industry by looking at solutions to overcome the problem of EMS/AHPND. Several projects are now being conducted in our R&D centre in Austria, focussing on strategies to reduce or eliminate the agent that causes EMS/AHPND (*Vibrio parahaemolyticus*). These include studies evaluating substances that inhibit bacteria, reduce its virulence capacity (quorum quenching) and eliminate or reduce of the effects of the toxin.

Partners

BIOMIN also actively participates in major shrimp exhibitions and organises technical seminars with local farmers where our aqua experts explain the mode of actions of our products and discuss the latest issues regarding good farming techniques. Working together with the farmers for their success, we have been able to grow our business sustainably.

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