NITRIFICATION IN MARINE AQUARIA

A FRESH LOOK AT THE MICROBES IN HEALTHY REEF SYSTEMS

Every thriving reef aquarium system depends on a complex, unseen community of bacteria that detoxify nitrogenous wastes. Aquarium of Heinz Hartwig.

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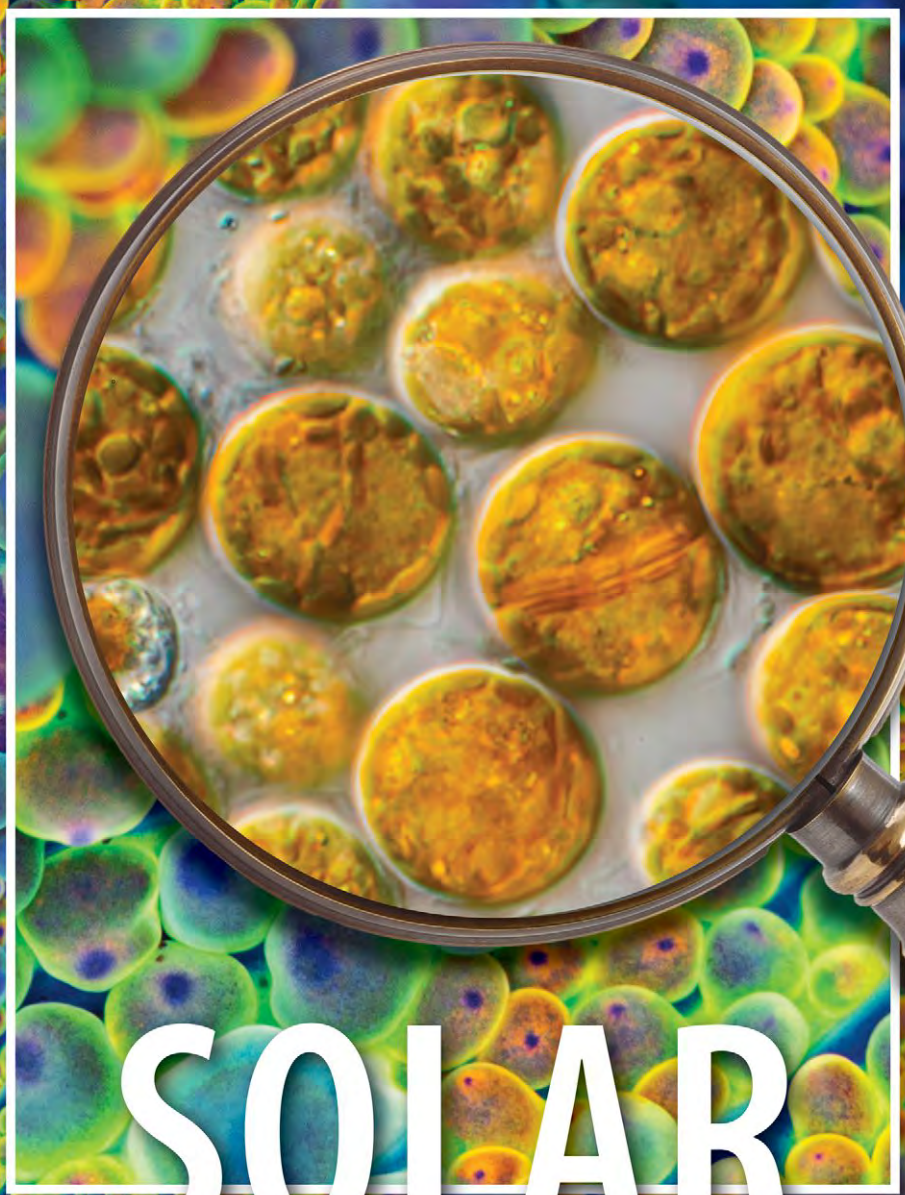

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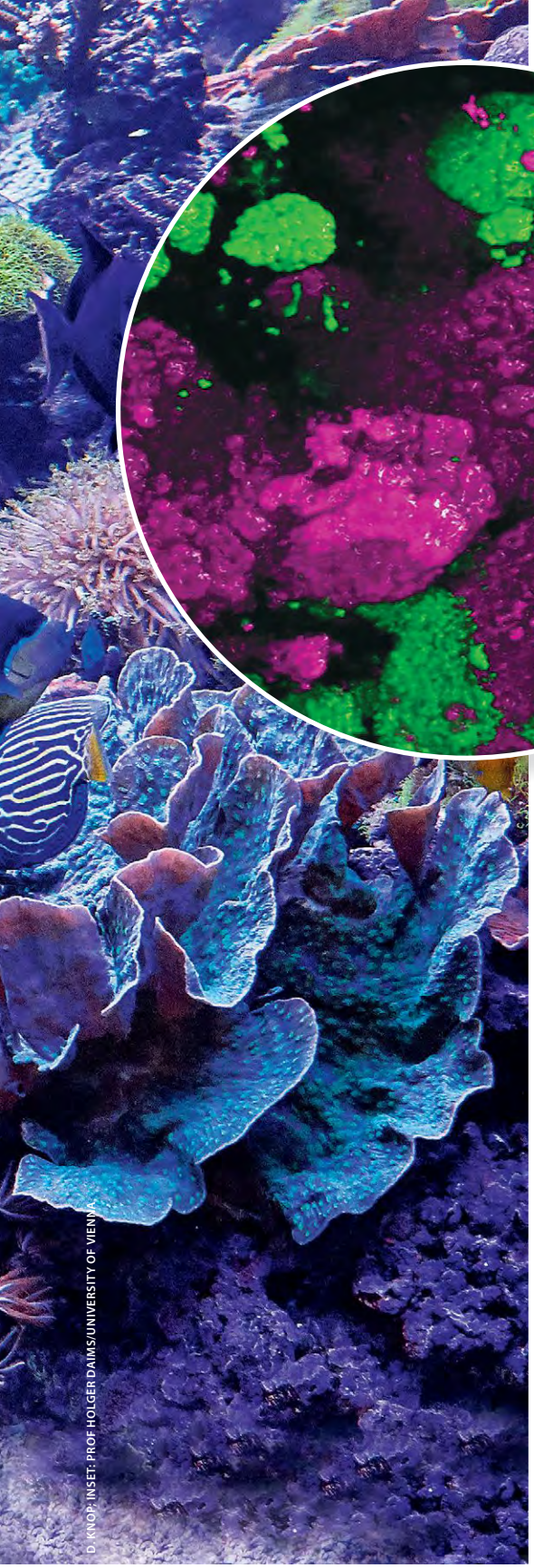

D. KNOP; INSET: PROF HOLGER DAIMS/UNIVERSITY OF VIENNA

Inset: *Nitrospira* sp. bacteria, a key player in the nitrogen cycle in aquatic systems. Image captures microscopic aggregations detected by fluorescence in wet biofilm.

"For every complex problem, there is an answer that is clear, simple… and wrong."

—H.L. Mencken

A long, long time ago, when all things seemed simpler, nitrification in the marine aquarium was known as vital for success—but an easy-enough process to understand:

[1] Ammonia and nitrite in water are toxins originating as animal wastes and uneaten food, able to kill life in an aquatic system. (At high enough levels, a poisoned fish can suffer hyperexcitability, loss of equilibrium, increased respiratory rate, and convulsions, and it may lapse into a coma and die.) In the early days of marine aquarium keeping, it was called "new tank syndrome."

[2] Through a process known as the Nitrogen Cycle, ammonia (potentially deadly) is converted to nitrite (less toxic, but still a threat), nitrite is then converted to nitrate (harmless).

Done. Straightforward. Simple. It was known that this nitrification was done by bacteria: *Nitrosomonas europaea* was **the** ammonia-oxidizing bacteria (AOB) and the nitrite it produced was converted to nitrate by **the** nitrite-oxidizing bacteria *Nitrobacter winogradskyi* (NOB). It didn't matter whether the aquarium was freshwater, brackish or marine—this was the process and that's who did it. End of story.

The problem is that everything written above is wrong when it comes to nitrification in aquatic environments—and aquaria in particular. Even though much of what I am about to describe has been known for 15 to 20 years or more, most authors writing in the aquarium literature, even today, gloss over nitrification using a version or variation of the classic, obsolete Nitrogen Cycle scenario. A rewriting of the textbooks and countless digital reference works is long overdue.

Before delving into what we know today, it's important to get something clear at the start: new research is published seemingly every month in the scientific literature about nitrifying bacteria and nitrification, with results from samples obtained from aquaria and aquaculture systems, and the story of nitrification and nitrifiers is not settled. Here and in the next issue we will present a good general overview of the current knowledge, but some details are bound to change in the near and distance future; that's how science works.

Consider some of the old mythology and stock answers to basic questions regarding the nitrification process in aquaria and the organisms responsible that still abound today:

- Q: Is nitrification in aquaria done by bacteria?
  A: Yes… *but*….
- Q: Is nitrification a two-step process done by two different organisms?
  A: Yes… *but*….
- Q: Do *Nitrosomonas europaea* oxidize ammonia and *Nitrobacter winogradskyi* oxidize nitrite?
  A: Yes… *but*….

Ammonia poisoning, along with lack of dissolved oxygen, is a leading immediate factor in many tank crashes and fish deaths in which nitrifying bacteria are overwhelmed or unable to cope with the biological load.

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The "yes... but...." answers add complications to the story. Humans like simple organization and linear trends, which is why we classify things and try to rearrange complex patterns, but this is biology, which means it can be messy.

The aim of this article is to bring us all up to date with the process of nitrification, name the major players, dispel some myths, and show how understanding the process and the players better can make cycling more efficient, saving you time and money.

