

Flaming as part of aseptic technique increases CO₂ (g) and decreases pH in freshwater culture media

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Abstract

Aseptic technique has historically served as a fundamental practice in microbiology, helping to maintain culture purity and integrity. This technique has been widely encouraged and employed for use with cultures of heterotrophic bacteria as well as freshwater and marine algae. Yet, recent observations have suggested that these approaches may bring their own influences. We observed variations in growth among replicate experimental cyanobacterial cultures upon flaming of the culture tube opening during sample transfer and collection. Investigation revealed the pH of culture media had decreased from the initial pH established during media preparation. Flaming of sterile culture media alone confirmed a significant decrease, by as much as 1.7 pH units, and correlated with increased flaming events over time. We hypothesized that the causative factor was the introduction of carbon dioxide (CO₂) into the media. To test this hypothesis, qualitative and quantitative analyses were used to determine the primary driver of pH decline. We further assessed the direct effects of flaming and subsequent pH changes on *Microcystis aeruginosa* cultures, showing flame-driven pH changes and/or the introduction of CO₂ influenced experimental results. Our observations provide a cautionary tale of how classic and well-accepted approaches may have unintended consequences, suggesting new approaches may be necessary in research areas assessing pH or carbon-related effects on microbial communities.

Aseptic technique has been a foundational method of microbiological research for decades (Harrigan and McCance 1966). Defined as steps to prevent contamination during manipulation of microbial cultures or sterile culture media (Madigan et al. 2015), aseptic technique commonly includes flame sterilization of the opening of culture vessels (Bykowski and Stevenson 2008). Flame sterilization involves passing the opening of a culture vessel through a flame to prevent the introduction of microbial contaminants to a sample and is an approach used in a variety of laboratories, hospitals, and industrial facilities. Yet, upon review of the literature, we found a general lack of consensus within the scientific community as to the details of the method. While flaming immediately before and after subsampling or culture transfer is specified in a variety of publications (Andersen 2005; Sanders 2012; Madigan et al. 2015), the exact number of times required for the mouth of the vessel to pass through a flame is

generally not specified. Additionally, while some argue flaming must be performed by passing the tube through the inner blue section of the flame (Bykowski and Stevenson 2008), others instruct the tube to be passed above the flame (Coté 1998). Further variances in flaming technique arise in the recommendation of a wait time prior to recapping the culture postflaming (Coté 1998), while others recommend immediate recapping to minimize introduction of airborne contaminants (Harrigan and McCance 1966). In fact, the primary purpose of flaming in itself is often disputed within the literature, as some sources claim it is to combust any contaminants located on the mouth of the vessel (Coté 1998), while others indicate it is to create an upward convection current to prevent atmospheric contaminants from entering the tube (Sanders 2012; Madigan et al. 2015). These variances among techniques create a lapse in methodological consistency within the scientific community. Additionally, the discrepancies may be exacerbated by confounding factors that are unintentionally applied because of flaming. For example, the practice of flaming in aseptic technique is mandated in the handling of freshwater media and phytoplankton cultures (Andersen 2005). Yet, previous studies have demonstrated aseptic flaming may have detrimental implications on algal cultures, such as decreased growth rates and increased cell death in cyanobacterial *Prochlorococcus* spp.

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cultures linked to flame-generated peroxides (Morris and Zinser 2013).

In the present study, we assessed the effects of flaming as part of aseptic technique on the pH of freshwater culture media and subsequently cyanobacterial cell growth. Effects were assessed as follows: (1) aseptic flaming on different freshwater growth media from the scientific literature and (2) aseptic flaming on the growth of *Microcystis aeruginosa* cultures. Based on our observations, recommendations are made in the form of media modifications to effectively sterilize while eliminating the potential introduction of confounding variables.

Materials and procedures

Freshwater media selection and preparation

Four freshwater culture media commonly used for phytoplankton culturing (C, CB, CT, and CSi) were selected: recipes were taken from a publication of the *NIES-Microbial Culture Collection* (Watanabe et al. 2000). The components and concentrations for each medium are identical except for the buffer type and concentration, allowing for direct comparisons of the specific buffers in response to flaming (Table 1). Briefly, the major constituents of C medium include 1.5×10^{-4} g mL⁻¹ Ca(NO₃)₂·4H₂O, 1.0×10^{-4} g mL⁻¹ KNO₃, 5.0×10^{-5} g mL⁻¹ Na₂ β-glycerophosphate, 4.0×10^{-5} g mL⁻¹ MgSO₄·7H₂O, and trace metals (Steffen et al. 2015). To maintain comparability, silica was not included in CSi media used in this study. Beyond these media, BG-11 medium (Andersen 2005), one of the most commonly used for cyanobacterial culture work, was also tested. We also assessed the effects of flaming on three heterotrophic bacterial growth media: Lysogeny broth (LB) (Bertani 1951), Nutrient broth (Sambrook et al. 1989), and Minimal media (M9) (Miller 1972). All media were prepared in 1 L volumes, titrated to a pH of 8.2 with 1 mol L⁻¹ NaOH and autoclaved. Samples were cooled at room temperature (20.5°C) and gases allowed to equilibrate (for 1 d). The pH of each medium was confirmed via the subsampling of a 10 mL volume and analyzed using a temperature compensating laboratory pH meter (Mettler Toledo Seven Compact™ pH/Ion meter S220, fitted with a Mettler InLab Expert Pro-ISM electrode with a temperature range of up to 100°C). The pH meter was calibrated daily using three pH standards (pH 4.0, 7.0, and 10.0) to yield an efficiency of 97% or higher prior to all measurements.

