METHODS

Optimised protocols for RNA extraction from a broad taxonomic range of algae

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Abstract

Despite advancementsin RNA extraction methods, RNA extraction from sources rich in polyphenols and polysaccharides such as algae and seagrasses remains a challenge. Here we present a RNA extraction strategy using a hexadecyltrimethylammonium bromide (CTAB) extraction buffer and demonstrate its effectiveness on a broad range of red, green, and brown algae, as well as on the cyanobacterium *Arthrospira platensis* and the seagrass *Zostera marina*. For the vast majority of tested samples we achieved high yields of RNA comparable to those obtained from higher plants by commercially available kits (ranging from 3.9 to 125.9 μg RNA g^{-1} fresh weight). Analysis by UV/Vis spectrometry and capillary electrophoresis revealed high purity and integrity of obtained RNA extracts. For highly challenging species of brown algae like *Fucus vesiculosus*, *Fucus serratus* and *Dictyosiphon foeniculaceus*, we established an alternative procedure using a sodium dodecyl sulfate (SDS) extraction buffer in combination with a commercial kit. With this protocol, even higher RNA yields up to 317.0 μg g^{-1} fresh weight were extracted from polysaccharide-rich brown algae tissues. This study can serve as a guideline and starting point for the development of RNA extraction protocols for so far unstudied algal species from very diverse taxa.

Keywords RNA extraction · Algae · CTAB · SDS · Polyphenols · Polysaccharides

Introduction

The analysis of gene expression is an important task in biological research. Therefore, high-quality RNA extracts are essential for common techniques used in RNA analysis and transcriptomics, including Northern blotting, hybridisation, RT-PCR and cDNA library construction. Whereas commercially available extraction kits or standardised extraction methods can easily extract animal tissues and bacteria, the extraction of RNA from plants rich in polysaccharides and polyphenols remains a challenge (Loomis 1974; Schneiderbauer et al. 1991; Salzman et al. 1999; Gehrig et al. 2000; Wang et al. 2008). Especially the cell walls of marine algae often contain high amounts of polyphenolic compounds and polysaccharides that interfere and co-precipitate with RNA in low-ionic strength buffers (Dos Reis Falcão et al. 2008; Sim et al. 2013; Greco et al. 2014).

In the past several authors achieved positive results using a hexadecyltrimethylammonium bromide (CTAB) lysis buffer and organic solvents for protein removal. To our best knowledge, RNA extraction from algae using a CTAB buffer was first published on the brown alga *Macrocystis pyrifera* and the red alga *Aglaothamnion neglectum* in the early 1990s (Apt and Grossman 1993; Apt et al. 1995). This method underwent multiple adaptations for the purpose of better extraction results or simplification on different species (Chan et al. 2002; Pearson et al. 2006; Heinrich et al. 2012; Sim

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et al. 2013). Chan et al. (2002) varied the precipitation procedure by using lithium chloride (LiCl) for the second RNA precipitation instead of re-precipitating with ethanol (EtOH) and sodium acetate and significantly reduced the precipitation time. Pearson et al. (2006) introduced further changes to the extraction buffer composition by increasing the concentrations of ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl) and dithiothreitol (DTT). At higher concentrations, DTT prevents the oxidative cross-linking of RNA by phenolic compounds and effectively inhibits RNase activity (Pearson et al. 2006). Higher NaCl concentrations increase the ionic strength, which was shown to facilitate the removal of contaminating polysaccharides (Fang et al. 1992; Chang et al. 1993; Wang et al. 2008). By incorporating these and a few other modifications, the group around Pearson reported impressive results on the brown algae *Fucus vesiculosus* and a few other species while effectively reducing buffer consumption and avoiding toxic chaotropic agents such as guanidinium salts and phenol.

However, when applying these protocols to novel algal species, in our hands RNA yields and qualities obtained by these methods were often lower to those achieved by the respective authors. In general, the effectiveness of most published RNA extraction methods was demonstrated only on a handful of species and did not cover a broad taxonomic diversity. Establishing and examining several extraction protocols for multiple species, however, can be time-consuming and expensive.