Let's start first with: *Why?* It is important to understand nitrification because:

A. Controlling ammonia, the principal waste product of fish and the major source of nitrogen input to aquaria, is key to balancing your tank's water quality over the long-term.
B. Microorganisms are key to having a successful aquarium, and nitrifiers can make up a high percent of the microorganisms in your tank.
C. Every tank must go through nitrification (i.e., cycle). Understanding the organisms responsible can save you time, money, and headaches during this period.
D. Fishless cycling is a very popular process to start your tank, and the difference between success and frustration is understanding the process and the bugs.
E. If you decide to use a bacterial starter or booster product, you'll be better informed on how to proceed.

THE BASICS

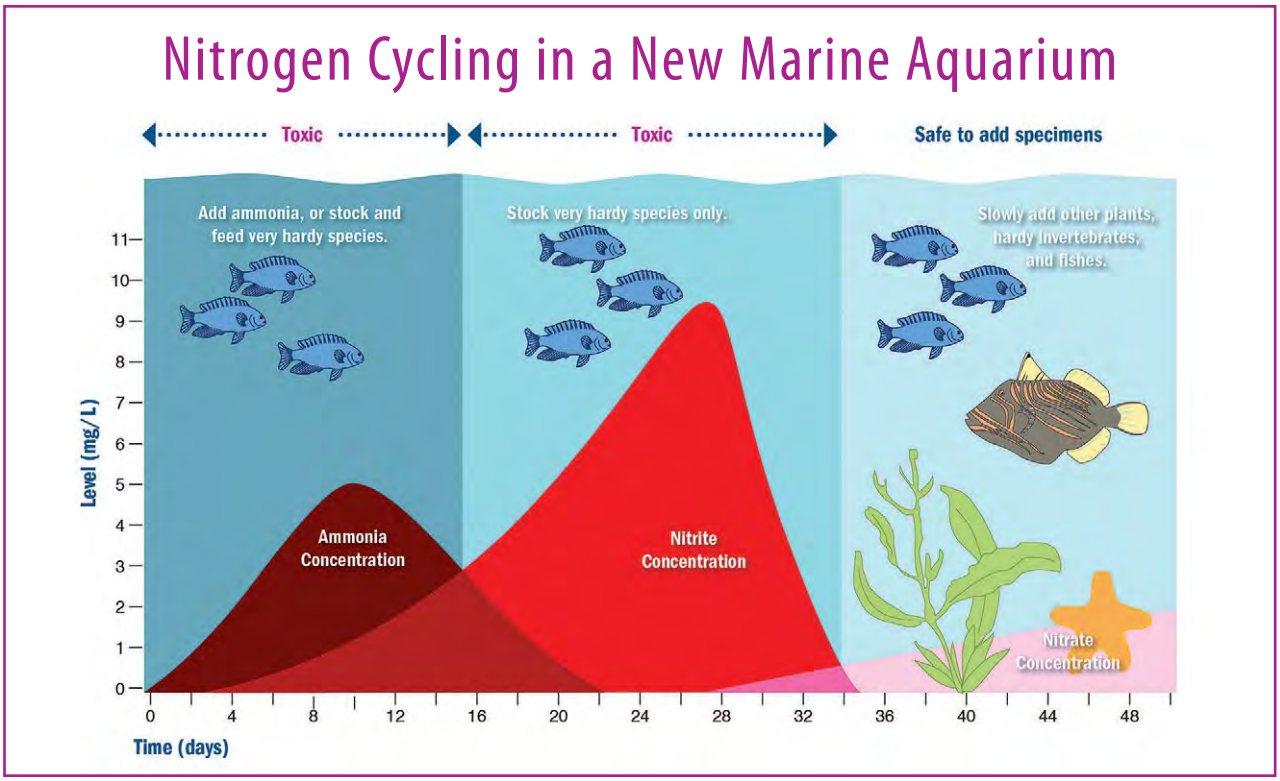
The vast majority of marine fish we keep in aquaria are classified as teleosts or bony fish—animals that excrete ammonia as their primary nitrogen waste product. This contrasts with sharks, skates, and rays (elasmobranchs), which produce urea much like humans. Ammonia (as the gaseous form [NH_3]) passes through their gills into the water via a process called passive diffusion. This is an energy-saving mechanism—the fish do not have to spend energy converting ammonia to urea or getting rid of a majority of their nitrogenous waste.

Once the ammonia (NH_3) enters the aquarium water, it forms its conjugate pair ammonium (NH_4^+). Ammonia is considered a base because it can accept hydrogen ions from the water, while ammonium is the conjugate acid to ammonia, as ammonium can release one of its hydrogen ions reforming ammonia. The accepting and releasing of hydrogen goes back and forth constantly.

The pH of the aquarium water is the main factor that determines the percentages of ammonia-ammonium present, with water temperature playing a minor secondary role. At low pH values (6.5 and lower), 99.7 percent or more of the total ammonia is in the ammonium form, while at high pH values (11.5 and higher), 98 percent or more of the total ammonia is in the form of ammonia. For convenience, many authors use "ammonia" to mean all the ammonia present, not just the NH_3 faction. Here I will use "total ammonia" when discussing the sum of the ammonia (NH_3) and ammonium (NH_4^+) factions, to be clearer. Thus:

Total Ammonia = Ammonia (NH_3) + Ammonium (NH_4^+)

Ammonia (NH_3) is a gas, which is why you can smell ammonia in cleaning solutions made with ammonium hydroxide (the pH is >12), but there is no scent of ammonia from ammonium chloride solutions (pH 4.6–6.0). Further, ammonia is un-ionized, meaning it has no charge, so it is neither a cation (positively [+] charged



The typical scenario for nitrogen cycling in a new marine aquarium, starting with a few hardy fish (or dosing with ammonia). "Cycling" turns highly toxic ammonia into nitrite, then into relativly benign nitrate as beneficial bacteria populations become established. New research is revealing that the microbial populations responsible for the detoxification of ammonia are more complex than previously believed.

ion) nor an anion (negatively [-] charged ion). Ammonium (NH_4^+) is a cation due its positive charge.

This distinction is important because ammonia, having no charge, can easily pass through cell membranes, which is what makes ammonia the toxic form of total ammonia. As previously mentioned, ammonia is the form passively excreted by fish, but the direction of flow can be reversed. If the ammonia (note the form) concentration in the aquarium water is higher than the ammonia concentration in the fish, ammonia will flow from the water into the fish and lead to ammonia poisoning.

This presents us with a short side road into practical advice: One of many controversial topics in the marine fishkeeping hobby is acclimating new fish arrivals. Think about what is happening in the bag. The fish are respiring, producing carbon dioxide that lowers the pH of the water, while at the same time excreting ammonia. So, the bag water has high total ammonia but a low pH, which means most of the total ammonia is in the non-toxic ammonium form, so the fish are relatively safe from ammonia poisoning. Now we open the bag and start dripping clean seawater into it. This causes the pH to rise, which shifts more of the total ammonia into the toxic ammonia form and starts to stress the fish. Depending on the length of acclimation time in the bag, you could be doing more harm than good to the fish. While there are always exceptions, in most cases getting the fish out of the bag water quickly and into fresh seawater (without thermal shock, of course) is better for the fish than a slow drip acclimation, at least in this author's opinion and experience.

As previously stated, ammonia from fish is the main source of nitrogen going into the aquarium. Ammonia (not ammonium) is also the form the nitrifying bacteria utilized, contrary to what most authors write. Again, this is because ammonia (being un-ionized) can flow across the cellular membrane, while ammonium can't. The oxidation of ammonia to nitrate is a two-step process:

$NH_3 + 1.5\ O_2 \rightarrow NO_2 + H_2O + H+$

$NO_2- + H_2O \rightarrow NO_3- + 2H+$

As the equations show, at each step of the nitrification process hydrogen ions (H+) are produced. The hydrogen ions are initially neutralized by the buffer capacity of your water (the common carbonate buffering system in seawater accepts the H+), but inevitably the pH of the water will drop, due to the constant input of hydrogen ions produced by nitrification. pH measures the concentration of hydrogen ions in the water, and it is a negative number, so adding more hydrogen ions means a less negative number. That is why a pH of 7.8 has more hydrogen ions than a pH of 8.2.

The average pH of the ocean is about 8.1, with minor differences due to depth or location. Marine fish, along with most organisms in the ocean, are not well adapted to changes in pH, because there was no evolutionary reason. This contrasts with freshwater fish, which can experience large pH changes over the course of the year. (This is also why acidification threatens the majority of

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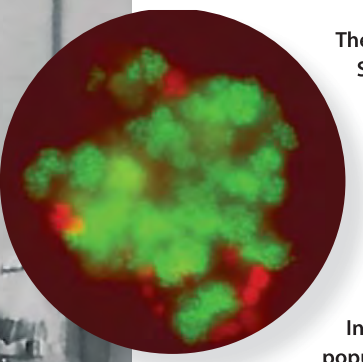

The great Russian microbiologist, Sergei Winogradsky, standing at center, with fellow researchers in his St. Petersburg lab, circa 1890. His pioneering work was a guiding influence for many decades.

Inset: Microscopic bacterial populations fluoresce in this image of Ammonia Oxidizing Bacteria (AOB-green) and Nitrite Oxidizing Bacteria (NOB-red) living in an aquatic biofilm including species of *Nitrosomonas* and *Nitrospira* bacteria.

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life in the Earth's oceans, as organisms from seaweeds to invertebrates to fish are not well adapted to the lower pH values that will result from the seas absorbing the excess carbon dioxide produced by human activities.)

Now that we have a basic understanding of the process, let's mess it up a little. It has been considered a basic "rule" that nitrification was done by two different organisms: one to convert the ammonia, another to convert the nitrite. In 2015, this was shown not to be the case; a single organism was isolated that could convert ammonia to nitrate, sometimes forming the nitrite intermediate.

Before that, it was demonstrated that ammonia-oxidation was not done solely by bacteria but that microorganisms from the Domain Archaea were important ammonia-oxidizing organisms. These newly discovered ammonia-oxidizing archaea (AOA) were, in fact, found and isolated from marine aquaria!

So, let's rewind a little and go from the process to the players responsible.

