IDEXX Summary 15A

Topic	Measuring Fecal Coliforms in Wastewater
Title	**A Simpler Method for Measuring Fecal Coliforms in Wastewater**
Source	Solutions: Covering Analytical Methods and Practices
Date	February/March 2011 – Volume 18, Number 1

Highlights

- Colilert-18 and Quanti-Tray will be recommended for the list of approved methods at 40 CFR 136.3 by the U.S. Environmental Protection Agency (USEPA) – this method meets the requirements for measuring fecal coliforms in wastewater.
- This test provides laboratories with a more straightforward and easy-to-use method over the alternative and more traditional, membrane filtration and multiple tube methods.
- This article highlights the benefits that Wastewater Treatment Plants and Laboratories will receive by using IDEXX's Colilert-18/Quanti-Tray method for fecal coliform detection in wastewater.

** EPA Microbiological Alternate Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Method, April 2004

Water Environment Laboratory

Solutions

Covering Analytical Methods and Practices

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A simpler method for measuring fecal coliforms in wastewater

Alternative process requires less training than traditional methods

A new alternative test procedure for measuring fecal coliforms in wastewater has the advantage of simplicity and removes the uncertainty associated with the membrane filter method.

The Colilert-18 media with Quanti-Tray, manufactured by IDEXX Laboratories (Westbrook, Maine), will be recommended for the list of approved methods at 40 *CFR* 136.3 by the U.S. Environmental Protection Agency (EPA). After reviewing test and supporting validation data submitted by IDEXX, the agency determined that the new method meets the requirements for measuring fecal coliforms in wastewater.

In the interim, laboratories can contact their state and/or regional authority to begin using Colilert-18 for fecal coliform detection, said Peter Madden, associate marketing manager at IDEXX.

"The new method is appropriate for any situation where a laboratory or facility is required to test wastewater effluent for fecal coliform bacteria," Madden said. "Approximately half of the states in the U.S. continue to use fecal coliforms as the indicator bacteria in determining effluent quality, while the other half target *[Escherichia] coli.* As such, a large number of facilities and laboratories are in position to benefit from IDEXX's Colilert-18."

Pricing is volume-based and dependent on the number of tests run in the laboratory or facility, as well as whether other IDEXX-tests are being used, Madden said.

"When factoring the labor savings and consumable costs, our pricing is comparable to membrane filtration," Madden added.

The new method provides wastewater treatment facilities with a more straightforward alternative for measuring fecal coliforms in wastewater, compared to the previously approved membrane filtration and multiple-tube methods, which involve a considerable amount of analytical skill and judgment, said Keith Chapman, laboratory program manager at the Willow Lake Treatment Plant (Salem, Ore.).

"Chlorinated wastewater effluent samples can be difficult to work with, since they often produce atypical colonies on the membrane filters that are hard to identify as fecals," Chapman said.

However, the new process effectively removes the uncertainty

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In This Issue

Group aims to improve use, understanding of field sensors

Water quality data collected by sensors deployed in the field have become increasingly important as a means of assessing environmental conditions in streams, rivers, and other waterbodies. Compared to traditional laboratory methods, field instrumentation can generate vast amounts of data more easily and cheaply. However, such sensors must be deployed and maintained properly in order to generate useful, reliable data.

To help ensure that the benefits associated with field instrumentation live up to the promise, a group comprising experts from government, academia, and industry is developing tools to assist sensor users in generating useful data of known quality.

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A simpler method for measuring fecal coliforms in wastewater

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associated with the membrane filter method, since wastewater samples no longer have to be filtered, according to Chapman.

"The determination is noticeably simpler, and much less judgment comes into play."

"Simply add a powder to a Quanti-Tray sample, and if fecal coliform bacteria are present, the sample will turn another color," Chapman said. "The determination is noticeably simpler, and much less judgment comes into play."

Bacteria from the coliform family are the only organisms that will react to the medium, Chapman added. "In this way, the method has the distinct advantage of being a defined substrate process," he said. "It is organism-specific."

The new method is a slight modification of an existing methodology for detecting total coliforms and *E. coli* that was developed more than 20 years ago for the drinking water industry, Chapman said. It uses the "same media, except at a higher temperature, which will specifically select fecal coliforms," he said.

"Instead of 35 degrees [F, or 2ºC], samples are incubated at 44.5 degrees [F, or 7ºC], which favors growth of fecal coliforms and is, in fact, the same temperature at which fecal coliform membrane filters are incubated," Chapman explained.

Less training needed

Another benefit to wastewater treatment plants includes the ease of training associated with the new IDEXX method, according to Madden. "Facilities don't need to have a dedicated microbiologist on staff as they do for running the traditional filtration method," he said. "Multiple operators are capable of handling the IDEXX method."

"The traditional method is also known for producing results that are subject to interpretation, since bacteria colonies need to be interpreted and counted," Madden added. "There are more time-consuming steps and more quality control performed with traditional methods. However, quality control is only recommended once per lot of Colilert-18 media, saving time in the laboratory."

Facilities should be able to move

easily to using the new fecal coliform testing method, as most will already have a Quanti-Tray sealer and Colilert-18 media on hand, according to Bennett Osborne, president of Valley Environmental Laboratory (Yakima, Wash.).

"These same items may have been used for monitoring total coliform counts in wastewater effluent," Osborne said. "Laboratories will also be familiar with the new testing method, as it is very similar to the existing process that is currently used for monitoring total coliform. In addition to generating more accurate results, implementing this process is anticipated to save lab facilities time and money."

— ***Jeff Gunderson**, Solutions*

Group aims to improve use, understanding of field sensors **Continued from page 1**

Known as the Aquatic Sensor Workgroup (ASW), the group was formed under the auspices of the Methods and Data Comparability Board, a partnership of water quality experts from federal agencies, states, municipalities, and private organizations.

The board, in turn, is a product of the National Water Quality Monitoring Council, a federal panel created to provide a forum for participants in efforts to monitor and assess water quality.

In 2008, members of the Methods and Data Comparability Board formed ASW in recognition of the fact that field sensor technology is "changing quickly," said Daniel Sullivan, co-chair of the board and a hydrologist at the U.S. Geological Survey (USGS). In particular, members of the board sought to address issues related to quality assurance and data management.

Ensuring proper data collection

In part, the growing interest in continuous water quality monitoring prompted ASW to develop products intended to improve the use and understanding of field sensors, said Charles ("Chuck") Dvorsky, continuous water quality monitoring network coordinator for the Texas Commission on Environmental Quality (CEQ) and a group member.

"There are a lot of people out there that are trying to do [continuous monitoring]," Dvorsky said, "and there are a lot of mistakes to be made."

Problems can result from not properly deploying a sensor or leaving it unattended in the field for an extended period, Dvorsky said. For example, biofouling or silt deposition on a sensor can diminish effectiveness and compromise data quality, particularly if an instrument is not serviced within appropriate intervals. By making information publicly available regarding sensor selection, deployment, and use, ASW seeks to "help people make good decisions about doing continuous monitoring," Dvorsky said.

The absence of standards for field sensors also prompted the formation of ASW, said Rob Ellison, global market and business development manager for YSI Inc. (Yellow Springs, Ohio) and another group member.

"There's a lot of gaps right now" regarding how data from sensors are used and how use of the instruments is controlled or regulated, Ellison said. By helping to close these gaps, ASW aims to "improve environmental monitoring overall and gain wider acceptance globally for this type of instrumentation," he said.

In deciding which types of field sensors to focus on initially, ASW opted to address "proven, widely used technologies," Sullivan said. To this end, the group is focusing on discrete sampling and continuous monitoring in fresh, brackish, and saltwater environments for sensors for dissolved oxygen, conductivity, temperature, pH, turbidity, depth, and oxidation–reduction potential.

Offering guidance to sensor users

In April 2010, ASW released its first products for sensor users, the *Field Deployment Guide* and the *Quality Assurance Matrix*. The guide is "one of the most practical products for a typical user," Ellison said.

