

## NOTE

# GAS COMPOSITION OF DEVELOPING PNEUMATOCYSTS IN BULL KELP *NEREOCYSTIS LUETKEANA* (PHAEOPHYCEAE)<sup>1</sup>

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The subtidal kelp *Nereocystis luetkeana* (hereafter *Nereocystis*) maintains an upright stature by producing a single gas-filled float (pneumatocyst) that provides buoyancy. The ability of *Nereocystis* pneumatocysts to inflate with gas underwater is peculiar, and the gas composition of pneumatocysts has been the topic of several studies over the last 100 years. Past studies of pneumatocyst gases only examined large sporophytes, leaving open questions about the origins of these gases and how gas composition may change during development. In this study, we use developmental time as a means to understand the origin and physiological mechanisms that give rise to different gases within *Nereocystis* pneumatocysts. Total gas composition was measured across a range of pneumatocyst sizes (5–725 mL). Contrary to previous studies that documented pneumatocyst gas concentrations to be similar to air, this study found internal gas levels of CO, CO<sub>2</sub>, and O<sub>2</sub> to be 1.2 ± 0.8%, 0.6 ± 0.2%, and 59.9 ± 13.6%, respectively. Our data suggest that the composition of gases does not change as pneumatocysts grow and that the rate of each gas added is approximately proportional to changes in pneumatocyst volume. Therefore, cells constituting the pneumatocyst wall are likely producing more gas (per surface area) to fill pneumatocysts as they expand, maintaining proportional gas composition and corresponding internal pressure.

**Key index words:** buoyancy; carbon dioxide; carbon monoxide; development; gas bladder; macroalgae; oxygen; photosynthesis; physiology; respiration

**Abbreviations:** C<sub>1</sub>, measured gas concentration; C<sub>2</sub>, unknown pneumatocyst gas concentration; C<sub>gas</sub>, gas concentration; g<sub>gas</sub>, grams of gas; kPa, kilopascals; M<sub>gas</sub>, molar mass of gas; P<sub>gas</sub>, gas partial pressure; R, gas constant; V<sub>1</sub>, volume of the flask; V<sub>2</sub>, volume of gas sample; V<sub>p</sub>, pneumatocyst volume; η<sub>gas</sub>, mols of gas

The bull kelp *Nereocystis luetkeana* (hereafter *Nereocystis*) builds unique subtidal forests in the Northeast Pacific by having a singular gas-filled float called a pneumatocyst, which enables the kelp's flexible thallus to remain upright and vertical in the water column (Arzee et al. 1985). The ability of this seaweed to create an air-tight reservoir and inflate underwater is remarkable, and the gas composition of *Nereocystis* pneumatocysts has been the topic of several studies during the first half of the 20<sup>th</sup> century (Frye et al. 1915, Langdon 1917, Langdon and Gailey 1920, Rigg and Henry 1935, Rigg and Swain 1941). Past studies found that 20–25% of gas sampled from pneumatocysts was oxygen (O<sub>2</sub>) and ~70% or more was nitrogen (N<sub>2</sub>), with both gases present in concentrations similar to air (Rigg and Henry 1935, Rigg and Swain 1941). In addition, up to 13% of the gas was CO, thought to be a product of cell degradation (autolysis) from the rapidly expanding pneumatocyst during early development (Foreman 1976), and 2–7% of the gas was carbon dioxide (CO<sub>2</sub>), shown to be a product of cellular respiration (Langdon and Gailey 1920, Rigg and Swain 1941).

Gas-filled structures are commonly found in Laminariales and Fucales, where the structure provides mechanical support to flexible thalli, allowing blades to efficiently access sunlight near the waters' surface (Dromgoole 1981a, Stevens et al. 2002, Stewart 2006, Burnett and Koehl 2017). Most macroalgal pneumatocysts develop from meristematic blade tissue, forming an organ that generally subtends the blades (Dromgoole 1981a). However, some genera such as *Durvillaea*, *Turbinaria*, and *Eualaria* modify their blades, by having tissue that separates to form hollow spaces within the blade itself (Smith 2002, Stevens et al. 2002, Stewart 2004, Stewart et al. 2007). Though pneumatocyst morphology and position varies greatly between species and groups, the same buoyant function is achieved. *Nereocystis* thalli, like most other kelps, initially develop a simple holdfast, stipe, and blade. Pneumatocysts begin to form at the transition zone between the stipe and the blade when sporophytes are no less than 15 cm, in length, about 2 weeks after germination (Nicholson 1970, Dromgoole 1981a, Liggen and Martone 2018). Medullary cells along the transition zone

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begin tearing, releasing gas into the newly opened space (Dromgoole 1981a).

