THE MICROBIOLOGICAL QUALITY OF WATER

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The microbiological quality of water: the nature of the problem

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Improvements in methods for the detection and enumeration of microbes in water, particularly the application of techniques of molecular biology, have highlighted shortcomings in the "standard methods" for assessing water quality. Higher expectations from the consumer and increased publicity associated with pollution incidents can lead to an uncoupling of the cycle which links methodological development with standard-setting and legislation.

The new methodology has also highlighted problems within the water cycle, related to the introduction, growth and metabolism of microbes. A greater understanding of the true diversity of the microbial community and the ability to transmit genetic information within aquatic systems ensures that the subject of this symposium and volume provides an ideal forum to discuss the problems encountered by both researcher and practitioner.

Introduction: the water quality cycle

The phrase "microbiological quality of water" is, in itself, meaningless unless we define the purpose for which we intend to establish a quality standard. In other words, we are attempting to establish Water Quality Objectives based on intended use. For the purposes of this meeting and this article, these objectives will relate largely to drinking water quality, although it would be foolish to overlook the much increased interest in bathing water quality. It is claimed that the Bathing Water Directive is one of the most popular in Europe and higher public expectations of amenity waters and environmental water quality standards should be anticipated. Unfortunately, quality standards and legislation have consistently lagged behind advances in microbiological techniques and, inevitably, will continue to do so for some time to come.

This assertion is, perhaps, best explained by reference to Figure 1. The Quality Cycle for water is depicted as one in which an appropriate standard for water quality is established and legislation passed. The purpose of the standard and legislation is to control the numbers of a given microbe in water, the organism in question being either the causative organism of disease or an indicator of conditions under which disease might be transmitted (e.g. faecal pollution). This, in turn, assumes a link between the presence of the microbe and the incidence of disease, whether or not there has been a firm, quantitative assessment of what comprises a minimum infective dose. The publicity associated with outbreaks of disease (whether associated with drinking water supply or bathing water) alerts regulatory and other public bodies which possess statutory powers to take legal action. However, in an increasingly litigious society, and with the rise in single-issue politics, we are already experiencing greater public expectations with regard to the quality of our drinking and bathing water. The fact that water supply and sewage treatment is now in the private sector has probably also contributed to this higher expectation, decreased tolerance and tendency to turn to legal action.

Against this background the law-makers and standard-setters are working during a period of unprecedented progress in microbiological methodology. The application of the techniques of

