APPENDIX B
ANALYTICAL METHODS AND NOMINAL QUANTITATION LIMITS

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The analytical methods described in this appendix were used to determine pollutant levels in wastewater samples collected by EPA at a number of aquatic animal production facilities (sampling efforts are described in Chapter 3). In developing the rule, EPA sampled aquatic animal production facilities to determine the levels of *Aeromonas*, ammonia as nitrogen, 5-day biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), chloride, *E. coli*, *Enterococcus faecium*, fecal coliform, fecal streptococcus, 27 metals, *Mycobacterium marinum*, nitrate/nitrite, oil and grease (measured as hexane extractable material (HEM)), pH, settleable solids, semivolatile organics, sulfate, total chlorine, total coliform, total dissolved solids (TDS), total Kjeldahl nitrogen (TKN), total organic carbon (TOC), total orthophosphate, total phosphorus, total solids, total suspended solids (TSS), volatile organics, volatile residue, and whole effluent toxicity (WET). As explained in Chapters 2 and 8, EPA is regulating TSS for all facilties, and regulating total phosphorus and BOD_5 for some facilities.

Section B.1 of this appendix provides an explanation of nominal quantitation limits. Section B.2 describes the reporting conventions used by laboratories in expressing the results of the analyses. Section B.3 describes each analytical method and the nominal quantitation limits associated with each method.

B.1 NOMINAL QUANTITATION LIMITS

The nominal quantitation limit is the smallest quantity of an analyte that can be reliably measured with a particular method, using the typical (nominal) sample size. The protocols used for determination of nominal quantitation limits in a particular method depend on the definitions and conventions that EPA used at the time the method was developed. Printouts in Section 10 of the proposal record list the nominal quantitation limit as a 'baseline value.'[1] The nominal quantitation limits associated with the methods addressed in this section fall into five categories.

1. The first category pertains to EPA Methods 1624, 1625, and 1664, which define the minimum level (ML) as the lowest level at which the entire analytical system must give a recognizable signal and an acceptable calibration point for the analyte. These methods are described in Section B.3.1.

2. The second category pertains specifically to metals, and is explained in detail in Section B.3.2.

[1] EPA used two different methods to analyze for ammonia as nitrogen and TKN, and only one method for the remaining pollutants of concern. The printout lists the nominal quantitation limit for the analytical method that was used most frequently for ammonia as nitrogen (Method 350.1) and TKN (Method 351.2).

3. The third category pertains to the remainder of the chemical methods (classical wet chemistry analytes) in which a variety of terms are used to describe the lowest level at which measurement results are quantitated. In some cases the methods date to the 1970s and 1980s when different concepts of quantitation were employed by EPA. These methods typically list a measurement range or lower limit of measurement. The terms differ by method and, as discussed in subsequent sections, the levels presented are not always representative of the lowest levels laboratories currently can achieve.

   For those methods associated with a calibration procedure, the laboratories demonstrated through a low-point calibration standard that they were capable of reliable quantitation at method-specified (or lower) levels. In such cases, these nominal quantitation limits are operationally equivalent to the ML (although not specifically identified as such in the methods).

   In the case of titrimetric or gravimetric methods, the laboratory adhered to the established lower limit of the measurement range published in the methods. Details of the specific methods are presented in Sections B.3.3 through B.3.19.

4. The fourth category pertains to all microbiological methods. This category pertains to the membrane filtration test and multiple tube fermentation procedures and are explained in detail in Section B.3.20.

5. The fifth category pertains to all whole effluent toxicity methods. The whole effluent toxicity methods are explained in detail in Section B.3.21.

B.2 ANALYTICAL RESULTS REPORTING CONVENTIONS

Most of the analytical chemistry data were reported as liquid concentrations in weight/volume units (e.g., micrograms per liter [g/L]), except for settleable solids data., which were reported in volume/volume units (e.g., milliliters per liter [mL/L]), and the pH data, which were reported in "standard units" (SU). In the case of solid samples such as sediments, the results were provided in weight/weight units (e.g., milligrams per kilogram [mg/kg]). Bacteriological data generated using membrane filtration techniques were reported as colony forming units (CFU) per 100 mL volume of sample or for data generated using multiple-tube fermentation techniques were reported as most probable number per 100 milliliters (MPN/100 mL). Whole effluent toxicity data endpoints measured were lethality in 50% of the organisms (LC50) for the fathead minnow and the *Ceriodaphnia*, growth in the larval fathead minnow and *Selenastrum*, and the number of offspring produced in the *Ceriodaphnia.*

The laboratories expressed results of the analyses either numerically or as not quantitated[2] for a pollutant in a sample. If the result is expressed numerically, then the

[2] Elsewhere in this document and in the preamble to the proposed rule, EPA may refer to pollutants as "not detected" or "non-detected." This appendix uses the term "not quantitated" or "non-quantitated" rather than non-detected.

pollutant was quantitated[3] in the sample. For the non-quantitated results, for each sample, the laboratories reported a "sample-specific quantitation limit."[4] The sample-specific quantitation limit was used as a reporting limit for this industry. Two reporting examples are provided below.

