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ABSTRACT
Pigment variation within and among algal species may have important ecological consequences because small changes in the concentration and composition of pigments can influence the photosynthetic efficiency and rate as well as the spectra of light utilized. Toward the goal of developing a rapid method for comparing pigment composition among algal thalli, we characterized the relationship between visual color information taken from photographs (e.g., red, green, and blue color values) and photopigment composition in the non-native red alga *Agarophyton vermiculophyllum* (Ohmi) Gurgel, J.N.Norris & Fredericq. We used a set of 19 thalli, collected from across the known native and non-native range in the Northern Hemisphere, which exhibited substantial color variation at the time of field collection, and sustained this variation after being maintained in a common garden. We identified a set of ecologically interesting pigment traits that are readily predicted by color information, including chlorophyll *a* and phycobilin concentration. Finally, we demonstrated the repeatability of estimating color phenotypes from photographs of thalli taken under a range of light conditions in order to evaluate the utility of this approach for field studies. We suggest this method could be useful for the rapid, high-throughput phenotyping of photopigments in other red algae as well.

KEY WORDS
intraspecific variation, ecology, photopigments, phycobilin proteins, Rhodophyta.

RÉSUMÉ

Utilisation d'informations photographiques sur les couleurs pour le phénotypage à haut débit de la composition de pigments chez Agarophyton vermiculophyllum *(Ohmi) Gurgel, J.N.Norris & Fredericq.*

La variation intra et interspécifique des pigments au sein des espèces d'algues et entre celles-ci peut avoir des conséquences écologiques importantes, car de faibles variations dans la concentration et la composition des pigments peuvent influer sur l'efficacité et la vitesse de la photosynthèse, ainsi que sur le spectre de la lumière utilisée. Afin de développer une méthode rapide de comparaison de la composition en pigment parmi les thalles d'algues, nous avons caractérisé la relation entre les informations de couleur visuelles tirées de photographies (valeurs des couleurs rouge, verte et bleue) et la composition de photopigment dans l'algue rouge non native *Agarophyton vermiculophyllum* (Ohmi) Gurgel, J.N.Norris & Fredericq. Nous avons utilisé un ensemble de 19 individus, provenant de l'aire de répartition connue des autochtones et des non-autochtones de l'hémisphère Nord, qui présentait une variation de couleur substantielle au moment de la récolte sur le terrain et a maintenu cette variation après avoir été placé dans un jardin commun. Nous avons identifié un ensemble de caractéristiques pigmentaires intéressantes sur le plan écologique qui sont facilement prédites par les informations sur les couleurs, notamment la concentration de chlorophylle *a* et de phycobiline. Enfin, nous avons démontré la répétabilité de l'estimation des phénotypes de couleur à partir de photographies d'algues prises dans diverses conditions d'éclairage afin d'évaluer l'utilité de cette approche pour des études sur le terrain. Nous suggérons que cette méthode pourrait être utile pour le phénotypage rapide et à haut débit de photopigments dans d'autres algues rouges.

MOTS CLÉS
variation intraspécifique, écologie, photopigments, protéines de phycobiline, Rhodophyta.

INTRODUCTION

Light-sensitive pigments, also known as photopigments, play a key role in defining the ecological niche of photosynthetic organisms (Falkowski & Raven 2007). The composition of photopigments determines the wavelengths of light an organism can use for photosynthesis (Lemasson *et al.* 1973; Gantt 1981). Additional accessory pigments may also protect sensitive photosynthetic machinery against damage from high intensity visible or UV radiation (Siefermann-Harms 1985; Ben-Amotz *et al.* 1989; Bailey & Grossman 2008). Thus, variation in pigment composition might act as a key predictor of performance under differing environmental conditions.

Visible color variation has been linked to heritable differences in the concentration and relative composition of phycobilin pigments in color mutants found in both natural and lab-reared populations (e.g., *Porphyra yezeoensis* Ueda: Niwa *et al.* 2009; *Chondrus crispus* Stackhouse: van der Meer 1981; Cornish *et al.* 2013; *Gracilaria tikvahiae* McLachlan: Kursar *et al.* 1983; *Gracilaria birdiae* E.M.Plastino & E.C.Oliveira: Plastino *et al.* 2003; Costa & Plastino 2011). In some cases, pigment mutations have been linked to changes in growth rates under laboratory conditions (Plastino *et al.* 2003; Yokoya *et al.* 2007; Kim *et al.* 2015; but see also Ramus & van der Meer 1983), supporting the potential for pigment traits to be shaped by natural selection. Indeed, variation in pigment composition has long been recognized as an important driver of algal niche diversity at higher taxonomic levels, offering a potential mechanistic explanation of light resource niche partitioning (Stomp *et al.* 2007; Stockenreiter *et al.* 2013). Because heritable variation in pigment composition may be an important target for selection under changing environmental conditions, pigment variation could be a key trait for predicting responses under climate change or during range expansion. Thus, there is an impetus for these data to be gathered more routinely during ecological studies.

Photopigment composition can also be highly dynamic within individuals. Thalli can respond to changes in irradiance, spectral quality, and other environmental variables by rapidly modulating the concentration and composition of accessory pigments (e.g., Rosenberg & Ramus 1982; Beach & Smith 1996; Grossman 2003; Kim *et al.* 2015). Such rapid responses suggest that changes in pigments may serve an acclimatory function over the course of a day, or across seasons for algae living in highly dynamic light environments, such as the intertidal zone (López-Figueroa 1992; Beach & Smith 1996; reviewed by Falkowski & Chen 2003). Measuring pigment variation through time is essential to our understanding of the role that plasticity plays in local acclimation, yet tracking changes in individual thalli is difficult without non-destructive protocols for characterizing pigments.

