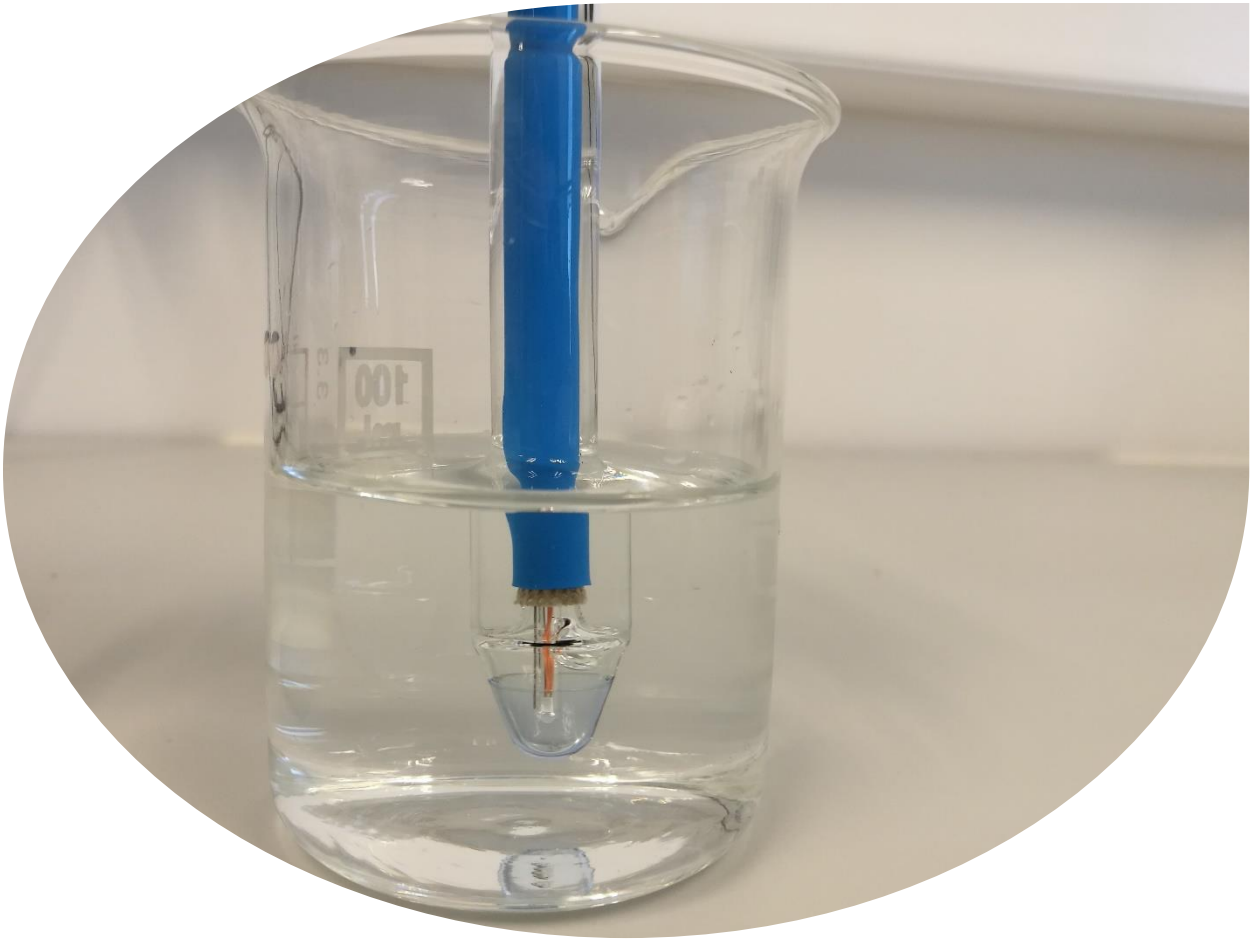
How to measure pH_T in biological experiments

TRIS buffer preparation, pH probe calibration, sampling and calculations



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Introduction

The biological response of marine organisms to ocean acidification has caught a lot of attention amongst researchers over the last decades. This often requires complex perturbation experiments and manipulation of the seawater carbonate chemistry. To ensure relevance, reproducibility and comparison between studies, it is critical to measure and report at least two variables of the carbonate system of seawater (e.g. dissolved inorganic carbon, total alkalinity, pH, or partial pressure of carbon dioxide). In addition, it is also necessary to measure and report salinity, temperature, the hydrostatic pressure (if the experiments were not performed at atmospheric pressure) and concentrations of total dissolved inorganic phosphorus and total dissolved inorganic silicon whenever possible. Using this information, it is then possible to calculate all the other parameters of the carbonate chemistry using one of the many available software packages and following the recommendations made by Orr et al. (2015). Additional recommendations for measurements and reporting of the carbonate system can be found in Gattuso et al. (2015) and IOC-UNESCO (2018).

While well-established procedures and protocols for precise monitoring of the seawater carbonate chemistry parameters are available (e.g. Dickson et al., 2007), these procedures are not always convenient (and are even not necessary) for experimental biologists as the level of precision and accuracy should be adapted to the question and needs (IOC-UNESCO, 2018). One of the parameters frequently measured is pH. pH can be measured on different scales (NBS, free, total, or seawater). Following the recommendations from the *Guide to best practices for ocean acidification research and data reporting* (Riebesell et al., 2011), seawater pH should be monitored and reported on a total scale, pH_T.

There are two frequently used methods to determine the pH_T of a sample. Spectrophotometric analysis is precise, but can be expensive, time-consuming, and requires trained personnel to perform analysis. The other method is based on potentiometry and involves the use of glass electrodes. These should be calibrated with TRIS buffer prepared in synthetic seawater approximating the ionic strength of seawater samples. The method and rationale are described in detail in the *Guide to Best Practices*

for Ocean CO_2 Measurements, SOP 6 - *Determination of the pH of seawater using a glass/reference electrode cell* (Dickson et al., 2007).