Example 1: For a hypothetical pollutant X, the sample-specific quantitation limit is 10 g/L. When the laboratory quantitated the amount of pollutant X in the sample as being 15 g/L, the result would be reported as "15 g/L."

Example 2: For the hypothetical pollutant X, the sample-specific quantitation limit is 10 g/L. When the laboratory could not quantitate the amount of pollutant X in the sample, the result would be reported as "<10 g/L." That is, the analytical result indicated a value less than the sample-specific quantitation limit of 10 g/L. The actual amount of pollutant X in that sample is between zero (i.e., the pollutant is not present) and 10 g/L. If a pollutant is reported as non-quantitated in a particular wastewater sample, this does not mean that the pollutant is not present in the wastewater. It means that analytical techniques (whether because of instrument limitations, pollutant interactions, or other reasons) do not permit its measurement at levels below the sample-specific quantitation limit.

In its calculations, EPA generally substituted the reported value of the sample-specific quantitation limit for each non-quantitated result.

B.3 ANALYTICAL METHODS

EPA analyzed all of the aquatic animal production facility wastewater samples using methods identified in Table B-1. (As explained in Section Z, EPA is proposing to regulate only a subset of these analytes.) Except for the volatile and semivolatile organics and total organic carbon, EPA used either EPA methods from *Methods for Chemical Analysis of Water and Wastes* (MCAWW) or the American Public Health Association's *Standard Methods for the Examination of Water and Wastewater*. EPA methods are identified in the sections that follow by their method number, e.g., EPA Method 1624. Methods from *Standard Methods for the Examination of Water and Wastewater* are prefaced by "SM." All of the chemical methods cited from Standard Methods (SM) are from the 18th edition; the biological methods cited from Standard Methods are from the 20th edition.

In analyzing samples, EPA generally used analytical methods approved at 40 CFR Part 136 for compliance monitoring or methods that had been in use by EPA for decades in support of effluent guidelines development. Exceptions for use of non-approved methods are explained in the method-specific subsections that follow. All EPA-proposed limitations or standards are based upon data generated by methods approved at 40 CFR Part 136.

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[3] Elsewhere in this document and in the preamble to the proposed rule, EPA may refer to pollutants as "detected." This appendix uses the term "quantitated" rather than detected.

[4] Elsewhere in this document and in the preamble to the proposed rule, EPA may refer to a "sample-specific quantitation limit" as a "sample-specific detection limit" or, more simply, as a "detection limit."

Each of the following sections states whether the method is listed at 40 CFR Part 136 (even if the pollutant was not proposed for regulation), provides a short description of the method, and identifies the nominal quantitation limit. Methods listed at 40 CFR Part 136 are approved for use in wastewater compliance monitoring under the NPDES process.

Table B-1 Analytical Methods

	Method	CAS Number	Nominal Quantitation Limit	Unit
Aeromonas	1605	C2101	1	CFU/100 mL
	9260L	C2101	2	MPN/100 mL
Ammonia as Nitrogen	350.1	7664417	0.01	mg/L
	350.2	7664417	0.05	mg/L
	4500-NH_3 H	7664417	0.02	mg/L
BOD_5	405.1	C003	2.0	mg/L
Ceriodaphnia Dubia Chronic	1002.0	N/A	100	%
COD	410.1	C004	5.0	mg/L
	410.4	C004	3.0	mg/L
	5220C	C004	50.0	mg/L
Chloride	325.1	16887006	1.0	mg/L
	325.3	16887006	1.0	mg/L
	4500Cl^- B	16887006	1.5	mg/L
Dissolved Phosphorus	365.2	14265442D	0.01	mg/L
E. coli	1604	68583222	1	CFU/100 mL
Enterococcus Faecium	9230C	68876788	100	CFU/100 mL
Fathead Minnow	1000.0	N/A	100	%
Fecal Coliform	9222D	C2106	1	CFU/100 mL
Fecal Streptococcus	9230C	C2107	1	CFU/100 mL
	9230B	C2107	2	MPN/100 mL
HEM	1664	C036	5.0	mg/L
Metals	1620	†		
	200.7	†		
	200.9	†		
	245.1	†		

