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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

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

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Abstract

In industrial-scale microalgal 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. Among 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* sp. '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, including in the presence of concentrated mixed cultures of other microalgae. A simple direct qPCR approach was also investigated to avoid classic DNA extraction and to reduce total assay time to approximately 2 h. The objective was to design strain-specific tools able to confirm and quantify the presence of different strains in whatever microalgae culture so as to achieve maximal productivity and quality of the produced biomass.

Keywords Microalgae · *Scenedesmus almeriensis* · Quantification · Identification · Direct qPCR

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 and feed industries (Spolaore et al. 2006), while a large diversity of secondary metabolites are yet to be fully exploited (Cardozo et al. 2007). However, for all commercial applications, the monitoring of contaminant and unwanted microalgae in outdoor or non-sterile bioreactors is of great importance (Dawidziuk et al. 2017). Non-target microalgae compete with the desired species for nutrients and CO_2 (Wang et al. 2013), reducing the growth rate of the target alga or even predominating within the whole culture; this, consequently, lowers overall productivity and biomass quality (Mingazzini et al. 2015).

Microalgae species have historically been discriminated by their morphology 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 (Ebenezer et al. 2012). In particular, DNA barcoding has been very useful in providing rapid, accurate, and automatable species identification using short, standardized gene regions as internal species tags (Hebert and Gregory 2005). The most promising candidates for green microalgae barcoding are the

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internal transcribed spacers 1 and 2 (ITS1 and ITS2) of the nuclear rDNA and the *ribulose bisphosphate carboxylase* (*RuBisCO*) large subunit (*rbcL*) gene (Hadi et al. 2016). 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 (Ebenezer et al. 2012). The main advantage of using quantitative PCR is that it is highly sensitive, specific, accurate, and cost-effective; furthermore, it can also be applied to a large number of experimental samples at the same time (Toyoda et al. 2010).

The objective of this study was to design a simple real-time PCR assay to precisely quantify the presence of *Scenedesmus* sp. '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 (Sánchez et al. 2008b). Subsequently, 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 (Sánchez et al. 2008a).

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 (Woodman et al. 2016). This technique is widely used for bacteria and yeast (Fode-Vaughan et al. 2001), but because algal cell walls are structurally stronger (Kim et al. 2016), direct PCR for microalgae is more difficult. Nevertheless, direct PCR for microalgae has been reported, initially with *Chlamydomonas* (Cao et al. 2009) (Zamora et al. 2004), thanks to its weaker cell wall (Imam et al. 1985), and subsequently also on other microalgae such as *Chlorella* (Wan et al. 2011), *Scenedesmus* (Radha and Fathima 2013), and *Nannochloropsis* (Liu et al. 2014).

Materials and methods

Microorganism and culture conditions

The freshwater microalga *Scenedesmus* sp. 'almeriensis' CCAP 276/24 (NCBI taxonomy ID: 2037726) 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 microalgae species used in this study, was grown as monoalgal continuous culture in Arnon medium (Arnon et al. 1974) 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-h light cycle was provided by fluorescent tubes giving up to 500 $\mu E/m^2$ s. The cell culture concentration was assessed via dry weight determination, with 1-μm pore size paper filters, or by cell counting with a Neubauer chamber. The obtained biomass was conserved at − 80 °C until the use.

DNA extraction

To remove the culture medium, 2–10 mL of fresh concentrated culture were centrifuged at 2500*g* for 5 min 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 the Soil DNA Isolation Plus Kit (Norgen Biotek Corp.) and the PureLink Plant Total DNA Purification Kit (Invitrogen). The total genomic DNA extracted was quantified with the Qubit dsDNA HS Assay Kit (Molecular Probes).

Sequencing

The ITS1-5.8S-ITS2 region was amplified through PCR using the primers ITS1 and ITS4 (White et al. 1990) described in 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 followed by High Resolution Melting (HRM) analysis.

To amplify a 1380 nt amplicon of the *rbcL* marker, two new primers were designed (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 was composed of 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 and lowering 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 PCR amplification protocol used was 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.

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

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

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 s, 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). The fluorescently labeled DNA was subsequently precipitated by ethanol precipitation (Sambrook et al. 1982). Sequences of both positive and negative strands were determined by capillary electrophoresis in the AB 3500 Genetic Analyzer to obtain a minimum twofold coverage for each sequenced nucleotide. Forward and reverse sequences were aligned and manually edited to generate consensus sequences. The new and first-time reported *Scenedesmus* sp. 'almeriensis' sequences were deposited in GenBank under the following accession numbers: MF977406 (ITS1-5.8S-ITS2) and MG257492 (*rbcL*).

