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Abstract

The fluorescent stain Nile Red has been used extensively for the quantification of lipids in phytoplankton, including microalgae, because it preferentially stains neutral lipids and it is economical and sensitive to use for screening purposes. Although its basic application has not changed for several decades, recent improvements have been made to improve its utility across applications. Here we describe additional refinements in its application and interpretation as a high-throughput method for the rapid quantification of neutral lipids in liquid cultures of marine phytoplankton. Specifically we address (1) inter-species comparisons, (2) fluorescence excitation and emission wavelengths and (3) the time-course of the Nile Red signal in the context of using bulk or cell-specific fluorescence to quantify neutral lipids of live or preserved cells. We show that with proper caution in its interpretation across species and physiological states that the quantity of lipid in hundreds of small volume samples can be reliably assessed daily using a refined Nile Red protocol.

Key Words

Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one); Fluorescent probe; Neutral lipids; Microalgae; Phytoplankton; High-throughput screen

79 **Abbreviations**

80 DAG, diacylglycerol; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol;
81 SQDG, sulfoquinovosyl diacylglycerol; PG, phosphatidylgycerol; PE, phosphatidylethanolamine;
82 PC, phosphatidylcholine; DGTS, diacylgycerol-N-trimethylhomoserine; DGTA, 1,2-diacylglyceryi-
83 O-2'-(hydroxymethyl)-(N,N,N-trimethyl)-β-alanine; DGCC, 1,2-diacylglyceryl-3-(O-
84 carboxyhydroxymethylcholine); TAG, triacylglycerol.

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1. Introduction

Along with proteins, carbohydrates and nucleic acids, lipids represent one of the four major biochemical components of cells and the ratio of these components varies widely among cell types, taxa and physiological state. In particular, the lipid composition of phytoplankton, including microalgae, is strongly sensitive to nutritional status (Marchetti et al. 2010; Shifrin and Chisholm 1980) and has been shown to vary greatly among different species of phytoplankton including microalgae (Ben-Amotz et al. 1985; Shifrin and Chisholm 1981). In addition to such basic research characterizing the quantity and classes of lipids, recently there is renewed interest in lipids from microalgae because of potential biotechnological uses; fast growth rates, lack of cellulosic cell wall in most species, and high lipid content make microalgae a top candidate for the production of biofuels and other bioproducts (DOE 2016; Gordon and Polle 2007; Greene et al. 2016; Huntley et al. 2015; Walsh et al. 2016; Williams and Laurens 2010). Towards this goal, rapid but accurate lipid quantification of microalgae is required (1) to identify which strains have the most desirable lipid profiles, (2) to identify the growth conditions that optimize the production of lipids and (3) to develop microalgae strains through genetic modifications that increase lipid production (Greenwell et al. 2009; Mcbrewster et al. 2009).

Direct extraction of total lipids is most commonly done with either the Folch method (Folch et al. 1957) using an 8:4:3 $CHCl_3$:MeOH:H_2O solvent mixture or the refined Bligh and Dyer method (Bligh and Dyer 1959) using a 1:1:0.8 $CHCl_3$:MeOH:H_2O solvent mixture to extract lipids from cells (or tissue). Both of these classic methods, which require the harvesting of dilute cells from their growth liquid medium via filtration or centrifugation, rely on solvent phase separation of the lipids; the organic (lower) phase is removed and concentrated by evaporation, leaving behind lipids. Total lipids may then be determined by gravimetry. While this approach is generally robust and has been

112 used for more than 50 years, but it is not compatible with screening efforts involving large numbers
113 of samples because of processing time involved with extraction. Further, relatively large volumes
114 (i.e. mass) of samples are required for quantification by gravimetry because the basis of the
115 measurements is mass difference between a sample and blank, and is therefore limited by the
116 precision of the balance. If specific lipid classes are of interest, other expensive and time consuming
117 chromatography (gas, high pressure liquid, thin layer, etc.), nuclear magnetic resonance, or mass
118 spectrometry based methods are required (Choi et al. 1993; Gurr and James 1980; Harvey 1991;
119 Holčapek et al. 2003).
120
121 Lipid-specific stains have been developed and offer an alternative to extraction based
122 protocols because they require neither large amounts of material nor solvent extractions. Nile Red
123 (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one) (Greenspan et al. 1985), LipidTox™
124 (Invitrogen™) and 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene-2,6-disulfonic
125 acid, disodium salt (BODIPY® 492/515 disulfonate Invitrogen™) are common fluorescent stains
126 used to characterize and quantify lipids in cells. Nile Red, which was originally developed as a
127 modification of Nile Blue, has an intense fluorescence signal and has been used as an economical
128 method to quantify the abundance and location of lipids in different cell types since its introduction
129 30 years ago (Greenspan et al. 1985). Because microalgae are naturally fluorescent, modifications by
130 Cooksey (Cooksey et al. 1987) broadened its general applicability for microalgae. Specifically,
131 fluorescence is quantitatively related to lipid content and highly correlated with both gravimetrically
132 determined total- and neutral lipids. This method has been further refined for the purposes of
133 screening microalgae in small volumes (Chen et al. 2009; Elsey et al. 2007), however as its
134 application has grown, additional uncertainties have been raised (Natunen et al. 2014; Rumin et al.
135 2015). Specifically, although the fluorescence intensity has been reported to increase in bound

versus free Nile Red (Cooksey et al. 1987; Greenspan et al. 1985; Pick and Rachutin-Zalogin 2012), there have been no reports documenting the relative excitation and emission spectra of bound and free Nile Red in aqueous phase even though this information is essential in selecting the proper wavelengths and optimizing fluorescence detection in the presence of a strong background fluorescence signal of phytoplankton. Also, recent advances in molecular level lipid class quantification allow a comprehensive comparison between Nile Red quantification and more laborious, but detailed solvent-based extraction protocols across different phytoplankton strains (Chen et al. 2009). Finally, recent reports show the importance of temporal variability of the Nile Red signal for intact model microalgae and their plasma membrane or lipid globules, suggesting a role of physiology and potentially taxonomy in modulating the Nile Red signal (Pick and Rachutin-Zalogin 2012). These considerations lead to a reassessment of the application and interpretation of the Nile Red technique for the rapid quantification of lipids in low biomass samples of phytoplankton reported here.

