1995 Clarke Lecture

Clean Water Hardly Anywhere and *That* Not Safe to Drink

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The lament of the Ancient Mariner, *"Water, water, everywhere, nor any drop to drink,"*[1] always comes to mind when I go hiking, forget my canteen, and remember what I learned about *Giardia* in medical school. Our modern civilization, with its exponentially growing needs for clean fresh water, prompts the revision I used in the title. Compounding the increasing demand for clean fresh water is the lack of appreciation and investment in conservation and water science and technology. The National Water Research Institute (NWRI), in its short history, has become the major catalyst in changing the conception and perception of water science and technology throughout the world. The opportunity to become a member of the NWRI Research Advisory Board has been a singular honor and responsibility for me. To be chosen as the recipient of the Athalie Richardson Irvine Clarke Prize for 1995 stands as the culmination of my career in science! From this bully pulpit, I want to thank the Clarke family, both for their vision of clean water and in nurturing the truly remarkable administration of NWRI. What began for me as environmental activism in the 1960s, using confrontation on local television to get the City of Lexington, Kentucky, to repair a sewer leaking into a stream, has led to vastly more productive efforts via the application of sophisticated interdisciplinary science for the conservation and renewal of this most precious resource through the resources provided by NWRI.

The Problem

The problems with freshwater are the limitations in its availability and the degradation of its quality as it is recycled. The earth is the blue water planet when observed from space, but 97 percent of the water is salt water — too concentrated for our kidneys or the roots of most terrestrial plants. Of the 3 percent that is fresh water, only a tenth is available for human ecosystem utilization. The bulk of the freshwater is locked in polar icecaps and groundwater aquifers, technologically or economically beyond our reach. But, water is a renewable resource and almost 45,000 cubic kilometers per year (km^3/year) are returned fresh and pure to the land whence it winds to the oceans as river water and groundwater. This freshwater could provide each of us with more than 8,000 cubic meters per year (m^3/year) if it were evenly distributed[2] and is the crucial resource for all of the terrestrial biosphere. Unfortunately, it is not evenly distributed.

Twenty percent comes to the ocean from the Amazon. All of Europe accounts for 7 percent and Australia for 1 percent of the global runoff. Thirty percent of the runoff in Africa comes from the single Congo/Zaire basin. Many regions get nearly all their rainfall in a short rainy season with floods that are followed by a drought.[2] Californians know the flood/drought cycle all too well.

Freshwater quality is also a major problem. Water is the most universal solvent we know and dissolves much of what it contacts. The result is a solution that may not be compatible for the uses we

Dr. White revolutionized the practice of environmental microbiology. His groundbreaking approaches and methods for studying microorganisms in their natural environments opened up the possibility of applying cutting edge analytical technology to microbial ecology. He has applied his expertise to many areas, including deep subsurface microbiology, sewage microbiota, marine sediment communities in polar and temperate regions, and waterborne pathogens.

One of Dr. White's greatest assets is his willingness to collaborate with other applied and environmental microbiologists, often spending more time helping others with their research than on his own projects. He is now applying analytical focus on on-invasive monitoring of diseases with breath condensate analysis. Currently, he is a Distinguished Professor in the Institute of Applied Microbiology at the University of Tennessee, Knoxville.

require. Unfortunately heavy metals, nitrates, pesticides, and organic materials from human processes contaminate the water and aquatic biosphere. Weddell seals in the Ross Sea, Antarctica, now have body burdens of polychlorinated biphenyls (PCBs), and the Cree Indians of the Canadian High Arctic have significantly higher mercury contamination than most of us.