Structured as a checklist, the guide is arranged to assist new and experienced users of sensors with four tasks: deciding the type of monitoring system needed; selecting the optimal sampling location; designing, installing, and maintaining platforms; and developing documentation pertaining to installations and site visits.

Also organized as a checklist, the matrix "provides recommendations for the minimal array of actions deemed necessary for collection of usable data of known and documented quality," according to the document.

Actions covered in the matrix are intended to enable sensor users to properly control, test, document, and report the quality of their sensors' measurements. In addition to a general set of actions that pertain to all sensors, the matrix lists dedicated actions specific to the particular parameter to be monitored. If disparate groups conducting water quality monitoring around the country are to be able to share their data effectively, "we have to have a process in place so that we know what the quality of the data is — good or bad," Ellison said. The matrix offers just such a process for these groups to follow, he said.

At press time, ASW was preparing to release its *Sensors Data Elements* list. Essentially, the list describes the "metadata," or the "data that you have on the data," Dvorsky said, to

answer such questions as when, where, why, and how the monitoring data were collected. "It's one thing to collect a set of measurements," he said. "It's another to collect it correctly and to be able to document that your instrument was working, that you followed procedures, and that the instrument was calibrated and post-calibrated."

In addition to USGS, Texas CEQ, and YSI, ASW includes members from the U.S. Environmental Protection Agency, In-Situ Inc. (Fort Collins, Colo.), Hach Co. (Loveland, Colo.), the U.S. National Park Service, the U.S. Department of Energy, Virginia Polytechnic Institute and State University (Blacksburg, Va.), and the U.S. National Oceanic and Atmospheric Administration. Membership in the group is open, Sullivan said.

For more information or to download copies of the *Field Deployment Guide and the Quality Assurance Matrix*, visit the group's Web site at http://watersensors.org/.

— ***Jay Landers**, Solutions*

An optimized method for low levels of selenium

Linnea Hoover and Peter Morrissey

Sec. 303(d) of the Clean Water Act requires states to develop a list of waterbodies that do not meet water quality standards and to develop total maximum daily loads (TMDLs) for them. Selenium is included on the 2010 Sec. 303(d) list as a trace element primary pollutant/stressor for the San Francisco Bay Estuary. Selenium is a pollutant of concern in the bay because it accumulates in wildlife to concentrations that pose a human health risk and may impair or kill juvenile fish. Currently, a TMDL for selenium is in development by the state water board. The TMDL will identify loads that must be decreased to meet targets in the bay and may require analysis of selenium species, as well as total selenium.

Historic data collected on selenium in the bay focus on total selenium. The average concentration of total selenium in the bay in 2009 was 0.16 µg/L, which is slightly higher than the long-term baywide average (0.13 µg/L). However, selenium in its dissolved form has varying potential for aquatic toxicity, depending on how efficiently selenium enters the food chain. More than two-thirds of the selenium in bay water is present in the dissolved form, mostly as selenate. Effluents from municipal dischargers contain selenium mostly in the form of selenate (60%), followed by selenite (25%), and organic and elemental selenium (15%).

The total selenium concentration in East Bay Municipal Utility District (EBMUD) wastewater treatment plant effluent is typically <0.3 µg/L. A project was undertaken to optimize the current method for selenium analysis to quantify the dissolved fraction of selenium in wastewater effluent.

Forms of selenium

Selenium exists in the 2^- (Se^{-2}, or selenide), 0 (Se^0, or elemental selenium), 4^+ (SeO_3^{-2}, or selenite), and 6^+ (SeO_4^{-2}, or selenate) oxidation states. Each oxidation state exhibits a different chemical behavior. The concentra-

Table 1. Modified graphite furnace–hydride parameters for low-level selenium analysis

Modified parameter	Current method	Low-level method	Reason for modification
Sample loop	130 µg/L	500 µg/L	A larger sample loop loads more analyte onto the graphite platform.
Graphite tube	Iridium-treated standard tube	Iridium-treated end-capped tube	An end-capped tube retains analyte in the light path longer, resulting in increased signal. Typically, end-capped tubes yield twice the signal of standard tubes.
Sample loading	Single injection	2X injection	Loads twice the amount of sample prior to firing the furnace. This doubles the signal but also adds 1 minute per sample to the analysis time (from 4 minutes to 5).
Calibration standards	µg/L Se 4.0 µg/L Se 8.0 µg/L Se 12.0 µg/L Se	0.1 µg/L Se 0.5 µg/L Se 1.0 µg/L Se 2.0 µg/L Se	Lower calibration concentrations are needed to stay within the reduced linear range of the instrument.

Table 2. Analytical results for total and filtered selenium

Replicate sample number	Total selenium, µg/L	Dissolved selenium, µg/L
1	0.25	0.21
2	0.27	0.22
3	0.28	0.22

tion, speciation, and association of selenium in a particular environment depend upon pH and redox conditions, the solubility of its salts (selenates are more soluble than selenites), the complexing ability of soluble and solid ligands, biological interactions, and reaction kinetics. Selenide and elemental selenium occur in acidic, reducing, and organic-rich environments. Metallic selenides, seleniumsulfides, and elemental selenium are insoluble and, therefore, biologically unavailable. For the pH and redox conditions of most soil and aquatic environments, selenite and selenate should be the dominant forms.

Methodology

Samples analyzed at EBMUD are digested by microwave with potassium persulfate and sulfuric acid and analyzed according to Standard Method 3114B using a Perkin–Elmer 600 graphite furnace with FIAS 100 hydride generation module. The current method detection limit (MDL) for this method is 0.3 µg/L, calculated from 20 of the most recent batch quality control samples spiked at 1.0 µg/L.

EBMUD's research chemist was assigned to modify the current method for selenium to obtain a detection limit equal to or lower than 0.1 µg/L. Modifications made to the method result in an increased amount of analyte in the light path of the spectrometer, therefore enabling detection at a lower concentration. Table 1 (p. 4) shows current and low-level instrument parameters.

To determine the MDL of the modified method, seven replicates of 0.1-µg/L spike solution were analyzed. Results for the seven replicates were 0.095, 0.088, 0.111, 0.086, 0.085, 0.09, and 0.089 µg/L, respectively. The calculated standard deviation was 0.00898, and the MDL was 0.028 µg/L.

EBMUD treatment plant effluent was analyzed in triplicate using the low-level method for both total and filtered selenium. The sample aliquot for total selenium was digested by microwave with potassium persulfate and sulfuric acid. The sample aliquot for dissolved selenium was passed through a 0.45-µm filter and then digested before analysis. Analytical results are shown in Table 2 (p. 4).

Discussion

Approximately 80% of selenium in EBMUD effluent is dissolved. The ratio of dissolved-to-total selenium in EBMUD wastewater treatment plant effluent is consistent with regional monitoring program data, which show that approximately 85% of municipal discharge effluent is dissolved selenate and selenite.

The low-level method for selenium can be used to determine total and filtered selenium in wastewater effluent at concentrations less than the current detection limit of 0.3 µg/L. Dissolved selenium provides an estimate of combined selenite and selenate, the two most prevalent oxidation states of selenium.

Linnea Hoover *is a laboratory supervisor and* ***Peter Morrissey*** *is a research chemist at East Bay Municipal Utility District (Oakland, Calif.).*

Links to further reading

The Pulse of the Estuary 2010, RMP No_618.

Technical Memorandum 2: North San Francisco Bay Selenium Data Summary and Source Analysis (July 2008).

Water Quality Standards; Establishment of Numeric Criteria for Priority Toxic Pollutants for the State of California | Federal Register Environmental Documents | USEPA.