	Method	CAS Number	Nominal Quantitation Limit	Unit
Mycobacterium marinum	9260M	C2119	4	CFU/100 mL
Nitrate/Nitrite	353.1	C005	0.01	mg/L
	353.3	C005	0.01	mg/L
	4500-NO_3 E	C005	0.01	mg/L
Oil and Grease	5520E	C036	5.0	mg/L
Orthophosphate	365.2	C034	0.01	mg/L
pH	150.1	C006		SU
	9045C	C006		SU
Selenastrum Growth Test	1003.0	N/A	100	%
Semivolatile Organics	1625	†		
Settleable Solids	2540F	N/A	0.1	mL/L
Sulfate	375.3	14808798	10.0	mg/L
	375.4	14808798	1.0	mg/L
Total Chlorine	Test Strip	7782505	0.05	mg/L
Total Coliform	1604	E10606	1	CFU/100 mL
	9221B	E10606	2	MPN/100 mL
Total Dissolved Solids	160.1	C010	10.0	mg/L
Total Kjeldahl Nitrogen	351.2	C021	0.5	mg/L
	351.3	C021	1.0	mg/L
	4500-N_{org} C	C021	0.02	mg/L
Total Organic Carbon	415.1	C012	1.0	mg/L
	Lloyd Kahn	C012	100	mg/kg
Total Phosphorus	365.2	14265442	0.01	mg/L
Total Solids	160.3	C008	10.0	mg/L
Total Suspended Solids	160.2	C009	4.0	mg/L
Volatile Organics	1624	†		
Volatile Residue	160.4	C030	10.0	mg/L

N/A There is no CAS Number for this analyte.† The method analyzed a number of pollutants

B.3.1 EPA Methods 1624, 1625, and 1664 (Volatile Organics, Semivolatile Organics, and HEM)

Laboratories used EPA Methods 1624, 1625, and 1664 to measure volatile organics, semivolatile organics, and *n*-hexane extractable material (HEM). EPA Methods 1624, 1625, and 1664 are approved at 40 CFR Part 136.

These methods use the minimum level (ML) concept for quantitation of pollutants. The ML is defined as the lowest level at which the entire analytical system must give a recognizable signal and an acceptable calibration point for the analyte. When an ML is published in a method, the Agency has demonstrated that the ML can be achieved in at least one well-operated laboratory. When that laboratory or another laboratory uses that method, the laboratory is required to demonstrate, through calibration of the instrument or analytical system, that it can achieve pollutant measurements at the ML.

The nominal quantitation values are equal to the MLs listed in the methods for each analyte. The MLs for majority of volatile and semivolatile organics are 10 g/L, with a small number of higher values for pollutants that are more difficult to analyze. The ML for HEM determined by EPA Method 1664 is 5 mg/L.

B.3.2 EPA Methods 1620, 200.7, 200.9, and 245.1 (Metals)

Laboratories used EPA Methods 1620, 200.7, 200.9, and 245.1 to measure the concentrations of 27 metals. While EPA Method 1620 is not listed at 40 CFR Part 136, it represents a consolidation of the analytical techniques in several 40 CFR 136-approved methods such as EPA Method 200.7 (inductively coupled plasma atomic emission (ICP) spectroscopy of trace elements) and Method 245.1 (mercury cold vapor atomic absorption technique). EPA Method 1620 was developed specifically for the effluent guidelines program. This method includes more metal analytes than are listed in the approved metals methods and contains quality control requirements that are at least as stringent as the approved methods. EPA Method 200.9 is not listed at 40 CFR Part 136, but represents a consolidation of the graphite furnace analytical methods approved at 40 CFR Part 136, such as EPA Methods 206.2 and 279.2.

EPA Method 1620 employs the concept of an instrument detection limit (IDL). The IDL is defined as "the smallest signal above background noise that an instrument can detect reliably." Data reporting practices for EPA Method 1620 analyses follow conventional metals reporting practices used in other EPA programs, in which values are required to be reported at or above the IDL. In applying EPA Method 1620, IDLs are determined on a quarterly basis by each analytical laboratory and are, therefore, laboratory-specific and time-specific.

Although EPA Method 1620 contains MLs, these MLs pre-date EPA's recent refinements of the ML concept described earlier. The ML values associated with EPA Method 1620 are based on a consensus reached between EPA and laboratories during the 1980s regarding levels that could be considered reliable quantitation limits when using EPA Method 1620. These limits do not reflect advances in technology and instrumentation since the 1980s. Consequently, the IDLs were used as the lowest values for reporting purposes, with the general understanding that reliable results can be produced at or above

the IDL. The nominal quantitation values are the MLs listed in EPA Method 1620, except for two instances. The published ML for lead in EPA Method 1620 is 5 g/L for graphite furnace atomic absorption (GFAA) spectroscopy analysis. However, for the purposes of this effluent guideline study, EPA determined that it was not necessary for the laboratories to measure lead to such low levels, and permitted the analysis of lead by ICP spectroscopy. Consequently, the nominal quantitation limit for lead was adjusted to 50 g/L, the ML for the ICP method. Boron has an ML of 10 g/L, but historical information indicates that laboratories could not reliably achieve this low level. As a result, EPA only required laboratories to measure values at 100 g/L and above; this is the nominal quantitation limit used here.

