Total Nitrogen/Total Phosphorus Sample Preparation for Colorimetric Flow Analysis

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Purpose: This procedure outlines the steps to prepare and digest HCl acidified standards and samples for the determination of total nitrogen and total phosphorus via micro-segmented flow analysis.

Concepts: The alkaline potassium persulfate digestion begins at a pH of 12, which is necessary to break nitrogen bonds. It is important to note that in an alkaline environment different forms of nitrogen are oxidized to nitrate, however, nitrogen compounds with triple and double bonds may not be oxidized. Next, the high temperature during the autoclave process causes the thermal decomposition of potassium persulfate. This leads to an acidic environment where the different forms of phosphorus are hydrolyzed to orthophosphate. This can be quantified by a pH drop to 2.3, which is necessary to break organic phosphorous bonds. Nitrate-N and orthophosphate are determined simultaneously via automated colorimetric analysis on the Astoria 2 flow analyzer.

Sample Holding Time:
≤ 1 year, room temperature, ~100 mL sample preserved with 1mL concentrated HCl acid in 125mL LDPE bottles. Acidify samples as soon after collection as possible.

Materials Required for Sample Preparation (see materials for regents in step 5):

- Type I water (≥18.2 MΩ-cm)
- 1% v/v Optima Hydrochloric Acid Solution
- Test-tube racks
- Prepared TNTP Standards in 1% v/v HCl (See API SOP for details)
- 0.5-5mL Bottle Top Dispenser
- 5mL adjustable pipette
- 5mL Pipette tips
- Avery 5160 Labels
- Disposable Round Bottom Threaded Culture Tubes (16x100mm)
- Phenolic GPI Screw Caps
- Autoclave able to achieve 121C and 18-20 psi for an hour

Material Preparation: All glassware and plastic bottles used to prepare and store solutions required for TNTP analysis (1% v/v HCl, digestion reagent, and standards) must be properly acid washed and dried before use to ensure absence of contamination. For best results, prepare a fresh acid bath to be used for bottles/glassware used for low level TNTP analysis.

Personal Protective Equipment: Nitrile or vinyl gloves and eye protection should be worn while prepping TNTP samples. Always use chemical resistant gloves (not latex), safety glasses, lab coat, and work in a fume hood while using concentrated acids to prepare the 1% v/v HCl. This is not only for your protection, but also to prevent contamination of samples.

Note: Nitrile gloves are a known source of nitrogen contamination, rinse gloves well with DI water before prepping samples/standards/reagents for TN analysis.

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Quality Assurance/Quality Control:

- Duplicate every 10th sample for analysis (lab duplicates)
- Duplicate sample collected from one randomized depth from each body of water within a sampling event (field duplicate/blind)
- 5 check standards prepared with a secondary source commercial stock solution: LOQ, ICV-L, ICV-M, ICV-H, and LRV. These check standards are run immediately following the calibration curve.
- A 2000ppb Nitrate-N solution and a 2000ppb Nitrite-N solution are prepared using the bisulfate sampler wash solution as the diluent to check TN cadmium column reduction efficiency at the start and end of every run. (No digestion required).
- A digestion efficiency check is included in every autoclave batch to be analyzed for % recovery of Nitrogen and Phosphorus.
- C1 is a calibration blank that largely determines the detection limit of the TN/TP assay. Digestion reagent is the primary source of background Nitrogen contamination. Nitrogen contamination is removed from potassium persulfate via recrystallization (see Section 3.2.1) To ensure the lowest C1 response possible, use 1% v/v HCl freshly prepared in a brand-new Nalgene LDPE bottle.

Waste Disposal: Prepped samples before autoclaving are basic (pH ~12), neutralize with weak acetic acid solution and check with pH paper before sanitary sewer. Acidified samples both pre and post digestion are very acidic (~2 pH), neutralize with sodium bicarbonate solution and check with pH paper before sanitary sewer.

Consumables Ordering Info:

Item	Catalog #	Item	Catalog #
Optima HCl (500 mL)	Fisher A466500	Corning Phenolic GPI Screw Caps	99999-15
Corning Disposable Round Bottom Threaded Culture Tubes	99447-161	Sigma Potassium Persulfate	60489
Fisher Sodium Hydroxide	7708-10	Thermo Finntip 1-5mL Pipette Tips	9402030
Nitrate-N, Nitrite-N, and Phosphorus Commercial Stocks	See Section 3.0	Avery Easy Peel Address Labels 1” x 2-5/8”	5160

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1.0 Sample Preparation

1.1 Remove standards for analysis from the fridge to allow them to warm up to room temperature prior to analysis. (Samples are stored at room temperature, it is important the standards warm-up to room temperature for pipetting accuracy.

