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Project Summary

Nutrients for Bacterial Growth in Drinking Water: Bioassay Evaluation

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The regrowth of bacteria in drinking water distribution systems can lead to the deterioration of water quality. Pathogenic bacteria are heterotrophs and heterotrophs are probably the dominant bacteria associated with the regrowth phenomenon. Only a portion of the total organic carbon (TOC) in drinking water is biologically labile to heterotrophic bacteria, and a bioassay developed to quantify this assimilable organic carbon (AOC) has been proposed as an index of the regrowth potential of drinking water. We have evaluated both biological and chemical assays for determining AOC as related to regrowth of bacteria in drinking waters from surface water and groundwater sources. *Pseudomonas fluorescens* strain P-17 was used in bioassays for AOC. Dissolved organic carbon (DOC), uv-labile DOC, DOC < 10,000 daltons, monosaccharides, and primary amines were the chemical assays used to predict concentrations of AOC. Growth of P-17 was enumerated as viable and total cells with spread plates and direct epifluorescence microscopy, respectively. AOC concentrations in surface waters ranged from 48 to 607 μg liter^{-1} and in a groundwater supply from 40 to 146 μg liter^{-1}. AOC remained relatively constant or declined in distribution systems with distance from the source. Incubation vessel surface to volume ratio influenced the AOC value by enhancing wall growth of reversibly attached cells. The bioassay assumes that (1) organic carbon limits growth of the bioassay organism, (2) the yield of the bioassay organism on naturally occurring AOC is constant and equal to yield on model organic compounds, and (3) the bioassay organism is an appropriate surrogate for the native microflora of distribution systems in utilizing AOC. We have found that phosphorus additions to some test waters were required to generate carbon limitation and that yield on naturally occurring AOC approximates the yield on acetate. Correlations of the bioassay AOC with chemical determinations were poor, but with improvements we have made in the handling of the test water, glassware, and P-17, we suggest that the bioassay holds promise for a simple, routine measure of drinking water regrowth potential.

This Project Summary was developed by EPA's Risk Reduction Engineering Laboratory, Cincinnati, OH, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

The regrowth of bacteria in drinking water distribution systems can lead to the deterioration of water quality and even non-compliance of a supply. Bacterial growth occurs on the walls of the distribution system, and in the water either as free living cells or cells attached to suspended solids. Regrowth of bacteria is a multi-faceted phenomenon

influenced by temperature, residence time in mains and storage units, the efficacy of disinfection, and nutrients. Secondary parameters probably include redox potential, pH, inoculum size, shear stress, main construction material, and chlorine residual.

Regrowth within distribution systems has largely been associated with heterotrophic bacteria, organisms which oxidize reduced carbon compounds for energy and also require these organic molecules as a source of carbon "building blocks" for biosynthesis. Some of those bacteria are opportunistic pathogens, and the growth of heterotrophs may establish conditions conducive to the growth of pathogenic bacteria. Additionally, large numbers of heterotrophs have been shown to interfere with the detection of coliforms, generate anaerobic conditions conducive to corrosion of pipe materials, and increase chlorine demand.

Most of the energy and carbon for heterotrophs in distribution systems presumably comes from dissolved organic molecules in the source water, and quantifying that nutrient supply was a major focus of the research reported here. The heterogeneous mixture of DOC in groundwaters and surface waters used for drinking water supplies ranges in complexity from large molecules of humic, fulvic, and hydrophilic acids, to relatively simple compounds such as carbohydrates, carboxylic acids, amino acids, and hydrocarbons. It is the simple compounds which are most susceptible to microbial decomposition and they have been collectively referred to as biologically labile DOC in the ecological literature.

The concept of labile DOC developed in the ecological literature has been applied to drinking water studies as the AOC concept. The need to quantify AOC has arisen, in part, because an easily quantified chemical parameter used in the drinking water industry, TOC, has not been found to be a good predictor of bacterial regrowth. The failure of TOC to predict regrowth is understandable because the ratio of AOC to TOC is not a constant. However, AOC must be viewed as one variable in a complex regrowth equation, and the ability to quantify AOC will be one step in the process of understanding and eventually predicting when and where regrowth will occur.

The research which is reported here was undertaken as a cooperative agreement with the U.S. EPA in an attempt to develop a refined and field validated AOC assay which could be used by water utility technicians. The research included an evaluation and modification of the bioassay technique of D. van der Kooij and associates of the Netherlands, and an exploration of possible chemical assays as an alternative to the bioassay. We have also generated data which describe changes in AOC between raw and finished water, and AOC changes during the transport of finished water through two distribution systems.

Procedure

Preparation of Inoculum for AOC Bioassay—Two different types of inocula were used in these studies, log phase cells and stationary phase cells of *Pseudomonas fluorescens* strain P-17. An inoculum required to yield 1000 cells ml^{-1} was added with a sterile microliter pipette to each bioassay vessel. Prior to inoculation of a bioassay vessel with P-17, a direct microscopic count was performed to determine the appropriate volume of inoculum.