THE PLAYERS

Nitrifying bacteria have been known since the 1890s from the efforts of the legendary Russian soil microbiologist Sergio Winogradsky to develop both an understanding of the process and isolate the organisms involved. As was the norm in microbiology until the late 1980s, most of the original work on nitrifiers involved the arduous task of trying to develop pure cultures, which is time-consuming and, in many cases, resulted in failures. However, Winogradsky, as far back as the 1920s, preached working with "environmental" samples rather than pure cultures of single species. But this process still involved manipulating the culture environment to try to simulate the natural environment.

Microbiologists knew that these culture-dependent methods resulted in some level of bias, as the methods would select for organisms that could grow best with the medium or environment provided. But there weren't yet alternatives, and the results, while not perfect, were assumed to be a good representation of what organisms were most numerous or important in the environment sampled. Nitrifying bacteria are a classic example of this.

Thus, the accepted scientific explanation became that ammonia was oxidized (technically losing an electron) to nitrite by a bacterium called *Nitrosomonas europaea* (Winogradsky 1891 and 1892), and nitrite was further oxidized to nitrate by another bacterium, *Nitrobacter winogradskyi*, which was officially described in 1917 but known earlier.

This essentially became the story of nitrification. A few other nitrifying organisms were found, but they were generally ruled unimportant because they were

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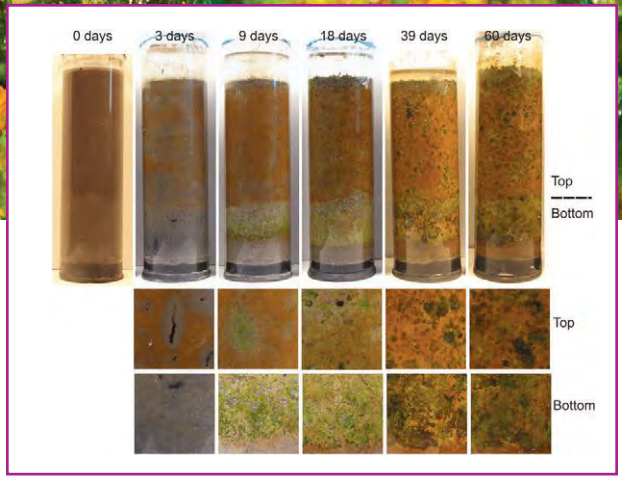

The Winogradsky Column, inset, is a classic method of culturing microbes in the lab. A transparent vessel is filled with a biological sample, such as pond mud, and the microbial communities are allowed to grow and stratify. Above: Close-up view of the complex development of micro-organisms in a Winogradsky Column exposed to light and driving photosynthesis.

from extreme or unusual environments. The reason for this is that in nearly every case the same general culture method was utilized to isolate or at least increase the number of nitrifiers in the target sample. The problem was that the lab culture method favored the growth of *Nitrosomonas europaea* and *Nitrobacter winogradskyi*, so that was the answer one got. Whether looking at sewage treatment, soil, or fish ponds, these two bacteria kept showing up as the answer.

There were clues that these culture-dependent methods were inadequate. Johnson and Sieburth in 1976 pointed to this conclusion when they were unable to find species of *Nitrobacter* in the nitrifying environment of a fish culture system's biofilter that they examined with transmission electron microscopy. Then the story took another hit when research showed that bottled, commercially available mixtures of nitrifying bacteria used to accelerate the start-up of nitrification either did not work or were of limited use.

As is common in any good tale, there were a few side plots that one needs to know about. Starting in the late 1970s and exploding in the 1990s, new analytical methods for microbiology and its sister science of microbial ecology (studying microorganisms in their environment) opened up a whole new micro-world and level of understanding.

THE THREE DOMAINS OF LIFE: ENTER ARCHAEA

At the cellular level, what makes a species a species, different from another species, is its genes. More specifically, it's the DNA and RNA of the genes, or, diving deeper

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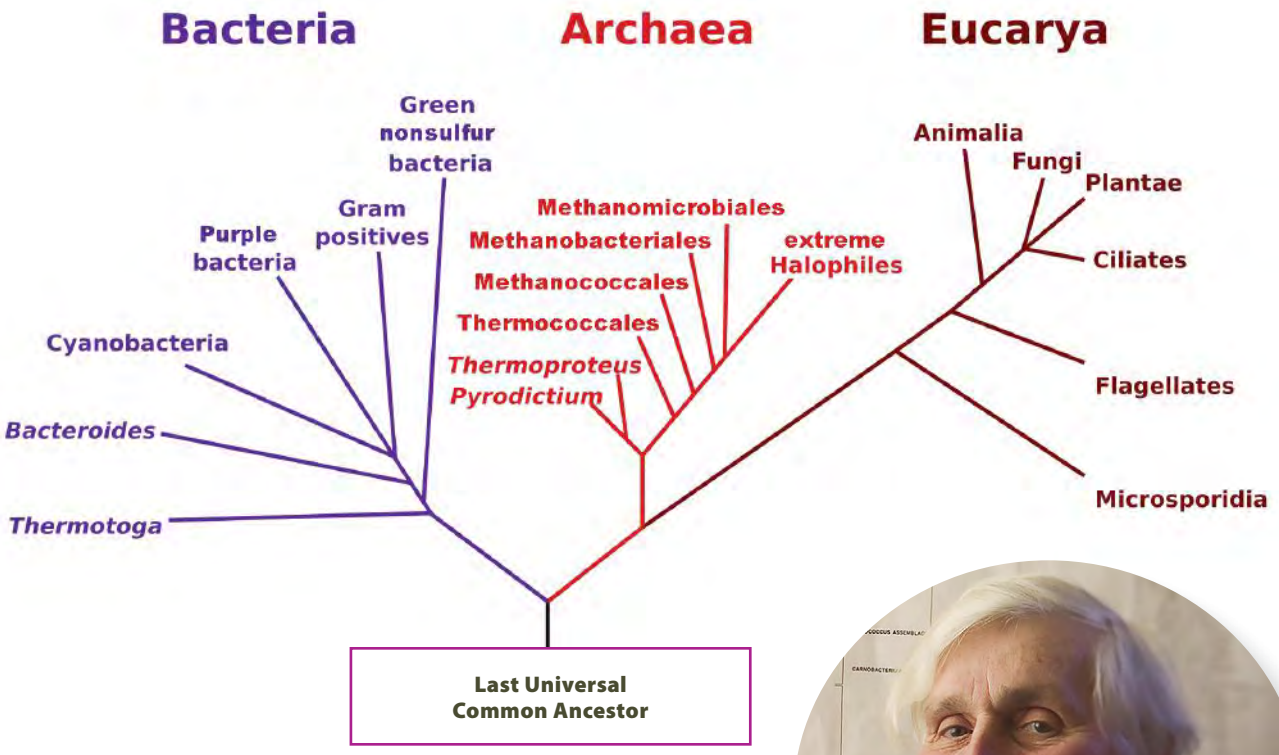
Phytogenetic Tree of Life



DON HAMERMAN, 2004/WIKI COMMONS

In 1977, Carl Woese and his research associate George Fox announced the discovery of ancient, bacteria-like microbial life they called the "archaebacteria" or Archaea, which radically changed the Tree of Life, expanding into three Domains or Kingdoms. This phylogenetic tree is based on rRNA analysis. All life arises from the last universal common ancestor (LUCA), represented by the vertical trunk at bottom.

Inset: American microbiologist Dr. Carl R. Woese (1928–2012) at University of Illinois.

into molecular biology, the sequence of the nucleotides that make up the DNA/RNA (which is as deep as we go in this dive). One ribosomal RNA gene, the *16S*, shortened to *16S rRNA*, occurs in all self-replicating organisms and holds the key to our story.

Most of us learned in an early biology class that are were five Kingdoms of Life: Animals, Plants, Fungi, Protists and *Monera*, the latter two being simple, single-celled organisms, such as algae, amoebas, and bacteria. However, this classification was not really based on any underlying scientific criteria but more on a thinking that it is easy to tell a plant from an animal—and, for those that can't easily be thrown into one or the other, we'll create a few catch-all groups. At a slightly more scientific level, all organisms can be classified as eukaryote (containing a membrane-bound nucleus inside a cell) or prokaryote (lacking a cell nucleus or any membrane-bound organelles in a cell). But, again, this classification is lacking in some scientific respects.

In 1977, a new Three Domain classification system was proposed by American microbiologist Carl Woese. It was based on the nucleic acid sequences of the 16S rRNA gene. This system was based upon the science that every extant organism has this gene, which is not too long and which can be sequenced (yielding phylogenetically-based data). Without getting too technical, there are regions on the gene where the nucleic acid sequence is exactly the same for all organisms, and other regions that are the same for all bacteria, or all eukaryotes and the same sequence for a third group—the Archaea. Thus, Woese proposed a new classification and called the Domains *Eubacteria* ("true bacteria"), *Archaea* (single-celled organisms without cell

nuclei), and *Eukarya* (all the higher taxonomic groups, all with membrane-bound organelles and cell nuclei).

Woese's concept was not immediately accepted, especially the part about Archaea being a separate domain. However, this is not the place to go further into phylogenetics (for brevity and clarity of concept, I am simplifying), but I encourage the curious reader to Google or do a library search for Carl Woese and learn more about the discovery of the Third Domain of Life, which now dominates how we think and classify organisms. Much remains to be learned about Archaea, but it is increasingly clear that they play important roles in the microbiota of different organisms and habitats, including coral reefs.

What is important to understand is that:

1) Now the classification of organisms could be done based on genetic information rather than what they look like (which is quite hard for bacteria/archaea, which do not look that much different from each other)
2) There is no need to culture the organisms—instead, *in situ* samples can be taken and processed without any further manipulation (called culture-independent methodology)
3) Tools could be developed that target specific regions of the *16S rRNA* gene at the genus and species level. One set of these tools is molecular probes.

