1.0 Procedure title

Measuring Alkalinity of Algae Culture using Methyl Orange indicator 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.

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

The dilution of the concentrated hydrochloric acid must be performed in a chemical fume hood.

Sodium Carbonate is toxic, unlike baking soda (sodium bicarbonate), and can be harmful if swallowed or inhaled. Oral rat LD50: 4090 mg/kg

Methyl Orange is toxic, and must be handled with care. Oral, rat: LD50 = 60 mg/kg.

2.2 Quality

Use ultra pure water for diluting samples and making reagents to obtain the most accurate results.

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 30 minutes has not been studied. To assure valid results read the plate before 30 minutes of color development.

The pH 4.01 KHP stock must be acidified with dilute HCl before adding Methyl Orange, or the calibration curve will not be linear. Use pH color strips to verify the pH.

2.3 Delivery

N/A

2.4 Environmental

Methyl Orange will eventually settle out in environmental streams.

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

2.5 Cost

The most expensive reagent per experiment was the Gibco Ultra Pure water. Making only the minimum volume of reagents for the required workload will decrease the cost of performing this assay by minimizing waste.

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

Filtering algae pond samples for water chemistry	TBD
Use of the plate reader for Absorbance Endpoint	TBD
Using the Water Chemistry Template Macro	TBD

4.0 Responsibilities

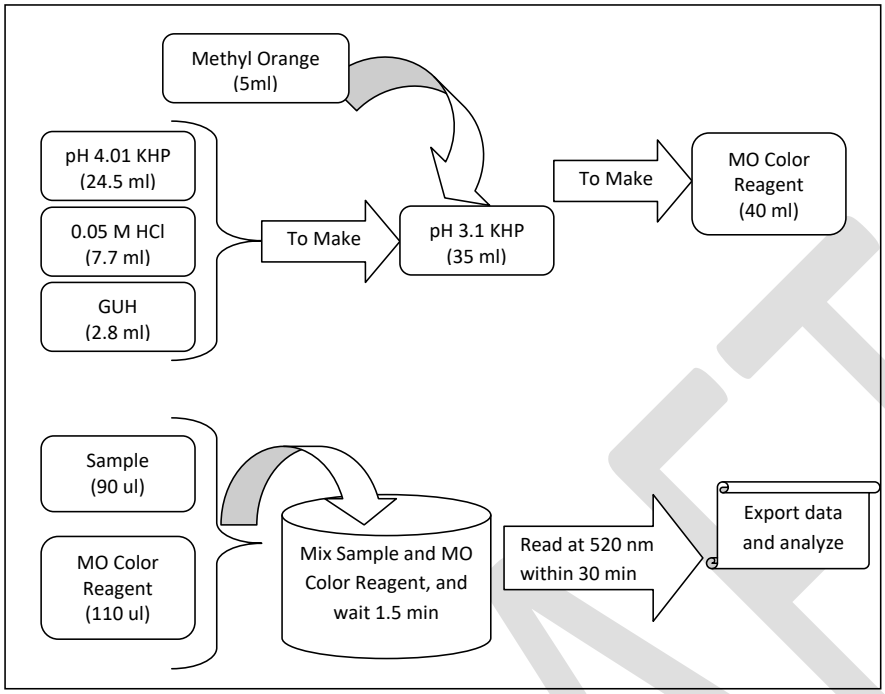
Document Owner	Manage content and distribution	Jeremy Ferrara
Process Owner	Responsible for content and process validation	Jeremy Ferrara
Plant Manager	Responsible for implementation and conformance	Nicole Heaps

