



# COMPARISON OF A NEW RAPID TOTAL VIABLE BACTERIA METHOD vs. R2A CULTURES

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## INTRODUCTION

The development of real-time data sensors for drinking water utilities has undergone vast expansion since the terrorist attacks of September 11, 2001. Mohawk Valley Water Authority (MVWA) has been a leader in the industry in testing and implementing several rapid techniques to ensure system integrity and enhance its water quality database. Since no single technology can offer surveillance of the entire array of contaminants, a toolbox approach to distribution system monitoring must be developed that employs different technologies to monitor for different events, chemical, biological, radiological, or physical.

The RBD3000 from Advanced Analytical (Ames, IA) is one such technology currently in use at MVWA. The RBD3000 allows for rapid, accurate identification of Total Viable Organisms (TVO) in distribution waters. Mainly used in the pharmaceutical and food/beverage industry for quality control purposes, Advanced Analytical has crossed into the water industry to help more rapidly and accurately assess drinking water samples. The technique involves tagging organisms in a sample with a nucleic acid dye in the presence of a buffer and adding an inhibitor to reduce background fluorescence of non-viable organisms. The sample is then passed through the RBD3000 and registers a total viable count. The entire process takes approximately fifteen minutes, thereby providing real-time quantitative data for utilities to assess system integrity.

Compared to traditional culture plate techniques, which have extended incubation times, the RBD provides results in a much more timely manner. Further, counts from Heterotrophic Plate Counts (HPC) are obtained by summing the total number of colony forming units (CFU's; APHA 1998). Colony forming units do not necessarily emerge from a single bacterium, which is detected by the RBD. Rather, a single CFU can represent multiple bacteria in many different shapes and arrangements (APHA 1998). Further, bacteria in distribution waters exposed to disinfection may become damaged and may not grow on culture plates (McFeters et al. 1986). All these factors: speed, sensitivity, and specificity make the RBD a promising tool in the water industry.



## MATERIALS/METHODS

Two datasets were developed for this study: one from samples collected from various points within the MVWA distribution system and a second from a simulated distribution system pilot study. An effort was made to pick sites that offered differing aspects of the distribution system, including sites with varying residence times in open finished water reservoirs. The pilot study was part of a larger research initiative consisting of four test trains that, in part, focused on the control of bacterial growth within the mock distribution mains using different disinfectant concentrations. The pilot distribution system was a plug-flow reactor constructed from approximately 400-feet of flexible black irrigation pipe with a needle valve to control flow. The flow was regulated to simulate a detention time of 4 days, which is the average water retention in the MVWA system.

Samples from MVWA's distribution system were collected three days per week and analyzed for HPC on R2A media, and for TVO on the RBD3000. Pilot samples were collected weekly from the outflow of the plug-flow system and processed in the same manner. HPC's were performed following Standard Methods 9215 using R2A media, which is a low-nutrient agar that is reported to improve recoveries of stressed organisms in drinking water systems (APHA 1998). Spread plates for the R2A agar were prepared prior to use and then 1mL of sample was pipetted onto the plate and spread across the surface with a glass rod. Sterile technique was employed throughout the analysis. Incubation of the samples was standardized at 7 days at room temperature (22°C) in the dark after which samples were enumerated by MVWA laboratory staff.

The RBD3000 is a flow cytometer that detects both fluorescence and side scatter (size). It can process up to 42 samples per batch. The technician loads the trays with the sample vials with a volume of approximately 3mL, and one disposable sterile syringe for each sample. The instrument decaps and maneuvers the syringe to draw up the sample from its respective vial and inject it into the sample port. The entire process is performed via automation, with a robotic arm maneuvering the sample vial to the proper reagent port injectors. The arm also places the disposable sterile syringe into the syringe loader arm, which pulls up 1mL of sample and then injects 0.250mL of sample into the injection port. Final results are extrapolated out to a per mL basis.

