Water Technologies & Solutions
application note

cleaning validation: microbial total organic carbon recovery and linearity

introduction

Effective cleaning of equipment is a critical to quality process when manufacturing consumer products. The goal of any cleaning process is to reduce the risk of product contamination, and an effective cleaning process reduces risk to an acceptable level to ensure product quality. Without a method to measure and verify cleaning process effectiveness, manufacturers cannot know the risks to product quality and consumer safety.

According to the Food and Drug Administration (FDA), in 2017 the leading cause for product recall in the Food and Beverage Industry was product contamination from microorganisms.[1] A robust cleaning process is critical to the reduction and elimination of microbial contamination. By extension, how cleaning process effectiveness is monitored is equally as critical.

Total Organic Carbon (TOC) analysis is a non-specific method widely adopted by consumer product manufacturers for measuring residual products, cleaning agents, and other potential contaminants, including microorganisms.

To demonstrate that TOC is an appropriate method for its intended use, recovery and linearity studies are performed on potential residues that may remain after cleaning. Chemical contaminants and compounds are commonly tested, however there is less information related to the recovery of microorganisms via TOC analysis. This application note aims to address whether TOC analysis can demonstrate acceptable recovery and linearity of microbial contamination for cleaning validation and verification.

experimental design and setup

In collaboration with the University of Colorado Boulder, 100 mL of *Bacillus subtilis* bacteria was inoculated and incubated overnight in tryptic soy broth. Ten milliliter aliquots of the final culture were centrifuged at 4,500 rpm for 10 minutes to form a cellular pellet.

Between each centrifugation, the supernatant was decanted, and the cellular pellet was resuspended with 10 mL of ultrapure water via vortex mixing. This procedure was repeated for seven cycles. These rinse cycles were designed to remove any TOC contamination from the growth media that the cells were cultured in. After the seventh rinse cycle, the cells were resuspended, diluted, and counted using an established 4,6-diaminidino-2-phenylindole (DAPI) staining protocol[2] (**Figure 1**).

After determining cellular density, a Sievers* M9 TOC Analyzer was used to measure a 1 ppm verification standard set followed by the three cellular concentration dilutions. After the TOC measurement, the remaining sample was filtered through a 0.45 µm sterilization filter to remove any bacteria (**Figure 2**). An additional TOC measurement was then taken to determine the non-cellular contributing background TOC from each sample (**Figure 2**).

Figure 1: Fluorescent microscope imaging of *Bacillus subtilis* during cell counting.

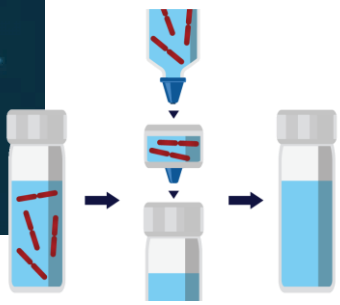

Figure 2: Filtration process of *Bacillus subtilis.*

results

Table 1: Microbial cell density to TOC correlation results.

Cell Density (cells/ml)	Raw TOC (ppb)	Std. Dev. (ppb)	Filtered TOC (ppb)	Std. Dev. (ppb)	Corrected TOC (ppb)	Std. Dev. (ppb)	% RSD
5.80E+05	262	4.04	191	1.71	71	5.75	8.10
5.80E+06	1650	38.3	349	7.85	1301	46.15	3.55
5.80E+07	12400	1450	3270	35.1	9130	1485.1	16.27
Filtered MilliQ H_20			27	1.83			

The results from the microbial TOC correlation study are displayed in **Table 1** and **Figure 3**. The linear trend line has an R^2 value of 0.9981 indicating a good linear trend across the cellular densities measured. Based on the linear fit trend line equation shown in **Figure 3**, the Level of Detection (LOD), defined as 3 times the noise, is determined to be 2.74E+06 cells/mL. Additionally, based on the linear fit trend line and M9 instrument specifications, the maximum instrument quantitation limit of 50 ppm corresponds to 2.49E+08 cells/mL.

After microbial TOC quantitation, 1 milliliter of each cellular density solution was deposited onto a stainless-steel coupon and allowed to dry. The goal of the coupon soiling was to examine the LOD for visual inspection relative to the microbial TOC correlation results. Images of the microbial coupon soiling are shown in **Figure 4**.

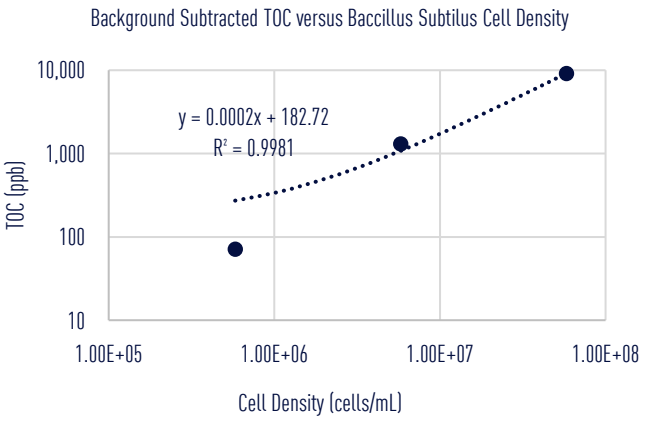

Figure 3: Linearity of microbial cell density versus TOC.