5.0 Process

5.1 Process description

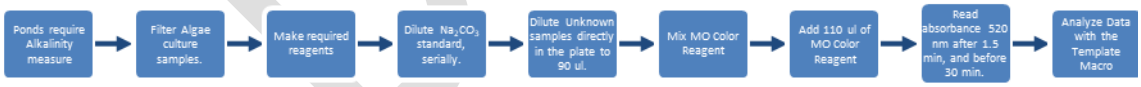
The method was based on Seal Analytical AQ1 method Alkalinity – Total Ground, Surface and Saline Waters, Domestic and Industrial Waste. The procedure was simplified to mixing an unknown sample with a Methyl Orange (MO) Color Reagent and reading color development at 520 nm. Methyl orange was used as the indicator in this method because its pH range is in the same range as the equivalence point for total alkalinity, and it has a distinct color change that can easily be measured, Abs 520 nm. The methyl orange was dissolved in a weak buffer at pH 3.1, just below the equivalence point, so that any addition of alkalinity caused a loss of color directly proportional to the amount of alkalinity. The transition pH range for Methyl Orange is 3.1 – 4.4, changing from red orange to yellow.

The AQ1 assay is documented to be useful for analyte concentration between 50 to 500 mg/L $CaCO_3$, estimated reporting level 100 mg/L $CaCO_3$. However, when validating the assay in a micro environment, 96-well format, the useful range was found to be wider, between 50 mg/L and 750 mg/L $CaCO_3$ equivalent. Then, the minimum limit of detection was 50 mg $CaCO_3$ eq/L. Note that since $CaCO_3$ is not very soluble in water, the carbonate standard was made using anhydrous sodium carbonate, Na_2CO_3, at a concentration equivalent to the molarity of 1 g $CaCO_3$/L solution; this is a factor of 1.06 more mass than required for the hypothetical 1 g $CaCO_3$/L solution. Then, the Na_2CO_3 stock can be referred to as $CaCO_3$ equivalent (mg $CaCO_3$ eq/L).



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. Then, one would mix 90 ul of diluted sample with 110 ul of MO Color Reagent for color development. The color development proceeds for at least 1.5 minutes before reading the plate at 520 nm in a plate reader. The entire process from start to finish can be completed for two full plates in about 1 hour and 15 minutes; averaging about 0.5 minutes per 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 $0.61, with Gibco Ultra Pure Water as the most expensive reagent. Since Row A of the plate has been designated for standards, each plate would accept up to 84 dilutions of samples, or 42 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 Nitrate (Fisher Cat# 09-761-102)

Magnetic stir plate and stir bar

1L Beaker

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 520 nm) to read 96-well plates, Molecular Diagnostics SpectraMax M2

5.4 Reagents

EMD colorpHast* pH Strips > Range: Narrow, 2.5 to 4.5 (Fisher Cat# M95813)

Gibco Ultra Pure Distilled H20 (Invitrogen Cat# 10977-015)

Methyl Orange (0.25 g/L) (Fisher Cat # AC15142-1000) (NFPA 2, 0, 0) – Stable 3 months

Potassium Hydrogen Phthalate (10.2 g/L) (Fisher Cat# AC42406-1000) (NFPA 1, 1, 0) – Stable 10 weeks

Sodium Carbonate Stock (1 mg/ml) as Na_2CO_3 (Sigma Cat# S2127) (NFPA 1, 0, 1, 2) – Stable 2 weeks

0.05 M HCl in ddH_2O (NFPA 3, 0, 0)

Alkalinity QC Check Standard (1000 mg eq $CaCO_3$/L) (Accustandard Cat# WC-ALK-10X-1)

5.5 Process steps

5.5.1 Preparation an algae culture filtrate.

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

5.5.2 Prepare dilutions of 1000 ppm Na_2CO_3 standard in microcentrifuge tubes.

Each 96-well assay plate used in the experiment must include duplicate rows of calibration dilutions, diluent check, media check, and QC validation check samples. For the standard curve, the stock of 1.06 g/L Na_2CO_3 is to be diluted to form an 8 point calibration curve spanning the working range of the assay, between 50 mg/L and 750 mg/L. The dilution method is presented in the table. Use larger volumes for use in more plates. However, achieving accurate dilutions is more difficult at larger volumes due to pipetting errors.

