# Environmental microbiology

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Volume 2, pp 682–688

in

Encyclopedia of Environmetrics
(ISBN 0471 899976)

Edited by

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Microorganisms are critical to life on earth. They are at the very bottom of the food chain (*see* **Community food webs**), providing the nutrients required to sustain other forms of life. Scientists are only now discovering the extent to which microorganisms control broad biochemical processes, such as the cycling of carbon, nitrogen, and sulfur [16, 46] (*see* **Ecosystem element cycling**). Bacteria live in all habitats accessible to any form of life – from Antarctic lakes to deep oceanic hot water vents [20] – even 2000 m below the earth's surface, where no fresh organic input has been experienced for millennia [1].

Rigorous investigation of environmental issues must recognize, and take account of, microorganisms. For example, consider a leaking oil storage tank that has contaminated the subsurface water. To guide the choice of a remediation strategy, environmental scientists and engineers drill holes, take measurements, and construct computer models to help them infer the characteristics of the underground plume of contaminated water (*see* **Groundwater monitoring**). Their investigation should consider the potential role of microorganisms living naturally underground in the path of the plume. Some of the microbes can metabolize the hydrocarbon contaminant, converting it to carbon dioxide and water. This natural, albeit slow, bioremediation process is of potential significance. Moreover, the hydrocarbon food source promotes bacterial replication and biofilm formation. The biofilm grows on the sand, or other porous media, and it can alter the porosity and permeability of the flow region; in fact, a large amount of biofilm can plug the pore spaces, thereby slowing, blocking, or redirecting the plume. Environmental engineers sometimes purposely promote such microbial growth to create subsurface flow barriers [8]. For these reasons, it is prudent to consider the microbiology when making environmental decisions about a site of subsurface contamination.

Until recently, environmental microorganisms were studied by observing batches of planktonic bacteria. However, the vast majority of microbial activity does not occur in liquid suspensions of individual organisms – it occurs on surfaces in structured communities called *biofilms* [3]. Of all microbial activity in an open ecosystem, 99% is in biofilms on surfaces [41]. The importance of biofilm processes is now well established in environmental microbiology [4, 7, 12, 14, 41].

Biofilms are formed as follows. Microbial cells that exist in a flowing or moist environment attach to an available surface. If there are sufficient nutrients, the cells multiply and move across the surface, eventually aggregating into small colonies. The colonies grow into a three-dimensional structure held together by a protective slime, a spaghetti-like mesh of proteins and exopolysaccharide substances produced by the bacteria. When the mature community gets overcrowded, chunks of the biofilm break off and float away. Individual cells are also continually eroding from biofilms, and may establish new biofilm communities on downstream surfaces. Bacteria are not the only inhabitants of biofilm. Fungi may establish their own territory, as may algae. Protozoans that consume bacteria may feed on the biofilm [7]. Protozoan cysts and virus particles can become entrapped in a biofilm and detach later to return to the environment.

Recent advances in molecular biology and laboratory instrumentation have provided the tools to show that a biofilm is not just a random collection of cells stuck to a surface, but rather, it is a dynamic, organized, cooperative community (*see* **Community, ecological**). Each bacterium possesses a genetic plasticity that allows it to alter its phenotypic state in response to an altered environment. Biofilm bacteria exhibit phenotypic characteristics that are very different from their planktonic state, and bacteria in the interior of a biofilm may well express different phenotypes than when on the exterior.

Microbiological processes are inherently stochastic, heterogeneous, and difficult to measure; consequently, statistical thinking is necessary. The associated challenges to statisticians are especially intriguing because the subjects under study are available in multitudes. In nutrient-rich environments, bacteria divide often; the population doubling time can be just a few hours. Statisticians seldom get to work with such large sample sizes or with subjects that produce many generations quickly. Unfortunately, bacteria are so small ($<5\,\mu m$) that they are impossible to see, and their activity is impossible to measure, except with specialized instruments such as microscopes and electrochemical

probes. Indeed, it is difficult to determine whether a cell is even alive or dead. A microbe's morphology and biochemical activity may change so much in response to its microenvironment that it looks and behaves like a completely different organism from one observation time to the next. Often, the best available data are the aggregate responses from samples containing huge numbers of microorganisms. Because a biofilm is a complicated, three-dimensional, dynamic community that is difficult to measure, the study of biofilms presents statisticians with a vast new set of challenges. Moreover, modern, high-technology laboratory tools employed by microbiologists create a corresponding need for new statistical theory and methods. Those needs encompass all the established branches of statistical science.