These three traits combined to break open not only the study of nitrifying bacteria but the entire field of microbial ecology—along with our revised understanding of the role of bacteria and other microbes in the successful startup and operation of marine aquariums.

Editor's note: *In Part II, Dr. Tim Hovanec will describe his own work in isolating the nitrifying microbes active in marine aquarium systems and will discuss how the aquarist can better manage aquarium cycling and maintain healthy populations of beneficial nitrifiers.*

Timothy A. Hovanec, Ph.D., *is microbiologist and founder of Dr. Tims Aquatics in Moorpark, California. For 17 years, he was the Chief Science Officer of Aquaria Inc., the parent company of Marineland Aquarium Products, Aquarium Systems (Instant Ocean) and Perfecto Manufacturing. Dr. Hovanec has authored numerous scientific papers in aquatic microbial ecology and in public aquaria and aquaculture fields, and he is a former President of the Pet Industry Joint Advisory Council.*

REFERENCES

All references and a digitized version of this article can be found online, where comments may be posted: https://www.reef2rainforest.com/2021/07/17/nitrification-in-marine-aquaria

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Shoal of healthy Threadfin Anthias, *Nemanthias carberryi*, in a reef aquarium. These are delicate fish that come from pristine waters and require stable conditions that healthy populations of beneficial microbes help provide. Photographed at Palmetto Reef, West Columbia, South Carolina.

Inset: Archaea (*Methanosarcina* sp.) as captured in a scanning electron micrograph (SEM). These archaebacteria have unusual cell walls and are now recognized as playing a role in detoxifying ammonia in marine aquarium systems.

NITRIFICATION IN MARINE AQUARIA

A MICROBIAL MYSTERY TOUR OF BENEFICIAL BACTERIA & ARCHAEA

MICHAEL VARGAS/MVARGAS PHOTOGRAPHY

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"If an alien visited Earth, they would take some note of humans, but probably spend most of their time trying to understand the dominant form of life on our planet—microorganisms like bacteria…"

—Nathan Wolfe

By training I am an ecologist. I want to look at the entire environment and understand: (1) how energy flows through it; (2) the balance between all the organisms in the environment; and (3) how these organisms change the environment. But seeing the whole picture is hard for microbes —in a wild habitat or in an aquarium—because you can't easily see them or count them, the two standard techniques in ecology.

In the fall of 1993, I was a new Ph.D. student at the University of California, Santa Barbara, while working full-time at Marineland, where we had recently come out with the *BioWheel* filter. Of course, from my days as a tropical fish keeper and then working in aquaculture I knew that microbes were the main players in detoxifying ammonia in captive aquatic systems. However, there were many unknowns, and I wanted to answer some basic questions: where did nitrifiers live, how did their population change over time and with changes to the system, etc.?

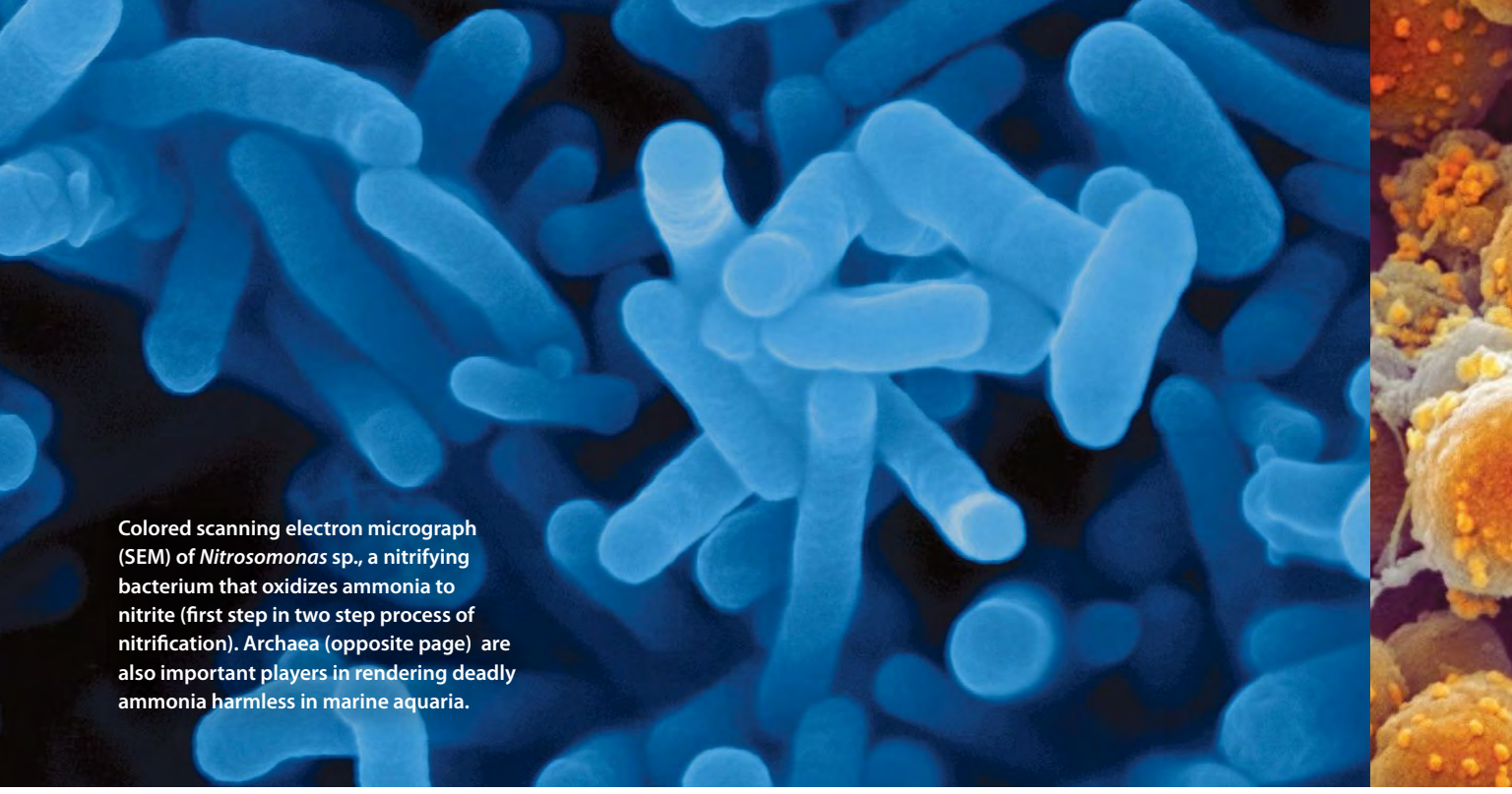
I soon found myself working in the lab of Professor Edward Delong, now considered a pioneer in marine aquatic microbiology, who had developed a novel method to identify single cells using 16S rRNA phylogenetics—aka the molecular probe. Using different molecular probes in the same sample one can distinguish different microbes at the species level, as well as establishing the presence or absence and relative quantity of the target microorganisms without the need for culturing. Thus, the samples are not manipulated in any fashion—the prior standard procedure that had produced much misinformation about aquatic bacteria.

Working in Ed's lab, I developed molecular probes for *Nitrosomonas europaea* and closely related ammonia oxidizing bacteria (AOB) and *Nitrobacter winogradskyi*. I started applying them to samples from various sections of freshwater and marine aquaria (biofilter material, substrate, and the bulk water). The results were unexpected—*Nb. winogradskyi* was not detected in any sample while *N. europaea*-type AOBs were only detected in marine aquaria. Furthermore, if I took a functioning freshwater aquarium and switched it to saltwater by only adding a synthetic salt mix, nitrification halted—as evidenced by the sudden appearance of ammonia and then nitrite (so the tank was going through another cycling period). When I re-probed the tanks, once nitrification became re-established, a positive signal for the presence of *N. europaea*-type AOBs was detected.

Here was the first solid evidence that ammonia oxidation was done by different organisms in freshwater aquaria compared to saltwater aquaria and *Nitrobacter winogradskyi* was not the nitrite-oxidizing bacteria in either situation. The obvious next steps were to determine the freshwater ammonia-oxidizing bacteria and the nitrite-oxidizing bacteria in both systems.

BEDROCK FINDINGS

After a couple of years research, using a variety of molecular methods such as clone library development and denaturing gradient gel electrophoresis (DGGE), we were the first to show conclusively that novel nitrite-oxidizing



Colored scanning electron micrograph (SEM) of *Nitrosomonas* sp., a nitrifying bacterium that oxidizes ammonia to nitrite (first step in two step process of nitrification). Archaea (opposite page) are also important players in rendering deadly ammonia harmless in marine aquaria.

bacteria (NOB) closely related to *Nitrospira moscoviensis* were the actual NOB in freshwater aquaria. Again *Nb. winogradskyi* was not detected in any aquarium, freshwater or marine, sampled.

In fact, 1998 was a watershed year in terms of NOB research in particular and nitrifying bacteria in general. After my paper, demonstrating the importance of *Nitrospira* rather than *Nitrobacter* in aquaria, a paper was published a few months later showing *Nitrospira* was the important NOB in domestic wastewater treatment systems (Burrell et. al. 1998). Following that, another report demonstrated that not only was *Nitrospira* the dominant NOB in the activated sludge of an industrial wastewater facility in which *Nitrobacter* was not detected, but that *Nitrosococcus mobilis* not *Nitrosomonas europaea* was the dominant AOB.

The lack of presence (so importance) of both *N. europaea* and *Nb. winogradskyi* in a fluidized bed reactor which was dominated by members of the *Nitrosospira* and *Nitrospira* continued the trend of showing that the long-assumed importance of *N. europaea* and *Nb. winogradskyi* as the nitrifying bacteria in aquatic systems was misplaced.