Cal #	Target Conc (mg CaCO3/L)	Source Conc (mg CaCO3/L)	Dilution Factor	Conc (mEq)	Source (uL)	GUH (uL)
1	750.0	1000	1.33	15.0	750	250
2	510.0	1000	1.96	10.2	510	490
3	346.8	1000	2.88	6.9	347	653
4	235.8	1000	4.24	4.7	236	764

5	160.4	1000	6.23	3.2	160	840
6	109.0	1000	9.17	2.2	109	891
7	74.2	1000	13.48	1.5	74	926
8	50.4	1000	19.84	1.0	50	950

5.5.3 Prepare the QC check standards – Hi and Lo

Dilute the Alkalinity QC check standard (AccuStandard Cat # WC-ALK-10X-1) in GUH according to the following table. Makes enough for 5 plates using duplicate measures of the QC check standard.

Alkalinity	**Stock Source Conc (ppm)**	**Vol Stock (uL)**	**Vol Dil H_2O(uL)**	**Final Conc (ppm) $CaCO_3$**	**Final Conc Alk (mEq)**
QC Hi	1000	923	350	725	14.99
QC Lo	1000	75	925	75.0	1.50

5.5.4 Acidify the pH 4.01 KHP with 0.1N HCl to pH 3.1

KHP stock solution pH 4.01	35 mL
Hydrochloric acid, 0.1 N (Fisher Cat # AC12420-0010)	Titer (approximately 5.5 mL)
GUH water	Dilute to 50 mL

The buffer system in the assay uses KHP at a slightly acidic initial condition. Adding acid to this buffer is critical for obtaining a linear calibration curve.

i. To a 50 mL conical tube add 35 mL KHP stock solution.
ii. Add enough 1.0 N hydrochloric acid to adjust the pH to 3.1.
iii. Transfer volume to a 50 mL volumetric flask.
iv. Dilute to 50 mL with GUH water.
v. Check pH prior to use.

5.5.5 Prepare the Methyl Orange Color Reagent

KHP solution, pH 3.1	35 mL
Methyl orange stock solution	5 mL

i. Add Methyl Orange Stock to the pH 3.1 KHP as described in the table above.
ii. Cap and vortex to mix.

5.5.6 Deliver GUH diluent Blank and Media Source to A1/B1 and A2/B2, respectively.

i. Add 90 ul of GUH diluent to wells A1 and B1 for a quality control check for contaminated water source.
ii. Add 90 ul of the media source used in the algae culture to wells A2 and B2 for a quality control check on the media.

Plate Map

Reference the Plate Map during the

	Col1	Col2	Col3	Col4	Col5	Col6	Col7	Col8	Col9	Col10	Col11	Col12
A	GUH dil	Media	Cal1	Cal2	Cal3	Cal4	Cal5	Cal6	Cal7	Cal8	QC Lo	QC Hi
B	GUH dil	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

next steps.

5.5.7 Deliver calibration standards to Row A and Row B.

i. Add 90 ul of each of the 8 calibration standards from step 5.5.2 to Row A and Row B in decreasing concentration starting with Well A3/B3 at 750 mg $CaCO_3$ eq / L.

5.5.8 Deliver QC Lo and QC Hi check standards.

i. Add 90 ul of QC Lo check standard to wells A11 and B11 for data validation.
ii. Add 90 ul of QC Hi check standard to A12 and B12 for data validation.

5.5.9 Dilute samples in 2 mL deep well plates.

Dilute the filtered unknowns to 500 uL in a 2 mL deep well plate using GUH diluent.
Recommended dilutions for pond samples are listed below.

i. 1:20 – Add 25 uL of sample to 475 uL of GUH water – use for high alkalinity media types.
ii. 1:10 – Add 50 uL of sample to 450 uL of GUH water – use for low alkalinity media types.

5.5.10 Add the MO Color Reagent.

i. Add 110 uL of Methyl Orange Color Reagent to each of the assay wells.
ii. Pipette up and down briefly, 3X, to mix upon delivery.

