A new approach for the detection and quantification of microalgae in industrial-scale microalgal cultures

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Abstract

In industrial-scale cultures, non-target microalgae compete with the desired species for nutrients and CO_2, thus reducing the growth rate of the target species and the quality of the produced biomass. Microalgae identification is generally considered a complicated issue although, in the last few years, new molecular methods have helped to rectify this problem. Of the different techniques available, DNA barcoding has proven very useful in providing rapid, accurate and automatable species identification; in this work, it is used to assess the genomic identity of the microalga species *Scenedesmus almeriensis*, a common strain in industrial-scale cultures. Barcode markers *rbcL* and ITS1-5.8S-ITS2 were sequenced and the obtained genomic information was used to design a quantitative PCR assay to precisely quantify the *S. almeriensis* concentration in microalgal cultures of industrial interest. TaqMan chemistry was used to quantify down to 1 µg/L dry weight of *S. almeriensis* cells, as well as to detect the presence of other concentrated microalgae cultures. A simple direct PCR approach was also investigated to avoid classic DNA extraction and to reduce total experiment time to approximately 2 hours. The objective was to design strain-specific tools able to confirm and quantify the presence of different strains in any microalgae culture so as to achieve maximal productivity and quality of the produced biomass.

Keywords

Microalgae, *Scenedesmus almeriensis*, Quantification, Identification, Direct PCR, qPCR

1. Introduction

Over recent years, interest in microalgae has increased due to the wide range of biotechnological application in which they are involved. Microalgal primary metabolites such as proteins, starch and lipids, are greatly valued in the food [1] and feed [2] industries, whilst diverse secondary metabolites are yet to be fully exploited [3]. Nonetheless, the human exploitation of microalgae has a long history; for instance, *Spirulina* has been harvested for food by the indigenous peoples of Mexico and Chad since ancient times because of its high protein content and excellent nutritive value [4]. Japan also has a long tradition of large-scale commercial production and consumption of *Chlorella* species as health food supplements [5] given their high content of proteins, carotenoids and vitamins [6]. Nowadays, microalgae are far more widely exploited and an extensive range of applications has been developed [7]. Of these, the carotenoid pigment astaxanthin, produced in high concentrations from the *Haematococcus* species, is considered to be one of the most valuable algal compounds and is used in many applications in the food, feed, cosmetics and pharmaceutical sectors [8]. However, for all commercial applications, the monitoring of contaminant and unwanted microalgae in outdoor or non-sterile bioreactors is of great importance [9]. Non-target microalgae compete with the desired species for nutrients and CO_2 [10], reducing the growth rate of the target alga or even predominating within the culture; this consequently lowers overall productivity and biomass quality [11].

Microalgae species have historically been discriminated by their morphological and pigment profiles even though they often display few morphological features that are useful for identification. In such cases, molecular methods are far more effective [12]. DNA barcoding in particular has been very useful in providing rapid, accurate and automatable species identification using short, standardized gene regions as internal species tags [13]. The most promising candidates for green microalgae barcoding are the Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2) of the nuclear rDNA and the *Ribulose Bisphosphate Carboxylase* (*RuBisCO*) large subunit (*rbcL*) gene [14]. Nonetheless, DNA sequencing of the barcode markers is very time consuming and not suitable for the daily monitoring of microalgal cultures. In contrast, quantitative real-time PCR meets the necessary requirements and may be considered the best method for the molecular quantification of a target microalgae species [12]. The main advantage of using quantitative PCR

is that it is highly sensitive, specific, accurate and cost-effective; it can also be applied to a large number of experimental samples at the same time [15].

The objective of this study was to design a simple real-time PCR assay to precisely quantify the presence of *Scenedesmus almeriensis*, a common microalga, at the Almería microalgae facility (Estación Experimental Las Palmerillas, Fundación Cajamar) in southern Spain. *S. almeriensis* was firstly isolated in an agricultural greenhouse under high temperature and irradiance conditions [16]. This strain has proven to be a common contaminant in industrial-scale cultures of *Chlorella*, *Haematococcus* and *Spirulina* (personal communication) due to its high growth rates and ability to flourish under a wide range of culture conditions while easily adapting to stressful conditions [17] [18].

