

What can shrimp farmers do about EMS (AHPNS/AHPND)?

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Vibrios inhabit most aquatic ecosystems including freshwater. As of late 2013, there are at least ninety-eight (98) recognized species with many more candidates. Vibrios species are common inhabitants of aquatic environments where they serve critical functions in the recycling of nutrients including polymeric n-acetylglucosamine, a molecule related to glucose that is commonly known as chitin, the primary structural component of arthropod (including shrimp) skeletons. Most vibrios are benign. Many have the ability to degrade chitin (by the production of chitinases) and readily attach to chitinous substrates (such as shrimp and other chitinous exoskeletons common to invertebrates). They are also often associated with algal and zooplankton species and indeed for some this is an important means of transport within aquatic environments and contributes to their ecological stability.

Shrimp in the wild, contrasted with most farms, are at lower densities and can move to environments where they are not stressed (or stressed less). In farms this is not usually the case. Stress is frequently an inherent component of the farming process. A relatively small number of vibrios cause disease in farmed shrimp. Most are opportunistic pathogens. They are able to cause disease because the host animals

are stressed and impaired in their ability to fight them off. Only a relatively few are obligate pathogens that cause disease just by being present. Among the obligate pathogens there are a wide range of species and a wide range of pathogenicity. Obligate pathogens cause disease in healthy animals. The most virulent of these kill shrimp at very low levels of exposure in the water or when consumed. Contrary to the mythology that pervades the industry not all of them are unable to ferment sucrose thus forming green colonies on the media that is typically used to select for vibrios, TCBS. Many form yellow colonies on TCBS. Vibrios readily exchange genetic material with each other as well as with other bacterial species and they are undergoing a constant process of evolution, thus the rapid changes seen in antibiotic resistance (due to the acquisition of genes encoding for these traits) and no doubt a myriad of other phenotypic characteristics including possibly those observed in the etiologic agent(s) of AHPND.

V. parahaemolyticus is a common inhabitant of most marine, estuarine and indeed some fresh water ecosystems. Most strains are not pathogenic and pose no threat. Given that a major cause and the reported number one cause of seafood poisoning in the USA and in many other countries is due to toxigenic strains of this bacteria from fisheries caught product it is constantly being tested for in final product. In fact the bacteria is commonly found in seafood and when it does not contain the appropriate toxin producing genes is not of any concern. It is harmless to ingest.

The advent of genomic taxonomy has been instrumental in allowing differentiation between isolates that are phenotypically and biochemically apparently identical as determined by conventional biochemical testing. These tools are no longer considered to be sufficient for accurate identification of some species and in order to determine a scientifically agreed upon name sufficient genomic characterization must occur or there is a significant risk of misidentifying some species. The group of bacteria that the etiologic agent(s) of AHPND is a member of (*Vibrio parahaemolyticus*) is particularly prone to this. Developing tools that are specific for the species that is causing a problem can only be done when bacteria can be reliably identified and characterized. Even very small differences that are consistent can be exploited to develop tools that are critical to the ability to localize the pathogen and come to a more complete understanding of where it is in the environment, where it comes from and how it might be controlled. Until these tools are available care must be taken in ascribing all cases of the disease to a single strain of bacteria and the possibility always exists that the strain might be sufficiently unique to merit becoming another vibrio species (#99).

Farmers should appreciate that it is very difficult if not possible to totally eradicate these or any other group of bacteria. Nor is this necessarily desirable. As most vibrios are benign and serve a useful purpose, eliminating them opens up niches for other species that may not be benign. There are many species of bacteria that can cause mortality in shrimp, not just vibrios.

Probably the most widely known member of the species is *Vibrio cholerae*, some strains of which cause cholera, a serious life threatening disease. There appears to be many similarities between this organism and the etiologic agent(s) of AHPND. As with the other members of the vibrio taxon there are many sub-species of *V. cholerae* that are not virulent. The same goes for *V. parahaemolyticus*. Only a few strains of *V. cholerae* are highly virulent (for humans) and they cause a serious diarrheal disease when people drink contaminated water and ingest typically more than a million bacteria at one time. A number of strains of *V. parahaemolyticus* are highly virulent for humans and can also cause an acute diarrheal disease (indeed this bacterial species has been implicated as the number one cause of seafood related food poisoning globally). Proper treatment to restore the electrolyte imbalance along with common antibiotics to lessen the chances of complications easily cures cholera. Severe cases of *V. parahaemolyticus* food poisoning are treated the same way.

