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Application of the standard addition method for the absolute quantification of neutral lipids in microalgae using Nile red

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ABSTRACT

Microalgae are considered one of the best candidates for biofuel production due to their high content in neutral lipids, therefore, an accurate quantification of these lipids in microalgae is fundamental for the identification of the better candidates as biodiesel source.
Nile red is a fluorescent dye widely employed for the quantification of neutral lipids in microalgae. Usually, the fluorescence intensity of the stained samples is correlated to the neutral lipid content determined with standard methods, in order to draw a standard curve and deduce the neutral lipids concentration of the unknown samples positioning their fluorescence intensity values on the curve.
Standard methods used for the neutral lipids determination are laborious and often implying solvent extraction and/or other transformation (*i.e.* saponification or transesterification) of the sample. These methods are also time consuming and may give rise to an underestimation of the lipid content due to variable extraction yields.
The approach described in this paper combines the standard addition method and the fluorometric staining using Nile red, avoiding the association of traditional neutral lipids quantification methods to the fluorometric determination. After optimization of instrument parameters and staining conditions, a linear correlation between the fluorescence intensity of each sample stained with the Nile red and its neutral lipids content deduced with the standard addition method was identified. The obtained curve allowed the direct determination of neutral lipids content maintaining a linearity range from 0.12 to 12 μg of neutral lipids per ml of sample, without need of pre-concentration. This curve was then used in the quantification of the neutral lipids content in culture of *Skeletonema marinoi* (Bacillariophyceae) at different days from the inoculum. This method was also successfully applied on *Chaetoceros socialis* (*Bacillariophyceae*) and *Alexandrium minutum* (*Dinophyceae*).

1. Introduction

The continuing increase in fuel demands, the dramatic situation in climate changes and the global warming are bringing the worldwide attention to the identification of alternative energy source for the production of combustibles that can replace fossil fuel. In last years, a lot of potential sources have been identified: the first potential biofuel feedstock that have been evaluated were oleaginous plants, but the utilization of vegetable, or vegetable oils, that may also be used for human feeding, could lead to the increase of food-grade oils costs and also generate ethic questions. Nevertheless, also using as biofuel sources not-edible oils, like oils from jatropha, tobacco seed or jojoba, the common problem for both edible and not-edible crops is the need to subtract arable land from agriculture and food industry (Ahmad et al., 2011).

In this context, the utilization of aquatic microorganisms like microalgae and cyanobacteria as substrate for the production of biofuel seems to be the better solution (Chisti, 2007; Mata et al., 2010, Dismukes et al., 2008). Microalgae are easy to cultivate and can grow with little or no attention, they can grow in fresh, brackish or salt water and in non-arable lands, moreover they are not edible vegetables with no consequences on food industry, and the oil productivity, respect to the other potential biofuel sources, can be higher (Chisti, 2007; 2008). In addition, the intensive cultivation of microalgae for biodiesel production could also play an important role in CO_2 mitigation (Wang et al., 2008).

Biodiesel production is based on the transesterification reaction between triglycerides and methanol that, in the presence of a catalyst,

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produces fatty acid methyl esthers with glycerol as a by-product (Alonzo and Mayzaud, 1999). For this reason, a rapid and accurate method for the quantification of neutral lipids in microalgae can be important for the evaluation of optimal species and/or culture conditions to be considered for biodiesel production.

Traditionally, the analysis of lipid content, require solvent extraction of the lipids from the samples and the subsequent quantification by gravimetric (Bligh and Dyer, 1959) or spectrophotometric (Wawrik and Harriman, 2010) analyses. These techniques are time-consuming and do not allow high throughput; moreover, the steps required by the traditional protocols (saponification, solvent extraction, *etc.*) may strongly affect yields and finally the characterization of lipid content of the considered microalgae.

Nile red (9-diethylamino-5H-benzo[α]phenoxa-phenoxazine-5-one) is a lipid-soluble fluorescent probe which allows the *in situ* staining of lipids also in microalgal samples (Cooksey et al., 1987; Elsey et al. 2007). Nile red is photostable and intensely fluorescent in organic solvents and hydrophobic environments. Its emission maximum is blue-shifted as the polarity of the surrounding environment decreases (Cooksey et al., 1987; Laughton, 1986; Lee et al., 1998); therefore, it is possible to differentiate between neutral and polar lipids by accurate selection of excitation and emission wavelengths. Usually, for quantification purposes, the fluorescence intensity of the sample is correlated to lipid content through a calibration curve. To construct this curve, the relative fluorescence intensity is correlated to the intracellular lipid content previously determined with other techniques such as gravimetry (Huang et al., 2009; Chen et al., 2009) or chromatography (Alonzo and Mayzaud, 1999; Kimura et al., 2004) that often imply, for the neutral lipids extraction, the use of hazardous solvents (Halim et al., 2011).

A simpler calibration curve, with serial dilution of a lipid standard, may facilitate the analysis but it is challenging to realize due to the need of considering fluorescence interferences caused by algal medium and/or cellular components as pigments and chlorophylls. The identification of a unique background (*i.e.* blank) for all microalgal samples applicable for standard curve construction can be near impossible: in fact, the environment where microalgal cells are resuspended is very variable in function of cellular age, death cells, secreted substances and salinity.