Pneumatocyst walls are thick and composed of tissues that are specialized in resisting compression, preventing pneumatocysts from buckling under hydrostatic pressure (Liggan and Martone 2018). In addition, pneumatocysts have internal pressures that are always less than atmospheric pressure, which maintains a positive pressure gradient and keeps the walls under compression as they rise toward the surface (Liggan and Martone 2018). As pneumatocysts continue to grow and enlarge more than two orders of magnitude in volume, internal pressure stays remarkably constant (Liggan and Martone 2018). Because pneumatocyst development involves the active growth of some cells and the degradation of others (Dromgoole 1981a), it is unknown which gases fill this newly opened space and which cells contribute to gas composition. In addition, it is unclear whether changes in pneumatocyst size and corresponding physiological processes influence internal gas composition and concentration. Because past studies of *Nereocystis* pneumatocyst gas composition have limited their examination to large, mature sporophytes, this study aims to investigate ontogenetic changes in gas composition by examining pneumatocysts of varying size.

There are three main processes that likely contribute to gas composition as pneumatocysts increase in size. One is cellular physiology, (i.e., photosynthesis and respiration) where every living cell that releases gases into or absorbs gases from the pneumatocyst contributes to gas concentration (Rigg and Swain 1941). If gas composition is entirely dependent on the by-products of photosynthesis and respiration, the combined concentration (% or ppm) of biologically produced gases such as O<sub>2</sub> and CO<sub>2</sub> would be proportional to tissue surface area and volume of the pneumatocyst (SA:V). The second process that may influence pneumatocyst gases is cellular degradation, tearing of medulla tissue and the subsequent release of CO, when the stipe opens during pneumatocyst development (Foreman 1976). Foreman (1976) developed hypotheses regarding the biological processes that influence CO production, suggesting that CO is a by-product of autolytic cell metabolism, which is active during pneumatocyst formation, and inherently linked to respiration by a requirement of energy in the form of ATP (Nicholson 1970, Duncan 1973, Foreman 1976). If true, then we would expect CO concentrations to decrease post-development as tearing subsides and pneumatocysts expand from cellular division (Nicholson 1970, Duncan 1973) and we would expect mature pneumatocysts to have lower CO concentrations than young, developing pneumatocysts.

This study used developmental time as a means to understand the origin and physiological

mechanisms that give rise to different gases within pneumatocysts and determined if gas composition changed throughout sporophyte development. We hypothesized that O<sub>2</sub> and CO<sub>2</sub> concentrations were influenced by pneumatocyst SA:V such that gas concentrations (% or ppm) and the amount of gas produced (mmol) would decrease (relative to increases in volume) as pneumatocysts grow. We assumed that CO is purely a product of early pneumatocyst development, and hypothesized that concentrations of CO would initially increase as medulla tissue tears (roughly proportional to pneumatocyst length), but ultimately decrease when cellular division primarily expands the growing pneumatocyst.

*Nereocystis* thalli ( $n = 29$ ) of varying pneumatocyst volume (5–725 mL) were haphazardly collected between April and August 2015 at four sites off the coast of British Columbia, Canada: Scott's Bay (48°50'04.9" N 125°08'50.0" W), Aguilar Point (48°50'22.0" N 125°08'27.2" W), Stanley Park (49°18'03.5" N 123°07'00.0" W), and Helby Island (48°51'19.6" N 125°10'07.4" W). Kelp were collected no deeper than 2 m below chart datum and all samples were detached from the substratum at the holdfast so that pneumatocysts were fully intact. 12 mL of gas was extracted with a 26-gauge (0.45 mm) needle and syringe from each pneumatocyst and stored in an Exetainer (Labco Limited 12 mL, Lampeter, UK). All Exetainers were vacuum sealed and contained a septum to allow gas to be stored without air contamination. Exetainers were punctured no more than three times to avoid air contamination and were stored upside down in distilled water to further prevent air contamination (Glatzel and Well 2008, Sturm et al. 2015). Samples were cooled to 5°C during transit and in the laboratory prior to gas analyses.