2. Materials and Methods

2.1 Cultivation and storage of phytoplankton samples

Marine phytoplankton cultures were grown in 40 mL Pyrex culture tubes or polystyrene tissue culture flasks using modified f/2 or H medium (Andersen 2005; Guillard and Ryther 1962) (100 μM NO_3^-, 36.3 μM PO_4^{3-}, 250 μM $SiOH_4$ (that was neutralized to pH = 7.0 prior to addition to medium (Mclachlan 1973)), f/20 metals, 0.2 g L^{-1} sodium bicarbonate). Cultures were grown at 30°C under high light (photosynthetically active radiation ~550 μmol quanta m^{-2} sec^{-1}) provided by cool-white fluorescent bulbs with a 14:10 h light:dark cycle. Additional marine phytoplankton cultures for lipid class experiments were grown in standard f/2 medium in 2 L Pyrex bottles

aseptically bubbled with air and stirred with a magnetic stir bar. These cultures were grown in the laboratory window using natural sunlight and at ambient laboratory temperatures of ~25°C. Phytoplankton cultures did not exceed 3 mM C biomass. The strains used in this part of the study were obtained from the University of Hawaii culture collection and represent a range of taxa and species that include: *Opephora* sp. C003, *Opephora* sp. C010, *Nanofrustulum* sp. C015, *Desmochloris* sp. C046, *Bacillariophyta* sp. C077, *Skeletonema* sp. C129, *Nitzschia* sp. C315, *Staurosira* sp. C323, *Chlorella* sp. C596, *Chlorella* sp. 599, *Thalassiosira* sp. C-D, *Chaetoceros* sp. MT16, *Chaetoceros* Ch60.

Cultures for flow cytometry were grown in 40 mL Pyrex tubes in a growth medium that consisted of Artificial Seawater (Goldman and Mccarthy 1978; Mclachlan 1973) with modified f/2 nutrients (100 μM NH_4, 36 μM PO_4, 106 μM $SiOH_4$, 1 mM TAPS, f/20 metals, f/2 vitamins). Cultures were grown at 25 ± 0.2 ºC with ~350 μmol quanta m^{-2} s^{-1} provided by cool-white fluorescent bulbs with a 14:10 h light:dark cycle. Three strains of marine phytoplankton including *Staurosira* sp. C323, *Chlorella* sp. C596 and one strain isolated from samples provided by the Sahara Forest Project in Qatar and identified as *Navicula sp.* based on microscopy observation. For all experiments, phytoplankton strains were harvested at different stages of their growth phase (e.g. exponential, early stationary, late stationary) to obtain populations that were in different stages of nutrition-stress, physiology and carbon allocation and therefore different lipid content (Bittar et al. 2013).

"Live" samples for Nile Red lipids were measured within 1 h after sub-sampling from culture flasks. "Frozen" samples of 2 mL culture were immediately frozen at either -80°C or -196°C in cryovials (VWR # 16001-102) until later analysis, typically within 1 week. No difference was detected between frozen storage at different temperatures. Longer term storage (up to a month)

also did not influence the results. "Preserved" samples were treated similarly to "Frozen", except the preservative glutaraldehyde (Tousimis #1057A) was added (0.125% final concentration) (Vaulot et al. 1989).

2.2 Nile Red solutions

Nile Red (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one) (Sigma # N2013) was dissolved (1 mg mL^{-1}) in either acetone or dimethyl sulfoxide (DMSO) (Greenspan and Fowler 1985; Greenspan et al. 1985; Rumin et al. 2015). Nile Red stock solutions were stored in air-tight Pyrex tubes at room temperature in the dark and made fresh weekly.

2.3 Nile Red Determined Lipids using Microplate Reader

Nile Red solution (1 mg mL^{-1}) was added to phytoplankton cultures, final concentration 10 μg Nile Red mL^{-1}, and thoroughly vortexed. Higher concentrations (up to 30 μg Nile Red mL^{-1}) did not significantly modify the signal. In pilot studies, the choice of solvent (acetone or DMSO) did not affect the magnitude or variability of the signal. Treated samples were placed into a black 96-well microplate (VWR #82050-728) (300 μL each well) and fluorescence quantified using a Synergy 4 multimode microplate reader equipped with a Xenon flash lamp. Fluorescence was measured every 5 min for a total of 30 min. For each microplate well, the peak fluorescence signal over this 30 min time period was used as the reported signal. Fluorescence was excited at 540 ± 12.5 nm (Synergy part #7082249) and emission measured at 590 ± 10 nm (Synergy part #7082225). To account for non-specific fluorescence, Nile Red signals were corrected by subtracting a medium blank with Nile Red and then subtracting the difference between the phytoplankton with solvent (with no Nile Red) and medium with solvent. Fluorescence excitation and emission spectra were

measured similarly except that excitation or emission was provided and measured in dual monochromator mode with 5 nm resolution.