To shorten the experiment time needed for sample analysis, we also investigated the feasibility of a direct PCR (or colony PCR) methodology. Direct PCR is a simple method in which a single colony or culture sample replaces the template DNA for amplification, requiring no preparation of pure DNA [19]. This technique is widely used for bacteria and yeast [20] but because algal cell walls are structurally stronger, direct PCR for microalgae is more difficult. Nevertheless, direct PCR for microalgae has been reported, initially with *Chlamydomonas* [21] [22], thanks to its weaker cell wall [23]; subsequently it was performed on other microalgae such as *Chlorella* [24], *Scenedesmus* [25] and *Nannochloropsis* [26].

2. Materials and methods

2.1. Microorganism and culture conditions

The freshwater microalgae *Scenedesmus almeriensis* CCAP 276/24 (Chlorophyta) was chosen as the model organism for this study because of its ability to grow easily in freshwater cultures. *S. almeriensis*, along with the other microalgal species used in this study, was grown in Arnon medium [27] enriched with 0.850 g/L $NaNO_3$. Approximately 600 mL of sterile medium was used for each 1 L round flat-bottom flask. Filtered air was continuously bubbled through the medium and a 24-hour light cycle was provided by fluorescent tubes giving up to 500 $\mu E/m^2 \cdot s$. The cell culture concentration was assessed via dry weight determination, with 1-µm pore size paper filters, or by cell counting using a Neubauer chamber. The obtained biomass was conserved at -80°C prior to use.

2.2. DNA extraction

To remove the culture medium, 2-10 mL of fresh concentrated culture were centrifuged at 4000 rpm for 5 minutes at room temperature. The pellet was resuspended in 1 mL of nuclease-free water and re-centrifuged under the same conditions. Genomic DNA was extracted from the pelleted microalgae using a Soil DNA Isolation Plus Kit (Norgen Biotek Corp.) and other commercial kits (such as the PureLink Plant Total DNA Purification Kit by Invitrogen) or by non-commercial methods (CTAB [28]). The total genomic DNA extracted was quantified with the Qubit dsDNA HS Assay Kit (Molecular Probes).

2.3. Sequencing

The ITS1-5.8S-ITS2 region was amplified through PCR using the primers ITS1 and ITS4 [29], as described in Table 1~~Table 1~~. The 25 µL PCR reaction mix comprised 12.5 µL of SensiFAST SYBR No-ROX Kit (Bioline), 5 µL of nuclease-free water, 2.5 µL of each primer (2 µM) and 2.5 µL of genomic DNA (10 ng/µL). Amplification was carried out using the MyGo Pro thermocycler (IT-IS Life Science Ltd.) under the following conditions: 94°C for 5 min, 45 cycles of 95°C for 30 s, 50°C for 1 min and 72°C for 1 min, then a final extension step at 72°C for 7 min and a High Resolution Melting (HRM) analysis.

To amplify a 1380 nt amplicon of the *rbcL* marker, two new primers were designed (Table 1~~Table 1~~) using the NCBI primer-BLAST tool. Suitable conserved regions were identified by aligning different Chlorophyta sequences mined from GenBank in order to design primers capable of amplifying the *rbcL* marker in a wide range of microalgae species. The 25 µL PCR reaction mix comprised 12.5 µL of SensiFAST SYBR No-ROX Kit, 5 µL of nuclease-free water, 2.5 µL of each primer (2 µM) and 2.5 µL of genomic DNA (10 ng/µL). Amplification was carried out with the MyGo Pro thermocycler using a touchdown approach, starting from an annealing temperature of 65°C, which was then lowered by 0.4°C/cycle down to 55°C over the first 25 cycles; the subsequent cycles were then conducted at an annealing temperature of 55°C. The temperature conditions used were: 95°C for 5 min, 45 cycles of 95°C for 30 s, 55-65°C for 1 min and 72°C for 1 min then a final extension step at 72°C for 7 min followed by HRM analysis.