Concurrent rapid identification of filamentous bacteria using reverse-line blot hybridization

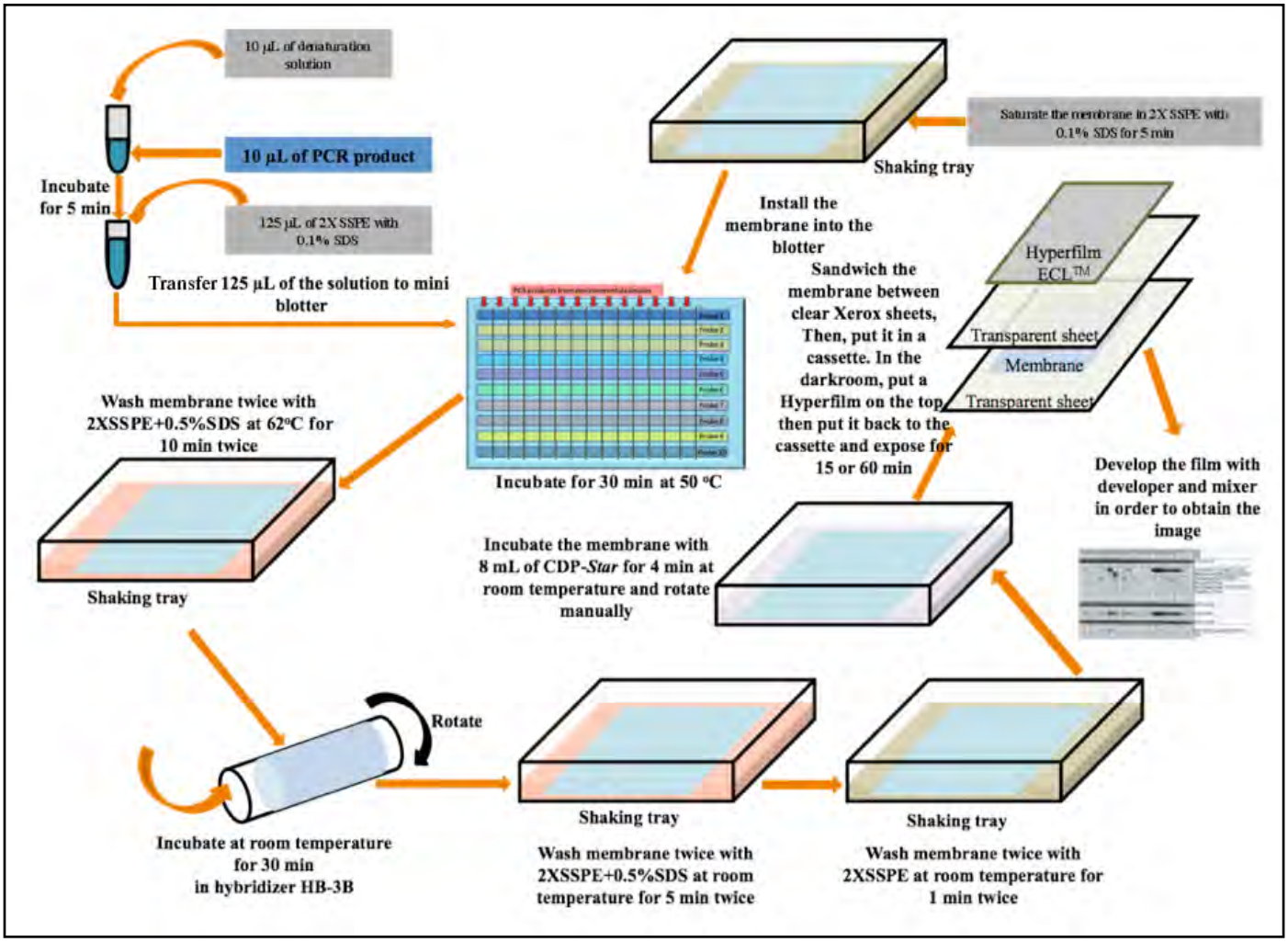
Pitiporn Asvapathanagul, Hyeeun Bang, Hyeyoung Lee, and Betty H. Olson

Foaming events have been explained as the result of excessive filamentous bacterial growth. To quantify filamentous bacteria, the numerical filament index, total extended filament length, and filament counting can be applied. However, such quantifying methods rely on light microscopy, which is time-consuming, requires technical expertise, and can be inaccurate.

Since the cause of filamentous bacterial growth in wastewater treatment plants (WWTPs) is still unclear, foaming incidents are difficult to prevent. The most important factors contributing to this issue are the lack of easy, cost-effective organism-identification tools.

Polymerase chain reaction–reverse-line blot (PCR–RLB) hybridization is a molecular technique that has been widely applied in the medical and public health fields. The method's high specificity and ability to process several samples simultaneously, as well as its use of multiple probes to identify different filamentous bacteria, are the advantages of this technique. Also, a broad range of species within one genus can be captured. For example, the *Nocardia* probe captures 42 different species within the genus, based on testing of pure cultures and blast analysis. Another advantage of the assay is

Figure 1. PCR–RLB methodology



PCR = polymerase chain reaction.
RLB = reverse-line blot.
SDS = sodium dodecyl sulfate.
SSPE = saline–sodium phosphate–EDTA.

the ability to add multiple primers and probes to identify more species at the genus level. PCR–RLB is useful for monitoring bulking and foaming bacteria in WWTPs, because these organisms' occurrence is unpredictable, and if the wastewater facility does not know which foaming or bulking organism is involved, the utility cannot relate operational conditions to its occurrence for prevention purposes.

Methodology

The method consists of two steps: preparing the PCR master mix, and performing the RLB assay (see Figure 1, above).

PCR for DNA extracts

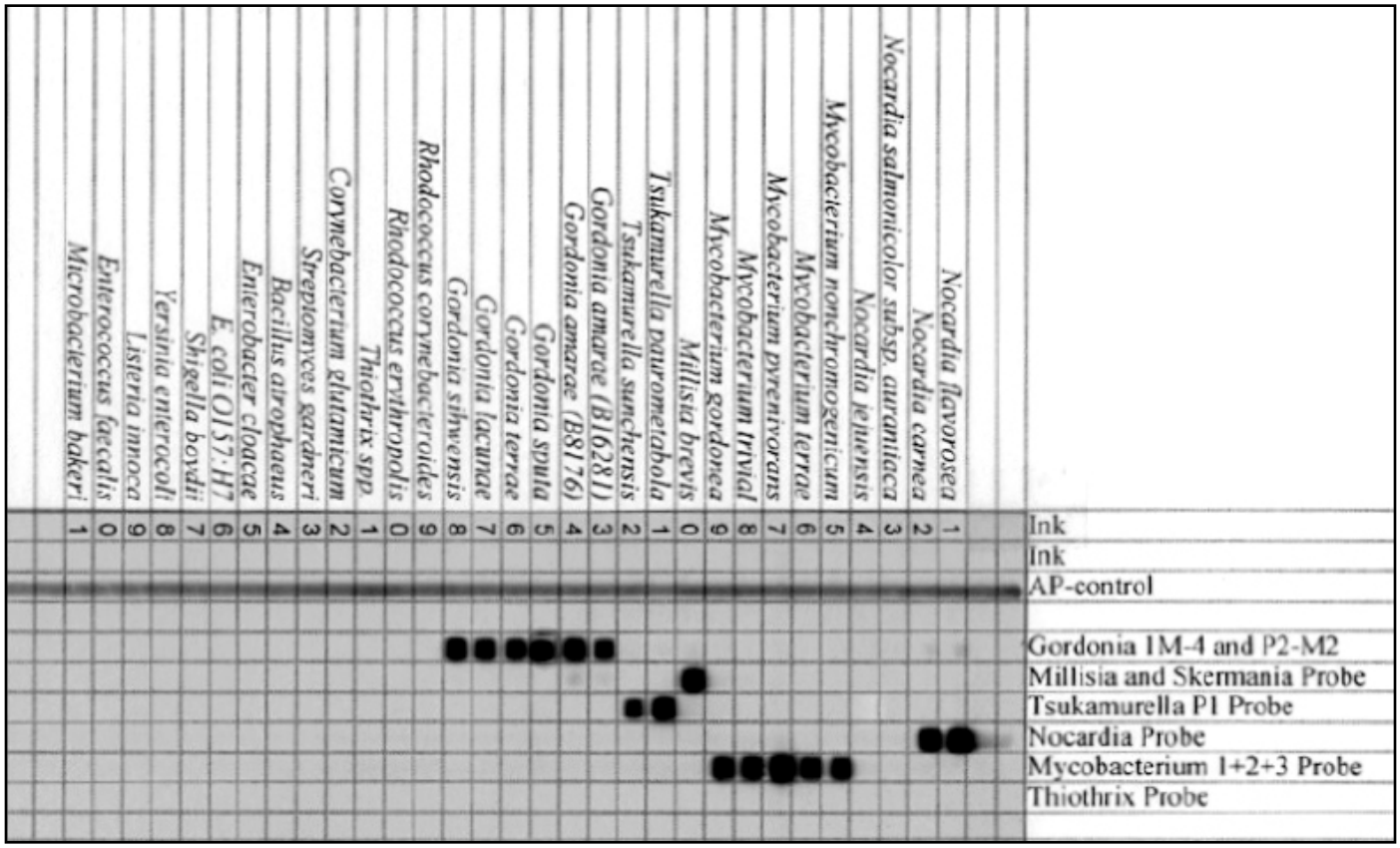
Primers used in this study were provided by B&F Diagnostics (Irvine, Calif.). PCR assays were conducted using a GeneAmp® PCR System 2700 (Applied Biosystems [Carlsbad, Calif.]) with a 5-minute holding at 94ºC, 20 seconds of denaturing at 94ºC, and 40 seconds of annealing at 53ºC for each of 35 cycles, followed by a 7-minute final extension at 72ºC. Then, amplified samples were cooled to 4ºC and then stored at –50ºC until the PCR–RLB technique was used.