There are three reagent ports located on the RBD3000. For TVO analyses, port #1 contains the nucleic acid (NA) dye (Syo 62), port #2 is BRAG3, and port #3 contains a buffer. The NA dye stains nuclear material in the sample, the BRAG3 is an inhibitor stain proprietary to Advanced Analytical that serves to inhibit background fluorescence from non-viable cells. The buffer aids the uptake of the NA dye. After the addition of each reagent the sample is mixed automatically within the instrument by a vortexer.

## QA/QC

Both HPC and TVO analyses require a sterile technique. Prior to analysis of any sample for TVO, a calibration of the instrument was performed using same-sized tagged calibration beads provided by Advanced Analytical. Two blanks of sterile phosphate buffer were performed with each batch. Duplicate samples were collected on each sample day and samples were processed and analyzed on the same day as collected.

## RESULTS

Unpublished data collected on purified strains of several (n=9) microorganisms at Advanced Analytical provided excellent correlations, ranging from 0.8425 to 0.9991, on serial dilutions of tested species in filtered deionized water (Figure A). Similar studies to validate reproducibility of the RBD with 10<sup>4</sup> concentrations of *E. coli* yielded a 2.29% deviation (n=10; Figure B). A representative intensity plot from a sample is shown in Figure C.

Figure A

Correlation of RBD 3000 TVO Counts vs. Plate/Expected Counts of Microbes Diluted in Water and Stained with Advanced Analytical's TVO Kit

Microbe	Microbial Concentration* (cfu/mL)	RBD 3000 Counts/mL	Number	Correlation Coefficient (R <sup>2</sup> )
<i>E. coli</i> (EC)	13 - 13200	32.14468	4	0.9991
<i>S. aureus</i> (SA)	26 - 24850	19.9919	4	0.9908
<i>C. albicans</i> (CA)	29-28750	12.7919	4	0.9946
Mixed (EC+SA+CA)	34-421000	61-262548	5	0.9814
<i>Ps. aeruginosa</i>	4.547000	13.324268	19	0.9551
<i>P. pickettii</i>	2.57000	13.28760	16	0.8425
<i>E. coli</i>	32.191500	49.257601	5	0.9951
<i>R. terrigena</i>	3-126500	35-124018	6	0.9703
<i>C. parvum</i>	7.6710**	30.7343	4	0.9932
<i>G. lamblia</i>	13-12500**	40.14520	4	0.9843

\* Microbial concentration based on plate count; obtained counts in this range through serial 10-fold dilutions in filtered de-ionized water  
\*\* Microbial concentration based on stock concentration multiplied by dilution factor; obtained counts in this range through serial 10-fold dilutions in filtered de-ionized water.

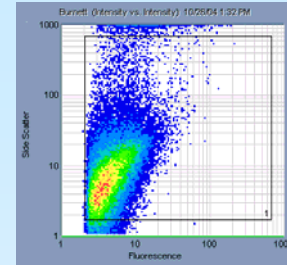
Figure B

E. coli ATCC 25922 Diluted in Sterile Purified Water (SPW) - Reproducibility of RBD 3000

Sample	RBD 3000		Plate Counts
	Counts/0.25mL	Counts/mL*	CFU/mL
SPW	1	4	-----
10 <sup>-5</sup> <i>E. coli</i> in SPW-1	2957	13006	11300
10 <sup>-5</sup> <i>E. coli</i> in SPW-2	2861	12584	11550
10 <sup>-5</sup> <i>E. coli</i> in SPW-3	2979	13103	10700
10 <sup>-5</sup> <i>E. coli</i> in SPW-4	2815	12382	9900
10 <sup>-5</sup> <i>E. coli</i> in SPW-5	2927	12874	10700
10 <sup>-5</sup> <i>E. coli</i> in SPW-6	2933	12991	11550
10 <sup>-5</sup> <i>E. coli</i> in SPW-7	3062	13468	10900
10 <sup>-5</sup> <i>E. coli</i> in SPW-8	2810	12360	9300
10 <sup>-5</sup> <i>E. coli</i> in SPW-9	2866	12606	11550
10 <sup>-5</sup> <i>E. coli</i> in SPW-10	2841	12496	10400
Average	2905.10	12778.04	10770.00
Average Deviation	66.50	292.60	870.00
Percent Deviation	2.29	2.29	5.29

\* RBD Counts/mL are corrected for the addition of the reagents (0.1mL Buffer + 0.1mL NA Dye (0.1mL BRAG3) using the following equation: (RBD counts/0.25mL) \* 4) / (3mL sample volume + 0.3mL total volume of reagents added) / (3mL sample volume).