On the basis of these observations, the standard addition method (Bader 1980) could be a valid approach for the absolute quantization of neutral lipid content in microalgal samples. This method is particularly useful when standard and samples differ in properties like ion strength, salinity, viscosity or other types of impurity and interferences. In this method, different amount of standard are directly added to some aliquot of the sample and the concentration of the analytes is then deducted via the intersection of the derived curve with the negative X-axis (Bader 1980).

In this paper, a new method based on the use of Nile red and the standard addition method for the quantification of neutral lipids in microalgal cultures is described.

The microalgae used as model in this work was the marine diatom *Skeletonema marinoi*. *S. marinoi* is typically represented in the Northern Adriatic Sea (Sarno et al., 2005). This characteristic allowed us to consider this algae as model because a possible scale-up in bioreactors or open ponds won't require particular adaptation of the diatom to photoperiod or temperature. Moreover, among microalgae, marine diatoms are particularly rich in triglycerides (Leonardos and Lucas, 2000; D'Souza and Lorenagan, 1999; Brown et al., 1998).

For the optimization of the Nile red-multiple addition method different parameters have been evaluated (excitation and emission wavelengths, emission slit, linearity range of fluorescence intensity and concentration of Nile red staining solution) in order to obtain a final set up valid for all the determinations. After optimization of instrument parameters and staining procedure, the same samples have also been analyzed with the spectrophotometric method (Wawrik and Harriman, 2010) to validate the obtained results.

After this preliminary phase of setting parameters and protocol optimization, it was possible to directly correlate the neutral lipids content and the fluorescence intensity of the samples before the addition of lipid standard, improving sensitivity, saving time and reducing amount of culture needed for the analysis. The obtained curve was used for the quantification of neutral lipids in the marine diatom *S. marinoi* in different growth phases.

The Nile red-standard addition method was also used for neutral lipids quantification of samples from two other microalgal species from the Mediterranean Sea: the diatom *Chaetoceros socialis* and the dinoflagellate *Alexandrium minutum.*

2. Materials and methods

2.1. Microalgal culture

S. marinoi CBA4, *C. socialis* CBA2 e *A. minutum* CBA5 have been isolated by the laboratory of the Environmental Biology Section of the University of Urbino, from Mediterranean Sea. Diatoms have been grown in f/2 medium and *A. minutum* has been grown in f/2 without silicate (Guillard and Ryther, 1962; Guillard, 1975), with a dark:light cycle of 12:12 h at light intensity (Photosynthetic Active Radiation, PAR) of 1.5 W/m^2. The temperature was 21 ± 1 °C. The inoculum were effectuated at a final concentration of 50,000 cells/ml.

2.2. Optimization of instrument parameters and staining conditions

The spectrofluorometric determinations were performed using a spectrofluorophotometer RF-5301PC (Shimadzu, Japan). The fluorescence of the samples was always measured before and after Nile red addition, in order to subtract the intrinsic fluorescence value of the sample. Moreover, fluorescence intensity of Nile red-stained f/2 medium alone was subtracted. Excitation and emission wavelength were identified with synchronous scan on *S. marinoi* CBA4 samples stained with Nile red and spiked with lipid standard.

As lipid standard, a 10 mg/ml isopropanol solution of Triolein (1,2,3-Tri(cis-9-octadecenoyl)glycerol) (Sigma-Aldrich) was used. For the optimization of Nile red staining solution concentration and instrument parameters, different concentrations of triolein solution were prepared as described in Section 2.3.1, using f/2 medium alone instead of *S. marinoi* culture.

Stock solution of Nile red (9-diethylamino-5H-benzo[[a]phenoxa-]phenoxazine-5-one) was prepared dissolving 0.5 mg of Nile red per ml in acetone. During optimization procedure three different dilutions in acetone (0.1, 0.05 and 0.025 mg/ml) have been tested.

The optimal size of the emission slit (3 and 5 nm) was also investigated.

2.3. Standard addition method and fluorometric analysis

2.3.1. Sample preparation

For the analysis of neutral lipids content with the standard addition method, a 20 ml volume of each sample was centrifuged for 20 min at 2200×g, at 15 °C and the pellets were resuspended in another 20 ml of fresh f/2 medium, in order to eliminate impurities potentially present in the medium used for cell growth and to ensure the same salinity level for all the samples.

From each 20 ml resuspended sample, 5 aliquots of 1.98 ml were transferred in different 15 ml-polypropylene tubes (VWR Collection); then isopropanol and lipid standard solution were added in order to obtain a final volume of 2 ml. Since the final concentration of triolein changed for each point of the curve, the added volumes of triolein standard and isopropanol varied for each aliquot (*e.g.* for the "0" point of the curve, 20 µl of isopropanol were added; for the 2.5 mg/l point of the curve, 0.5 µl of the triolein standard solution and 19.5 µl of isopropanol were added; *etc.*). After the triolein addition, the fluorescence of each