To account for potential machine and stain variability, a secondary solid standard plate (Synergy part # 7092092) was run weekly to ensure machine stability. Nile Red stain efficiency and stability was verified with each microplate analysis using a standard solution made from Chemically Defined Lipid Concentrate (Invitrogen # 11905-031) preserved with 0.125% glutaraldehyde and stored in cryovials at -196°C (in liquid nitrogen) until each standard vial was sacrificed.

2.4 Total Lipids

Samples for lipid analysis (50–100 mL) were collected by filtration onto combusted 47-mm glass fiber filters (nominal porosity of 0.7 μm) and gently rinsed thrice with 0.5 M ammonium formate to remove external salts (Zhu and Lee 1997). Samples were stored at -80°C prior to express mail shipping on dry ice to the University of Hawaii. Upon arrival, samples were stored at -80°C until further analysis. Prior to extraction, the frozen sample filters were freeze-dried overnight using a bench top Labconco lyophilization unit, set at -40ºC and a vacuum of at least 0.06 mbar for a minimum of 12 hr. The samples were freeze-dried to eliminate the water barrier and allow organic extraction solvents to permeate the sample matrix during the following accelerated solvent extraction. A microscale analytical approach was developed for the determination of total lipid concentration as only small amounts of microalgae were available for analysis. The lipid extraction procedure is based on that described by Bligh and Dyer (Bligh and Dyer 1959). Total lipid concentrations are determined via gravimetric analysis following extraction using a DIONEX ASE 200 system and HPLC-grade solvents (VWR International). The ASE 200 flow-through solvent extraction system allows rapid extraction of up to 24 solid or semisolid organic matter samples using

11-mL stainless-steel extraction cells with independent control of temperature and pressure using organic or aqueous solvents. The sample extract and solvent mixture of each cell were pumped into pre-cleaned 40-mL glass extraction vials. The total lipid content of each sample was calculated by weighing the glass extraction vials before and after the accelerated solvent extraction.

The following steps were carried out in preparation for extraction. The pre-cleaned and uncapped extraction vials were equilibrated for 30 min in a rack using a timer. Following the equilibration, the vials were weighed on a five-place analytical balance (Mettler Toledo, XS105DU). The weight value (g) was recorded as pre-weight to 0.01 mg. Internal and external calibration was carried out daily. The DIONEX extraction cells were cleaned using a programmed cleaning cycle (pre-heat = 0 min, heat = 5 min, N2 pressure = 1500 psi, temperature = 60°C, static time = 0 min, flush % = 5, purge = 20 sec, cycle = 1, and solvents = 35% chloroform and 65% methanol. The 11-mL DIONEX extraction cells were lined with 19.88 mm DIONEX cellulose filters. The folded (in-half) sample filters were transferred individually in each cell with the crease facing the inside wall of the DIONEX cell. To ensure even contact of the sample material with the extracting solvents, the folded filters were carefully opened with forceps. Once the filter was positioned in the cell, Ottawa sand (20-30 mesh) was poured into the void volume up to 1-2 mm under the rim of the cell. Dispersing with an inert material allows increased permeation of the sample matrix, prevents sample adhesion and compaction and hence maximizes extraction efficiency. For each set (24 cells) we ran three combusted 47-mm GFF filters as blanks in non-sequential order. For the lipid extraction, DIONEX instrument settings were as follows: pre-heat = 0 min, heat = 5 min, N_2 pressure = 1500 psi, temperature = 60°C, static time = 5 min, flush % = 20, purge = 60 sec, cycle = 2, and solvents = 35% chloroform and 65% methanol.

Following the accelerated solvent extraction, the glass vials containing the sample extract were loaded in a Genevac EZ-2 evaporator for 1.75 hr at 40°C using the 'low BP mixture' setting to evaporate the contents of the pre-weighed extraction cells under vacuum to dryness, and then placed in a rack uncapped for a 30 min equilibration time. For gravimetric "total lipid" analysis, the weight of the dry residue in the collection vials was used to calculate the amount of lipids in the original sample. The average blank value was subtracted from the final weight. For storage and further analysis the dried lipid material was re-dissolved in 2 mL dichloromethane (HPLC-grade, Fisher Scientific) and transferred into 4-mL glass vials. One mL of dichloromethane was added twice to each sample and then sonicated for 30 sec, for a final volume of 2 mL. Once all samples were transferred into the 4-mL storage vials, they were purged with nitrogen gas for 10 sec and closed with Teflon caps. Each vial was sealed with Teflon tape and stored at -80ºC until further analysis.

The precision of the method was determined in triplicate on two different days by extracting a 47-mm GF/F filter plus 0 (blank), 10, 20, 30, 40 and 50 mg of dried Chlorella (SOLGAR #K-1250, broken cell-wall dietary supplement) in triplicate using the procedure described above. Blank values were subtracted and the two data sets were combined. The extraction efficiency was linear ($R2 = 0.987$) over this range of dry weights, and yielded a mean and SD of 17.4 ± 1.4 weight percent (WT %) total lipids (CV = 8.0%). This value is not statistically different ($P > 0.05$) from that (15.9 ± 0.4 WT% total lipids) determined for Chlorella samples run in parallel using a Folch et al. (1957) validated (Des Moines, IA; www.eurofins.com/) DIONEX macroscale protocol that requires an individual sample size of ~500 mg.

