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Development of a Rapid Assimilable Organic Carbon Method for Water

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A rapid method for measurement of assimilable organic carbon (AOC) is proposed. The time needed to perform the assay is reduced by increasing the incubation temperature and increasing the inoculum density. The ATP luciferin-luciferase method quickly enumerates the test organisms without the need for plate count media or dilution bottles. There was no significant difference between AOC values determined with strain P17 for the ATP and plate count procedures. For strain NOX, the plate count procedure underestimated bacterial levels in some samples. Comparison of AOC values obtained by the Belleville laboratory (by the ATP technique) and the Stroud Water Research Center (by plate counts) showed that values were significantly correlated and not significantly different. The study concludes that the rapid AOC method can quickly determine the bacterial growth potential of water within 2 to 4 days.

The assimilable organic carbon (AOC) test was first proposed by Van der Kooij in 1978 (10, 11, 17). Briefly, the test involves collection of water in very clean, AOC-free glassware. The water sample is heated to kill the indigenous bacterial population and inoculated with one or more test organisms. The sample is incubated, and growth of the test organisms is monitored. The stationary-phase level (N_{max}) of bacteria is proportional to the amount of limiting nutrient in the water. The nutrient level in a sample is converted into carbon equivalents with an empirically derived yield coefficient of the organism for a selected growth substrate (13). Cell yield for different carbon compounds may vary (11), and the limiting nutrient may not be carbon. Thus, the AOC test is best thought of as an indication of the growth potential of the water and not as a direct measurement of biodegradable carbon (13).

The AOC test has been found to be a useful tool for predicting growth of coliform and heterotrophic plate count bacteria in water (1–3, 6–8, 12, 13, 16). Growth of heterotrophic plate count bacteria is limited in unchlorinated drinking water at AOC levels of <10 to 20 μg/liter (12, 13, 16). Problems with excessive levels of coliform bacteria in chlorinated water may be limited at AOC levels ranging between 50 and 100 μg/liter (6–8).

There are problems that impede the widespread application of the AOC test including production of AOC-free glassware, the long incubation time, dilutions, media, and time associated with plate counts of the target organisms. Some advances, however, have been made. Kaplan et al. (4, 5) have suggested the use of precleaned, commercially available 40-ml vials as a means of simplifying the glassware portion of the procedure. The current research proposes procedures to reduce the time needed to perform the assay by increasing the incubation temperature, increasing the inoculum density, and using the ATP luciferin-luciferase method to quickly enumerate the test organisms.

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MATERIALS AND METHODS

Bacterial strains. Cultures of *Pseudomonas fluorescens* P17 and *Spirillum* strain NOX were obtained from D. Van der Kooij, KIWA, Nieuwegein, The Netherlands. Cultures were stored in a solution of 20% glycerol–2% peptone at −70°C.

Prior to use, the cultures were retrieved from the freezer and streaked for purity on R_2A agar (Difco Laboratories, Detroit, Mich.) and incubated at room temperature (20 to 22°C) for 3 to 5 days. The cultures were adapted to low-nutrient growth by inoculating an isolated colony into 100 ml of sterile, chlorine-neutralized tap water. The tap water was incubated at room temperature for 7 days. An aliquot (0.1 ml) of the tap water-adapted culture was used to inoculate 100 ml of a sodium acetate solution. The sodium acetate solution contained 11.34 mg of sodium acetate (2,000 μg of acetate carbon per liter) in the following buffer: 7.0 mg of K_2HPO_4, 3.0 mg of KH_2PO_4, 0.1 mg of $MgSO_4 \cdot 7H_2O$, 1.0 mg of $(NH_4)SO_4$, 0.1 mg of NaCl, and 1.0 μg of $FeSO_4$ (all quantities are per liter). The sodium acetate solution was incubated at room temperature for 7 days. Bacterial counts were generally in the range of 8.2×10^6 to 2.4×10^7 CFU/ml for strains P17 and NOX, respectively. The sodium acetate solution was used as a working stock culture to inoculate AOC experiments. Cell counts were generally stable for 30 days at room temperature.