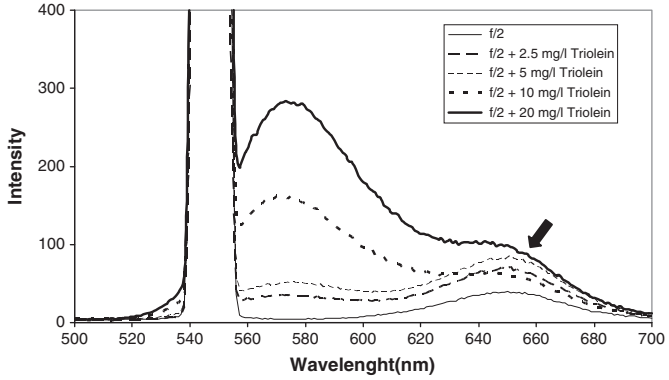

Fig. 1. Emission spectra of f/2 medium spiked with five different amount of triolein standard solution. Triolein final concentrations were: 0–2.5–5–10 and 20 mg/l. Nile red concentration in all samples was 1 μg/ml (Kimura et al., 2004). Spectrofluorometer parameters were: Excitation slit: 5 nm; Emission slit: 5 nm; Excitation at 547 nm. The black arrow indicates the unexpected emission peak at 650 nm.

sample was detected at the selected excitation and emission wavelengths (547 nm and 580 nm, respectively).

For staining procedures, each 2 ml-aliquot of *S. marinoi* CBA4 (or f/2-triolein solution in optimization procedures) was stained with 20 μl of Nile red solution, mixed for 1 min and incubated 5 min at room temperature in the dark.

2.3.2. Standard addition curve

A curve was designed for each sample by plotting the fluorescence value at each point (obtained by the difference between the fluorescence of the sample before and after Nile red addition) *versus* the corresponding triolein concentration used for the spike. The neutral lipids concentration in the sample, expressed as triolein equivalents, was determined on the basis of the intercept of the curve on X-axis.

2.4. Sensitivity test

To test the sensitivity of the method, serial dilutions of the same sample have been analyzed with the standard addition method. A 20 ml aliquot of *S. marinoi* CBA4 culture was centrifuged at 2200×g for 15 min and resuspended in the same volume of fresh f/2 medium. A 10-ml aliquot was used for the analysis with the standard addition method and the remaining 10 ml have been diluted 1:2 using f/2 medium. The dilution procedure was then repeated to obtain 1:4, 1:8 and 1:16 dilutions. A further 1:20 dilution has been prepared taking a 2 ml aliquot of undiluted culture to a final volume of 40 ml.

2.5. Correlation curve

The data obtained with the optimized method have been correlated by plotting on the Y-axis the fluorescence intensity of the samples before addition of triolein and, on the X-axis, the concentrations obtained with the standard addition method.

2.6. Sample preparation for the neutral lipids analysis using the correlation curve

At day 8, 10 and 15, 1.98 ml aliquots of culture were centrifuged for 20 min at 2200×g, at 15 °C. Pellets were resuspended in 1.98 ml of fresh f/2 medium and 20 μl of isopropanol were added to the samples. The staining procedure with Nile red was performed accordingly to the protocol described in Section 2.3.1 and the sample concentrations have been obtained positioning their fluorescence value on the correlation curve described in Section 2.5.