Freshwater safety is another problem. Water is the universal medium of life. Its physical chemistry sets the extremes within which life on earth is compatible. The conditions allowing the existence of liquid water sets the boundaries of life: pH between 1 and 13, temperature from –20°C to ~110°C (at atmospheric pressure, probably higher in the deep sea), ionic strengths between saturated sodium chloride (NaCl) and distilled water. Consequently, it is not surprising that water would become the matrix for all the wonderful interactions of the biosphere. When our ancestors slithered onto the mudflats, they brought the relics of the primordial ocean within their bodies or cells along with them and evolved an elaborate system to maintain the homeostasis of the internal sea. Thus, it is no wonder that parasitism should capitalize on the aqueous milieu. One of the greatest contributions of Western Civilization to humanity was the separation of sewage from drinking water. Our human ancestors formed the city-states of Jerico, Ur, Memphis, Babylon, Athens, Carthage, and Alexandria by relying on carefully managed systems for drinking water. By 500 B.C., the Romans had built extensive aqueducts and an enclosed sewage system that functioned better than many systems available today. Despite this continual demonstration of the importance of keeping the freshwater supply clean and safe, many of the hospitalized humans in the world are there because of contaminated water. Cholera is a price of defective sanitary engineering. It has been clear since 1849 when Dr. John Snow, Queen Victoria's obstetrician, against strong opposition, removed the handle from the Broad Street pump so that sewage should not contact drinking water, and thereby stopped a cholera epidemic in London. Clearly, our American society does not consider water a most critical resource in much of its planning activities, until the tap runs dry or *Cryptosporosis* appears.

Why Do We Take Clean Freshwater for Granted?

In his elegant 1994 Clarke Prize lecture, Dr. Bruce Rittman showed how water is a greatly undervalued product. Many of us take it for granted — it is somehow always there. All we do is turn on the tap. We gladly pay $1.00 for 12 ounces of Coca-Cola, but will not support tertiary treatment of sewage. We import bottled water from France rather than clean up our rivers and lakes. Unfortunately, this cavalier attitude must change rapidly. We just cannot continue to deliver diluted, partially treated sewage to the drinking water mains. We only require that our water meet drinking water standards for the indicator bacteria *E. coli* and not that it contain relatively high concentrations of the most egregious contaminants. In many cities, what we drink is actually a dilute hypochlorous acid solution. The halohydrocarbon products of chlorine treatment are carcinogens. These halohydrocarbons have become readily detectable as they give especially bright mass spectra after separation by a capillary gas chromatograph. We now know when they are present in our water. The sensible application of elementary biology should indicate to us that disinfectants that can disrupt membrane-bound electron transport systems of bacteria could also affect our own cells. Recycling drinking water by inhibiting the human pathogenic microorganisms with cellular poisons should be only an emergency treatment. We must insist on, and be willing to pay for, better means to protect drinking water.

Along with the incredible views of our beautiful planet Earth that NASA has given us comes the profound realization of how much water we waste. The high cost of lift-off energy for space flight, which must include the water necessary for life, has forced recycling, purification, and reuse on scales not considered possible in the past. There are still major problems in water and waste disposal in the space station. Total recycling of water in space has not yet been achieved, and resupply from earth is still required. There will be no "manned" interplanetary travel until a lot more water science and technology research has been supported.

In 1994, Dr. Bruce Rittman also detailed the problems in water science and technology as both a triumph of technological skill in producing a universally available low-cost product and the resulting long-term problem of insufficient intellectual and financial capital for new and more subtle dilemmas. The problems in water supply are obvious to all, but the problems in water quality, which are much less obvious, may be more significant and the problems more insidious. My point is that if we can marshall the resources and realize a deeper appreciation of the diversity in the biosphere, we will find an underexploited microbial world that can be of enormous benefit in improving our stewardship of this planet's freshwater resources.

The Unexpected, Underutilized Resource

The largest and most diverse biomass on earth is unseen and certainly underappre-

ciated. The unseen microbes have exclusively comprised the biosphere for almost all but the last 6.0×10^8 of 3.8×10^9 years that life existed on the earth and are responsible for the geochemical cycles that allow life to continue. The microbes are important in water science and technology, as they render drinking water infectious, facilitate through-wall corrosion in distribution systems, foul and seriously impair membrane-based water reclamation, greatly influence the water cycle through their activity on plants and soils, can clean up or intensify groundwater pollution, and can be used to quantitatively assess toxicity.

