1.0 Procedure title

Measuring Urea and Ammonium in Algae Culture using o-phthaldialdehyde in 96-well Plates

2.0 Procedure impacts and concerns

2.1 Safety

The reagents used in this assay are potentially harmful to human health. Especially important are the use of chemical resistant gloves, and safety glasses for eye protection.

2-mercaptoethanol: Skin, rabbit: LD50 = 150 uL/kg

Review the MSDS for each of the reagents used before starting the assay.

2.2 Quality

Wiping the pipette tip before delivering small volumes, < 10 ul, was essential for accuracy, as verified by the validation.

The effect of reading the color developed plate after 60 minutes has not been studied. To assure valid results read the plate before 60 minutes of color development.

2.3 Delivery

N/A

2.4 Environmental

All waste and unused reagents must be collected in a common container for professional disposal.

2.5 Cost

Cost of reagents per plate was estimated to be about $0.85 per plate for each ammonium assay. Cost was estimated to be about $2.76 per plate for each Urea assay, which applies the *Urease* enzyme.

2.6 Compliance

Use of the Water Chemistry Template Macro helps to ensure valid data results, as well as expedite the analysis process.

3.0 Related Procedures

TBD	TBD

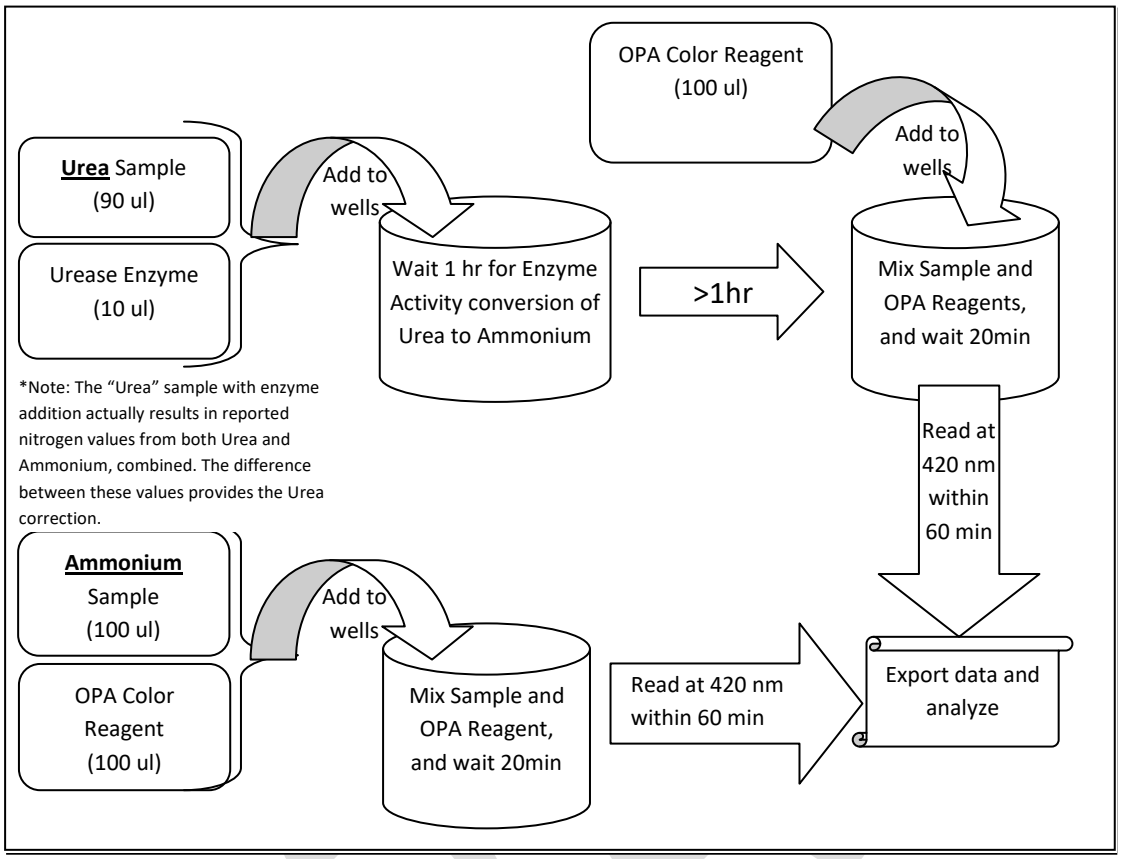
4.0 Responsibilities

Document Owner	Manage content and distribution	Jeremy Ferrara
Process Owner	Responsible for content and process validation	Jeremy Ferrara
Plant Manager	Responsible for implementation and conformance	Nicole Heaps

5.0 Process

5.1 Process description

Ammonium reacts with o-phthaldialdehyde (OPA) to form a light yellow –brown compound. Urea can be converted to ammonium using *Urease* enzyme from Jack bean. The difference between results using enzyme and without enzyme calculates the amount of urea in the sample.