TRIS buffer that is used as a calibration standard for seawater pH_T is made from the base species (2-amino-2-hydroxymethyl-1,3-propanediol) and its conjugate acid: $TRIS{\cdot}H^+$, prepared in synthetic seawater, to keep activity coefficients similar between the buffer and a sample of seawater. TRIS buffers are commercially available from Dr. Andrew Dickson's laboratory at the Scripps Institution of Oceanography, California. However, access to this buffer can be difficult due to a continuously increasing demand as well as costs including shipping, customs fees, and taxes, making them less available for countries and laboratory with limited funds. A simplified buffer preparation method is described in Paulsen & Dickson paper (2020) making the use of TRIS buffers available to a wider range of researchers.

The aim of this document and associated material (xls sheets and videos) is to help experimentalists entering the field of ocean acidification to make their own TRIS buffer, calibrate their glass electrodes for pH measurement on the total scale, take water samples and calculate pH_T.

How to prepare TRIS buffer?

The methodology described in this section is based on Paulsen&Dickson (2020) and all the step are summarized in the video ***1. How to make your own TRIS buffer.mp4***.

LIMNOLOGY
and
OCEANOGRAPHY: METHODS

ASLO

Limnol. Oceanogr.: Methods 2020

doi: 10.1002/lom3.10383

Preparation of 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS) pH_T buffers in synthetic seawater

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Abstract

Buffers of known quality for the calibration of seawater pH_T measurements are not widely or commercially available. Although there exist published compositions for the 0.04 mol kg-H_2O^{-1} equimolar buffer 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS)-TRIS · H^+ in synthetic seawater, there are no explicit procedures that describe preparing this buffer to achieve a particular pH_T with a known uncertainty. Such a procedure is described here which makes use of easily acquired laboratory equipment and techniques to produce a buffer with a pH_T within 0.006 of the published pH_T value originally assigned by DelValls and Dickson (1998), 8.094 at 25°C. Such a buffer will be suitable for the calibration of pH measurements expected to fulfil the "weather" uncertainty goal of the Global Ocean Acidification Observation Network of 0.02 in pH_T, an uncertainty goal appropriate to "identify relative spatial patterns and short-term variation."

List of chemicals

- 0.1% methyl red indicator in alcoholic solution
- hydrochloride acid, 37% stock solution, HCl
- 2-amino-2-hydroxymethyl-1,3-propanediol, TRIS solid
- natrium chloride, NaCl
- natrium sulphate, Na_2SO_4
- potassium chloride, KCl
- 1M magnesium chloride, $MgCl_2$
- 1M calcium chloride, $CaCl_2$
- deionized water

Alternatively:

- $MgCl_2$ x $6H_2O$
- $CaCl_2$ x $2H_2O$
- $CaCl_2$ anhydrous

List of materials

- magnetic stirrer
- stir bar
- analytical balance (0.0001g)
- glass beakers (250 ml, 100 ml)
- disposable pipettes (4.5 mL, 15 mL)
- glass rod
- volumetric flasks
- funnel
- weighing dishes
- spatulas
- automatic pipette
- storage bottles

Alternatively:

- analytical balance (0.001g)

STEP 1. Preparation of chemicals

Calculations for the preparation of chemicals can be found in the Excel document ***2. Preparing chemicals.xlsx***. We used analytical balance readable to 0.001 g, but more precise results could be obtained with the analytical balance readable to 0.0001 g.

Chemicals and materials

- HCl concentrated stock solution
- 1 L volumetric flask
- 250 mL glass beaker
- glass rod
- automatic pipettes(s)
- 1 L storage bottle
- deionized water

1 M Hydrochloric Acid, HCl

Procedure:

(! to be performed in a fume hood)

For 1 L of ~1 M HCl solution dilute 83.30 mL of stock solution HCl, 37% with deionized water:

1. fill approximately half of the volumetric flask with deionized water (always add acid to water)
2. carefully transfer over the glass rod approximately 100 mL of concentrated HCl, 37% into the small beaker
3. with an automatic pipette add 83.30 mL of HCl from the beaker to the volumetric flask
4. carefully fill up the volumetric flask to the line with deionized water
5. transfer ~1 M HCl to the storage bottle over the funnel, label it

1 M Magnesium chloride, $MgCl_2$ & 1 M Calcium chloride, $CaCl_2$

Whenever it is possible, commercially available $MgCl_2$ and $CaCl_2$ solutions (1 mol dm^{-3}) should be used (Paulsen & Dickson, 2020).

It is also possible to make the 1 molar solutions from salts ($MgCl_2$ x 6 H_2O, $CaCl_2$ x 2 H_2O), but due to their hygroscopic nature, it is of utmost importance to take care of the chemicals storage, otherwise, the number of H_2O molecules could be much higher than it was taken into consideration for calculations.

A new batch of salts was used for making 1M solutions, and the resulting buffer performance was similar to the one purchased from Scripps laboratory (See *Home-made buffer performance*)

Chemicals and materials

- $MgCl_2$ x $6H_2O$
- $CaCL_2$ x $2H_2O$
- analytical balance (readable to 0.001 g)
- magnetic stirrer
- stir bar
- spatula x2
- beaker 250 mL x2
- volumetric flask x2
- storage bottle x2

Procedure:

For 1L of 1M $MgCl_2$ solution, a 145.6 g of $MgCl_2 \times 6H_2O$ salt needs to be dissolved in deionized water.