2.5 Neutral lipids

Neutral lipids in the lipid extracts (see above) were determined by high-performance liquid chromatography (HPLC) coupled with evaporative light scattering detection (ELSD) using a

modified version of the Silversand and Haux (1997) procedure (Silversand and Haux 1997). Lipid extracts (50 µ L) were injected onto an Agilent Technologies 1200 HPLC system (left and right column temperatures: 30µ C, flow rate: 1 mL min-1) equipped with a Supelguard LC-DIOL guard column (4 x 20 mm, 5 µm particle size), a Supelguard LC-DIOL analytical column (4 x 250 mm, 5 µm particle size), and a Varian 385-LC ELSD (evaporator temperature: 35°C, nebulizer temperature: 35°C, N2 flow rate = 1.5 SLM). A binary solvent system was employed for the separation of neutral lipids: eluent A (hexane:acetic acid, 99:1, v:v) and eluent B (hexane:2-propanol:acetic acid, 84:15:1, v:v:v). The following solvent gradient program was used for analyte separation: t = 0 min (100% A), t = 6 min (30% A, 70%B), t = 7 min (100% B), t = 9 min (100% B), t = 11 min (100% A), and t = 14 min (100% A). Eluting peaks were identified by comparing their retention times (Rt, min) with those of standards obtained from Sigma-Aldrich: monoolein, 1,2-diolein, 1,3-diolein, and triolein mix (1787-1AMP, certified reference material) and cholesterol (C8667). Neutral lipids were quantified by peak area (EZChrom Elite software) and expressed as percentage of total peak area. Triacylglycerols (TAGs) were quantified using C18:1 TAG (triolein, Sigma-Aldrich, T7140) as a reference standard over a concentration working range of 0.48 - 2.86 µg injection-1. The on-site precision for this method (2.1% RSD; n = 30, over > 15 days) was established using a diatom reference material (Staurosira sp.) prepared in 1-g quantities by High-Purity Standards (Charleston, SC; www.highpuritystandards.com/).

2.6 Lipids Classes

Twelve samples were selected for molecular-level lipid analyses by HPLC mass spectrometry (HPLC/MS). Lipids were extracted from phytoplankton filtered onto glass fiber filters (VWR, Grade 691) which were extracted using a modified Bligh and Dyer solvent extraction protocol (Bligh and Dyer 1959). Briefly, filters were immersed in dichloromethane, methanol, and phosphate-

buffered saline (PBS) in a ratio of 10:20:8, sonicated for 15 min, and let to stand overnight under nitrogen at -20°C. The next day, dichloromethane and water were added in a ratio of 10:10; samples were centrifuged for 10 minutes, and the organic phase was removed and concentrated as the total lipid extract; this step was repeated twice more. Phosphatidylethanolamine-N-(2,4-dinitrophenyl) (DNP-PE; Avanti Polar Lipids) and triheptadecenoin (17:1/17:1/17:1, NuCheckPrep) were added as internal standards at the first step of the extraction.

Total lipid extracts were analyzed for polar-diacylglyceride lipids (polar-DAGs) by using HPLC/electrospray ionization mass spectrometry (HPLC/ESI-MS) as described by Sturt et al. (2004), Van Mooy et al. (2006, 2009) and Levitan et al. (2015) on an Agilent 1100 HPLC and Thermo LCQ Deca XP ion trap MS (Levitan et al. 2015; Sturt et al. 2004; Van Mooy et al. 2009; Van Mooy et al. 2006). The molecular ion chromatograms were extracted for each individual IP-DAG species at their appropriate retention time, integrated, and applied to external standard curves. The standard curves were composed of triplicate measurements at four concentrations of the following IP-DAGs: the recovery standard, DNP-PE; PG, PE, and PC, which were obtained from Avanti Polar Lipids Inc; MGDG and DGDG, which were obtained from Matreya, LLC; SQDG and DGTS, which were isolated from phosphorus-starved cultures of *Synechococcus* WH8102 (kindly provided by E.A. Webb, University of Southern California) and *Chaetoceros gracilis* (kindly provided by S. Dyhrman, WHOI), respectively, by using preparative HPLC (Van Mooy et al. 2009); DGTS was applied as the standard for DGTA and DGCC (Van Mooy et al. 2009), since none of the betaine lipids are commercially available.

Total extracts were analyzed for TAGs and DAGs by using reversed-phase non-aqueous HPLC/atmospheric pressure chemical ionization mass spectrometry (NARP-HPLC/APCI-MS) on

an Agilent 1200 HPLC and Agilent 1200 single quadrupole MS. We identified 31 distinct TAGs by their [M+H]+ and [M+H-ROOCH]+ mass spectra using previously described identification protocols (Holčapek et al. 2003; Holčapek et al. 2005). External standard curves were composed of triplicate measurements of the following TAGs at five concentrations: trihexadecenoin (16:1/16:1/16:1), triheptadecenoin (17:1/17:1/17:1; recovery standard), trioctadecenoin (18:1/18:1/18:1), trioctadecatrienoin (18:3/18:3/18:3), and trieicosapentaenoin (20:5/20:5/20:5). Response factors for the TAG standards were linearly regressed against both retention time (r^2 = 0.94) and equivalent carbon number (ECN = acyl carbon atoms-2(acyl double bonds); r^2 = 0.91). Response factors for unknown TAGs were calculated from the regression against retention time, and then used to determine the concentrations of the unknown TAGs. DAGs were observed in a few samples, and these were quantified as TAGs.

2.7 Microscopy

Phytoplankton cultures for lipid observation and localization were placed in flat bottom 24 place cell wells (Becton Dickinson # 351147). Nile Red solution (0.1 mg mL^{-1}) in acetone was added to cultures for a final concentration of 1 μg Nile Red mL^{-1}. Cultures were allowed to stain for ~5 min before observation. Cells were viewed with a 100 W epifluorescense Olympus IX71 inverted microscope, and pictures were acquired with a MacroFire digital camera and PictureFrame 2.3 software. Cells were initially viewed with a filter set typical for chlorophyll observation (excitation (ex) 460-490 nm, dichroic mirror = 500 nm, emission (em) >520 nm) in which lipid bodies stained a bright yellowish-green in contrast to the red autofluorescence of the chlorophyll. Several other combinations of excitation and emission filters were examined, designed to target both neutral and polar Nile Red stained lipid bodies. For comparison with the microplate method, cultures were

viewed with a similar filter combination (ex. 546 ± 13 nm, DM = 560 nm, em. 585 ± 20 nm) and yellow fluorescing neutral lipid bodies accounted for the majority of the signal in most strains.