The PCR products were purified with the PureLink PCR Purification Kit (Invitrogen) and quantified with the Qubit dsDNA HS Assay Kit (Molecular Probes). Approximately 10-20 ng of PCR product were amplified with the GeneAmp PCR System 9700 (Applied Biosystems) thermocycler using the BigDye Terminator v.3.1 Cycle Sequencing Kit with the following program: 96°C for 1 min, 25 cycles of 96°C for 10 s, 50°C for 5 sec and 60°C for 4 min. An additional reverse primer (rbcLR_14) was used to sequence the first nucleotides of the *rbcL* marker (Table 1~~Table 1~~). The fluorescently labelled DNA was subsequently precipitated by ethanol precipitation [30]. Sequences of both positive and negative strands were determined by capillary electrophoresis in the AB 3100 Genetic Analyzer to obtain a minimum 2-fold coverage for each sequenced nucleotide. Forward and reverse sequences were aligned and manually edited to generate consensus sequences. The new, first-time reported *Scenedesmus almeriensis* sequences were deposited in GenBank under the following accession numbers: MF977406 (ITS1-5.8S-ITS2) and MG257492 (*rbcL*).

Name	Sequence (5' > 3')	Ta (°C)	Amplicon length	Source
RbcL_13F	AATGGCTCCACAAACAGAAAC	50-55	1380 nt	This study
RbcL_8R	TCACAAGCAGCAGCTAATTC	50-55		This study
RbcL_14R	ATCAAGACCACCACGTAAACA	50		This study
ITS1	TCCGTAGGTGAACCTGCGG	50	≈700 nt	White et al.
ITS4	TCCTCCGCTTATTGATATGC	50		White et al.
SalmF	ACCCTCACCCCTCTTTCCTTT	63	74 nt	This study
SalmR	TTGGGAAAGCCAGATCCACC	63		This study
SalmProbe	6FAM-GTTAGCTTCTCAGCTGG	63		This study
UnivF	TTGGAGGGCAAGTCTGGT	63	83 nt	Hayden et al.
UnivR	CGAGCTTTTTAACTGCAACAA	63		Hayden et al.
UnivProbe	VIC-CGGTAATTCCAGCTCC	63		This study

Table 1. List of primers used in this study, including primer sequences, annealing temperatures (Ta), amplicon length and primer references.

2.4. Real-time PCR assay specifications

S. almeriensis-specific primers (SalmF and SalmR) and an internal TaqMan FAM-labelled MGB probe (SalmProbe) were designed within the internal transcribed spacer region 2 (ITS2) of the previously

sequenced ribosomal DNA cluster (Table 1~~Table 1~~; **Figure 1**~~Figure 1~~). ITS2 sequences of the most similar microalgal species were mined from GenBank and aligned in order to design primers and probes for maximum differentiation from non-target algae. The probe was designed to have a minimum of two mismatches from all the sequences analysed. An additional set of primers (UnivF and UnivR [31]) and a VIC-labelled MGB probe (UnivProbe) were used to amplify an 83-nucleotide region of the 18S rDNA, which is present in all eukaryotes. The two sets of primers along with the probe can be used in the same well for a multiplex assay - the universal set is used as the positive control, while the *S. almeriensis* set is used to quantify the fraction of total DNA belonging to this microalgae species.

The reactions were performed in a 15 µL reaction mix comprising 1 µL of each of the four primers (3 µM), 1 µL of each labelled probe (3 µM), 1.5 µL of the sample DNA and 7.5 µL of the SensiFAST Probe No-ROX Kit (Bioline). Amplifications were carried out using the MyGo Pro thermocycler (IT-IS Life Science Ltd.) under the following conditions: an initial hold step of 95°C for 10 min and 45 PCR cycles of 95°C for 15 s and 63°C for 1 min. The specificity of the Salm set for the target alga was assessed using a variety of different microalgae species (*Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Parachlorella kessleri*, *Spirulina platensis*, *Haematococcus rubens*, *Haematococcus pluvialis*, *Nannochloropsis oceanica* and *Nannochloropsis gaditana*), most of which have important commercial applications, with special attention being given to the most genetically similar species in the ITS2 region (*Scenedesmus obliquus*, *Scenedesmus bajacalifornicus*, *Scenedesmus rubescens* and *Coelastrum proboscideum*). The naming of the microalgae strains used in this study was genetically confirmed through DNA sequencing of the barcode markers ITS1-5.8S-ITS2 and *rbcL*, as described in paragraph 2.3.