Table 1. Buffer concentration (mmol L⁻¹), buffering pH range, and pKa for each of the five freshwater culture media used in this study (CT, C, CSi, CB, BG-11).

Media	[Buffer]	pH range	pKa at 20°C
CT	1.64 mmol L ⁻¹ TAPS	7.7–9.1	8.49
C	4.13 mmol L ⁻¹ Tris	7.0–9.0	8.20
CSi	2.10 mmol L ⁻¹ HEPES	6.8–8.2	7.55
CB	3.06 mmol L ⁻¹ Bicine	7.6–9.0	8.35
BG-11	1.75 mmol L ⁻¹ K ₂ HPO ₄ ·3H ₂ O	8.2–9.6	7.21

Standard vitamin additions for freshwater media in the forms of vitamin B₁₂, thiamine HCl, and biotin were omitted from all media.

Effects of flaming on media types

Twenty-five milliliters of each medium were aliquoted into 50 mL sterile, acid-washed glass culture tubes (sterilized, acid-washed tubes were used in all following experiments). For each growth medium, triplicate control and flame-treated replicates were generated. The flaming technique used in this experiment was performed in accordance with protocols expected during standard lab-based subsampling (for cell number, etc.) during a typical cyanobacteria growth study. Subsampling consisted of the removal of a small volume of culture daily for cell enumeration, though subsampling was not performed in all of the experiments of this study to decrease risk of heterotrophic contamination. Flame replicates were inverted three times (normally done to ensure cell resuspension) and held at a 45° angle while passed above the blue cone of the flame for four passes through the flame. After a brief pause, during which one would collect a small volume for determining cell number or inoculating cells into fresh media, the mouth of the culture tube was again passed through the flame four times (thus a total of eight passes through the flame corresponds to a single subsampling event). The cap was then immediately replaced, and the tube inverted three times before it was replaced in the rack (see aseptic flaming demonstration video, <https://youtu.be/Bvn6OvM8JpM>). In this study, the treatment of replicates using an open flame will be denoted as “flamed.” The controls in this study are subject to the same aseptic procedure as denoted above, with the exception of the presence of a flame: they have been assigned the terms “unflamed or air-flamed.” All samples were stored at 20.5°C and approximately 15–20 μmol photons m⁻² s⁻¹. This mock sampling of uninoculated growth media was performed daily for 10 d. Prior to the daily flaming, pH was measured for each replicate utilizing a pH probe, with sterilization of the probe performed prior to each sampling using 70% EtOH.

Effects of buffer age on media pH

We assessed whether buffer age had any effect on the buffering capacity. Twenty-five milliliters volumes of CT media containing aged TAPS buffer stock (TAPS stock solution aged approximately 3.5 months and stored at 4°C) and CT media containing fresh TAPS buffer (TAPS stock solution prepared 3 d prior and stored at 4°C) were aliquoted into 50-mL glass culture tubes in triplicate manner for the control treatments. The culture tubes were air-flamed in the same manner as mentioned prior. All replicates were stored on a lab bench at ~ 20.5°C. The pH of replicates was monitored daily for 10 d.

Influence of gas-exchange on media pH

The potential implications of gas exchange with the atmosphere were analyzed, with 25 mL volumes of CT media aliquoted

into 50-mL glass culture tubes in triplicate. The lids were closed firmly for one treatment group, while the lids were left loose for the second treatment. Replicates were stored on a lab bench at 20.5°C and 15–20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The pH of all replicates was monitored daily for 10 d. Efforts to explore potential mitigating-practices in reducing flaming effects on media pH were performed by abstaining from any type of inversion or simulated shaking of the media. Twenty-five milliliter volumes of CT media were aliquoted in triplicate into 50-mL glass culture tubes. Control and flamed treatments were applied daily for 10 d, without inversion of the tubes or any other form of disturbance. All replicates were stored on a lab bench at 20.5°C and approximately 15–20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Consequences of photooxidation on media pH

It has been demonstrated that light exposure causes photo-oxidation (and the inherent generation of reactive oxygen species, such as the weak acid HOOH) within a variety of media utilizing buffers such as HEPES, TAPS, Bicine, and TRIS (Morris and Zinser 2013). To determine the effects of photooxidation on media pH, 25 mL volumes of CT media were aliquoted into 50-mL glass culture tubes in triplicate for air-flame treatments. All replicates were stored on a lab bench at 20.5°C and approximately 15–20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The “non-photooxidation” treatment replicates were wrapped in foil (kept in dark conditions) to prevent superoxide generation. The pH of all replicates was monitored daily for 10 d.