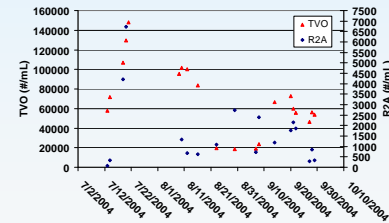
Figure C



A standard correlation cannot be drawn between sample results for HPC and TVO counts. However, generalized trending specific to sample sites were similar using either method. TVO counts consistently are higher than those observed with the traditional culture method. When reading HPC plates, colony-forming units are enumerated. These CFU's may represent not just a single bacterium, but also multiple bacteria in many different arrangements and configurations (APHA 1998). Further, bacteria in distribution waters exposed to disinfection may become damaged and may not grow on culture plates (McFeters et al. 1986). The flow cytometric method is able to enumerate individual bacteria as they are pulled by the sheath through the laser and detector, thereby lending to the greater numbers observed in both datasets compared to the R2A plates. Examples from each dataset are represented in Figures D & E.

Figure D

MVWA Distribution Samples  
Burnett St.

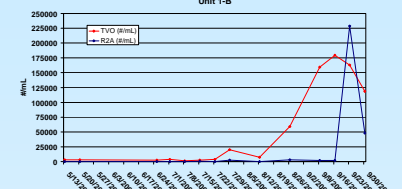


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- LeChevallier, M., Olson, B., and McFeters, G. 1990. Assessing and controlling bacterial growth in distribution systems. AWWAWE.
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Figure E

Distribution Pilot Study  
Unit 1-B



Results of 10 replicate samples of sterile phosphate buffer carried through each method yielded average counts of 1351 counts per mL for TVO and 3 colonies per mL using the R2A method. This further demonstrates the increased sensitivity of the TVO method over the traditional plate technique. Results from duplicate samples are indicative of the increased precision of the TVO method over HPC. The mean RPD for duplicate samples (n=24) in the distribution dataset was 9% 6% with the TVO method and 31% 27% using the R2A plate technique (n=20). The pilot dataset displayed a similar trend for duplicates, 11% 6% RPD (TVO; n=13) and 31% 31% (R2A; n=12). Baseline levels of TVO are currently being established to serve as a benchmark for future samplings and to further develop the MVWA distribution dataset. If an event arises that necessitates speedy microbiological analysis, the RBD3000 allows for quick insight into the quality of the distribution system in near real time. It is imperative that baseline data be established from a number of points in the distribution system and that data should not be compared across sites as results may vary; i.e. bacterial counts will fluctuate across a distribution system. Factors such as temperature and low disinfectant residuals will contribute to bacterial growth in distribution systems (LeChevallier et al. 1990). Because of this, a monitoring network establishing a number of water quality parameters should be established across the distribution system in order to gain an overall perspective of expected counts across distribution sites.

The authors feel that the speed of the TVO test coupled with a comprehensive water quality database of sites within the distribution system provides for a significant security tool in the event of a contamination. The RBD3000 allows for a rapid assessment to determine if a system has been compromised microbiologically and has the capability to determine the reach of the contamination event. It is unlikely that a good correlation can be established between the TVO method and culture plates. While purified strains of bacteria did produce good correlations between the two methods, environmental samples will undoubtedly contain positive and/or negative interferences that may separate the two methodologies. It is possible that methods like the TVO method may more accurately enumerate what is viable in a distribution system than traditional methods. This flow cytometric method was not developed for use in the water industry (Hoefel 2004), but real time technology, the need for continuous monitoring, and 21<sup>st</sup> century events have forced the water industry to consider these methods as standard operating procedures. ■