Microbes tend to be ignored because they are difficult to study. The classical methods of isolation and culture of microbes that are taught in most microbiology courses and required by law at water distribution facilities do not work when applied to the environment, as less than 1 percent of what can be detected in stained microscopic preparations can be cultured. Staining microbes in environmental samples can be difficult as many are attached to soil granules or are in biofilms and may be hidden. Agents that release attached microbes are often selective and do not release them quantitatively. The morphology of the microbes does not often reflect the function or activity, so very little insight into the community structure or nutritional status is possible. Measurements of metabolic processes are complicated by the facts that most microbes in the environment exist in an inactive state, but are poised for activity when nutrients appear. Growth rates may vary from minutes to centuries.[3]

There exists a comprehensive, quantitative technology by which the microbial community can be defined. Detection of cellular components that are universally distributed can provide estimates of biomass. Detection of cellular components that are restricted to subsets of the community can be used to describe the community structure and, if the proportions of specific structures change with changes in the nutritional or physiological status of the community, then analysis of these cellular components can define the microbial community and give insights into its ecology. Our laboratory has focused on the analysis of the cellular membrane lipids. Analysis of the cellular lipids provides a satisfactory way to gain insight into the critical attributes of microbial communities. Lipids are cellular components recoverable by extraction in organic solvents, and the extraction provides both a purification and concentration. Lipids are essential components of the membrane of all cells and play a role as storage materials. The signature lipid biomarker analysis provides quantitative insight into important attributes of microbial communities.

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- *Viable Biomass:* Viable microbes have an intact membrane that contains polar phospholipids. Cellular enzymes hydrolyze (release) the phosphate group within minutes to hours of cell death[4] and yield the non-polar diglyceride lipids.
- *Community Structure:* Specific groups of microbes often contain unusual lipids[5] and these can be utilized to define functional groups of microbes.
- *Nutritional/Physiological Status:* Just like us, microbes can get fat when they accumulate carbon, but cannot reproduce. The difference is that their fat is a polyester. Detection of the polyester is a good measure of the nutritional status. There are other lipid biomarkers that have been shown to provide detailed insights into precise conditions of pH, bioavailablity of phosphate, redoxactivity and availability of terminal electron acceptors, adequacy of micronutrients, presence of toxicants, and inhibitors in the microenvironments that specific microbes occupy.[6] Recent developments have shown that DNA from microbiota can be recovered in the same process as the lipids. DNA recovered with the lipid extraction is clean and readily amplified enzymatically for specific gene probing. DNA probing allows detection of functional groups of microbes and the potential for enzymatic activities that do not happen to have significant signature lipid biomarkers.

With this signature biomarker technology in hand, it has become possible to gain a much deeper appreciation of the ecology of microbial communities and to examine the consequences of manipulations of these communities in their environments and on the water we need to conserve.

Bioremediation

Groundwater contamination is an ever-growing problem as soil is impacted by the disturbances of civilization. Signature lipid biomarker analysis of the subsurface microbiota show a rich but starving microbial community able to subsist on

refractory polymerized substrates that have been worked over by surface microbes. This community is deceptively simple yet contains the propagules of amazing diversity that stand ready to utilize the nutrient flush induced by a rainstorm, the disturbance of a mining earthworm, or some other disruption in the soil ecology. When a metabolizable contaminant mixture is introduced into the subsurface, there is a rapid increase in the viable biomass, a profound shift in the community structure to the gram-negative heterotrophic "weeds" capable of rapid growth, and all the metabolic attributes of growth. This lasts until some essential nutrient, or a terminal electron acceptor like oxygen, runs out. The altered microbial community then stops and begins a long waiting period during which the slow growing components of the subsurface community gradually again become the dominant microbiota.