Real-time PCR assay specifications

S. almeriensis-specific primers (SalmF and SalmR) and an internal TaqMan FAM-labeled MGB probe (SalmProbe) were designed within the internal transcribed spacer region 2 (ITS2) of the previously sequenced ribosomal DNA cluster (Table 1; Fig. 1). ITS2 sequences of the most similar microalgal species were mined from GenBank and aligned in order to design primers and probes with maximum differentiation from non-target algae. The probe was designed to have a minimum of two mismatches from all the sequences analyzed. An additional set of primers (UnivF and UnivR (Hayden et al. 2006)) and a VIC-labeled MGB probe (UnivProbe) were used to amplify an 83-nucleotide region of the 18S rDNA, which is conserved in all eukaryotes. The two sets of primers along with the probes 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 labeled 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 commercially important microalgae species (*Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Parachlorella kessleri*, *Spirulina platensis*, *Haematococcus pluvialis*, *Nannochloropsis gaditana*, and *Nannochloropsis oceanica*), 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 Sequencing.

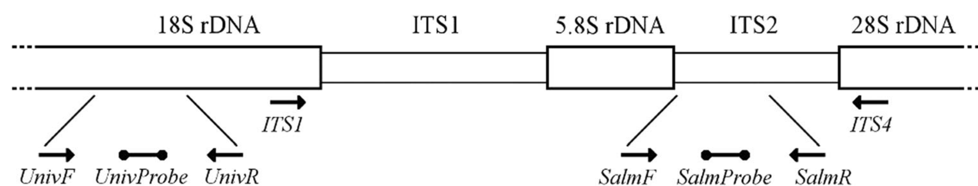

Fig. 1 Relative locations of primers and fluorogenic probes within the nuclear ribosomal DNA region (not to scale). Primers are indicated with single-headed arrows and probes with double-headed bars. ITS, internal transcribed spacer

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Standard curves (SC) were assembled for both the Salm and Univ sets through linear regressions of tenfold 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 (DNA extraction or Direct qPCR). 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: $(|SC\ value - Exact\ value| / |Exact\ value|) \times 100$; where the "SC value" was obtained using the standard curve equation and "Exact value" was calculated knowing the applied dilution of a culture whose concentration was determined by dry weight.

Direct qPCR

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 Fig. 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 PCR assay as previously described. Different vortex protocols (6 m/s for 40 or 120 s) 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 qPCR results from DNA obtained from classic DNA extraction and the supernatant obtained from this method, using the same culture but with the different matrices and vortex protocols tested.

Direct qPCR 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.

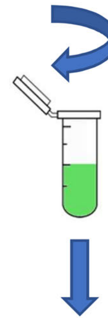

Fig. 2 Experimental procedure for medium removal and subsequent cell rupture by means of strong vortexing. The use of nuclease-free water is recommended

Results

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 (Carreres et al. 2017) and NEDT01000001–NEDT01002707 (Starkenburg et al. 2017) 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* sp. 'almeriensis' diversity between algal sequences present in the GenBank database until now. These sequence differences were sufficient to design a set of species-specific primers and probe for this microalgal strain.

Extraction methods

DNA extractions were performed on eight microalgal strains with two different commercial kits—the Soil DNA Isolation

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Plus Kit by Norgen Biotek and the PureLink Plant Total DNA Purification Kit by Invitrogen—obtaining yields ranging from 1.6 to 41.4 times higher with the first one. Thus, 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 setup, 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 qPCR assays directly on the lysate supernatant (Fig. 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 vortexing protocols tested, since in qPCR, 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 break down a number of *S. almeriensis* cells ranging from 30 to 3×10^7 (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 2 h of total experiment time, while maintaining the same qPCR 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* (Online resource 1).

Real-time PCR assay

For both methodologies to obtain template DNA, the Salm set was able to correctly quantify *Scenedesmus* sp. '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 1.7×10^{-6} ng/μL extracted genomic DNA or 15 cells/mL in the direct qPCR 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 (Dyhrman et al. 2006) (Coyne et al. 2005).

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 (Online resource 2), especially in the closest relatives (Online resource 3), 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* (Online resource 4). In contrast, the Univ set amplified all the algae tested without interfering with *S. almeriensis* detection, confirming its suitability as a positive control (Online resource 1).

Standard curves were assembled for both the Salm (Figs. 3 and 4) and Univ (data not shown) sets through linear regressions of tenfold 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 (Roa and Guerra 2012). 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 ensure 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) with the direct qPCR method. The lower efficiency for the direct qPCR assay was probably due to inhibitor molecules that remained in the PCR mix during the reaction (Schrader et al. 2012); 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, 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 qPCR 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 qPCR gave a percentage error significantly lower than that observed with DNA extraction. This observation can be explained by the direct qPCR's simpler experimental protocol and thus the fewer calculations that need to be carried out; this leads to lower probability of making errors and to a more precise result.