The master mixture for PCR was composed of 1X buffer with 20 millimolar of magnesium chloride, 200 mM of deoxyribonucleotide triphosphate, 2.5 units of AmpliTaq DNA polymerase, and 10 picomole of each primer. The mixture then was brought to a final volume of 20 µL with high-performance liquid-chromatography water, to which 5 µL of each sample was added. The final amount of pure culture extracts of DNA and environmental DNA extracts for the reaction were 5 ng and 0–10 ng, respectively.

RLB hybridization assay

The membrane was soaked in a shaking tray for 5 minutes in a 2X saline–sodium phosphate–EDTA (SSPE) buffer with 0.1% sodium dodecyl sulfate (SDS). While the membrane was immersed, 10 µL of PCR product was mixed with 10 µL of denaturation solution and incubated for 5 minutes at room temperature. Then, 125 µL of 2X SSPE with 0.1% SDS was added to the same tube. This mixture was left at room temperature until the membrane was prepared. The membrane was placed

Figure 2. Validation of bulking and foaming filamentous bacterial probe using reverse-line blot hybridization



Note: *Microthrix spp.* and *Thiothrix* data not shown (15-minute exposure).

in a miniblotter. All remaining liquid in the miniblotter was removed before the denatualized PCR products were added onto the membrane, one slot for each sample. The membrane hybridization was performed at 50ºC for 30 minutes in the HB-3B hybridizer (Techne [Staffordshire, England]). Afterward, the membrane was washed twice in washing solution (2X SSPE, 0.5% SDS) at 62ºC for 10 minutes before being transferred to the HB-3B hybridizer. The membrane was incubated with 1:2000 of diluted streptavidin-conjugated alkaline phosphatase in washing solution at room temperature for 30 minutes in the hybridizer. The membrane was again washed twice with washing solution at room temperature for 5 minutes for each washing and then washed twice with 2X SSPE at room temperature for 1 minute per wash.

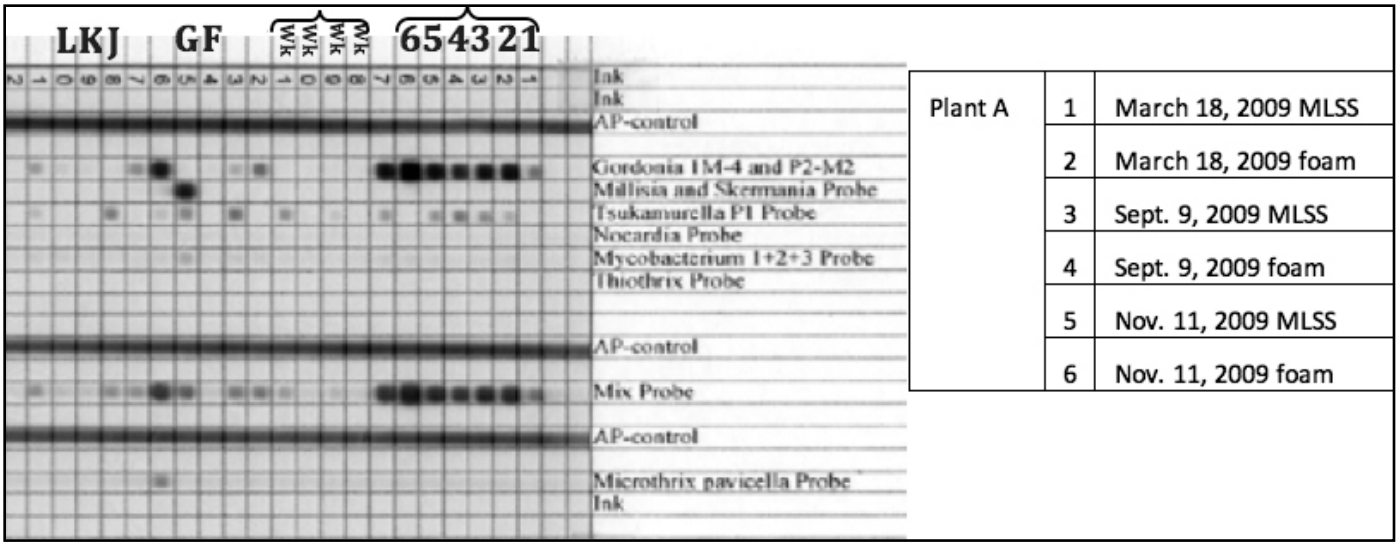
The membrane then was incubated using CDP-*Star* Detection Reagent ($C_{18}H_{19}C_{l2}O_7PNa_2$; Amersham Biosciences, GE Healthcare [Buckinghamshire, England]) for 4 minutes at room temperature. The membrane was removed from the solution with forceps, marked so that the probe and PCR product side could be identified, and placed between two clear sheets. Afterward, it was put into a cassette. In the darkroom, the film (Amersham Hyperfilm ECL™) was placed on the clear sheet on the marked side of the membrane. The film was exposed for 15 and 60 minutes for pure culture and environmental DNA extracts, respectively. Probes and membranes were provided by B&F Diagnostics.

Validation of filamentous bacteria probes

Primers and probes for seven filamentous organisms — including genera *Gordonia*, *Millisia*, *Skermania*, *Microthrix*, *Nocardia*, *Mycobacterium*, and *Tsukamurella* — were developed and successfully tested (see Figure 2, above). The *Nocardia spp.* probe accurately identified *Nocardia flavorosea* and *Nocardia carnea* and also produced negative results for *Nocardia jejuensis* and *Nocardia salmonicolor subsp. aurantiaca*, because *Nocardia jejuensis* had greater than 24% base-pair mismatches to the DNA sequence of the constructed probe. The similarity of *Nocardia salmonicolor subsp. aurantiaca* to *Nocardia spp*. was not reported, since the DNA sequence was not available in GenBank, but at least five mismatches had to be present in the probe region to produce a negative result.

The *Mycobacterium spp*. probe precisely identified all five *Mycobacterium* species tested in this assay, including *Mycobacterium pyrenivorans, Mycobacterium marinum, Mycobacterium trivial, Mycobacterium nonchromogenicum,* and *Mycobacterium terrae*. The

Figure 3. PCR-RLB of samples from 12 wastewater treatment plants using AF primers (60-minute exposure)



PCR–RLB = polymerase chain reaction–reverse-line blot hybridization.
MLSS = mixed liquor suspended solids.

Mycobacterium genus was included in this study because it was found to be in most of the wastewater tested and is closely related to foaming organisms. Thus, it was important to ensure that it did not interfere with the assay.