Effects of increased buffer concentration on media pH

Attempts to minimize the effects of flaming upon media pH were made by increasing the TAPS buffer concentration in CT media by 10-fold and 100-fold (the later serving as the positive control) and monitoring the effects of flaming and air-flaming upon the triplicated experimental groups. All replicates were stored on a lab bench at 20.5°C and 15–20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The pH of all replicates was monitored daily for 10 d.

Testing drivers of pH decline as a result of flaming

In a preliminary investigation into the potential of flame-generated CO₂ as the primary driver of pH decline, limewater (Ca(OH)₂) was prepared following standard protocol (Shakhashiri 1983). Aliquots (25 mL) were dispensed in triplicate to test the effects of flaming across the following treatments: flamed 8 times, 16 times, 24 times, and 32 times. Note 8 passes through the flame corresponded to one subsampling event, or day 1 in a growth study, with 16 passes corresponding to 2 subsamplings, or day 2 in a growth study, and so forth. Negative controls included three tubes which were air-flamed, and positive controls were exposed to 10 s of CO₂ bubbling (administered via exhalation into straw submerged in limewater). Qualitative assessment of turbidity due to the formation of CaCO₃ as an indirect proxy for CO₂ introduction was made using a spectrophotometer (Thermo Spectronic Genesys 20) at 600 nm.

Quantification of the net CO₂ (total carbon [TC]) incorporated into media due to flaming was measured by the Water Quality Core Facility at the University of Tennessee Knoxville utilizing a Shimadzu Carbon/Nitrogen analyzer (TOC-L Shimadzu, detection limit of 4 $\mu\text{g L}^{-1}$ according to the manufacturer’s specifications). Volumes of CT media (25 mL) were aliquoted into 50 mL acid-washed, sterilized tubes in triplicate per treatment. Control (unflamed), flamed 8-times, flamed 16-times, flamed 24-times, and flamed 32-times treatments were administered, then immediately analyzed for TC with technical replicates of three performed per sample.

Effects of flaming on freshwater cyanobacterial cultures

To determine the consequences that flaming may have on freshwater cyanobacterial cultures, axenic *M. aeruginosa* NIES 843 was inoculated in triplicate into CT media at a pH of 8.2 and flamed daily for 10 d to mimic subsampling during growth assays. Note one mock-sampling event corresponds to eight passes through the flame according to standard aseptic protocol performed in the video demonstration. Cultures were incubated at 26°C and approximately 55–60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ in incubators on a diel cycle (VWR low temperature diurnal illumination incubator). Chlorophyll *a* auto fluorescence (fluorescence signal units [FSU]) was quantified daily as a proxy for relative biomass and cell health utilizing a fluorometer (Turner Designs TD-700), equipped with a blue mercury bulb, a #10-050R excitation filter (340–500 nm) and a #10-115 (680 nm) emission filter. The instrument was standardized before each use with Turner’s solid standard (#7000-994). Flaming/air-flaming was performed post-FSU measurements. Final pH values were measured on day 10 after the completion of the experiment.

Effects of increased buffer concentration on freshwater cyanobacterial cultures

We assessed the effects of a 10-fold increase in the concentration of TAPS buffer in CT media on *M. aeruginosa* NIES 843 growth. Cultures were inoculated into standard TAPS-buffered CT medium as well as a 10X TAPS-buffered CT medium for comparison. Post-inoculation, the cultures were incubated at 26°C and approximately 55–60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ as mentioned previously for 10 d. FSU (Turner Designs TD-700) and cell number (BD FACSCalibur flow cytometer) were assessed every 2 d for additional comparison of the techniques themselves.

Statistical analyses

Multiple *t*-tests corrected for multiple comparisons (Holm-Sidak method) were used to statistically assess the following: flame assays of freshwater media (Fig. 1), *M. aeruginosa* growth rates (Fig. 4b), flame assays of heterotrophic media (Supporting Information Fig. S1), external influences on media pH (Supporting Information Fig. S2), buffer concentration effects on media pH (Supporting Information Fig. S4), effects of shaking on media pH (Supporting Information Fig. S5), and