1.2 Fresh 16x100 mm Pyrex Brand Disposable Culture Tubes with Threaded End should be used for each analysis. Label each tube with the appropriate description of its contents (sample number, standard, etc).

- Print barcodes from Chemlab for LTER or IDAutomation Software for graduate student projects.
- Orientation of labels on vials is important, make sure labels are placed along the vial and not around it. Barcodes cannot be scanned around curves of vials.

1.3 Pipette 5 mL of sample, blank, standard, etc. into tubes.

- Be sure to work on a clean surface, and practice good pipetting technique.
- Rinse the pipette tip once with 1% v/v trace metal grade concentrated HCl, and then twice with type I water between different solutions.
- When working with filtered and unfiltered versions of a sample, you can pipette the filtered version before the unfiltered version with the same pipette tip (no rinse required).
- Pipette tips should be changed whenever the performance is found to be unsatisfactory, or solution droplets are sticking to tip.

1.4 Deliver **2.5 mL** of Digestion Reagent (see Section 3.2 for preparation) to each tube with a repeat pipettor dedicated to that use.

- The repeat pipettor is 3x rinsed and filled with type I water for storage to prevent corrosion. It should always be labeled with tape indicating whether it is filled with MQ or digestion reagent. Be sure to dry out the glass amber container the best you can before adding digestion reagent from stock bottle stored in cabinet (to protect from UV).
- Repeat pipettor should be routinely calibrated to ensure it is reliably delivering 2.5mL. Tare out an empty beaker on scale in B102 and add one pump of water, reweigh the beaker. It should weigh 2.5g (+/- 0.01g). Multiple additions can also be made, and the average of the number additions should be 2.5g (+/- 0.01g).

1.5 Tightly cap tubes after the addition of the digestion reagent and invert **3x** to mix.

1.6 See **Figure 1** for proper organization of samples in rack.

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2.0 Digestion via Autoclave

2.1 Procedure

1. Turn on POWER switch – top right of front right panel (**Figure 3**)
2. Fill water reservoir - gray plastic cap on top of machine
    a. Use DEIONIZED WATER ONLY
    b. Tighten drain spigot **(Figure 5),** or water will drain into sink
    c. Make sure the bottom dial is in the upright position
    d. Fill to bottom of the wire loop on right end of the pressure safety valve
    e. Water should NOT touch pressure safety valve **(Figure 4)**
3. Load autoclave with samples in sample rack on the sample tray.
    a. **Figure 2** shows how to organize the samples in the autoclave rack. This is an attempt to physically distance the C1 calibration blank from the higher level calibrants and checks to reduce possible contamination.
4. Close door
    a. Bring latch from left side to the front
    b. Hand tighten until door is firmly shut
    c. Run may not start if door isn't properly tightened
5. With Bottom dial in the upright position**, Turn the bottom dial (clockwise) to Fill.** Let fill until water reaches notch on bottom of chamber.
6. Turn bottom dial to **STERILIZE** to stop filling, this is "run" mode
7. Verify Temperature setting – middle dial
    a. TN/TP digestion= 250°F/121°C
8. Set Time dial to desire length of run – top dial
    a. TN/TP = 60 min
9. Heat light should come on, indicating start of run
10. Do ***NOT*** open door/latch after the run until machine has slowly exhausted heat and pressure completely. If multiple batches are needed in a day, bottom nob can be turned to the left position (EXH-DRY) to exhaust pressure quickly. You must still wait a bit for the temp to cool before opening (be careful of steam).
11. Drain reservoir with spigot/barbed fitting (turn counterclockwise to loosen after attaching short white segment of tubing) **(Figure 6)** on the bottom left of the front of the unit (with door open) and leave door open when done.