Therefore, we present here an adaptable strategy for RNA extraction from sources rich in polysaccharides and polyphenols based on the CTAB method from Pearson et al. (2006) and others and demonstrate its effectiveness on a broad range of marine algae and seagrasses commonly found in the Baltic Sea. Adaptations in the buffer composition and experimental procedures were explored to ensure robust and reliable extraction results. We were able to apply the method to a variety of taxonomically diverse species without further optimisation procedures. Additionally, an alternative protocol using a sodium dodecyl sulfate (SDS) extraction buffer was established for a selection of brown algae species of which RNA extraction with the modified CTAB led to insufficient results.

Materials & methods

Sample collection and preparation

All macroalgae and seagrass samples were collected between September and May in the coastal waters of the Kiel fjord, Germany, and identified based on morphological characteristics. Macroalgae samples were collected from single individuals, if possible, and immediately quick-frozen on dry ice. We collected eight species of red algae (*Furcellaria lumbricalis*, *Dumontia*

contorta, *Ahnfeltia plicata*, *Callithamnion corymbosum*, *Dasya baillouviana*, *Polysiphonia stricta*, *Ceramium virgatum*, *Porphyra purpurea*), four species of green algae (*Bryopsis plumosa*, *Cladophora rupestris*, *Spongomorpha aeruginosa*, *Ulva lactuca*), six species of brown algae (*Saccharina latissima*, *Chorda filum*, *Pylaiella littoralis*, *Dictyosiphon foeniculaceus*, *Fucus vesiculosus*, *Fucus serratus*), and the seagrass *Zostera marina*. All samples were stored at -80 °C until use. Fresh microalgae (*Chlorella vulgaris*) and cyanobacteria (*Arthrospira platensis*) cultures were obtained from the Botanical Institute of Kiel University. After culturing, microbes were separated from the culture media by centrifugation, frozen in liquid nitrogen and stored at -20 °C until use. Commercial dried *A. platensis* ("Spirulina") food supplement powder was bought from Borchers Fine Food GmbH & Co. KG, Germany. All samples were ground in a mortar with liquid nitrogen until a fine powder was achieved. Aliquots of approx. 1 g of powder were transferred into 50-mL tubes, quick-frozen in liquid nitrogen and stored at -80 °C until extraction.

RNA extraction with the modified CTAB method

The final protocol used for RNA extraction using CTAB is summarized in Fig. 4a. A CTAB extraction buffer containing 2% (w/v) CTAB, 2% (w/v) polyvinylpyrrolidone 40 (40 kDa; PVP40), 100 mM trishydroxymethylaminomethane (Tris; pH 8.0), 50 mM EDTA (pH 8.0), and 2 M NaCl in RNase-free ultra-pure water was prepared. 50 mM DTT were always freshly added to the buffer prior to extraction. 4 mL of the freshly prepared extraction buffer were then added to 1 g of frozen algae powder and incubated for 15 min at room temperature while thoroughly vortexing every 2-3 min. Phenolic separation was conducted by adding 1 volume of ROTI®-Aqua-PCI containing phenol, chloroform and isoamylalcohol [25:24:1] (PCI) with a pH of 4.5 to 5 and centrifugation ($3350 \times g$, 30 min, room temperature). The supernatant was transferred into a new tube and polysaccharides were precipitated by a 5 min long incubation with 0.3 volumes of absolute EtOH. After removing the precipitate by centrifugation ($3350 \times g$, 10 min, room temperature), a second PCI separation was performed. LiCl (2.5 M final) and β-mercaptoethanol (1% (v/v) final) were added to the supernatant, which was then mixed and aliquoted into 1.5 mL tubes. We performed RNA precipitation at 4 °C overnight and carried out subsequent centrifugation at 4 °C and $16,000 \times g$ for 30 min. Obtained RNA pellets were washed with 500 μL of pre-cooled (-20 °C) EtOH per tube, air-dried, resuspended in a total volume of 80 μL of RNase-free water, and pooled. RNA extracts were polished using the OneStep PCR Inhibitor Removal Kit (Zymo Research Europe GmbH, Germany) according to the manufacturer's instructions. All samples were extracted once except *F. lumbricalis* ($n=3$, biological replicates), *S. latissima* ($n=3$, biological replicates) and *B. plumosa* ($n=3$, technical replicates).