B.3.3 EPA Methods 350.1 and 350.2, and SM 4500H (Ammonia as Nitrogen)

Ammonia, as nitrogen, was measured using EPA Methods 350.1 and 350.2, and SM 4500H, all of which are approved at 40 CFR Part 136. Methods 350.1 and SM 4500H are automated methods using a continuous flow analytical system with a phenate/hypochlorite color reagent that reacts with ammonia to form indophenol blue that is proportional to the ammonia concentration. Method 350.2 utilizes either colorimetric, titrimetric, or electrode procedures to measure ammonia.

Method 350.1 has a lower measurement range limit of 0.01 mg/L. SM 4500H has a lower measurement range limit of 0.02 mg/L. Method 350.2 has a lower measurement range limit of 0.20 mg/L for the colorimetric and electrode procedures, and a lower measurement range limit of 1.0 mg/L for the titrimetric procedure.

B.3.4 EPA Method 405.1 (Biochemical Oxygen Demand)

Biochemical oxygen demand (BOD_5) was measured using EPA Method 405.1, which is approved at 40 CFR Part 136. The sample and appropriate dilutions are incubated for five days at 20 C in the dark. The reduction in dissolved oxygen concentration during the incubation period is the measure of the biochemical oxygen demand.

The nominal quantitation limit for Method 405.1, which is expressed in the method as the lower limit of the measurement range, is 2 mg/L.

B.3.5 EPA Methods 410.1 and 410.4, and SM 5220C (Chemical Oxygen Demand)

Chemical oxygen demand (COD) was measured using EPA Methods 410.1 and 410.4, and SM 5220C, all of which are approved at 40 CFR Part 136. Methods 410.1 and SM 5220C are titrimetric procedures designed to measure mid-level concentrations of COD and are associated with a nominal quantitation limit of 50 mg/L. Method 410.4 is a spectrophotometric procedure that measures COD and is associated with a nominal quantitation limit of 3 mg/L.

B.3.6 EPA Methods 325.1 and 325.3, and SM 4500B (Chloride)

Chloride was measured using Methods 325.1 and 325.3, and SM 4500B, all of which are approved at 40 CFR Part 136. Method 325.1 is an automated colorimetric method that uses a ferricyanide reagent color for development. Method 325.3 is a titrimetric

procedure that uses mercuric nitrate as the titrant. SM 4500B is also a titrimetric procedure, but it uses silver nitrate as the titrant.

Methods 325.1 and 325.3 measure concentrations greater than 1 mg/L, so the nominal quantitation limit is 1 mg/L. SM 4500B measures concentrations greater than 1.5 mg/L, so the nominal quantitation limit is 1.5 mg/L.

B.3.7 EPA Methods 353.1 and 353.3, and SM 4500E (Nitrate/Nitrite)

Nitrate/nitrite was measured using EPA Methods 353.1 and 353.3, and SM 4500E, all of which are approved at 40 CFR Part 136. Method 353.1 is based on a colorimetric technique (i.e., adding reagents to a sample that form a colored product when they react with the nitrate/nitrite and measuring the intensity of the colored product). Method 353.1 uses hydrazine to reduce the nitrate (NO_3) present in the sample to nitrite (NO_2). Methods 353.3 and SM 4500E use granulated copper cadmium to reduce nitrate to nitrite. The nitrite is determined by reaction with sulfanilamide and coupling with N-(1-naphthyl)-ethylene diamine dihydrochloride to form a highly colored azo dye that is measured spectrophotometrically.

The nominal quantitation limit associated with Methods 353.1, 353.3, and SM 4500E is 0.01 mg/L.

B.3.8 SM 5520E (Oil and Grease)

SM 5520E was used to measure oil and grease in the sediment samples from the aquatic animal production facilities because EPA Method 1664 is only applicable to aqueous samples. SM5520E is not approved at 40 CFR Part 136 because this method is applicable only to solid samples and not wastewater samples. SM 5520E is a gravimetric method in which the sediment is dried, the oil and grease is extracted with *n*-hexane, and the extract is weighed to obtain the concentration of oil and grease in the sample. The only difference between SM5520E and Method 1664 is the preparation of the sample for extraction. The solid sample is dried and magnesium sulfate added before extraction. There is no nominal quantitation limit associated with this method.