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3.0 Preparation of Standards and Reagents			
Item	**Catalog #**	**Item**	**Catalog #**
Ricca 1000ppm Nitrate-N Stock for Calibrants	5459-4	Ricca 1000ppm Nitrite-N Stock (OTCR Reduction Check Solution)	5461-4
Ricca 1000ppm Phosphorus Stock for Calibrants	5839.1-4	Glycine Hydrochloride (Organic N Source)	AC411011000
Inorganic Ventures 1000ppm Nitrate-N Stock for ICVs	ICNNO31	Glycerophosphate (Organic P Source)	3567550GM
Inorganic Ventures 1000ppm Phosphorus Stock For ICVs	AAP1	D-(+)-Glucose (Carbon Source)	G004825G

3.1 Preparation of TNTP Calibrants and ICVs

3.1.1 <u>See pages 17-19 in Astoria2 Analyzer (API) SOP</u>

3.2 Preparation of Digestion Reagent

Safety Overview:

Sodium Hydroxide (NaOH): Causes severe burns by all exposure routes. Water reactive.
Potassium persulfate ($K_2S_2O_8$): Contact with combustible/organic material may cause fire. Harmful if swallowed. Irritating to eyes, skin and respiratory tract. May cause allergic reactions.

Digestion Reagent Recipe :

Sodium Hydroxide (NaOH) 12.0 g
Potassium persulfate ($K_2S_2O_8$)* 30.5 g
Milli-Q water

1. Dissolve sodium hydroxide in 970 ml of fresh Milli-Q water.
2. Dissolve the twice recrystallized potassium persulfate in NaOH solution.
3. Dilute to 1 L with MQ.
4. Store in bottle in dark cabinet.

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**Twice recrystallized potassium persulfate. Sigma Aldrich potassium persulfate is currently used but needs to be recrystallized twice to remove nitrogen contamination (any ACS grade may be used, "Low N" in product name is even better.*

3.2.1 Potassium Persulfate Recrystallization

1. Pre-heat 300mL of Type I water in a 1L Erlenmeyer flask and 200mL of Type I water in small beaker **to 60˚ C**.
2. Add medium stir bar to 1L flask, place on stir plate, and add 100g potassium persulfate
3. While stirring, slowly add 60˚ C water from small beaker until all persulfate dissolves (The less solvent used, the more crystals you can recover).
4. Filter the solution rapidly through a sintered glass funnel into a clean 1L side-arm flask.
5. Place filter flask on metal benchtop slowly cool to room temperature.
   5.1 If the crystals need help forming, lightly scratch the bottom of the flask with a glass stir rod.
6. Cool solution to about 4°C by placing the flask in an ice water bath (A *slush of water with plenty of ice in it*. This has much better heat transfer properties than just ice alone.) Swirl the flask occasionally to prevent the solution from freezing. Cool a squirt bottle of Type I water in ice bath to about 4°C for rinsing.
7. Vacuum filter the 4°C potassium persulfate solution using a new sintered glass funnel and a new clean 1L side-arm flask. Rinse the flask with 2 squeezes ice-cold Type I Water. Save the white potassium persulfate crystals in the filter.
8. Discard the filtrate from the 1L flask.
9. Repeat steps 1 through 8 a second time using the crystals from the filter and a clean 1L flask.
10. Allow crystals to vacuum dry in the filter-top, covered with a watch glass until crystals appear flaky and shiny (approx. 10 min). Alternatively, put the crystals on a pie plate and leave in a desiccator to dry. Yield is about 80% = 80g dry potassium persulfate.
11. Store at room temperature in vacuum desiccator.

Note: New clean glassware for the second recrystallization is *best practice, but triple rinsing filter/flasks with Type I water between batches will do if 2x of all glassware/filters/funnels are not available.*

3.3 Preparation of Digestion Efficiency Solution

3.3.1 Glycine digest-check stock solution (1 mL = 1.0 mg-N): Dissolve 3.98 g glycine (C2H5NO2«HC1, FW=111.5) in about 400 mL of Type I water in a 500-mL volumetric flask. Dilute this solution to the mark with Type I water and mix it thoroughly by manual inversion and shaking. Transfer the stock digest-check solution to a 500-mL Pyrex™ media bottle in which it is stable for 6 months at 4°C.

3.3.2 Glycerophosphate digest-check stock solution (1 mL = 0.4 mg-P): Dissolve 1.976 g glycerophosphate (C3H7O6PNa2«5H2O, FW=306.1) in about 400 mL of Type I water in a 500-mL volumetric flask. Dilute this solution to the mark with Type I water and mix it

thoroughly by manual inversion and shaking. Transfer the stock digest-check solution to a 500-mL Pyrex™ media bottle in which it is stable for 6 months at 4°C.