Use Nitrogen-free media as a diluent in this assay to achieve the best results.

The schematic shown in the figure can be accomplished in a standard 96-well culture plate using a final volume of 200 ul per well. The "Urea" plate applies the Urease enzyme activity to convert all urea to ammonium. For a Urea plate, 90 ul of diluted sample is mixed with 10 ul of enzyme, and allowed to react for 1 hour. For Urea plate, 100 ul of sample is added to the required wells. Then, 100 ul of OPA Color reagent mix is added to each well. Color development is allowed to proceed 10 to 60 minutes before reading at 420 nm. The entire process from start to finish can be completed for three full plate applying both Urea and Ammonium assay in about 2 hours; averaging about 1.0 minutes per duplicate sample where most of the time would be spent mixing the samples at various steps. Any given mixing event can take about 5 to 10 seconds. Cost of reagents per plate was estimated to be about $0.85 perplate applying Ammonium acquisition only, and $2.76 per plate applying Urea with the enzymatic conversion. Since Row A and Row B of the plate has been designated for standards and QC check samples, each plate would accept up to 72 dilutions of samples, or 36 dilutions of samples in duplicate.

5.2 Process diagram

5.3 Equipment

Vortexer
15 ml PP Conical Tube (Fisher Cat# 05-539-12)
Filter Unit, 0.22 um Cellulose Urea (Fisher Cat# 09-761-102)
Pipette aid
Pipette and Pipette tips (20, 200, and 1000 ul)
Pipettes (5, 10, 25 ml)
1.5 ml micro centrifuge tubes (Fisher Cat# 05-408-129)
Clear 96-well culture plates (BD Falcon Cat# 353072)
Kimwipes (Fisher Cat# 06-666-11C)
Spectrophotometer (Abs 420 nm) to read 96-well plates, Molecular Diagnostics SpectraMax M2

5.4 Reagents

Ultra Pure water (milliQ or equivalent)
Urea (Sigma Cat # U5378)
Urease from Jack Bean (Sigma Cat # U1875)
Ammonium Chloride (Fisher Cat # A687-500)
Phthaldialdehyde (OPA) (Sigma Cat # P1378)
2-mercaptoethanol (Sigma Cat # M6250)
100% Ethanol (Sigma Cat # E7023)
Potassium Phosphate Monobasic (Fisher Cat # P284)
Potassium Phosphate Dibasic (Fisher Cat # P290-212)
Ammonium QC Standard, 100 ppm NH_4^+ (Ricca Chemical Cat # r0692500)

5.5 Process steps

5.5.1 Preparation an algae culture filtrate.

i. Refer to procedure TBD, Filtering algae pond samples for water chemistry. Ref: TBD

5.5.2 Prepare the 10% phthaldialdehyde (OPA) in 100% Ethanol

i. In a chemical safety hood, weigh the required amount of o-phthaldialdehyde into a 15 ml conical tube. Refer to the table.
ii. Carefully add the 100% Ethanol to the tube to QC to required volume. Estimated actual volumes of ethanol are presented in the table.
iii. Cap tightly and vortex extensively for several minutes to dissolves all the OPA.

Weigh the OPA and transfer ethanol in the hood.

Stable 24 hrs.