CO<sub>2</sub> and CO concentrations were analyzed at room temperature (in ppm) using a Q-trak indoor air quality monitor 7565 (TSI Inc. Shoreview, MN, USA). Q-trak sensors were calibrated to 50 ppm of CO and 447 ppm of CO<sub>2</sub> (CO<sub>2</sub> in air). Pneumatocyst samples were analyzed by injecting 0.5 mL of gas through the sensor where CO<sub>2</sub> and CO concentrations were electronically read after the gas sensor ran for 45 s, recording a peak concentration. Nitrogen (N<sub>2</sub>) was used to flush out any excess air in the sensor that would alter measurements.

Neofox probes (Ocean Optics Inc., FL, USA) were used to measure O<sub>2</sub> concentrations (in %) in gas samples extracted from pneumatocysts. Before each measurement, a 30 mL flask was flushed with 100% N<sub>2</sub> to evacuate any residual air. 1 mL of gas was extracted from the Exetainers and was injected into the flask. Each sample was mixed with the residual N<sub>2</sub> using a magnetic stir bar during the reading. Data were recorded by measuring the peak concentration of O<sub>2</sub>. Percent O<sub>2</sub> was converted to ppm by multiplying measured values by 10,000 (where 1% = 10,000 ppm).

Total gas concentration (ppm) for 2 mL of pneumocyst gas was calculated using the following equation:

$$C_2 = \frac{C_1 V_1}{V_2} \quad (1)$$

where  $C_1$  = the measured gas concentration (ppm),  $V_1 = 1$  mL sample,  $C_2$  = unknown pneumocyst concentration (ppm), and  $V_2$  = pneumocyst volume (mL). Pneumocyst volume was recorded by carving a hole in each pneumocyst, filling it with water, then pouring the water into a graduated cylinder three times, taking an average of the three replicates.

Molar concentration of pneumocyst gases was calculated in two steps:

$$g_{\text{gas}} = (C_g)(V_p) \quad (2)$$

and

$$\eta_{\text{gas}} = \frac{g_{\text{gas}}}{M_{\text{gas}}} \quad (3)$$

where  $g_{\text{gas}}$  = grams of gas (g),  $C_g$  = concentration ( $\text{ppm} \cdot \text{g}^{-1} \cdot \text{m}^{-3}$ ), and  $V_p$  = pneumocyst volume ( $\text{m}^3$ ),  $\eta_{\text{gas}}$  = mols of gas (mol), and  $M_{\text{gas}}$  = molar mass ( $\text{g} \cdot \text{mol}^{-1}$ ). Partial pressures of pneumocyst gases were calculated using the Ideal Gas Law:

$$P_{\text{gas}} = \frac{\eta_{\text{gas}} RT}{V_p} \div 1,000 \quad (4)$$

where  $P_{\text{gas}}$  = gas partial pressure (kPa),  $R$  = gas constant ( $8.314 \text{ L} \cdot \text{Pa}^{-1} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ ),  $T$  = seawater at  $11^\circ\text{C}$  (284.15 K), and  $V_p$  = pneumocyst volume (L). Data published in Liggan and Martone 2018 were used to determine the correlation between total internal pneumocyst pressure (kPa) and volume (between 5 mL and 725 mL).

Raw data (mmol, %, ppm and kPa) were tested for normality using a Shapiro-Wilk test and then normalized by applying a square root transformation where necessary. Linear regression analyses were conducted to determine significant changes in relative gas composition, concentrations and pressure with volume. All statistical analyses were conducted using R version 3.6.0 (R Core Team 2017) for Mac OS X.