While it can be quite deadly, cholera is easily treated (and prevented). The pathogen acts primarily through the gut as apparently does the etiologic agent of AHPND. *V. cholera* has been widely studied and much is known about how it produces disease and what its role and niches are in the aquatic ecosystems it lives and thrives in. It is ubiquitous and is associated with phyto and zooplankton species that provide a ready vehicle for spreading it. It also is a biofilm former as are many other vibrio species (and many other bacteria). *V. parahaemolyticus* has also been well studied and occupies a niche very similar to that of *V. cholerae*.

There are many similarities between cholerae and AHPND. Cholera typically becomes problematic when drinking water and sewage systems are not properly segregated. In many parts of the world the all too common practice of shrimp farms in many of the affected areas using effluents as influents is analogous. In China where the first reports of the disease are documented (although that does not mean that it did not appear elsewhere earlier) this is a common practice. In some cases this is blatant and in others not readily so. Currents from discharged waters carry vectors that act to move the pathogen(s) far and wide.

In the early 1990's cholera rapidly spread along more than a 1000 miles of Peruvian coast in a very short period of time and, it is well documented that as it followed rivers and streams, much of Latin America was affected. There is reliable evidence that shows that the etiologic agent of AHPND has also been spread similarly. The disease has moved in a manner that strongly suggests that this may be an important route of transmission. As with *V. cholera*, *V. parahaemolyticus* tolerates a wide range of salinities, pH, temperatures and nutrient conditions. *V. parahaemolyticus* will grow at a wide range of salinities (although most strains apparently cannot survive in fresh water) and a pH range from 6 to 10. It is well documented that both species of bacteria readily piggyback on many different potential vectors.

V. cholerae and *V. parahaemolyticus* are both commonly found associated with many different species of marine plankton. Producing a wide range of enzymes as

with most of the other vibrios they readily attach to chitin, the surfaces of algae (many of which are slimy by nature) and other similar substrates. Vibrios can be found attached to blue green algae (cyanophytes or more accurately cyanobacteria), diatoms, dinoflagellates and many other algae species as well as macrophytes, aquatic plants, etc. Zooplankton is thought to be one of if not the most important means by which *V. cholerae* is spread. *V. parahaemolyticus* also readily attaches to and is moved by zooplankton. Clearly the etiologic agent of AHPND occupies many niches. This could explain how it seems to move so easily and why eradication and/or control will not be a simple matter. To compound matters pathogenic strains of cholerae have also been recovered from bilge in ships that readily move between ports. It is highly likely that the same can be said of *V. parahaemolyticus*. The extremely wide range of *V. parahaemolyticus*, its ready filtering by bivalves and its presence in fish are all factors that could be important in what we are currently observing.

Biofilms occur everywhere. Probably the most familiar to humans is why we brush our teeth. They are implicated in a myriad of disease states and are an effective mechanism for bacteria to spread. A biofilm is an assemblage of organisms that have attached, typically to a surface of some kind (such as in the case of shrimp farming detritus on pond bottoms, the liners present on lined ponds and in the case of AHPND the stomach of the shrimp). This biofilm protects the bacteria within from the action of antibiotics and other bacteria that would seek to occupy the niche themselves.

Given the extremely common nature of biofilms they have been widely studied and characterized. Typically there are 5 stages involved in the process. The first is where the bacteria cells attach in a manner that is reversible to a cell surface. In the case of AHPND this would be when the bacteria first attach to the chitinous stomach and gastric mill surfaces in the shrimp. After this first step the bacteria form sticky materials, exopolymers that in a sense glue the bacteria to the surface. They are no longer easily detached and as the biofilm subsequently matures by the formation of micro-colonies these exopolysaccharides can protect the bacteria against antibiotics, disinfectants, herbal extracts, etc. The biofilm matures in a complex arrangement of bacterial cells that acts to protect the integrity of the biofilm while still allowing normal metabolic activity of the cells. In the final state the biofilm begins to detach and the cells in the film disperse as a new biofilm develops. These detached cells can spread in the environment.