Standard curves (SC) were assembled for both the Salm (Figure 4~~Figure 3~~ and Figure 4) and Univ (data not shown) sets through linear regressions of 10-fold dilution series ranging from 10 to 10^{-5} ng/µL [DNA] or from 1 to 10^{-4} g/L [cells] according to the method used to obtain the DNA (paragraphs 2.2 and 2.5). Four replicates for each concentration were amplified to obtain reliable threshold cycle (Ct) values; the average value was used to create the curve. Standard deviation (SD) of the four measurements was also calculated and reported in the figures. Both standard curves and relative methods to obtain DNA were laboratory tested using known concentrations of *S. almeriensis* cells (ranging from 1 µg/L to 1 g/L) diluted in microalgal cultures of commercial interest with concentrations ranging from 1 to 2 g/L dry weight. For each

assay the percentage error was calculated using the following formula: $\left(\frac{|SC\ value - Exact\ value|}{|Exact\ value|}\right) x100$; where the SC value was obtained using the standard curve equation and the exact value was calculated knowing the applied dilution of a culture whose concentration was determined by dry weight.

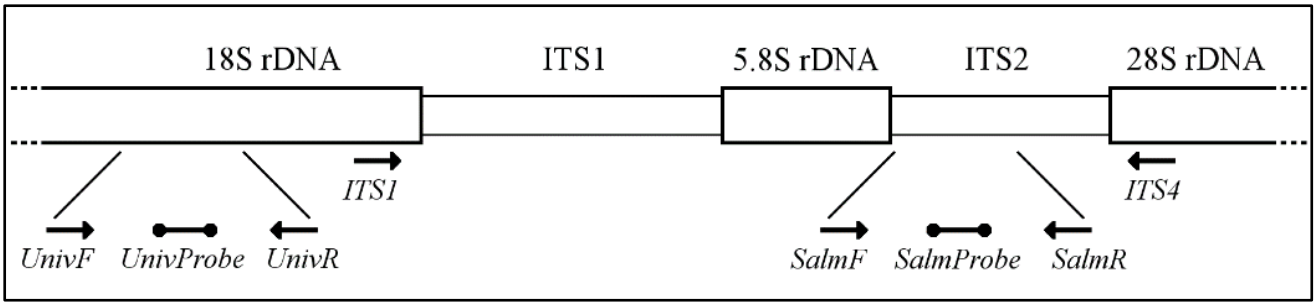

Figure 1. Relative locations of primers and fluorogenic probes within the nuclear ribosomal DNA region (not to scale). Primers are indicated with single-headed arrows and probes with double-headed bars. ITS: internal transcribed spacer.

2.5. Direct PCR

A simple method was developed to break down the *S. almeriensis* cells and rapidly perform the real-time PCR assays, avoiding DNA extraction. The FastPrep-24 instrument (MP Biomedicals) and Lysing Matrix B 2-mL tubes (MP Biomedicals) were used as described in Figure 2~~Figure 2~~. The first steps were intended to remove the culture medium; subsequently the sample was vortexed and centrifuged to separate the suspended DNA from the matrix particles and the cellular residuals. After this short procedure, 1.5 µL of supernatant was immediately used for a real-time assay, as described in paragraph 2.4. Different vortex protocols (6 m/s for 40 or 120 sec) and matrix types (B and C) were tested: Lysing Matrix B contained 0.1 mm silica beads, while Lysing Matrix C contained 1 mm silica beads. To assess the method's validity, real-time PCR assays were carried out to compare the DNA results obtained from classic DNA extraction, as

described in paragraph 2.2, and the supernatant obtained from this method, using the same culture but from the different matrices and vortex protocols tested.

Direct PCR assays were also performed on other microalgal strains belonging to the genera *Chlorella*, *Haematococcus*, *Scenedesmus* and *Nannochloropsis*. Using the VIC-fluorescence signal of the control UnivProbe, it was possible to estimate whether cell rupture occurred or not.