In order to incorporate pigment data into ecological and biogeographic studies, however, these data may need to be gathered from hundreds of sampled specimens (i.e., high throughput phenotyping), or under field conditions. Pigment extractions require destructive sampling, which may limit the use of tissues for characterizing additional traits (e.g., protein content) or evaluating changes at the individual level through time. Moreover, extractions are time consuming and require laboratory equipment (e.g., a spectrophotometer and liquid nitrogen), making it difficult to generate these data in the field, or for a large number of samples in a short time period. Nevertheless, intraspecific variation in phenotypic traits, including variation in pigment composition, may be

Table 1. — Collection site information. Region designation as Native (non-source), Native (source), EUSA, EU, and WNA are based Krueger-Hadfield *et al.* (2017). * : kie and nor were sampled twice, once for the color variation and once for testing the repeatability of color estimates under different lighting conditions.

Code	Site name	Country	Region	Latitude	Longitude	Sampled by	Date
qin	Qingdao	China	Native (non-source)	36.053085	120.35950	F. Weinberger & G. Wang	03 Sep. 2017
ron	Rongsheng	China	Native (non- source)	37.112682	122.345719	F. Weinberger & G. Wang	01 Sep. 2017
ahc	Ape Hole Creek	USA	EUSA	37.957186	-75.024576	S. A. Krueger-Hadfield & G. Bonthond	08 Sep. 2017
fdm	Dinan	France	EU	48.514599	-1.9698	F. Weinberger	21 Sep. 2017
kie*	Kiel	Germany	EU	54.368052	10.150223	F. Weinberger	13 Sep. 2017
						F. Weinberger	22 June 2018
nor*	Nordstrand	Germany	EU	54.454571	8.8748466	F. Weinberger	11 Sep. 2017
						G. Bonthond	27 June 2018
sou	Soukanzan	Japan	Native (source)	38.352839	141.059694	F. Weinberger & T. Bayer	27 Aug. 2017
tmb	Blake's Landing	USA	WNA	38.17976	-122.90857	S. A. Krueger-Hadfield & G. Bonthond	21 Sep. 2017

essential to understanding local adaptation and performance variation in natural populations of algae.

Here, we describe the wide range of color variation we observed in a subset of thalli from across the extant range of *Agarophyton vermiculophyllum* (Ohmi) Gurgel *et al.* (2018). We demonstrate that color variation is strongly predictive of some facets of photosynthetic pigment composition, and propose a rapid, non-destructive, and inexpensive method of predicting pigment composition from photographic color data to facilitate the high-throughput phenotyping of algae and to encourage the collection of these data in ecological studies.

MATERIAL AND METHODS

Sample collection and identification

Whole algal thalli were collected from eight sites across the Northern Hemisphere range of *A. vermiculophyllum* in 2017 (Table 1). The wet mass of each alga varied depending on the genotype, but an algal thallus was divided into equal parts and shipped to the Weinberger lab at GEOMAR in Kiel, Germany or the Krueger-Hadfield lab at UAB in Birmingham, AL, USA. Three sites were from the native range, and sampled along the coastlines of China (qin, ron) and Japan (sou). Five sites were sampled from the non-native range, including one site from the west coast of the United States (tmb), one site from east coast of the United States (ahc), and three sites from Europe (fdm, kie, nor; see also Krueger-Hadfield *et al.* 2017). At all sites, thalli were collected with at least one meter separating each putative genet (i.e., unique genotype) to minimize the chance of sampling the same genet twice (Guillemin et al. 2008; Krueger-Hadfield *et al.* 2013, 2016).

Maintenance of samples prior to phenotyping

Prior to phenotypic analyses conducted at the University of Alabama at Birmingham, all thalli were maintained in an environmental control chamber (I-36LL, Percival, Perry, IA, USA) for approximately two months to homogenize environmental influences on color and pigment composition (see also Sotka *et al.* 2018). Thalli were kept in individual 50 ml conical polyethylene vials filled with 45 ml of filtered, natural seawater collected from Charleston Harbor, South Carolina, USA (salinity: 30 ppt) at a temperature of 15°C, and an irradiance of approximately 60 µmol photons $\cdot m^{-2} \cdot s^{-1}$ with a 12:12 light:dark cycle. Fouling was minimal and the water was changed two times per week.

Selection of thalli for experiment

Approximately 50 algal cultures were available at the time of the experiment. In order to maximize the range of color morphs used, available samples were roughly clustered by visual similarity into four color groups (Red, Green, Yellow, and Black). Five thalli from each group were selected without taking site or origin (i.e., native vs non-native) into consideration. The concept of visual color groups was dropped after sample selection in favor of analyzing color as a continuous variable to facilitate a regression approach. One sample was removed prior to analyses because there was too little tissue to accurately measure phycobilin protein composition, leaving 19 thalli in total (Fig. 1).