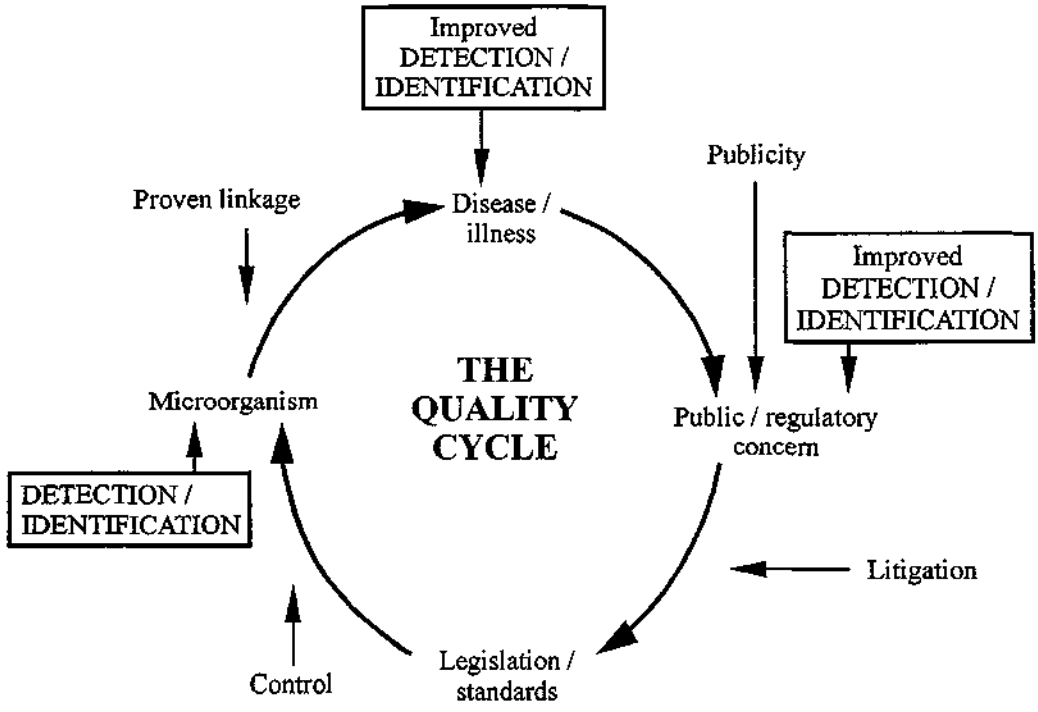

Figure 1. The quality cycle for water.

modern molecular biology, in particular (Pickup & Saunders 1997), have highlighted the shortcomings in the standard methods recommended for the assessment of water quality. Microbiologists have always embraced advances in methodology for detection and enumeration with enthusiasm. There can be no doubt that a major factor contributing to this search for improvement in environmental microbiology has been the inadequacy of methods used in the past. Microbial ecologists have lagged behind their "macrobiological" counterparts in the study of population and community ecology and hence in the development of ecological theory.

The net result has been that the newer methodology, and the results obtained, now run ahead of legislation and standard-setting. Thus the improvements in our ability to detect, identify and enumerate potentially harmful microbes in surface waters, groundwaters and the supply system (Jones 1996), have acted as forcing functions at the three stages in the quality cycle which precede the setting of standards and the passing of legislation. We should, however, exercise care in changing standard methodology to accommodate the improved sensitivity provided by modern technology. Does the ability to detect larger numbers of a given organism imply a greater risk of infection? It seems to me that we are only beginning to determine a quantitative relationship between the microbiological quality of water, the incidence of infection, and the epidemiology of waterborne diseases. The concept of the minimum infective dose (whether through consumption or amenity use) requires rigorous examination.

The purpose of the joint FBA/IWSA Specialised Conferences is to bring together scientists who are conducting research at the cutting edge of their subject, with practitioners who are working at an equally sharp cutting edge in the application of that knowledge to water quality and supply. In examining the microbiological quality of water we are, I believe, providing a forum for discussion of one of the most rapidly expanding and changing subjects in the field of water supply and treatment.

Methodological problems and assumptions

In determining the microbiological quality of water, the investigator is, essentially, asking the following simple questions: which microbes are present; how many are there; are they alive/active; do they constitute a health threat?

Which microbes are present?

The method used to identify and enumerate a given microbe must provide an acceptable level of reliability. This, in turn, will depend on the efficiency of recovery achieved. It is likely that more space has been devoted to the inadequacies of the so-called viable count techniques in aquatic microbiology than any other aspect of the subject. This section is, therefore, reduced to a minimum, but the subject cannot be ignored totally as the methods are still amongst those recommended in the Drinking Water and Bathing Water Directive of the European Commission.

How many microbes are there? Viable count techniques

Viable count techniques include plate counts, membrane-filter counts and most-probable-number dilution counts. In all but exceptionally well-researched circumstances, none of these techniques is truly quantitative; (the often-used euphemism of "semi-quantitative" is somewhat meaningless). In each, the microbe of concern is detected by the production of visible growth (colonies or turbidity) on a growth medium usually designed to be selective for a particular group or narrow range of organisms. To be truly quantitative it would be necessary to determine the efficiency of recovery from every water sample tested (usually by the introduction of a known number of the organism in question). Lack of detection would not, of course, imply absence, as is discussed later when the phenomenon of "viable but non-culturable organisms" is considered. The use of the most-probable-number (MPN) dilution technique often yields higher "counts" than those obtained on agar plates or membrane filters. Unfortunately the statistical error terms associated with MPN methods (which are dependent on the dilution ratio and the number of replicates used (Jones 1979)) are so large, often spanning an order of magnitude or more, that confidence in the mean count is insufficient for the purpose of standard testing procedures.