In 2001, my research group at Marineland published the first paper showing that the ammonia-oxidizing bacteria (AOB) in freshwater aquaria were AOBs closely related to *Nitrosomonas marina* rather than *Nitrosomonas europaea*. Not only did we show the presence of these bacteria in active aquarium biofilters, but we had developed enriched cultures containing these AOBs and *Nitrospira* that when added to a newly set-up aquarium established nitrification in record time compared to controls with no bacteria added. This led to the development and commercialization of the bacterial starter product called *BioSpira*® and several US and foreign patents on these novel microorganisms.

Another important conclusion reached in Burrell et. al. in 2001 was the role the ambient ammonia concentration played in selecting which group of AOBs would dominate. In fact, maximum ammonia tolerance was found to be one criterion when classifying several newly discovered AOB. Other studies showed that ammonia concentration was a major factor in determining which AOBs would dominate in various of environments. For aquariums, the critical ammonia level was 5 mg/L ammonia-nitrogen. Concentrations above this would push the system to favor AOBs that would not survive once lower ammonia concentrations prevailed. The obvious importance of this to aquariums was when cycling a new aquarium. In order to establish the correct AOBs for long-term stability, you need to make sure the ammonia concentration does not exceed this 5 mg/L NH_3-N threshold for extended periods.

A NITRIFIER FOUND: AMMONIA OXIDIZING ARCHAEA (AOA)

So that's it, right? Science had identified the bacteria involved in detoxifying ammonia in aquatic systems, but even with different names the process is still more or less the same. Two groups of bacteria in a stepwise progression: **AMMONIA > NITRITE > NITRATE.**

Well no, not so fast. This tale is not over! Veteran aquarists and aquaculturists knew from experience that marine aquaria and cold-water aquariums didn't behave like their warm freshwater counterparts. And, in some cases even tropical-temperature aquaria were known to "act weird." Cold tanks, especially cold saltwater tanks, took *a lot* longer to cycle—months and months some-

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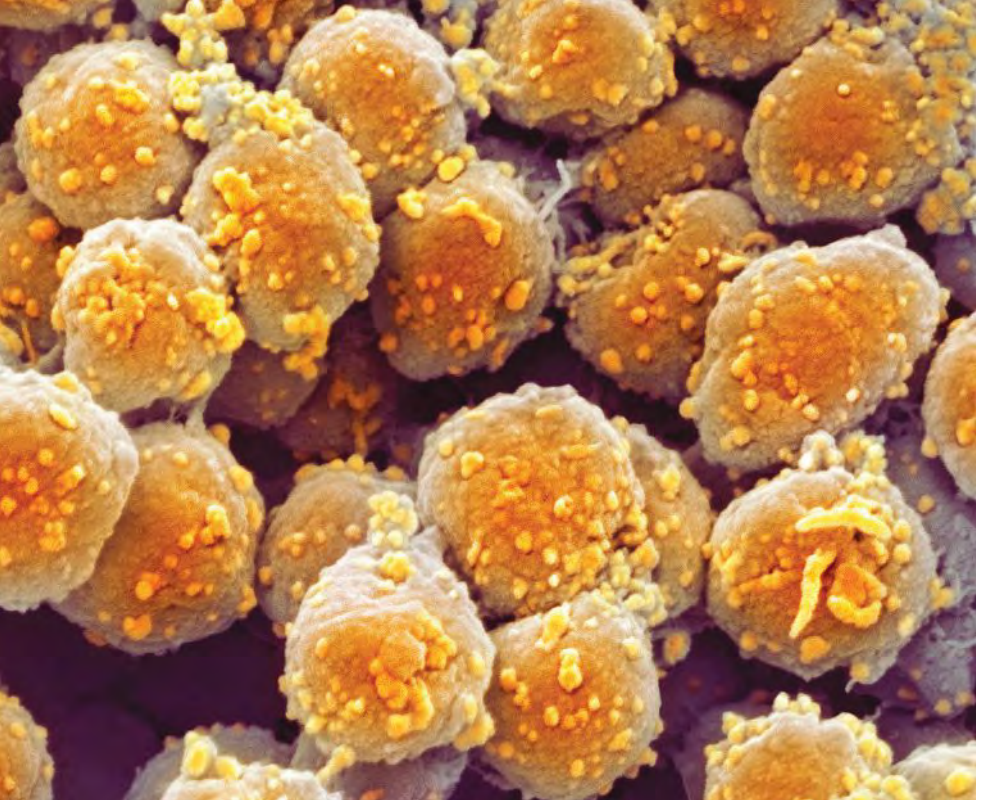

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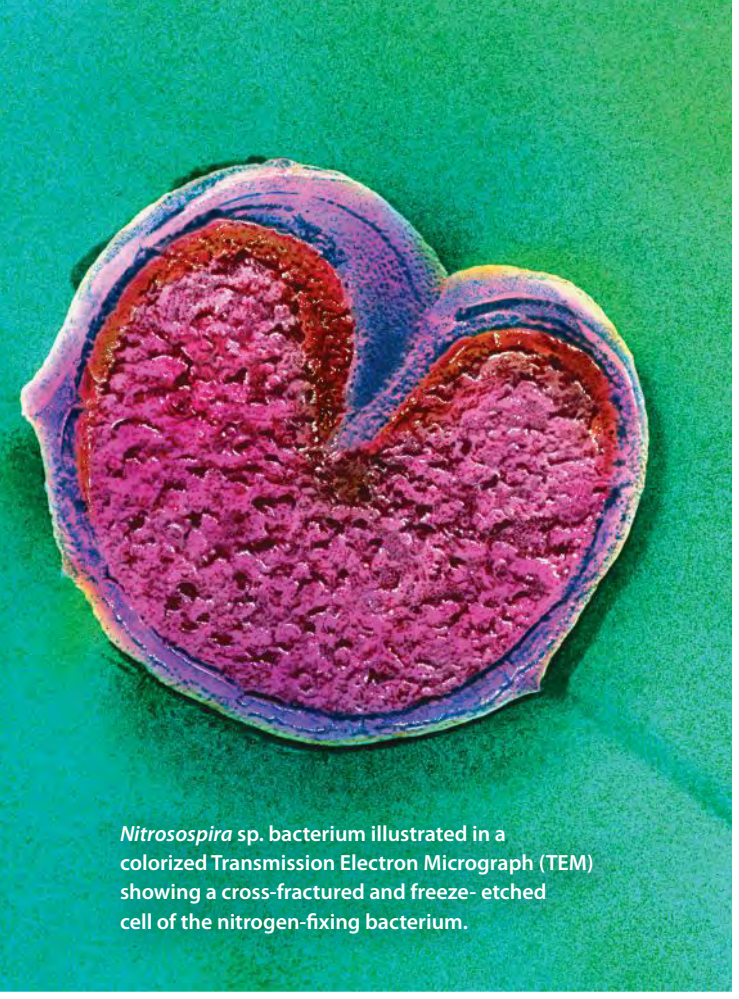
times—rather than weeks. Was this because the bacteria are just growing slower in cold water and/or the fact that more cellular energy is required to maintain the cell as opposed to cellular division as the salt concentration increases?

What was known by this time was that in cold, oxygenated ocean waters Archaea, specifically Crenarchaeota, were numerically dominant, rather than bacteria, but cultures of these organisms were not available in the early 1990s. During gene surveys of samples from two marine public aquaria (the Shedd Aquarium in Chicago and Seattle Aquarium in Seattle), sequences of group 1 Crenarchaeota were detected. A research group led by German microbiologist Martin Könneke was able to obtain pure, highly concentrated cultures of the target Archaea by isolating the Archaea via standard serial dilution methods in culture medium containing only ammonia and bicarbonate. They had found and isolated a novel organism that converted ammonia to nitrite with only inorganic carbon available—a nitrifier! This was the first known ammonia-oxidizing archaea (AOA), and they had done it from marine aquarium samples.

Two interesting findings about this AOA were that even very low concentrations of organic compounds inhibited the growth of the AOA and it only grew in low ammonia concentrations (500µM or 0.014 mg/L NH_3-N).

Questions that immediately came to mind were: 1) who were the dominate ammonia-oxidizing organisms in marine aquaria: ammonia-oxidizing bacteria (AOBs) or ammonia-oxidizing archaea (AOAs) and what conditions, in any, favored one type over another?

Part of the answer came in 2008 in a study that examined the diversity of AOA and AOB in bio-filters on three marine tanks located at a public aquarium in Japan. Two of the tanks had water temperatures from 19 to 20° C while the third was 5.5° C. These researchers found the same AOA in the two marine aquariums near 20° C as Könneke along with a good diversity of AOB. However, the diversity was comparably less in the very cold third tank (5.5° C) with AOA's dominating. While they were not able to determine all the factors that influence the diversity of AOA and AOB in the marine aquarium biofiltrations systems, they did conclude that temperature was an important factor in determining species richness. They also concluded that,



Nitrosospira sp. bacterium illustrated in a colorized Transmission Electron Micrograph (TEM) showing a cross-fractured and freeze- etched cell of the nitrogen-fixing bacterium.

DR KARI LOUNATMAA/SCIENCE PHOTO LIBRARY

at low temperatures, AOA were the primary ammonia-oxidizing organisms.

There have been multiple studies comparing AOBs and AOA from biofilters in marine aquaculture facilities and the general conclusion is that AOBs are the primary ammonia-oxidizing organisms (Brown et al 2013, Bartelme et al 2017, Huang et al 2018). This conclusion was reached in a comprehensive study of nine moving-bed biofilters in aquaculture systems that including freshwater, brackish and marine salinities. All these were considered higher organic systems due to the feeding levels and had been operating from 1.5 to 9 years at temperatures from 17 to 22° C. AOAs were not detected in any of the systems. Results also show that the freshwater nitrifying community lost 95 percent of its function at a salinity of 15 ppt. On the other hand, the marine nitrifying community maintained 85 to 88 percent of its activity at a salinity of 5 ppt. In completely freshwater, the marine AOB lost 77 percent of their activity, but the marine NOB retained 84 percent of their nitrite-oxidizing ability.