The *Tsukamurella spp.* probe identified *Tsukamurella paurometabola* and *Tsukamurella sunchoensis* correctly. The *Millisia spp.* and *Skermania spp.* probes showed positive results on *Millisia brevis. Millisia spp.* and *Skermania spp*. have only one nucleic-acid base-pair difference in the probe region of the DNA sequence, so this technique was unable to differentiate between these two genera. The *Gordonia spp.* probe displayed a truly positive result on *Gordonia sputa, Gordonia terrae, Gordonia lacunae, Gordonia sihwensis*, and *Gordonia amarae* (B-8176 and B-16281).

PCR–RLB results from 12 WWTPs

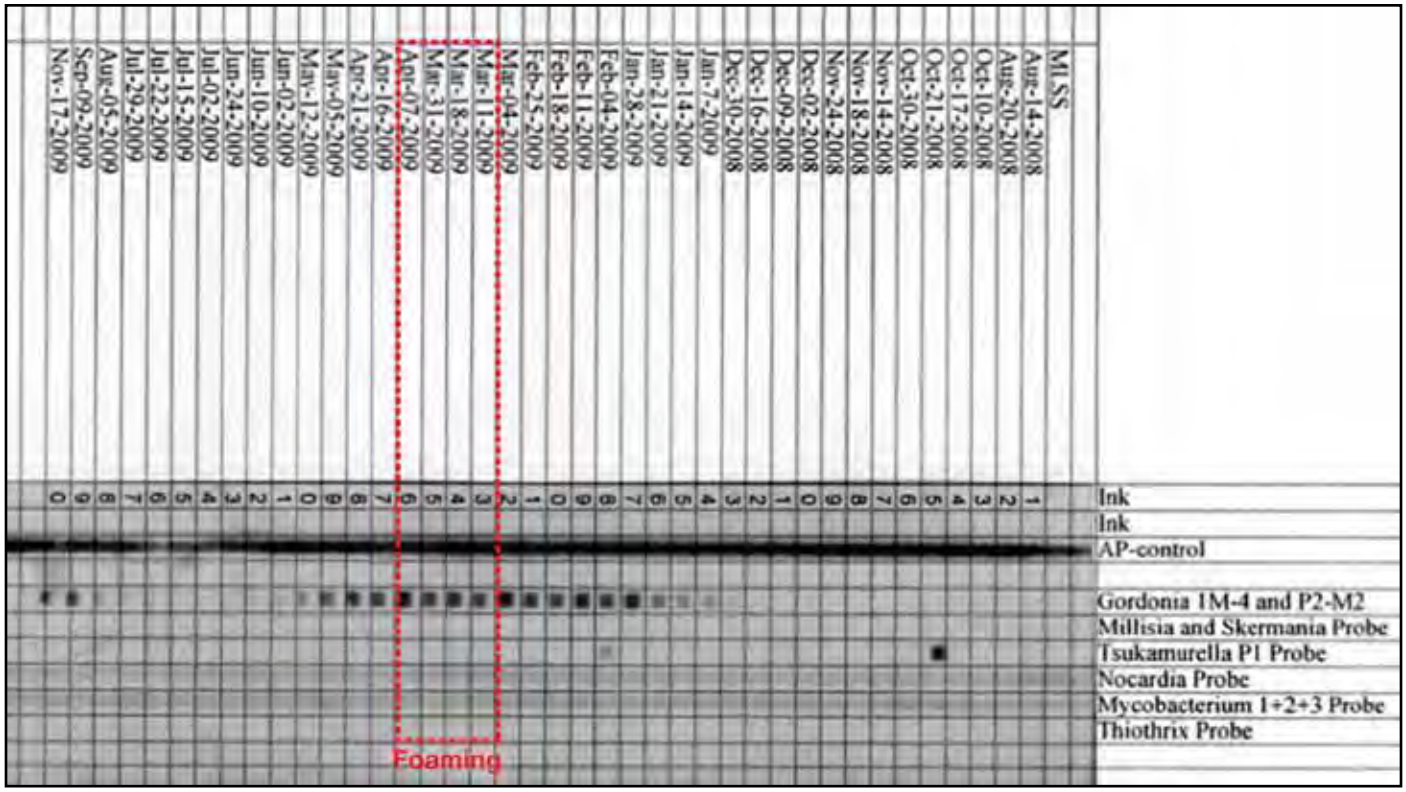
The results for samples from 12 WWTPs are shown in Figure 3 (above). The samples were taken during foaming events at Plant A (March 18, Sept. 9, and Nov. 17, 2009). *Gordonia spp*. were the majority of the filamentous organisms. *Tsukamurella spp*. also were detected during the foaming events at this WWTP but in lower concentrations than *Gordonia spp.* The samples obtained from aeration tanks of Plant B during a bulking event contained *Gordonia spp*. and *Tsukamurella spp*. as most of the nocardioforms.

Most gram-positive filamentous bacteria were below the minimum detection limit using this primer set in Plant C samples (<100 copies), except *Tsukamurella spp.*, which were slightly positive in samples taken during weeks 2 and 4. Plant C encountered bulking by Type 021N on Week 3, and chlorine was added during this time, which was why filamentous bacteria were below the detection limit in the Week 3 sample. Samples from plants D, E, and F were taken during nonfoaming or bulking events. *Gordonia spp.* were detected in the Plant D sample. Plant E had *Gordonia spp*. and *Tsukamurella spp*., and these filamentous bacteria were below the detection limit in Plant F.

Plant A (foaming) results, compared to those from plants D, E, and F (nonfoaming/nonbulking), showed greater signal intensity, which was an indication of higher organism concentrations in foaming samples. The sample from Plant G contained *Millisia spp*. or *Skermania spp*. A recognized expert on bulking and foaming who had sent these samples identified these as nocardioforms using light microscopy. PCR–RLB identified *Tsukamurella spp*. as the secondary filamentous bacteria (mycolata nonnocardioform organisms) and *Mycobacterium spp*., which are not filamentous but have a similar cell-wall composition. *Gordonia spp*. were likely the cause of foaming at Plant H, along with a low concentration of *Microthrix parvicella.* On the other hand, foam from Plant I showed only a slightly positive result for *Gordonia spp.*, which indicated that there were other nocardioforms associated with this foaming event, suspected to be *Rhodococcus spp*. However, the details of probe sequence are still being tested at this time, so the data are not shown. *Tsukamurella spp*. were found in the Plant J mixed liquor suspended solids sample (nonbulking/nonfoaming). The Plant K sample (nonfoaming/nonbulking) contained *Tsukamurella spp.*, and Plant M (nonfoaming/nonbulking) had both *Gordonia spp*. and *Tsukamurella spp*. at low concentrations.

The PCR–RLB technique indicated there was more than one specific bacterium associated with prefoaming events and solid-separation problems. This finding indicates why it is often difficult to predict when a foaming or bulking event will occur. The differences among plants are likely due to different treatment processes. To under-

Figure 4. PCR–RLB on 1-year Plant A mixed liquor suspended solids samples (60-minute exposure)



PCR–RLB = polymerase chain reaction–reverse-line blot hybridization.

stand the environmental factors and operating procedures that promote or suppress growth, it is crucial to apply reliable and reproducible detection procedures to assess the abundance of filamentous bacteria populations.

PCR–RLB proved to have many advantages, compared to existing technologies. One advantage of the extraction and storage of DNA is that it is stable over a number of years. Although this assay is not quantitative, it can be adapted to a quantitative format. Conversely, after the organisms are known, qPCR can be used to follow population abundance during the different seasons and relate these to other chemical and physical conditions in the plant or aeration basin.