Preparation of Incubation Water—Finished drinking water used for bioassays was collected in organic carbon free glassware containing 33.3 mg $liter^{-1}$ sodium thiosulfate and processed either by pasteurization or filtration. Pasteurization was performed by placing the incubation vessels into a water bath heated to 60°C for 0.5 h.

Preparation of Bioassay Vessels and Other Glassware—Glassware cleaning involves a detergent wash, four rinses with hot tap water, three rinses with 0.1N HCl, four rinses with deionized water, and heating to 550°C for 6 h. The pipettes were treated in the same manner, except that there was a 4 h cold tap water rinse in a pipette washer between the detergent wash and the hot water rinses. Three different incubation vessels were used, 1 liter Erlenmeyer flasks with ground glass stoppers, BOD bottles, and 45 ml borosilicate vials with teflon lined silicone septa. Commercially cleaned 45 ml borosilicate vials were used without any prior treatment.

Enumeration of Bioassay Organism—Incubation vessels were sampled using asceptic technique and organic carbon free sterile pipettes. Viable count samples were placed into test tubes containing sterile phosphate buffer and serially diluted to 10^{-2}, 10^{-3} and 10^{-4}. Repeated samplings over several days were taken from the Erlenmeyer flasks and the BOD bottles. The small size of the vials allowed sufficient replication such that each vial was sampled once, and ferent vials were used to follow change in population numbers over tir

Measurement of Cell Size and Ca lation of Biovolume—Cell size was de mined on formalin fixed samples stai with acridine orange. P-17 is rod shaf so the volume was calculated from formula for a prolate spheroid, V = 4, (L/2) $(W/2)^2$. A minimum of twenty c from a given bioassay vessel were me ured.

Determination of Cellular Carbon C tent—Cells from incubation vessels w filtered onto an organic carbon free gl fiber filter (Gelman A/E). Filters w oven dried at 60°C for 1 h a combusted in a Carlo Erba Model 1 Elemental Analyzer* set up for carb nitrogen, and hydrogen analysis. Divid the amount of carbon on a filter by number of cells per filter resulted in estimate of carbon per cell.

Chemical Characterization of As Water—All glassware used for orga analyses was muffled at 550°C to eli inate organic carbon contamination. D was analyzed in either a Dohrmann 80 which used uv-promoted wet oxidat or an OI 700 which used persulfa oxidation at elevated temperatur (100°C). Low molecular weight DOC w determined by ultrafiltration through p cleaned membranes in a 80 ml stir cell. Uv-labile DOC was determined by 1 h uv irradiation of water samples held quartz tubes without any additior oxidant. DOC was measured before a after the irradiation step, with t difference being uv-labile DOC. Prima amines were measured by a fluoromet technique using fluorescamine, a monosaccharides were assayed using spectrophotometric MBTH method.

Estimation of AOC Concentrations Two different experimental approach and designs were used to estimate AC concentrations, (1) a bioassay base upon the work of van der Kooij, and the direct measurement of DOC upta by P-17. Both approaches require growth of P-17 to stationary phase and estimation of cell numbers. The bioass approach further required an estimate yield while the carbon uptake approa required an estimate of carbon per cell.

The experimental design for the bi assay approach initially involved sar pling the incubation vessels on five da during stationary phase. Triplicate ve

* Mention of trade names or commercial product does not constitute endorsement or recom mendation for use

sels were used for each of five acetate concentrations. Each vessel was subsampled in duplicate for both direct microscopic counts and viable counts. The subsamples for viable counts were serially diluted, and a single spread plate prepared for each dilution used. A single filter was prepared from each subsample used for direct microscopy. Beginning in May of 1987, direct microscopic counts were limited to a single sampling date and the 0 and 250 μg C liter^{-1} acetate concentrations, and after May of 1987, viable counts were determined on 4 rather than the original 5 sampling dates. In the bioassay approach, yield of P-17 on acetate was empirically derived for viable and total cells through November of 1987. Following that, only unamended test water was used and the mean of all previous yield values was applied to the cell number data.

The direct measurement of DOC changes in incubation vessels required separating the organic carbon contained in P-17 from solution without artifact. Technical problems in accomplishing sterile filtration without organic carbon contamination necessitated the determination of TOC (including P-17 carbon) and the calculation of DOC changes from estimates of P-17 densities and carbon per cell. The experimental design used with this approach involved four replicate vessels of unamended test water and duplicate determinations of TOC concentrations in the vessels just prior to inoculation and on the last sampling date for stationary phase. Measurements of organic carbon changes within the incubation vessels and the densities of P-17 at stationary phase were used to calculate the yield of P-17 on naturally occurring AOC.