3.3.3 Glucose digest-check stock solution (1 mL = 1.25 mg-C): Dissolve 1.564 g glucose ($C_6H_{12}O_6$, FW=180.2) in about 400 mL of Type I water in a 500-mL volumetric flask. Dilute this solution to the mark with Type I water and mix it thoroughly by manual inversion and shaking. Transfer the stock digest-check solution to a 500-mL Pyrex™ media bottle in which it is stable for 6 months at 4°C.

3.3.4 Mixed digest-check solution - nominal concentration 2000ppb-N, 800ppb-P, and 25 mg-C/L): Dispense 1 mL each of glycine and glycerophosphate stock digest-check solutions and 10 mL of the glucose digest-check stock solution into a 500-mL volumetric flask that contains about 400 mL of Type I water. Add 5 mL Concentrated Optima HCl to flask. Dilute the contents of the flask to the mark with Type I water and mix it thoroughly by manual inversion and shaking. Transfer the stock digest-check solution to a 500-mL Pyrex™ media bottle in which it is stable for 1 month at 4°C.

3.3.5 Notes: Smaller batches can be made of all solutions.

- Most samples for LTER contain less than 25mg/L organic carbon, this solution is matched to be near the top of the TN and TP calibration curves as well as the higher end of OC values seen in LTER samples (only Trout Bog samples at 7m reach ~30mg/L on average).
- Treat this solution the same as a sample and include one in every autoclave batch. This procedure tests digestion efficacy by calculating percent recovery of N and P post digestion to determine if the digestion was completed successfully. The check solution of organic compounds containing known concentrations of N and P are lysed into inorganic Nitrate and orthophosphate in solution via digestion which we should recover on the API as +/- 10% of the prepared concentration.

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References

US Geological Survey. (2003). *Methods of analysis by the U.S. Geological Survey National Water Quality Laboratory-Evaluation of alkaline persulfate digestion as an alternative to Kjeldahl digestion for determination of total and dissolved nitrogen and phosphorus in water*.

US Environmental Protection Agency. (August 1993). *Method 353.2, Revision 2.0: Determination of Nitrate-Nitrite Nitrogen by Automated Colorimetry*.

US Environmental Protection Agency. (August 1993). *Method 365.1, Revision 2.0: Determination of Phosphorus by Semi-Automated Colorimetry*.

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Kalff, J., and E. Bentzen. 1984. A method for the analysis of total nitrogen in natural waters. Can. J. Fish. Aquat. Sci. 41: 815-819.

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Nydahl, F. 1978. On the Peroxodisulphate oxidation of total nitrogen in waters to nitrate. Water Res. 12: 1123-1130.

Solorano, L., and J.H. Sharp. 1980. Determination of total dissolved nitrogen in natural waters. Limnol. Oceanogr. 25: 751-754.

Figure 1:

Figure 2:


CALIBRANT
QC
SAMPLE
LRV
C10
ICV-H
ICV-M
ICV-L
C4
DC
C9
C8
C7
C6
C5
S1
S10 DUP
S20
S29
S38
S45
S2
S11
S20 DUP
S30
S39
S46
S3
S12
S21
S30 DUP
S40
S47
S4
S13
S22
S31
S40 DUP
S48
S5
S14
S23
S32
S41
S49
S6
S15
S24
S33
S42
S50
S7
S16
S25
S34
S43
S50 DUP
S8
S17
S26
S35
S44
C3
S9
S18
S27
S36
C2
BLK
S10
S19
S28
S37
LOQ
C1

Figure 3:

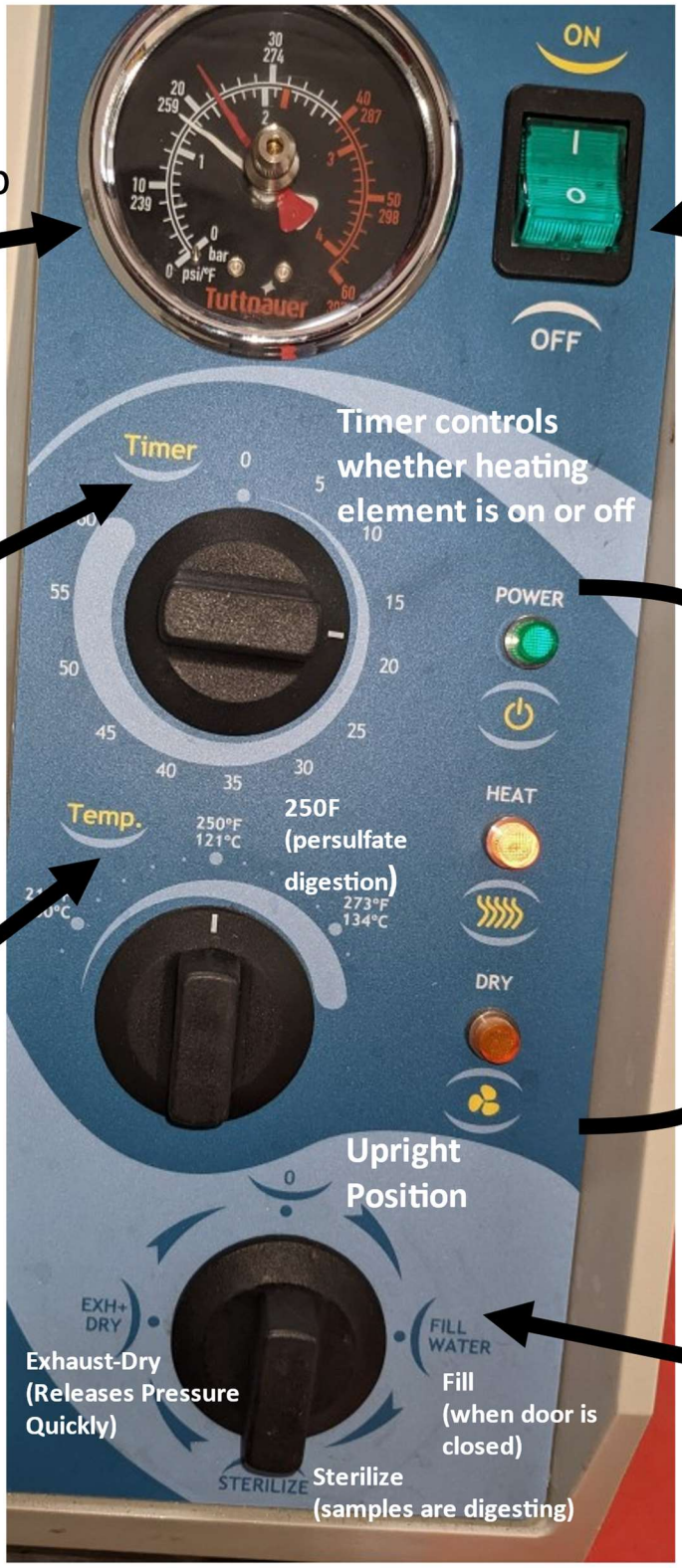

Pressure and Temp Gauge
Power Switch
ON
OFF
Timer
Timer controls whether heating element is on or off
Timer Dial
POWER
Indicator Lights
HEAT
DRY
Temp.
250°F 121°C
250F (persulfate digestion)
273°F 134°C
Temp Setting Dial
Upright Position
EXH+ DRY
Exhaust-Dry (Releases Pressure Quickly)
FILL WATER
Fill (when door is closed)
Bottom Dial
STERILIZE
Sterilize (samples are digesting)

Figure 4:

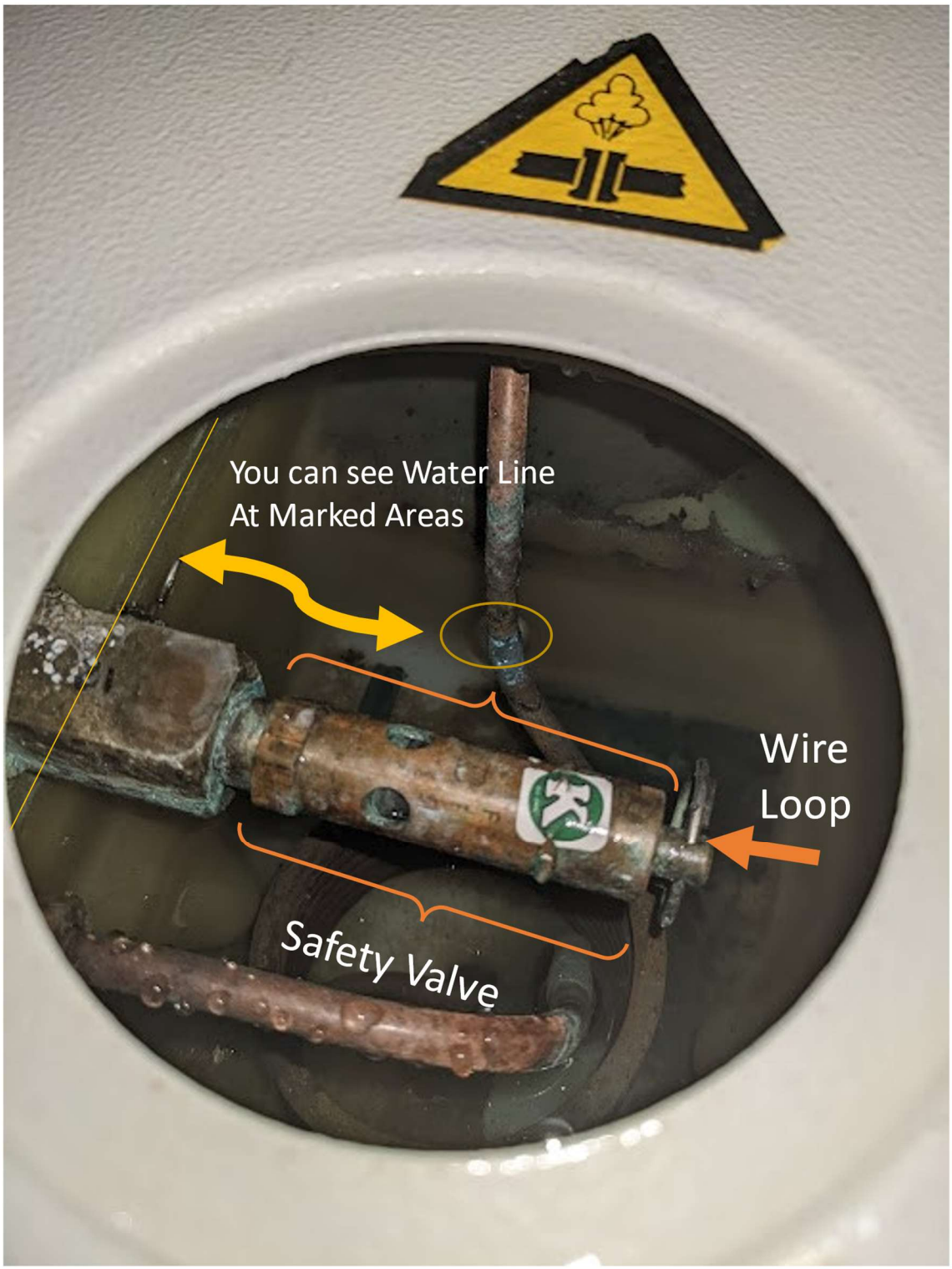
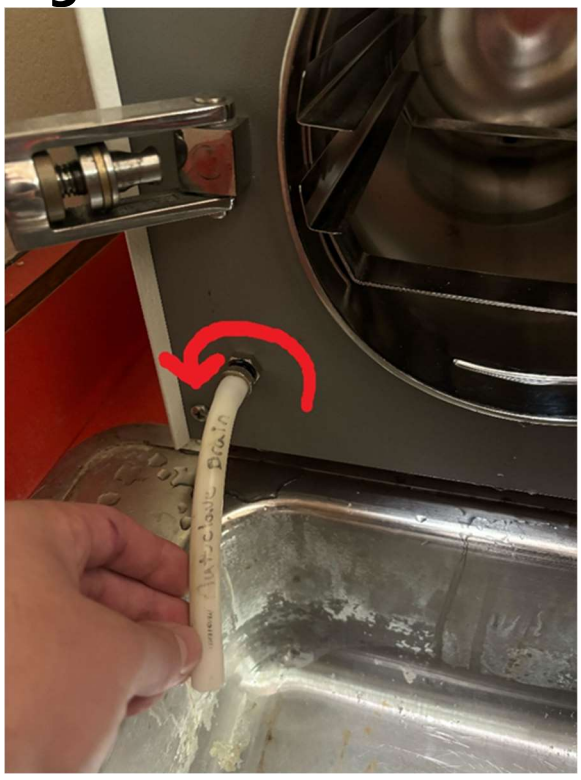

You can see Water Line
At Marked Areas
Wire
Loop
Safety Valve

Figure 5:

Figure 6:



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Revision Tracking Table

Version	Rev. date	Changes Made	Rev. author
1.0	11/15/24	1. Compiled and edited “Total Nitrate and Total Phosphate Procedure”, “Autoclave Instructions”, and “K-Persulfate Recrystallization” into this SOP	James Sustachek