1. place a beaker on the analytical balance, tare
2. weigh 145.6 g of salt
3. add deionized water to the beaker for salt to start dissolving (for easier transfer to the volumetric flask)
4. pour the content into the flask over a funnel
5. keep rinsing the beaker with deionized water until all the residue is removed, and fill up to the mark
6. put a flask on the magnetic stirrer, let it dissolve
7. transfer it to the storage bottle over a funnel

This protocol is the same for all the salts, exact amounts for the desired volumes can be found in the Excel document ***2. Preparing chemicals.xlsx****.*

STEP 2. Calibrating the buffer ratio

In this step, we are determining the exact content of ~1M HCl solution with colorimetric end-point acid-base titration and ensuring a 1:1 ratio of HCl:TRIS.

To reduce possible errors in the resulting pH_T of a buffer, make sure you practice the titration method before moving on to mixing the buffer.

Repeat the following steps as many times as needed, until you get the *relative standard deviation* of 0.1% or better for four replicates.

Chemicals and materials

- 1M HCl solution (see STEP 1)
- TRIS solid
- 0.1 methyl red indicator in alcoholic solution
- magnetic stirrer
- stir bar
- 250 mL beaker x 4
- small beaker
- disposable pipettes
- white paper
- analytical balance
- spatula
- deionized water

Procedure:

1. put a 250 mL beaker on the balance, tare
2. weigh 1 g of TRIS solid, record accurately
3. add deionized water to a total weight of about 80 g
4. put a beaker on a magnetic stirrer, place a sheet of white paper underneath
5. add 6 drops of methyl red indicator – the solution will turn yellow (Fig.1. (a))
6. fill disposable pipettes* with 1M HCl, put them in a small beaker, and weigh
7. while the TRIS solution is stirring, add the HCl from a pipette with a wider opening until you notice that a hint of pink persists for more than a couple of seconds
8. now add HCl from the pipette with a narrow tip, drop by drop, until one drop changes the solution color from orange-ping to a distinct pink (magenta) (Fig.1. (b))
9. weigh the beaker with 1M HCl pipettes – for 1M solution of HCl and 1 g of TRIS solid the weight of HCl used should be about 8 g
10. insert the weight of TRIS, initial and final weight of beaker with HCl into the Excell document ***3. Preparing TRIS buffers*** provided by Paulsen & Dickson (2020)

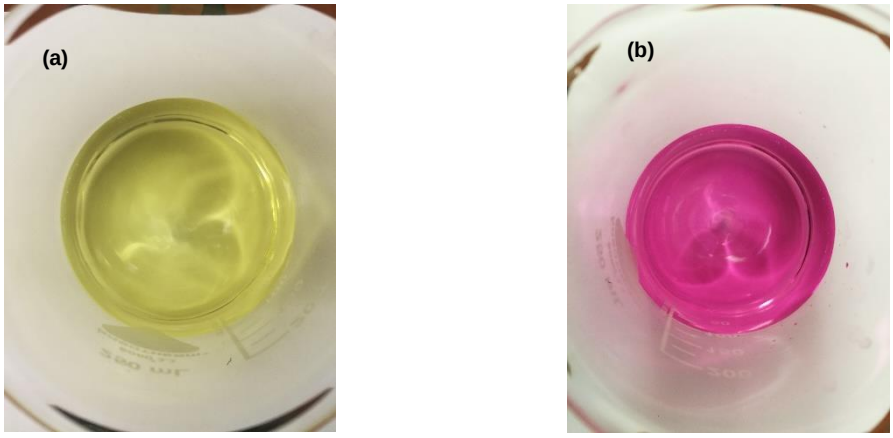


Fig.1. Color of TRIS solution after addition of methyl red indicator (a) and after reaching the titration endpoint (b)

* Depending on what you have in your laboratory, a modification can be made with disposable pipettes. For, example Paulsen & Dickson (2020) used pippets of 4.5 and 15.3 mL capacity (Fig. 2) and modified 4.5 mL pipette by melting and stretching the tip over the ethanol flame to deliver a smaller drop size for final titration steps – until the color change occurs.

If you do not have a 15 mL disposable pipette, pipettes with smaller capacity can be used instead (Fig. 3. (a)). A disposable glass dropper (Pasteur pipette) with a fine tip can be used instead of a modified 4.5 mL pipette (Fig. 3. (b)).

Whichever pipette is used, make sure that you can deliver a small size drop, because otherwise the amount content of HCl would not be estimated precisely (relative standard deviation too high).

A modified cap should be used to prevent the HCl evaporation from the disposable pipettes. A safety check can be made with placing the pipettes on a balance to see if there is any evaporation for the time needed for titration. It will depend on the type of the pipette and the amount of time needed for titration.

Fig. 2. (a) a 15.3 mL pipette, b (tubing cap), (c) modified tip of a (d) 4.5 pipette

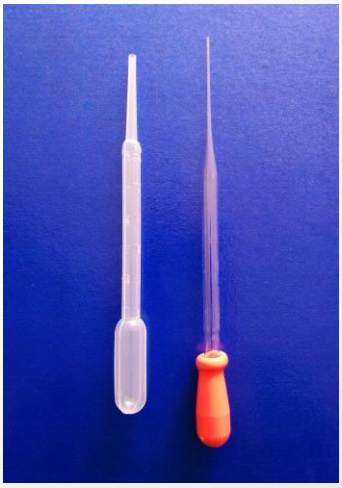


Fig.3. (a) a disposable 3 mL pipette and (b) a glass Pasteur pipette with narrow tip

Example calculation

Use the sheet ***#1 Calibrating HCl and TRIS*** in the Excel document ***3. Preparing TRIS buffers***

Insert TRIS weight

Spreadsheet to calibrate HCl against TRIS				
Supplementary spreadsheet 1 to Paulsen and Dickson, 2020				
				Info. for user
Your initials	S.G.			
Date				User input
TRIS manufacturer				
Approx. [HCl]	1	mol kg-sol^{-1}		
	#1	#2	#3	#4
w(TRIS)	1,006	1,004	1,007	1,001
w(de-ionized H_2O)	80 g	80 g	80 g	80 g
dye amount	6 drops	6 drops	6 drops	6 drops
$w(HCl)_{initial}$	125,113	126,228	126,327	122,796
$w(HCl)_{final}$	116,273	117,404	117,481	114,004
$[HCl]_{titr}$	0,9392	0,9390	0,9395	0,9396
Average $[HCl]_{titr}$ =	0,9393	mol kg-sol^{-1}	±	0,03%