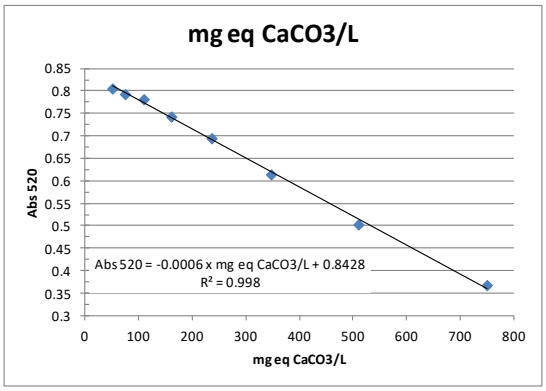
5.5.11 Measure Absorbance 520 nm after 1.5 min and before 10 min of color development.

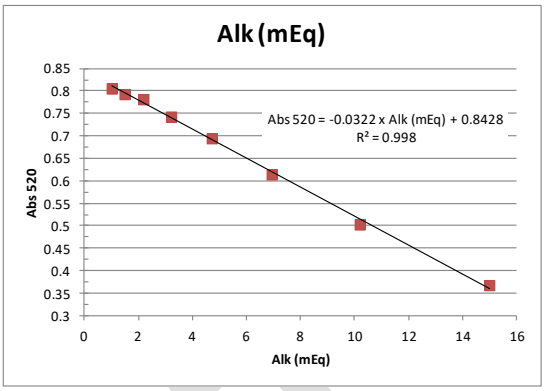
i. Measure the absorbance using a 96-well plate reader. Ref: TBD
ii. Export data to text file.

5.5.12 Workup the data using the Water Chemistry Template Macro. Ref: TBD

5.6 Example Data

5.6.1 Example of Calibration Standard Curve





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.

	QC lo		QC hi	
	ppm	Alk (mEq)	ppm	Alk (mEq)
Target	75	1.50	725	14.99
Mean				
Std Dev				
3 SD	15.00	0.30	145.00	3.00
Upper Bound	90.00	1.80	870.00	17.99
Lower Bound	60.00	1.20	580.00	11.99
%RSD	20%	20%	20%	20%
%DIF	0.0%	0.0%	0.0%	0.0%

Note: Hypothetical 3 SD of 20% of the target ppm was used to set upper and lower limit until enough historical data can be collected to calculate a representative standard deviation.

5.7 Reagent Recipes

Use reagent grade chemicals, certified for analytical or general laboratory use. Use gibco ultra pure reagent water that is free from organic contamination. Use glassware that has been acid washed and rinsed with nanopure water. The use of glassware contaminated with detergents will adversely affect the assay.

5.7.1 Methyl Orange (0.25 g/L)

Methyl Orange Sodium Salt (Fisher Cat # AC15142-1000)	0.152 g	Stable 3 months
GUH water (Invitrogen Cat# 10977-015)	Dilute to 500 mL	(NFPA 2,0,0)

i. Add 0.125 g methyl orange sodium salt to approximately 450 mL GUH water in beaker.
ii. Add stir bar, stir at least 2 hours, until dissolved, on stir plate.
iii. Transfer to a 500mL volumetric flast and dilute to 500 mL with GUH water.
iv. Filter sterilize into filter unit (Fisher Cat# 09-761-102).
v. Store at 4°C for up to 3 months.

5.7.2 pH 4.01 KHP (10.2 g/L)

Stable 10 weeks

Potassium hydrogen phthalate (Fisher Cat# AC42406-1000	5.105 g
GUH water	Dilute to 500 mL

(NFPA 1,1,0)

i. Add 5.10 g , Potassium hydrogen phthalate, anhydrous to 450 mL GUH water.
ii. Add stir bar, and stir to dissolve on magnetic stir plate.
iii. Transfer to a 500 mL volumetric flask and bring to 500 mL volume with GUH water.
iv. Filter sterilize into filter unit (Fisher Cat# 09-761-102).
v. Store at 4°C for up to 4 weeks.
vi.