Because of the inherent biological variability and measurement variability in microbiological studies, summary statistics often exhibit large **standard errors** or coefficients of variation. When microbiological experiments are repeated on different occasions in a laboratory, there is usually significant variation among those experiments. When the same experiment is replicated by different laboratories (*see* **Interlaboratory studies**), the variation among laboratories is also significant; see, for example, [52]. It seems impossible at present to identify and control all the environmental factors that affect microbes. Therefore, it is particularly important for statisticians to recommend replication of microbiological experiments or field studies so that the statistical uncertainty can properly be ascertained.

The goals of this entry are to review some of the historical statistical contributions to environmental microbiology and list some developing issues and associated research strategies in environmental microbiology. Other articles and books dedicated to statistics in microbiology include [21], [23], [24], [40], [48] and [51].

## Counting Planktonic Bacteria

Since the 1880s, when the terms *bacteriology* and *microbiology* were first used, it has been important to enumerate microbes in environmental samples, to classify the microbes into species according to form and function, and to measure the biochemical activities of each consortium. Conventional methods have focused exclusively on planktonic bacteria, regardless of the source of the sample. If the sample were a semi-solid, such as sediment, soil, or food, it would be blended in with a liquid, thereby creating a suspension of disaggregated microbes. In biofilm studies, it is common practice to remove the biofilm from a known area of the surface, suspend and disaggregate the bacteria, and count them using a conventional method.

### *The Most Probable Number and Limiting Dilution Assays*

Fisher et al. [18] and Student [49] showed that the Poisson distribution is a useful model for the random number of particles in a sample from a well-mixed suspension. Using the Poisson model, McCrady [33] invented in 1915 the most probable number (MPN) method for estimating the density (number of microbes per milliliter) based on serial dilutions and presence–absence data (*see* **Binary data**). When a homogeneous solution is divided into samples by serial dilution, some of the samples eventually contain such a small amount of the original solution that they are absent of the microbes. That 'absence' is observable because laboratory methods have been devised to detect 'presence', indicating that the sample contains one or more microbes. Since McCrady, serial dilution assays have been a subject of continual statistical attention [6, 15, 17, 32, 43].

Limiting dilution assays are so closely related to the MPN problem that they can be treated with the same statistical approaches. Some notation is required to describe the relationship. Let $\lambda$ denote the density of microbes in the suspension. Let $P_+$ denote the probability that a dilution tube exhibits 'presence'. Suppose that (a) the dilution tube contains $N$ microbes, (b) each microbe produces a random, binary response (positive or negative) to the laboratory test, where the probability of positive, denoted by $p$, is the same for each microbe, (c) the responses of the microbes are statistically independent, and (d) the tubes are statistically independent.

For an MPN assay, 'presence' indicates at least one viable cell, and $\lambda$, the parameter to be estimated, denotes the density of viable cells. It is assumed that $N$ is a Poisson (mean $= \lambda d$) random variable, where $d$ is a known dilution fraction, and $p = 1$. In this case, $P_+ \doteq 1 - \exp(-\lambda dp) = 1 - \exp(-\lambda d)$. The MPN is the maximum likelihood estimator of $\lambda$ based on presence/absence in all the tubes over all the dilutions.

For the limiting dilution assay, $p$ is to be estimated and it is assumed that $N$ is a Poisson (mean $= \lambda d$) random variable, where both $d$ and $\lambda$ are known. In this case, $P_+ \doteq 1 - \exp(-\lambda dp)$. The statistical model and estimation problem are the same as for the MPN; however, the practical problem is different. In a limiting dilution assay, 'presence' may not be a measure of viability, rather it may indicate some other characteristic, such as cells of a specific species or a specific phenotype within a species. The parameter $p$ denotes the fraction of the suspended microbes possessing that specific characteristic [43].