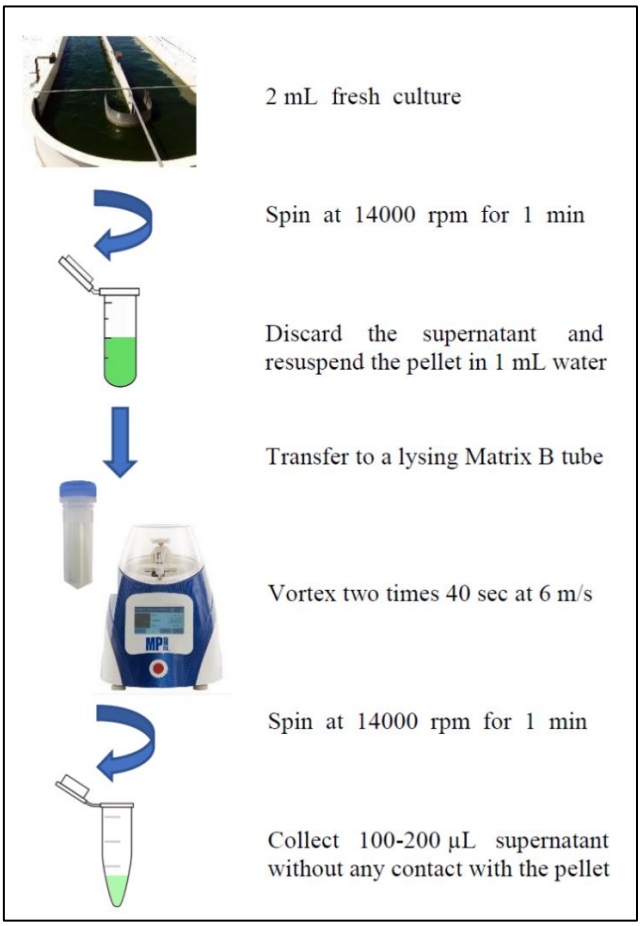

Figure 2. Experimental procedure for medium removal and subsequent cell rupture by means of strong vortexing. The use of nuclease-free water is recommended.

3. Results and discussion

3.1. Barcode marker sequencing

The ITS1-5.8S-ITS2 and *rbcL* barcode markers were successfully sequenced, assembled and uploaded onto the GenBank database (MF977406 and MG257492). The obtained 655-nt and 1312-nt sequences were compared with the other sequences in GenBank using the BLAST tool and the closest species were identified as *Scenedesmus obliquus* and *Scenedesmus bajacalifornicus*. Comparisons were made with *S. obliquus*

complete genomes FNXT01000001–FNXT01001368 [32] and NEDT01000001–NEDT01002707 [33] finding only 93% sequence similarity for both markers. Moreover, sequence similarity with *S. bajacalifornicus* was 97% for the ITS1-5.8S-ITS2 marker and 93% for the *rbcL* marker. Overall, these data demonstrate *Scenedesmus almeriensis* diversity between algal sequences present in the GenBank database, confirming the previous identification conducted by Sánchez et all [16].

3.2. Extraction methods

Scenedesmus almeriensis DNA extraction was performed with different commercial kits (Soil DNA Isolation Plus Kit by Norgen Biotek, PureLink Plant Total DNA Purification Kit by Invitrogen) and non-commercial methods (CTAB) [28] obtaining similar yields (data not shown). From these, the Soil DNA Isolation Plus Kit was selected and used routinely, starting always from a 2-mL culture sample with a concentration ranging from 0.5 to 2 g/L dry weight. With this set-up we were able to maintain a constant DNA extraction efficiency (43.2 ng DNA/mg of sample), avoiding column saturation and improving reproducibility.

Nevertheless, complete DNA extraction is a very time-consuming step in sample analysis. To solve this problem, a rapid method was developed to disrupt *S. almeriensis* cells by strong vortexing and to perform direct PCR assays directly on the lysate supernatant (Figure 2~~Figure 2~~). The best conditions to efficiently break down the microalga cells were obtained with Lysing Matrix B and a vortex protocol of: 40 s vortexing at 6 m/s, 40 s rest followed by another 40 s vortexing at 6 m/s. However, similar results were obtained from both matrices and the vortexing protocols tested; such as in qPCR, where a maximal 1.7-threshold cycle (Ct) difference was observed between the direct PCR samples and the average Ct of the control samples treated with classic DNA extraction.