When BOD bottles were used as incubation vessels, samples were poured from the BOD bottles into vials for carbon analysis. When vials were the incubation vessels, replicate vials were sacrificed after the heat fixation step for an initial carbon analysis. An equivalent P-17 inoculum was added to carbon blanks to verify that the inoculum was not a source of measurable carbon. Controls for the abiotic adsorption of TOC by the carbon free glassware were carried through heat treatment, but not inoculated.

Evaluation of P-17 as a Surrogate for the Native Microflora of Water Distribution Systems—Sterile, carbon free microscope slides were placed into a test tube rack and suspended in a 100 liter polyethylene tank continuously fed by finished water from the distribution system. After 4 months of incubation, slides were removed and placed into a rack submerged in site water. The slides were transported back to our laboratory and scraped with an organic carbon free razor blade. The scrapings and rinses were combined in a Corex tube, concentrated by centrifugation, sampled for direct and viable cell enumeration, and used as inocula in heat treated, dechlorinated test water.

Study Sites

Three different water supplies were sampled during the course of this investigation, the Chester Water Authority reservoir on the Octoraro Creek in Lancaster County, PA, Well number 18 in Chester County, PA from the Great Valley Division of the Philadelphia Suburban Water Company, and Pickering Creek main pump station in Delaware County, PA of the Philadelphia Suburban Water Company (Figure 1).

Results and Discussion

Bacteriology of Water Used for AOC Bioassays—None of the test water used for bioassays had persistent coliform problems. The Maximum Contaminant Level established by the U.S. Environmental Protection Agency is 1 coliform (100 ml)$^{-1}$ as a quality limit and 4 (100 ml)$^{-1}$ as an action limit. The only detected and verified coliforms in finished water were associated with Pickering Creek water in January when a taste and odor problem occurred.

Chemistry of Drinking Waters Used for AOC Bioassays—The correlation of organic constituents with AOC concentrations was tested using data from 10 separate dates and 2 different water sources. None of the correlations explained more than 16% of the variability in the AOC data (Table 1).

Table 1. *Correlation of Organic Constituents in Bioassay Water with AOC*

Organic Constituent	*Correlation Coefficient (r)*
DOC	*0.397*
UV-Labile DOC	*0.268*
DOC < 10,000 Nominal Molecular Weight	*0.032*
Primary Amines	*0.162*
Monosaccharides	*0.381*

Apparently the source of uv irradiation used in this study was much more effective at oxidizing organic carbon than was P-17, and the molecular weight cut-off of 10,000 daltons may have been too high to have had much biological significance. Primary amines and monosaccharides can both be used by P-17, and the carbon equivalents in these organic compounds can be used as a check on AOC determinations as minimum estimates, but the plethora of additional carbon molecules available in drinking water to P-17 probably kept these classes of compounds from being good predictors of AOC concentrations.

Because simpler, faster chemical alternatives to the AOC bioassay were not found, we proceeded to work on validation of the bioassay. The bioassay assumes that (1) organic carbon is limiting to the growth of the bioassay organism, (2) the yield of the bioassay organism on naturally occurring AOC is constant and equal to yield on model organic compounds, and (3) the bioassay organism is as capable as the native microflora in a water distribution system in utilizing AOC.

Influence of Incubation Vessel Size—The 1 liter Erlenmeyer flasks with ground glass stoppers are relatively expensive and awkward to handle. BOD bottles and eventually 45 ml vials were substituted for the flasks. Changing the size of the incubation vessel did not diminish the precision of the assay, as shown in Table 2. In Table 2, the replicate spread plate variation represents duplicate spread plates prepared from a single dilution tube. This was performed as a quality control measure on approximately 10% of the spread plates. The estimate of within vessel variation was derived from all duplicate samples from vessels, identified by level of acetate addition, vessel replicate, and sample date. The between vessel variation was derived from the triplicate vessels, separated by level of acetate addition and sample date, and the between incubation day variation was derived from the estimates of P-17 densities at stationary phase over several days, separated by site, initial sample date, vessel type, and level of acetate addition.

Although the number of data for the vials are limited, the current data indicate that within vessel, between vessel, and between sampling day variation were unaffected by reducing the size of the incubation vessel. Several benefits were gained by using the vials, especially the simplification of glassware preparation. The vials are inexpensive, so different vials can be used for each sampling