2.8 Flow cytometry

Cellular Nile Red fluorescence was quantified with a FACSCalibur flow cytometer using argon-ion laser excitation at 488 nm and an emission wavelength of 585 ± 42 nm (FL2 channel) as previously described (Bittar et al. 2013; Johnson et al. 2010). Both live and preserved samples were measured. Preserved samples were stored at -80 ºC and measured within two days of the live samples. Samples for flow cytometry were stained with Nile Red to a final concentration of 6 μg L^{-1} and vortexed, then kept in the dark. Aliquots were taken from the sample and measured with the flow cytometer at 5-minute intervals from 0 to 30 minutes, with the sample vortexed before each interval. Fluorescent microspheres (Fluoresbrite Microspheres, Polysciences, Inc.) were added as internal fluorescence standards. Measurement duration lasted an average of 40 seconds. Unstained cells were also used to check that no background fluorescence occurred in the same range as Nile Red fluorescence. When comparing microplate reader and flow cytometer results directly, flow cytometrically determined cell concentrations and Nile Red fluorescence per cell were multiplied to calculate the total fluorescence of a given culture and compared to bulk estimates from the microplate reader.

3. Results

The Nile Red stain was initially examined using qualitative analyses to determine the localization of the staining within lipid-containing marine microalgal cells. Using a broad excitation and emission filter set for chlorophyll *a* fluorescence optimized for visual inspection, cells that are

large and have defined organelles and cellular bodies show that the majority of Nile Red fluorescence occurs in defined regions of the cells (e.g. Figure 1A). This specificity of staining is also apparent when using excitation and emission filters that have been optimized for neutral lipid (i.e. TAGs) bound Nile Red fluorescence detection (Figure 1B). Cells that were too small to observe specific sub-cellular features using standard transmission microscopy did not show any specific regions of staining, but still stained and fluoresced similarly. When viewed with the appropriate filter sets, the sub-cellular patterns of staining of Nile Red were nearly identical to the patterns observed with other commercially available lipid stains that target neutral lipids such as LipidTox™ and BODIPY® and are consistent with specific staining of lipid vesicle within the cells.

To determine the influence of non-specific fluorescence in high-salt aqueous phase (i.e. saltwater medium) across wavelengths, excitation and emission spectra were measured for a variety of microalgae cultures and medium controls in the presence and absence of the Nile Red stain (Figure 2). Excitation spectra (with emission measured at 590 nm, the peak of triacylglyceride-bound fluorescence) of stained microalgae show a significant excitation peak at 530 nm while unstained cells and stained and unstained medium controls are all 3 orders of magnitude below this signal (Figure 2 top panel) demonstrating that the contribution of non-specific fluorescence is not dependent on the excitation wavelength. Emission spectra (with excitation provided at 545 nm) of stained microalgae have broad peaks at 660 nm (Figure 2 middle panel) that are substantially longer the 590 nm peak expected for pure Nile Red bound-triacylglyceride (Invitrogen). But unlike excitation spectra, emission spectra of the stained medium (i.e. no cells) control also have a significant broad peak at ~660 nm. Further, unstained microalgae have a minor fluorescence peak at ~680 nm that corresponds to the presence of chlorophyll *a* (Jeffrey et al. 1997) while unstained medium does not have any significant emission fluorescence. Taken together, the excitation spectra

corrected for the presence of natural (i.e. unstained) fluorescence and non-specific fluorescence (i.e. Nile Red stained medium) are essentially identical to the raw Nile Red-stained cell cultures (Figure 2 bottom panel) and have one major excitation peak at 530 nm. However, correcting for Nile Red stained medium and, to a lesser degree natural fluorescence of microalgae, significantly changes the dominant peak of the emission wavelength spectra to ~590 nm. This wavelength is significantly lower than the raw Nile Red emission peak wavelength at ~660 nm, but has the least interference from non-specific Nile Red fluorescence; it also more closely matches the emission peak of Nile Red bound to triacylglycerides. Thus, previous reports that show emission maxima at wavelengths >600 nm could be influenced by the presence of significant background fluorescence of Nile Red (i.e. unbound stain) in the aqueous phase (Cooksey et al. 1987) and potentially misinterpreted to indicate high levels of phospholipids, which has ~50-75 nm red-shifted excitation and emission peaks for Nile Red. This may be of particular importance when using marine microalgae because of the presence of high levels of salt in the medium. Based on the peak-corrected excitation and emission wavelengths, the availability of filters for microplate readers and the desire to target neutral lipids (i.e. triacylglycerides), an excitation of 540 ± 12.5 nm and emission of 590 ± 10 nm were used for further plate reader based assays.