### *Plate (Colony Forming Unit) Counts*

Techniques for actually counting microbes have been routinely used for many decades [18, 49]. To find the density of viable bacteria, a sample of the suspension is incubated on a plate of agar-based media that contains essential nutrients. It is presumed that each viable bacterium divides many times and forms a colony that is relatively easy to identify and count. The most common laboratory plate counting techniques are the pour plate, spread plate, and drop plate methods [13, 24]. There are established statistical methods for converting the counts into density estimates [40]. Plate counting techniques can be used with microbes other than bacteria; for example, a virologist might count 'plaques' formed on a lawn of susceptible mammalian cells.

The colony counting approach possesses some potential deficiencies. One is that a colony may actually represent more than one viable organism, especially if the bacteria were not well disaggregated in the suspension. For this reason, each count is called a *colony forming unit* (cfu) instead of a bacterium. Another reason is that a bacterium may be metabolically alive and eventually capable of cell division under appropriate conditions, but it will not form colonies under the conditions of the assay [34]. Therefore, a 'nonviable' cell is not an absolute term; it simply means a microbe that cannot grow a colony under the defined assay conditions.

### *Direct Counts, Total Cell Counts, Membrane Filter Counts*

Instead of counting colonies, a sample of the suspension can be passed through a membrane filter, thereby capturing the microbes on the surface of the filter. With the aid of a microscope, one can make direct counts of the cells. To visualize them, it is standard practice to stain the microbes with a fluorescent chemical, such as acridine orange, a fluorochrome that causes the bacteria to fluoresce green or shades of red through orange. Such stains do not differentiate dead from live cells, hence the resulting counts are called 'total cell counts' [34].

At the magnification required to see the cells, each field of view seen through the microscope is a small fraction ($<0.001$) of the area of the filter. It is not practical to count the whole filter using this approach. Therefore, a few (5–20) sample fields are observed and the counts are scaled up accordingly [13, 26, 27]. It is very important that the fields be selected at random, but the field selection method is seldom described in publications.

### *Current Status of Counting Methodology*

For each of the conventional counting methods, statisticians have proposed various interval estimation procedures. For the MPN method, the best approximate confidence interval methods are based on the **likelihood ratio test** or the Wald method (*see* **z-statistic**) for ln(MPN) [19, 43]. Exact, but computationally demanding, confidence interval methods are available [31, 43]. Bayesian interval methods have also been proposed [5]. For cfu counts or direct counts, Poisson model confidence intervals are often appropriate. However, the counts may exhibit extra-Poisson variability across replicate plates or microscope fields, in which case, interval estimates could be based on a **negative binomial distribution** [24].

Suppose the source of the microbial suspension being counted is an environmental sample, and the goal is to characterize the density of microbes in that environment. In this case, the interval estimates discussed above are not appropriate because they depend only on the variability within the counting assay, and they do not take account of sample-to-sample variability. Consider, for example, samples from recreational waters. Tillett [50] found that variation at the water source is usually much greater than the variability introduced by laboratory counting procedures. This sample error can be estimated only by replicate sampling. Natural variation over time and place can be 10 or 100 fold in excess of

Poisson variation and laboratory variation. Variance components and cost analysis show that the optimal study design is to take multiple environmental samples and expend less effort counting each sample [24, 25, 50].

Investigators have found that across samples, the densities approximately follow a **lognormal distribution**. Therefore, the conventional statistical summary is based on normal theory methods applied to the log-transformed densities, no matter whether they are based on MPN, viable counts, or direct counts. Note that the base 10 logarithm is used for count data in microbiology, and that the results are typically expressed on the log scale; e.g. 'the mean log density for the biofilm was 6.0 per $cm^2$' instead of the equivalent statement, 'the **geometric mean** density for the biofilm was $10^6$ per $cm^2$'.

## Laboratory Tests of Germicides

Environmental microbiologists use laboratory assays to assess the efficacy of germicides, such as chlorination of swimming pool water or spray disinfectants applied to kitchen surfaces. These assays are similar in concept to animal bioassays used in toxicology and pharmacology (*see* **Toxicology, environmental**). There are three basic test systems, (a) planktonic assays, which emulate treatment of recreation water, drinking water, etc., (b) dried surface assays, which emulate the treatment of a liquid spilled and dried on a nonporous surface such as a floor or counter top, and (c) various biofilm assays, which emulate treatment to prevent biofilm formation, treatment to remove an existing biofilm, or treatment to kill microbes in a biofilm. In general, a germicide will be most effective in the planktonic assay and least effective in the biofilm assay. There are standard germicide tests for application to planktonic and surface-dried organisms. The traditional qualitative laboratory tests, such as the use-dilution method, have serious deficiencies and they are being replaced with new quantitative tests [44, 45]. Although no standard laboratory tests exist for application to bacterial biofilms, there has been substantial progress toward the development of such tests [12, 14].