Collection and preparation of water samples. To determine AOC, nine 45-ml organic carbon-free borosilicate glass vials (Scientific Specialities Service, Inc., Randallstown, Md.) with Teflon-lined silicon septa (septraseal) were filled to the shoulder (approximately 40 ml) with the water sample. Prior to filling the vials, raw water was prefiltered through organic carbon-free, 25-mm glass fiber filters (type GF/F; Whatman Ltd., Maidstone, England) with an in-line filter (model 4320; Gelman Sciences Inc., Ann Arbor, Mich.). Experiments showed that turbidity in the raw water samples could interfere with accurate ATP determinations and bacterial plate count enumerations (data not shown). Vials containing chlorinated water samples were neutralized with 0.1 ml of a sodium thiosulfate solution (13.2 g/liter) (prepared with

AOC-free water). Kaplan and Bott (4) found that the addition of thiosulfate did not significantly stimulate the growth of P17 or NOX.

To kill vegetative cells in the water, vials were placed in a rack or basket and put into a 70°C water bath for 30 min. After cooling, vials were placed in a foam mailing carton (Safesend vial kit, Scientific Specialities Service, Inc.) and shipped to the laboratory via overnight delivery. No significant bacterial growth occurred in the pasteurized vials during the 24-h shipping time.

In the laboratory, six vials were inoculated with approximately 10^4 CFU of either strain P-17 or strain NOX per ml, final concentration (three vials of each strain). Inoculated vials were incubated at room temperature for 1 to 3 days. Three vials were set aside as uninoculated controls. These controls were stored at 4°C to prevent growth of indigenous bacteria surviving pasteurization.

Bacterial enumeration. (i) Plate counts. Strains P-17 and NOX were enumerated by spread plating decimal dilutions of phosphate buffer (American Public Health Association) on R_2A agar. Plates were incubated at room temperature for 3 to 5 days.

(ii) Determination of ATP. The contents of the vial were filtered through a polyvinylidene difluoride membrane filter (25 mm; pore size, 0.22 μm) (Durapore GC; Millipore Corp., Bedford, Mass.) under a vacuum of 152.4 mm (6 in.) of Hg. Without being allowed to dry, the filter was placed into a solution of 0.5 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.75) and 0.5 ml of ATP releasing agent with phosphatase inhibitor (Turner Designs, Sunnyvale, Calif.) and vortexed for 10 s. Care was taken to ensure that the filter was completely immersed in the buffered releasing agent. After 20 min of contact time, 150 μl was placed into a polypropylene test tube (8 by 50 mm), and the tube was placed into the model TD-20e luminometer (Turner Designs). Luciferin-luciferase (100 μl) was injected into the sample, and after a 5-s delay, the full integral of light was recorded (10-s measurement). ATP standards were obtained from Turner Designs and used according to the manufacturer's instructions.

Quality assurance. Additional vials of water were collected for quality assurance tests. Growth control vials were spiked with 100 μg of acetate carbon per liter and 0.1 ml of a mineral salts solution (per liter of high-pressure liquid chromatography-grade water, 17.1 mg of K_2HPO_4, 76.4 mg of NH_4Cl, and 144 mg of KNO_3). The growth control vials were used to detect growth-inhibitory substances in the water. A blank vial containing just the mineral salts buffer without added carbon was used to detect carbon contamination of the glassware. A yield vial containing 100 μg of acetate carbon per liter and mineral salts buffer was used to check the growth yield of the cultures. For calculation of AOC, the yield values of 4.1×10^6 CFU of acetate-C per μg for P17 and 1.2×10^7 CFU of acetate-C per μg for NOX, as determined by Van der Kooij et al. (14, 17), were used.

Evaluation of membrane filters and reagents. Experiments evaluated the release of ATP from bacteria on three different membrane filters. Cultures of P17 and NOX were filtered through 0.22-μm polyvinylidene difluoride, 0.2-μm polycarbonate (Nuclepore Corp., Pleasanton, Calif.), and 0.22-μm cellulose acetate (Sartorius Inc., Hayward, Calif.) filters. The filters were placed in the ATP releasing buffer and processed as described above.