Efforts to control this disease began long before the identity was established. What was probably AHPND was first reported in China 4 years or so ago and has spread to a number of other countries. Its effect has been very severe. I am personally of the opinion that this strain of vibrio may already be in most shrimp farming countries (with some exceptions) and that it will spread slowly but inexorably into those environments that allow it to dominate at the expense of the other bacterial species that are present. Given the complex nature of the vibrio taxon there may be a

number of things going on that make this a less than a straight forward problem to address.

This particular strain is an unusual pathogen (in shrimp) in terms of how it produces disease. Further analogies with cholera are apparent as it appears that it is a toxin based disease process where the bacteria colonize a limited surface and the toxin does the damage. Most other pathogenic vibrios invade the animal and though various toxins and their cell wall structural component, lipopolysaccharides (LPS) overwhelm the ability of the animal to defend itself with ensuing decline and death. It does not appear to be invasive in the sense of finding its way into the animal through injuries or other mechanisms into the hemolymph. This explains why antibiotics do not stop the infection. If the antibiotics are not able to come in contact with the pathogen at sufficient levels to impact it by stopping it from growing or killing it, then they will not work. Since the abuse of antibiotics in shrimp farming has been a point of concern for some years now, although there is still and should be room for appropriate use of antibiotics, this is one example where any use of antibiotics would be considered inappropriate. Furthermore if it is present in a biofilm in the stomach this can serve to protect it from the action of many other types of compounds that could theoretically kill it. This will pose a serious challenge to those trying to develop treatment modalities.

There are a number of commonalities that have been observed about this disease process between the disease in SE Asia and in Mexico (where its presence was only

recently confirmed despite its nearly destroying the industry over the course of many months). These observations provide some clues about what is going on and what potential approaches towards dealing with it might be.

It has been noted that when shrimp are reared in cages off of the pond bottoms in ponds where shrimp are dying from the disease they are unaffected. This suggests strongly that the disease is not being transmitted through the water column. If it were then it would make no difference. It has also been noted that when animals are held in nursery raceways even with the same water that is in the pond they do not typically develop the disease. Again the inference is that it does not spread via the water column in the ponds. This could be for any number of reasons although the most logical is that it never reaches high enough levels to be infective via the water column. Bacterial loads in ponds vary considerably. Ponds develop very complex ecosystems with many species of bacteria, algae, phytoplankton and zooplankton. Vibrios are always present at some levels, although even in the middle of outbreaks from obligate pathogens the levels are not typically very high (there are some exceptions to this). Attachment of this pathogen to various substrates could readily explain transmission.

Since there is no evidence to support that this bacteria causes a septicemia it is highly likely that molting disrupts the biofilm process. Given that it takes time for the bacteria to progress through the various stages of biofilm formation, this explains why we do not see the disease in hatcheries (although there does not

appear to be any proof that it is even in the hatcheries). The fact that strains of *V. parahaemolyticus* are ubiquitous so it is not surprising to find them in hatcheries, in production tanks, in Artemia and algal production systems as well. No one has reported mortality in broodstock held in nuclear breeding facilities either, although there is no reason why, if the pathogen were present in larger animals, it could not kill them.

Another interesting observation from the field is that co-cultivating the shrimp with Tilapia seems to lessen the incidence and severity of the disease. It is common knowledge that Tilapia produce substances that are inhibitory to a variety of bacteria including vibrios. This is one of the attractive features of green water culture. The exact nature of these materials remains to be elucidated and while there are theories it seems that this is not an absolute effect. Some farms still report problems even when they co-cultivate with Tilapia.