The log reduction is the most commonly employed quantitative measure of germicide efficacy. It is found by subtracting the typical log density of viable microbes in a treated system from the typical log density in an untreated, or control, system. The densities could be determined by the MPN method or the viable plate count method. There is more than one mathematical definition of log reduction, and there are various alternative statistical methods available for estimating the log reduction [10, 11, 22]. In many applications, the log reduction values approximately follow a normal distribution, and conventional normal theory statistical analysis can be used.

## Microbial Ecology

Fluid dynamics, mass transport, surface characteristics, and spatial patterns are some of the phenomena that are important to a biofilm, but do not necessarily affect planktonic organisms. Essentially, every conventional measurement used to study planktonic microbes can also be applied to biofilm microbes. However, in undisturbed biofilms, the measurements will usually display important spatial or temporal patterns (*see* **Point processes, spatial–temporal**). The microbes in a biofilm are held in place long enough for visualization by microscopes and surface science instruments, and statistical image analysis can be used to elucidate the spatial and temporal configurations [30, 39].

### *Microbial Taxonomy*

Traditionally, microbiologists have isolated organisms from the field and then grown them up in culture. This approach has given researchers a markedly skewed picture of the diversity of bacteria and other microorganisms because it ignores organisms that cannot be cultured. Only about 1% of bacteria can be grown in culture. Now, using genotyping techniques that directly sample and compare gene sequences from different organisms, microbiologists are finding that the bulk of genetic diversity on earth (*see* **Species diversity**) lies not with plants and insects, but with single-celled microbes. Such findings suggest that untold millions of species lie hidden in the microbial world [47]. Statisticians, using established techniques in multivariate analysis, as well as those from the emerging fields of computational biology [53] and bioinformatics [2], have much to contribute to the development of the new taxonomy.

### Species Interactions and Niches

Different species of bacteria can collaborate to make the microbial community much more powerful; e.g. some microbes release hydrogen, while others ingest it in order to reduce carbon dioxide to methane; still other bacteria feed on dead cells. A species may prefer a specific environment, or an environment may be so extreme that only a few species can survive. New tools, such as genetic probes, physiological indicators, and heritable fluorescent labels have made it feasible for microbiologists to locate, identify, and track environmental microbes [29, 35–38, 42]. Statistical techniques devised by terrestrial ecologists for describing and modeling spatial data (*see* **Spatial analysis in ecology**) are of potential use to environmental microbiologists.

### Cell–Cell Signaling

The structure of the biofilm and the biochemical activity of biofilm bacteria, as well as the activity of planktonic bacteria, can be modulated by 'signaling molecules', which are both produced and sensed by the microbes. Molecular biologists are now identifying the signaling molecules, discovering the genes that are activated when the cell senses high concentrations of the molecules, and measuring the induced effects on the cell's biochemical activities [28]. By interfering with the signaling process, one might be able to control microbial activity. There is a high chance that cell–cell signaling researchers will discover alternatives to antibiotics and germicides. There is even evidence that signaling molecules, or analogs of such molecules, can be used to prevent surface colonization in marine systems [9]. The use of signaling molecules to modify bacterial behavior, instead of attempting to kill bacteria, is potentially an environmentally friendly approach. It is expected that such a treatment (a) leaves the desirable bacteria undamaged, (b) does not cause bacteria to initiate resistance mechanisms, and (c) is not generally toxic to the broader environment. Cell–cell signaling is an exciting area of research in environmental microbiology.

### Acknowledgments

This work was supported by Cooperative Agreement EEC-8907039 between the US National Science Foundation Engineering Research Centers Program and Montana State University – Bozeman. Thanks to John Lisle and Louise Ryan for helpful comments.

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(*See also* **Ames assay; Aquatic toxicology; Ecotoxicology; Water quality**)

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