

## Microbiological Requirements Relating to the Ground Water Rule and Calculations for MPN Sludge Testing

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## Objectives

- Over view of the Ground Water Rule
  - Source water monitoring requirements
  - Methods
- Calculations for sludge testing
  - MPN
  - MF
- Q & A

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## Ground Water Regulation

- Federal Register –Nov 2006 overview of rule
  - To reduce the risk of illness by microbial contamination in public ground water systems (GWS)
  - Applies to all PWSs that use GW
    - Rely on one or more GW sources
    - Mixed surface and ground water, where GW is added to directly to the distribution system and delivered to consumers without treatment equivalent to SW
    - Purchasing of GW

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## General Information

- There are approx. 147,000 GWS
- Estimates that 114 million people consume GW

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## Ground Water Regulation con't

- What are the basic requirements of the GWR?
  - Sanitary surveys
  - Source water monitoring (triggered)
  - Compliance monitoring
  - Corrective actions
- Sanitary survey requirements
  - Requires states to conduct the survey (all GWSs) to identify deficiencies that could make a system susceptible to microbial contamination
    - After initial survey, state conducts survey every 3 years for CWSs and every 5 years for NCWSs
    - CWSs that provide a 4-log (99.99%) treatment of viruses or have outstanding performance records can be surveyed every 5 years determined by the state

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## Ground Water Regulation con't

- Eight elements to the survey and if there is a deficiency it must be corrected
  - Source
  - Treatment
  - Distribution system
  - Finished water storage
  - Pumps, pump facilities and controls
  - Monitoring, reporting and data verification
  - Operator compliance with state requirements

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### Compliance Monitoring

- GWS can notify the state that it provides at least 4-log reduction of viruses via inactivation or removal
- Begins compliance monitoring to show the effectiveness of the treatment process

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### Triggered Source Water Monitoring

- GWS that do not conduct compliance monitoring and find a total coliform positive routine sample collected in compliance with the TCR must conduct triggered source water monitoring
- Must collect at least 1 GW sample from each source in use at the time the TC + was collected
- If the triggered water sample is fecal positive must either
  - Take corrective action as directed by the state
  - If corrective action not required and the sample is not invalidated by the state, then additional sampling of SW is required
    - Must collect an additional 5 SW samples from the same source, using the same indicator within 24 hours

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### Types of Notification Required by the GWR

- Tier 1 Notification is required within 24 hours to the state and public
  - GW fecal positive not invalidated by the state
- Tier 2 Notification is required within 48 hours to state and within 30 days to the public
  - Failure to comply with a state approved corrective action plan
  - Failure to complete required corrective action plan
- Tier 3 for all other violations not included in Tier 1 & 2
  - Notice is required within 12 months
  - Mainly monitoring and reporting violations

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## Microbiological Methods for TCR and GW Testing

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## Methods Approved for Detection of Total Coliforms under the TCR and for the Detection of E.coli Under the GWR

Method	TC detected	E.coli detected	TCR/GWR approval
LTB/PA → BGLB	X	----- -	X
EC-MUG	-----	X	X
NA-MUG	-----	X	X
Colilert, Colilert-18, Colisure	X	X	X
m-Endo → LTB/BGLB	X	-----	X
EC-MUG	-----	X	X
MI Agar	X	X	X
m-ColiBlue	X	X	X

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## Other Approved Parameters/Methods under the GWR

- Enterococci
  - Enterolert
  - EPA 1600 method
- Coliphage (Standard Methods)
- E.coli will be the most likely used indicator under the GWR to full fill monitoring requirements

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# Multiple Tube Fermentation (MTF) Most Probable Number (MPN)

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### Most Probable Number Assay