Additional observations show that farms that use well water in some ponds and seawater (water not from wells) in other ponds only have the disease in the ponds where water from non-well water is used. This further supports the theory that the primary mechanism of movement of the pathogen is not between shrimp but through a host of vectors similar to what we see in cholera. Furthermore the incidence of the disease in farms that use water that is less than 5 ppt is much lower than in those with higher salinities. In that *Vibrio parahaemolyticus* strains do not

typically grow in water that is less than about 1% sodium chloride (10 ppt), this is expected.

The role of a toxin-producing gene is of paramount importance and determining what the nature is of the toxin and how its production is controlled could be important in minimizing the impact. Toxins are often produced as the result of a phenomenon known as quorum sensing. Simply put this is a means by which bacteria communicate with each other. Toxins are often substances that are involved in the nutrition of the bacteria. The fact that they are toxic to specific hosts is not their primary function. It is a side effect of their being present. *V. parahaemolyticus* is a very well studied bacterium. There are thousands of publications in peer-reviewed literature that report on many different aspects of it. Many toxins have been identified and characterized and it is probable that the toxin that is damaging the HP is not something new.

Producing shrimp in stressful environments and using pseudoscientific cultural practices can play a role in the disease and its spread. The evidence suggests that many of the so-called probiotics that are widely marketed have little if any impact on the loads of vibrios in the environment. Eliminating the bacteria is impractical although one can develop strategies that favor the growth of more (a generality) benign vibrios (yellow) over those that have a greater potential for causing problems (green). Optimizing this while addressing the presence of potential vectors could lessen the impact. It is highly likely that AHPND will continue to

spread. Successful control strategies will entail the use of a variety of tools and must include a far better understanding of where it is in the environment and how it moves.

What are the options that the farmer has? At this time they are very limited. Despite press to the contrary this problem continues to persist in SE Asia. Some individuals and groups are claiming that broodstock and hatcheries are where the problem comes from. While there is no doubt that *Vibrio parahaemolyticus* strains are present in broodstock and throughout poorly biosecurity managed hatcheries, given the virtual ubiquitous nature of this bacteria strain and the very strong evidence that suggests that the etiologic agent of AHPND is being moved by vectors, it is not likely that the source of the disease is either of these sources. That does not mean that one should ignore biosecurity. Reasonable precautions should always be taken to lessen the loads of potential pathogens in these environments and indeed the technology to do this is well established. Tools have been available to impact vibrio loads in these environments for many years. Failure to be used is a result of a proliferation of pseudoscience and snake oil salesmen.

So for the sake of discussion if one can eliminate the hatchery as the source of the pathogen if not simply for the fact that shrimp molt daily in hatcheries making a stable biofilm problematic, the real issue becomes how does one control the vibrio on the farm. Considering the similarity to *V. cholerae* it makes sense that controlling ingestion of materials that contain the bacteria, whether it is detritus on

pond bottoms, zooplankton or algae becomes part of an apparent solution. This could be confirmed experimentally by looking at the difference in susceptibility between traditional culture systems, semi-intensive and intensive to super intensive systems where there is no natural food present. If in super intensive systems the disease is not present this would be further evidence that the disease is not a waterborne problem .

Ideally the farmer needs to create conditions on their farm and in their animals that make it harder for the *Vibrio* to colonize the animal's stomach and for the toxin to produce the pathology that it does. This is unfortunately not as easy as it sounds. Theoretically, this can be done by changing the production paradigm and eliminating the niches that the bacteria occupies or making it more difficult for the shrimp to ingest high enough loads of the bacteria for the acute disease to develop. One possible approach might be to use much higher water exchange rates as this will flush out nutrients and bacteria. The widespread use of bacterial amendments in ponds apparently has had little if any impact and there are anecdotal reports that suggest that this may make the problem worse in some cases. This is complicated and may relate to the species of bacteria used, the delivery methods as well as timing and frequency of application as well as the use of supplements such as molasses which quite likely can also stimulate the growth of the etiologic agent of AHPND.