Bagchi et. al. (2014) on the basis on testing one marine aquarium multiple times over a 35-day period found that AOB *amoA* gene abundance was 3 to 5 orders higher than AOA amoA genes (measuring certain genes is an analog for species magnitude). Thus, they conclude AOB are the dominant ammonia-oxidizing organism in marine aquaria. They also found that AOA greatly preferred adhering to fine sponge filters compared to rough sponges, ceramic or sintered glass media.

PATHWAYS TO AMMONIA DETOX

But there's still more! So far, the process of nitrification has been the traditional two-steps of ammonia > nitrite > nitrate involving two phylogenetically distinct groups of organisms: ammonia-oxidizers and nitrite-oxidizers. But theoretically there are two other pathways that a single organism could use to remove ammonia from the environment.

The first pathway, proposed in 1995, is to combine ammonium and nitrite to form nitrogen gas (N_2). The process is called ANAMMOX (ANaerobic AMMonium OXidation) and found to occur in anaerobic conditions as the name implies.

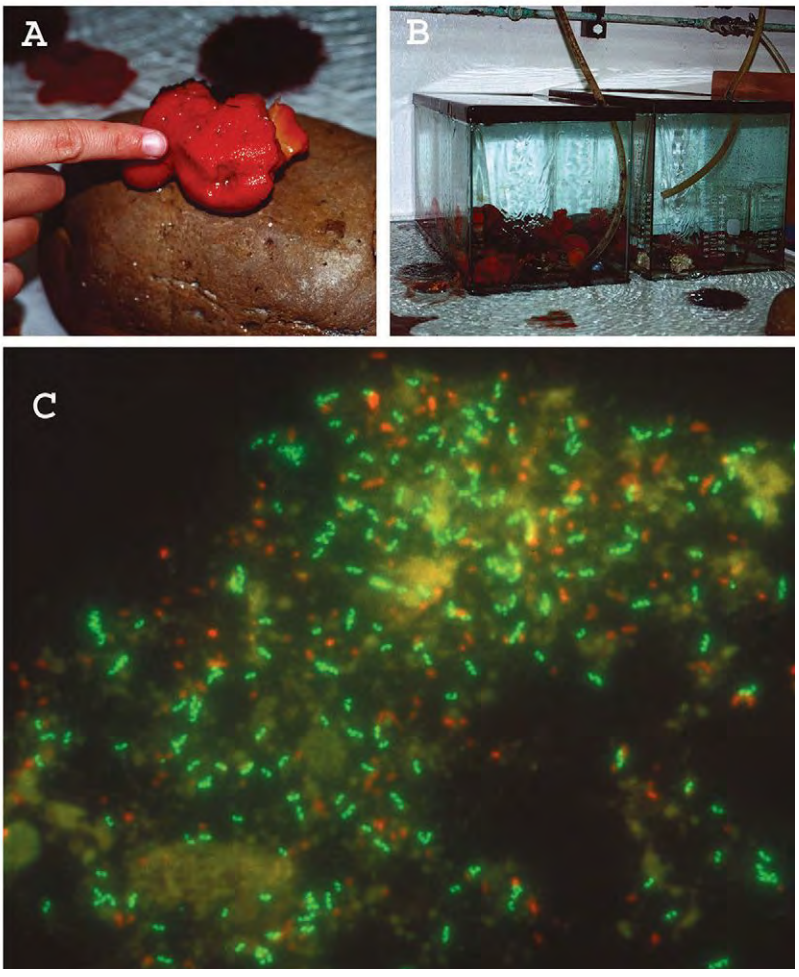
An organism, a bacterium in the order Planctomycetes, capable of this was found in 1999 in freshwater systems. It wasn't until 2008 that a marine Planctomycetes, *Scalindua erythraensis*, was isolated that could perform anammox. This organism grows very slowly and is anaerobic.

The second pathway is for a single organism to oxidize ammonia to nitrate, with no production of nitrite; basically, a single-step nitrifier.

Two research groups, publishing in the same volume on the scientific journal Nature in 2015, reported independently discovering such organisms. And the organisms are members of the *Nitrospira* clade of bacteria! Yes, you read that correctly; the one-step nitrifiers are a special group of *Nitrospir*a and they are in fish culture systems.

A team led by Dutch microbiologist Maartje van Kessel 2015 isolated two of these novel *Nitrospira* from samples taken from the anaerobic section of a recirculating trickle filter connected to a freshwater aquaculture facility. A second group isolated this special *Nitrospira* from samples taken from the wall of a freshwater well pipe rising from 1,200 m (3,937 ft) where the water temperature in the pipe was 56° C (132° F). Not all *Nitrospira* have this ability, and the ones that do are termed comammox *Nitrospira*. Comammox stands for COMplete AMMonia OXidizer.

So far comammox *Nitrospira* have not been found in marine systems and one study in wetlands showed that as salinity increased the percentage of comammox *Nitrospira* decreased to zero at seawater salinity while traditional (non-comammox) *Nitrospira* increased. Comammox *Nitrospira* have been found in significant numbers in freshwater recirculating aquaculture systems (Bartelme et al 2017). Recently, comammox *Nitrospira* were detected on biofilters in a brackish water fish culture system but it was not clear how much on a contribution they made to nitrification in this system (Hüpeden et al 2020). These organisms are included in this marine review to show that the complete picture of nitrification has not been painted.



CHRISTINA PRESTON/DELONG LAB

Experiments conducted by pioneering microbiology Prof. Edward DeLong and Dr. Christina Preston (then a graduate student, now at Monterey Bay Aquarium Research Institute), showed the presence of archaea (*Cenarchaeum symbiosum*) in red marine sponges (A and B) from coastal California. Archaea glow green in micro image (C). Their landmark paper was published in *Oceans of Archaea*, ASM News, Volume 69, Number 10, 2003.

A final, rather amazing point is that a great percentage of the work, samples and discoveries on nitrification and the responsible nitrifying organisms—research that has completely changed how scientists look at nitrification—have been from aquaria. Maybe you'll view your tank a little differently now!

SUMMATION

At this point, let's go back to the beginning of this article and answer some of the "Yes... buts" we started with.

Is nitrification in aquaria done by bacteria? Yes... *but*... Solid evidence shows that ammonia-oxidizing archaea

(AOA) play a role in ammonia-oxidation in aquaria. How big a role is not known at this time. Are they *the* major and only ammonia-oxidizing organisms in marine aquaria? The answer is no. At this point, ammonia-oxidizing bacteria seem to be more important, as they can tolerate the higher levels of organics present in most aquaria, along with the increased salinity of marine aquaria. Most likely there is an ebb and flow over time between AOA and AOBs numbers as conditions change in the closed aquatic aquarium environment.

Is nitrification a two-step process done by two different organisms? Yes… *but*… In many cases, yes — it is still a two-step process. However, evidence shows freshwater culture systems contain members of comammox *Nitrospira* that can perform nitrification in one-step. Will more research find these organisms in marine tanks? What is certain is that marine biofilters are dominated by different species of AOB and NOB compared to freshwater systems.

Do *Nitrosomonas europaea* oxidize ammonia and *Nitrobacter winogradskyi* oxidize nitrite? Yes… *but*… The importance of these two particular species for nitrification in marine aquaria has long been over-stated. Numerous studies under many conditions have shown that the dominant AOB in marine biofilters are generally *Nitrosomonas marina*-like and unidentified *Nitrosospira* spp. In terms of *Nitrobacter winogradsky*i it is absolutely clear that this bacterium plays virtually no role in nitrite-oxidation in aquaria. Authors, hobbyists and others need to start naming *Nitrospira* as the correct NOB in marine culture systems including aquaria and *Nitrosospira* spp./*Nitrosomonas marina*-like organisms as the AOB.

Now that the reader is up-to-date with the history and current knowledge of nitrifying organisms in aquaria, our attention can turn to putting this collective information to practical use in marine aquaria.

The third and final installment of this series will appear in the next issue of *CORAL*, January/February 2022.

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All references and a digitized version of this article can be found online, where comments may be posted: www.CORALmagazine.com/nitrification-in-marine-aquaria-2021

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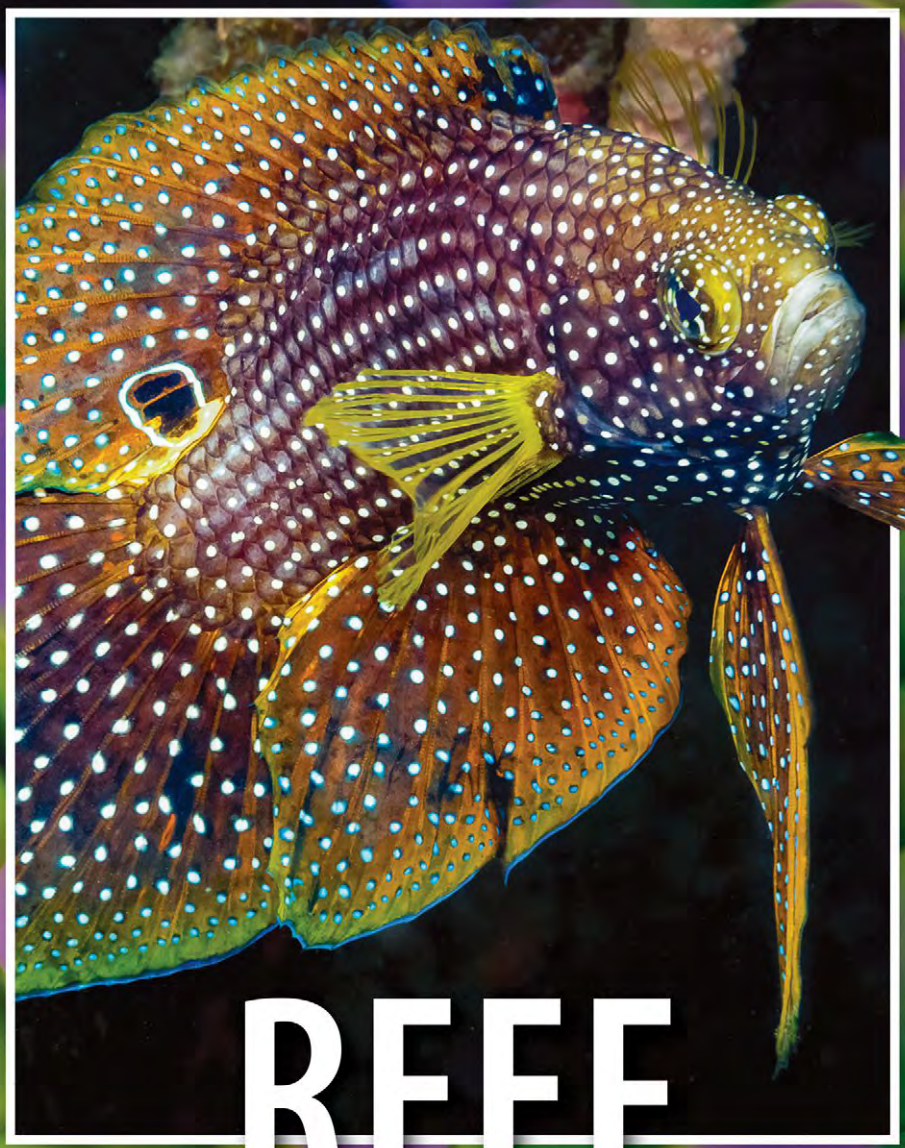