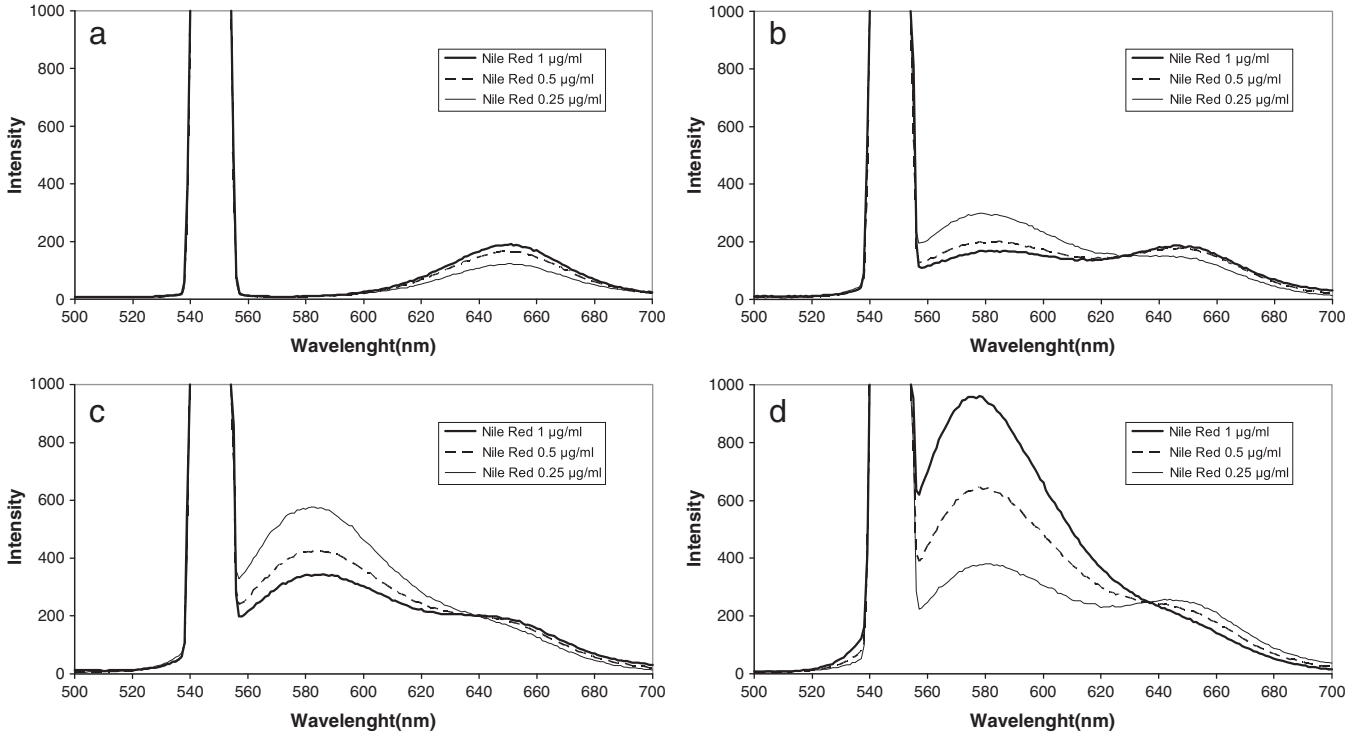

Fig. 2. Emission spectra of four different triolein standard solutions in f/2 medium, stained with Nile red at three different concentrations. Final concentrations of Nile red in the samples were: 1, 0.5, and 0.25 μg/ml. a) f/2 medium without triolein; b) triolein solution 2.5 mg/l concentrated; c) triolein solution 5 mg/l concentrated; d) triolein solution 20 mg/l concentrated. Instrument parameters were: Excitation wavelength: 547 nm; Excitation and emission slit: 5 nm.

2.7. Dry weight determination

At day 8, 10 and 15 a 50 ml aliquot of culture was filtered through preweighed, precombusted (450 °C; 2 h), glass-fiber filters (Whatman GF/C, 47 mm, nominal pore size 1.2 mm). The filters were washed with 30 ml of 0.5 M ammonium formate and dried at 100 °C for 16 h to volatilize the ammonium formate. The filters were then reweighted to determine the dry weight. (Brown et al., 1998).

2.8. Spectrophotometric analysis of lipids

The spectrophotometric analysis of lipids content was performed as described in Wawrik and Harriman (2010), with slight modifications. Briefly, a 5 ml aliquot of culture was centrifuged at 2200×g for 20 min and washed once with f/2 medium. The pellet was resuspended in 200 µl of saponification reagent (NaOH 1 N in 25% MetOH) and cell lysis was performed by incubation for 30 min at 42 °C (Myklestad and Swift 1998). The determination of lipids concentration was carried out with no more exception respect to the standard protocol (Wawrik and Harriman, 2010). For the spectrophotometric determinations, a spectrophotometer UV-2401 PC (Shimadzu, Japan) has been used.

3. Results

3.1. Optimization of instrument parameters and staining conditions

The synchronous scan of *S. marinoi* CBA4 samples stained with Nile red identified a specific emission peak at 580 nm, when samples were excited at 547 nm. Based on this data, subsequent analyses were performed at the excitation and emission wavelengths of 547 nm and 580 nm, respectively.

In the first attempt to quantify the neutral lipid content in *S. marinoi* CBA4 culture, the standard addition method was combined with the Nile red staining protocol (Kimura et al., 2004), but the correlation among fluorescence intensity and triolein concentration failed to be linear ($R^2 = 0.899$). In order to optimize the method and to understand the reasons of this lack of correlation, the subsequent optimization experiments were performed on triolein solutions in f/2 medium alone, to avoid the introduction of all the variables related to the biological samples.

The emission scan of different triolein concentrations in f/2 medium, stained with 20 µl of 0.1 mg/ml Nile red solution, are reported in Fig. 1. The emission scan revealed an unexpected peak at 650 nm. To investigate if this peak was related to an excess of Nile red, three different staining solutions have been tested on different triolein concentrations in f/2 medium. The emission spectra showed that the peak at 650 nm was related to the presence of an excess of Nile red, strongly affecting the intensity of the signal at 580 nm and demonstrating that a lower concentration of Nile red in the staining solution reduced that interference (Fig. 2).

Using a Nile red staining solution of 0.025 mg/ml (0.25 µg/ml in the sample) the peak at 650 nm was strongly reduced but it did not disappear. For this reason, a further optimization step, regarding the emission slit, was performed: on the basis of the obtained emission spectra, emission slit = 3 nm was selected. In this conditions, the peak at 650 nm was not present (Supplementary data, Fig. A1) and, even if the signal at 580 nm was lower, its intensity ensured a good sensitivity (see Section 3.2). Therefore, the optimized instrument parameters and staining conditions have been set as follows: excitation wavelength, 547 nm; emission wavelength, 580 nm; excitation slit, 5 nm; emission slit, 3 nm; Nile red staining solution concentration, 0.025 mg/ml.