B.3.9 EPA Methods 150.1 and 9045C (pH)

EPA Method 150.1 was used to analyze aqueous samples. Method 150.1 is approved at 40 CFR Part 136. Method 9045C, from *Test Methods for Evaluating Solid Waste, Physical/Chemical Methods* (SW-846), was used to analyze the sediment samples. Although Method 9045C is not approved at 40 CFR Part 136, it is approved for analyses of solid samples under the RCRA regulations at 40 CFR Part 261.

For Method 150.1, the pH of a sample is determined electrometrically using either a glass electrode in combination with a reference potential or a combination electrode. For Method 9045C, the sample is mixed with reagent water and the pH of the resulting aqueous solution is measured electrometrically.

There are no nominal quantitation limits for either Method 150.1 or 9045C.

B.3.10 SM 2540F (Settleable Solids)

Settleable solids was determined by SM 2540F in the field by the samplers at the aquatic animal production facilities. SM 2540F is a volumetric method which uses an Imhoff cone. An Imhoff cone is filled to the 1-L mark with a well-mixed wastewater sample. The solids in the sample are allowed to settle in the cone for 45 minutes. The sample is agitated near the sides of the cone with a rod or by spinning and allowed to settle for an additional 15 minutes. The volume of the settleable solids in the cone is recorded as milliliters per liter (mL/L).

SM 2540F is approved at 40 CFR Part 136 under "residue-settleable." The method lists a lower limit of the measurement range of 0.1 mL/L; this value is also the nominal quantitation limit.

B.3.11 EPA Methods 375.3 and 375.4 (Sulfate)

Sulfate was measured by EPA Methods 375.3 and 375.4, both of which are approved at 40 CFR Part 136. Method 375.3 is a gravimetric method that measures the amount of barium sulfate formed by reacting the sample with barium chloride. Method 375.4 measures the turbidity created by the insoluble barium sulfate in solution. A dispersant/buffer is added to the solution to aid in creating uniform suspension of the barium sulfate.

The nominal quantitation limit (also the lower limit of the measurement range) for Method 375.3 is 10 mg/L. The nominal quantitation limit for Method 375.4 is 1 mg/L.

B.3.12 Test Strip Kit (Total Chlorine)

Total chlorine was determined by SenSafe™ total chlorine test strips in the field by the samplers at the aquatic animal production facilities. SenSafe™ total chlorine test strips range in sensitivity from 0.05 mg/L to 80 mg/L. The test strip from each sample is compared to a color chart to determine the result of the total chlorine.

B.3.13 EPA Method 160.1 (Total Dissolved Solids)

Total dissolved solids (TDS) were measured by EPA Method 160.1, which is approved at 40 CFR 136 under "residue-filterable." Method 160.1 is a gravimetric method with a lower limit of the measurement range of 10 mg/L; this value is also the nominal quantitation limit.

B.3.14 EPA Methods 351.2 and 351.3, and SM 4500C (Total Kjeldahl Nitrogen)

Total Kjeldahl nitrogen (TKN) was measured by EPA Methods 351.2 and 351.3, and SM 4500C, all of which are approved at 40 CFR Part 136. For Method 351.2, the sample is digested in a strong acid and a metal ion catalyst solution, taken to dryness, then reconstituted with an alkaline solution. The ammonia is the solution is determined by indophenol colorimetry using an automated continuous flow system. For Methods 351.3 and SM 4500C, the sample digestion is performed using a strong acid reagent with a metal ion catalyst. After the digestion period is complete, the solution is made alkaline and the ammonia in the digestate is distilled off into a borate buffer solution. Methods 351.3 and SM 4500C offer three different quantitation technique options for determining

the ammonia concentration: titrimetric, iodide colorimetric, or NH_3 ion selective electrode.

The nominal quantitation limit (also the lower limit of the measurement range) for Method 351.2 is 0.1 mg/L. The nominal quantitation limit for Method 351.3 is 0.05 mg/L and the nominal quantitation limit for SM 4500C is 0.02 mg/L.

B.3.15 EPA Method 415.1 and the "Lloyd Kahn" Procedure (Total Organic Carbon)

Total organic carbon (TOC) was determined by EPA Method 415.1 and the "Lloyd Kahn" procedure. Method 415.1 is a combustion (or oxidation) method with a lower limit of the measurement range is 1 mg/L; this value is also the nominal quantitation limit. The Lloyd Kahn procedure is similar to Method 415.1, but allows for a pyrolitic method that uses an elemental analyzer to determine carbon concentration. The nominal quantitation limit for the Lloyd Kahn procedure is 100 mg/kg.

Method 415.1 is approved at 40 CFR Part 136 and was used to analyze aqueous samples. However, this method only applies to aqueous samples. Therefore, the Lloyd Kahn procedure was used to analyze the solid samples. The Lloyd Kahn procedure applies only to solid samples and therefore is not approved at 40 CFR Part 136.