REEF GHOSTS

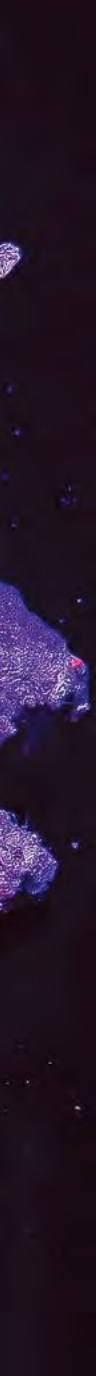
- Comets & Assessors
- Urchin Renewal
- *Euphyllia* Torch Corals

JANUARY/FEBRUARY 2022

The gold standard for "seeding" a new system: live rock that has been collected from a reef or mariculture farm, such as this Australian rock from Unique Corals (learn more at www.CORALmagazine.com/aussierock2021).

NITRIFICATION IN MARINE AQUARIA

SMARTER—AND FASTER—CYCLING IN A NEW AQUARIUM SYSTEM



"If you look at the ecological circuitry of this planet, the ways in which materials like carbon or sulfur or phosphorous or nitrogen get cycled in ways that makes them available for our biology, the organisms that do the heavy lifting are bacteria."

—*Andrew H. Knoll*

If starting a new marine aquarium is closer than ever to the dream of a "plug-and-play" experience, there remains the reality that every system must "cycle"—have nitrogen cycling established—before it is safe or smart to start adding livestock.

As we have discussed in this series, there is no shortage of advice from Internet sources, but sadly many of the facts presented are outdated or simply wrong. And, as we now know, scientific studies have shown that nitrification in aquariums is not as simple as many authors would have you believe.

Nitrification—the detoxification of lethal ammonia, breaking it down to more benign nitrogen products—is a complex process. Nitrification is carried out by microbes, and several different organisms (bacteria and archaea) are responsible, depending on the water conditions: physical (i.e., temperature), chemical (i.e., trace elements), and biological (i.e., heterotrophic competitors). While it can seem daunting, and you might want to throw your hands up and say, "Whatever!" there are ways to use information to your advantage, especially when cycling a new tank.

In simplest terms:

- Be patient—the process takes days or weeks, not hours.
- Think of nitrifiers as fish or coral—they are living organisms.
- Nitrifiers are not human—they are hardier; they can survive your mistakes!
- Less is better—don't make the process complicated.
- Test kits are needed—but don't chase "ideal" numbers.
- When all else fails during an aquarium startup, do a water change.

CYCLING A NEW AQUARIUM

Let's jump in! The control of ammonia is central to success with marine aquaria. But too often bacteria, bacterial additives, and the microbial ecology of aquarium are treated as some sort of "aquatic field of dreams"—if you build it, or if you add it, they (the desirable microorganisms) will come and stay. This is not the case.

Microorganisms such as bacteria and archaea, especially nitrifying microorganisms (nitrifiers, for short), need to be thought of and treated more like fish. One wouldn't add a freshwater fish to a saltwater aquarium, nor would one add a small damselfish to a tank swarming with large groupers, as surely it would be quickly eaten. The point is, not all microorganisms can survive in all conditions. If the correct nutrients are missing, or the salinity is wrong, or space isn't available, or a variety other conditions exist that we don't yet know about, then the microorganisms will not survive.

STARTER MICROBES

When setting up a new marine aquarium, the system will need to be inoculated ("seeded") with nitrifying microbes to kick start the establishment of nitrogen cycling. The established gold standard is cured live rock, either from the ocean or another healthy saltwater tank. Hobbyists also commonly use rubble or coral sand from a friend's tank, which can be fine if you know for sure the tank is disease-free. Getting rubble or sand from a dealer is trickier because new fish are constantly being added to the dealer's system increasing the chances of introducing disease to your tank. (Rock or rubble from an "inverts-only" system, if your dealer has one, is much less risky than material from tanks that have a rotating population of fishes.)

"Live" filter media such as sponges, media chips, or bio-balls can also be a good source—with the same caveats about introducing disease as above. A poor source is water from another tank as nitrifiers are on surfaces not in the water.

Another "traditional" but lesser-known source of nitrifying bacteria for is actually your tap water. Nitrifiers grow on the inside of the water distribution pipes leading to your home. For years, municipal water companies have added chloramine to treat drinking water, and the chloramine breaks down as it goes through the system and forms ammonia. This ammonia promotes the growth of nitrifying bacteria on the pipe walls. The nitrifying biofilm will slough-off and cells of nitrifiers reach your tank when you filled it. However, the use of RO/DI systems will greatly reduce the chance of "natural" seeding of nitrifiers, another reason cycling a marine tank can take so long.

Of course, there are bottled bacteria "starter" products that have been around since the 1970s or so. There are few rigorous studies on these products, but an early one showed the lack of efficacy of some of these

products, and most experts agree that some work better than others. We will discuss packaged commercial "bottle" bacterial mixtures below, but the advice given here applies to newly set-up aquaria, whether or not you are not going to add a bacterial starter mixture. A marine aquarist's first real encounter with essential microorganisms is when cycling his or her tank for the system to handle the ammonia excreted by the fish. There are certain things one can do from the start to make this process faster with less stress.

FASTER CYCLING

The fact is, nitrifiers grow very slowly compared to many other aquatic microbes, including the non-nitrifiers, which are generally lumped under the term "heterotrophic bacteria." The oxidation of ammonia (or nitrite) provides the energy nitrifiers' cells needs to live. The issue is the amount of energy produced by these reactions is very small. Most of the energy produced goes towards maintaining the cells, with the balance towards replication. So, anything you can do to reduce the energy required to maintain the nitrifiers' cells means more energy for replication.

Depending on whether you are cycling a new tank with durable fish (e.g., a hardy damselfish or two) in the tank or doing a fishless cycling by adding an inorganic ammonia source and maybe a bacterial starter product, these tips will help you cycle faster with less stress.

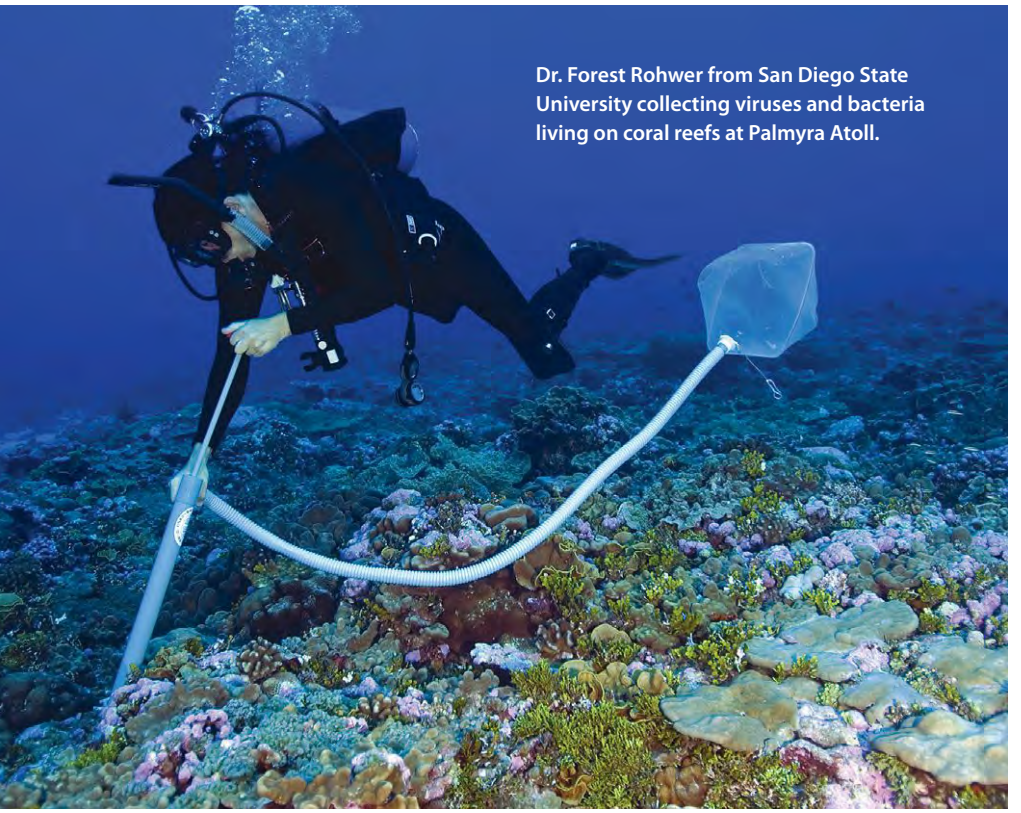
1) Lower the salinity. Nitrifying bacteria activity decreases with increased salt concentration, and one study showed that the maximum activity of marine nitrifiers was more than 40 percent less in seawater versus freshwater (Hüpeden et al, 2020). So, assuming you are cycling with ammonium (fishless cycling), consider an initial salinity around 20 ppt (1.015 sg). This will increase the activity of the nitrifiers, decreasing the cycling time. Once the tank is cycled, you can slowly increase the salinity back to the normal range without loss of nitrifiers. Nitrite-oxidizing organisms are especially slow-growing, as the salinity increases due to inherent physiological challenges of the marine environment and the requirements to maintain cell turgor pressure (Oren, 1999).