3.2. Linearity range

A linearity test was effectuated on f/2 medium spiked with different concentration of triolein. The correlation between fluorescence intensity and triolein concentration was linear up to a concentration of 45 mg/ml of triolein in f/2 medium (Fig. 3). The same test was repeated spiking different aliquots of *S. marinoi* CBA4 culture. The linearity was maintained up to the spike with 40 mg/l of triolein, but the fluorescence intensity was very high respect to the one obtained on f/2 medium. This confirms that the fluorescence response in culture samples and in f/2 medium alone is very different, but also shows that the linearity of the signal is not affected.

3.3. Standard addition curve

After the optimization of instrument parameters and staining conditions, the standard addition method analysis was effectuated on *S. marinoi* CBA4 cultures. The emission scan of spiked samples is shown in Fig. 4a. A strong linear correlation among fluorescence intensity and triolein concentration was obtained ($R^2 = 0.9994$) (Fig. 4b).

3.4. Sensitivity test

In order to test the sensitivity of the method, the neutral lipids content of serial dilutions of the same culture has been quantified. For each sample a standard addition curve was designed (Supplementary data, Fig. A2). The method allowed the neutral lipids quantification of the diluted samples up to the concentration of 0.12 mg/l of triolein equivalents. A strong correlation between the neutral lipids concentration and the corresponding dilution factor was obtained ($R^2 = 0.9932$) (Fig. 5).

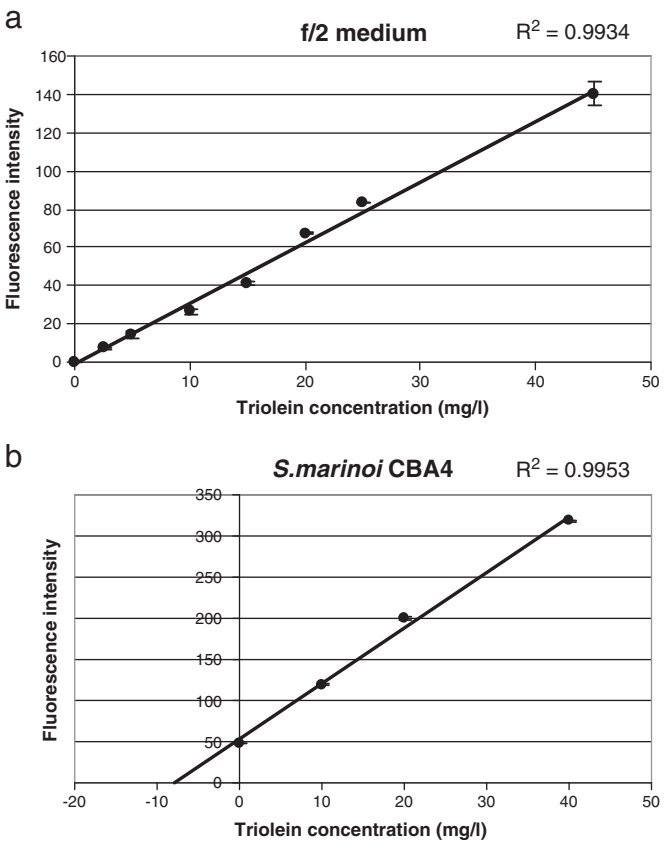

Fig. 3. a) Linear correlation between fluorescence intensity and triolein concentration in f/2 medium spiked with different amount of triolein standard solution. b) Linear correlation between fluorescence intensity and triolein concentration in *S. marinoi* CBA4 samples spiked with different amount of triolein standard solution. Nile red concentration in the samples: 0.25 µg/ml; Excitation slit: 5 nm; Emission slit: 3 nm. Excitation wavelength: 547 nm; Emission wavelength: 580 nm. For linear correlations reported values are means ± standard deviation of three technical replicates.

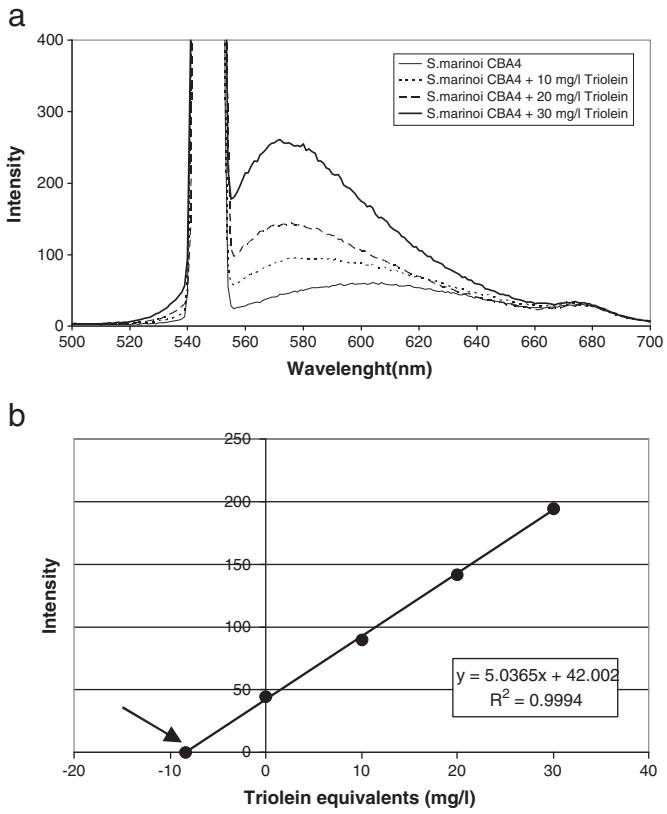

Fig. 4. a) Emission spectra of four aliquots of *S. marinoi* CBA4 culture used for the analysis with the standard addition method. The peak at 668 nm is the characteristic autofluorescence peak of chlorophyll. Nile red concentration in the samples: 0.25 μg/ml; Excitation slit: 5 nm; Emission slit: 3 nm; Excitation at 547 nm; b) Correlation among fluorescence intensity of the four aliquots of *S. marinoi* CBA4 culture and the triolein equivalents used for the spike. The black arrow indicates the deducted neutral lipids concentration in the sample expressed as triolein equivalents.