B.3.16 EPA Method 365.2 (Dissolved Phosphorus, Total Orthophosphate, and Total Phosphorus)

Dissolved phosphorus, total orthophosphate and total phosphorus were measured by EPA Method 365.2, which is approved at 40 CFR Part 136. Total phosphorus represents all of the phosphorus present in the sample, regardless of form, as measured by the persulfate digestion procedure. Dissolved phosphorus results were obtained by filtering the sample prior to this step. Total orthophosphate represents the inorganic phosphorus (PO_4) in the sample determined by the direct colorimetric analysis procedure.

Method 365.2 is a colorimetric method and measures concentrations greater than 0.01 mg/L, which is also the nominal quantitation limit, for dissolved phosphorus, total orthophosphate, and total phosphorus.

B.3.17 EPA Method 160.3 (Total Solids)

Total solids were determined by EPA Method 160.3, which is approved at 40 CFR Part 136 as “residue-total.” Method 160.3 is a gravimetric method with a lower limit of the measurement range of 10 mg/L; this value is also the nominal quantitation limit.

B.3.18 EPA Method 160.2 (Total Suspended Solids)

Total suspended solids (TSS) were determined by EPA Method 160.2, which is approved at 40 CFR Part 136 as “residue-nonfiltrable.” Method 160.2 is a gravimetric method with a lower limit of the measurement range of 4 mg/L; this value is also the nominal quantitation limit.

B.3.19 EPA Method 160.4 (Volatile Residue)

Volatile residue was determined by EPA Method 160.4, which is approved at 40 CFR Part 136. Method 160.4 is a gravimetric and ignition method with a lower limit of the measurement range of 10 mg/L; this value is also the nominal quantitation limit.

B.3.20 EPA 1604 and SM 9221B, SM 9222D, SM 9230 B and 9230C, EPA 1605 and SM 9260L, SM 9260M (total coliform, fecal coliform, *E. coli*, fecal Streptococcus, *Enterococcus faecium, Aeromonas*, and *Mycobacterium marinum*)

Laboratories measured the densities of total coliform, fecal coliform, *E. coli*, fecal Streptococcus, *Aeromonas*, and *Enterococcus faecium* using membrane filtration methods specified in Standard Methods. EPA used multiple-tube fermentation procedures methods approved at 40 CFR Part 136 for ambient water for fecal coliform (SM9222D), fecal streptococcus (SM9230B and SM9230C), *Enterococcus faecium* (EPA Method 1106.1), total coliforms (EPA Method 1604 and SM9221B), and *E. coli* (EPA Method 1604). There are no 40 CFR Part 136-approved methods for *Aeromonas* or *Mycobacterium marinum*. For these microorganisms, EPA Method 1605 and SM 6260L was used for *Aeromonas*, and SM 9260M was used for *Mycobacterium marinum*.

1. **Total coliforms and *E. coli*** (EPA Method 1604 and SM 9221B). For EPA Method 1604, samples are filtered utilizing 0.45 m filters, placed onto MI agar, and incubated for 24 ± 2 hours. Plates are read using ambient light and UV light to obtain total coliform and *E. coli* counts. Blue colonies are recorded as positive for *E. coli* and all colonies that fluoresce under UV light are recorded as total coliforms. Total coliforms were also measured by SM 9221B. Samples were inoculated into a presumptive medium (lauryl tryptose broth) and incubated. Tubes positive for growth and gas production were transferred into confirmatory media, brilliant green bile broth for total coliform. Tubes with growth and gas production in this media were recorded as positive.

2. **Fecal coliforms (9222D)**. Samples are filtered and placed onto mFC plates and incubated for 24 ± 2 hours in a water bath at 44.5°C ± 0.2 C. All blue colonies are considered positive for fecal coliforms.

3. **Fecal streptococcus (SM 9230 B and 9230C)**. Samples are filtered and placed onto mEnterococcus plates and incubated for 48 ± 3 hours for SM 9230C. All light and dark red colonies are considered positive for fecal streptococcus. For SM 9230B, samples were inoculated into a presumptive medium (azide dextrose broth) and incubated. Tubes positive for turbidity (growth) were confirmed by streaking onto bile esculin agar plates. All plates with typical growth were recorded as positive for fecal streptococcus.

4. ***Aeromonas* (EPA Method 1605 and SM 9260L)**. For EPA Method 1605, samples are filtered and placed onto ADA-V plates. All yellow colonies are isolated on nutrient agar and confirmed as *Aeromonas* if they are oxidase- and indole-positive and are able to ferment trehalose. *Aeromonas* densities were also determined by SM 9260L, followed by the confirmation steps in EPA Method 1605 to minimize false positive results. Samples were inoculated into a presumptive medium (TSB30) and

incubated. Tubes with growth were streaked onto ampicillin-detxtrin agar (ADA). All yellow colonies were isolated on nutrient agar and confirmed as Aeromonas if they were oxidase positive and were able to ferment trehalose. In addition to the biochemical confirmation, colony morphologies from ADA and nutrient agar were recorded and used to differentiate between *Aeromonas* and *Bacillus*.