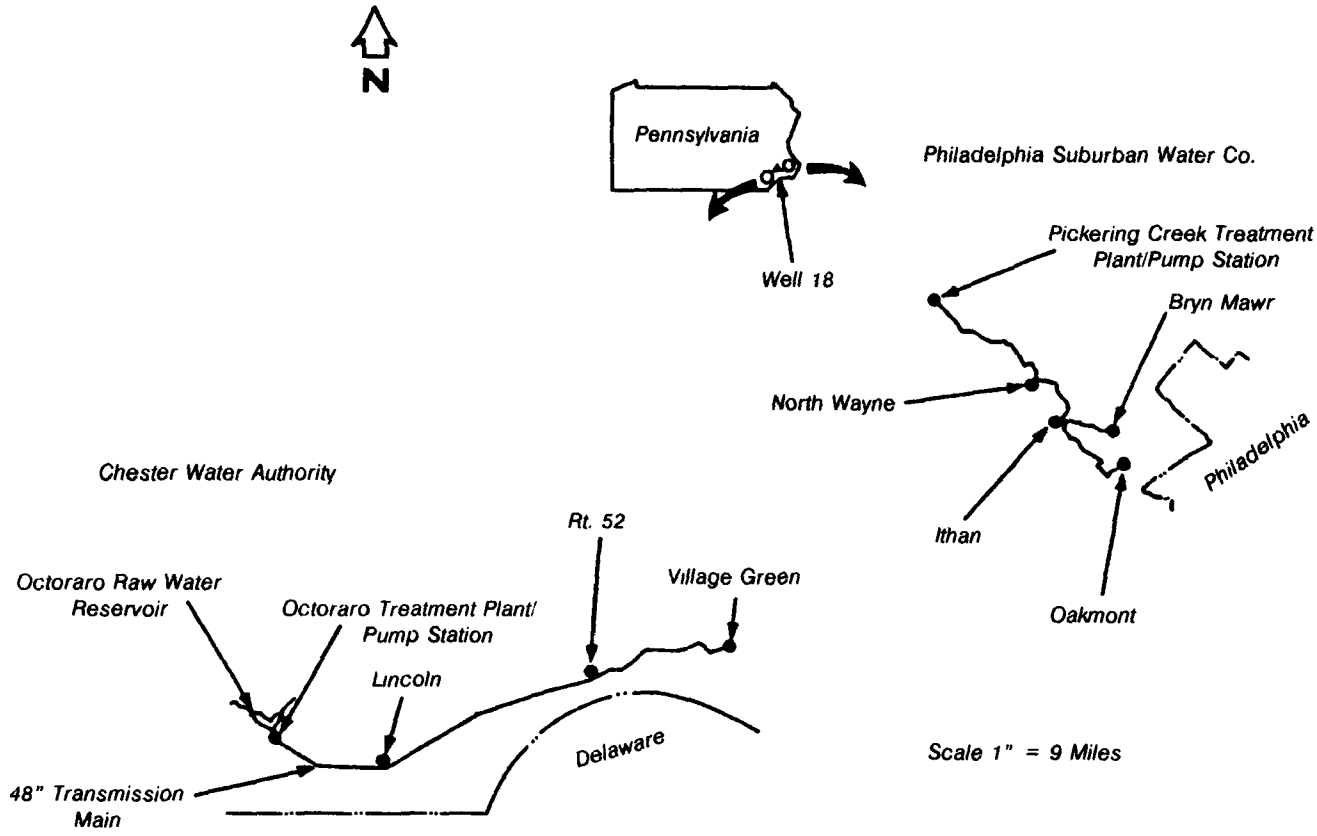

Figure 1. *Map of study sites.*

Table 2. *Estimates of Precision for Different Vessels Used in the AOC Bioassay*

	Coefficient of Variation*			
Vessel Type	*Replicate Spread Plates*	*Within Vessel Variation*	*Between Vessel Variation*	*Between Incubation Day Variation*
Flasks	*9.9 ± 6.7 (150)*	*11.2 ± 9.0 (280)*	*13.7 ± 9.5 (95)*	*11.4 ± 7.7 (20)*
BOD Bottles		*10.8 ± 9.9 (821)*	*15.6 ± 11.7 (249)*	*15.7 ± 7.5 (62)*
Vials	*12.1 ± 10.5 (102)*	*25.0 ± 19.1 (24)*	*13.4 (1)*	

**Coefficient of variation is calculated from (x ± SD) 100, and the data are expressed as x ± SD (n).*

during stationary phase. This avoids repeated sampling of the same bioassay vessel over a period of days, eliminating the need for organic carbon free pipettes and reducing the risk of carbon or bacterial contamination. Preliminary tests with commercially available precleaned vials and teflon faced silicone septa show less than 20 µg C liter^{-1} of contamination.

The use of the smaller vessels with higher surface to volume ratios yielded higher densities of P-17 in stationary phase (Figure 2). The differences between cell densities in the vials, BOD bottles, and the flasks are believed to be related to wall effects. The dimensions of the vials, BOD bottles, and flasks are such that the surface to volume ratio of the vial is twice that of the BOD bottle and three times that of the flask. The benefits to bacteria of association with surfaces in nutrient poor environments, i.e. higher concentration of nutrients, was demonstrated for *E. coli* using glass beads 40 years ago and it appears that increased surface area is advantageous for P-17 under our culture conditions.