Blocking attachment of the bacteria to the stomach wall and gastric mill warrants a closer look at as does the use of compounds that will kill the bacteria as they enter the host or even during the early stages of attachment. Likely a combination of several approaches might prove useful in limiting the impact. Unfortunately we cannot treat this like cholera. We can however, when and if probes that are specific for it become available, get some idea of where it is in the environment. There is strong evidence that strains of *Vibrio parahaemolyticus* occupy many of the same niches that *V. cholerae* does. The observation that this bacteria colonizes the stomach would at first thought suggest that one approach might be to feed the shrimp compounds that inhibit the bacterial growth. This would include antibiotics, monoglycerides and a host of other substances that are potentially inhibitory. Whether or not this will work may be related to the nature of the biofilm on the stomach. If the biofilm is typical of that noted in other bacteria then is it not likely that this approach will work. The bacteria will be protected by virtue of the biofilm. Timing of delivery would be critical and problematic.

There is much speculation about where this came from and what the reservoirs are in the environment. Shrimp farming by its very nature encourages the growth of vibrios. They are present naturally in all environments and there are complex mechanisms in place that typically moderate them. This balance has been disturbed and this could explain why this bacterium is able to proliferate at the expense of others. The widespread use of chlorination to eliminate the virus that causes WSSV and the vectors that might be present in the incoming water may be a contributing

factor since this practice is altering the pond microbial ecology. It is well documented that chlorine increases the ease of which organic matter is assimilated and there are reports that this may stimulate bacteria that form biofilms. The irony in this is that the use of chlorine is not the most effective approach towards controlling the viral loads and that the role of secondary bacterial infections in animals that are weakened by the virus may be more important in determining the outcome of the disease process than the presence of the virus itself. Some of the vectors produce cysts that are buried deep in sediments and it is common knowledge that within a few weeks post-chlorination the virus is easy to find in vectors and the environment again. Whether or not active disease ensues is environment dependent.

The preponderance of evidence to date suggests that exclusion where possible and appropriate management of ecosystems might offer some hope. Many people are dumping anything into the ponds that they can get their hands on and into the shrimp as well. This shotgun approach, even if it were successful on a limited scale does not allow a ready determination of what if any approach is successful in limiting the disease process. What is clear is that the problem is likely moved through many different vectors in the water and that shrimp consuming the bacteria plays a critical role in the disease process. I would not expect that all shrimp would be universally affected to the same extent. Affected animals may not all necessarily die although they may grow poorly, have poor feed conversions, etc.

Aquatic ecosystems are complex and manipulation of these systems along with what most experienced aquaculturists would know is a foolish practice regardless of purported safeguards (using effluent for influent), likely allowed this bacteria to dominate in a few ponds. From there it readily spread much as cholera has and will again. However unlike cholera the problem appears to be chronic and as long as animals are recolonized with this particular pathogen(s) the problem will persist. Any practice that is aimed at impacting this process should be useful in lessening the impact the disease.

To conclude, there are many analogies between the etiologic agent of AHPND and *V. cholerae*. Foolish cultural practices could have led to the initial transfer of genetic material that allowed a strain with this particular properties to develop and propagate itself. The pathogen is readily spread through the water by a myriad of vectors. The pathogen has been able to establish itself and will continue to spread. As things of this nature go, it is not inconceivable (although unlikely) that the problem will self limit. It is not likely that genetic selection will allow the development of shrimp strain(s) that are able to tolerate the toxin and since it binds to chitinous surfaces it is not likely that this will change.

Controlling the pathogen will require a combination of environmental manipulation techniques that allow the balance to be restored, changing the production paradigm in areas where the problem is endemic and the balance can not be restored. Tools that lessen the overall load of vibrios at any and all stages of the shrimp production

process might be useful in lessening the impact of the disease and ultimately allowing an ecological shift to occur back to a more favorable outcome. In that this bacterial pathogen is unlike anything that has been reported in shrimp farming to date, many of the classic strategies for controlling bacteria are not likely to work. It may be in the long run the the process of elimination will winnow out those farms that are marginal at best and those farmers who consistently fail to use the tools of proactive disease management and science to ensure that they produce a sustainable and consistent crop. The end result will be a more robust and healthier shrimp farming industry.