Others have reported significant variability in the fluorescence signal of Nile Red as a function of time (Chen et al. 2009; Cooksey et al. 1987; Natunen et al. 2014) and therefore fluorescence is routinely measured over the course of 30 min or more (Chen et al. 2009; Elsey et al. 2007). The dynamics of these changes are variable and influenced by multiple factors including microalgae taxa, physiological conditions, and composition of extracted lipids that together result in fluorescence maxima from 0 to >30 min after the addition of Nile Red (e.g. Fig 3). For the majority (78%) of more than 500 assays representing numerous strains, physiological conditions and

presumably cellular biochemical composition, there is a time delay between the addition of Nile Red and the fluorescence maximum (Fig 3). The subsequent decreases over time were not due to the decay associated with the measurement process (i.e. photobleaching) as the decrease was independent of the number of times Nile Red fluorescence was quantified during the time course. To further investigate these temporal patterns, we compared standard time-course measurements from bulk estimates (i.e. microplate reader based, e.g. Figure 3) to integrated cell-specific Nile Red fluorescence (i.e. flow cytometery) (Figure 4) for both fresh and preserved samples. Although some differences exist in the relative fluorescence between methods as well as in the timing of the fluorescence maxima, perhaps due to differences in the temporal precision, overall bulk and cell-specific techniques show similar trends suggesting that the bulk signals are quantifying cellular properties (and not medium or other non-specific extracellular binding artifacts).

While the bulk and cell-specific approaches show similar temporal trends, there are notable differences between some of the live and preserved samples, which can be different between the strains. For example, live *Chlorella* had fluorescence maxima near time zero, whereas preserved *Chlorella* had fluorescence maxima that were more stable in the first 15 min (Figure 4). *Chlorella* also had the greatest temporal dynamics (specifically for fresh samples), which are driven in part by two populations of cells (stained and unstained) that begin to emerge after 5 min (Figure 4). Preserving the cells prevented formation of two distinct populations, dramatically reducing these temporal dynamics. Conversely, *Staurosira* was relatively constant over the time course, regardless of treatment and did not have multiple populations. Finally, *Navicula* also had substantial temporal dynamics that were greatly reduced when the cells were preserved. However, unlike for *Chlorella*, the patterns for *Navicula* are not driven by heterogeneous cellular responses (i.e. multiple populations), but rather consistent increases and decreases among the cells (Fig 5). Thus, temporal dynamics

446 observed for bulk Nile Red signals can be manifest by both changes in population frequencies (i.e.
447 either stained or unstained such as with *Chlorella*) or from changes in the relative efficiency of the
448 stain such as with *Navicula*.
449
450 We compared our refined Nile Red protocol that monitors the temporal dynamics, which
451 includes monitoring the temporal dynamics and selecting wavelengths optimized for detection, to a
452 recently developed high precision technique for quantifying lipid classes. As expected, an individual
453 strain grown under a variety of environmental conditions and sampled at different points in the
454 growth curve to maximize the biochemical heterogeneity, had substantial variability in its relative
455 total and TAG lipid contents (Figure 5). Moreover, Nile Red fluorescence was highly correlated
456 with both total and TAG lipid content ($r = 0.94$, $n = 32$, $p < 0.01$; $r = 0.97$, $n = 32$, $p < 0.01$,
457 respectively). This relationship was investigated across numerous strains and showed that Nile Red
458 fluorescence is positively correlated with total cellular lipids quantified by gravimetry (Figure 6), but
459 with reduced fidelity ($r = 0.76$, $n = 57$, $p < 0.01$) compared to the relationship for an individual
460 strain. Similarly, Nile Red fluorescence across strains are most strongly correlated with the TAG
461 lipids ($r = 0.84$, $n = 57$, $p < 0.01$). However, Nile Red lipids were poorly correlated ($r = 0.45$, $n =$
462 57, $p < 0.01$) with the non-TAG lipids suggesting that the increased variability across species in the
463 Nile Red – total lipids relationship was due, in part, to differences in these non-TAG lipids.
464
465 This relationship was investigated in more detail by quantifying the molecular lipid classes of
466 selected strains (Table 1). As expected, the concentration of the various specific lipid classes varies
467 widely, but is mostly dominated by TAGs (Table 2). In general, Nile Red fluorescence is most
468 highly correlated with the most abundant TAG classes including 16:1/16:1/20:5, 14:0/16:1/20:5,
469 16:1/16:0/20:5, 16:1/16:1/16:1, 16:1/16:1/14:0, 16:0/18:0/20:4, 16:1/16:0/16:1 and

16:0/16:1/14:0. Another abundant TAG (16:0/16:1/16:0) is not strongly correlated with Nile Red (but this low correlation is driven by one point and would otherwise be >0.85). Other polar and non-polar diacylglycerols (DAGs) are also not significantly correlated with Nile Red fluorescence. Nile Red fluorescence measured on live, frozen or preserved samples generally showed the same correlations with the various lipid classes and there were no statistically significant differences between the treatments as an ensemble, even though there are differences among some of the molecular lipid classes. Summing the specific lipid classes into major categories of polar-DAGs, non-polar DAGs, TAGs or total gravimetric lipids and comparing to Nile Red fluorescence shows similar patterns to the larger data set using higher throughput approaches (Figure 6). Overall there are no statistically significant differences between Nile Red fluorescence and major lipid classes for samples that were live, frozen or preserved (Table 2). In summary, Nile Red fluorescence is most strongly correlated with TAGs and less strongly correlated with other lipid components, and is in bulk unaffected by common sample preservation methods.

4. Discussion

Here we show that non-specific binding of the Nile Red stain can lead to substantial fluorescence emission at longer wavelengths. In particular, in a high ionic strength water matrix (such as the saltwater used here or standard laboratory cell buffers like phosphate buffer saline) the majority of the fluorescence emission signal above 600 nm is from unbound Nile Red and therefore not quantitative for lipids. Proper selection of wavelengths for lipid quantification (i.e. neutral vs. polar), or consideration of blanks and background corrections are essential.