PCR–RLB on weekly aeration tank samples

Plant A experienced three foaming events in its aeration tank in 2009 (see Figure 4, above). The samples were collected before, during, and after foaming incidents from Aug. 14, 2008, to Aug. 5, 2009, and on Sept. 9 and Nov. 17, 2009 (during foaming events). *Tsukamurella spp.* were observed in Oct. 21, 2008, and Feb. 4, 2009, samples. *Gordonia spp*. were detected from Dec. 30, 2008, to May 12, 2009. The first foaming incident started March 18 and lasted until April 7, 2009 (foam covered the surface of the aeration tank). These membranes can be treated so that they can be reused, but as can be seen in Figure 4 (see *Mycobacterium spp.* and *Nocardia spp.*), this can result in breakdown of the probes and the associated amino acid group such that a partial reaction occurs. This can be distinguished from a true reaction because there is a smear across all samples tested instead of individual dots, as can be seen in *Gordonia spp.*

PCR–RLB can provide early warning signals to treatment plant operators. In Figure 4, the first foaming incident positive for *Gordonia* can be seen on Dec. 30, 2008 — approximately 2 months before the first event — and again slightly more than 1 month before the second foaming incident (September 2009). Additionally, the relative concentrations of the bacteria were shown with increasing signal intensity (Figure 4). *Nocardia spp.*, *Millisia spp*., and *Skermania spp*. were absent (Figure 4) in this plant, and the only other member of the nocardioforms present was *Tsukamurella spp. Gordonia amarae* persisted in Plant A through June (very faint reactions) but were below the minimum detection limit and did not cause foaming. When wastewater conditions or environmental parameters were favorable, *Gordonia amarae* caused foaming problems.

A review of operational data showed that the solids retention time was being increased from Dec. 2 until Dec. 30, 2008, and then was decreased steadily until Feb. 7, 2009, but almost no corresponding decrease in intensity was evident. Changes in intensity would enable operators to see if increased wasting or other operational strategies had the desired effect of decreasing foaming populations. Sporadic chlorination of the return activated sludge was used in late March and early April, which finally

controlled the foaming. The periodic occurrence of other foaming organisms is also interesting, suggesting that plant conditions were unfavorable.

The data in this work clearly show the value of applying the PCR–RLB technique when monitoring filamentous bacteria in WWTPs, especially the nocardioform group, which is difficult to identify to the genus level using light microscopy. However, the level of sensitivity of the technique could have been increased by running the PCR for 40 cycles instead of 35. Nevertheless, the PCR–RLB technique can provide an early warning to WWTP operators of potential bulking or foaming events and indicate how a population is responding to different operation strategies.

Pitiporn Asvapathanagul *is a graduate research assistant, and* ***Betty H. Olson*** *is a university professor in the Henry Samueli School of Engineering at the University of California–Irvine.* ***Hyeeun Bang*** *is a postdoctoral fellow, and* ***Hyeyoung Lee*** *is a university professor in the College of Health Science in the Biomedical Laboratory Science Department of Yonsei University (Wonju-si, Kangwon-do, Korea).*

Microconstituents: What to expect in your permit

Sarah Reeves and Peter Littlehat

Microconstituents are beginning to make their way into discharge permits in the United States. Removal of microconstituents often requires tertiary treatment, and utilities are interested to know what permit limits they might expect in the future as they plan for capital improvements. The U.S. Environmental Protection Agency (EPA) is currently focusing on developing methods for determining the endocrine-disrupting impact of particular compounds. EPA has developed some criteria for endocrine-disrupting compounds (EDCs), but the states have been slow to turn these criteria into standards, and their inclusion into permits is much slower. While several states are moving forward with developing standards for diazinon, polychlorinated biphenyls (PCBs), nonylphenol, atrazine, and tributyltin, permit holders probably will not see EDCs in permits in the near future.

Sources of EDCs

Microconstituents enter the environment from many sources. The Water Environment Federation (WEF; Alexandria, Va.) defines "microconstituents" (also known as "compounds of emerging concern") as "natural and manmade substances, including elements and inorganic and organic chemicals, detected within water and the environment, for which a prudent course of action is suggested for the continued assessment of the potential effect on human health and the environment" (WEF, 2007).

Microconstituents include pesticides, a variety of residual trace contaminants in treated wastewater, pharmaceutical and synthetic hormones, and EDCs. EDCs have

Table 1. Example nonylphenol standards and environmental water levels

Parameter	Nonylphenol concentration, µg/L	Source
Acute comparison		
National recommended water quality criterion (aquatic life protection)	28	Levels in fresh water (EPA)
EU surface WQS, maximum allowable	2.0	Directive 2008/105/EC
Colorado acute surface WQS	28	Acute aquatic life protection
D.C. Department of the Environment WQS	28	Class C CMC
Rivers/streams measured maximum concentration	40	Kolpin *et al.,* 2002
Secondary-treated wastewater effluent	34	Loyo–Rosales *et al.,* 2007
Tertiary-treated wastewater effluent	10	Loyo–Rosales *et al.,* 2007
Chronic comparison		
National recommended water quality criterion (aquatic life protection)	6.6	Levels in fresh water (EPA)
EU surface WQS, annual average	0.3	Directive 2008/105/EC
Colorado chronic surface WQS	6.6	Chronic aquatic life protection
D.C. Department of the Environment WQS	6.6	Class C CCC
Rivers/streams median concentration	1	Kolpin *et al.,* 2002

CCC = criteria continuous concentration.
CMC = criteria maximum concentration.
EPA = U.S. Environmental Protection Agency.
EU = European Union.
WQS = water quality standard.

Table 2. Example diazinon standards and environmental water levels

Parameter	Diazinon concentration, µg/L	Source
Acute comparison		
National recommended water quality criterion (aquatic life protection)	0.17	Levels in fresh water (EPA)
Colorado acute surface WQS	0.17	Acute aquatic life protection
Maximum concentration to specific water bodies	0.10	California Environmental Protection Agency, 1998
Rivers/streams measured maximum concentration	0.35	Kolpin *et al.,* 2002
Treated wastewater levels, maximum concentration	1.7	Burkhard and Jensen, 1993
Chronic comparison		
National recommended water quality criterion	0.17	Levels in fresh water (EPA)
Colorado chronic surface WQS	0.17	Chronic aquatic life protection
Maximum concentration to specific water bodies	0.16	California Environmental Protection Agency, 1998
Rivers/streams median concentration	0.007	Kolpin *et al.,* 2002

EPA = U.S. Environmental Protection Agency.
WQS = water quality standard.

Table 3. Example tributyltin standards and environmental water levels

Parameter	Tributyltin concentration, µg/L	Source
Acute comparison		
National recommended water quality criterion	0.46	Levels in fresh water (EPA)
EU surface WQS, annual average	0.0002	Directive 2008/105/EC
Colorado acute surface WQS	0.73	Acute aquatic life protection
Secondary treated wastewater effluent	8.3	Kent *et al.*, 1991
Chronic comparison		
National recommended water quality criterion	0.072	Levels in fresh water (EPA)
EU surface WQS, maximum allowable	0.0015	Directive 2008/105/EC
Colorado chronic surface WQS	0.0002	Chronic aquatic life protection
Coastal waters concentration	0.5	Alzieu *et al.,* 1989

EPA = U.S. Environmental Protection Agency.
EU = European Union.
WQS = water quality standard.

been receiving attention because of their apparent impact on aquatic life events at low concentrations detected in wastewater and in rivers and streams. As a result, EPA has been charged with standardizing methods to characterize EDCs.

Regulation of EDCs

In 1996, the Food Quality Protection Act directed EPA to establish test methods for chemical ingredients in registered pesticides that may alter endocrine system activity. That same year, amendments to the Safe Drinking Water Act mandated a screening program to study EDCs in drinking water. In response, EPA organized the Endocrine Disruptors Screening and Testing Advisory Committee to help implement a policy that protects humans and wildlife from suspected endocrine disruptors. The committee's primary objective was to recommend chemical screening and testing programs to EPA for chemicals of potential concern.

EPA screening program

Under the advisory committee's recommendations, EPA developed the Endocrine Disruptor Screening Program, which prioritizes chemicals to be tested as endocrine disruptors. At press time, EPA had selected 67 pesticides as part of Tier 1 of the screening program, which would determine whether a compound has the potential to disrupt the estrogen, androgen, or thyroid hormone system. The compounds were selected based on their high production volume and exposure

Table 4. Example PCB standards

Parameter	PCB concentration, µg/L	Source
Water quality objectives	0.3 – 0.5	California state and regional water boards
WQS based on human health protection	0.07	California — Los Angeles region plan
WQS based on aquatic life protection (fresh water)	0.014	California — Los Angeles region plan
WQS based on aquatic life protection (saltwater)	0.03	California — Los Angeles region plan
National recommended water quality criterion	0.014	Chronic levels in fresh water (EPA)

EPA = U.S. Environmental Protection Agency.
PCB = polyychlorinated biphenyl.
WQS = water quality standard.