Five-Tube MPN Table											
Number of Tubes Containing Positive Reactions		MPN per 100 ml					MPN per 100 ml				
		0	1	2	3	4	0	1	2	3	4
5	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	1	0	0	0	0	0	0
4	0	0	0	1	0	0	0	0	0	0	0
4	0	0	1	0	0	0	0	0	0	0	0
4	0	1	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	1	0	0	0	0	0	0	0
3	0	0	1	0	0	0	0	0	0	0	0
3	0	1	0	0	0	0	0	0	0	0	0
3	1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	1	0	0	0	0	0	0	0
2	0	0	1	0	0	0	0	0	0	0	0
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0	1	1	0	0	1	0	0	0	0	0	0
0	1	1	0	1	0	0	0	0	0	0	0
0	1	1	0	1	1	0	0	0	0	0	0
0	1	1	1	0	0	0	0	0	0	0	0
0	1	1	1	0	1	0	0	0	0	0	0
0	1	1	1	1	0	0	0	0	0	0	0
0	1	1	1	1	1	0	0	0	0	0	0
0	1	1	1	1	1	1	0	0	0	0	0

NOTE: 1. A number of double tubes showing gas after inoculation with 1 ml sample.  
2. Number of double tubes showing gas after inoculation with 0.1 ml sample.  
3. Number of double tubes showing gas after inoculation with 0.01 ml sample.

MPN of 240 organisms/100ml pond water

NOTE: Modified from the 2<sup>nd</sup> edition of Water and Wastewater, 1980, American Public Health Association, Inc.

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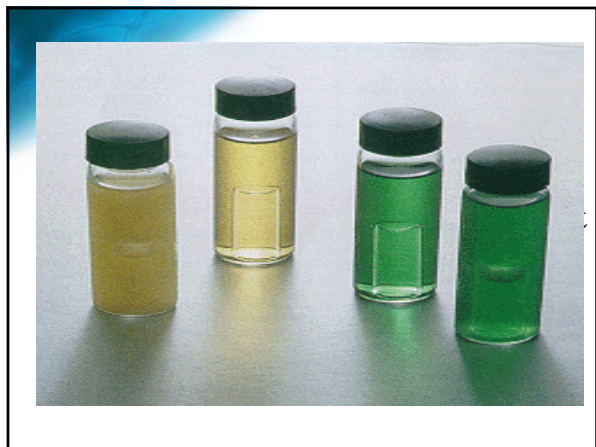
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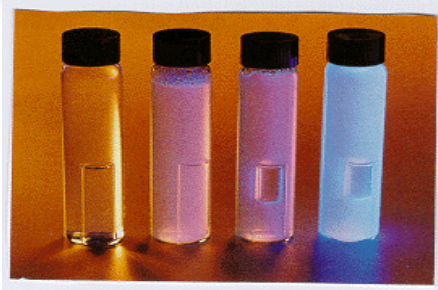
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Fecal Coliform/E. coli Multiple Tube  
Method -  
EC medium + MUG



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Membrane  
Filtration  
  
Methods

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Membrane Filter Apparatus



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### Single Unit MF



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### Removing the Filter



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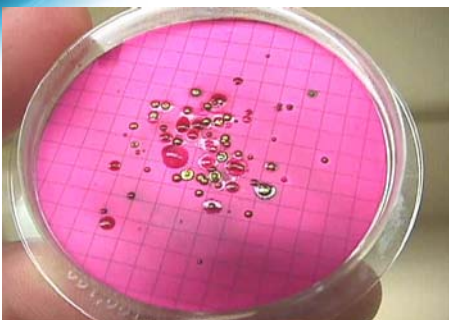
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### m-Endo Plate



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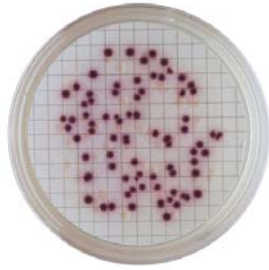
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### Modified m-TEC for E. coli

- Contains 5-bromo-6-chloro-3-indolyl- $\beta$ -D-glucuronide
- Selective inhibitory chemicals that can effect the growth of sub-lethal injured bacteria.
- 2 hours of incubation at 35°C followed by 22 hours at 44.5°C
- Positive reaction is red to magenta color colonies
- Extensive QC requirements