3.5. Correlation between fluorescence intensity and neutral lipids concentration

The correlation between the neutral lipids concentrations, obtained applying the standard addition method, and the fluorescence intensity of the Nile red-stained samples without the addition of triolein standard, is shown in Fig. 6.

The strong linearity obtained ($R^2 = 0.9948$) allowed us to perform all the successive determinations just reading the stained sample, avoiding triolein spiking. The neutral lipids concentrations deduced from the equation of the curve were not significantly different ($p > 0.05$) from the ones obtained with the standard addition method applied to the same samples (Table 1).

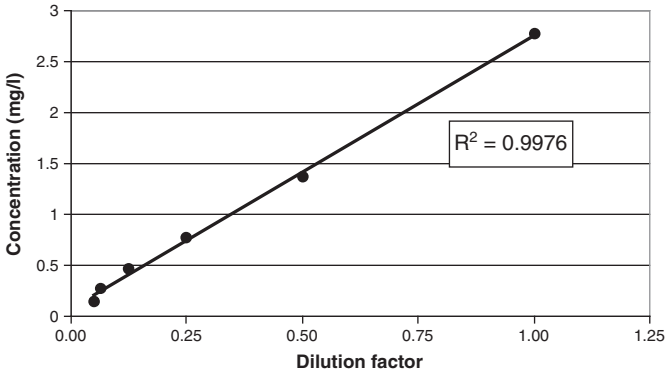

Fig. 5. Sensitivity test on serial dilution of *S. marinoi* CBA4 culture. The dilution factor of solutions are reported on the X-axis while the neutral lipids concentration obtained with the multiple addition method on Nile red stained samples are reported on the Y-axis. R^2 value is 0.9976.

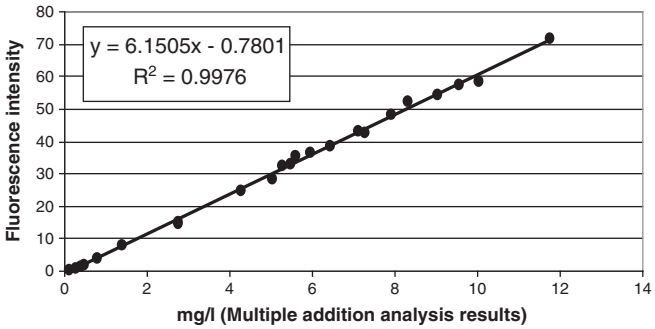

Fig. 6. Linear correlation among fluorescence intensity of the samples without spike and the neutral lipids concentration obtained with the multiple addition method on the same samples. R^2 value is 0.9976.

3.6. Correlation with spectrophotometric analysis

To validate the results obtained with the standard addition method, the same samples were tested with spectrophotometric analysis as described in Section 2.8. The comparison among the results obtained with the two methods is shown in Fig. 7. Although the neutral lipid concentration values obtained with the spectrophotometric method were always lower than values obtained with the Nile red method, a strong linear correlation was demonstrated ($R^2 = 0.9958$).

3.7. Interday variability of neutral lipids content

The optimized method was then applied for the determination of neutral lipids in *S. marinoi* CBA4 cultures to evaluate variation in neutral lipids content at different days from the inoculum (see Section 2.6). The results showed that the neutral lipids concentration increased with the culture age. The percentage of neutral lipids content respect to the dry weight from day 8 to day 15 went from 6% to 20%, demonstrating that the higher neutral lipid content was not correlated to the biomass increase (Fig. 8).

3.8. Application of the Nile red-multiple addition method to other species

The Nile red-multiple addition method has also been applied on two other microalgal species: the diatom *C. socialis* and the dinoflagellate *A. minutum*. In both cases a strong linear correlation ($R^2 = 0.9969$ and $R^2 = 0.9954$ respectively) between the fluorescence intensity and the amount of lipid standard added to the sample was demonstrated (see

Table 1
Comparison between the neutral lipids concentrations deduced from the equation of the curve (Fig. 6) and the concentrations obtained, on the same samples, with the standard addition method. The *T*-test response indicates that there are no significant differences ($p > 0.05$). Reported values are means ± standard deviations of three technical replicates. Neutral lipids concentration is expressed as mg/l of triolein equivalents.