#Plates:	1	2	4	6	8	10
#Duplicate Samples:	36	72	144	216	288	360
o-Phtaldialdehyde (g)	0.10	0.20	0.40	0.60	0.80	1.00
100% EtOH (ml)	1.0	1.9	3.8	5.8	7.7	9.6
10% phthaldialdehyde Total Volume (ml)	1.0	2.0	4.0	6.0	8.0	10.0

5.5.3 Prepare the OPA Color Reagent in 500 mM phosphate, pH 7.4.

i. Refer to the table for required volumes.
ii. Mix equal amounts of 1 M Potassium Phosphate, pH 7.4 and milli-Q water, or equivalent, in a 50 ml conical tube or glass bottle.
iii. In a chemical safety hood, add 5 ul of 2-mercaptoethanol per 10 ml of required reagent.
iv. Mix the 10% o-phthaldialdehyde made in the previous step with the mixture.
v. Cap and vortex/mix thoroughly.
vi. Allow to sit at least 1 hour. Mix again before use.

The color reagent will have a slight yellow tinge. The color clears with time, and affects the background of your assay reads in the spectrophotometer. One hour of wait time is sufficient for required results.

#Plates:	1	2	4	6	8	10
#Duplicate Samples:	36	72	144	216	288	360
milli-Q H_2O(ml)	4.5	9	18	27	36	45
1M Potassium Phosphate Buffer, pH 7.4 (ml)	4.5	9	18	27	36	45
2-mercaptoethanol (ml)	0.005	0.010	0.020	0.030	0.040	0.050
10% (w/v) Phtaldialdehyde in 100% EtOH (ml)	1.000	2.000	4.000	6.000	8.000	10.000
OPA Color Reagent Total Volume (ml)	10	20	40	60	80	100

5.5.4 Dilute the 500 mM stock source of Urea and Ammonium Standards **1:250 to 2 mM** in nitrate-free media

i. Add 8 ul of unknown pond filtrate samples to 1992 ul (=2 X 996 ul) of nitrate-free media in a 15 ml conical tube.
ii. Cap and vortex to mix.

The 1:250 dilutions are required for use in making the standards in the following step.

5.5.5 Prepare serial dilutions of standard in microcentrifuge tubes using nitrate-free media.

i. Refer to the table to make the 8 required concentrations of either *Urea* or *Ammonium* standards using the 2 mM NH_4Cl or 2 mM Urea.
ii. Cap and vortex thoroughly to mix.

There will be enough volume of each standard to accommodate 9 plates each for both urea and ammonium.

				NH_4Cl or Urea		NH_4Cl	NH_4^+	N by NH_4^+	Urea	N by Urea
Cal#	Volume **2mM** NH_4Cl or Urea Stock	Volume Media Diluent (ul)	df	mM	Concentration [uM]	ppm	ppm	ppm	ppm	ppm
1	**5**	**995**	200.0	0.010	10	0.53	0.18	0.14	0.60	0.28
2	**50**	**950**	20.0	0.100	100	5.35	1.80	1.40	6.01	2.80
3	**100**	**900**	10.0	0.200	200	10.70	3.61	2.80	12.01	5.60
4	**150**	**850**	6.7	0.300	300	16.05	5.41	4.20	18.02	8.40
5	**250**	**750**	4.0	0.500	500	26.75	9.02	7.00	30.03	14.01
6	**350**	**650**	2.9	0.700	700	37.44	12.62	9.80	42.04	19.61
7	**450**	**550**	2.2	0.900	900	48.14	16.23	12.61	54.05	25.21
8	**550**	**450**	1.8	1.100	1100	58.84	19.84	15.41	66.07	30.81

Be sure to wipe the pipette tip before adding the 5 ul stock to 995 ul of diluent for accurate transfer.

5.5.6 Prepare dilutions of the algae culture filtrates in nitrogen-free media, 1:10

Recommended dilutions for pond samples expected to be up to 160 ppm N by either urea or ammonium.

Always use nitrogen free media as a diluent in this assay.

i. 1:10 – Add 100 ul of sample to 900 ul of nitrogen-free media – Rinse tip.
ii. Cap the 1:10 microcentrifuge tube, and vortex thoroughly to mix.

5.5.8 Deliver Nitrate-free media Blank and Media Source to A1/B1 and A2/B2, respectively.

i. Add 90 ul of Nitrate-free media to wells A1 and B1 of the *Urea* plate for a quality control check against contaminated water source.
ii. Add 90 ul of the media source used in the algae culture to wells A2 and B2 of the *Urea* plate for a quality control check on the media.
iii. Add 100 ul of Nitrate-free media to wells A1 and B1 of the *Ammonium* plate for a quality control check against contaminated water source.
iv. Add 100 ul of the media source used in the algae culture to wells A2 and B2 of the *Ammonium* plate for a quality control check on the media.