RNA extraction with the SDS method

The final protocol is given in Fig. 4b. An SDS extraction buffer containing 0.5% (w/v) SDS, 25 mM Tris, 35 mM EDTA, 35 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and 50 mM LiCl was prepared and adjusted to a pH of 7.5 with hydrochloric acid. As an optional step, 2% of PVP40 were added in half of the extractions performed with this method. Samples were incubated in 6 mL of the extraction buffer at room temperature for 15 min while vortexing every 2–3 min. The first organic separation was conducted using 1 volume of PCI, whereas the second separation was conducted with 1 volume of chloroform-isoamylalcohol [24:1] (CI). Polysaccharide precipitation was performed with 0.6 volumes absolute EtOH. All centrifugation steps during organic separation, polysaccharide precipitation, RNA precipitation and polishing were performed as described for the CTAB method.

RNA analysis

The absorbance of extracted RNA samples was measured by UV/Vis spectrometry using an Eppendorf BioPhotometer 6131 (Eppendorf SE, Germany) at 230 nm, 260 nm, and 280 nm. Samples were diluted in ultrapure water in a 1:8 to 1:30 ratio in order to fit the quantitative range of the device. Furthermore, UV/Vis spectra were collected using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). Quantity and integrity of RNA samples were determined by capillary electrophoresis with a 2100 Bioanalyzer (Agilent Technologies Inc., USA) using the RNA 6000 Nano Kit according to the manufacturer's instructions. Except the RNA extracts of *A. platensis*, which were analysed by the Prokaryote Total RNA Nano assay, all samples were analysed by the Plant RNA Nano assay in Agilent's 2100 Expert software.

RNA extracts of *F. lumbricalis*, *C. corymbosum*, *C. virgatum*, *B. plumosa*, *S. aeruginosa*, *C. vulgaris*, *S. latissima*, *P. littoralis*, *F. serratus*, and *Z. marina* were examined by a PCR of 18S and 16S ribosomal DNA regions to detect DNA contamination of eukaryotic and prokaryotic origin, respectively. RNA extracts were diluted to concentrations between 1.5 and 7 ng μL^{-1} and, per reaction, 1 μL of diluted RNA extract was added to a master mix, finally containing 1×Colorless GoTaq reaction buffer, 0.2 mM dNTP's, 1 unit GoTaq DNA polymerase, and 4 nM per forward and reverse primer. Genomic DNAs of *Mnemiopsis leidyi* and *Muribaculum intestinale* YL27, which were isolated using the Wizard Genomic DNA Purification Kit (Promega, USA), were used as positive controls for the 18S and 16S PCR, respectively, while water was used as negative control. The primer pairs are given in Table 1. The PCR program was performed as follows: initial denaturation for 5 min at 95 °C, followed by 30 cycles of denaturation (95 °C, 30 s), annealing (50 °C, 45 s), and extension (72 °C, 90 s), as well as one final extension step at 72 °C for 10 min. PCR products were examined on a 1% agarose gel stained with ethidium bromide.