As pneumocyst volume increased from 5 mL to 725 mL, total pneumocyst pressure did not change significantly (linear model:  $F_{1,53} = 3.56$ ,  $R^2 = 0.05$ ,  $P = 0.06$ ; Fig. 1). Measured relative gas composition and calculated partial pressures of CO, CO<sub>2</sub>, and O<sub>2</sub> did not change significantly with pneumocyst volume (linear model:  $P > 0.05$  for all gases; Fig. 2, A–C, Table 1). Average concentration for CO, CO<sub>2</sub>, and O<sub>2</sub> were  $12,212.7 \pm 7,764.7$  ppm,

$6,018.1 \pm 1,486.8$  ppm, and  $599,250.0 \pm 136,584.2$  ppm, while average partial pressures were  $1.0 \pm 0.7$  kPa,  $0.3 \pm 0.1$  kPa, and  $44.2 \pm 12.7$  kPa, respectively (Fig. 2, A–C). In addition, the percent concentration of CO, CO<sub>2</sub>, and O<sub>2</sub> was  $1.2 \pm 0.8\%$ ,  $0.6 \pm 0.2\%$ , and  $59.9 \pm 13.6\%$  (Table 1). The amount of CO increased ~280-fold from 1.66 mmol to 473 mmol as pneumocysts increased in volume (linear model:  $F_{1,28} = 23.43$ ,  $R^2 = 0.47$ ,  $P < 0.001$ ; Fig. 2D); CO<sub>2</sub> increased ~70-fold from 1.54 mmol to 103 mmol (linear model:  $F_{1,28} = 126.8$ ,  $R^2 = 0.82$ ,  $P < 0.001$ ; Fig. 2E); O<sub>2</sub> increased ~60-fold from 219.7 mmol to 13,037.5 mmol (linear model:  $F_{1,28} = 249.7$ ,  $R^2 = 0.90$ ,  $P < 0.001$ ; Fig. 2F).

Contrary to our hypotheses, gas composition did not change (i.e., all gases are added in the same proportions) over the lifetime of growing pneumocysts. In particular, the concentration of all gases is stable as pneumocysts become larger, suggesting that CO<sub>2</sub> and O<sub>2</sub> produced by photosynthesizing cells do not directly scale with SA:V. Instead, our data suggest that gas composition is tightly regulated to maintain a constant internal pressure, and that all cells constituting the pneumocyst wall likely contribute to all gases as volume increases and SA:V decreases. Pneumocyst walls become thicker with age through active cell division in the meristoderm (Nicholson 1970, Foreman 1976). We speculate that dividing cells within the thickening pneumocyst walls not only aid in creating a non-permeable barrier to the outside environment but also contribute more gas than originally hypothesized to compensate for rapid increases in volume.

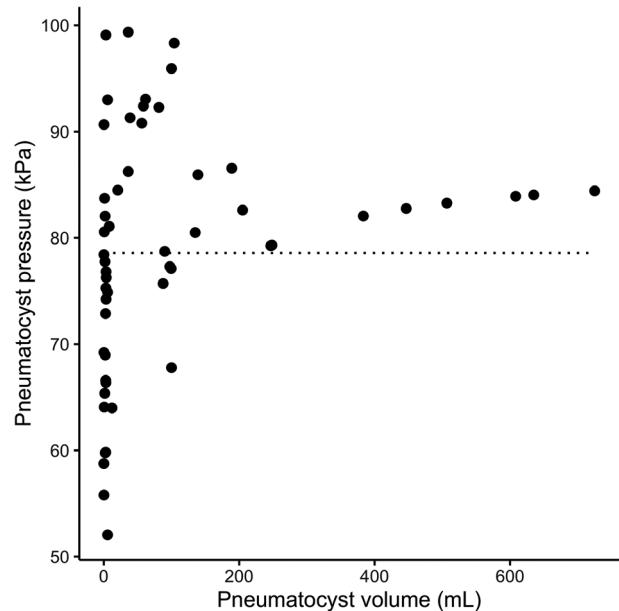


FIG. 1. Pneumocyst internal pressure (kPa) as a function of pneumocyst volume (mL). Dotted line represents the calculated mean across all samples. Average = 79 kPa near the dotted line.