Sample	Neutral lipids concentration (mg/l) (1)	Neutral lipids concentration (mg/l) (2)	Two tailed p value ($\alpha = 0.05$)
1	9.524 ± 0.372	10.374 ± 1.169	0.456
2	8.843 ± 0.426	9.275 ± 2.298	0.884
3	8.150 ± 0.221	11.306 ± 1.128	0.060
4	12.819 ± 0.904	14.860 ± 1.337	0.216
5	6.485 ± 0.070	6.420 ± 0.321	0.806
6	8.597 ± 0.462	8.297 ± 0.996	0.736

(1) Obtained using the correlation curve (see Fig.6).
(2) Obtained with the standard addition method.

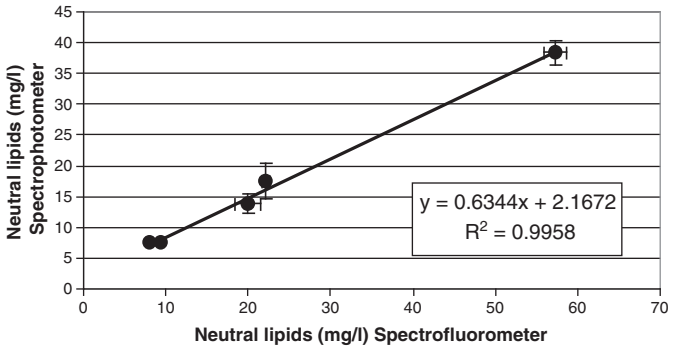

Fig. 7. Linear correlation between the neutral lipids concentration obtained with the fluorescence analysis with the multiple addition method on Nile red stained samples and the same samples analyzed with the spectrophotometric analysis (Wawrik and Harriman, 2010). R^2 value is 0.9958. Reported values are means of three technical replicates.

Fig. 9), allowing the determination of neutral lipids content in the samples using the standard addition method (for emission spectra see Supplementary material Fig. A3). For *A. minutum* samples, the analysis have been performed using a 0.0083 mg/ml Nile red staining solution, in order to remove the interferences related to the excess of Nile red.

4. Discussion

This paper described a new approach for neutral lipids quantification in microalgae that associates the fluorometric determination of neutral lipids using Nile red to the standard addition approach (Bader 1980). Usually, the correlation between the fluorescence intensity of Nile red and the specific amount of neutral lipids, require the association of other methods for the quantification of lipids (Chen et al., 2009; Halim et al., 2011). Traditional methods often require an extraction step or other manipulations that imply the use of organic solvents.

The method described in this paper allowed the quantification of neutral lipids in microalgal samples with no needs to associate any other kind of lipids quantification to Nile red staining procedure, saving time and avoiding purifications and other kind of manipulations with consequent employ of hazardous solvents and possible loss of material.

In particular, it has been shown that a careful standardization and optimization of instrument parameters and staining conditions are essential for the reliability and robustness of the method. It is worth noting that the excess of Nile red that did not react with neutral lipids generated an emission peak at 650 nm which interfered with the peak related to the dye bound to neutral lipids, affecting the quantification of the samples (Fig. 2).

After the optimization of all the parameters, it was also possible to design a curve that correlate the neutral lipids concentration, determined with the standard addition method, with the fluorescence of the samples before the spiking. This curve was used for subsequent quantification of the samples, just registering the fluorescence of the stained sample and deducing the neutral lipids concentration from the curve, saving time and material and, as shown in Table 1, giving results that are not significantly different from the ones obtained with the multiple addition procedure. The method was applied in a test for interday variability of neutral lipids content in *S. marinoi* CBA4 cultures. The results showed that the neutral lipids content increases with the culture age, confirming previously studies conducted on other microalgae (Chiu et al., 2009) and supporting the common idea that the nutrient consumption of growing cells induce a nutrient deficiency that finally bring to lipids accumulation (Roessler 1988).

The method has also been validated using the spectrophotometric method (Wawrik and Harriman, 2010). The results obtained with the Nile red-standard addition method showed a strong correlation with the spectrophotometer results (Fig. 7), even if the absolute value of neutral lipids content is always higher than the ones obtained with the spectrophotometer method. In fact, the sensitivity was widely increased: the spectrophotometric method showed linearity from 0.125 mM to 5 mM concentration of C_{14} and C_{16} fatty acid (Wawrik and Harriman, 2010). Considering three moles of fatty acid for each triglyceride and converting the concentration in mg/l, the sensitivity of the spectrophotometric method can be estimated from 30 mg to 1.2 g per liter of culture. The method described in this paper allowed the quantification of neutral lipids from 0.12 mg/l of triglycerides (Fig. 5), processing the samples without any step of pre-concentration and saving time and material.

To prove the applicability of the Nile red-multiple addition method with other microalgae, two other species from Mediterranean Sea were used: *C. socialis* and *A. minutum*. In both cases it was possible to determine the neutral lipids concentration using the standard addition method.

In particular, for *C. socialis*, the same protocol employed for *S. marinoi* was maintained with no exception. Instead, in the case of *A. minutum* neutral lipids quantification, a lower concentration of Nile red staining solution was used.