Insert initial and final weight of HCl (weighted beaker with pipettes)

Relative standard deviation should be at least 0.1 (preferably less)

STEP 3. Mixing the buffer

The amount content of HCl $[HCl]_{titr}$ calculated in the previous step (yellow underline) is used to determine the weight of HCl that will be needed to make the buffer. This calculation can be done on the sheet **#2 *Mixing the buffer*** in the Excel document ***3. Preparing TRIS buffers***. The calculated $[HCl]_{titr}$ value from a previous sheet should be copied in the *Before mixing the TRIS buffer* section.

Chemicals and materials

- ~ 1M HCl
- TRIS solid
- NaCl
- Na2SO4
- KCl
- MgCl2
- CaCl2
- deionized water

- volumetric flask 1 L
- magnetic stirrer, stir bar
- small beakers or weighing dishes x 7
- disposable pipettes
- spatula
- analytical balance
- funnel

Procedure:

1. put a funnel into the volumetric flask
2. put a small beaker on the analytical balance, tare
3. weight HCl to within 0.3 g of the calculated value (see spreadsheet)
4. adjust to a final weight with a disposable pipette
5. insert actual weighed HCl to the spreadsheet to get the weights of other components
6. add each component by weighing it in a small beaker and rinsing it with a ~ 100 ml of deionized water to the volumetric flask.
7. 1M solutions of $MgCl_2$ and $CaCl_2$ can also be adjusted to a final weight with a disposable pipette
8. after adding all of the components, rinse the funnel with deionized water into the flask and fill the flask to a few centimeters below the line. put a flask stopper and mix the solution to dissolve the majority of salts.
9. carefully fill the flask to the line
10. put a stir bar and leave it stirring for a minimum of four hours
11. store buffer in a borosilicate glass bottle.

Example calculation

Use the sheet **#2 *Mixing the buffer*** in the Excel document ***3. Preparing TRIS buffers***

Insert $[HCl]_{titr}$ from the previous spreadsheet

Add HCl to within 0.3g of this calculated value

Spreadsheet to prepare TRIS buffers in synthetic sea water						
Supplementary spreadsheet 2 to Paulsen and Dickson, 2020						
					Info. for user	
Your initials	S.G.					
Date	Jan 10 2021				User input	
Before mixing the TRIS buffer						
Buffer information			What you need of each component			
Buffer volun	1	L	HCl	41,8080	g	
Salinity	35		TRIS	9,5169	g	
			NaCl	22,2544	g	
$c(HCl)_{titr}$	0,9393	mol kg-sol^{-1}	Na_2SO_4	4,0847	g	
$c(MgCl_2)$	1	mol L^{-1}	KCl	0,7748	g	
$c(CaCl_2)$	1	mol L^{-1}	$MgCl_2$	58,8618	g	
			$CaCl_2$	11,7261	g	
Mixing the TRIS buffer						
Add HCl:	41,810	g				
Actual weights			***Re-calculated weights, scaled to HCl***			
TRIS	9,5173	g	TRIS	9,5173	g	
NaCl	22,2546	g	NaCl	22,2554	g	
Na_2SO_4	4,0848	g	Na_2SO_4	4,0849	g	
KCl	0,7749	g	KCl	0,7748	g	
$MgCl_2$	58,8668	g	$MgCl_2$	58,8646	g	
$CaCl_2$	11,7265	g	$CaCl_2$	11,7267	g	

Insert weight of HCl actually added

Weights of other components you need to add (scaled to the amount of HCl added)

Home-made buffer performance

Home-made buffer performance was compared to the one purchased from Scripps laboratory (Fig. 4.).

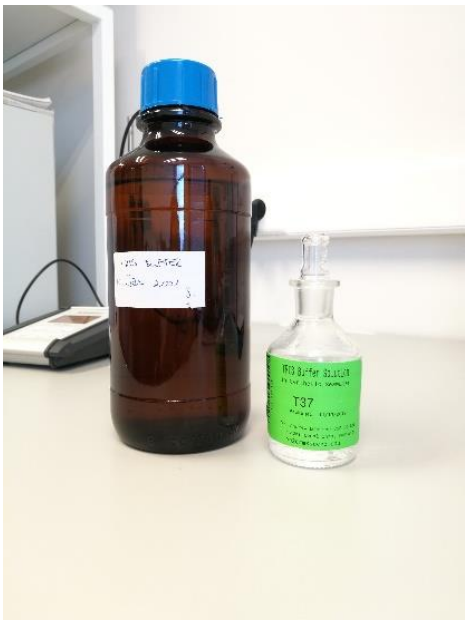
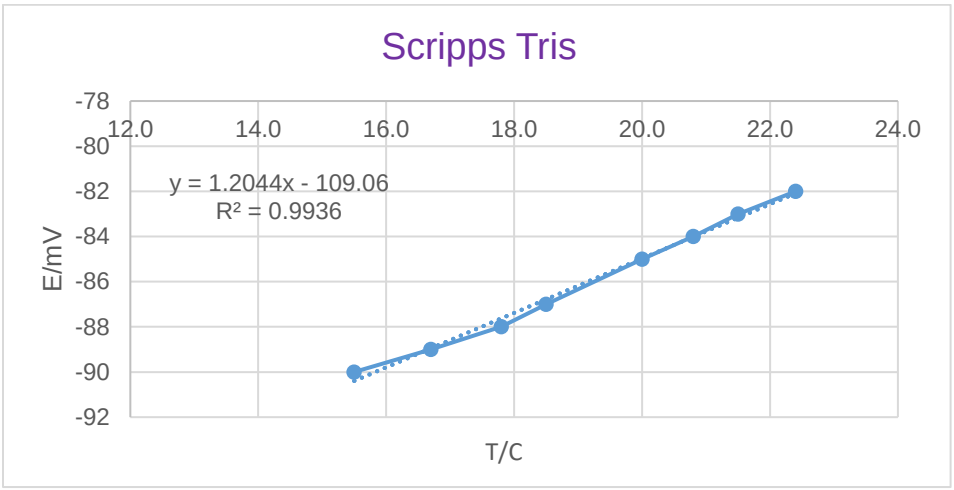