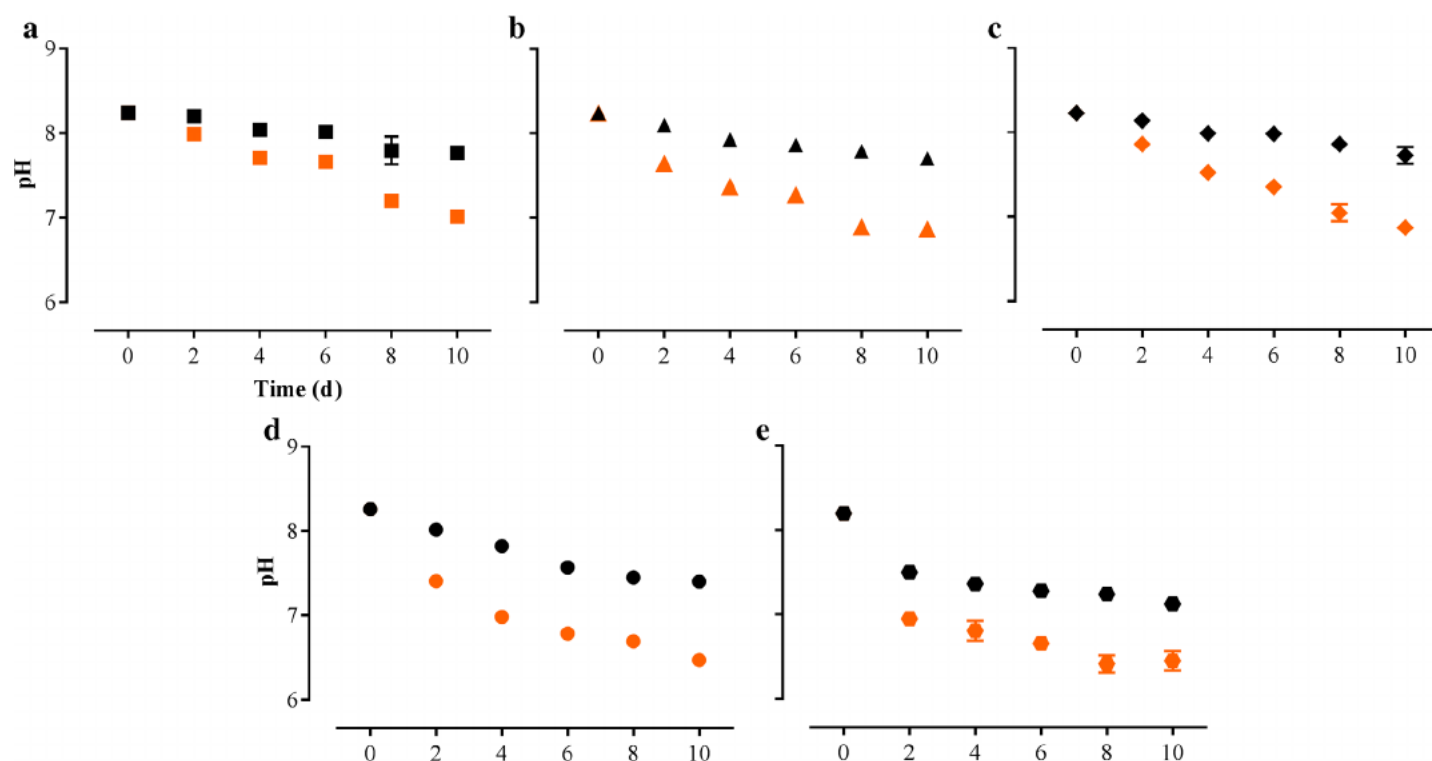


Fig. 1. Flame-induced declines in pH in control (black) and flamed replicates (orange) of freshwater media. Data present mean \pm SEM. Where not shown, error bars are within the symbol. (a) C media/Tris buffer (indicated by squares). (b) CSI/HEPES media (triangles). (c) CB/Bicine media (diamonds). (d) CT media/TAPS buffer (circles). (e) BG-11/inorganic phosphate buffer (hexagons).

the comparison of *M. aeruginosa* biomass accumulation in standard CT vs. 10X TAPS-buffered CT medium (Supporting Information Fig. S7). One-way ANOVAs corrected for multiple comparisons (Tukey method) were used to statistically assess the following: net pH decline by buffer type (Fig. 2), TC (mmol L^{-1}) incorporation as a result of flaming (Fig. 3), and limewater turbidity as an indicator for CO_2 incorporation (Supporting Information Fig. S3).

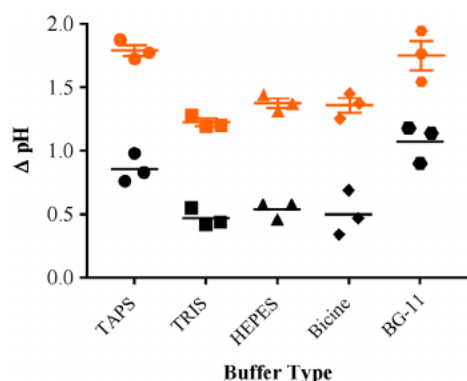


Fig. 2. Net change in pH ($T_0 - T_{10}$ pH) observed in each media after 10 d of aseptic flaming. Net change in pH observed in the control replicates of each media indicated in black. Net change in pH of the flamed replicates for each media indicated in orange.