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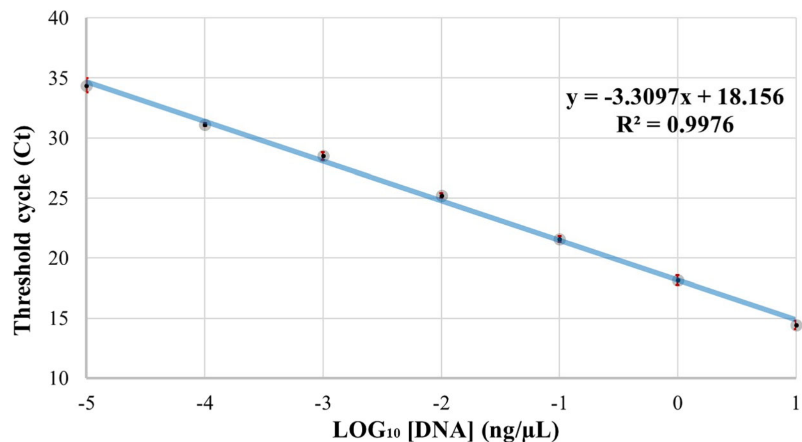

Fig. 3 Standard curve obtained assessing threshold cycles with the Salm probe at different DNA concentrations, ranging from 10 to 10^{-5} ng/μL. DNA was obtained via classic DNA extraction. Data shown as mean +/-SD, $n = 4$. Linear regression and coefficient of determination are also reported

Discussion

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 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 labor intensive (Handy et al. 2008). The *S. almeriensis* ITS1-5.8S-ITS2 genomic region was sequenced and used to design a qPCR assay that not only allows us to identify the presence of *Scenedesmus* sp. 'almeriensis' cells in any microalgal culture but also enables us to quantify down to 1 μg/L microalgal biomass 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 (Hyka et al. 2013); 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. Furthermore, the qPCR assay enables to overcome flow cytometry problems related with the quantification of colony-forming species, like in *Scenedesmus* spp. (Peniuk et al. 2016).

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 2 h. Compared to the other direct PCR methods previously reported, our approach excels for its versatility with regard to the number and species of cells as well as its rapidity and simplicity—since it does not need long incubation periods or extraction buffers—thus demonstrating its suitability for this and further applications.

Overall, the proposed methodology is highly versatile. In this work *S. almeriensis* was used as model organism, but the same technique can be applied to identify and quantify virtually any microalgae species, by developing specific primers and probes for each organism. Not only microalgae could be

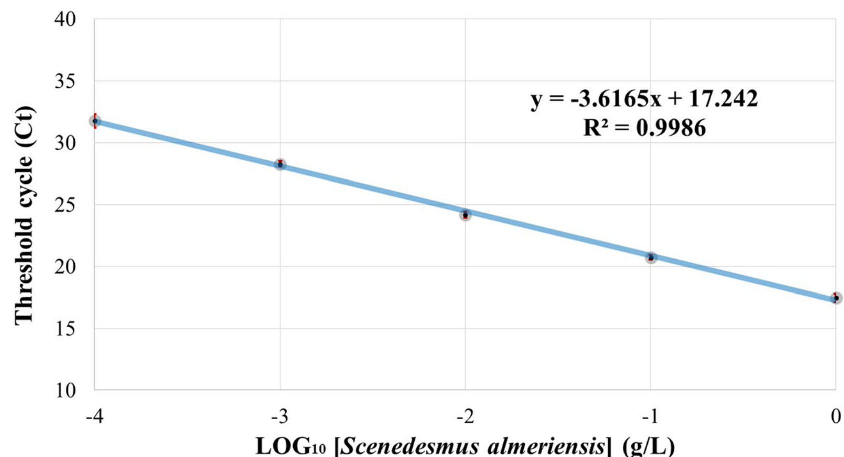

Fig. 4 Standard curve obtained assessing threshold cycles with the Salm probe at different concentrations of *S. almeriensis* cells, ranging from 1 to 10^{-4} g/L. DNA was obtained via vortexing as explained in paragraph Direct qPCR. Data shown as mean ± SD, $n = 4$. Linear regression and coefficient of determination are also reported

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monitored; with the creation of appropriate standard curves for each organism, also rotifer, microcrustacean, and protist concentrations could be assessed, in order to cover the most common contaminant species of a culture of interest. If well designed, all the probes can be used in the same qPCR run, making the assay rapid and highly informative, allowing mitigating the negative effects of the contaminating organisms. 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 of the biomass produced.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal studies This article does not contain any studies with human participants or animals performed by any of the authors.

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