Fig.4. Home-made Tris buffer and Tris buffer from Scripps laboratory (T37)

Change in a glass pH probe potential difference measured in millivolts (mV) with a change in temperature (C°) was compared for both buffers, and a resulting pH_T for several samples was calculated.

Comparison of a home-made TRIS buffer (made from salts $MgCl_2x\ 6H_2O$, $CaCl_2\ x\ 2H_2O$) with TRIS buffer from Scripps laboratory (T37)



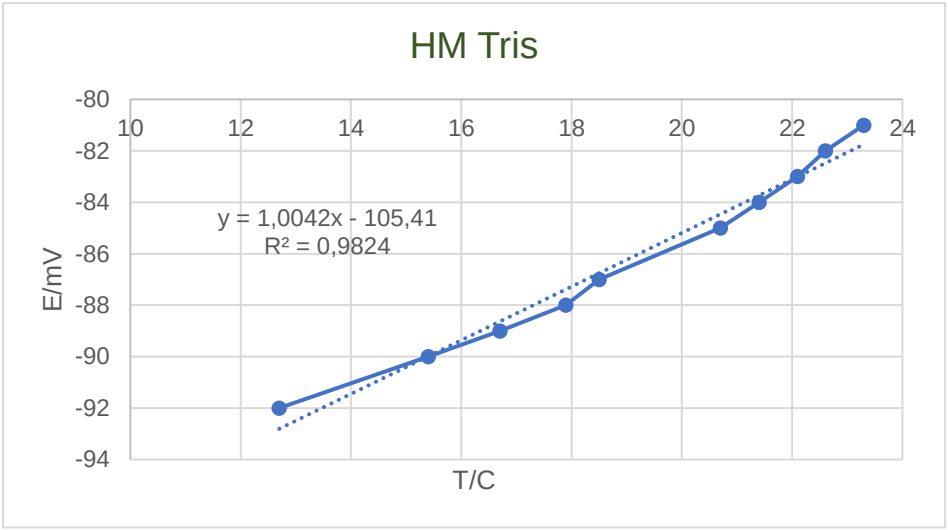


Fig.5. mV/T calibration of Scripps Tris buffer and home-made (HM) buffer made with 1M $MgCl_2$ and $CaCl_2$ solutions made from salts ($MgCl_2x\ 6H_2O$, $CaCl_2\ x\ 2H_2O$)

The calculated pH_T after calibration with homemade buffer was on average 0.004 units smaller than after calibration with Scripps buffer.

Comparison of a home-made TRIS buffer made from purchased 1M $MgCl_2$ solution and 1M $CaCl_2$ solution with TRIS buffer from Scripps laboratory (T37)

We did two comparisons of home-made TRIS buffer with purchased TRIS, over several days.