90 ul => Urea Plate

100 ul => Ammonium Plate

Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	N-free Media	Media	Cal1	Cal2	Cal3	Cal4	Cal5	Cal6	Cal7	Cal8	QC Lo	QC Hi
B	N-free Media	Media	Cal1	Cal2	Cal3	Cal4	Cal5	Cal6	Cal7	Cal8	QC Lo	QC Hi
C	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
D	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
E	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
F	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
G	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36
H	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36

Reference the Plate Map during the next steps.

5.5.9 Deliver calibration standards to Row A and Row B.

i. Add 90 ul of each of the 8 calibration standards to wells A3 through B10 of the *Urea* plate.
ii. Add 100 ul of each of the 8 calibration standards to wells A3 through B10 of the *Ammonium* plate

5.5.10 Deliver QC Lo and QC Hi Check Standards.

i. Add 90 ul of each of the QC Lo and QC Hi Check standards to wells A11/B11 and A12/B12 of the *Urea* plate, respectively.
ii. Add 100 ul of each of the QC Lo and QC Hi Check standards to wells A11/B11 and A12/B12 of the *Ammonium* plate, respectively.
iii. Concentrations of the QC check standards are shown in the table.

Dilutions made in nitrogen-free media

Ammonium: Ricca (100 ppm NH_4^+)	Source Conc (ppm)	Vol Stock (ul)	Vol Dil Media (ul)	Actual Conc (ppm) NH_4^+	ppm N by NH_4^+
Hi	100	180	820	18.0	14.0
Lo (Serial from Hi)	18	14.7	1000	0.261	0.203

Urea QC standard made in lab to 500 mM in water.

Urea: Sigma (made to 500 mM in GUH water)	Source Conc (mM)	Vol Stock (ul)	Vol Dil Media (ul)	Actual Conc Urea (mM)	ppm Urea	ppm N by Urea
Hi	500	2.6	1300	0.998	59.9	28.0
Lo (Serial from Hi)	0.998	0.250	12.5	0.0196	1.2	0.5

5.5.11 Deliver the diluted algae culture samples to the plates.

i. Add 90 ul of each of the sample dilutions to the required wells of the *Urea* plate.
ii. Add 100 ul of each of the sample dilutions to the required wells of the *Ammonium* plate.
iii. Cover the *Ammonium* plate with 2 square X 3 square parafilm
   a. Remove the lid.
   b. Lay the cup piece of parafilm gently over the top of the wells - flat.
   c. Gently wedge the plastic lid over the plate to create a seal.

Parafilm is used in the event that the sample is basic, which would allow ammonia to form. This gas can then move to adjacent wells to cause error.

5.5.12 Deliver the *Urease* Enzyme Mix to the *Urea* plate.

i. Deliver 10 ul of the Enzyme using a multichannel pipette to each well in the *Urea* plate
ii. Rinse the tip in the well 3X upon delivery.
iii. Cover the *Urea* plate with parafilm as in the previous step for the ammonia plate.
   a. Remove the lid.
   b. Lay the cup piece of parafilm gently over the top of the wells - flat.
   c. Gently wedge the plastic lid over the plate to create a seal.

Use the Enzyme directly from the Sigma source bottle (transferred to a multichannel through).

5.5.14 Wait 1 hour for enzyme activity – conversion of *Urea* to *Ammonium*.

Seal plates with parafilm during the incubation step.

5.5.15 Add the OPA Color Reagent Mix to each well.

i. Add 100 ul of OPA Color Reagent Mix to each of the assay wells in both the *Urea* AND *Ammonium* plates.
ii. Pipette up and down briefly, 3X, to mix.

5.5.16 Allow color development to proceed for between 20 and 60 minutes sealed in dark.

i. Allow color development to occur in the dark.
ii. Cover the plates with parafilm during the color development step.
   a. Remove the lid.
   b. Lay the cup piece of parafilm gently over the top of the wells - flat.
   c. Gently wedge the plastic lid over the plate to create a seal.

5.5.17 Measure Absorbance 420 nm.

i. Measure the absorbance using the 96-well plate reader.
ii. Export data to text file.