For some experiments, duplicate samples were processed with releasing agent and luciferin-luciferase from Turner Designs and Lumac bv (Lumit PM, Landgraaf, The Netherlands). The samples were processed as described above.

TABLE 1. Influence of inoculum density on maximum cell density

Strain	Inoculum density (CFU/ml)	Maximum cell density (CFU/ml)[a]
NOX	8.7×10^2	$(4.85 \pm 0.57) \times 10^5$
	4.4×10^4	$(4.65 \pm 2.26) \times 10^5$
P17	4.3×10^2	$(5.61 \pm 1.19) \times 10^5$
	2.2×10^4	$(6.43 \pm 1.62) \times 10^5$

[a] Values are means ± standard deviations; $n = 5$.

Comparison of AOC results between two laboratories. A comparison of AOC results between the Belleville and Stroud laboratories was made. For the trials, sample vials were filled by utility personnel, heat treated, and shipped via overnight delivery to each laboratory. The Belleville laboratory performed the AOC procedure by the ATP method described above. The Stroud Research Center performed the procedure by the plate count method. In addition, the Stroud laboratory inoculated both bacterial strains into the same vial. The P17 and NOX colonies could easily be distinguished from each other by colonial morphology on the plating medium.

Statistical tests. Statistical comparison of different treatments was performed with the Stat-Pak software package (Northwest Analytical, Inc., Portland, Oreg.).

RESULTS

Inoculum density and incubation temperature. To decrease the time required for the culture to reach N_{max}, the inoculum density was increased from the low level (50 to 500 CFU/ml) recommended by Van der Kooij et al. (17) to 10^4 CFU/ml. Results shown in Table 1 indicate that the N_{max} values for the higher inoculum were not statistically different from the low-inoculum values. The time required to reach N_{max} was shortened by five generations (36 to 120 h, depending on temperature and carbon source).

Increasing the incubation temperature is another means of reducing the time required to reach N_{max}. Temperature, however, can have multiple effects, increasing the growth rate but also increasing endogenous respiration rates and decreasing yields. Data shown in Fig. 1 and 2 indicated that

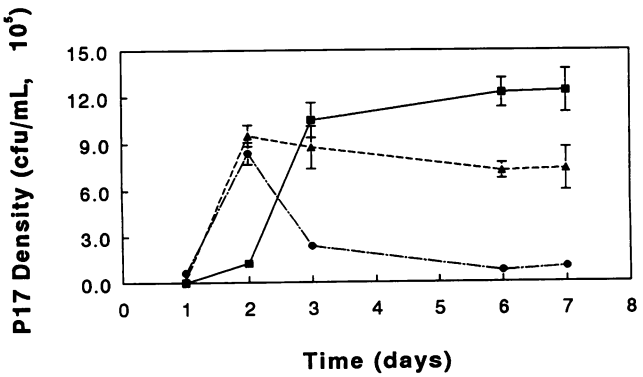

FIG. 1. Growth of strain P17 in water at 15°C (■), 25°C (▲), and 30°C (●). Data are plotted as means ± standard deviations ($n = 3$).

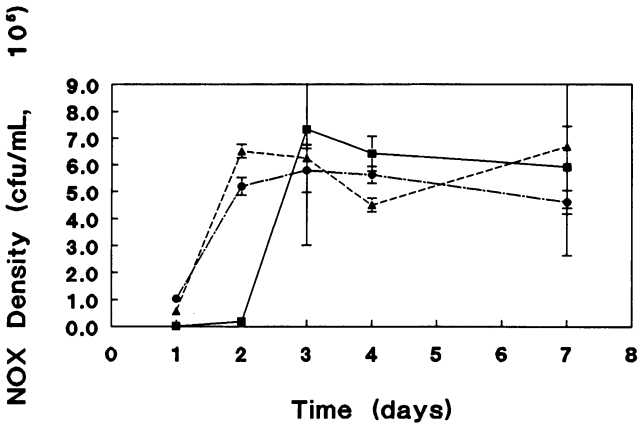

FIG. 2. Growth of strain NOX in water at 15°C (■), 25°C (▲), and 30°C (●). Data are plotted as means ± standard deviations (n = 3).