5. ***Enterococcus faecium* (EPA Method 1106.1)**. Samples are filtered, placed onto mE agar, and incubated for 48 ± 3 hours. All filters with growth are transferred to EIA plates and incubated for an additional 20 minutes. All pink to red colonies on mE agar that produced a black or reddish brown precipitate on EIA agar are considered positive for *Enterococcus*. This effluent guideline study required that five positive colonies from each plate be submitted to biochemical identification to speciate and determine the levels of *Enterococcus faecium*.

6. ***Mycobacterium marinum* (SM9260M)**. Samples are screened for acid-fast bacteria prior to culturing. If acid-fast bacteria are present, the samples are decontaminated to remove organisms that may out-compete and overgrow the mycobacterium. After decontamination the samples are cultured in duplicate and incubated for 3-8 weeks at 37°C. Biochemical tests were then used to speciate the *Mycobacterium*.

The nominal quantitation limits are based on the actual sample volume filtered for the membrane filtration technique. The nominal quantitation limits for all the microbiological methods, except *Enterococcus faecium* and *Mycobacterium marinum*, are 1 CFU/100 mL. The nominal quantitation limit for *Enterococcus faecium* is 100 CFU/100 mL; the nominal quantitation limit for *Mycobacterium marinum* is 4 CFU/100 mL. For example, if a 100 mL volume is filtered, the nominal quantitation limit would be 1 CFU/100 mL. If a 10 mL volume is filtered, the nominal quantitation limit would be 10 CFU/100 mL.

Data were also generated using the most probable number (MPN) approach specified in Standard Methods. The MPN of each target organism per 100 milliliters was calculated based on the positive and negative results from the analysis of multiple replicates at multiple dilutions for each sample (see Table 9221.IV of Standard Methods). Based on the tables in Standard Methods, the nominal quantitation limit for the analytes analyzed by the multiple fermentation technique was 2 MPN/100 mL.

Table II at 40 CFR 136.3 specifies holding times of six hours for some pathogens. In collecting data supporting this rule, EPA measured counts in samples that had been retained longer than the six hours specified in Table II. In its data review narratives (located in Section 6.20 of the proposal record), EPA has identified those samples that were retained longer than eight hours at the laboratory (includes the six-hour holding time allotted for delivery to the laboratory plus an additional two hours at the laboratory). Standard Method 9221E, the 40 CFR Part 136 approved method for fecal coliform, states that “Water treatment and other adverse environmental conditions often place great stress on indicator bacteria, resulting in an extended lag phase before logarithmic growth takes place.” EPA conducted a holding time study to assess potential changes in pathogen concentrations in effluents over time (i.e., 8, 24, 30, and 48 hours after sample collection). This study evaluated total and fecal coliforms, *Escherichia coli*, *Aeromonas*

species, and fecal streptococci for the aquatic animal production facilities industrial effluents.

EPA conducted this holding time study for possible revisions to Table II. EPA notes that if the holding time can be extended to longer periods, overnight shipping of samples would be possible for compliance monitoring. However, EPA has not established any limitations and standards for these analytes. The report for the holding time study is located at DCN 62398 in Section 6.20 in the public record for the Notice of Data Availability (NODA).

B.3.20.1 Holding Time Study

When EPA conducted its own sampling episodes at the facilities, it exceeded the required holding time for sample samples. Although laboratories qualified to conduct total coliform, fecal coliform, and *E. coli* analyses might have been within driving distance of the facilities being evaluated, laboratories qualified to perform fecal streptococcus, *Aeromonas*, and *Enterococcus faecium* analyses generally were not available, because analysis for these analytes is more complex than coliform analyses. As a result, for most sampling episodes, EPA decided to ship samples overnight to a laboratory capable of performing all of the bacterial analyses. Because these samples would exceed the holding time requirements in 40 CFR 136, EPA performed a holding time study to evaluate the possible effects of analyzing samples at different holding times.

To determine whether or not the results for samples with longer holding times were consistent with results for samples analyzed within 8 hours (i.e., the time period consistent with 40 CFR 136 for compliance monitoring), for total coliforms fecal coliforms, *E. coli*, *Aeromonas*, fecal streptococcus, and *Enterococcus faecium* from CAAP facilities, EPA conducted a holding time study to evaluate sample concentrations at 8, 24, 30, and 48 hours after sample collection for wastewater effluent samples from two freshwater CAAP facilities. The study report , which contains results for all target bacteria, is DCN 62398 in Section 6.20 in the public record for the Notice of Data Availability (NODA).