Assessment

Effects of flaming on pH of freshwater media

After flaming the mouths of the culture tubes, a decline in pH was observed in all the freshwater media analyzed in this study compared to the controls (Fig. 1). After 10 d, CT ($p = 0.01$) and BG-11 ($p = 0.02$) experienced the greatest decline in pH after flaming, dropping from 8.2 to average final pH values of 6.47 and 6.45, respectively (Fig. 1d,e). TRIS ($p = 0.03$), HEPES ($p = 0.01$), and Bicine buffered-C media ($p = 0.01$) had average final pH values of 7.02, 6.86, and 6.87, respectively (Fig. 1a-c). Differences between the controls and flamed replicates manifested after only 2 d of daily flame sterilization (or 16 passes through the flame resulting from two standard aseptic flaming events). A comparison of net decline in pH of each media/buffer indicated that there were no differences between the pH declines in CT and BG-11 ($p = 0.99$) when flamed, and neither were different from the control ($p = 0.27$) (Fig. 2). This data confirm that CT (TAPS buffer) and BG-11 (inorganic phosphate buffer) demonstrated the greatest decline in pH when flamed (1.5–1.7 pH units). TRIS, HEPES, and Bicine-buffered media that were flamed exhibited similar trends as in the unflamed control (Fig. 2). The data demonstrated a consistent trend among the five freshwater media/buffers tested, in which flaming resulted in a decline in pH after just 2 d (16 passes through the flame) and continuing up to 10 d (80 passes through the flame).

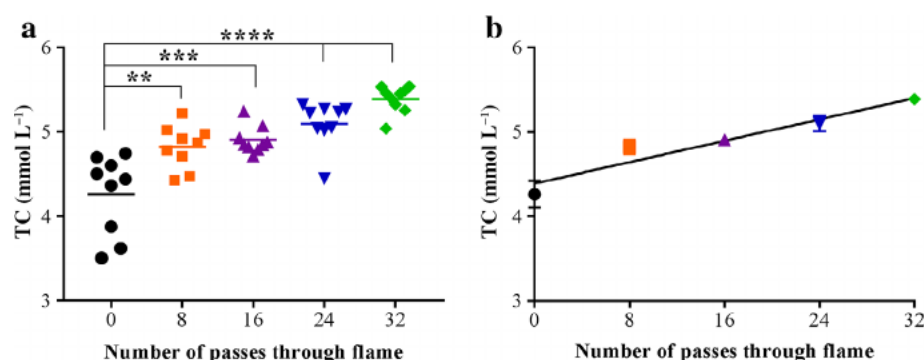


Fig. 3. TC (mmol L⁻¹) in control and flamed CT media replicates. **(a)** Net TC (mmol L⁻¹) incorporated into CT media control and flame treatments. Eight passes through the flame indicated by orange squares, 16 passes through the flame indicated by purple triangles, 24 passes through the flame indicated by blue inverted triangles, and 32 passes through the flame indicated by green diamonds. **(b)** Linear trend of mean TC (mmol L⁻¹) introduced per flame treatment ($y = 0.06323 [\text{number of passes through flame}] + 4.391$, $R^2 = 0.93$).

This trend was observed (albeit to a lesser extent) when the experiment was replicated by flaming three growth media used for heterotrophic bacteria culture work: LB, Nutrient broth, and M9 (Supporting Information Fig. S1). In the case of the unbuffered media (LB and Nutrient broth), the declining pH trend was observed on a smaller scale, with flamed LB decreasing in pH from 8.2 to an average of 7.5 after 10 d of flaming and Nutrient broth to an average pH of 7.42 (Supporting Information Fig. S1a,b). M9 media demonstrated a similar decline in pH upon flaming (Supporting Information Fig. S1c). However, all time points of the M9 flamed replicates (T2–T10) were significantly different from the controls ($p < 0.01$) whereas this was not the case for LB and Nutrient broth. The data demonstrated that flame sterilization resulted in pH declines of lower magnitude in unbuffered media compared to buffered media, with freshwater media experiencing the highest decline in pH due to flaming.