N_{max} was achieved 24 h faster by growth at 25 and 30°C but that the N_{max} level was reduced, especially at 30°C. The lower growth yields were more apparent for strain P17 than for strain NOX.

Evaluation of the high inoculum level (10^4 CFU/ml) and the 20 to 22°C growth temperature for 54 raw and filtered water samples showed that N_{max} was achieved on day 2 in 28% of the samples and day 3 in 72% of the samples for strain NOX. For strain P17, N_{max} was achieved on day 2 in 18% of the samples and on day 3 in 82% of the samples. N_{max} was never achieved for either strain after only 1 day of incubation.

Filtration and releasing time. Because of the significant level of free ATP in some water samples, it was necessary to separate cellular ATP from non-cell-associated ATP. It was observed that filtration could lower background ATP levels in uninoculated blank water samples by an average of 4.23-fold (range, 0.6- to 15.1-fold). Filtration of the test strains also increased cell densities and permitted a lower limit of detection.

Comparison of ATP levels from bacteria on different filters showed no significant difference between the types of filter material. ATP levels from 10^6 P17 cells on polyvinylidene difluoride, polycarbonate, and cellulose acetate filters averaged 74, 169, and 196 luminescence units, respectively. ATP levels from 10^6 NOX cells averaged 21, 44, and 74 luminescence units for the polyvinylidene difluoride, polycarbonate, and cellulose acetate filters, respectively. Although the results were not statistically different (P ranged between 0.06 and 0.66; n = 4), the cellulose acetate filter generally tended to yield the highest ATP levels. There was no difference in ATP levels when P17 cultures were vacuum filtered (152.4 mm [6 in.] of Hg) or pressure filtered with a syringe and a Swinnex filter holder (Fig. 3). Similar results for pressure and vacuum filtration were obtained for strain NOX (data not shown).

The luminometer manufacturer's instructions recommended evaluating the optimal releasing time for each bacterial culture. The results of releasing time experiments for bacteria on polyvinylidene difluoride (Durapore) and cellulose acetate filters showed that optimal contact time ranged between 10 and 20 min for strain P17 (Fig. 4 and 5). The effect was less dramatic for strain NOX, although the 20-min releasing time tended to be higher than the conventional

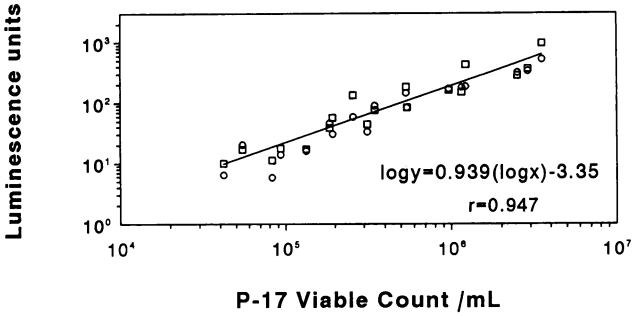

FIG. 3. Comparison of ATP levels in P17 cells processed by pressure (□) and vacuum (○) filtration. ATP levels are expressed in relative luminescence units.

contact time recommended by the manufacturer (30 to 60 s). Comparison of data in Fig. 4 and 5 shows that the cellulose acetate filter averaged slightly higher luminescence levels than the polyvinylidene difluoride filter, although the differences were not statistically significant.

ATP determinations. The relationship between ATP levels and luminescence units showed that the method had a theoretical detection limit of 10^{-12} g of ATP/ml. However, the method had a practical detection limit in the range of 10^{-11} g of ATP/ml. Data shown in Table 2 indicate that the standard error of analysis was 11 to 13.4% for ATP levels ranging from 10^{-8} to 10^{-10} g of ATP/ml. Near the level of detection (10^{-11} g of ATP/ml), the standard error of analysis increased to 30%.