Photographing thalli and retrieving color data

To standardize the light conditions among samples, thalli were submerged in a Petri dish (100 × 15 mm) filled with seawater and photographed with a mounted camera (Canon SX710 HS; Canon Inc., Tokyo, Japan) inside a tent of lightweight, white muslin, which diffused light and minimized glare. Aperture (f 3.2), exposure time (⅛ second), and white balance (manually adjusted using the white background before the first picture) were identical for all photographs. To allow for better visual discrimination of thallus color, the brightness was increased slightly and uniformly for all images in Photoshop ver. CC 2015 (Adobe Systems Inc., San Jose, CA, United States). We did not digitally color correct using a color card as the images were all taken under the same light conditions.

To quantify the visual color of each thallus, RGB values were recorded for ten randomly chosen points, on the thallus in each photograph using the Color Picker Tool add-on

in ImageJ (Schneider *et al.* 2012). A unique grid of 100 randomly numbered points was overlaid onto each image of the thallus. Points were checked sequentially and the first ten points identified that met the following quality control criteria were used to collect color information. Points were rejected if they: i) occurred near the thallus edge; ii) occurred where tissue overlapped; or iii) occurred where obvious glare or shadow obscured the color.

Pigment extraction and analysis

We quantified the concentration of chlorophyll *a* (chl *a*) in all thalli following the protocol of Torres *et al.* (2014). Briefly, approximately 50 mg of fresh tissue was frozen with liquid nitrogen, and ground with a pestle in a 2 ml tube. Pigments were extracted in 1.5 ml of methanol for 9 minutes, at which point tissues had lost their color, followed by centrifugation at 13 500 x g for 5 minutes. Extraction time was standardized across samples to minimize error while processing samples sequentially. The supernatant was transferred to a cuvette and absorbance was read at 665 and 750 nm on a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Background absorbance at 750 nm was subtracted from values to account for variation in light scatter from debris or other impurities. The concentration of chl *a* was calculated as:

$$[\text{chl } a] = 12.61 \cdot A_{665} \quad (1)$$

We extracted and quantified two classes of phycobilin proteins following the protocol of Rosenberg (1981). Approximately 0.25 g of fresh tissue was frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. The powder was transferred to a 2 ml tube with 750 ml of a 0.5% solution of Triton-X in a 0.1M phosphate buffer (6.8 ph). Samples were frozen and defrosted twice before centrifugation at 13 500 x g for 5 minutes. We, then, transferred the supernatant to a cuvette and read absorbance values at 565, 615, 650 and 750 nm. Background absorbance measured at 750 nm in each sample was subtracted from all absorbance measurements to account for variation in light scatter from debris or other impurities. Then, values were used to calculate the concentrations of phycoerythrin (PE) +and phycocyanin (PC) in microgram per gram of tissue wet weight using the following formulas:

$$[\text{PE}] = 123.5 \cdot A_{565} - 73.5 \cdot A_{615} + 16.3 \cdot A_{650} \quad (2)$$

$$[\text{PC}] = 163.2 \cdot A_{615} - 117.1 \cdot A_{650} \quad (3)$$

We summed the concentrations of the two phycobilin types in order to obtain total phycobilin protein concentration. To describe the relative composition of the two phycobilin proteins, we calculated the percent contribution of each phycobilin protein relative to the total concentration of phycobilins.

We also calculated the ratio of total phycobilins to chl *a* concentration in order to assess how changes in the relative composition of these pigments influenced thallus color. We added the chl *a* concentration to the total phycobilin value for a measure of total pigment concentration for each thallus.

Analyses of pigment and color variation

To describe the extent of color and pigment variation among the thalli, we calculated the median and range of each color trait [red (R), green (G), and blue (B) values], as well as each pigment trait (chl *a*, PE, and PC concentrations). To estimate the major axes of variation in color and pigment across traits and among thalli, we performed a Principal Components Analysis (PCA) on the scaled and natural log transformed R, G, and B values using the *prcomp* function in *R* (R Core Team 2017). To understand the relative contribution of each variable to overall variation, PCA loadings were derived from the *prcomp* function, and were plotted onto the scatterplot of PC1 versus PC2. The same routine was used separately to describe variation among pigment components, including the concentrations of chl *a*, PE, and PC. All variables were log transformed to meet the assumptions for normality for PCA.

To understand the relationship between variation in color traits and pigment composition, we evaluated a set of linear models using the first and second principle components axes (color PCA), and their interaction, as predictors of the first and second principle component axes estimated for pigment variables (pigment PCA). Step-wise model selection using the Akaike Information Criterion (AIC) was performed to identify the most informative combination of predictor variables for each pigment PCA axis in *R*. The model with the lowest AIC value was selected. When two models had similar AIC values ($\Delta AIC < 2$), the model with the fewest parameters was chosen (Burnham & Anderson 2004).

Variation in photo-based color estimates under different light conditions

To estimate the variation in color information caused by differing light conditions, we used five individual thalli (three originating from nor and two from kie; Table 1) which had been maintained in the laboratory for ten weeks. Thalli were cultivated in aerated artificial seawater enriched with Provasoli's Enrichment at 15°C. All five thalli maintained color differences exhibited at the time of collection during these ten weeks. Each thallus was placed on a white cloth background with a color card (DGK Digital Kolor Kard, Digital Image Flow, Boston MA, USA). Thalli were then photographed under five different light conditions, while maintaining the same orientation of each thallus in each image. The five light conditions were: Direct midday sunlight (direct), indirect sunlight through a window (indirect), cool white light from a 680 nm wavelength neon light (white), interior incandescent light (yellow), and a 50:50 mix of cool white and blue LED light (blue; Fig. 1). Light conditions were chosen to create an extreme range of conditions on which to test the ability of digital color correction techniques to standardize extracted thallus color data.