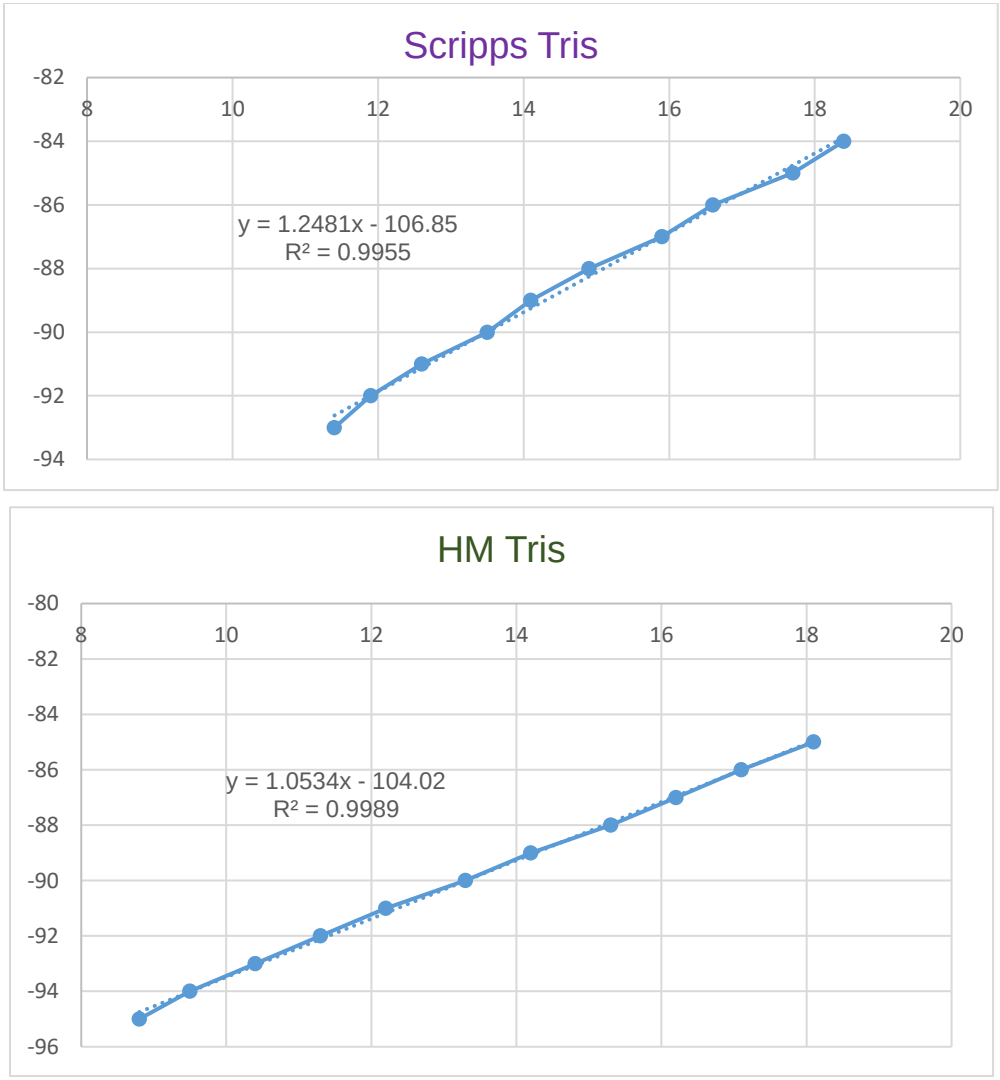
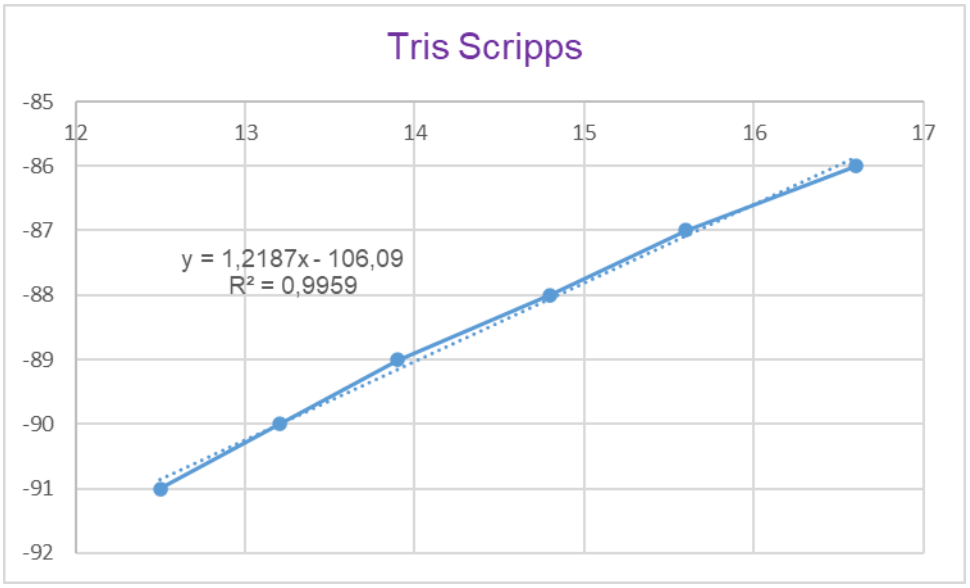


Fig.6. 1st mV/T calibration of Scripps Tris buffer and home-made (HM) buffer made with 1M $CaCl_2$ solution made from anhydrous salt

The calculated pH_T after the first calibration with homemade buffer was on average 0.02 units larger than after calibration with Scripps buffer.