Ref: TBD

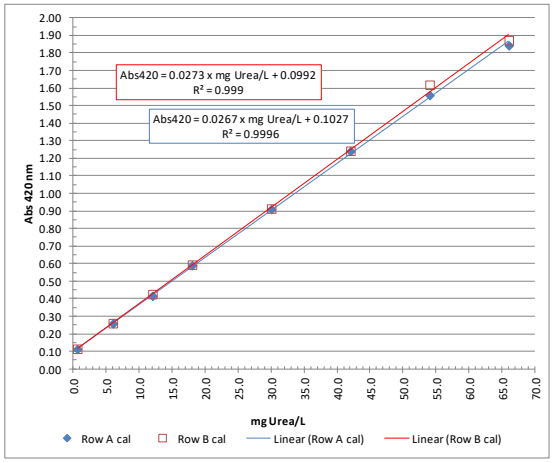
5.5.18 Workup the data using the Water Chemistry Template Macro.

Ref: TBD

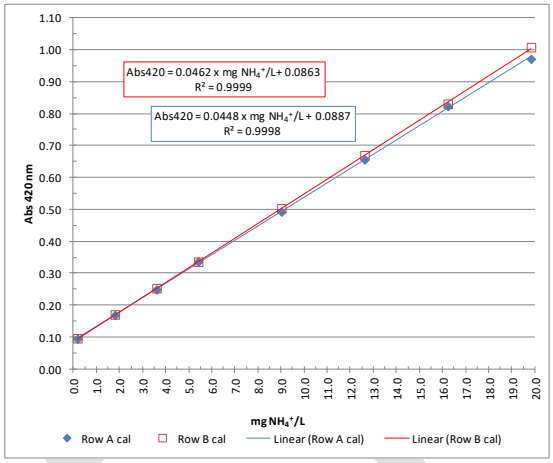
5.6 Example Data

5.6.1 Example of Calibration Standard Curve

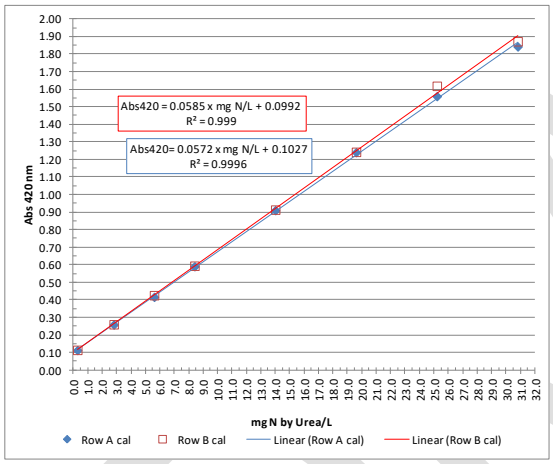
Urea Plate

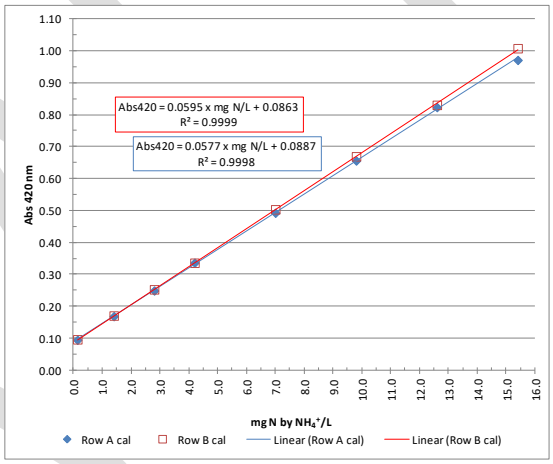


Ammonium Plate



mg Urea/L
mg Ammonium/L





mg N/L

5.6.2 Example of QC check standard results with Upper and Lower Bound at +/-3s, respectively.

The QC check standards are used to validate that the experiment was performed accurately using quality reagents. QC check standard results must be "within spec", between the Upper and Lower Bound determined during quality control studies.