To digitally standardize the light environment, images captured under different light conditions were white balanced using the custom white balance tool in Photoshop Lightroom v. 5. 7.1 (Adobe Systems Incorporated, San Jose, CA, United States). The 80% grey square on the color card was used as a reference standard in each image.

A randomized grid of points was then laid over each image and RGB data were collected using the same technique as described above. The same grid was used for all images of the same thallus to standardize the points measured on the thallus

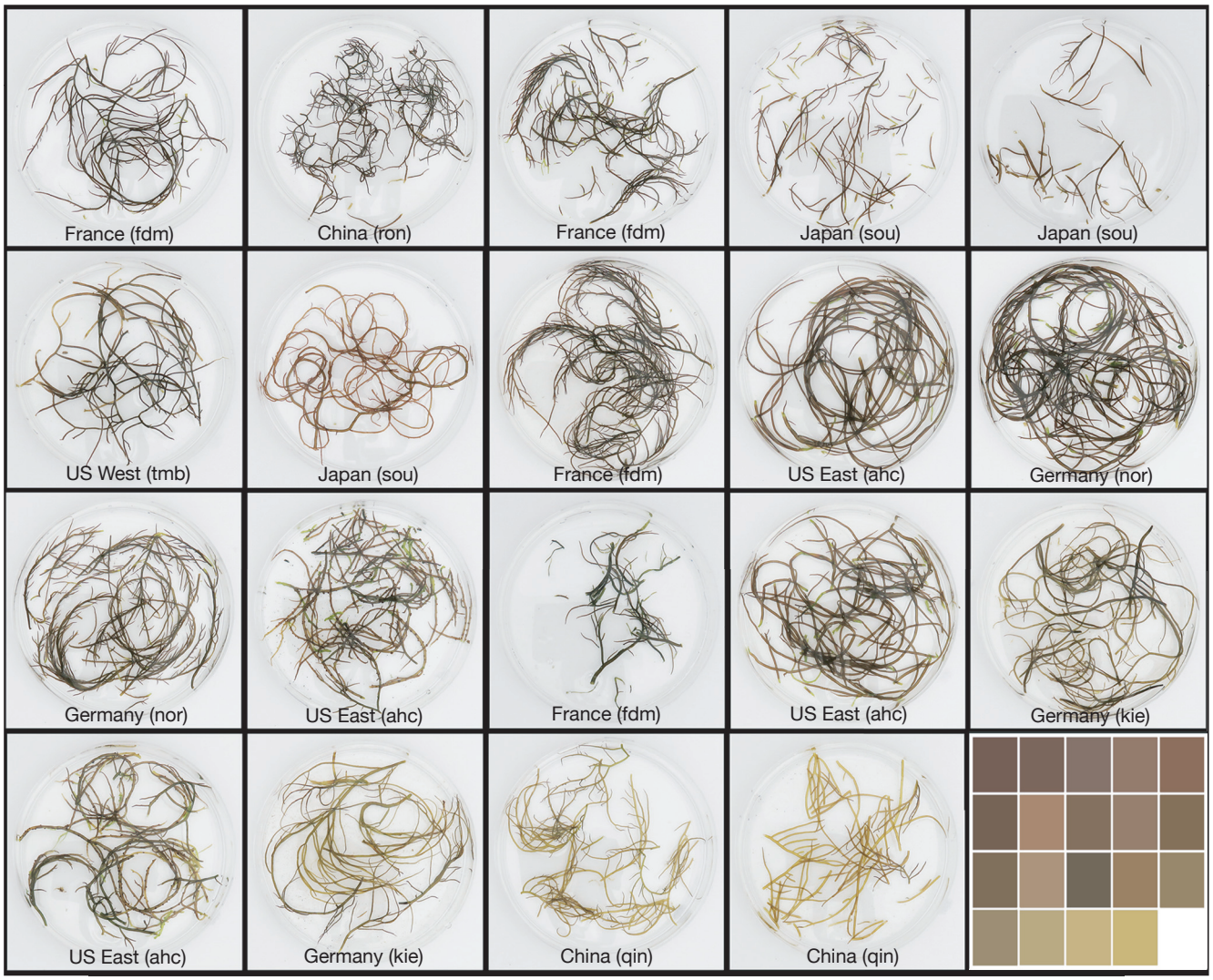

FIG. 1. — *Agarophyton vermiculophyllum* color variation. Photographs used for color information arranged (from top left) in rank order of increasing hue value. Lower right panel shows the color of the average RGB values estimated from each image. Labels indicated the region and site of origin for each thallus. Diameter of dish in each image is 100 mm.

among light conditions. Color data were also taken from four points on the color card (black, white, red, blue) to facilitate further color correction.

We then used an iterative process to perform a linear color correction in order to determine the correction factor that best reduced the variation in RGB values measured for the color card swatches. Briefly, the grand mean of R, G, and B values of the color card were estimated using each possible combination of values from the red, black, white, and blue squares, pooled across all images. Each of these possible grand means was used in the correction factor (see below) to generate a data set of R, G, and B values for each square in each image. PCA was run on each data set. The correction factor that produced the most similar estimates of color card color across light conditions was chosen.