Influence of external conditions on the pH of freshwater media

A pH decline was observed in the control replicates in all five freshwater media, though to a much lesser extent than the flamed replicates (Fig. 2). Factors associated with media handling and storage were analyzed to identify potential confounding variables or contributing factors to this pH decline observed in controls. Buffer age appeared to have no immediate implications corresponding to pH (Supporting Information Fig. S2a). The effects of light exposure (i.e., photooxidation) on pH revealed no differences ($p = 0.99$) between CT subjected to photooxidation and those exposed to photooxidation inhibition (Supporting Information Fig. S2c). Influence of gas exchange (i.e., tight vs. loose caps) revealed no effect on pH ($p = 0.90$) throughout the 10 d sampling (Supporting Information Fig. S2b). Efforts to minimize pH decline in CT media were made by forming a CT-TAPS buffer concentration gradient. Daily flaming events over the course of 10 d revealed the largest pH declines in the control (protocol-standard

concentration of TAPS), with 10-fold and 100-fold increases in TAPS resulting in more stable pH (Supporting Information Fig. S4a,b). While a 10-fold increase in TAPS buffer did not result in statistical differences between the unflamed and flamed treatments ($p = 0.07$) ($p = 0.35$), the decline in pH was observed to a lesser extent compared to the standard buffer media. Additional means of minimizing flame-induced pH decline was accomplished by abstaining from tube inversion/disturbance throughout 10 d and revealed a substantially lower net decline in pH in CT media (Supporting Information Fig. S5).

Analysis of carbon dioxide as driver of flame-induced pH declines

Preliminary qualitative means for determining the absence/presence of CO₂ in flamed media were conducted utilizing a limewater turbidity assay (Shakhashiri 1983). After flaming, evidence of CO₂ incorporation was observed via the visible formation of a Ca(CO₃) precipitate in the corresponding manner: negative controls or “air-flamed” replicates depicted no change in turbidity formation of precipitates, while 8, 16, and 24 passes through the flame each resulted in increased turbidity and precipitate formation, serving as a visual proxy for CO₂ introduction to the media (Supporting Information Fig. S3a). Spectrophotometric analyses further support the limewater analyses (Supporting Information Fig. S3b). Although variable, as the number of passes through the flame increased, a parallel rise in turbidity and precipitate formation of Ca(OH)₂ occurred. Increased variability of Ca(OH)₂ formation among replicates was observed as the number of passes through the flame increased. Quantification of TC input revealed an average of 170 ± 6.4 mmol L⁻¹ C (or 4.26 mmol C per 25 mL) present in the unflamed controls, 193 ± 3.60 mmol L⁻¹ C present in the 8 passes through the flame replicates, 196 ± 2.0 mmol L⁻¹ C present in the 16 passes through the flame replicates, 204 ± 3.6 mmol L⁻¹ C present in the 24 passes through the flame replicates, and 216 ± 2.0 mmol L⁻¹ C present in the 32 passes through the flame replicates (Fig. 3a). It was determined that all flaming treatments resulted in an increase of C

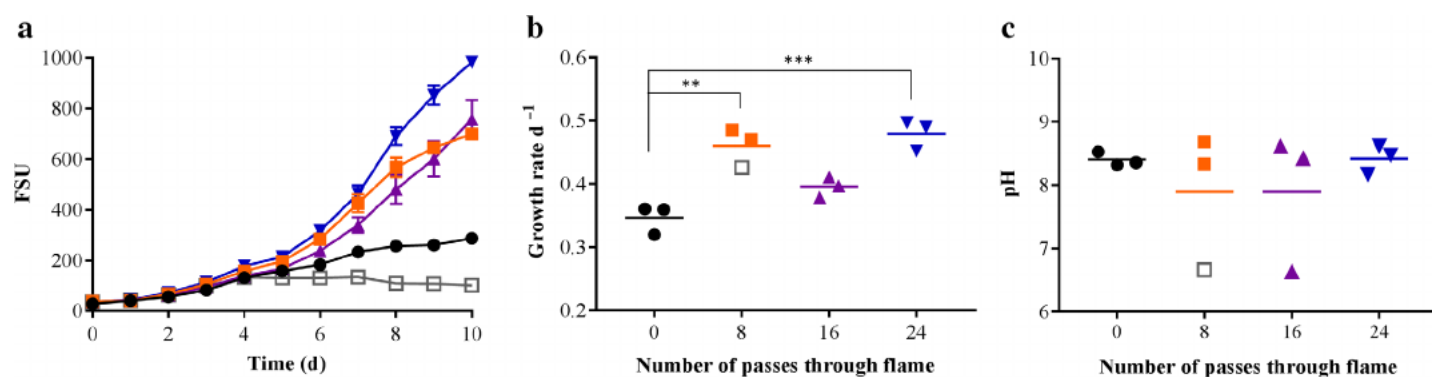


Fig. 4. Aseptic flaming effects on CT media + axenic *M. aeruginosa*. **(a)** *M. aeruginosa* growth treated with flaming: control replicates (0 daily passes through the flame) indicated by solid black dots, 8 daily passes through the flame indicated by solid orange squares, 16 daily passes through the flame indicated by solid purple triangles, 24 daily passes through the flame indicated by solid blue inverted triangles, and dead replicates indicated in gray open symbols. **(b)** Growth rate of each replicate + mean growth rate. **(c)** pH of replicates at the completion of the experiment (after 10 daily treatments): control (0 daily passes through the flame), 8 daily passes through the flame, 16 daily passes through the flame, or 24 daily passes through the flame, respectively.

to the media compared to the control ($p = 0.002$, $p = 0.0002$, $p = 0.0001$, $p = 0.0001$, with p values listed corresponding to tubes passed through the flame 8–32 times in consecutive order), and increased flaming highly correlated with the increase in TC (mmol L^{-1}) ($R^2 = 0.93$, slope = 0.06, Fig. 3b).