The biggest problem associated with viable count methods is that they are inherently selective. In very general terms, a direct count (microscopic observation) of the bacteria in any water sample will yield numbers that are three orders of magnitude higher than those obtained by the best viable count technique. There are several reasons for this. No medium can support the growth of all the species of bacteria present and therefore it will inevitably select for the few that can. Of course, we do not yet know how many species of bacteria are present in water; many remain to be isolated, characterised and classified. The difference between the viable and total count will become smaller the more clearly defined the target group of organisms becomes. The difference quoted above was in relation to all detectable species of bacteria. If the direct and viable counts of coliforms, *Escherichia coli* and a particular strain of *E. coli* were to be compared, we would find that the differences between the two methods would become progressively smaller. However, the chances are that the direct count would be consistently higher than the viable count. One of the reasons for this is that many bacteria are capable of entering a shutdown state, particularly in dilute solutions such as water. This state, variously known as starvation, resting, shutdown, cryptoform, viable but not culturable (Cervantes *et al.* 1997, this volume), etc., is usually characterised by metabolism of carbohydrate intracellular reserves and eventually of protein and RNA. If the process reaches these final stages, the cells may be metabolically active but will not be recoverable on growth media regardless of the care

taken in their formulation. More importantly, in aquatic systems, bacteria may enter this state but retain the ability to infect animals or plants, thus becoming culturable once more (Colwell *et al.* 1985). This transition may be an important part of the life cycle of the organism and is a characteristic of several pathogens, including *Vibrio cholerae*, *Aeromonas salmonicida* (Morgan *et al.* 1991) and *Legionella pneumophila* (see later in this article).

Are the microbes alive/active?

Bacteria in the shutdown state may exhibit several characteristics including size reduction (usually to form small spheres) and resistance to antibacterial substances, but the ability to re-infect implies a positive answer to our third simple question, i.e. they are potentially active and viable (see also Pickup & Rhodes 1997, this volume). A common characteristic of such stressed bacteria is a change in protein patterns, including the synthesis of what have become known as "heat shock proteins" (the phenomenon was first observed in bacteria that were stressed by heat). Starvation of *Vibrio* sp. produced not only a change in proteins but resistance to a wide range of challenges including heat, ultra-violet light and cadmium chloride (Nystrom *et al.* 1992).

We are therefore faced with the problem that research has demonstrated that conventional viable count techniques will not detect all the organisms present, a simple direct count will not provide an identification, and neither will tell us whether they are active in the field sample. On the other hand, the techniques of modern molecular biology can provide these answers (Pickup & Saunders 1996) but are often either prohibitively expensive or too complex to be applied in routine testing. The ability to amplify genetic material, through the application of the polymerase chain reaction to water samples, has provided a level of sensitivity hitherto beyond our imagination. Although these methods, combined with the application of gene probes, require some assumed knowledge of the genomic material involved, they have been used successfully to detect a range of microbes. These have included enteroviruses and hepatitis viruses (Jothikumar *et al.* 1993; Kopecka *et al.* 1993), *Legionella* spp. (Palmer *et al.* 1993) and *Shigella dysenteriae* (Islam *et al.* 1993). The additional application of fluorescent antibody techniques, immunomagnetic capture (Pickup & Saunders 1991) and electrorotation assay (Coghlan 1993) has raised sensitivity to a claimed 1 in 10^{15}. However, it is probably the application of flow cytometry and cell sorting (Edwards *et al.* 1992, 1996; and Taylor 1997, this volume) that has done most to raise hopes that reliable quantification of specific microbes in natural water samples is an achievable aim.

For such procedure to become part of routine testing the production of affordable diagnostic kits will be essential (Fricker & Fricker 1996). These will require not only mass production of the necessary enzymes, probes and fluorescent antibodies, but also the development of simpler dedicated flow-cytometric instruments. The rewards would, however, be significant, particularly as several techniques for the determination of viability and metabolic activity at the single cell level now exist. Ribosomal RNA fluorescent probes have been used in combination with digital microscopy to quantify the activity of single cells (Poulsen *et al.* 1993) and the transfer of genes has been used as an indicator of active populations in aquatic systems (Barkay *et al.* 1993). However, the most rapid advances have been achieved through the use of a range of conjugates and dyes to assess enzyme activity and viability. Fluorescein-, naphthol- and coumaryl-linked substrates can be used to determine specific enzyme activity (Kell *et al.* 1991; Watkins & Jian 1997, this volume), viability may be detected with compounds such as Chemichrome B and carboxy fluorescein (Pinder *et al.* 1993), and cells that exhibit a membrane potential are identified by the application of Rhodamine 123 (Diaper *et al.* 1992; Kaprelyants & Kell, 1992).