ATP determinations provided an accurate measure of P17 and NOX cell densities (Fig. 6 and 7). A linear relationship with a slope of 0.9 to 1.0 between cell densities and ATP luminescence units was observed. Analysis of the data showed that the average P17 cell contained 1.85×10^{-15} g of ATP and that the average NOX cell contained 2.13×10^{-16} g of ATP (assuming complete extraction). The practical limit of detection, therefore, is approximately 5×10^3 cells per ml for P17 (or an AOC_{P17} of 1.5 μg/liter, based on the yield factors for cells grown on acetate carbon [14, 17]) and 5×10^4 cells per ml for NOX (AOC_{NOX} of 4 μg/liter).

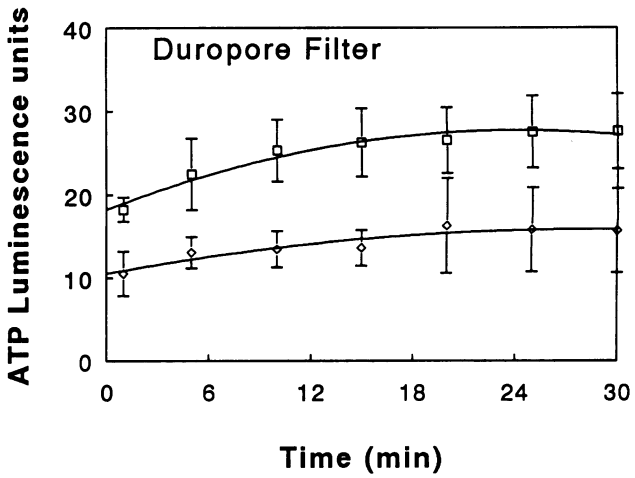

FIG. 4. Effect of releasing time on ATP levels for cells on polyvinylidene difluoride (Duropore) filters. Values are means ± standard deviations (n = 9). □, strain P17; ◇, strain NOX.

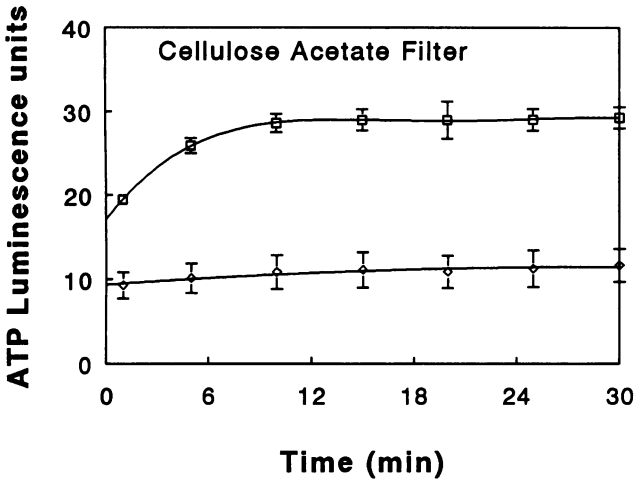

FIG. 5. Effect of releasing time on ATP levels for cells on cellulose acetate filters. Values are means ± standard deviations (n = 9). □, strain P17; ◇, strain NOX.

Reagents. Evaluation of two commercial sources of ATP releasing agents and luciferin-luciferase showed that the two products were not significantly different (P ranged from 0.07 to 0.53). The Turner reagents produced a luminescence value of 80.7 ± 43.9 and 21.3 ± 12.9 for 10^6 cells of P17 and NOX, respectively. The Lumac reagents produced luminescence values of 32.7 ± 22.8 and 16.3 ± 6.2 for 10^6 cells of P17 and NOX, respectively.