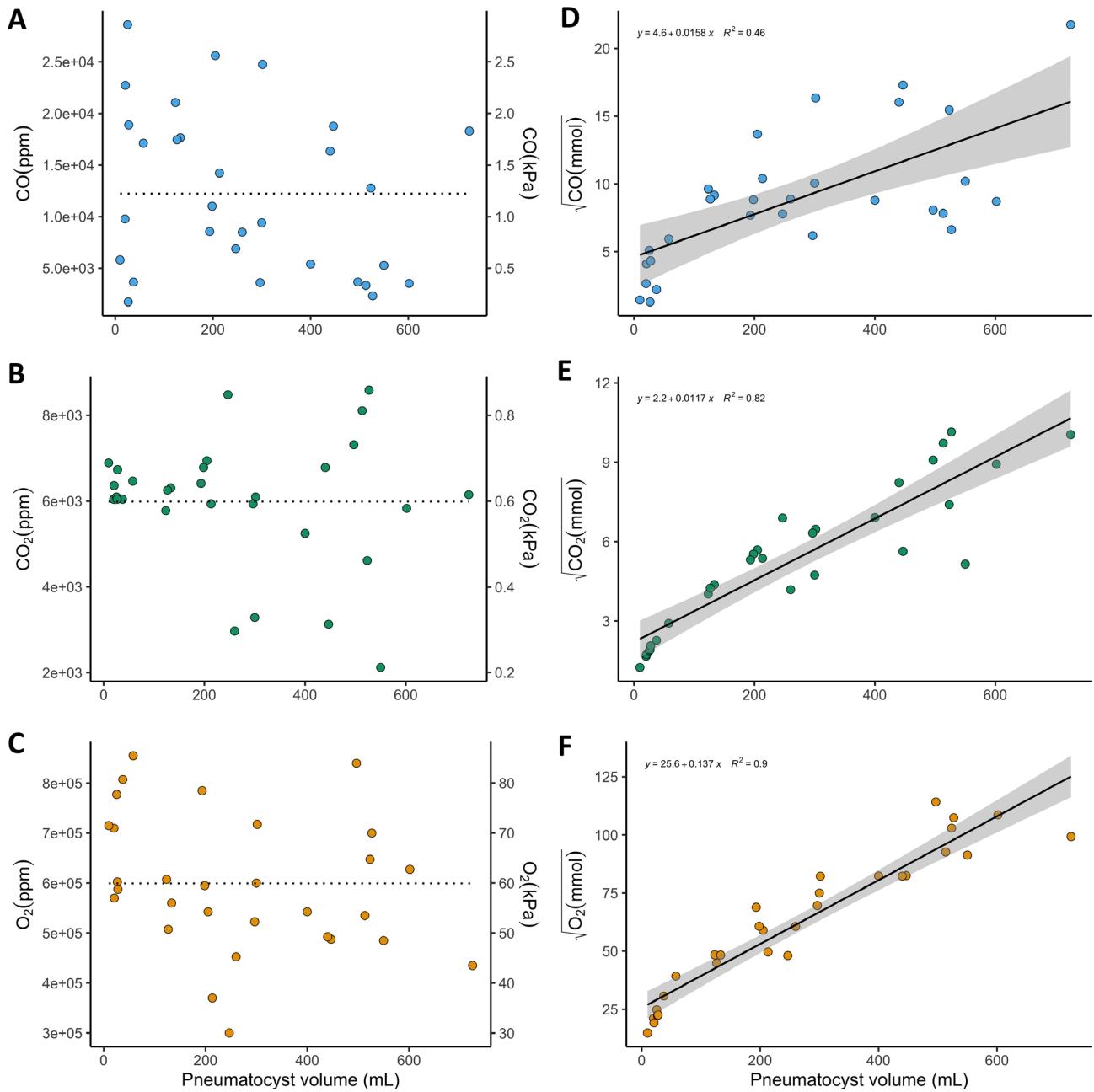


FIG. 2. Measured gas concentration (ppm) and partial pressure (kPa) of (A) CO, (B) CO<sub>2</sub>, and (C) O<sub>2</sub> as functions of pneumocyst volume. Dotted lines represent a calculated mean. Total amount (mmol) of (D) CO, (E) CO<sub>2</sub>, and (F) O<sub>2</sub> as functions of pneumocyst volume.

TABLE 1. Measured concentrations and partial pressures of CO, CO<sub>2</sub>, and O<sub>2</sub> (%), ppm, kPa) of varying pneumocyst volumes between 5 and 725 mL.