Do the microbes constitute a health threat?

In spite of these advances in methodology, the final "simple" question on whether the organisms present in water constitute a health threat and what range of organisms are involved (Lightfoot 1997, this volume) has yet to be investigated with the same degree of thoroughness. Whilst the standards set for drinking water have, generally speaking, stood the test of time, those for bathing water clearly require monitoring followed by modification if this proves to be necessary. The stages in the assessment of the performance of any given test might be as follows: (1), efficiency of detection of the target organism; (2), correlation of numbers with outbreaks of disease (epidemiology); (3), characterisation of the causative organism isolated from diseased individuals and comparison with the original sample.

Modern molecular methods will have a significant role to play at stages (1) and (3) but care must be taken to ensure that the results obtained by the newer, more sensitive techniques, are not used as a reason for tightening standards beyond the point of necessity. For example, a viable count may detect x organisms per ml, a count which falls within the approved standard for a particular water sample. Molecular techniques may detect $n(x)$ organisms per ml, but does this make the sample inherently less safe? The answer to this can only be obtained by detailed studies of epidemiology and what is, in effect, the application of Koch's postulates. Whilst the database for drinking water provides a reasonable degree of confidence in the outcome (except possibly for the newer nuisance microbes such as some protozoa) there is a long way to go with regard to bathing water. Surveys of a selection of bathing beaches and follow-up questionnaires on the health of bathers have taken place for marine sites. Precise identification of the microbial strains involved by genetic analysis has yet to be undertaken and, in spite of increasing pressures, freshwater bathing sites have been studiously ignored.

The present situation may be summarised as follows. Much of the methodology required to complete a realistic assessment of the microbiological quality of water now exists (Jones 1996; Saunders & Saunders 1997, this volume). However, further effort is required to bring this methodology into the framework of standard-setting and legislation. The outstanding problems are often associated with events in the "field" and the adequacy of sampling regimes.

Problems inherent in the system

The water cycle can be represented in several ways, but for the purposes of microbiological water quality I have summarised it as seven interconnecting compartments (Fig. 2). The problems encountered in these compartments generally relate to the introduction, growth and metabolism of microbes. However, more recently we have also come to appreciate the diversity of microbes relevant to the assessment of microbiological water quality. It is no longer a matter of confining the search to indicators of faecal pollution (and usually only bacterial indicators at that); each major microbial group presents its own problems when assessing water quality. The greatest diversity of microbes is found in the natural environment, in this case the freshwater and marine systems. Whereas freshwater and sewage systems may transport offending microbes into marine bathing areas the reverse process is negligible. We can assume there is little transport from the sea via evaporation, although droplet infection may carry over some distance and saltwater ingress of groundwater presents localised problems, but not necessarily because of inoculation with pathogenic or nuisance microbes.

Primary producers

The population dynamics of primary producers (in the form of phytoplankton) has long been of concern to the water industry, and significant progress in methodology can be reported (Wilhelm & Lohmann 1997, this volume). However, the organisms are no longer considered to