Table 5. Example atrazine standards and groundwater levels

Parameter	Atrazine concentration, µg/L	Source
EU surface WQS, annual average	0.6	Directive 2008/105/EC
EU surface WQS, maximum allowable	2.0	Directive 2008/105/EC
Water quality objectives	0.3 – 0.5	California state and regional water boards
Colorado surface water quality standard	3.0	For potable water supplies
Concentrations in groundwater	0.007	Dawson *et al.,* 2008

EU = European Union.
WQS = water quality standard.

potential. The results obtained from the Tier 1 program will be validated in the Tier 2 screening program, which involves a more comprehensive set of bioassays to determine whether each compound reveals endocrine-disrupting capacity. Pesticides are being used as a "test case" that likely will help future compound analysis to be completed more quickly; the methods developed during this process will focus on pesticides first, and other types of compounds will follow.

The current challenge of the Tier 1 screening program is that target compounds being evaluated are only high-volume-production pesticides. Other types of compounds — such as steroidal compounds, pharmaceutical and/or personal care products, and other specific compounds, such as PCBs, bisphenol-A (BPA), and nonylphenol — are not being evaluated. Regulatory agencies have no standard methodologies to determine endocrine-disrupting impacts. The Tier 2 screening program should be able to establish standard methods in determining whether a compound exhibits endocrine-disruption capabilities, but establishing the Tier 2 program likely will take a much longer time than the Tier 1 program, since the methods may involve subjecting two or more generations of organisms to the test compounds.

Priority pollutants

Microconstituents can become regulated through water quality standards established by the Clean Water Act (CWA). Under CWA, EPA establishes water quality criteria for selected compounds based on their potential to affect aquatic and human health. CWA also provides concentration-based guidance to states and tribes for adopting the criteria into standards. The selected compounds are referred to as "priority pollutants." Most states have implemented standards for all priority pollutants, many of which are considered endocrine disruptors. However, the EDCs included in the priority pollutant list generally do not have standards associated with the endocrine-disrupting effects (at low levels); instead, the standards are generally for higher concentrations and are designed to protect from toxic effects.

A closer look: nonylphenol, diazinon, and tributyltin

In addition to the compounds listed as priority pollutants, EPA has identified other compounds for their potential to affect aquatic life, including three endocrine disruptors: nonylphenol, diazinon, and tributyltin.

Nonylphenol

Nonylphenol primarily comes from household detergents. This compound has been of concern due to its biotransformation in the wastewater treatment process. Of the 85 rivers and streams sampled by the U.S. Geological Survey across the United States, approximately half contained nonylphenol of various congeners, with a median concentration of 1 µg/L (Kolpin *et al.*, 2002). However, a maximum concentration of 40 µg/L was detected. Secondary- and tertiary-treated wastewater effluents can have concentrations as high as 34 µg/L and 10 µg/L, respectively. The EPA criteria for nonylphenol are 28 µg/L for acute aquatic life protection and 6.6 µg/L for chronic aquatic life protection (see Table 1, p. 10). The European Union recently established stricter surface water quality standards. The standards, through Directive 2008/105/EC (2008), are 0.3 µg/L and 2.0 µg/L for annual average and maximum allowable concentrations, respectively.

Table 6. Priority substances listed in Directive 2008/105/EC that are known endocrine disruptors

Chemical	AA-EQS inland surface water, µg/L	AA-EQS other surface water, µg/L	MAC-EQS inland surface water, µg/L	MAC-EQS other surface water, µg/L
Atrazine	0.6	0.6	2.0	2.0
DDT total	0.025	0.025	n/a	n/a
Di(2-ethylhexyl)-phthalate	1.3	1.3	n/a	n/a
Endosulfan	0.005	0.005	0.01	0.01
Nonylphenol (4-nonylphenol)	0.3	0.3	2.0	2.0
Octylphenol ((4-(1,1',3,3'-tetramethybutyl)-phenol))	0.1	0.01	n/a	n/a
Pentachlorophenol	0.4	0.4	1.0	1.0

AA = annual average.
EQS = environmental quality standard.
MAC = maximum allowable concentration.
PCB = polyychlorinated biphenyl.

Table 7. Contaminants in drinking water regulated in California

Contaminant	California MCL, µg/L
1,1 dichloroethane	5.0
1,3 dichloropropene	0.5
Methyl tert butyl ether (MTBE)	13.0
1,1,2,2 tetrachloroethane	1.0
Trichlorofluoromethane	150
1,1,2 trichloro 1,2,2 trifluoromethane	1,200
Bentazon	18
Molinate	20
Thiobencarb	70
Perchlorate	6.0

EU = European Union.
MCL = maximum contaminant level.

The District of Columbia's Department of the Environment initiated a water quality standards triennial review process to add a nonylphenol water quality standard. The district will be adopting EPA's recommended water quality criteria of 28 µg/L and 6.6 µg/L for acute and chronic concentrations, respectively (see Table 1). The facilities operated by the D.C. Water and Sewer Authority (DC Water) will be required to comply with these standards in their revised permits.

Colorado has adopted the national criteria for nonylphenol with a delayed effective date. The state's Water Quality Control Commission set an effective date of Jan. 1 for nonylphenol standards. The Colorado Wastewater Utility Council had requested an extension of the effective date, citing the lack of an EPA-approved analytical method and the lack of ability to identify and control nonylphenol and its parent compounds in wastewater influent. Council members have been working to better understand sources of nonylphenol, identify options for source control, and develop an accurate analytical method.

At the time this article was written, EPA regional offices were not aware of any other states or cities that are implementing nonylphenol as a standard. However, as the District of Columbia and Colorado implement standards for nonylphenol, this compound likely will be controlled by more state environmental departments.

Diazinon

Diazinon, an insecticide, can affect the liver and pancreas, as well as the reproductive systems, of organisms. According to Kolpin *et al.* (2002), approximately 25% of the 85 rivers and streams sampled contained diazinon, with a median and maximum concentration of 0.007 µg/L and 0.35 µg/L, respectively. Diazinon was not part of Directive 2008/105/EC of the European Union (EU); however, it is monitored at wastewater treatment plants as part of Directive 2000/60/EC requirements. A numerical standard for diazinon has been established by EPA at 0.17 µg/L, which was developed to protect aquatic life. This standard has been adopted in some states across the United States, including Colorado.

At press time, EPA regional offices were unaware of references to diazinon in discharge permits. However, at the state level, one of the nine California regional boards in the Central Valley has implemented water quality standards for specific waters (see Table 2, p. 11). Water quality standards for diazinon in the other eight regional plans were not implemented. Therefore, it seems diazinon may not be covered in discharge permits unless agricultural activities are in the area.

Tributyltin

Tributyltin is an ingredient in biocide and is primarily used to prevent fouling in water pipelines. It is considered an extremely toxic compound to aquatic organisms. Concentrations as high as 500 ng/L have been detected in coastal waters. Tributyltin is considered an endocrine disruptor for aquatic organisms due to its ability to produce

male sexual characteristics in female abalones and gastropods.

Numerical standards for tributyltin exist in states such as Colorado and were established from EPA's national recommended water quality criteria (see Table 3, p. 11). Acute and chronic concentration standards for tributyltin are 0.73 µg/L and 0.0002 µg/L, respectively, for aquatic life protection. EPA has not established criteria for human health. The EU has established surface water quality standards at 0.0002 µg/L and 0.0015 µg/L for annual average and maximum allowable concentrations, respectively.

At press time, instances of tributyltin in discharge permits had not been identified, according to officials in EPA regional offices.