Under these conditions it was possible to efficiently break down a number of *S. almeriensis* cells, ranging from 30 to $3 \cdot 10^7$ (from 1 µg/L to 1 g/L) in a 2-mL culture, proving its ability to work at a wide range of concentrations, a mandatory requirement when working with unknown samples. This approach enables us to save approximately two hours of total experiment time while maintaining the same PCR assay sensibility and precision as that obtained from samples processed by classic DNA extraction. Furthermore, the proposed disruption methodology has shown itself able to efficiently break down not only *S. almeriensis* cells, but also

a wide range of other species belonging to the genera *Chlorella*, *Haematococcus*, *Nannochloropsis* and *Scenedesmus*. Compared to the other direct PCR methods previously reported, our approach excels from its versatility with regard to the number and species of cells as well as its rapidity and simplicity - it does not need a long incubation period or extraction buffers - thus demonstrating its suitability for this and further applications.

Real-time PCR assay

For both methodologies obtaining template DNA, the Salm set was able to correctly quantify *S. almeriensis* concentrations as low as 1 µg/L (dry weight) in a 2-mL concentrated culture (1-2 g/L dry weight) of another microalga. A 1 µg/L concentration of *S. almeriensis* corresponds to approximately $1.7 \cdot 10^{-6}$ ng/µL extracted genomic DNA or 15 cells/mL in the direct PCR assay; this equates to less than one cell per PCR reaction as only a fraction of the total sample is used for the assay. The ability in detecting one cell or less is due to the presence of multiple copies of the ribosomal genes in these organisms; comparable results have been observed in similar works [34] [35].

The specificity of the Salm set for target species was also checked but no amplification was observed in any of the non-target microalgae tested, especially in the closest relatives, so the chances of a false-positive identification of another species are remote using this method. All the algae checked were previously submitted for sequencing to confirm correct identification. The obtained ITS2 sequences were aligned in order to establish the similarity to *S. almeriensis* in the region where the probe was designed. *SalmProbe* demonstrated itself to be species-specific for the target alga as well as having only two mismatches, as was the case with *Coelastrum proboscideum* (data not shown). In contrast, the Univ set amplified all the algae tested, including the Cyanobacteria *Spirulina*, without interfering with *S. almeriensis* detection, confirming its suitability as a positive control.

Standard curves were assembled for both the Salm (Figure 4~~Figure 3~~; Figure 4) and Univ (data not shown) sets through linear regressions of 10-fold dilution series. Subsequently, cell density could be calculated by comparing Ct values from an unknown sample with the standard curve. However, experiments have shown that the standard curve obtained with the Univ set using *S. almeriensis* DNA cannot be used to reliably quantify the total eukaryote genomes in a multi-species sample, probably because of the different

repetition number of the 18S gene in the different species [36]. Therefore, this set will only be used as a PCR positive control and to approximately estimate algal concentration, with no precise quantification or ratio with the *S. almeriensis* concentration. Moreover, it provides a control that ensures the nucleic acid extraction or the vortexing protocol have worked successful.

The PCR reaction efficiency (E) was estimated from the standard curve slope (m) obtained for the Salm set using the two DNA preparation methods, employing the formula $E = 10^{(-1/m)} - 1$. A 100% reaction efficiency (-3.31 slope) was obtained using the classic DNA extraction method, and an 89% efficiency (-3.62 slope) using the direct PCR method. The lower efficiency for the direct PCR assay was probably due to inhibitor molecules that remained in the PCR mix during the reaction [37]; however, these did not affect the linearity of the standard curve in the selected range, making this method suitable for microalgae quantification.

Both standard curves (Figure 4~~Figure 3~~ and Figure 4), and the relative methods for obtaining DNA, were laboratory tested in order to assess the precision of the measurements. For both, the real-time PCR assay was shown to be species-specific and sufficiently precise to identify the correct order of magnitude for the *S. almeriensis* concentration, with an average percentage error of 122% for DNA extraction and 68% for the direct PCR approach, and with the maximum error detected at the lowest concentrations (1 µg/L). This magnitude of error is compatible with the intended applications of this methodology; nonetheless, direct PCR gave a percentage error significantly lower than that observed with DNA extraction. This observation can be explained by the direct PCR's simpler experimental protocol and thus the fewer calculations that need to be carried out - this lead to a lower probability of making errors and to a more precise result.