It is clear that the subsurface microbiota can be manipulated to promote *in situ* bioreclamation. This is much more rationally done if bioremediation engineers can ask subsurface microbes, which are their "customers," about the environment in the subsurface and monitor the conditions that best promote the bioprocessing. Signature lipid biomarker analysis of recovered subsurface sediments or groundwater filter-retentates provide a means to monitor and manipulate the remediation process. Degrading petroleum hydrocarbons, which can serve as substrates for microbial growth, is most effective when the microbes are not accumulating the polyester fat-like storage polymer and the analysis shows the signatures of growth of the gram-negative "weeds." Alternatively, there is even evidence that could be considered altruism in the subsurface. Some microbes degrade halohydrocarbons like trichloroethylene even though they cannot grow or derive much energy from the process. This so-called fortuitous metabolism, when done by the methane-oxidizing bacteria, goes best when the organisms — semi-starved for methane — are supplied oxygen and fertilizer and have accumulated large reserves of the fat-like polyester polymer.

Drinking Water Protection

Clean freshwater is not an ideal milieu for microbial life. In order to live, drinking water microbes form biofilms on surfaces and concentrate the rare nutrients that pass by whilst they remain fixed to the surface. Much of their nutrients can be supplied from gases in the atmosphere and, if light is available, the autotrophic process of photosynthesis will greatly increase the complex carbon and nutrient load of the water. As freshwater gets utilized and recycled, its amazing stating prowess gradually increases the dissolved and particulate organic content. This increases the opportunities for microbes to survive and prosper.

In the United States, water is not routinely monitored for total viable microbiota, but only for the "indicators" of contamination. The tradition that is still firmly in place in American water science and technology is that tests for microbes are done from the water (not the biofilms) and are only valid when these pelagic microbes are able to form discernible colonies when incubated on a appropriate growth medium. These are conditions when most of the microbes actually present will not be detected. Even when sampling the pelagic microbes, there are many important organisms that are totally not culturable such as *Giardia*, *Cryptosporidium*, and many viruses; there are many that are culturable only with difficulty such as *Legionella* and more viruses; and there are others that are readily rendered nonculturable by environmental conditions such as *Vibrio cholera*. Most of these are, however, infectious should they be in the water you drink. This infectious but nonculturable problem is compounded when the water is treated with biocides, which can obfuscate morphological features used to identify cysts of protozoa and damage bacteria so that they cannot recover on the "appropriate" growth media. Direct methods of detection, such as nucleic acid probes and polymerase chain reaction, provide powerful new tools in protecting the water drinking community. Signature lipid biomarker methods are particularly powerful as they provide DNA for gene probing, are quantitative, and provide insight into the phenotypic expression that reflects the specific microenvironments of the microbial communities. It is often important to have this phenotypic insight to fully understand the ecology of the microbial community.[7]

The viable biomass of the microbial community in drinking water can be determined as the polar lipid content in the signature lipid biomarker analysis. All viable microorganisms possess an intact cellular membrane. The microbial community that have lysed and are nonviable do not have intact cellular membranes. Their phospholipids have been transformed into diglycerides. The portion of the viable community that has been lysed can be defined by the fatty acid patterns of the diglycerides. Evidences for unbalanced growth, toxicity, and nutritional stress are apparent in the signature lipid biomarkers, and specific pathogens can be detected in the lipopolysaccharide hydroxy fatty acid patterns.[8] The *Mycobacteria* can be readily identified by their end-chained methyl branched and micocerosic acids, secondary alcohol, and methyl 3-hydroxy fatty acid patterns of neutral lipid components. Fungi, protozoa, and algae can be identified by the analysis of sterol patterns.