PCBs

PCBs have received considerable attention as endocrine disruptors, primarily due to low-level estrogenic activity, and have been linked to various thyroid-related abnormalities in humans. Before their ban in 1979, PCBs were used as lubricants and as ingredients in pesticides, paint, sealants, plastics, and flame retardants. The plastics industry employed PCBs because of their insulating properties and stability. Despite their ban, EPA lists PCBs as a priority pollutant. Many states have adopted the national recommended water quality criterion of 0.014 µg/L and 0.03 µg/L for fresh water and saltwater, respectively, as enforceable standards, or they have adopted pollution prevention programs for PCBs (see Table 4, p. 12).

In general, although there are regulations in place to control PCBs, PCBs are not covered in discharge permits, because they do not enter the environment from industrial, commercial, or residential sources. PCBs do persist in the environment and in rivers and streams from previous sources of contamination, and in some areas of the country, PCBs are making their way into collection systems as a legacy pollutant. For instance, the District of Columbia is currently establishing a criterion for PCBs in surface water (see Table 4), which will also be implemented in DC Water's discharge permit.

PCBs illustrate how product bans, along with public education, could be used to remove EDCs from sanitary wastewater streams. This approach may be particularly important for compounds that are extremely persistent or difficult to treat effectively, or for compounds that transform to a more harmful form during treatment.

Atrazine

Atrazine is used primarily in the corn production industry as an herbicide and has been detected in groundwater in California at 0.007 µg/L and in surface water at 14 µg/L. Atrazine is considered an endocrine disruptor due to its potential to reduce the production of androgen hormones and increase estrogen production. An aquatic life criterion for atrazine is currently being developed by EPA. The EU has developed standards for atrazine of 0.6 µg/L (chronic) and 2.0 µg/L (acute; see Table 5, p. 12). Colorado has developed a surface water drinking water supply standard, which is strictly for potable water. Some of California's regional boards, each with its own water quality objectives, have developed standards for atrazine: The Los Angeles region has a standard of 0.3 µg/L, and the San Francisco and San Diego regions have a standard of 0.1 µg/L.

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At this time, instances of atrazine coverage in discharge permits have not been identified, according to discussions with officials in EPA regional offices.

EDC regulation in Europe

The EU has developed environmental quality standards for priority substances. Thirty-three compounds have been identified as priority substances; seven of the compounds are known endocrine disruptors (see Table 6, p. 13). Annual average concentrations apply to long-term exposure, and maximum allowable concentrations apply to protection against short-term exposures.

Under Directive 2008/105/EC, new compounds are being considered as possible priority substances or priority hazardous substances. Of the compounds that are subject for review, six are known endocrine disruptors: BPA, dicofol, ethylenediaminetetraacetic acid, musk xylene, PCBs, and dioxin.

Drinking water and water reuse requirements as catalysts

All states must meet the requirements of the Safe Drinking Water Act. Although no state can relax federal standards, it can establish standards that are more stringent, as well as establish standards for compounds that are not regulated by EPA. With the increasing use of tertiary-treated wastewater as a source for potable water, drinking water standards can serve as a catalyst to establish numerical standards at wastewater treatment plants. This is especially true in dry areas where fresh water is scarce. For instance, California, the leading state in water reuse, has established numerical standards for contaminants in drinking water sources (see Table 7, p. 13).

Conclusions

It seems that, in general, the development and implementation of standards for EDCs is not imminent. EPA's current focus is on determining the actual endocrine-disrupting levels and impacts of 67 specific compounds. Presumably, EPA criteria will be developed based on the impacts observed during these studies, which the states may then adopt. There is also a need for standard, EPA-approved methods for measurement of the compounds. Although several states are moving forward with developing and implementing standards for EDCs, most have no plans to do so in the near future. Additionally, many compounds that are being adopted are pesticides and herbicides, which may not cause a reasonable potential determination for inclusion in permits for publicly owned treatment works but might be included in an industrial permit.

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WEBNOTES

Editor's Note: Questions and replies are taken from the technical discussion groups on the Water Environment Federation's Web site. Solutions *assumes no responsibility for claims or comments.*

Phosphorus method detection limit

Q: I would like to figure out our [lab's] method detection limit (MDL) for phosphorus (*Standard Methods* 4500-P), B (sample preparation), E (ascorbic acid). We use a Spec 20 to read the absorbance. My question is what value do I use for my lower instrumentation limit? My understanding is that the concentrations are only linear from 0.20 [mg/L-P] to 0.70 [mg/L-P]. I obviously can't use 0.20 [mg/L-P] as my lower limit, since 5 times this limit would give me 1.0 mg/L. Thanks.

Name withheld

A: I have not used the Spec 20 for some time, but if memory is correct, the MDL was about 0.02 mg/L-P. I do not know where the range 0.20 mg/L-P to 0.70 mg/L-P came from. Analyze a set of standards and see what you can generate for a detection limit with your equipment.

Jim Royer
Chief chemist
Urbana and Champaign (Ill.) Sanitary District

Q: Do I develop a standard curve around this low detection limit of 0.02 mg/L-P?

Name withheld

A: What you need to do is develop a curve ranging from about 0.005 mg/L to 1.5 mg/L. Read each of these points and graph your responses. You will need to visually inspect the resultant curve and find the range where your instrument, procedure, analyst, etc., produce a linear response.

You should try to shoot for having a detection limit of whatever the end user will require. If you are a commercial lab, you need to know the detection limits required by your clients. If you are doing this as a lab in a plant, you need to know the detection limit on your permits. Once you have established your curve, you can determine your MDL. Do your

seven replicates at the previously determined detection limit and apply the necessary statistical analysis.

Your final MDL should be less than your detection limit. The "5 times" you are referring to in your original post is a "5 times less than." I believe the actual requirement is less than half the detection limit, but greater than one-tenth the detection limits. If your detection limit is 0.2 mg/L (somewhat high in my opinion; you really ought to be able to get down to 0.05 mg/L), [then] your MDL should end up 0.02 [0.02 mg/L-P] to 0.1 [0.02 mg/L-P]. If your calculated MDL is consistently lower than one-tenth your detection limit, that tells you that your detection limit is too high.

David Smith
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BRIEFS

LabWrench.com launches comparison engine

LabWrench.com, a product-focused social networking Web site, has released a comparison engine on a number of key lab-equipment product categories. This feature gives lab workers the ability to filter through a defined set of product features based on their requirements and output a side-by-side comparison with comprehensive specifications and details.

LabWrench, produced by LabX Media Group (Midland, Ontario), is a product-focused social networking site that enables visitors to connect, discuss, and compare lab equipment, according to a company press release.

For example, said LabX Media Group President Bob Kafato, the centrifuge category enables users to "delve into the purchase process of a centrifuge and define [their] requirements for maximum spin speed, [as well as] requirements for refrigeration and tube capacity, and then sort the matching products side by side however [they] want. It puts all the details in front of the lab purchaser, and that helps them make smart, informed decisions."

Report revises U.S. EPA method holding times

The U.S. Environmental Protection Agency (EPA) has released a report titled *Holding Time Study for Pharmaceuticals and Personal Care Products, Sterols and Hormones*.

In this work, EPA describes a study conducted to revise the holding times and preservation conditions for EPA methods 1694 and 1698. This study tested a broad number and variety of chemicals, matrices, and preservation techniques under conditions expected in samples collected for Clean Water Act programs.

EPA methods 1694 and 1698 cover pharmaceuticals and personal care products, steroids, and hormones in wastewater influent, effluent, and sludge. Although researchers have published several holding-time and preservation studies, the studies have been limited in the number and variety of matrices, chemicals, and preservation techniques tested. Because these studies were also conducted with a range of different methods, comparing data between studies is difficult.

EPA has used the results of this study to revise the holding times and preservation conditions in EPA methods 1694 and 1698. The suggested holding times are precautionary, as they protect the most sensitive compounds. They are not universal holding times for pharmaceuticals and personal care products or steroids and hormones.

For more information, visit the EPA Web site at water.epa.gov/scitech/swguidance/methods/index.cfm, or call the EPA Office of Science and Technology at (202) 566-1000.

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