In the specific case of *A. minutum* neutral lipids quantification, a lower concentration of Nile red staining solution was used. In fact, in the Nile red concentration range used for *S. marinoi*, the analysis of emission spectra showed the presence of the peak at 650 nm related to the excess of Nile red (not shown). This may be due to a lower

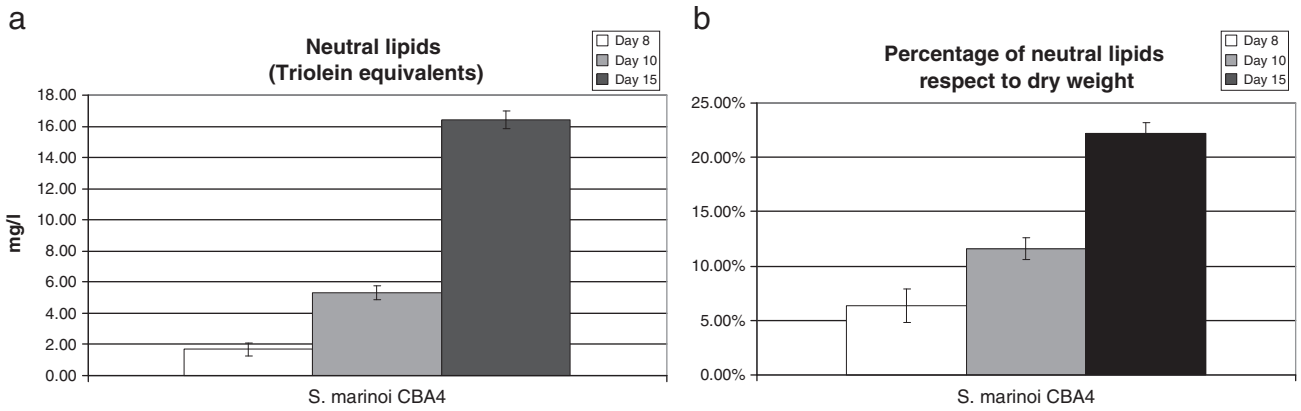

Fig. 8. a) Neutral lipids content of *S. marinoi* CBA4 culture at 8, 10 and 15 days from the inoculum. Values have been obtained by spectrofluorometric analysis using Nile Red solution with the multiple standard addition method. Values are expressed as means ± standard deviations of three replicates. b) Same data expressed as percentage of neutral lipids respect to the total dry weight.

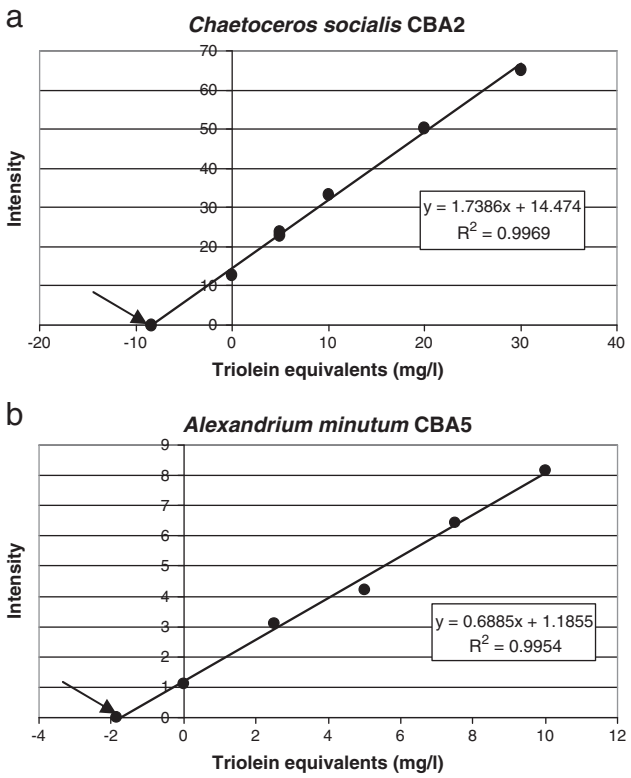

Fig. 9. Correlation among fluorescence intensity and triolein concentration of different aliquots of *C. socialis* CBA2 culture (Fig. 9a) or *A. minutum* CBA5 culture (Fig. 9b) spiked with different amount of 10 mg/ml triolein standard solution. The black arrows indicate the deducted neutral lipids concentrations in the samples expressed as triolein equivalents. Nile red concentration in the samples was: 0.25 μg/ml for *C. socialis* CBA2 and 0.083 μg/ml for *A. minutum* CBA5.

permeability of the dinoflagellate membrane to Nile red or to a lower concentration of neutral lipids in the cells.

In any case, after optimization of the concentration of Nile red staining solution, strong correlation between fluorescence intensity and triolein standard additions was identified ($R^2 = 0.9954$) allowing the determination of neutral lipids content also in this dinoflagellate.

5. Conclusion

This study showed that the combination of standard addition method and Nile red staining can be a good approach for the neutral lipids quantification in diatoms and dinoflagellates. In fact, this method did not require any extraction and purification of lipids, avoiding the use of hazardous solvents, it allowed the determination of neutral lipids content also at very low concentration, giving strong and reliable results in few minutes and requiring small amount of culture.

In fact, in the application of this method to *S. marinoi* CBA4 only 2 ml of culture have been used for the analysis performed on the correlation curve (Fig. 6).

Since the method itself minimizes the variability due to the environmental conditions or the nature of the samples, it could be potentially applicable to any microalgal species with slight adaptation to the single species.

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