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Nile Red assay development for the estimation of neutral lipids in *Chlorella emersonii* and *Pseudokirchneriella subcapitata*

Priyanka Priyanka[1], Gemma K. Kinsella[1], Gary T. Henehan[1], Barry Ryan[1*]

Abstract

Fluorescent dyes offer a useful method for the measurement of intracellular lipids. They are inexpensive and require simple optical measurement instrumentation, whilst simultaneously providing high throughput application. Nile Red is a hydrophobic, metachromatic dye which has been widely used for detection of intracellular lipids. However, Nile Red fluorescence depends on its concentration, microenvironment polarity, incubation time and, therefore, requires strain specific optimization. Hence, neutral lipids in *Chlorella emersonii* and *Pseudokirchneriella subcapitata* cannot be quantified using existing Nile Red methods developed for other microalgae strains and, therefore an optimised procedure for these strains is required. In this method development, the optimal excitation and emission wavelengths were selected based on the solvent used for Nile Red dissolution. The effect of Nile Red concentration, microalgae cell concentration, incubation time on fluorescence intensity was explored and optimised. Quintuplet assay repeats were executed for increased assay robustness for two microalgae strains, *Chlorella emersonii* and *Pseudokirchneriella subcapitata*, with protocol reliability confirmed by fluorescence microscopy. In brief, 20% (v/v) DMSO containing 10µg/ml and 5µg/ml Nile red was found to be ideal concentration for neutral lipid estimation in *Chlorella emersonii* and *Pseudokirchneriella subcapitata* respectively when an incubation time of 60mins and 40mins at 40°C was used. This optimised Nile Red protocol is a robust, simple and cost-effective method for neutral lipid quantification in *Chlorella emersonii* and *Pseudokirchneriella subcapitata*.

[1]School of Food Science and Environmental Health, College of Sciences and Health, Technological University Dublin, Dublin, Ireland

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Introduction

Spectral properties (excitation and emission characteristics) of Nile Red are highly dependent on the microenvironment polarity (1, 2). The peak emission of Nile Red is blue shifted as the surrounding polarity decreases, however, it shifts to red with an increase in microenvironment polarity (3,4,5). To enhance neutral lipid staining by Nile Red, solvents such as acetone, dimethylsulfoxide (DMSO), ethanol, isopropanol, hexane or chloroform have been used; with DMSO and acetone being the most commonly used solvents (Cooksey et al., 1987b; Chen et al., 2009; Satpati and Pal, 2015). Intracellular neutral lipids can be detected via a Nile Red fluorescence signal with a maximum emission at 570-580 nm. By choosing an excitation/emission setup of 480/570–580 nm, Nile Red was successfully used for staining neutral lipids from *Chlorella vulgaris* under nutrient starvation conditions (Morschett, Wiechert and Oldiges, 2016). In addition to the variability generated from hydrophobic environments, Nile Red fluorescence parameters also vary for microalgae species (6, 7, 8, 9). As the composition and structure of the microalgal cell wall varies, the time and Nile Red concentration required to generate optimal fluorescence varies too. Robust and thick cell walls (particularly in green algae and nutrient starved microalgae) act as a barrier; preventing efficient penetration of Nile Red in cells and neutral lipid staining and, therefore, increased staining time is required (10). Even after the establishment of appropriate staining conditions (e.g. dye concentration, tem-

perature, organic solvents) for a microalgae strain, the ability to measure fluorescence intensity remains dependent on the cell concentration used during the assay. A decrease in fluorescence (or saturation) for neutral lipid staining has been reported below and above the threshold of cell concentration at fixed Nile Red concentrations. Therefore, considering all the parameters that can affect the final neutral lipid quantification using Nile Red staining, it is important to establish an assay which is robust enough to addresses these factors. Here, for quantification of neutral lipids, such a standard Nile Red assay development, and optimisation, is detailed for *Chlorella emersonii* and *Pseudokirchneriella subcapitata*.