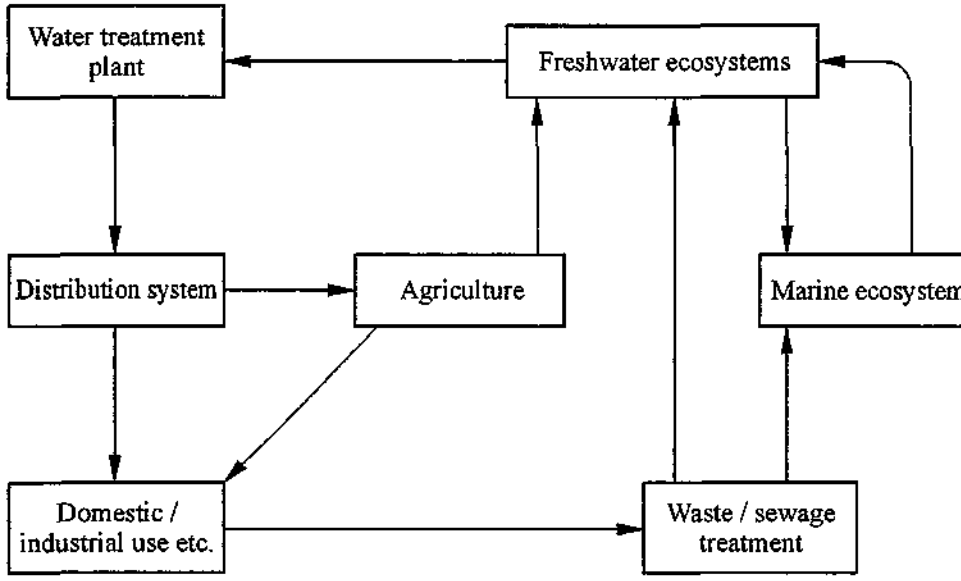

Figure 2. The distribution and transport cycle for water.

be a problem merely because they block filters and slow the process of water treatment. A combination of freshwater eutrophication and particular weather conditions has resulted in an increase in the incidence of nuisance blooms, particularly of cyanobacteria, most of which should now be considered as being potentially toxic. In the marine environment toxic dinoflagellates have been implicated in poisoning incidents associated with shellfish (see Taylor 1997, this volume). In both cases, treatment at source (i.e. the reduction of nutrient loading by sewage and other sources) should provide a solution, although incorporation of physical factors (mixing; weather conditions) is essential for the development of models and the successful prediction of such events (Reynolds 1993).

Cryptosporidium *and viruses*

As man makes progress in the fight against disease, so other threats to health assume prominence. This is seen clearly in the area of general health care where the eradication of many microbial diseases has ensured that cancer and heart disease assume a new prominence. We would be unwise, however, to ignore the ability of microbes (particularly bacteria) to develop multiple drug resistance and, possibly, to use parts of the water transport cycle as sites where this resistance can be transferred to different species or even genera. The (generally) successful treatment of bacterial contaminants of water has ensured that attention is now more keenly focussed on the protozoa and viruses. The fact that four speakers at this 2-day meeting are concerned with protozoa and the problems they cause the water industry is largely attributable to the rapid expansion in our fundamental knowledge and the apparent increase in the incidence (or at least the correct diagnosis) of *Cryptosporidium* infections (see the articles by Finlay 1997 and Smith 1997, in this volume). That one contributor was unable to report findings fully at this stage in his investigation is ample evidence of the increasingly litigious society alluded to earlier.

Cryptosporidium has presented the water industry with two problems: one of detection and correct identification of very low numbers of the organism, and the other of successful treatment of a resting stage which exhibits considerable resistance to chlorine. To some extent viruses present similar problems, although the articles by Cartwright (1997, this volume) and Butcher & Gould (1997, this volume) demonstrate that significant advances in methodology have been made in the last decade or so. It might be more relevant in this case to consider whether the standards being set are realistic and whether the sampling design is adequate. It is now generally accepted that surface waters used for abstraction or bathing will probably contain 10^6 bacteria ml^{-1} (well in excess of any count obtained using standard procedures). What is becoming increasingly clear is that direct counts of virus particles in the same water will exceed this number by one or two orders of magnitude. If we superimpose on these figures the observation that spatial variability of such counts in a single waterbody on a given day will be equal to, or greater than, that observed over the whole annual cycle at one site (Jones & Simon 1980), then we clearly need to reassess the validity of sampling intervals and designs recommended in standard protocols. In any event, there seems to be little logic in recommending that bathing water should be checked for enteroviruses "by the competent authorities when an inspection in the bathing area shows that the substance may be present or that the quality of the water has deteriorated". It is clearly not possible to detect the "substance" (viruses) by on-the-spot inspection and, if deterioration of the water is visible, then bathing would, clearly, be foolish. Why set standards which cannot be measured accurately when common sense provides the obvious guideline?

Fungi

Of the microorganisms under consideration in this symposium volume, the fungi receive little or no attention. This is probably because detailed studies have largely been confined to their role in decomposition and conditioning of larger detrital material and vegetation prior to its consumption by invertebrates. Some difficulty has also been experienced in distinguishing those fungi which are truly active in aquatic systems and those merely washed in from the surrounding soil. This difficulty has, to a considerable extent, reflected inadequate methodology and will be largely overcome when modern techniques are applied to this understudied group. We must, therefore, resort to speculation about the role of fungi in microbiological water quality except in cases of obvious gross contamination or interference in treatment plants. Fungi may well have a role to play as primary colonisers of surfaces, particularly when these are organic in nature. Pipe and jointing materials in distribution systems may be susceptible to fungal attack and, given the experience in terrestrial habitats, one might reflect on their possible role in the production of tastes and odours.