Implications of flaming for freshwater cyanobacteria cultures

The *M. aeruginosa* in the CT control consistently had the lowest FSU. Replicates flamed 24 times had the highest FSU (with FSU interpreted to be indirectly indicative of cell number and biomass, while directly suggestive of cell health Fig. 4a). Estimates of the relationship between FSU and cell density (cells mL^{-1}) confirmed the presence of a strong and significant relationship between the two ($R^2 = 0.92$, $p = 0.0001$, Supporting Information Fig. S6). Replicates that were flamed exhibited higher instances of culture death in comparison to the controls (death of replicates that were flamed 8 times graphed separately, Fig. 4a). The pH values of the dead replicates were considerably lower than replicates that survived, all of which remained close to the initial pH of 8.2 (Fig. 4c). One replicate that was flamed 16 times had a correspondingly low pH, though there was no indication of death. The *M. aeruginosa* cultures that were flamed 8 times and 24 times had higher growth rates than the control ($p = 0.002$ and $p = 0.001$, respectively), with replicates flamed 16 times falling short of statistical difference ($p = 0.072$) (Fig. 4b).

Discussion

There is little doubt of the importance of aseptic technique in microbiology and microbial ecology. In the current study, our efforts to monitor microscale changes in pH as drivers of phytoplankton community composition in lakes revealed a Heisenberg-like moment (Wheeler et al. 1983) in biology—where the process of making the measurement shapes the

actual measure one is making. In short, our use of aseptic technique used during experiments examining pH effects was itself affecting pH of our culture media. These observations are critical in face of the massive number of ongoing climate change studies in aquatic systems where pH is a major variable.

The data we collected suggested flaming as a part of aseptic technique had effects on five freshwater culture media as well as effects on the growth of the cyanobacterium *M. aeruginosa*. While flaming is a sterilization method which has been endorsed for decades (Andersen 2005; Madigan et al. 2015), our results indicate that media pH was altered by as much as 1.7 units via the introduction of CO_2 , serving as a confounding variable. In contrast, the assessment of other variables, including buffer age, photooxidation effects, and gas exchange, were found to have no effect on pH. These observations effectively ruled out additional contributors to the pH decline and indicated that flaming was solely responsible for the pH change. Previous studies have identified pH as a parameter of importance when culturing algae, with broad growth rate optima falling within pH values of 7–9, though many organisms exhibit growth outside of these ranges (Lavens and Sorgeloos 1996; Berberoglu et al. 2008; Huang et al. 2017). Changes in pH can affect algal cultures, with many algal species having growth optima at narrow pH ranges (Lavens and Sorgeloos 1996; Tsaloglou 2016). The cyanobacterium used in this study, *M. aeruginosa*, has an optimal pH for growth between 7.5 and 10 (Fang et al. 2018) with an optimal photosynthetic potential at $\text{pH} > 8.0$ (Bano and Siddiqui 2004). Flaming of culture vessel openings, specifically during the process of aliquoting media for inoculation, had the capacity to drive down the pH (Fig. 1a–e). Additionally, flaming open culture tubes postinoculation could further decrease pH levels if CO_2 is not assimilated by the cyanobacteria. BG-11 and CT media exhibited the highest net declines in pH (Fig. 2), which raises concern, as these are frequently utilized freshwater growth media. Random culture death in certain

flamed cultures (Fig. 4) may be due to the variability of CO₂ incorporation from flaming, as some cultures may have experienced larger-scale CO₂ inputs that resulted in pH decline if the CO₂ is not consumed by the culture. Previous studies have also found that up to 2 $\mu\text{mol L}^{-1}$ of HOOH may be generated as a result of flaming (Morris and Zinser 2013). While it is recognized that mmol L⁻¹ concentrations of HOOH are needed to cause cell death (Palenik et al. 1991; Alam et al. 2001), the 2 $\mu\text{mol L}^{-1}$ of HOOH generated from flaming results in physiological changes during the upregulation of intracellular peroxidases (Price and Harrison 1988). There may also be a role here for incomplete combustion products (although we did not investigate this).

Dissolved inorganic carbon (DIC) plays a significant role in the growth and development of phytoplankton species. CO₂ and HCO₃⁻ are the major chemical forms of carbon assimilated during photosynthesis (Schindler et al. 1971; Wetzel 2001). Previous studies have indicated that free CO₂ is labile and readily accessible to most algae and aquatic photoautotrophs (Wetzel 2001). *M. aeruginosa* isolates have been shown to have a high affinity for DIC which is reflective in its enzymatic low half-saturation constant, allowing it to outcompete other algal species due to its efficient carbon acquisition (Yamamoto and Nakahara 2005).