Materials and Methods

Culturing of microalgae strains

Bold basal medium, with 3-fold nitrogen and vitamins (3N-BBM+V modified) was prepared by mixing the components detailed in Table 1. The trace element solution mentioned in Table 1 was prepared by addition of chemicals outlined in Table 2 in the exact same order.

Stock solutions of Vitamin B1 (Thiaminhydrochloride) and Vitamin B12 (Cyanocobalamin) were prepared as follows; 0.12g of Vitamin B1 was dissolved in 100ml of ddH2O. 1ml of this Vitamin B1 was further diluted into 99ml ddH2O. For Vitamin B12 stock; 0.1g of Vitamin B12 was dissolved in 100ml of ddH2O. Both working stocks of vitamin B1 and B12 were filter sterilized through a 0.2µm sterile filter and stored at -20°C until required for use. Cultures were grown in 3N-BBM+V media at 18°C with 16h:8h (light:dark) cycle at 120rpm. Cell growth from was checked at O.D 590nm, and fixed number of cells (1 O.D@590nm = 107 cells/ml) were transferred into nitrogen deficient media (3N-BBM+V media, without NaNO3). Cells were maintained in the same nitrogen deficient media without any further supplementation for 7days at 18°C with 16h:8h (light:dark) cycle at 120rpm. After 7days, cells were harvested and were used for neutral lipid quantification.

Preparation of microalgae cell pellet

Table 1. Composition of 3N-BBM+V media for the synthesis of microalgal biomass

Chemical	MW (g/mol)	Stock solution (g/L)	Volume (ml) required for 1L medium	Final conc. (g/L)
$NaNO_3$	84.99	25	30	0.750
$CaCl_2$. $2H_2O$	110.98	2.5	10	0.025
$MgSO_4.7H_2O$	246.47	7.5	10	0.075
$K2HPO_4.3H_2O$	228.22	7.5	10	0.075
KH_2PO_4	136.09	17.5	10	0.175
NaCl	58.4	2.5	10	0.025
Trace element solution			6	
Vitamin B1			1	
Vitamin B12			1	

Table 2. Composition of trace element solution

Chemical	MW (g/mol)	mg/L
Na_2EDTA	372.24	750
$FeCl_3.6H_2O$	270.3	97
$MnCl_2.4H_2O$	197.91	41
$ZnCl_2$	136.286	5
$CoCl_2.6H_2O$	237.93	2
$Na_2MoO_4.2H_2O$	241.95	4

After 7 days of incubation in nitrogen deficient media; 10ml of culture was removed aseptically to a universal tube. The culture was centrifuged at 5,000*g for 5mins and the culture media (supernatant) was discarded. The microalgal cell pellet was later mixed thoroughly in 10ml of double distilled water. 50µl of this water dissolved pellet was used as one assay aliquot. Into a flat bottom, transparent 96-well microtiter plate, 100µl of double distilled water was added in quintuplet to which 50µl of microalgae culture prepared earlier was added. These diluted microalgae cultures were used for further assay development.

Selection of solvent and wavelength for excitation and emission

As based on Chen, and Huang and colleagues, (7 and 11) 10µg/ml of Nile Red solution was prepared over a concentration range (10-60% v/v) of the solvents examined (DMSO and acetone). To 150µl of diluted microalgae culture, 100µl of 10% (v/v) Nile Red prepared in DMSO was added in quintuplet. The process was repeated for all the solvent concentrations (10-60% v/v DMSO and acetone) containing 10µg/ml Nile red. The sample (ddH2O+microalgae+Nile Red) was incubated at room temperature in the dark for 30mins before monitoring the fluorescence intensity using a Perkin Elmer Lambda 900 UV/VIS/NIR Spectrometer at various excitation wavelengths (485nm-530nm) and at an emission wavelength of 580nm.