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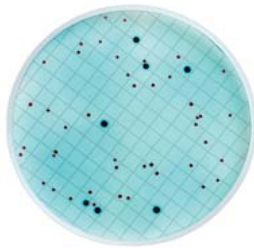
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### m-ColiBlue

- Reagent storage is at 2-8°C
- Incubate for 24 hours at 35±0.5°C
- Read at 24 hours
  - Red or blue colony = TC
  - Blue colony = *E.coli*
  - *Difficult to interpret colonies (very small)*
- *If count >200 for TC/EC, exclude from calculation*



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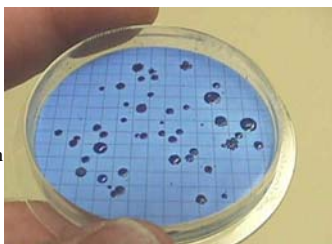
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### m-FC

- Enriched lactose medium
- Contains aniline blue
- Larger colonies than m-Endo; range is 20-60 colonies
- Incubate in water bath at 44.5±0.2°C for 24 hours in plastic bag
- Blue colonies are positive
- Atypical- grey to green colonies
- Confirm as per section 9020 Standard Methods



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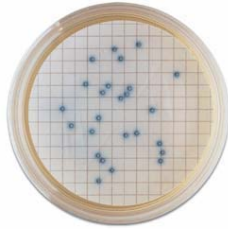
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### m-EI Method for Enterococci

- 24 hour MF Test
- All colonies regardless of color with a blue halo
- 20-60/plate
- Colonies <0.5mm difficult to read
- Media is expensive (Indoxyl- $\beta$ -D-glucoside)
- Magnification required to read plates



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### Colilert<sup>®</sup>, Colilert<sup>®</sup>-18, and Colisure<sup>®</sup>

- Colorimetric/fluorogenic tests for total coliforms and *E. coli* (18 and 24 hour tests, with Colisure up to 48 hours)
- Based on IDEXX's patented DST<sup>®</sup>

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### Colilert<sup>®</sup> and Colilert<sup>®</sup>-18



Add reagent to sample and incubate

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### Colilert and Colilert-18 P/A Demonstration



*E. coli* make sample  
fluoresce

Negatives remain  
colorless

Coliforms turn  
sample yellow

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### Colisure P/A Demonstration



*E. coli* make sample  
fluoresce

Negatives remain  
yellow

Coliforms turn  
sample magenta

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### Quanti-Tray Demonstration



Add Colilert to sample  
and shake to dissolve



Pour mixture into a  
Quanti-Tray

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## Quanti-Tray Demonstration

cont.



Seal and then incubate at 35°C for 24 hours



Count positive wells and refer to MPN table

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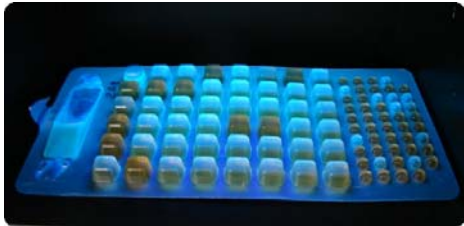
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## *E.coli*- Blue Fluorescence- Quanti-Tray under a 365nm UV Light



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## Enterolert™ Demonstration



Add reagent



Seal in a Quanti-Tray

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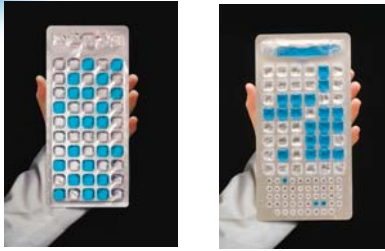
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## Enterolert™ Demonstration



Count fluorescent wells  
and refer to MPN table

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## Sludge Calculations

- Class B monitoring for fecal coliform
  - 40 CFR 503 requires 7 samples for testing be taken to demonstrate compliance required for bio solids.
  - Why 7 samples
    - Adequate to account for short term fluctuations in treated sludge quality
    - Allows determination of average performance
    - Variance for this test is high and if the 7 samples are averaged the error band about the mean is sufficiently compressed and allows to meet the standard
  - The fecal coliform geometric mean density of the 7 samples be < 2 million CFU or MPN/gram of total solids sewage sludge (dry weight basis)