Comparison of the ATP and plate count methods. It was the intent of the project to develop a rapid and simple AOC method that would produce results equivalent to those of the conventional plate count method. Evaluation of the ATP and the conventional plate count procedure was conducted by analyzing 54 raw, filtered, and finished drinking water samples from 19 water utilities. To convert ATP luminescence units to AOC values, we used the relationship between cell counts and luminescence units shown in Fig. 6 and 7 and the cell yield factors for acetate carbon reported by Van der Kooij et al. (14, 17). These calculations showed that 1 luminescence unit equaled 1.3 μg of AOC per liter for strain P17 and 3.7 μg/liter for strain NOX.

Data presented in Fig. 8 indicate that the results of the ATP and plate count procedures produced equivalent AOC results for strain P17. There was no significant difference (P = 0.83) between AOC values for the two methods for all samples or when the data were separated into raw and filtered water samples. The correlation coefficient for the two techniques was 0.91.

Results of the ATP and plate count procedures for strain NOX (Fig. 9) were significantly correlated (P < 0.001), although the ATP technique tended to produce higher values. Most noticeable are four datum points (from two sample sites) with ATP-AOC values of 540 to 1,100 μg/liter. The plate count values for these samples ranged between 60 and 80 μg/liter (off by one decimal dilution). When all the data were analyzed, the correlation coefficient was 0.73. When the four variant samples were removed, the correlation coefficient increased to 0.81. AOC analyses from filtered water were better correlated (r = 0.85) than analyses from raw water (r = 0.79).

TABLE 2. Summary of ATP results

ATP (g/ml)	n	Luminescence units	Range	SD	SE
10^{-8}	85	1,061	1,300–806	125	11.8
10^{-9}	94	103.7	131–82	14.8	11.0
10^{-10}	62	10.58	16.1–7.9	1.42	13.4
10^{-11}	62	1.15	2.43–0.35	0.34	30.0
Blank	113	0.34	0.92–0.08	0.16	47.8

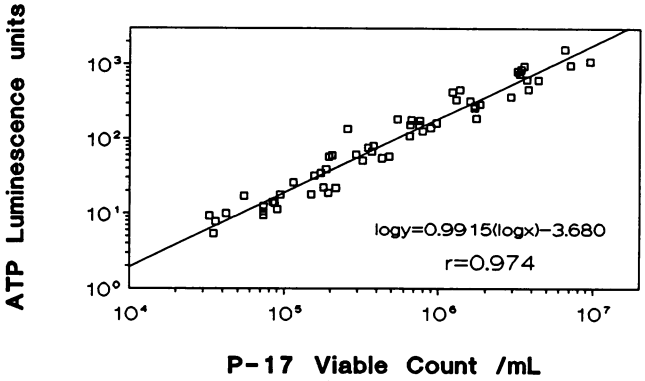

FIG. 6. Relationship between luminescence units and P17 viable counts.

Comparison of AOC results from two laboratories. Because of the difference in the amounts of ATP per cell between strains P17 and NOX, the rapid AOC procedure inoculates each strain into a separate vial. By cultural techniques, both strains are inoculated into the same vial and separated on the basis of colony morphology. Figure 10 shows the comparison between the Belleville laboratory, performing the ATP procedure, and the Stroud Water Research Center, which performed the plate count method. Overall, AOC results were significantly correlated (r = 0.708, n = 48, P < 0.01), and there was no significant difference between AOC concentrations for the two laboratories on the basis of paired t tests for all water types collectively or for each individual water type (raw, filtered, or plant effluent). A one-way analysis of variance showed no significant difference (P ranged between 0.09 and 0.9) among P17, NOX, or total AOC results for individual water types. For the collective water samples, the analysis of variance showed no significant difference (P = 0.23) when the four variant NOX results

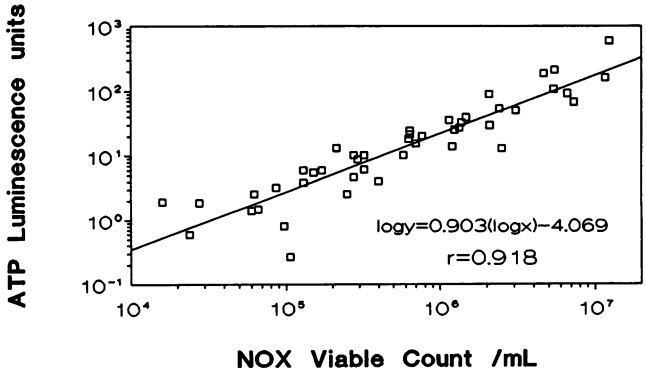

FIG. 7. Relationship between luminescence units and NOX viable counts.