2) Increase the water temperature. Nitrifiers prefer culture temperatures higher than a normal tropical marine aquarium. Try for a range of 83–86° F (28.3–30° C) during the cycling period. You can lower it once the cycle has finished.

3) Provide the proper surface area and good water flow, and keep it clean. All the nitrifying organisms discussed in this article prefer to adhere to a surface, as compared to being free-swimming in the aquarium water. Bare-bottom tanks are going to take a lot longer to cycle because the nitrifiers are not going to work at peak efficiency unless they are on a surface. Coral sand, gravel, and rubble provide a massive increase in surface area compared to bare glass. Furthermore, all the surface area in the world is worthless unless it is in contact with constantly flowing water, which brings nutrients and oxygen. If you are going to use a media with a high ratio of internal surface volume to external surface area, you need to make sure the water is forced to flow through it and that it does not clog with organics.

Also, be careful using live sand products. These products seem to contain mostly heterotrophic bacteria and a fair amount of organic material, rather than nitrifiers. What can happen is that a day or two after the material is placed in the tank and exposed to oxygen, the heterotrophic bacteria start breaking down the organics, producing ammonia and maybe even a bacterial bloom, which would cause cloudy water. This can lead to excess ammonia levels, especially if you are adding ammonia during the course of a fishless cycling program. Also, the high levels of heterotrophs can compete against the nitrifiers (see point 7).

4) Watch the ammonia and nitrite concentrations. As previously mentioned, the long-term ambient ammonia and nitrite concentrations play a major role in determining which species of nitrifiers colonize your system. Take my advice, as



Dr. Forest Rohwer from San Diego State University collecting viruses and bacteria living on coral reefs at Palmyra Atoll.

someone with 20-plus years of growing nitrifiers specifically for use in aquaria and aquaculture facilities (what I call "ultra-low ammonia environments"): your tank will cycle much faster if you keep both the ammonia and nitrite below 5 mg/L-nitrogen. Even if you must let the ammonia remain at zero for a few days while the nitrite drops, resist adding more ammonia, which will cause the nitrite to get too high. The nitrifiers are not going to starve without ammonia for several days.

5) Don't use chemicals to control ammonia. Resist the urge to dose ammonia-removing chemicals. It will just prolong the cycle. Yes, you need to get rid of chlorine and chloramines (assuming you are using tap water instead of RO/DI water). Use a simple dechlorinating agent. Any ammonia left will be taken care of by the nitrifiers. Of course, if it is an emergency and there is livestock in the tank and nowhere else to place them, use ammonia-removing chemicals.

6) The cycle is not working because "there is no nitrate." When starting a new tank, even if you are fishless cycling and adding ammonium chloride, don't expect your nitrate test kit to be much help in determining if the cycle is working. Instead, rely on a quality ammonia test kit and maybe a nitrite test kit. The issue with nitrate is that most nitrate test kits don't measure nitrate levels below 20 mg/L NO_3-N—very well, so you get a false negative (the reading says "zero nitrate," but you actually have some). The other issue is that it is easy to get a false positive (the reading says you have nitrate, but you really don't). The reason is that all nitrate tests measure both nitrate and nitrite. One of the first steps in the test procedure is to reduce nitrate to nitrite. This is the step where you are supposed to shake the vial for a few minutes. Do it—shake hard for several minutes to get a good reading. At the same time, you need to measure nitrite and subtract the nitrite reading from the nitrate reading. During cycling, if there is a lot of nitrite in the water and you do not measure and subtract it from your nitrate reading, you will be getting an incorrect value. Plus, nitrite values over 5 mg/L nitrite-nitrogen interfere with most nitrate tests, leading to incorrect values. It is best just to leave the nitrate test on your shelf and save it for later.

7) Provide a healthy environment and reduce competitors to the nitrifiers. Nitrifiers need phosphate and micro-nutrients and, as previously mentioned,

ZAFERKIZIKAYA/SHUTTERSTOCK

Beware the modern-day vendors of snake oil. Commercial bacterial starter products have a long, mixed history in the marine aquarium world, and are best purchased only from reputable companies that culture their own nitrifying bacteria. A recent (unpublished) test of bacterial products in the marine aquarium trade found several that contained virtually all heterotrophic species, which are cheaper and easier to produce, and virtually zero nitrifying bacteria. Other tests have shown that high-quality bottled nitrifying bacteria can accelerate the detoxification of nitrogenous compounds.

they are very slow-growing. Plus, they need surface area. They compete against heterotrophic bacteria (for our purposes, basically all the other bacteria in the system) for all these resources. Most heterotrophic bacteria can replicate in 20 to 30 *minutes*, versus 20 to 36 *hours* or more for nitrifiers. Heterotrophs need organics to grow, so during cycling do not add products intended to promote bacteria growth or get rid of nitrate and phosphate, as they contain sugars and other organic substances that can feed the wrong bacteria. Also, do not use phosphate-removing chemicals and media, such as GFO, during the cycling period.

BACTERIAL STARTER PRODUCTS

Typically, it takes 35 to 42 days for a complete cycle, which is defined as the complete lack of ammonia and nitrite in the aquarium under normal feeding and maintenance routine. Many times, novice aquarists are not familiar with cycling and might add too many fish during this period, which can result in fish deaths. It has been something of a holy grail to have a product that one could add to a newly set-up aquarium to instantly establish a functioning nitrification cycle.

An interesting result of one early study of nitrifying bacteria inoculation of new tanks was that introducing media or water from an established marine aquarium did help, but there was a difference between the sources. The best case was wet media, which was significantly better than using water from the tank. This demonstrated that there are insignificant numbers of nitrifying organisms in the water column itself. If you are going to cycle by getting some media from the tank of a friend or your local aquarium shop, get some substrate from right below the top layer. Rinse it lightly with tank water and keep it damp, but it does not have to be kept underwater while moving to your tank.

A second early study of bottled bacterial starter products showed decreased time for ammonia oxidation but no ability to decrease the time nitrite disappeared (Bower and Turner 1984). Over time the mythology became that bacteria couldn't live in a bottle and these products didn't work, because (a) the bacteria couldn't breathe in the bottle, (b) they were starving, because there was no food (ammonia or nitrite), and maybe (c) a special liquid medium was needed that hadn't yet been developed to preserve them in the bottle—plus a few other reasons.

None of the reasons for additive failure made real sense then, and they still don't. Nitrifiers don't have lungs: they don't breathe. They only need oxygen when oxidizing ammonia (or nitrite). Microorganisms are evolutionarily designed to survive extremely long periods without nutrients. The cell does not die like an animal cell. As long as the cell wall is intact, the cell itself is not poisoned, and the DNA is not damaged by radiation or something else, they are viable but nonculturable in routine testing (known as "VBNC") but can be revived with the right conditions. One thing that can kill nitrifiers is freezing. They do not form spores like heterotrophic bacteria and in the absence of some type of cryopreservative will not survive freezing solid. So far there has been no success at making a freeze-dried nitrifier product.

Of course, the real problem was some of these bacterial products that were tested didn't contain the right nitrifiers, but that wasn't known or considered. After all, the product labels said they had the right bacteria and they were growing nitrifiers. I should also point out that some of these manufacturers were providing real nitrifiers in their products and, based on their culture technique, they believed in the product. The issue was, as we now know, that adding just any nitrifiers doesn't

DINA BELENKO/SHUTTERSTOCK

mean they will colonize, replicate, and work in your aquarium. The product needs to contain the right ones.

So, even today, the old notion persists with some people who believe that bottled bacteria don't work and can't work, usually because they refuse to accept the science and/or they have a poor experience. (Obvious disclosure: I speak as a microbiologist whose company produces such products.) Part of the issue can be the handling of the bacteria, but another part of the problem is the industry itself.

Many companies market aquarium bacteria starter products, but a recent microbiome survey conducted by an independent testing laboratory on eight well-known bacterial starters and two live sand products showed that only three of the ten products contained nitrifiers (pers. comm).

So the consumer is right to be cautious and should consider only buying from reputable companies that grow their own nitrifying cultures and who are honest about their work and research. Even products that contain true nitrifiers for aquariums can fail, but that doesn't mean they are snake oil. As we know, nitrifiers are living organisms that can be negatively affected by many factors—some out of the control of the hobbyist, but others well within the aquarist's ability to manage these microbes successfully.

CONCLUSION

When all is said and done, the fact remains that nitrification and nitrifying organisms in marine aquaria demonstrate that real, important science can be done close to home and that citizen science can yield exciting, novel results and practical cost- and time-saving advice, and even new products. Light is being shed on the black hole of microbial ecology in closed aquatic systems and the cost of microgenomics to identify microbes properly is quickly dropping, which will lead to better studies that can help fill in the gaps, leading to an even better understanding of the process and ecology of nitrifiers in aquaria. The final chapter has yet to be written—stay tuned for the sequel! Good fishkeeping.

Timothy A. Hovanec, Ph.D., *is microbiologist and founder of DrTim's Aquatics in Moorpark, California. Dr. Hovanec has authored numerous scientific papers in aquatic microbial ecology and in public aquaria and aquaculture fields, and he is a former President of the Pet Industry Joint Advisory Council (PIJAC).*

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