The chosen factor was then applied across the images to reduce the variation imposed by light condition on the color values measured for each thallus. A correction factor specific to each image was calculated by dividing the grand mean by the R, G, and B values averaged across the color card in each image. The R, G, and B values measured from the thallus in each image were then multiplied by the correction factor to calculate standardized R, G, and B values, on which statistical analyses were performed. A multivariate analysis of variance (MANOVA) was performed on the color traits (PC1 and PC2) measured for the color card squares in each image to confirm that the color correction procedure had removed any systematic variation among light conditions.

The effect of light conditions on measured color was then estimated after color correction. To determine the reproducibility of color estimates, PCA was used to describe the major axes of color variation. A second MANOVA was performed to evaluate the influence of thallus identity and light condition on color variation described by the first two PC axes. Significant differences in color estimates among light condition groups were further described with univariate linear mixed models on each PC axis, with thallus identity as a random factor, using

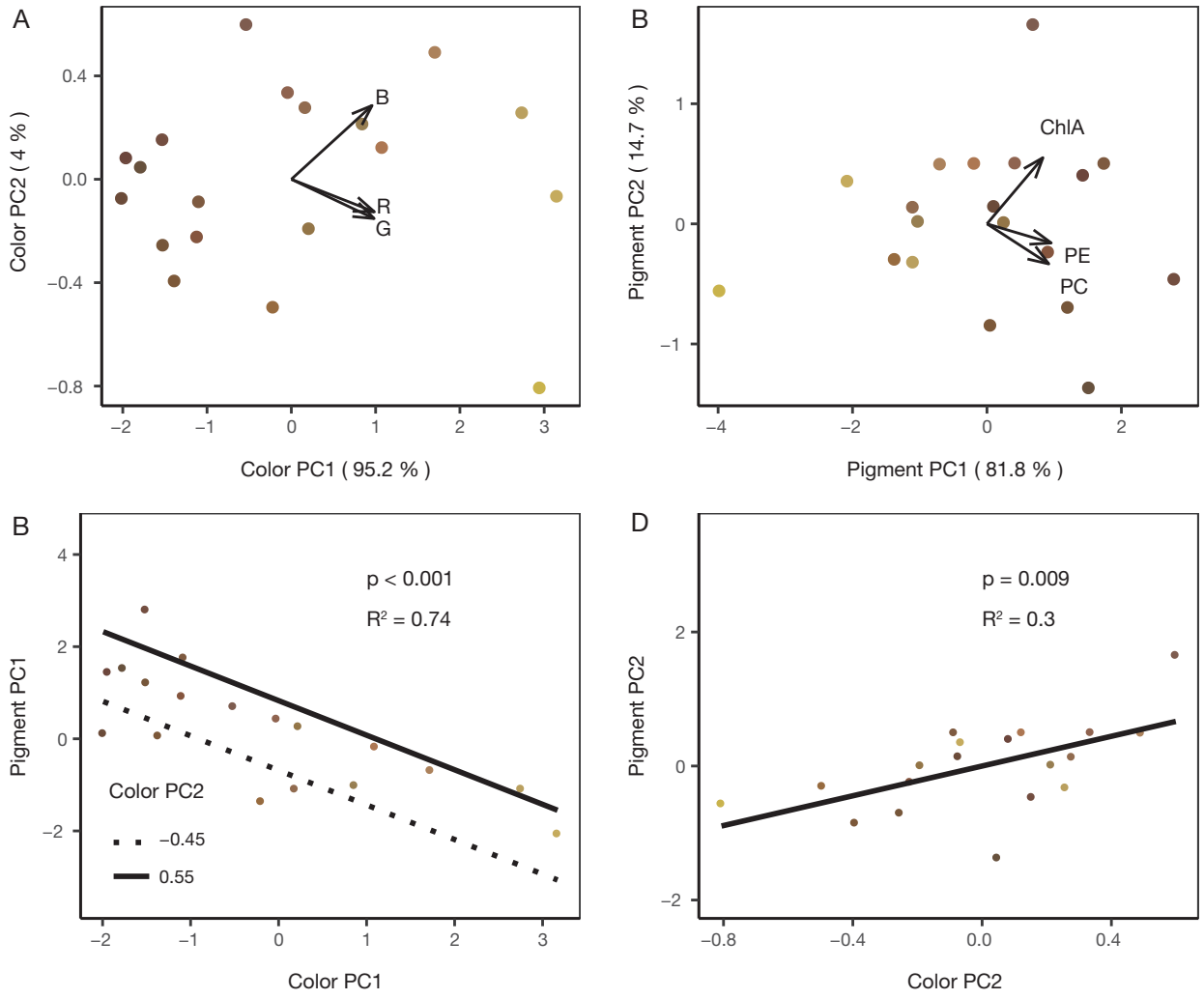

Fig. 2. — Principal components of variation for: **A**, R, G, and B values; **B**, pigment variables. Arrow directions indicate the association of each variable to PC 1 and PC 2. Arrow length indicates the relative strength of the contribution of each variable. Point color reflects the average color of each thallus; **C**, the major axis of variation in pigments (PC1) is best predicted by a combination of color PC1 and PC2. The dashed line represents predicted values when PC2 is low, compared to when PC2 is high (solid line); **D**, pigment variation along PC2 is best predicted by color variation along PC2.

the *lmer* function in the *lme4* package (Bates *et al.* 2015) in *R*. Significance values were compared to a Bonferroni adjusted alpha to account for multiple tests.