Comparison of Viable and Total Cell Enumerations—In general the density of total cells, determined by direct microscopy, exceeded the density of viable cells determined as colony forming units, by a factor of 1.6 (1.6 ± 0.6 (39) $\bar{x}$ ± S (n) (Figure 3)). This relationship seem reasonable in that P-17 is easily culture on nutrient agar and the population a stationary phase would be expected t contain a significant proportion of dea cells.

Cell Yield—The influence of all enum erating techniques on yield is shown i Figure 4. Assuming carbon limitation, th yield of P-17 on acetate should not var between test waters, and there are n reasons to expect variations in yiel based upon viable or direct enumeratio of P-17 or vessel size. Phosphorus limita tion was observed only at elevated level

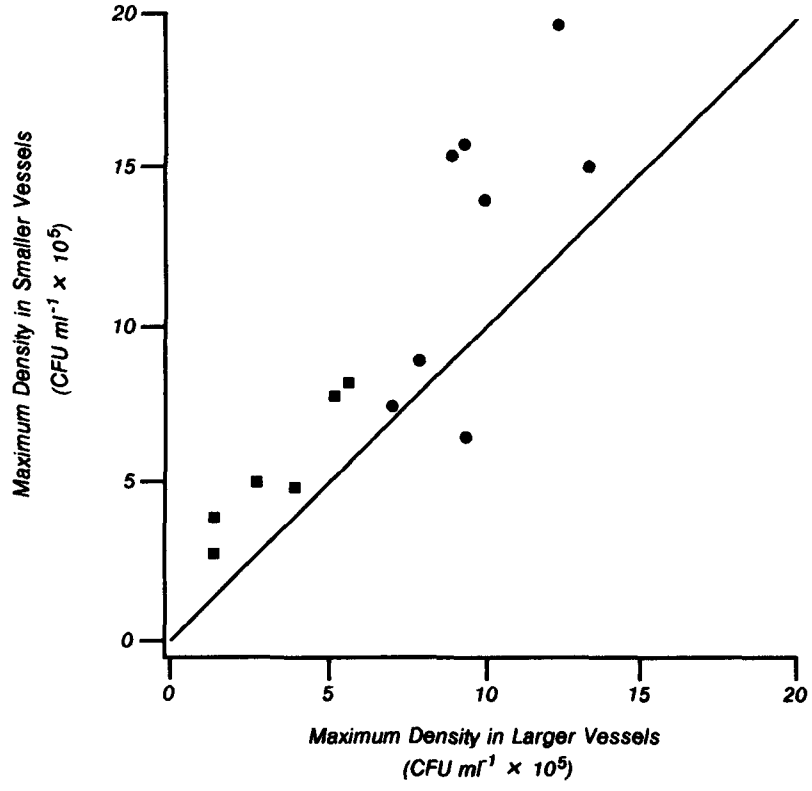

Figure 2. *Influence of vessel size on densities of P-17: (■) Flask versus BOD bottle (●) BOD bottle versus vial comparisons.*

of organic carbon when acetate was added to incubation vessels to measure yield. The assumption of carbon limitation was probably valid in all unamended test waters. Our empirically derived estimates of yield for the entire data set were similar for viable and total cells averaging $3.24 \pm 0.41 \times 10^6$ cfu (µg acetate-C)$^{-1}$ and $3.68 \pm 0.85 \times 10^6$ cell (µg acetate-C)$^{-1}$ ($\bar{x} \pm$ SD (n = 20)), respectively. Where yield was determined for more than one vessel type, cellular yield was found to be independent of vessel size. In those instances, the average yields based upon viable counts were $3.15 \pm 0.30 \times 10^6$ and $3.15 \pm 0.42 \times 10^6$ ($\bar{x} \pm$ SD (n = 7)), and based upon total cells were $3.67 \pm 0.86 \times 10^6$ and $3.53 \pm 1.46 \times 10^6$ ($\bar{x} \pm$ SD (n = 6)) for flasks and BOD bottles, respectively. We also used the estimates of cellular carbon in P-17 determined with the elemental analyzer ($1.73 \pm 0.03 \times 10^{-7}$ µg C cell^{-1} (n = 4)) to convert the cellular yield on acetate to cellular-C yield:

$$\frac{(1.73 \times 10^{-7} \text{ µg C cell}^{-1})}{(3.24 \times 10^6 \text{ cfu (µg acetate-C)}^{-1})} = 0.56 \text{ µg cell-C (µg acetate-C)}^{-1}$$

Theoretical yields of heterotrophic bacteria on acetate have been calculated from stoichiometry. With ammonia as the nitrogen source, the theoretical yield on acetate was 0.36, and when nitrate was the nitrogen source, 0.28. These values, however, are expressed as g cells (g substrate)$^{-1}$ and can be converted to units of C assuming that 50% of cell dry weight is C, and the knowledge that 40% of acetate is C. The adjusted values then become 0.45 and 0.38, respectively.