Based on the results of this study, it appears that *Aeromonas* and fecal coliform samples from aquaculture effluents may not be analyzed beyong 8 hours after sample collection and still generate data comparable to those generate at 8 hours after sample collection. With these exceptions noted, fecal streptococcus samples may be analyzed at 30 hours; and total coliform, *E. coli*, *Enterococcus*, and *E. faecium* may be analyzed at 48 hours after sample collection and still generate data comparable to those generated within 8 hours of sample collection, provided the samples are held below 10 C and are not allowed to freeze. (Results of all samples analyzed are discussed in section 7.3 and Table 5 of the report.) Notwithstanding this conclusion, bacterial samples collected from concentrated aquatic animal production effluents should always be analyzed as soon as possible to comply with requirements at 40 CFR Part 136.

B.3.21 EPA Methods 1003.0, 1000.0, and 1002.0 (*Selenastrum* growth test, Fathead Minnow Chronic, and *Ceriodaphnia Dubia* Chronic)

Whole effluent toxicity was measured using a suite of methods including the *Selenastrum* growth test (EPA Method 1003.0), the fathead minnow larval survival and growth test

(EPA Method 1000.0), and *Ceriodaphnia dubia* survival and reproductive test (EPA Method 1002.0). All three methods are listed in Table 1A at 40 CFR Part 136. Endpoints measured were lethality in 50% of the organisms (LC50) for the fathead minnow and the *Ceriodaphnia*, growth in the larval fathead minnow and *Selenastrum*, and the number of offspring produced in the *Ceriodaphnia.*

1. **Method 1003.0: *Selenastrum* growth test**. A population of the green algae, *Selenastrum capricornutum*, is exposed in a static system to a series of effluent concentrations for 96 hours. The response of the population is measured in terms of changes in cell density (cell counts per mL). The toxicity of the effluent is indicated by increases or decreases in algal growth in response to nutrients and toxicants, compared to a control group (unexposed) of algae.

   The test is run using a 50-mL aliquot of effluent solution in a 250-mL flask. The effluent solutions are 6.25%, 12.5%, 25%, 50%, and 100% effluent. Each effluent concentration is run in five replicates. Each flask is inoculated with 10,000 cells per mL and allowed to grow during a 96-hour time period. During this time, the flasks are swirled twice daily to homogenize the cells within the flasks to allow for optimum growth. After the 96 hours, cells are counted from each of the flasks by taking an aliquot and counting the cells under a microscope using an approved cell counting method.

2. **Method 1000.0: Fathead minnow chronic**. Larva of the fathead minnow, *Pimephales promelas*, are exposed to different concentrations of effluent for seven days in a static renewal system. Test results are based on survival and weight of the larvae. The toxicity of the effluent is indicated by changes in the survival rate and decreases in the growth of the larvae that survive the testing period, compared to a control group (unexposed) of larvae.

   The test is run using a 250-mL aliquot of effluent solution in a 500-mL beaker. The effluent solutions are 6.25%, 12.5%, 25%, 50%, and 100% effluent. Each effluent concentration is run in 4 replicates, each containing 10 minnows, with an initiation age of less than 24 hours old. Daily observations are made to record the number of surviving minnows when the effluent solution is renewed. At 96 hours the test is terminated, the final number of surviving minnows is recorded, and the surviving minnows are preserved in 70% ethanol, then dried and weighed. The survival of minnows at the different concentration levels is compared to the control group to determine if any statistical difference was observed and the results are reported as an LC50. The weight of the surviving minnows at the different concentration levels is compared to the control group to determine if any statistical difference was observed and the results are reported as the inhibition concentration with a 25% effect (IC25).

3. **Method 1002.0: *Ceriodaphnia dubia* chronic**. *Ceriodaphnia dubia* are exposed in a static renewal system to different concentrations of effluent until 60% of surviving control organisms have three broods of offspring. Test results are based on survival and reproduction. If the test is conducted properly, the surviving control organisms should produce 15 or more offspring in three broods.

The test is run using a 15-mL aliquot of effluent solution in a 30-mL beaker. The effluent solutions are 6.25%, 12.5%, 25%, 50%, and 100% effluent. Each effluent concentration is run in 10 replicates containing 1 female with an initiation age of less than 24 hours old. Daily observations are made to record the number of surviving organisms and the number of offspring when the effluent solution is renewed. When 60% of the surviving females produce 3 broods, the test is terminated. The survival of organisms at the different concentration levels is compared to the control group to determine if any statistical difference was observed and the results are reported as an LC50. The number of offspring produced by the surviving organisms at the different concentration levels is compared to the control group to determine if any statistical difference was observed and the results are reported as an IC25.