Gas	Concentration (%)	Concentration (ppm)	Partial pressure (kPa)
CO	1.2 ± 0.8	12,212.7 ± 7,764.7	1.0 ± 0.7
CO <sub>2</sub>	0.6 ± 0.2	6,018.1 ± 1,486.8	0.3 ± 0.1
O <sub>2</sub>	59.9 ± 13.6	599,250.0 ± 136,584.2	44.2 ± 12.7

Concentrations measured in this study roughly account for 48%–76% of gases present in *Nereocystis* pneumocysts, leaving 24%–52% of gas uncharacterized. Past studies found up to 83% N<sub>2</sub> in pneumocyst gas samples of *Nereocystis*, *Sargassum*, *Macrocystis*, and *Ascophyllum* species (Zeller and Neikirk 1915, Langdon 1917, Hurka 1971, Dromgoole 1981b), and suggested that N<sub>2</sub> found in pneumocysts is not produced by cellular metabolism, but is rather the result of passive diffusion (Dromgoole

1981b). If the concentration of  $N_2$  is a result of passive diffusion and given that internal pressure stays relatively constant throughout development (Liggan and Martone 2018), we speculate that  $N_2$  could passively move from the outside environment into the pneumatocysts, filling any remaining volume. Thus,  $N_2$  concentrations might behave similarly to the other gases directly measured in this study, where concentration stays constant and the total amount of gas increases, as pneumatocysts grow larger. Additional research is needed to completely identify and quantify other pneumatocyst gases.

The production of CO is well known within the plant kingdom (Loewus and Delwiche 1963, Foreman 1976); however, the CO concentrations measured in this study were remarkably high; ~1.5 times greater than CO concentrations found in Mars' atmosphere (King 2015). Contrary to previous studies, our data show that the total amount of CO (mmol) increases with increasing pneumatocyst size, suggesting that CO is continually produced after the medullary tissue tears during early development. We speculate that either cellular autolysis continues to occur during pneumatocyst expansion or else some other undescribed biological process is involved to produce CO proportional to increases in pneumatocyst volume and wall thickness. Interestingly, microbial activity on the surface of some marine macroalgae permits them to use CO for carbon fixation (King 2001, Tolli et al. 2006); however, the functional aspect of CO within *Nereocystis* pneumatocysts is currently unknown.

Contrary to previous studies, which noted that internal gas concentrations were remarkably similar to air, this study found that internal  $O_2$  concentrations were three times greater than those in air ( $59.9 \pm 13.6\%$ ; Table 1). We suspect that discrepancies could be explained by differences in  $O_2$  gas collection and analysis. Pneumatocysts have internal pressures less than atmospheric pressure, such that air is rapidly drawn inside once punctured (Liggan 2016, Liggan and Martone 2018)—a problematic circumstance when trying to avoid air contamination. We suspect that samples in previous studies were contaminated with air, skewing their results, and that the vacuum-sealed Exetainers used in this study mitigated contamination issues during extraction and analysis. Rigg and Swain (1941) extracted and stored pneumatocyst gas in wax sealed syringes prior to analyses, which may have been prone to leaking.

The high levels of CO within pneumatocysts were first noted by Langdon (1917), who determined the toxicity of CO concentrations by exposing animals to pneumatocyst gases and measuring their physiological effects. Subsequently, the statement familiar to many phycologists that “the pneumatocysts of *Nereocystis* have enough CO to kill a chicken” was a product of Langdon (1917). In the current study—

and without harming any animals—our data uphold this old phycological adage. Given that a CO concentration  $>100$  ppm (0.01%) could kill or render a person unconscious (Suner et al. 2008), the largest pneumatocyst analyzed in this study (725 mL) had a CO concentration of 1.6%, resulting in a total concentration of ~1,500 ppm, 15 times greater than the maximum concentration of CO someone with a 5.8 L lung capacity could tolerate before passing out.

In summary, and in contrast to previous studies, concentrations of CO,  $CO_2$ , and  $O_2$ , within *Nereocystis* pneumatocysts were  $1.2 \pm 0.8\%$ ,  $0.6 \pm 0.2\%$ , and  $59.9 \pm 13.6\%$ , respectively. All gas concentrations remained stable as pneumatocysts increased in volume and, in particular, physiological gases stored in the pneumatocyst do not scale with SA:V. This study demonstrates that gas composition does not change as pneumatocysts expand and that the rate of gases being added is proportional to changes in pneumatocyst volume, suggesting that the same relative proportion of gases is being added at all stages of development. We speculate that cells constituting the pneumatocyst wall continuously contribute to total gas composition and maintain internal pressure by producing more gas (per surface area) to fill pneumatocysts as they rapidly grow.

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