Screening Nile Red concentration

To identify the concentration of Nile Red required to obtain the maximum fluorescence intensity; different concentrations of Nile Red, ranging between 1µg/ml to 50µg/ml were prepared in 20% (v/v) DMSO. 100µl of this solution was added to 150µl of diluted microalgal culture in quintuplet. The assay plate was then statically incubated, at 40°C in the dark, for 60mins before recording the fluorescence intensity using a Perkin Elmer Lambda 900 UV/VIS/NIR Spectrometer at 530nm excitation and 580nm emission wavelength.

Incubation time

A 10µg/ml and 5µg/ml Nile Red solution was prepared in 20% (v/v) DMSO. 100µl of 10µg/ml and 5µg/ml of Nile red was added to 150µl of diluted *Chlorella emersonii* and *Pseudokirchneriella subcapitata* respectively in quintuplet. A kinetic study of the assay was performed by incubating the plate at 40°C in the dark and recording the fluorescence intensity using a Perkin Elmer Lambda 900 UV/VIS/NIR Spectrometer after every 13mins over a 90mins incubation period, with an excitation and emission wavelength of 530nm and 580nm respectively.

Cell concentration

Different cell concentrations (0.05-0.9 at OD590nm) of *Chlorella emersonii* and *Pseudokirchneriella subcapitata* were prepared in ddH2O. 50µl of these microalgae cultures was added to each 96-well plate, in quadruplicate, to which a further 100µl of ddH2O was added. 50µl of either 10µg/ml or 5µg/ml of the Nile Red solution (20% v/v, DMSO) was respectively added to 150µl of *Chlorella emersonii* and *Pseudokicrhnella subcapitata*. The mixture was statically incubated, at 40°C, for 60mins and 40mins respectively for *Chlorella emersonii* and *Pseudokicrhnella subcapitata* before recording the fluorescence intensity using a Perkin Elmer Lambda 900 UV/VIS/NIR Spectrometer at 530nm excitation and 580nm emission.

Triolein standard for neutral lipid quantification

A working range of triolein standard (between 1µg/ml to 15µg/ml) was prepared in neat chloroform. In a 96-well plate, 100µl of ddH2O, 100µl of 10µg/ml Nile Red (in 20% v/v DMSO) and 50µl of the respective triolein concentration was mixed in quintuplicate. The assay monitored fluorescence intensity at 530nm excitation and 580nm emission wavelength after 5mins incubation. The fluorescence intensity measured using a Perkin Elmer Lambda 900 UV/VIS/NIR Spectrometer for various concentrations of triolein was used for preparing a standard curve. The intensity of the unknown microalgal strain observed after optimization of the Nile Red assay was compared to the Triolein standard curve and neutral lipid content was quantified.

Results

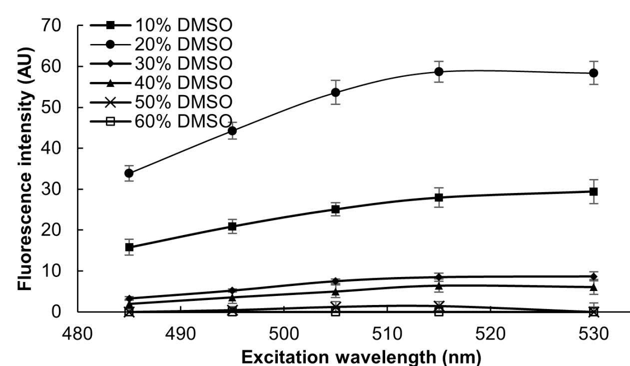

Figure 1. The effect of different concentrations (10-60% v/v) of DMSO on the fluorescence intensity of neutral lipids from *Chlorella emersonii*. A constant concentration of Nile Red was maintained (10µg/ml) for all the solvents. Different excitation wavelengths resulted in different fluorescence intensities for both types of solvent with 530nm being the wavelength for maximum fluorescence intensity from both solvents after 30mins of incubation at room temperature. Data represented here are the average of three independent experiments, with five assay values each, and the error bars shown are the standard deviation.