AOC Concentrations in Field Samples—Unlike the estimates of P-17 yield on acetate-C, the estimates of AOC concentration are very dependent upon both P-17 enumeration technique and vessel size. The influence of enumeration technique is fairly obvious. Direct enumeration of total cells, as discussed above, gives higher values for maximum cell densities which will translate into higher AOC concentrations when divided by a constant yield factor. The influence of vessel type is harder to explain, but probably involves wall effects as discussed above. The impact of these findings on the AOC bioassay is that they clearly require the assay to be operationally defined as to vessel size and enumeration technique if comparable data are to be generated.

Keeping those caveats in mind, the AOC values measured in this study ranged from 48 to 607 µg C liter^{-1} and were generally lowest for the Well 18 water, intermediate for Chester Water Authority, and highest for Philadelphia Suburban's Pickering Creek distribution system. No seasonal patterns were apparent in the surface water supplies, but there is some evidence of biological stabilization of the water within the distribution systems (Figure 5). This was most noticeable in the Pickering Creek system, especially in the January and February samples. In the Chester Water Authority system and the Pickering Creek system during the April sample, little change was observed in AOC beyond the raw water.

Yield on Naturally Occurring AOC—An important assumption in the AOC bioassay is that the yield of P-17 on acetate-C is equivalent to the yield of P-17 on naturally occurring AOC. Previous studies with P-17 have shown that the yield of P-17 on amino acids, carboxylic acids, carbohydrates, and aromatic acids ranged from 3.2 to 7.8 x 10^6 cells (µg carbon)$^{-1}$. Our approach to determining the yield on naturally occurring AOC was to perform carbon mass balance measurements in the incubation vessels. Rather than separate the P-17 cells from stationary phase cultures for the measurement of organic carbon uptake, we measured the TOC in the incubation water, thus including cellular carbon from the bioassay organisms present in the water. We also measured the carbon content of P-17 cells and applied that conversion factor to the calculation of carbon changes during incubation. The data presented in Figure 6 represent values which assume complete oxidation of the cellular carbon present during the TOC analyses. AOC concentrations based upon these separate estimation techniques were very similar, implying that the yield of P-17 on acetate-C is a reasonable approximation of the yield on naturally occurring AOC.

Distribution System Microflora—A final question addressed in this study was the suitability of P-17 as a surrogate for heterotrophic bacteria within distribution systems. The initial enumeration of inocula used in this comparison indicated that the density of bacteria on the slides incubated for 4 months at the Bryn Mawr site was 6×10^4 cell cm^{-2}, and that only