RNA extraction by external service provider, cDNA library construction & next generation sequencing

RNA samples of *S. latissima*, *C. filum*, *A. plicata*, and *F. lumbricalis* extracted by the modified CTAB method were send to an external service provider (StarSEQ GmbH, Mainz, Germany) for cDNA library construction and sequencing. As a reference, a frozen tissue sample of *S. latissima* was send to the same provider for external RNA preparation. StarSEQ performed tissue disruption using a bead mill homogenizer and performed RNA isolation using multiple protocols based on the peqGOLD Total RNA Kit (VWR International LLC, USA), the RNeasy Plant Mini Kit, and the RNeasy PowerPlant Kit. The RNA extract with the highest quality, which was obtained by the RNeasy Plant Mini Kit-based method, was chosen for cDNA library construction. StarSEQ performed mRNA enrichment using the NEB Next Poly(A) mRNA Magnetic Isolation Module and cDNA synthesis using the NEB Next Ultra II Dual Index Library Preparation Kit (New England Biolabs, USA). Sequencing was performed with an Illumina NextSeq 500 (Illumina, USA) generating paired-end 150 bp reads. The expected total throughput for five species was 250 million reads (forward & reverse).

Sequence data processing and quality control

Sequence data from the external supplier were adapter and quality trimmed with a quality threshold ≥ Q20. Remaining read pairs and single reads were deduplicated and used for de novo assembly. Minimum contig length was set to 120 bp and the read pair distance range was set to 100–700 bp. All steps were computed with the CLC assembly Cell software (v5.2.1, https://digitalinsights.qiagen.com/). The quality of raw and processed sequence data was examined using FastQC (Andrews 2010).

Table 1 Primers used for the 16S and 18S PCRs

PCR	Primer	Sequence	Reference
18S	EukA	5´-AAC CTG GTT GAT CCT GCC AGT-3'	(Wang et al. 2014)
18S	Euk1209	5´-CAG GTC TGT GAT GCC C-3'	
16S	27F	5´-AGA GTT TGA TGC TGG CTC AG-3'	(Lane 1991)
16S	1482R	5´-GGT TAC CTT GTT ACG ACT T-3'	

Results

Yield & purity of RNA extracts obtained by the optimised CTAB/SDS-based extraction methods

Due to the limitations of commercially available RNA extraction kits and methods for extraction of RNA from species used in this report, a CTAB-based RNA extraction method was optimised based on previously published work (Apt and Grossman 1993; Chan et al. 2002; Pearson et al. 2006; Sim et al. 2013) and tested on 22 taxonomically diverse algae,