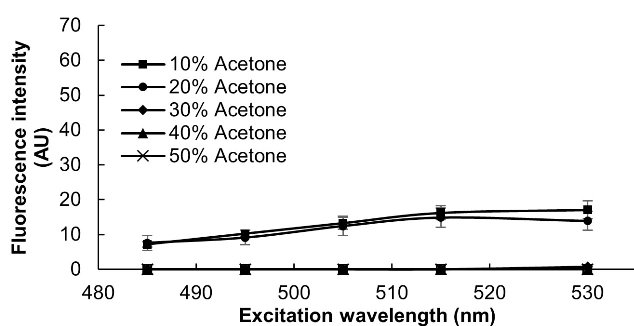

Figure 2. The effect of different concentrations (10-60% v/v) of acetone on the fluorescence intensity of neutral lipids from *Chlorella emersonii*. A constant concentration of Nile Red was maintained (10μg/ml) for all the solvents. Different excitation wavelengths resulted in different fluorescence intensities for both types of solvent with 530nm being the wavelength for maximum fluorescence intensity from both solvents after 30mins of incubation at room temperature. Data represented here are the average of three different experiments, with five assay values each, and the error bars shown are the standard deviation.

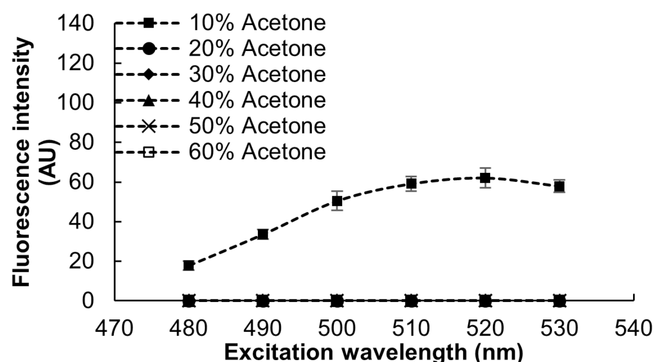

Figure 4. The effect of different concentrations (10-60% v/v) of Acetone on the fluorescence intensity of neutral lipids from Pseudokirchneriella subcapitata. A constant concentration of Nile Red was maintained (10μg/ml) for all the solvents. Different excitation wavelengths resulted in different fluorescence intensities for both types with 530nm being the wavelength for maximum fluorescence intensity from both solvents after 30mins of incubation at room temperature. Data represented here are the average of three independent experiments, with five assay values, each and the error bars shown are the standard deviation.

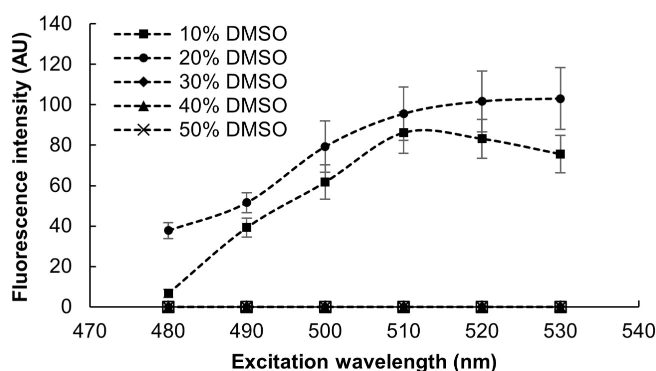

Figure 3. The effect of different concentrations (10-60% v/v) of DMSO on the fluorescence intensity of neutral lipids from *Pseudokirchneriella subcapitata*. A constant concentration of Nile Red was maintained (10μg/ml) for all the solvents. Different excitation wavelengths resulted in different fluorescence intensities for both types with 530nm being the wavelength for maximum fluorescence intensity from both solvents after 30mins of incubation at room temperature. Data represented here are the average of three independent experiments, with five assay values each, and the error bars shown are the standard deviation.