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## Sludge Calculations con't

- Class A monitoring for fecal coliforms
  - 40 CFR 503 requires sludge be monitored for fecal coliforms or salmonella spp. and be <1000 MPN/gram of total solids sewage waste (dry weight) or salmonella below 3MPN/4 gram
    - Recommended to take > 7 samples over 2 weeks to adequately represent sludge quality
    - Allows the monitoring of either fecal coliforms or salmonella in order to demonstrate compliance.
- All sludge samples are to be analyzed in accordance with SM 9221E Fecal Coliform MPN procedures or 9222D FC MF procedure (20-60 colonies/plate)

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### Sludge Calculations con't

- Procedure for solid samples: Class B
  - Weigh 30 grams of a well mixed sample.
  - Add this to a blender
  - Rinse any material with sterile buffer dilution water. total volume is 270 ml (1:10 dilution or 0.1g/mL)
  - Cover and blend on high speed for 2 minutes
  - With a sterile pipette transfer 11 ml from the blender to a screw cap bottle containing 99 ml of sterile buffer dilution water. Shake vigorously a minimum of 25 times (dilution A: 1:100 dilution or 0.01g/mL).

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### Sludge Calculations con't

- Pipette 1 ml of dilution A into a screw cap bottle containing 99 ml of sterile buffer and mix well. (dilution B: 1:1000 or 0.0001g/mL)
- Pipette 1 ml of dilution B into a screw cap bottle containing 99 ml of sterile buffer and mix well. (dilution C: 1:1,000,000 or 0.000001g/mL)

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### Sludge Calculations con't

- For MPN analysis:
  - Four series X 5 tubes
    - » 1<sup>st</sup> 5 tubes- pipette 10 ml of dilution B in each of the 5 tubes (0.001 mL)
    - » 2<sup>nd</sup> 5 tubes- pipette 1 ml of dilution B into each of the 5 tubes (0.0001mL)
    - » 3<sup>rd</sup> set of 5 tubes- pipette 10 ml of dilution C into each of 5 tubes (0.00001 mL)
    - » 4<sup>th</sup> set of 5 tubes- pipette 1 ml of dilution C into each of 5 tubes (0.000001 mL)
- Refer to the MPN table to estimate the MPN/100mL
  - Only ¾ series will be used estimating the MPN
  - Choose the highest dilution that gives positive results in all 5 tubes, and the next 2 higher dilutions

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Compute the MPN/g using the following formulation:

$$\text{MPN FC/g} = \frac{10 \times \text{MPN index} / 100\text{ml}}{\text{largest volume} \times \% \text{ dry solids}}$$

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Examples	0.0010 mL	0.00010 mL	0.000010 mL	0.0000010 mL	Combination of positives
a	5/5	5/5	3/5	0/5	5-3-0
b	5/5	3/5	1/5	0/5	5-3-1
c	0/5	1/5	0/5	0/5	0-1-0

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### Sludge Calculations con't

- Assume 4% total solids for each example
- **For a:** The MPN index/100mL is 80. Therefore
- $\text{MPN/g} = 10 \times 80 / 0.00010 \times 4.0 = 2 \times 10^6$
- **For b:** The MPN index/100mL is 110. Therefore
- $\text{MPN} = 10 \times 110 / 0.0010 \times 4.0 = 2.8 \times 10^5$
- **For c:** The MPN index/100mL is 2. Therefore
- $\text{MPN/g} = 10 \times 2 / 0.0010 \times 4.0 = 5 \times 10^3$

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## Membrane Filtration

- Can be used for fecal coliform testing and only be used if comparability with MPN has been established
- Three individual filtrations be used per SM 9222D:
  - Using 10 mL of dilution C = 0.000010 mL
  - Using 1 ml and 10 mL of dilution B = 0.00010 mL & 0.0010 mL
  - Incubate samples and count colonies as indicated by the method
  - May need to modify the dilution scheme to obtain between 20-60 colonies/plate