RESULTS

Color variation

Approximately 99.2% of variation in R, G, and B values among 19 thalli was described by the first two principal components axes (95.2 and 4%, respectively; Fig. 2A). PC1 was primarily associated with levels of R and G. Increasing PC1 values were associated with a shift from dark to light yellowish-green thalli. PC2 was primarily associated with B value, shifting from reddish to brownish as PC2 increases. Untransformed R values ranged from 100 to 197, with a median value of 137, G values from 73 to 197, with a median value of 101, and B values ranged from 61 to 105, with a median value of 78.

Pigment variation

Approximately 96.5% of the variation in absolute pigment concentrations (chl *a*, PE, and PC) was explained by the first two axes (81.8 and 14.7%, respectively; Fig. 2B). PC1 was positively correlated with an increase in the concentration of both phycobilin pigments, whereas PC2 reflected increasing chl *a*. The chl *a* concentration ranged from 77 to 558 µg/g wet weight, with a median value of 261 µg/g. The total concentration of phycobilin pigments ranged from 38 to 445 µg/g, with a median value of 145 µg/g. PE concentration ranged from 27 to 295 µg/g, with a median value of 100 µg/g. PC concentration ranged from 11 to 150 µg/g, with a median value of 45 µg/g. Additionally, the ratio of total phycobilin protein concentration to chl *a* concentration ranged from 0.48 to 2.66, with a median value of 0.98. The proportional contribution of PE and PC to total phycobilin composition ranged from 0.60 to 0.86 and 0.14 to 0.40, respectively, with median values of 0.67 and 0.33. While there was ample variation in the relative composi-

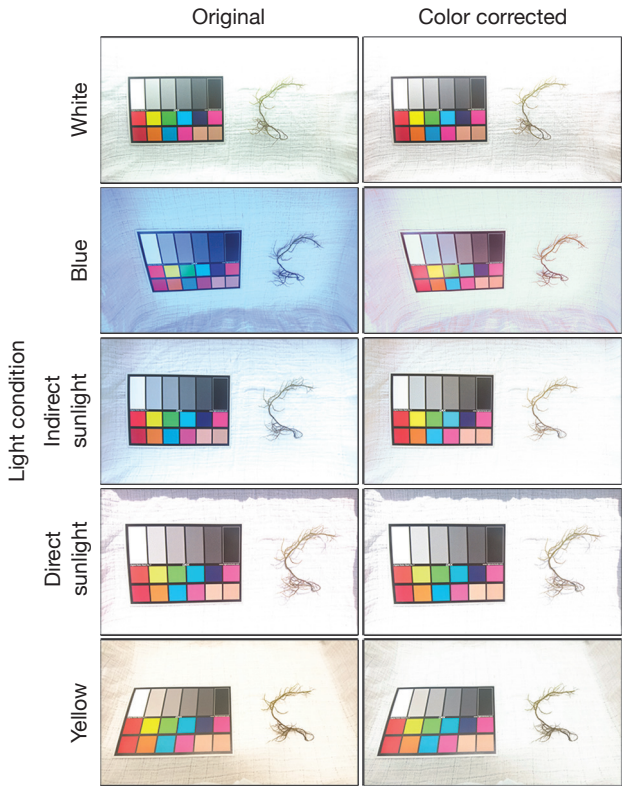

Fig. 3. — Examples of images taken of the same thallus and color card under five light conditions (left column), and those same images after white balancing (right column). Width of the color card in each image is 178 mm.

tion of photopigments, composition did not appear to vary systematically with changes in total pigment concentration (R^2 = 0.06, -0.05, -0.05, for total phycobilin/chl *a*, PE/total phycobilin, and PC/total phycobilin, respectively).

Relationship of thallus color to pigment traits

There was a strong association between thallus color and pigment composition. The major axis of pigment variation (pigment PC1) was best predicted by a combination of the color axes (color PC1 and PC2) (R^2 = 0.74, $p < 0.001$, Fig. 2C). In general, darker colored thalli had higher pigment concentrations. Pigment PC2 was best predicted by color PC2 (R^2 = 0.30, p = 0.009, Fig. 2D). Color PC1 was not included as a predictor in the best-fit model for pigment PC2. See model selection details in Table 2.

Reproducibility of thallus color estimates under different light conditions

Photos taken under differing light conditions showed a large amount of variation in the average color of the image (Fig. 3). However, a simple white balance procedure greatly improved the similarity of the images (Fig. 3). The additional correction factor that most reduced variation among color card measurements took into account the average R, G, and B values of the red, white, and black, but not blue, color card standards. Measurements of the blue color standard were highly variable among images, even after color correction, highlighting the disproportionate sensitivity of different parts of the color spectrum to ambient light conditions (Fig. 4A). After white balancing, there was no significant difference among the color of the color card standards, as described by the first two PC axes, in images taken under different light conditions (Pillai approximation = 0.09, $F_{4,95}$ = 1.09, p = 0.37; MANOVA), indicating that images taken under very different light conditions can be standardized effectively.

Despite effectively standardizing the average color across images, there was a significant effect of light conditions detected

Table 2. — Model selection details for linear models estimating the relationship between PC axes of color traits and the PC axes of pigment traits. Bolded values indicate the best-fit model.