5.7.3 Alkalinity Stock Standard Solution (1000 ppm)

Sodium carbonate, anhydrous (Sigma Cat# S2127)	0.530 g
GUH water	Dilute to 500 mL

(NFPA 1,0,1,2)

i. Add 0.530 g Na_2CO_3 to 450 mL GUH water in a beaker.
ii. Add stir bar, and stir to dissolve on magnetic stir plate.
iii. Transfer to a 500 ml in volumetric flask and dilute to 500 mL with GUH water.
iv. Store at 4°C.

6.0 Waste and Safety

Disposal of reagents

Use the recommended optimal volumes for minimized waste.

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.

6.1 Required PPE

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

6.2 Potential Health Effect of Reagents

6.3 6.3.1 Potential Health Effects of Methyl Orange, $C_{14}H_{14}N_3NaO_3S$

Inhalation: May cause respiratory tract irritation. The toxicological properties of this substance have not been fully investigated.

Ingestion: Harmful if swallowed. May cause gastrointestinal irritation with nausea, vomiting, and diarrhea.

Skin Contact: May cause skin irritation.

Eye Contact: May cause eye irritation. This product contains an anionic dye. Similar dyes have not caused injury to the cornea or conjunctiva in documented exposure cases with human or rabbit eyes.

Chronic Exposure: No information found.

6.3.2 Potential Health Effects of Sodium Carbonate, Na_2CO_3

Inhalation: Inhalation of dust may cause irritation to the respiratory tract. Symptoms from excessive inhalation of dust may include coughing and difficult breathing. Excessive contact is known to cause damage to the nasal septum.

Ingestion: Sodium carbonate is only slightly toxic, but large doses may be corrosive to the gastro-intestinal tract where symptoms may include severe abdominal pain, vomiting, diarrhea, collapse and death.

Skin Contact: Excessive contact may cause irritation with blistering and redness. Solutions may cause severe irritation or burns.

Eye Contact: Contact may be corrosive to eyes and cause conjuctival edema and corneal destruction. Risk of serious injury increases if eyes are kept tightly closed. Other symptoms may appear from absorption of sodium carbonate into the bloodstream via the eyes.

Chronic Exposure: Prolonged or repeated skin exposure may cause sensitization.
Aggravation of Pre-existing Conditions: No information found.

6.3.3 Potential Health Effects of Potassium Hydrogen Phthalate (KHP), $HOOC\text{-}C_6H_4\text{-}COOK$

Inhalation: May be harmful if inhaled. May cause respiratory tract irritation.

Ingestion: May be harmful if swallowed.

Skin Contact: May be harmful if absorbed through skin. May cause skin irritation.

Eye Contact: May cause eye irritation.

Chronic Exposure: No information found.

Aggravation of Pre-existing Conditions: No information found.

6.3.4 Potential Health Effects of Hydrochloric Acid, HCl

Inhalation: Corrosive! Inhalation of vapors can cause coughing, choking, inflammation of the nose, throat, and upper respiratory tract, and in severe cases, pulmonary edema, circulatory failure, and death.

Ingestion: Corrosive! Swallowing hydrochloric acid can cause immediate pain and burns of the mouth, throat, esophagus and gastrointestinal tract. May cause nausea, vomiting, and diarrhea. Swallowing may be fatal.

Skin Contact: Corrosive! Can cause redness, pain, and severe skin burns. Concentrated solutions cause deep ulcers and discolor skin.

Eye Contact: Corrosive! Vapors are irritating and may cause damage to the eyes. Contact may cause severe burns and permanent eye damage.

Chronic Exposure: Long-term exposure to concentrated vapors may cause erosion of teeth. Long term exposures seldom occur due to the corrosive properties of the acid.

Aggravation of Pre-existing Conditions: Persons with pre-existing skin disorders or eye disease may be more susceptible to the effects of this substance.

Required documents

7.0

Input documents

7.1 Assay protocol.

Output documents

7.2

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

Document control

8.0

Revision history

8.1

R0 – Initial Release – <Jeremy Ferrara>	<Date>
R1 –Added the use of pH color strips to verify the pH. <Jeremy Ferrara>	<4/18/2012>

Document approval

8.2

<Name>	<Approval date>

Document reviewers

8.3

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

Risk analysis

9.0 TBD