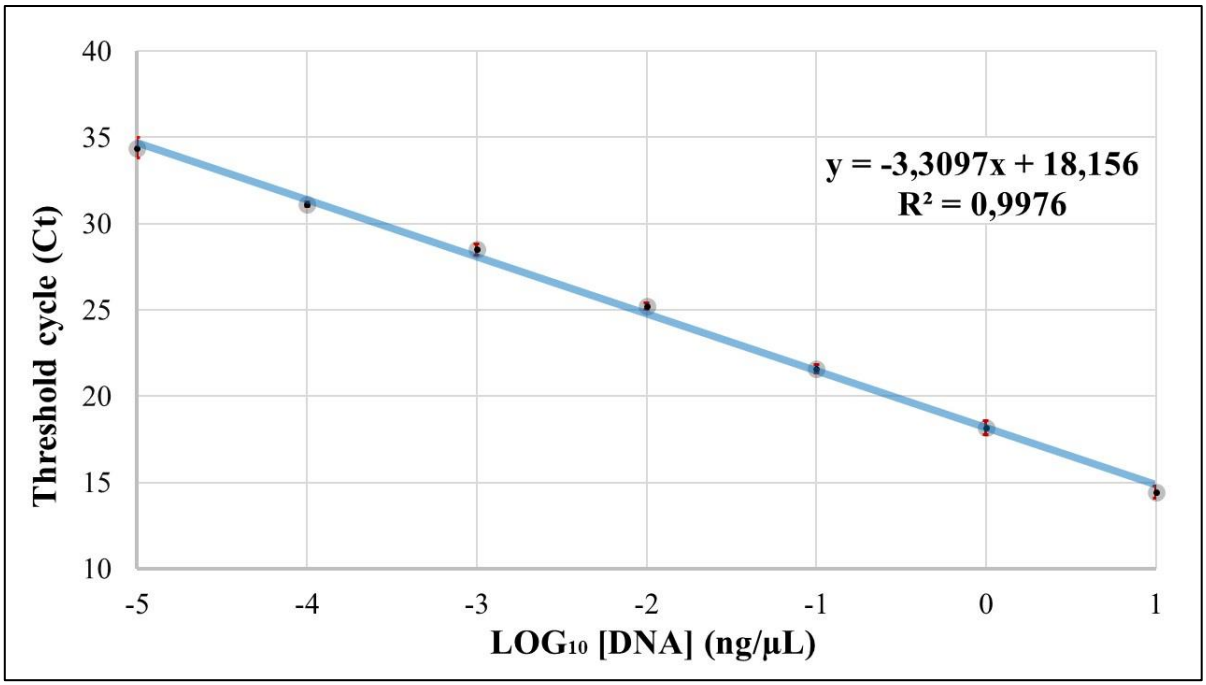

Figure 43. Standard curve obtained assessing threshold cycles with the Salm probe at different DNA concentrations, ranging from 10 to 10^{-5} ng/µL. DNA was obtained via classic DNA extraction. Data shown.