Model	AIC
Pigment PC1 ~ color PC1 * color PC2	51.79
Pigment PC1 ~ color PC1 + color PC2	**50.19**
Pigment PC1 ~ color PC1	55.70
Pigment PC1 ~ color PC2	73.68
Pigment PC2 ~ color PC1 * color PC2	37.33
Pigment PC2 ~ color PC1 + color PC2	36.75
Pigment PC2 ~ color PC1	42.82
Pigment PC2 ~ color PC2	**35.48**

Table 3. — Graphs taken of five thalli under five different light conditions: **A**, MANOVA results jointly comparing color traits estimated by PC1 and PC2 of a principle components analysis among photographs; **B**, **C**, linear mixed model results estimating the effect of each light treatment on either PC1 or PC2, respectively. Thallus was treated as a random factor. PC1 and PC2 were analyzed separately. Bonferroni adjusted alpha = 0.016.

A: MANOVA

Factor	DF	Pillai estimate	Approximate F	P value
Light	4	0.68	28.81	< 0.001
Thallus	4	1.34	113.33	< 0.001
L X T	16	0.50	4.67	< 0.001
Residuals	225			

B: Color PC1

Random factor	Variance
Thallus	1.27
Residual	0.67

Fixed factor	Estimate	DF	T value	P value	
Intercept	-0.31	4.34	-0.61	0.57	
Direct	-0.27	241	-1.67	0.10	
Indirect	**1.86**	**241**	**11.37**	**< 0.001**	*
White	0.15	241	0.91	0.37	
Yellow	0.17	241	-1.03	0.31	

C: Color PC2

Random factor	Variance
Thallus	0.51
Residual	0.17

Fixed factor	Estimate	DF	T value	P value	
Intercept	-0.18	4.21	-0.56	0.60	
Direct	-0.01	241	-0.18	0.86	
Indirect	**0.72**	**241**	**8.78**	**< 0.001**	*
White	0.01	241	0.13	0.90	
Yellow	**0.20**	**241**	**2.46**	**0.015**	*

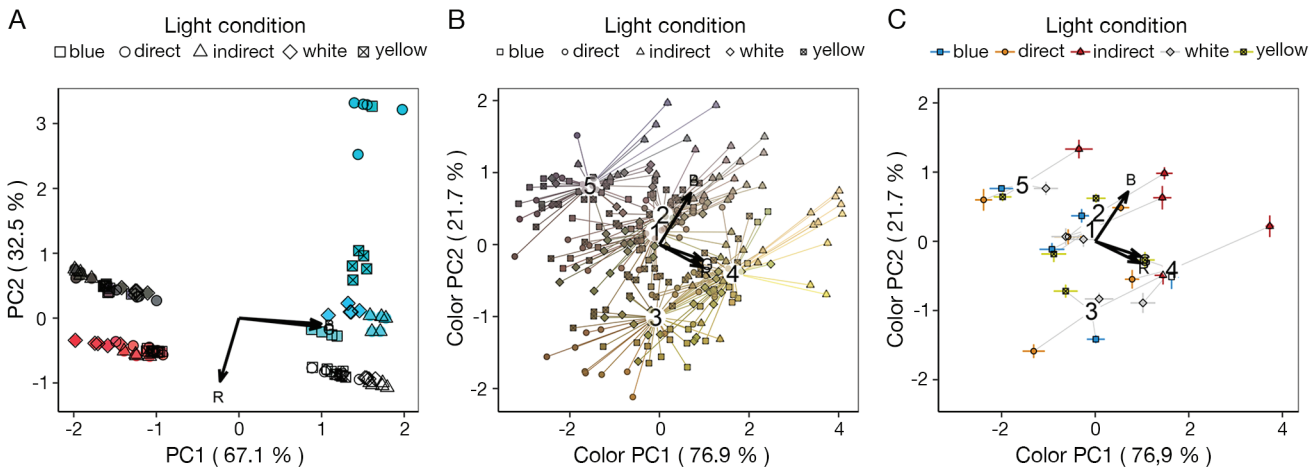

FIG. 4. — **A**, After color correction procedures, color values estimated for four areas on the color card (i.e., black, white, red, and blue swatches) included in each photograph showed no significant differences among light condition groups (shapes); **B**, After color correction using values standardized using a color card, color traits (RGB values analyzed with PCA) were more similar within thallus (numbered clusters) than among light conditions (shapes), with a few exceptions. **C**, Centroids estimated for each thallus (numbered clusters) and light condition (shape and color) combination showed that some light conditions could be effectively standardized with color correction procedures (e.g. blue, direct sunlight, and white light), whereas thalli photographed under other conditions showed different color profiles even after correction (e.g. indirect sunlight (triangles), and to a lesser extend yellow light (boxed Xs).

on the measurements of thallus color ($F = 28.81$, $p < 0.001$; Fig 4B, C; see Table 3 for statistical details). The effect of thallus identity was larger ($F = 113.33$, $p < 0.001$), indicating that color differences among thalli were recovered despite variation in light conditions. There was also a significant interaction between light conditions and thalli ($F = 4.67$, $p < 0.001$) suggesting that color estimates of lighter colored thalli were more variable under some light conditions than darker thalli. Subsequent analysis with linear mixed models showed that images taken under indirect light had significantly higher PC1 and PC2 values compared to other groups (both $p < 0.001$, Table 3B, C). Subjects under yellow light also showed slightly higher PC2 values ($p = 0.015$, marginally significant under adjusted $\alpha = 0.016$), whereas the other light conditions provide statistically indistinguishable estimates of thallus color (see Table 3 for full results).