Upon Nile Red addition to microalgae suspensions, the fluorescence intensity generally increased over time to a maximum and then decreased (Fig 3B) – most (65%) of the temporal peaks

494 in Nile Red fluorescence occurred between 5 and 25 min with an additional 22% at time zero.
495 These temporal patterns occur with both live and dead (frozen or preserved) for bulk suspensions or
496 cell-specific metrics (Fig 4). However, for some strains these dynamics are modified (or attenuated)
497 by preservation suggesting that the temporal patterns are related to a combination of active and
498 passive cellular processes (Figure 4). While the preservation of microalgae prior to dye addition
499 does not dramatically affect the interpretation of bulk Nile Red fluorescence for major lipid class
500 contents (e.g. Tables 1 and 2), cell-specific results can be impacted (e.g. Figure 4 – *Chlorella sp.*)
501 highlighting the importance of maintaining observations over a period of time and monitoring for
502 population heterogeneity. (In microtiter plates used here, cell settling will not affect the fluorescence
503 intensity since all contents of each well are equally quantified, regardless of their position within the
504 cell well.) Others have observed this temporal variability (Pick and Rachutin-Zalogin 2012)
505 including bi-modal peaks (Elsey et al. 2007) (which we did not observe) and shown that it can be
506 driven by a combination of biotic (e.g. active dye pumping) and abiotic factors (e.g. diffusion; dye-
507 dye or dye-matrix interactions). Beyond the specific mechanisms responsible for this temporal
508 variability, for practical purposes much of this variability can be reduced by preserving the cells and
509 monitoring fluorescence intensity over time, without the need for lipid separation via density
510 gradients or solvent extraction (Pick and Rachutin-Zalogin 2012).
511
512 Regardless of the wavelength pair used to visualize fluorescence, the majority of the Nile
513 Red signal originates from specific sub-cellular locations (namely the lipid vesicles) of the microalgae
514 (Figure 1). These lipid vacuoles, which are essentially 100% lipid, are comprised of specific types of
515 lipids, and in most strains Nile Red is targeting these lipids more strongly than other common
516 cellular lipids such as membrane associated lipids (phospholipids, glycolipids and sterols) or other
517 structural features. Cells that have been subject to various disruptions including freeze/thaw or

518 ultrasonic disruptions can show different staining patterns suggesting that this localization can be
519 altered by compromising sub-cellular membranes or by altering the fluidity permeability and binding
520 sites for Nile Red (Dempster and Sommerfeld 1998). This is evident in some differences among the
521 live, frozen and preserved samples in specific molecular classes of lipid (Table 1). Nevertheless, the
522 overall bulk Nile Red – lipid class relationship holds regardless of treatment (Table 2).
523
524 Consistent with the localization of stain within the cell, the Nile Red signal is most highly
525 correlated with neutral lipids and specifically TAGs within the cell (Tables 1 and 2). These lipid
526 classes are the dominant energy storage lipids within the cell. Other major structural lipids which are
527 present such as the polar glycolipids, are not well-quantified by Nile Red, making the stain a good
528 candidate for targeting the neutral lipids without significant interference from other potentially
529 abundant lipids. Using this approach there is a robust relationship between Nile Red fluorescence
530 and neutral lipids for a given strain (Figure 5). While this relationship is less strong across strains,
531 due to differences in cell lipid composition (Table 1) and other biological factors (e.g. Figure 4), Nile
532 Red is still sufficiently robust to identify taxa with elevated TAGs without the need to optimize
533 solvents or additives (Natunen et al. 2014). In turn, the conditions that optimize neutral lipids in
534 these candidate strains can then be more precisely defined because of the robust intraspecific Nile
535 Red-neutral lipid correlation. The Nile Red method to quantify neutral lipids in microalgae in
536 solution provides a rapid screening tool for comparing the lipid quantity of hundreds of samples in a
537 day. Because of the low volume (<300 μL per assay well) and biomass requirements (< 3 mM C),
538 numerous cell lines can be assayed in small volume microtiter plates. Moreover, the same cell lines
539 can be grown under a factorial of environmental conditions (temperature, light, growth medium,
540 etc.) that cause variation to identify conditions that optimize lipid profiles (Bittar et al. 2013). This
541 screening approach may be of particular use in comparing or developing (through genetic

modification by either random or targeted mutagenesis) microalgae strains for next generation biofuels where neutral lipids (i.e. TAGs) are of particular interest and where there is great potential for increasing lipid production through changes in environmental conditions or strain modification (Doe 2016; Georgianna and Mayfield 2012; Williams and Laurens 2010).

5. Research Needs

Nile Red has been used to quantify cellular lipids for ~30 years (Greenspan et al. 1985) and improvements over time have increased its utility for specific types of cell lines including microalgae (Chen et al. 2009; Cooksey et al. 1987; Elsey et al. 2007). Yet because of its increased use in the screening for lipids of collections of microalgae, additional refinements in protocols and interpretation may be necessary for broader application. In particular, the differences observed between the microalgae strains (e.g. Figure 4) point to additional areas where protocols can be refined to overcome the likely fundamental differences in biology. Comparing the responses of other lipid binding stains (e.g. BODIPY, LipidTox) to Nile Red in the context of temporal, live/dead and strain to strain variability may provide insight into the mechanisms of these varied responses. Finding solutions or work-arounds to these challenges will further enhance the utility of Nile Red as a tool for screening marine microalgae for lipids.

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673
674
675

676 Figure Legends:

677

678 Fig 1: Localization of Nile Red stain in microalgae. (A) Epifluorescence image using typical
679 chlorophyll filter set. Yellow bodies are stained lipid droplets and red hue is chlorophyll (auto)
680 fluorescence. (B) Epifluorescence image using narrow excitation and emission filters targeting
681 bound neutral lipid fluorescence and optimized for microscopy. Note the localization of the stain in
682 both images to the lipid droplets.