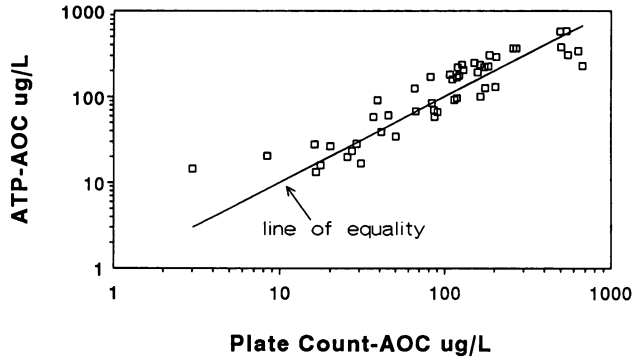

FIG. 8. Comparison of AOC values for strain P17 determined by the ATP procedure and those by the plate count technique. The line of equality represents equivalent results for both methods.

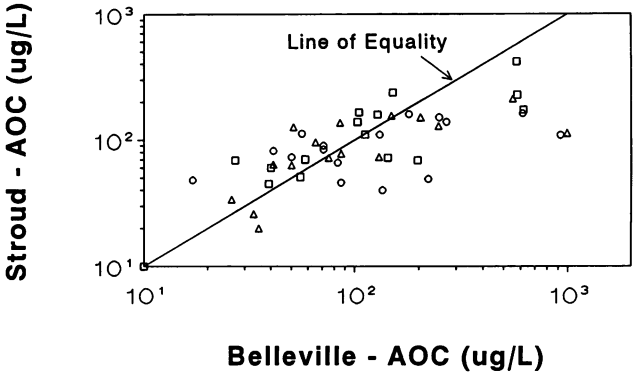

FIG. 10. Comparison of AOC values from the Belleville laboratory (using separate inocula) and the Stroud Water Research Center (using a mixed inoculum). The line of equality represents equivalent results for both techniques. □, raw water samples; ○, filtered water samples; △, plant effluent samples.

were excluded from the test (when they were included, the P value was 0.03).

DISCUSSION

The current research proposes a rapid method for determination of AOC. Use of commercially available glassware helped simplify the procedure. Kaplan et al. (4, 5) had previously suggested the use of 40-ml vials for the AOC assay. They found that the increased ratio of surface area to volume, relative to that of the 1-liter flask used by Van der Kooij et al. (17), produced higher AOC values. In addition, the vials are easier to handle and transport. The cleanliness of commercially available vials, however, may vary (4, 5). Quality assurance procedures outlined in the Materials and Methods section can help detect contaminated glassware.

Increasing the inoculum density and incubation temperature helped reduce the time required for the assay. Van der Kooij et al. originally recommended that growth of the cultures be monitored until N_{max} was achieved, sometimes as long as 5 to 25 days (13–15, 17). Changing the inoculum and temperature levels shortened the assay time to as little as 2 days. We found that N_{max} never occurred after 1 day of incubation. Using the average of the stationary-phase levels, rather than the N_{max} value, helped reduce the variability of the assay. Use of the N_{max} level was found to place emphasis on outlier values, whereas the average of stationary-phase counts takes advantage of the replicate analyses performed over a period of several days. We found that 65.1% of the P17 samples, and 85.9% of the NOX samples, had reached stationary phase after 2 days of incubation. Therefore, monitoring the growth of the cultures for 2 to 4 days would ensure that stationary-phase levels have been achieved.