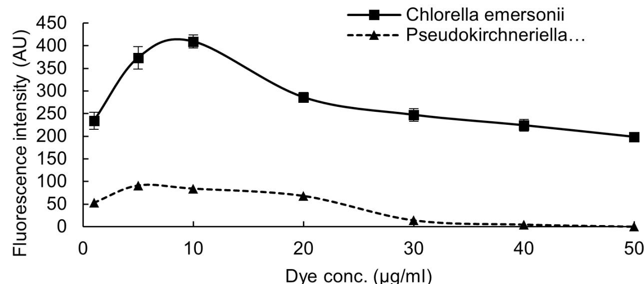

Figure 5. Fluorescence intensity from neutral lipids of *Chlorella emersonii* and *Pseudokirchneriella subcapitata* utilizing different dye concentrations (1-50μg/ml) in 20% (v/v) of DMSO, with an excitation wavelength of 530nm and an emission wavelength of 580nm. Microalgal strains were incubated at 40°C for 60mins before measuring fluorescence intensity. The data represented here are the average of three independent experiments, with five assay values each, and the error bars shown are the standard deviation.

Discussion

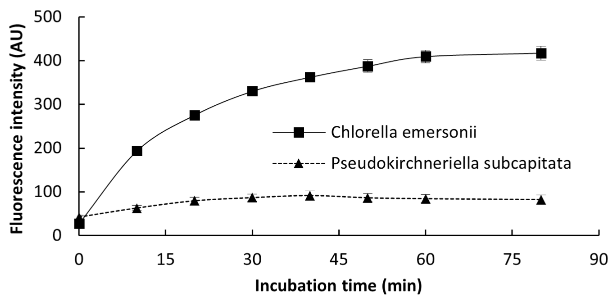

Figure 6. The effect of incubation time on the fluorescence intensity of neutral lipids from *Chlorella emersonii* and *Pseudokirchneriella subcapitata* when microalgal cells were incubated at 40°C with 10µg/ml and 5µg/ml of Nile Red dye in 20% (v/v) of DMSO respectively. A maximum fluorescence intensity in *Chlorella emersonii* was observed after 60mins of incubation and for Pseudokirchneriella subcapitata 40mins of incubation was ideal. Data represented here are the average of three independent experiments, with five assay values each, and the error bars shown are the standard deviation.

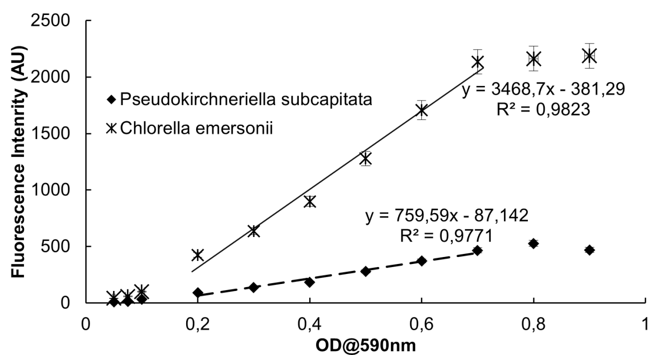

Figure 7. The relationship between fluorescence intensity and number of cells (OD590nm) when *Chlorella emersonii* and *Pseudokirchneriella subcapitata* were stained with 10µg/ml and 5µg/ml Nile red in 20% (v/v) DMSO respectively. Fluorescence was recorded after 40min and 60min of static incubation at 40°C respectively. Data represented here are the mean of three independent experiments and the error bars shown are the standard deviation.

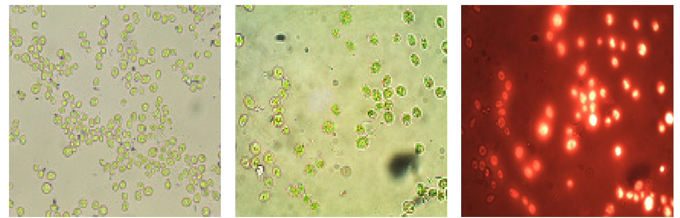

Figure 8. Microscopic observation of *Chlorella emersonii* under (a) 40X and (b) 100X magnification. Microalgal cells containing neutral lipids present in *Chlorella emersonii* after incubation with 20% (v/v) DMSO with 10µg/ml of Nile Red dye under 100X magnification (c). Cells without any fluorescence have either no neutral lipid or too few lipids to be observed visually.