683

684 Figure 2. Representative excitation and emission spectra of control and Nile Red stained medium
685 and cells. Top panel: excitation spectra (emission 590 nm) with stained cells (black solid), unstained
686 cells control (black dotted), stained medium control (red solid) and unstained medium control (red
687 dotted). Middle panel: emission spectra (excitation 545 nm) (lines as top panel). Bottom panel:
688 excitation (blue line) and emission (green line) spectra corrected for natural fluorescence of cells and
689 medium and non-specific binding.

690

691 Figure 3: (A) Representative temporal variability in the Nile Red fluorescence for microalgae. Error
692 bars (standard error, n = 24) are smaller than the symbols. (B) Fraction of observations (n = 515,
693 triplicate analyses) across different strains or physiological conditions with Nile Red fluorescence
694 maxima at a given time point.

695

696 Figure 4: Temporal variability of fluorescence per cell (left column), mean cellular fluorescence
697 (middle column) or microplate reader (bulk) (right column) with live (upper row) or preserved
698 (lower row) cells for *Chlorella* sp. C596 (top box), *Staurosira* sp. C323 (middle box), and *Navicula* sp.

699 SFP (lower box). Each curve is normalized to its maximum value. Histograms represent composite
700 population values. Means and standard error bars are for n≥9 technical replicates.
701
702 Figure 5: Relationship between Nile Red fluorescence vs. Total Lipids and TAGs for *Desmodesmus sp.*
703 C046.
704
705 Figure 6: Relationships between Nile Red fluorescence and total, non-TAGs and TAGs lipids for a
706 range of microalgae taxa poised at different physiological states.

707 **Table 1**: Correlation between Nile Red fluorescence and molecular lipid classes. Statistically
708 significant correlations (n=5, $p < 0.05$) are in bold.

709
710

Lipid Class			% Total[a]	CV (%)	r-live	r-frozen	r-preserved
Polar –DAGs							
	Glycolipids						
		MGDG	5.6	145	0.10	0.26	0.47
		SQDG	2.6	54	0.00	0.00	0.24
		DGDG	1.8	117	0.32	**0.96**	0.72
	Phospholipids						
		PC	2.0	137	0.46	**0.96**	0.71
		PG	1.4	91	0.36	0.74	0.36
		PE	0.5	88	0.22	0.39	0.00
	Betaine Lipids						
		DGCC	1.1	129	0.77	**0.97**	0.73
		DGTS	0.1	129	0.69	0.61	0.79
		DGTA	0.1	183	0.66	0.56	0.74
Non-Polar							
DAGs		20:0/20:0	0.6	224	0.14	n/a	n/a
		20:1/20:1	0.3	139	0.41	0.48	0.20
TAGs							
		16:1/16:0/16:1	9.7	79	**0.93**	**0.95**	**0.98**
		16:0/16:1/16:0	8.0	70	0.48	0.36	0.76
		16:1/16:1/14:0	7.4	132	**0.90**	**0.97**	0.75
		16:0/16:1/14:0	7.0	111	**0.89**	**0.98**	0.77
		16:1/16:0/20:5	4.3	77	**0.97**	**0.99**	**0.85**
		16:1/16:1/16:1	4.3	117	**0.87**	**0.99**	**0.82**
		16:0/18:2/20:4	4.2	136	**0.84**	**0.97**	0.74
		14:0/16:1/20:5	3.9	145	**0.92**	**0.96**	0.73
		16:1/16:1/20:5	3.8	130	**0.95**	**0.98**	0.77
		Unidentified TAGs	3.0	193	**0.86**	**0.97**	0.73
		16:1/16:1/20:4	2.1	121	**0.85**	**0.99**	0.80
		16:1/14:0/20:4	1.8	136	0.73	**0.97**	0.73
		16:0/16:0/20:5	1.3	63	0.45	0.57	0.76
		16:0/16:1/20:4	0.8	150	0.22	0.30	0.17
		16:0/16:0/20:4	0.6	94	0.35	0.30	0.71
		18:2/18:2/18:3	0.5	121	**0.92**	**0.97**	0.76
		14:0/14:0/18:2 + 14:0/14:0/17:1	0.5	147	**0.92**	**0.97**	0.74
		16:0/14:0/20:5	0.4	174	0.73	**0.91**	0.62
		18:3/16:0/16:1	0.4	176	**0.87**	**0.99**	**0.84**
		18:1/16:0/16:1	0.3	105	**0.98**	**0.96**	**0.98**
		20:4/16:0/20:4	0.2	120	0.00	0.39	0.00
		20:5/16:0/20:5	0.1	159	0.57	0.51	0.50
		17:1/17:0/14:0	0.1	154	0.14	0.10	0.39
		18:0/16:0/16:1	0.1	224	0.00	0.10	0.39
		16:0/16:0/16:0	0.1	224	0.00	0.10	0.39

711
712 [a] Percent of lipid class normalized to gravimetrically measured lipids, averaged across strains and
713 replicates.
714

715 **Table 2**: Correlation between Nile Red fluorescence and lipid class totals for five microalgae strains.

716 Statistically significant correlations (n=5, $p < 0.05$) are in bold.

717

Lipid Class	% Total	CV (%)	r-live	r-frozen	r-preserved
Polar –DAGs	15.1	110	0.24	0.60	0.24
Non-polar DAGs	0.3	139	0.41	0.48	0.20
TAGs	65.1	95	**0.97**	**0.99**	**0.88**
Gravimetric Lipids	100.0	58	**0.95**	**0.99**	**0.87**

718