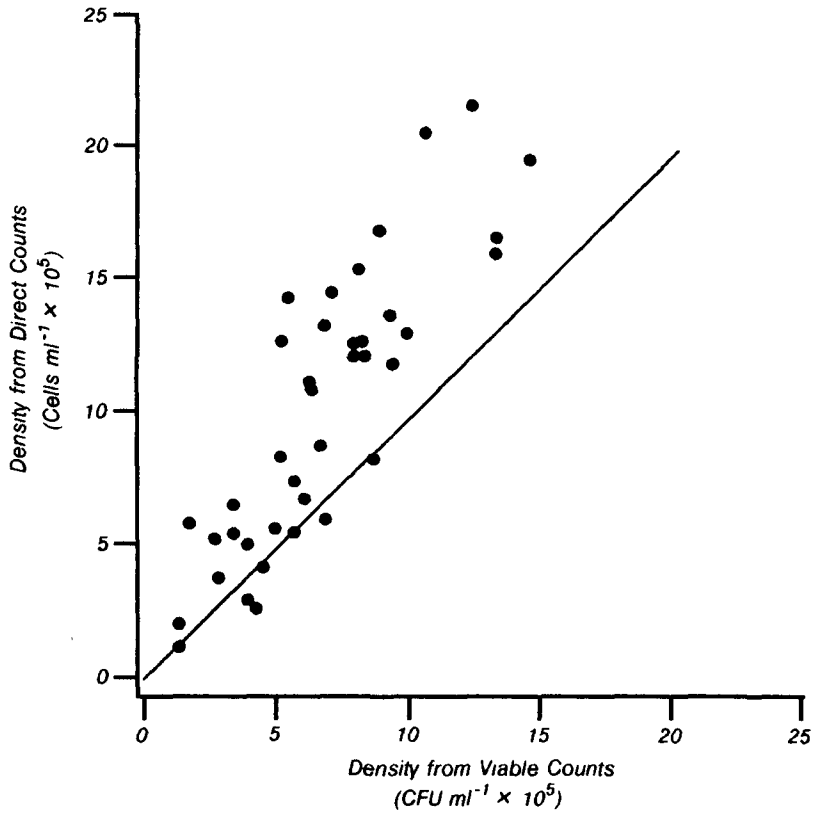

Figure 3. *Influence of cell enumeration technique on densities of P-17.*

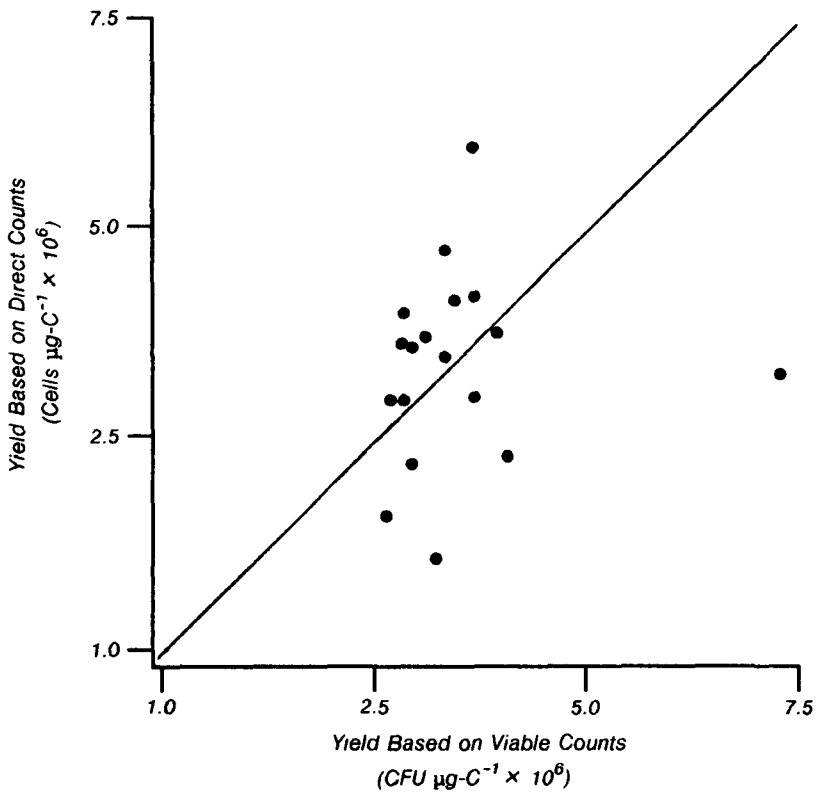

Figure 4. *Influence of cell enumeration technique on yield.*

0.5% of the cells were viable. In contras 56% of the P-17 inoculum consisted c viable cells. The viability figure for th native microflora must be viewed wit caution, as many of the organisms ma have been viable but not culturabl under the conditions provided. Durin the 9 day incubation period, P-1 densities in the incubation vessel increased from 1 x 10^3 to 1.81 x 10^6 cf ml^{-1}, while no growth was detected in th native microflora. TOC measurement performed along with the cell enum eration showed a decrease of 240 µg (liter^{-1} in the presence of P-17 and a most a 42 µg C liter^{-1} decrease in th presence of the native microflora. Man questions remain unanswered by thi initial experiment, not the least of whicl is the metabolic condition of the nativ microflora used as an inoculum.

Conclusions

1. The AOC bioassay of van der Koo can be simplified and the risk o carbon and bacterial contaminatio reduced by changing the type o incubation vessel used and changin the protocol for sampling incubatio vessels.
2. Simple chemical assays for constitu ents of labile DOC or assays pro posed as surrogates for the tota labile DOC pool do not correlate wel with the AOC bioassay results.
3. The bioassay organism proposed b van der Kooij, *Pseudomonas fluores cens* strain P-17, grows primarily i suspension rather than attached t the walls of incubation vessels However, incubation of P-17 i vessels with greater surface t volume ratios yields higher concen trations of the bioassay organism.
4. The yield of P-17 on acetate empir ically determined in various source waters is relatively constant an agrees with theoretical yields calcu lated on the basis of stoichiometry.
5. Empirical determinations of the yiel of P-17 on acetate are subject t phosphorus limitation in some drink ing waters.
6. The yield of P-17 on acetate is a reasonable approximation of th yield of P-17 on naturally occurrin AOC.
7. Preliminary data used to evaluat P-17 as a surrogate for the native heterotrophic microflora in a wate distribution system are inconclusive.
8. AOC concentrations were generall lower in groundwater than in surface waters.