It appeared that cultures which received CO₂ via flaming benefited substantially and exhibited higher growth rates in our study. This suggested that C availability may have been growth limiting in CT medium when cultures were not bubbled or shaken (Fig. 4). CO₂ is extremely soluble in aqueous solutions, demonstrating 200 times higher solubility relative to oxygen (Wetzel 2001). H₂CO₃ is a weak acid which readily and rapidly dissociates, losing its two protons in a two-step process (pK_{a1} = 6.43, pK_{a2} = 10.43 at 15°C) (Schindler 1971; Wetzel 2001). Hence, CO₂ that is not readily taken-up by *M. aeruginosa* in its initial gaseous form readily dissolves into the media and hydrates into H₂CO₃, as demonstrated in the flaming of freshwater media alone (Fig. 1). In addition, *M. aeruginosa*'s direct uptake of flame-generated gaseous CO₂ is further supported in the consistent pH values among control and flamed replicates, as healthy cultures did not experience any significant declines in pH, save for one replicate in the flamed 16-times treatment (Fig. 4c). Yet, when culture death did occur, the final pH was in the same range as CT media subjected to 10 d of flaming (Fig. 1d), indicating CO₂ had dissolved into the media. Should the concentration of dissolved CO₂ become too high, H₂CO₃ formation would yield low culture pH, which would inhibit growth and development in a variety of algal species, including *M. aeruginosa* (Weisse and Stadler 2006). Thus, while CO₂ influxes via flaming increased growth rate and biomass in carbon-limited cultures, if the CO₂ dissolution rate becomes too high, significant drops in pH will invoke detrimental effects on the culture. In total, it appears likely that the availability of CO₂ coupled to pH

decline shaped the inconsistency observed among replicates in previous experiments.

Comments and recommendations

We have demonstrated that flame sterilization of culture medium vessel openings can result in a significant decline in the pH of freshwater media due to the dissolution and hydration of CO₂. This decline in pH has several implications, as optimal pH ranges for algal photosynthesis and growth are often narrow and well defined (Huang et al. 2018). Moreover, under current climate scenarios, research into the effects of small pH shifts is common yet could be compromised by this observation. This pH shift would also affect the bioavailability and accessibility of macro- and micronutrients, including crucial trace metals within the media, many of which are associated with photosynthetic processes (Wetzel 2001). Low pH levels that result from CO₂ introduction, coupled with HOOH generation via flaming and excessive trace metal bioavailability, may result in randomized spontaneous cell and culture death, as seen in our previous flamed growth assays (Fig. 4). Flaming further effects cultures by serving as a variable yet direct source of CO₂, which is labile and thus readily accessible for uptake by *M. aeruginosa*. This CO₂ is likely consumed by *M. aeruginosa* cells, influencing cell growth rate and biomass accumulation (Fig. 4). This mechanism is further supported by the relatively constant pH levels observed in all *M. aeruginosa* cultures (flamed and nonflamed) as after 10 d the pH remains constant (excluding the “dead” replicates who demonstrated lower pH). These CO₂ inputs may result in a higher degree of replicate variability in unshaken *M. aeruginosa* cultures in contrast to shaken cultures, as unshaken cultures in potentially C-limiting media such as CT are likely to be C-limited.

There is a need for updated aseptic alternatives to flame-induced sterilization. It is advisable to abstain from flaming when feasible, and alter flaming practices when flaming is necessary. Refraining from inverting, shaking, or otherwise disturbing the media postflaming may decrease the dissolution of CO₂ into the media and mitigate pH decline, though this is not always feasible. When CT medium was subject to a 10 d flaming study without any inversion/shaking, it was found that the pH decline was drastically diminished (Supporting Information Fig. S5) and not significantly different ($p = 0.30$) from the unflamed controls. Additionally, exercising caution in media selections is advised, as the commonly utilized medium (BG-11) was shown to have the highest decline in pH overall. Ten-fold increases in TAPS buffer concentration within CT media were shown to mitigate pH shifts within unflamed replicates (Supporting Information Fig. S4) with no observed biological consequences (Supporting Information Fig. S6). However, the approach of simply increasing buffer concentrations must be pursued with caution: for example, TRIS is toxic to cells in high concentrations and HEPES

generates higher amounts of reactive oxygen species when present in higher concentrations (Morris and Zinser 2013). Our results indicate that a 10-fold TAPS increase in CT may serve as a suitable medium in future studies concerning pH (climate change research) but each specific case should be individually examined.

This study serves as a cautionary tale of the unintended effects of flaming of culture vessel openings upon the media and microorganisms. While flaming has served as an aseptic technique for decades, it may be time to put aside the Bunsen burner and pursue further alternatives to this classic practice.

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Conflict of Interest

None declared.

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