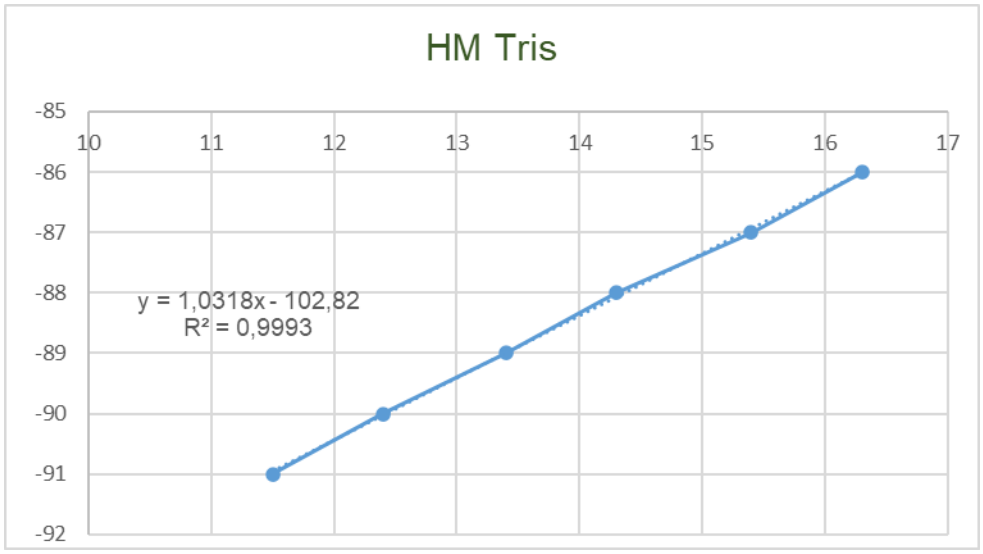


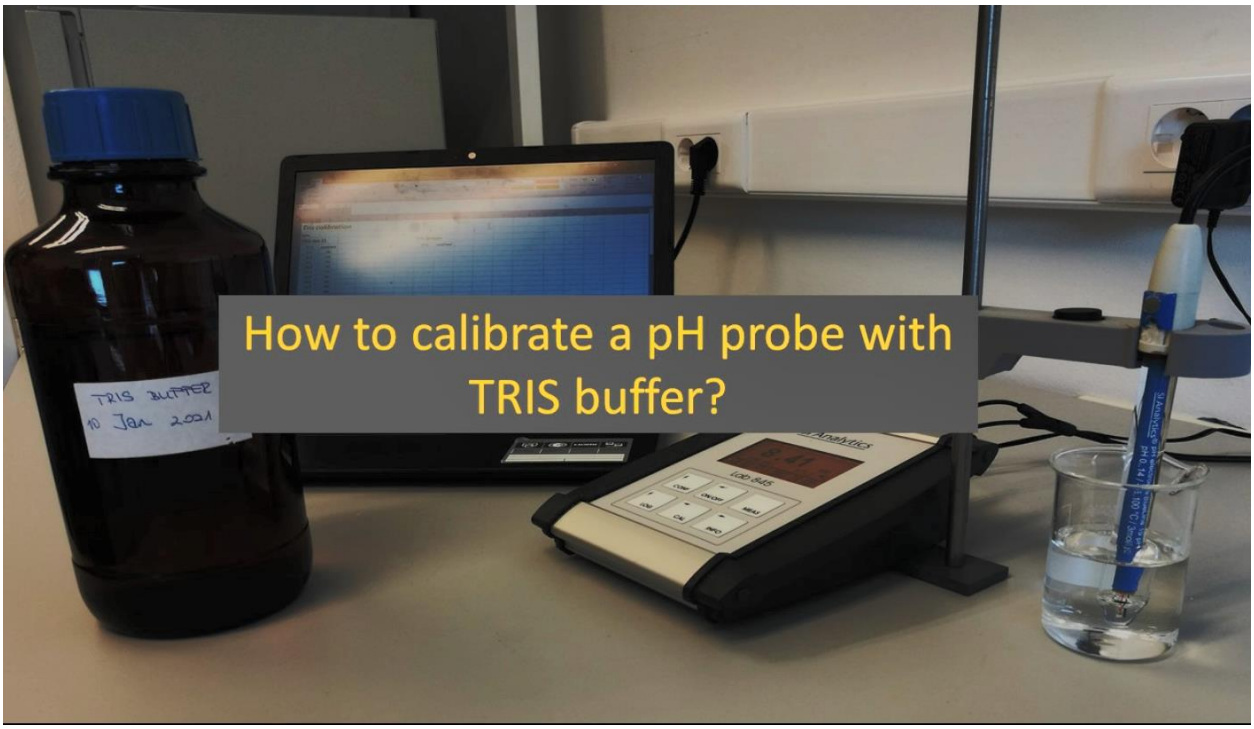
Fig.7. 2nd mV/T calibration of Scripps Tris buffer and home-made (HM) buffer made with 1M $CaCl_2$ solution made from anhydrous salt

The calculated pH_T after the second calibration with homemade buffer was on average 0.007 units larger than after calibration with Scripps buffer.

For the purposes of monitoring the carbonate chemistry during the biological experiment, these differences in accuracy are acceptable.

Calibration of pH probe with TRIS buffer

All the step are summarized in the video ***4. pHT_Calibration & sampling.mp4***.



To measure pH on the total scale (pH_T), you need a glass electrode allowing measurements in mV. A calibration curve of the TRIS buffer by recording the mV over a range of temperature and calculating the linear regression between the two parameters. Make sure that the temperature range for the calibration includes and extends beyond the temperature range of seawater in your experiment.

Procedure:

1. take TRIS buffer out of the fridge and pour it into a small beaker
2. put pH probe in the beaker, make sure that the tip of the probe is completely submerged
3. wait for the reading to stabilize
4. note the TRIS temperature together with mV reading
5. leave TRIS to slowly warm-up at the room temperature

6. remember to gently stir the sample (or you can put it on a magnetic stirrer)

7. mV reading is going to change with the rising temperature of a buffer

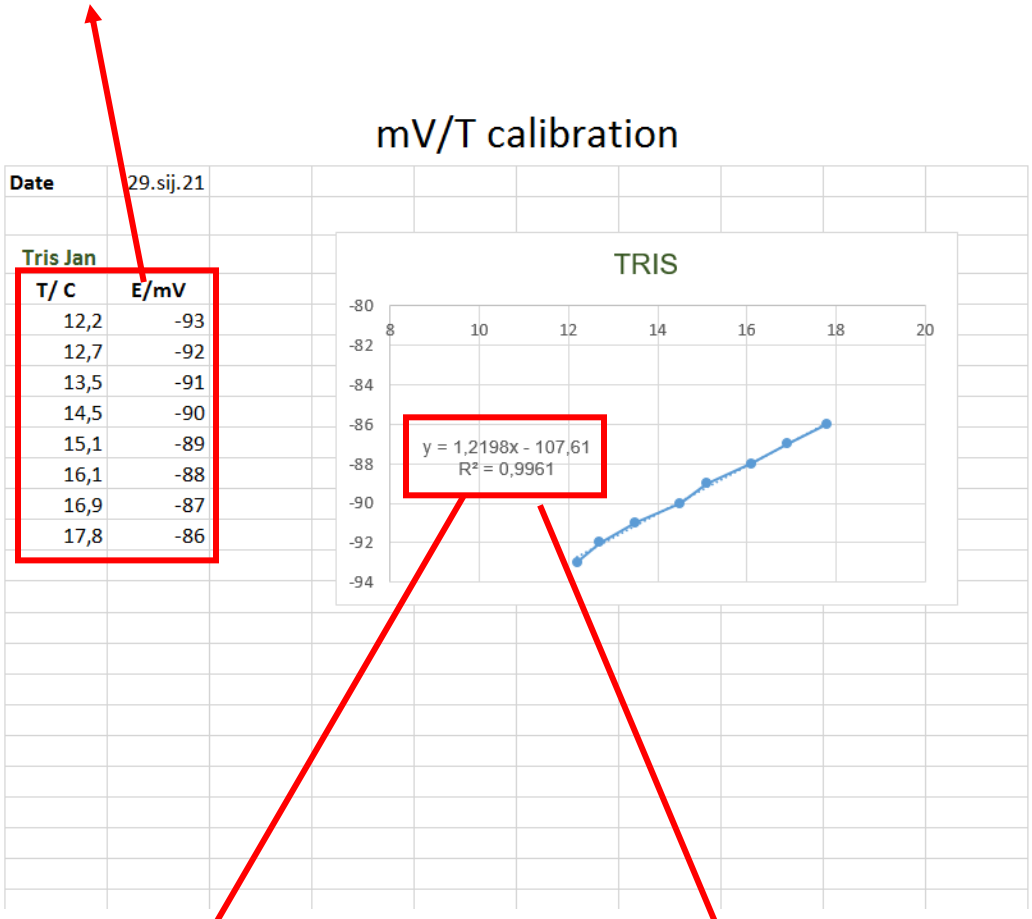
8. note the changes for 5-6 temperature points