Enumeration of the test organisms by measurement of ATP levels increased both the speed and simplicity of the assay. Because the test strains, particularly strain NOX, grow slowly, it can require 3 to 5 days before the colonies are countable by the plate count procedure. In addition, preparation of media and dilution bottles and cleanup add to the time required for the assay. Conversely, the ATP procedure takes only minutes to perform. The availability of commercially prepared reagents means that only rehydration is required prior to use. Autoinjection systems can even further simplify the task.

Stanfield and Jago (9) have proposed a similar AOC assay that uses an indigenous bacterial population. We found, however, that different strains contained different concentrations of ATP per cell. For example, strain P17 contained an average of 8.7 times more ATP per cell than strain NOX. In a mixed population, ATP determinations would be biased towards growth of P17 rather than NOX. Recalculation of the AOC test on the basis of cell carbon rather than cell density could allow the use of a mixed inoculum, provided that the ratio of ATP to carbon in the cell was constant. We also found that concentrating the cells on a membrane filter helped increase method sensitivity and reduce background levels of non-cell-associated ATP. In some samples, particularly raw waters, the non-cell-associated ATP level was high enough to obscure the AOC results.

We found no significant difference between various sources of ATP releasing agents and reagents. The Turner products performed slightly better than the Lumac reagents; however, the assay was performed with a Turner luminometer. Different results may be obtained with different detection systems. Investigators using different materials, however, should perform a standard curve (Fig. 6 and 7) to determine the relationship between luminescence units and cell density.

This research did not intend to develop a new AOC method but rather to simplify the existing AOC procedure. Results shown in Fig. 8 and 9 indicate that the ATP procedure produced results equivalent to those of the plate count

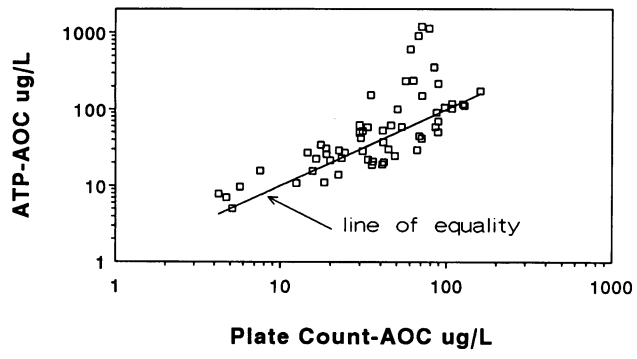

FIG. 9. Comparison of AOC values for strain NOX determined by the ATP procedure and those by the plate count technique. The line of equality represents equivalent results for both methods.

method. In several cases, the ATP procedure produced higher AOC values than the plate count method (Fig. 9). This difference was attributed to underestimation of bacterial levels by the plate count procedures. The observation that raw water values were more variable than those of filtered water samples also supports this hypothesis. It is well known that attachment of cells to turbidity or particulates can result in underestimation of bacterial levels. In addition, the plate count procedure provides the opportunity for numerous errors (preparation of dilutions, labeling plates, and counting, etc.). Likewise, there needs to be some caution in performing the ATP assay. Intensely colored water, for example, can interfere with the ATP assay. However, the use of an internal ATP standard can compensate for this effect.

Results presented in Fig. 10 show a good correlation between the rapid AOC technique (using separate inoculations of P17 and NOX) and the plate count procedure (using a combined inoculum). The fact that there were no significant differences between the two determinations suggests that the organisms do not have a wide overlap of compounds that each can metabolize. Van der Kooij has shown that P17 can grow on a wide variety of compounds but not on carboxylic acids including oxalate (11, 13). Strain NOX can utilize a wide range of carboxylic acids but not carbohydrates, alcohols, or aromatic acids and does not assimilate amino acids when grown on mixtures of compounds (13, 15). It is not surprising, therefore, that separate inoculations would give results similar to those for a mixed inoculation. In addition, for the compounds that both organisms can use, the yield values are different. In mixed cultures, organisms with the higher yield will dominate. The use of separate inoculations avoids competitive reactions that may yield variable results.

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