	Urea				
	QC Lo			QC Hi	
	mg Urea/L	mg N/L		mg Urea/L	mg N/L
Target	1.20	0.559	Target	61.1	28.5
Mean	1.17	0.543	Mean	61.9	28.9
Std Dev,s	0.11	0.049	Std Dev	1.1	0.5
3s	0.317	0.148	3s	3.3	1.5
Upper Bound	1.48	0.69	Upper Bound	65.1	30.4
Lower Bound	0.85	0.40	Lower Bound	58.6	27.3
%Err-Avg	-2.8	-2.8	%Err-Avg	1.2	1.2
%Err-Hi	23.7	23.7	%Err-Hi	6.6	6.6
%Err-Lo	-29.2	-29.2	%Err-Lo	-4.1	-4.1

	Ammonium, NH_4^+				
	QC Lo			QC Hi	
	mg NH_4^+/L	mg N/L		mg NH_4^+/L	mg N/L
Target	0.26	0.200	Target	18.0	14.00
Mean	0.29	0.229	Mean	18.12	14.07
Std Dev,s	0.04	0.029	Std Dev	0.16	0.13
3s	0.11	0.087	3s	0.49	0.38
Upper Bound	0.41	0.316	Upper Bound	18.61	14.45
Lower Bound	0.18	0.141	Lower Bound	17.63	13.69
%Err-Avg	14.3	14.3	%Err-Avg	0.52	0.52
%Err-Hi	58.0	58.0	%Err-Hi	3.22	3.22
%Err-Lo	-29.3	-29.3	%Err-Lo	-2.2	-2.2

5.7 Reagent Recipes

5.7.1 Nitrate-free media

All dilutions in the same media used for the algae culture, but made without any nitrogen source.

5.7.2 Urea QC Check Standards

500 mM Stock Urea

i. Weigh 1.5015 g Urea into a clean 50 ml Volumetric Flask
ii. Add 40 ml milli-Q water.
iii. Cap, or seal the volumetric flask with parafilm.
iv. Shake the flask vigorously until all Urea is dissolved.
v. Remove the cap.
vi. Add water to bring volume to the 50 ml mark on the flask.
vii. Cap, seal with parafilm.
viii. Invert to mix for at least two minutes.

Urea	
500	**mM**
0.50	M
60.06	MW
50	**Vol (ml)**
0.050	Vol (L)
0.0250	Mol
1.5015	**Mass Urea**

2 mM Stock of Urea

i. Add 40 ml of milli-Q water to a clean 50 ml volumetric flask.
ii. Transfer 200 ul of the 500 mM Urea stock to the 40 ml of water in the flask. Rinse the tip.
iii. Add milli-Q water to the flask to bring the volume to the 50 ml mark.
iv. Cap, seal with parafilm.
v. Invert to mix for at least two minutes.

5.7.3 1 M Potassium Phosphate buffer, pH 7.4

The method is derived from the use of buffer tables which mix both 1 M potassium phosphate, dibasic and 1 M potassium phosphate, monobasic solutions to produce the desired pH. Stable 3 months

i. Obtain 1 M solution of Potassium Phosphate, dibasic, K_2HPO_4.
ii. Obtain 1 M solution of Potassium Phosphate, monobasic, KH_2PO_4.
iii. Mix 400 ml of 1 M Phosphate, dibasic with 100 ml of 1 M Phosphate, monobasic.

6.0 Waste and Safety

6.1 Disposal of reagents

i. All unused reagents and reagent waste is to be collected into a single waste container for professional removal offsite.
ii. Fluid in plates is to be removed and collected with other unused reagents and reagent waste.
iii. Tips should be completely expelled into the assay wells, or into waste collection. When the tips are clear of any fluid, they can be placed in the trash.

6.2 Required PPE

i. Gloves and safety glasses are to be worn for the entire duration of the experiment.

6.3 Potential Health Effect of Reagents

6.3.1 Potential Health Effects of Potassium Phosphate, monobasic, KH_2PO_4

Inhalation: May be harmful if inhaled. May cause respiratory tract irritation.
Ingestion: Ingestion may cause gastrointestinal irritation and diarrhea. NFPA (1,0,0)
Skin Contact: May cause skin irritation.
Eye Contact: May cause eye irritation.
Chronic Exposure: No information found.

6.3.2 Potential Health Effects of Potassium Phosphate, dibasic, K_2HPO_4

Inhalation: May be harmful if inhaled. May cause respiratory tract irritation.
Ingestion: Ingestion may cause gastrointestinal irritation and diarrhea.
Skin Contact: May cause skin irritation. NFPA (1,1,0)
Eye Contact: May cause eye irritation.
Chronic Exposure: No information found.