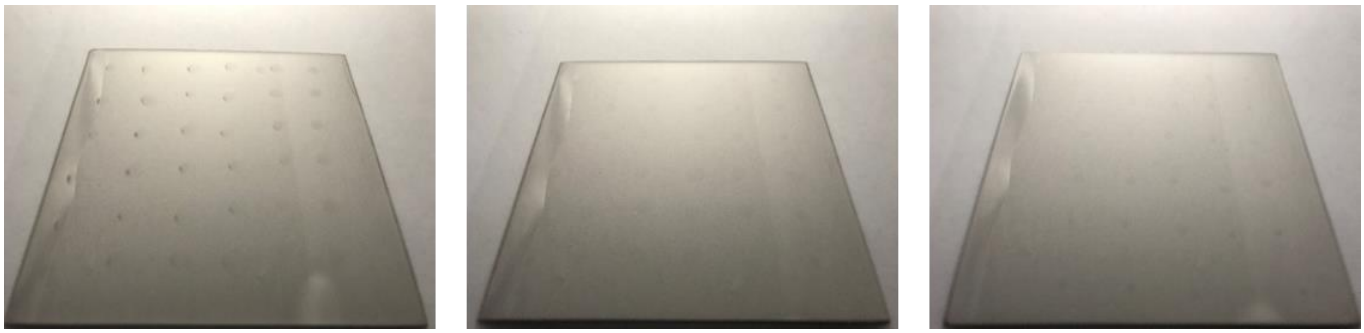

Figure 4: Microbial coupon soiling.
(A) 5.8E+07 cells/mL **(B) 5.8E+06 cells/mL** **(C) 5.8E+05 cells/mL**

discussion and conclusion

Both the microbial TOC correlation results and the coupon soiling images emphasize the importance of continuously monitoring the effectiveness of established cleaning processes. At the highest cellular density, the 5.8E+07 cells/mL soil is readily identified on the stainless-steel coupon surface under ideal lighting conditions. The lower cellular densities become much more difficult to identify on the surface, even under ideal lighting conditions. This demonstrates the importance of not only performing robust cleaning procedures but also measuring the effectiveness of those cleaning procedures beyond visual inspection.

Based on the data gathered, it is conceivable that the equipment used for producing consumer products could be released into production through visual inspection with significant microbial contamination still on the surface. Continuous monitoring of the effectiveness of established cleaning procedures is necessary to reduce risks to product quality and consumer safety.

Lastly, it is challenging to determine recovery of microbial solutions due to the uncertainty in molecular composition of microbes. This research attempts to determine theoretical recovery of the microbial solutions based on previous findings which established the carbon content in a live microbial cell.[3] **Figure 5** shows calculations for theoretical microbial TOC yield. Based on the referenced carbon atoms per cell, the theoretical TOC concentration for 5.8E+07 cells/mL is 11.6 ppm.

$$\left\{5.8x10^{7}\ \frac{\cancel{cell}}{mL}\right\}\ x\ \left\{1.0x10^{10}\ \frac{carbon\ atoms}{\cancel{cell}}\right\} = \left\{5.8x10^{17}\ \frac{carbon\ atoms}{mL}\right\}$$

$$\left\{5.8x10^{17}\ \frac{\cancel{carbon\ atoms}}{mL}\right\} \div \left\{6.022x10^{23}\ \frac{\cancel{atoms}}{mol}\right\} = \left\{9.67x10^{-7}\ \frac{mol}{mL}\right\}$$

$$\left\{9.67x10^{-7}\ \frac{\cancel{mol}}{mL}\right\} x \left\{12.01\ \frac{g\ of\ carbon}{\cancel{mol}}\right\} = \left\{1.16x10^{-5}\ \frac{g\ of\ carbon}{mL}\right\}$$

$$\left\{1.16x10^{-5}\ \frac{g\ of\ carbon}{mL}\right\} == \left\{11.6\ \frac{\mu g\ of\ carbon}{mL}\right\} == \{11.6\ ppm\}$$

$$\frac{9.13\ ppm\ measured}{11.6\ ppm\ expected}\ x\ 100 = 78.7\%\ Recovery$$

Figure 5: Dimensional analysis on theoretical microbial TOC yield.[3]

In the experiements performed for this application note, the actual recovery of TOC for 5.8E+07 cells/mL was measured at 9.13 ppm. This indicates a recovery of 78.7% which, for a challenging recovery compound, demonstrates a successful method.

In conclusion, using the Sievers M9 TOC Analyzer, this research successfully demonstrates the recovery of microbial TOC at cellular densities relevant to visual inspection limits of detection for cleaning validation and verification. These data support the use of Sievers TOC Analyzers for verifying equipment cleanliness and demonstrate the importance of considering a quantitative method for monitoring microbial contamination in addition to visual inspection. TOC analysis offers an effective way to measure residues, monitor cleaning processes, and reduce overall risks. Through its Sievers product line, SUEZ offers complete TOC solutions, services, and support for all your cleaning validation and verification needs.

References

1. Recall Index and Spotlight. Expert Solutions https://www.stericycleexpertsolutions.com/recall-index/
2. DAPI Protocol For Fluorescence Imaging Thermo-Fisher Scientific – US https://www.thermofisher.com/us/en/home/references/protocols/cell-and-tissue-analysis/protocols/dapi-imaging-protocol.html
3. Phillips, Rob, and Ron Milo. "A Feeling for the Numbers in Biology." *Proceedings of the National Academy of Sciences* 106, no. 51 (December 22, 2009): 21465. https://doi.org/10.1073/pnas.0907732106.