It is in the area of taste and odour that the microbiologist probably has least to offer the water manager at present. Whilst we are able to identify most of the offending compounds and their microbial source (see Kelly & Pomfret 1997, this volume), the solution is essentially one of water management (to dilute the offending compound) or treatment for removal before distribution. If cyanobacteria are the cause of the problem, then it is feasible to manage waterbodies to reduce their population size (nutrient reduction or physical mixing). If, on the other hand, the main source of the offending compounds are actinomycetes, then this could well present an insurmountable problem unless ever-dwindling resources of high quality water are used for dilution. Clearly, the association of such problems with emergency water transfer schemes will result in more careful analysis of source waters in future. It will, however, be more difficult to predict which microbial (especially actinomycete) populations will develop after mixing.

Indicator organisms

Given the diversity of microbes in freshwater systems and the advances currently being made in the identification, detection and enumeration of aquatic organisms, it will only be a matter of time (if it has not already arrived) before the concept and use of "indicator organisms" will be questioned more rigorously. Why should we test for *E. coli* when we are really interested in *Salmonella*? If we are concerned about faecal pollution, do faecal coliforms provide the best measure of its presence? Are they the most numerous faecal organisms? We are reaching the stage when we can seriously consider determination of the genetic "fingerprint" of the entire microbial population of faeces (and other contaminants), thus providing evidence of the source and the extent of populations. Eventually these techniques will be used to determine the exchange between the compartments of the cycle illustrated in Figure 2 and, hopefully, they will also provide insights into the phenomena of dormancy and selection within the water cycle.

Selection and survival of bacteria

The development of the shutdown (viable but not culturable) state, particularly in bacterial populations, has been mentioned above. I shall close this contribution by examining the role of this and related phenomena in the selection and survival of bacteria in supply, treatment and distribution systems. There now exists ample evidence that bacteria undergo a physiological change in dilute solutions which results in the closing down of many metabolic functions. In addition to reduction in size and metabolism a common feature has been the development of enhanced resistance to a wide range of antibacterial substances, this resistance being greater the lower the concentration of nutrients in the growth medium (Jones *et al.* 1986). Strains of *E. coli* and *Legionella pneumophila* have been shown to be more resistant to chlorine when grown at very low dilution rates or in tap water (Berg *et al.* 1982; Kuchta *et al.* 1985). However, such resistance is not only characteristic of growth limitation by nutrient concentration, but also by temperature (Harakeh *et al.* 1985). The range of antibacterial substances to which resistance develops is also wide, including cetrimide, chlorhexidine and benzalkonium chloride (Cozens & Brown 1983), 3- and 4-chlorophenol (Gilbert & Brown 1978) and phenylphenol (Abou-Shleib *et al.* 1983). In most cases the resistance was associated with changes in the composition of membrane proteins (Abou-Shleib *et al.* 1983; Chai 1983; Jones & Pickup 1989). Clearly, such results have important implications in the calculation of dosages for the disinfection of domestic and commercial water supplies, particularly as failures have resulted, e.g. in the chlorination of hospital water supplies to remove *Legionella pneumophila*, when the dosage has been based on responses observed in rich growth media. Resistance induced by some form of growth limitation may be equally important in the consideration of interactions and survival in the natural environment. However, it has been consistently recognised that it is difficult to detect antibiosis in nature and knowledge about its significance is lacking (Saunders 1984).

The bacterial responses described above deserve some consideration in the examination of waters used for amenity purposes, heated water in industrial and domestic systems and, most importantly, the system for treatment and supply of drinking water (see Block *et al.* 1997, this volume). It has been shown, for example, that chlorination may enhance the incidence of antibiotic resistance in sewage-related bacteria (Murray *et al.* 1984). Although the mechanism of resistance was not determined in this case, this is one of the many factors to be borne in mind when considering the microbiological quality of water. Hopefully, we shall soon see the application of advances in molecular biology not only to gain an understanding of these phenomena, but also to the framing and enforcement of increasingly sensible and realistic water quality standards.

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