6.3.3 Potential Health Effects of Urea, CH_4N_2O

Inhalation: Irritating to respiratory system. May be harmful if inhaled. NFPA (2,1,0)
Ingestion: May be harmful if swallowed. Ingestion may cause gastrointestinal irritation, nausea, vomiting and diarrhea.
Skin Contact: Irritating to skin. May be harmful in contact with skin.
Eye Contact: Irritating to the eyes.
Chronic Exposure: None known.

6.3.4 Potential Health Effects of *Urease* enzyme

NFPA (2,0,1)

Inhalation: Causes respiratory tract irritation. May be harmful if inhaled. May cause respiratory sensitization.
Ingestion: May cause irritation of the digestive tract. May be harmful if swallowed.
Skin Contact: Causes skin irritation. May be harmful if absorbed through the skin.
Eye Contact: Causes eye irritation.
Chronic Exposure: Repeated or prolonged exposure may cause allergic reactions in sensitive individuals.

6.3.5 Potential Health Effects of o-phthaldialdehyde, $C_8H_6O_2$

NFPA (3,1,0)

Inhalation: May be harmful if inhaled. Material is extremely destructive to the tissue of the mucous membranes and upper respiratory tract.
Ingestion: Toxic if swallowed. Causes burns.
Skin Contact: May be harmful if absorbed through skin. Causes skin burns.
Eye Contact: Causes eye burns.
Chronic Exposure: No data found.

6.3.6 Potential Health Effects of Ammonium Chloride, NH_4Cl

NFPA (2,0,1)

Inhalation: May cause irritation of respiratory tract. May be harmful if inhaled.
Ingestion: Harmful if swallowed. Ingestion may cause gastrointestinal irritation, nausea, vomiting and diarrhea.
Skin Contact: May cause irritation. May be harmful in contact with skin.
Eye Contact: Irritating to eyes.
Chronic Exposure: May cause adverse kidney effects.

6.3.7 Potential Health Effects of 2-mercaptoethanol, C_2H_6OS

NFPA (3,2,1)

Skin, rabbit: LD50 = 150 uL/kg

Inhalation: Inhalation of high concentrations may cause central nervous system effects characterized by nausea, headache, dizziness, unconsciousness and coma. May cause respiratory tract irritation. May cause dyspnea (difficult or labored breathing). Exposure to high concentrations of mercaptans can produce unconsciousness with cyanosis (bluish discoloration of skin due to deficient oxygenation of the blood), cold extremities and rapid pulse. Mercaptans may cause nausea and headache.
Ingestion: Harmful if swallowed. May cause gastrointestinal irritation with nausea, vomiting and diarrhea. May cause muscle paralysis, respiratory failure, and possible death.
Skin Contact: May be fatal if absorbed through the skin. May cause irritation with burning pain, itching and redness.
Eye Contact: May cause severe eye irritation. May result in corneal injury.
Chronic Exposure: Prolonged or repeated skin contact may cause defatting and dermatitis. Repeated or prolonged exposure may cause CNS stimulation.

6.3.8 Potential Health Effects of 100% Ethanol, C_2H_5OH NFPA (2,3,0)

Inhalation: May cause irritation of respiratory tract. May be harmful if inhaled. Inhalation may cause central nervous system effects.
Ingestion: May be harmful if swallowed. Aspiration hazard if swallowed - can enter lungs and cause damage. Ingestion may cause gastrointestinal irritation, nausea, vomiting and diarrhea.
Skin Contact: Irritating to skin. May be harmful in contact with skin.
Eye Contact: Irritating to eyes.
Chronic Exposure: This substance has caused adverse reproductive and fetal effects in humans. Substances known to cause developmental toxicity in humans. Tumorigenic effects have been reported in experimental animals. May cause adverse liver effects. May cause adverse kidney effects.

7.0 Required documents

7.1 Input documents

Assay protocol.

7.2 Output documents

i. Absorbance data file in text format with timestamp of the read associated with the file. TBD
ii. Saved data workup from Water Chemistry Template Macro. TBD

8.0 Document control

8.1 Revision history

R0 – Initial Release – <Editor name> <Date>
R1 – <Editor name> <Date>

8.2 Document approval

<Name> <Approval date>

8.3 Document reviewers

<Name> <Last reviewed date>
<Name> <Last reviewed date>

9.0 Risk analysis

<Owner> <RPN>