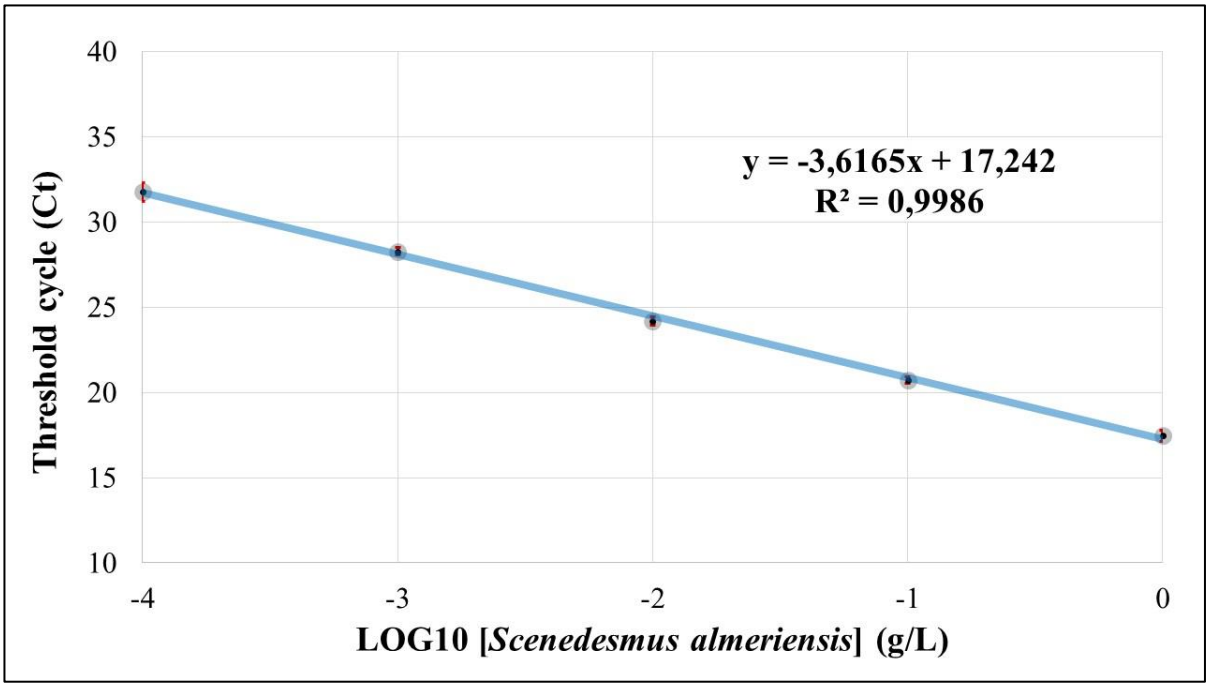

Figure 34. Standard curve obtained assessing threshold cycles with the Salm probe at different concentrations of *Scenedesmus almeriensis* cells, ranging from 1 to 10^{-4} g/L. DNA was obtained via vortexing, as explained in paragraph 2.5. Data shown as mean +/-SD, n=4. Linear regression and curve slope is also reported.

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4. Conclusions

Traditionally, microalgae species are recognized by morphological discrimination under an optical microscope. However, microalgae often display very few morphological features that can be used for identification, leading to uncertainty regarding the true identity of the routinely used microalgae species. Furthermore, when it comes to identifying small concentrations of contaminant microalgae in a concentrated culture of another alga, the task is even harder and more labour intensive [38]. The applied qPCR assay not only allows us to identify the presence of *Scenedesmus almeriensis* cells in any microalgal culture but also enables us to precisely quantify the population via a species-specific TaqMan probe. This outcome is almost unobtainable with other techniques. Among the possible alternatives, flow cytometry is a powerful method for counting cells [39]; nevertheless, when it comes to restricting the analysis to a single species mixed with many others, the task is hard to accomplish. In contrast, our technique has the advantage of simultaneously identifying and quantifying just the target species, even when its presence is minimal within the algal sample.

The methodology was initially developed using classic nucleic acid extraction but because of the time-consuming protocols of the commercially available kits, we set about optimizing a direct PCR approach based on strong vortexing and which had minimal experimental steps. This approach demonstrated how it provided the same sensitivity as classic DNA extraction but was more precise, cheaper and less time-consuming, given that the total experiment can be performed in approximately two hours. Furthermore, the proposed methodology is highly versatile and can be easily applied to virtually any microalgae species, even to new or non-identified species - this is because the nucleic acid sequence used to design primers and probes is obtained directly from the algae of interest, without the necessity of knowing its correct binomial name. ~~At the same time, the sequencing of barcode markers like ITS and *rbcL* enables to check the GenBank for similar sequences and to precisely identify the alga at genomic level, without any need of trained staff or taxonomists able to identify the species with traditional morphological discrimination.~~

The broad applicability of the proposed methodology promises to considerably expand our understanding of microalgal occurrence in economically important microalgal cultures and to support the achievement of maximal productivity and quality in the biomass produced.

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6. Additional information

The authors declare no competing interests with any existing national or international bodies. In this research, no conflicts, informed consent, human or animal rights are applicable. The authors mutually agree to submit this manuscript to Algal Research for peer review and publication.

7. Author contributions

FGAF, JAGC and PBL designed the research. CS grew the algal samples. PBL performed the molecular work. JAGC analysed the sequences. PBL wrote the paper. FGAF and JAGC reviewed the manuscript.

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