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### MF con't

Calculate the density of CFU from MF which yields counts within the 20-60 range  
 $\text{fecal coliform colonies/g} = \text{colonies counted} \times 100 / \text{mL sample} \times \% \text{ dry solids}$

Examples:

Seven samples of treated sludge had solids concentration as determined by SM

They were found to be:

Sample No.	Solids Concentration %
1	3.8
2	4.3
3	4.0
4	4.2
5	4.1
6	3.7
7	3.9

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### Number of Fecal Coliform Colonies on MF Plates

Sample No.	0.000010mL Filtration	0.00010 mL Filtration	0.0010 mL Filtration
1	0	1	23
2	2	18	TNTC
3	0	8	65
4	0	5	58
5	0	1	17
6	0	1	39
7	0	1	20

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Coliform density is calculated using only those MF plates which have between 20-60 colonies; samples 1, 3, 6 & 7.

For sample 1: 1 plate is within the 20-60 colony range

$$\text{Colonies/g} = \frac{23 \times 100}{0.0010 \times 3.8} = 6.0 \times 10^6$$

If the total number of colonies is less than or greater than 20-60, total the counts on all countable filter; samples 2, 3, & 5

For sample 2:

$$\text{Colonies/g} = \frac{(2+18) \times 100}{(0.00001+0.0001) \times 4.3} = 4.2 \times 10^6$$

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Calculate the coliform density of all the samples and convert to  $\text{Log}_{10}$  to obtain a geometric mean. Average the 7 samples and take the anti-log to obtain the final value for the 7 samples.

Sample No.	Coliform Density	$\text{Log}_{10}$
1	$6.0 \times 10^5$	5.78
2	$4.2 \times 10^6$	6.63
3	$1.6 \times 10^6$	6.22
4	$1.4 \times 10^5$	6.14
5	$4.0 \times 10^5$	5.60
6	$1.0 \times 10^6$	6.02
7	$5.1 \times 10^5$	5.71

The average value  $\text{Log}_{10} = 6.01$  and the antilog =  $1.03 \times 10^6$

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### Class B and Class A Sludge

- Class B pathogen requirement for fecal coliform density is  $< 2$  million/g
- Class A pathogen requirement is  $< 1000\text{MPN/g}$ (total solids dry weight) or *Salmonella* sp.  $< 3\text{MPN/4 g}$  (total solids dry weight)
  - Suggested sampling over 2 weeks and collect  $> 7$  samples for testing
  - MF cannot be used
  - MPN method using at least 4 series of 5 tubes using the ten fold serial dilution
  - Follow calculation as outline for Class B sludge

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## For Your Information

- Colilert-18/Quanti-Tray approved in UK for sludge for *E.coli*.
- A comparison of methods used to enumerate *E.coli* in conventionally treated sewage sludge-J.P. Eccles, J of Applied Microbiology 2004, 96, 375-383

Three methods tested (Colilert-18/Quanti-Tray, MGLA and CEC) gave comparable recoveries and did not vary by greater than 1 log

All methods had a false positive rate of <3%

Colilert/Quanti-Tray had a false negative rate of 3.8%, compared to 7.75% (CEC) and 35.5% (MLGA)

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## For Your Information

Enumeration of Coliform Bacteria in Wastewater Solids using Defined Substrate Technology- T.A. Kramer- Water Environment Research, Nov/Dec 2002, Vol. 74, #6

-Data indicates that Colilert/Quanti-Tray system is capable of producing equal population estimates for coliform and *E.coli* bacteria in WAS as the accepted technique of MTF

-This system offers numerous advantages over MTF, including ease of use, lower cost and rapid detection time

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## IDEXX Support

1-800-321-0207

- #1 Customer Support

- #2 Technical Service

-#3 Select extension

-[www.idexx.com/water](http://www.idexx.com/water)

IDEXX

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That's all folks!

Thank You

Questions

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