The detection of human fecal contamination in water is discernible with the detection of the steroid, coprostanol, which is formed in the human intestine from cholesterol by bacteria. Coprostanol is not found in significant quantities in the feces of domestic and feral mammals, birds, reptiles, amphibians, or fish. An isomer, epicoprostanol, is found in the feces of elephant seals and blue whales, but if it is human, it is coprostanol. Under proper conditions, coprostanol can persist, so it is a conservative indicator that human feces have been associated with the sample in question. Chlorination drastically decreased the *E. coli* viable cell count of a sewage discharge recovered from a mid-Western Great Lake without affecting the total phospholipid ester-linked fatty acids (PLFA) or the lipopolysaccharide hydroxy fatty acids (both strong indicators of gram-negative bacteria with intact membranes) or affecting coprostanol. The lipopolysaccharide hydroxy fatty acids are signatures for bacterial endotoxins. Endotoxins induce powerful responses in human immune systems. Our laboratory is presently developing a rapid and automatable system for signature lipid biomarker analysis that could be very useful in assessing the safety of drinking water supplies.

A recent experiment showed a microbial biofilm containing 2.8 picomoles (pmol) PLFA (equivalent to about 105 bacteria the size of *E. coli* per squared centimeter [cm^2]) was recovered after passing 600 liters (L) of potable tap water over a surface of 200 cm^2 of stainless steel spheres in 3 weeks. No viable *E. coli* or coprostanol was detected. The biofilm contained evidence of bacteria with short terminally branched saturated PLFA with an *iso/anteiso* ratio of 0.7, suggesting gram-positive *Micrococci* or *Arthrobacter* type organisms. The majority of the biofilm was formed of gram-negative heterotrophic bacteria showing evidence of starvation and 16 and 18 carbon oxirane (epoxy) and 9 and 11 carbon dioic acids, most likely derived from the monoenoic PLFA with unsaturation 7 carbons from the omega end of the molecule (which are typical of gram-negative heterotrophic bacteria). These unusual fatty acids are likely formed during alkaline methanolysis from halohydrin derivatives generated in the intact bacterial membranes with exposure to hypochlorite. These "death markers" may prove useful in studies of biocide efficacy.

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Microbially Influenced Corrosion

Except for gold and traces of copper, metals on earth are found as oxides or some other salt. The loss of electrons by metals with the formation of salts is detected as corrosion. It is not unreasonable then to expect that the iron, steel, and other metals that support our civilization might be susceptible to corrosion. Some of the most devastating types of corrosion are found where the process is localized and perforations through the metal wall result. Contents in tanks or pipes leak out or contaminants leak in. Unfortunately, biofilms of microbes foster localized corrosion in a very powerful way. The microbial biofilms have a very heterogeneous distribution of cell types and metabolic activities. To initiate pitting corrosion, an anode where the metal loses its electron must be formed. Microbes locally secrete acids that powerfully facilitate metal loss (oxidation) and then chelate the metal salts with their polymers and further shift the equilibrium towards dissolution. There is no corrosion unless there is a cathode where the electrons can be utilized in reduction reactions. Microbes facilitate reactions at the cathodes as well. The heterogeneity in the distribution of microbial metabolic activities in biofilms greatly facilitates the generation of anodes and cathodes, which form a circuit between the conducting metal and the dilute salts in the water phase. Engineers do not like their structures to degenerate because of the slimy biology they consider a messy science. Microbially influenced corrosion (MIC) is industrial venereal disease — it is destructive, can be painful in the costs for repair and prevention, and no one admits their facilities have the problem. As a consequence, there is almost no research into MIC, and we have come to expect that our bridges will degenerate as the microbes eat the rebar reinforcing rods or the concrete or that sewers will collapse as the *Thiobacilli* dissolve the concrete. It is difficult to maintain a water distribution system when the biofouling microbes are intent on filling its piping with holes. Remember, all the soil on the earth was bioprocessed largely by microbes from the igneous rock that formed our planet.