DISCUSSION

Color information extracted from photographs can be used to estimate both absolute and relative pigment concentrations for rapid, high-throughput phenotyping, supporting the utility of this technique as a tool for studying phenotypic variation in algae. Our methods provide a simple and reliable way to detect color variation on a large number of algal thalli, including under field-work conditions where laboratory-based pigment extractions would be challenging. The availability of field-ready techniques may encourage the incorporation of pigment information into ecological studies, where such data have been historically limited.

In the case of *A. vermiculophyllum*, color variation may be an important source of heritable variation within and among populations, and may have particular relevance to acclimation or adaptation under differing environmental conditions. The algal thalli used in this study were kept under laboratory conditions for eight to ten weeks prior to measurements. As a consequence, the substantial color and pigment variation observed (Fig. 1) likely reflects constitutive differences among algal thalli as algal thalli did not converge on the same color after two months under the same laboratory conditions. Algal thalli varied substantially in the amounts of chl *a* and total phycobilin concentration, which may be associated with differences in photosynthetic efficiency (Yokoya *et al.* 2007), growth (Plastino *et al.* 2003), or both under a range of light conditions. Such variation may be critical to understanding the success of *A. vermiculophyllum* in both its native and non-native ranges (see also: Yokoya *et al.* 1999; Raikar *et al.* 2001; Sotka *et al.* 2018, Lees *et al.* 2018).

Agarophyton vermiculophyllum inhabits shallow, estuarine habitats across a large range of latitudes (Krueger-Hadfield *et al.* 2016, 2017), which differ in turbidity, temperature, and seasonality (Sotka *et al.* 2018). Biological invasions will expose non-native thalli to novel conditions (i.e., average cloud cover, latitude, Sotka *et al.* 2018), which may dramatically influence daily and seasonal patterns of available light (Anthony *et al.* 2004). Evolutionary shifts in mating system (Krueger-Hadfield *et al.* 2016), stress tolerance (Sotka *et al.* 2018), and palatability (Hammann *et al.* 2013; but see Bippus *et al.* 2018) have been documented in non-native populations of this seaweed. Given the centrality of photosynthesis to the life of an alga, it is reasonable to expect that all facets of the photosynthetic machinery, including pigments, would also be under strong selection during a range expansion. While the replication in this study does not allow for a geographical analysis, anecdotal reports of site and regional differences in thallus color (S. A. Krueger-Hadfield, F. Weinberger, *pers. obs.*) suggest that geographic patterns are worthy of future investigation. Color estimates from photographs are a way to rapidly phenotype thalli in this alga and other species, which will allow for the

development of more detailed hypotheses based on observed variation.

Our results suggest that color estimates taken from photos are robust to a wide range of light environments, as long as a few simple precautions are taken. First, when images are taken at different times, or under differing conditions, a standardized color card must be included in every image to allow for nuanced color corrections above and beyond simple white balancing. Because different parts of the color spectrum are more or less sensitive to ambient light conditions, the choice colors to include as a reference standard need to be well-suited to the spectrum of colors to be estimated from the samples. Second, specimens must be evenly lit and relatively free of shadows and highlights. For example, the indirect sunlight treatment may have produced aberrant color estimates relative to other light environments because there was a subtle gradient in a light intensity across the specimen and photographed area. Such artifacts cannot be rectified by color correction algorithms and could lead to significant errors in estimating color properties. Constructing or purchasing a lightbox to diffuse light and minimize shadows and glare is a simple way to standardize photos to be analyzed for color, regardless of whether images are taken in the field or in the laboratory. As high-quality camera technology has improved and become more affordable, color data derived from digital imagery have become increasingly common in ecological research (e.g., Endler & Mielke 2005; Siebeck *et al.* 2006; Winters *et al.* 2009; Kobayashi & Fujita 2014). As such, literature is available to assist the development of accurate and repeatable work flows using imaging technology (e.g., Endler 1990, 2012; Akkaynak *et al.* 2014), which could aid in the design of algal sampling projects.

Given the potential value of photopigment variation data for our understanding of algal ecology and evolution, we advocate for the collection of pigment data to become a routine part of describing phenotypic variation in algae. Our method offers a way for these data to be rapidly and non-destructively collected from large amounts of material direct from the field or after laboratory cultivation. Estimating photopigment properties using comparative color data has been effective for studying coral reef health (e.g., Siebeck *et al.* 2006; Winters *et al.* 2009). Such techniques have formed the basis of large citizen science monitoring programs (e.g., coralwatch.org). Collecting similar data from field studies of algae would be valuable for generating initial hypotheses about the role of photopigment variation in ecological processes. In many cases, these data could help identify targets for subsequent sampling efforts with conventional pigment extraction techniques.

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Author contributions
WHR and SAKH framed hypotheses and experimental design; TB, GB, GW, FW, and SAKH performed field work; WHR, SH, MDC, FW and SAKH performed laboratory work; WHR, SH, MDC, CDA, and SAKH performed data analysis and interpretation; WHR and SAKH prepared the manuscript with help from all authors.

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