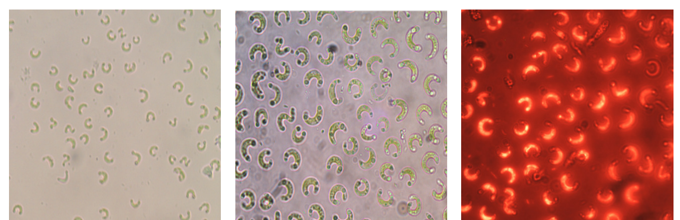

Figure 9. Microscopic observation of *Pseudokirchneriella subcapitata* under (a) 40X and (b) 100X. Microalgal cells containing neutral lipids present in *Pseudokirchneriella subcapitata* after incubation with 20% (v/v) DMSO with 5µg/ml of Nile Red dye under 100X magnification (c). Cells without any fluorescence have either no neutral lipid or too few lipids to be observed visually.

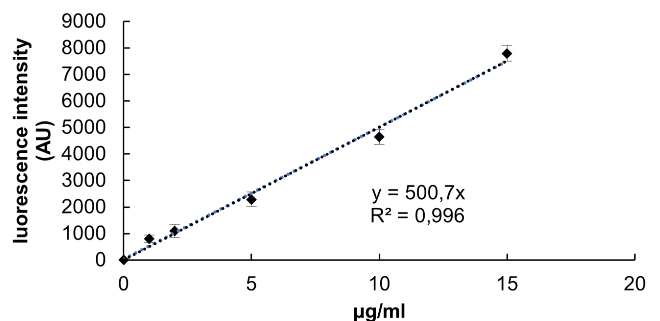

Figure 10. Triolein standard curve, used for the quantification of neutral lipids in *Chlorella emersonii* and *Pseudokirchneriella subcapitata.*

This study aimed to optimise the Nile Red assay for use with two microalgae strains, *Chlorella emersonii* and *Pseudokirchneriella subcapitata*, for the determination of neutral lipids using simple, but robust, protocol. Initially, the optimal wavelength for excitation and emission was determined and it was noted that irrespective of DMSO and acetone concentrations, an excitation emission wavelength of 530/580 nm was found to

be optimum for neutral lipid detection in *Chlorella emersonii* and *Pseudokirchneriella subcapitata*. This mirrors previous literature with similar microalgae strains (7, 12, 13, 14).

Subsequently, the optimum solvent to obtain maximum fluorescence intensity, for both *Chlorella emersonii* and *Pseudokirchneriella subcapitata*, was determined to be 20% (v/v) DMSO (see Figures 1, 3). In acetone a lower fluorescence intensity was attained in both microalgae strains (see Figures 2, 4) irrespective of acetone concentration. However, increased DMSO concentrations resulted in decreased fluorescence intensity, echoing seminal literature sources (6, 7, 12, 13). The decrease in fluorescence intensity at higher DMSO concentrations may be due to cell lysis/low cell survival and enhanced cellular debris (4, 10, 12).

The optimum concentration of Nile Red was explored, and the maximum fluorescence intensity was observed with 10µg/ml concentration of Nile Red in the case of *Chlorella emersonii* (Figure 5) and 5µg/ml for *Pseudokirchneriella subcapitata* (Figure 5). With a further increase in dye concentration, a decrease in fluorescence intensity was observed for both the microalgal strains. Pseudokirchneriella subcapitata displayed no significant difference ($p \geq 0.5$, t-test) in fluorescence intensity at 5µg/ml or 10µg/ml Nile Red dye. The mean fluorescence intensity increased with increasing dye concentration (>1µg/ml), indicated that at low Nile Red concentrations cellular lipids were not saturated (12). Furthermore, at low Nile Red concentrations (~0.5µM), quenching is minor compared to high concentrations (>5µM) (10). Conversely, with increased Nile Red concentrations, the dye not only interacts with neutral lipids, but also with phospholipidic coat and hydrophobic protein surfaces; generating a redshift of emission peak which interferes with neutral lipid fluorescence (9, 15). An optimum concentration of Nile Red (generally between 0.01 to 100µg/mL) is required to stain each individual microalgal species (7, 9). Hence, a concentration of 10µg/ml and 5 µg/ml Nile Red dye in 20% (v/v) DMSO was selected as the optimal parameters for neutral lipid quantification in *Chlorella emersonii* and *Pseudokirchneriella subcapitata* respectively.