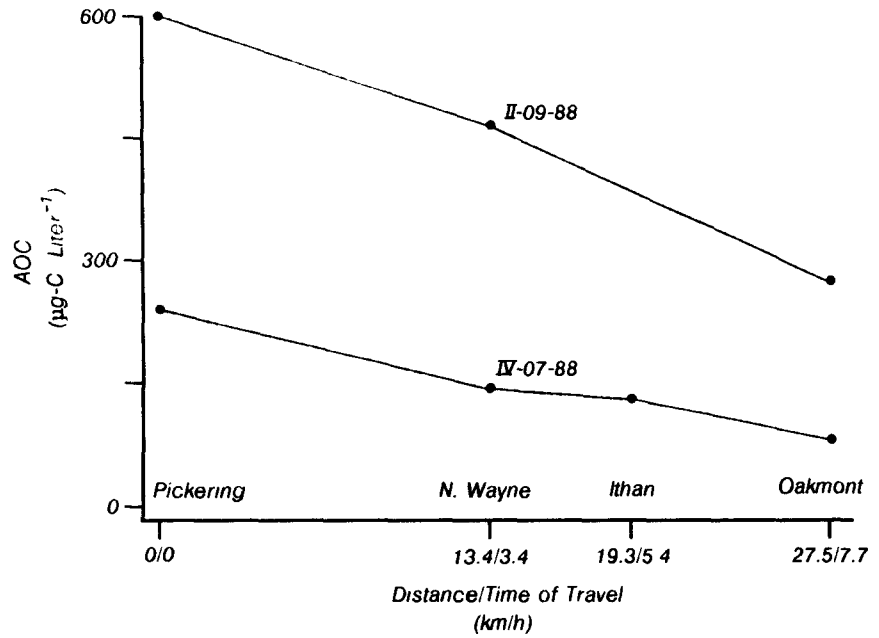

Figure 5. *AOC concentrations in a water distribution system.*

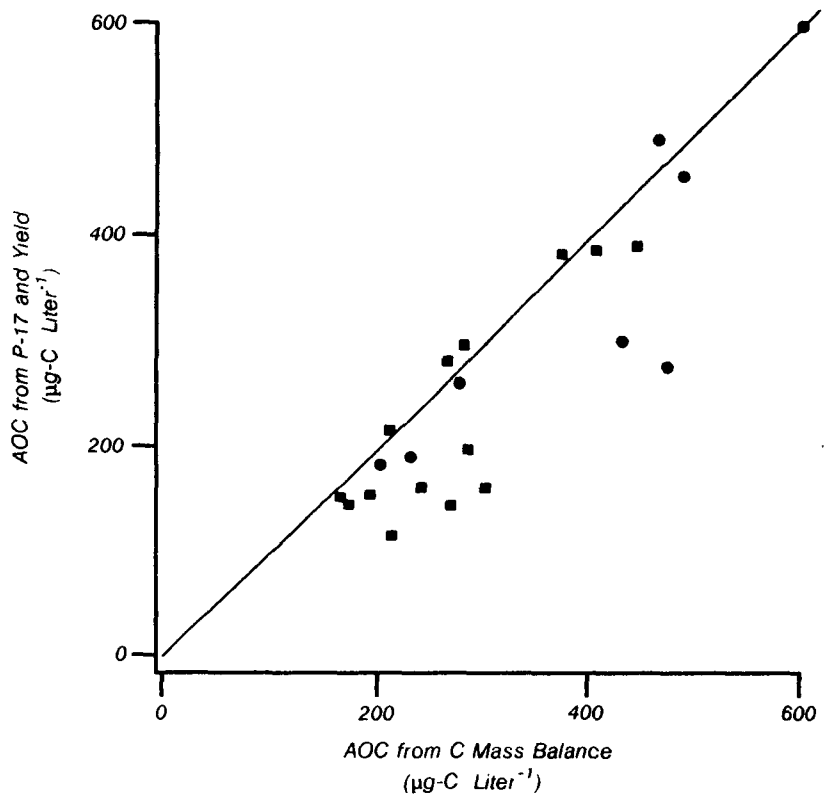

Figure 6. *AOC concentrations estimated from P-17 density and yield compared to estimated from carbon mass balance: (■) BOD bottles; (●) vials.*

9. No seasonal patterns in surface water AOC concentrations were apparent, but there was evidence of biological stabilization of water within a distribution system.

Recommendations

1. The simplified AOC bioassay developed as part of this research project needs to be tested with a larger number of water types and users.
2. The yield of P-17 on naturally occurring AOC needs to be measured in a greater number of water types.
3. Any AOC bioassay needs to be standardized to vessel type and method of organism enumeration.
4. The AOC bioassay should be performed over at least three separate days and criteria developed for acceptance or rejection of the bioassay results.
5. More research is needed as to the efficacy of P-17 versus the native microflora of water distribution systems in the utilization of and subsequent growth on AOC.

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The complete report, entitled "Nutrients for Bacterial Growth in Drinking Water: Bioassay Evaluation," (Order No. PB 89-213 995/AS; Cost: $15.95, subject to change) will be available only from:
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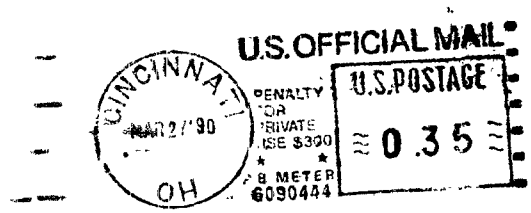

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