Microbial Biofouling

Some of the most effective ways of purifying water for recycling involve separating the salts and contaminants from water with a membrane. This is a wonderful system except that a membrane is a superb place to live if you are a microbe. Life on the scale of microns (a millionth of a meter) is very different from what we can sense. For us, it would be like living in dense maple syrup where viscosity makes movement difficult, and everything you need to subsist must diffuse ever so slowly to you. You cannot swim fast enough to eat enough to maintain the swimming. If you can sit in a biofilm, especially if it is a membrane, all the goodies come to you in the filtering water. You get fat, happy, and reproductive until your progeny grow and plug up the membrane. Our solution to this dilemma is to just kill the bacteria with biocide and control the problem. Unfortunately, there are bacteria that resist the biocides by forming a waxy outer coating. The biocide treatment makes the waxy-coated bacteria prosper as it removes their faster growing competition. Meet the waxy-coated bacteria — they are the *Mycobacteria*, close cousins of *Mycobacterium tuberculosis*. Just like their infamous cousin, some of the species recovered from water are capable of human infections, particularly in immunocompromised hosts. This cousin, *M. tuberculosis*, is estimated to be present in more than half the people on earth.

Water Cycles and the Soil

The water cycle depends on microbial activity in terrestrial plants (phylosphere) and soils. The microbiota of the soil, the roots (rhizosphere), as well as the fungi (mycorrhiza), can be quantitatively assessed with the signature lipid biomarker method. Specific increases in buried carbon in the living rhizosphere microbiota have been demonstrated on the roots of trees grown with increased levels of carbon dioxide. The amount of carbon buried as viable microbes could buffer the global climate from effects of greenhouse gases. The effect of tillage and agricultural practice on the soil microbiota markedly affect the soil quality in terms of subsequent productivity and photosynthetic activity of crop plants. Photosynthetic activity correlates with evapotranspiration and the return of surficial water to the atmosphere. Plants have another important function. Bacteria that live on the surface of the leaves can be readily released and are carried high into the stratosphere. In the stratosphere, specific molecules in these bacteria greatly accelerate the nucleation of raindrops. Where the land is deforested, the rains stop and the desert expands.

Indoor Air Biocontamination

We spend more and more of our time indoors in buildings sealed off from the outside world. Under these conditions, it becomes vitally important that reservoirs where molds can grow and produce mycotoxins be strictly controlled. Where there is sufficient moisture, the molds will grow and can form and release mycotoxins. These mycotoxins are powerful potentiators of the immune response and are the most efficacious carcinogens known. These mycotoxins are the mold's way of protecting its food supplies from other scavengers. You can find these reservoirs and define the conditions that prompt the molds to form their mycotoxins with the signature lipid biomarker analysis and show where in the building the water balance fosters their growth.

Toxicity Assessment

In water treatment, it is required that no toxicity be detected in the effluents of treatment facilities. In the Eastern United States, this is relatively easy as the effluent can be highly diluted with cleaner water. In the arid West, there is no extra clean water to use for dilution. Regulations defining no toxicity have focused on the lifecycles of water fleas as the test of toxicity. These tests have an enormous biological variability, so it is almost impossible to certify a treatment as safe. With the signature lipid biomarker analysis of subsurface sediments, it is possible to accurately define the impacts of pollution as they are reflected in the modifications of the microbial communities. We have shown that signature lipid biomarker analysis of the slime (periphyton) formed on rocks in running surface waters is a reproducible and quantitative multi-trophic level, multi-species toxicity assessment that correlates with standard measures of pollution and toxicity.[9] With this data in hand, several multi-million dollar artificial stream facilities have been built and used convincingly in setting limits of commercial product release rates into natural waters.

Implementation

Is there the leadership and resources to focus new thoughts on the vital issues confronting Water Science and Technology? I believe there is, and it is here at NWRI. The politic application of pressure, persuasion, and judicious funding has made the ponderous Federal United States Science Bureaucracy actually begin to consider basic water science and technology. The water industry and its research organizations may also begin to support new technologies and basic research. NWRI has been the leader in making the applications of advanced techniques in molecular biology focus on problems of safety in the water supply and has facilitated the application of signature biomarker analysis to the ecology of pathogens in biofilms of water distribution systems. I look forward to

each meeting of the NWRI Research Advisory Board as an opportunity to gain new collaborations and insights into research. To be chosen as the 1995 Clarke Prize recipient is a magnificent honor, and this prize will be used to further stimulate my research capabilities in this most vital arena of water science and technology.

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