An increase in fluorescence intensity was observed with increased incubation time for both the microalgal strains, to a maximum of 60mins (*Chlorella emersonii*) and 40mins (*Pseudokirchneriella subcapitata*, see Figure 6). However, extended incubation resulted in saturation, with no significant change, in fluorescence intensity ($p \geq 0.5$, t-test). The intensity of Nile Red fluorescence is generally not constant over time with any microalgal strain (3, 10); maximum fluorescence is not attained at the same time for all microalgal species, quenching of the fluorescence curve is species specific (10). After the addition of Nile Red to microalgal cells, fluorescence increases to reach a peak and then plateaus or decreases with time.

Therefore, since a minimum incubation of 60mins is required to attain maximum fluorescence intensity for *Chlorella emersonii* and no significant change was observed in fluorescence intensity for *Pseudokirchneriella subcapitata* after 40mins of incubation (Figure 6), 60min and 40min incubation at 40°C with 10µg/ml and 5µg/ml Nile Red (20% v/v DMSO) was used to identify the maximum fluorescence intensity in the case of *Chlorella emersonii* and *Pseudokirchneriella subcapitata* respectively.

The optimum range of cell concentration is species specific and varies between 5×10^4 to 1×10^6 cell/mL, as observed with *Chlorella vulgaris* (7). A linear relationship was observed between fluorescence intensity and number of cells when an OD590nm between 0.2-0.7 for both *Chlorella emersonii* and *Pseudokirchneriella subcapitata*. Below an OD590nm of 0.2 and above 0.7, a saturation in fluorescence intensity was observed. As microalgal biomass increased, fluorescence signal saturation occurred and is possibly caused by one or a combination of the following; limited dye transport to lipid droplets, adsorption to other hydrophobic compartments like membrane phospholipids, depletion of Nile Red or a negative effect in the cell permeation/staining reaction. Furthermore, Nile Red diffusion from the hydrophobic cell membrane, via the aqueous cytoplasm towards the liposomes, as well as its uptake by the liposomes itself, could be rate limiting step (16).

Finally, microscopic examination of microalgal cells indicated the shape of *Chlorella emersonii* to be spherical and *Pseudokirchneriella subcapitata* to be sickle shaped (Figure 8). Treatment of *Chlorella emersonii* and *Pseudokirchneriella subcapitata* with 20% (v/v) DMSO containing 10µg/ml and 5µg/ml Nile red respectively generated fluorescence when the cells were observed in the green excitation zone (530nm) and a red emission zone (604nm; Figure 8c and 9c). The presence of fluorescence after the excitation of Nile Red treated microalgal cells indicated the presence of neutral lipids in the microalgal strains and verified this optimised protocol.

Conclusion

Fluorescent dyes offer a useful method for the measurement of intracellular lipids in microalgae as they are inexpensive and require simple optical measurement instrumentation while simultaneously providing the opportunity of high throughput application. In this study, a standard and bespoke fluorescent Nile Red assay was developed and optimised for two microalgae strains; *Chlorella emersonii* and *Pseudokirchneriella subcapitata*, with a specific focus on the reduction of background interference to enhance neutral lipid quantification. Future intracellular microalgae lipid quantification optimisation will benefit from the quick, robust and cost-effective protocol detailed here.

Acknowledgements

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Declaration of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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