9. input the data in the sheet ***Regression*** sheet of the Excel document **5. TRIS calibration and pHT calculation**

10. at this point make sure that $R^2 > 0.95$

Example calculation

Input temperature and mV data



Linear regression equation

R^2 has to be above 95% (0.95)

Sampling during experiment

After calibrating with TRIS buffer, the pH probe is ready for taking measurements in the experiment.

Procedure:

1. take a sample of seawater from your experiment with a beaker
2. rinse the probe with a little bit of sample
3. immerse the probe into the beaker
4. wait for the reading to stabilize
5. record: mV, temperature and salinity
6. move on to the next sample

It is important to take the measurements as soon as possible after sampling as the pH of the seawater can quickly change over time. The frequency of sampling depends on the experimental design, question and level of variability.

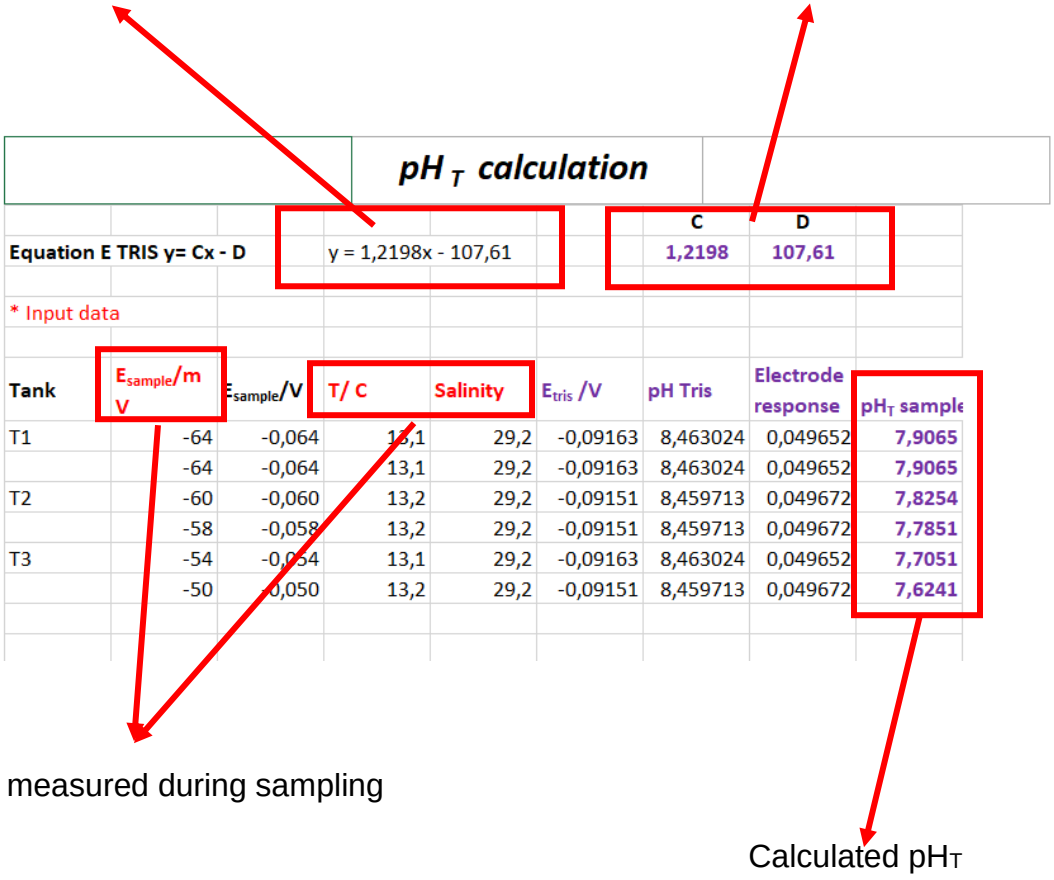
Calculation of pH_T

Use the sheet ***Calculation*** in the Excel document ***4. pHT_Calibration & sampling.mp4***. Copy the equation of the linear regression from the sheet ***Regression*** and insert coefficients under cells C and D. Input the sample values of salinity, temperature and mV reading from your samples and the pH_T is automatically calculated in the last column.

Example calculation:

Linear regression equation

Insert coefficients



				pH_T calculation					
							C	D	
Equation E TRIS y= Cx - D				y = 1,2198x - 107,61			1,2198	107,61	
* Input data									
Tank	E_{sample}/mV	E_{sample}/V	T/ C	Salinity	E_{tris} /V	pH Tris	Electrode response	pH_T sample	
T1	-64	-0,064	13,1	29,2	-0,09163	8,463024	0,049652	7,9065	
	-64	-0,064	13,1	29,2	-0,09163	8,463024	0,049652	7,9065	
T2	-60	-0,060	13,2	29,2	-0,09151	8,459713	0,049672	7,8254	
	-58	-0,058	13,2	29,2	-0,09151	8,459713	0,049672	7,7851	
T3	-54	-0,054	13,1	29,2	-0,09163	8,463024	0,049652	7,7051	
	-50	-0,050	13,2	29,2	-0,09151	8,459713	0,049672	7,6241	

Values measured during sampling

Calculated pH_T

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