cyanobacteria and seagrass samples. Quality and yield of RNA obtained by LiCl precipitation were determined. Then, the RNA was polished using a PCR Inhibitor Removal Kit. Overall, RNA was successfully extracted from 8 species of Rhodophyta (red algae), 5 species of Chlorobionta (green algae), 3 species of Phaeophyceae (brown algae), as well as from the seagrass *Z. marina* and the cyanobacterium *A. platensis*, indicating the broad applicability of the modified CTAB method on a variety of marine and non-marine algal and plant species (Figs. 1a and 2a, Table S1). UV/Vis absorbance spectra did not display shifted maxima (data not shown) and $A_{260nm/280nm}$ and $A_{260nm/230nm}$ ratios indicated good purity
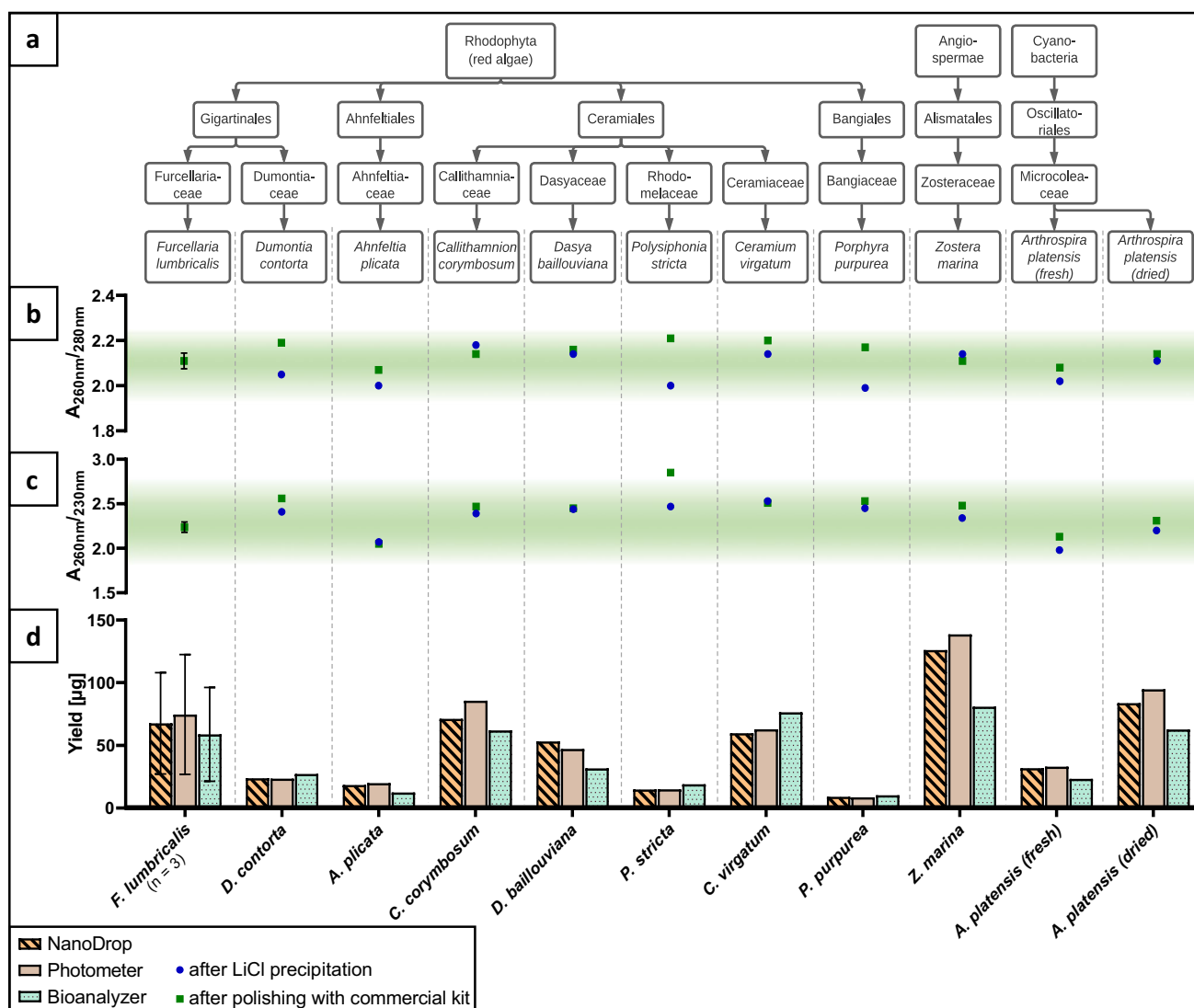
Fig. 1 RNA quantity and quality data of red algae, seagrass, and cyanobacteria samples extracted by the modified CTAB method. (**a**) Taxonomic distribution of tested samples. (**b**) A_{260nm}/A_{280nm} UV/Vis ratios. (**c**) A_{260nm}/A_{230nm} UV/Vis ratios. Samples were measured with a NanoDrop 2000 spectrophotometer after LiCl precipitation (blue dot) and polishing (green square). Green bands indicate the range of acceptable RNA purity. Absorbance ratios of *F. lumbricalis* were only

measured after polishing, but not after LiCl precipitation. (**d**) RNA quantity measured with the NanoDrop spectrophotometer (orange), UV/Vis Eppendorf BioPhotometer (violet) and Bioanalyzer (light blue). RNA yield per g fresh weight from dried *A. platensis* powder was calculated based on an assumed initial water content of 80% (Seghiri et al. 2021). Values for *F. lumbricalis* display means ± SD (biological triplicates)
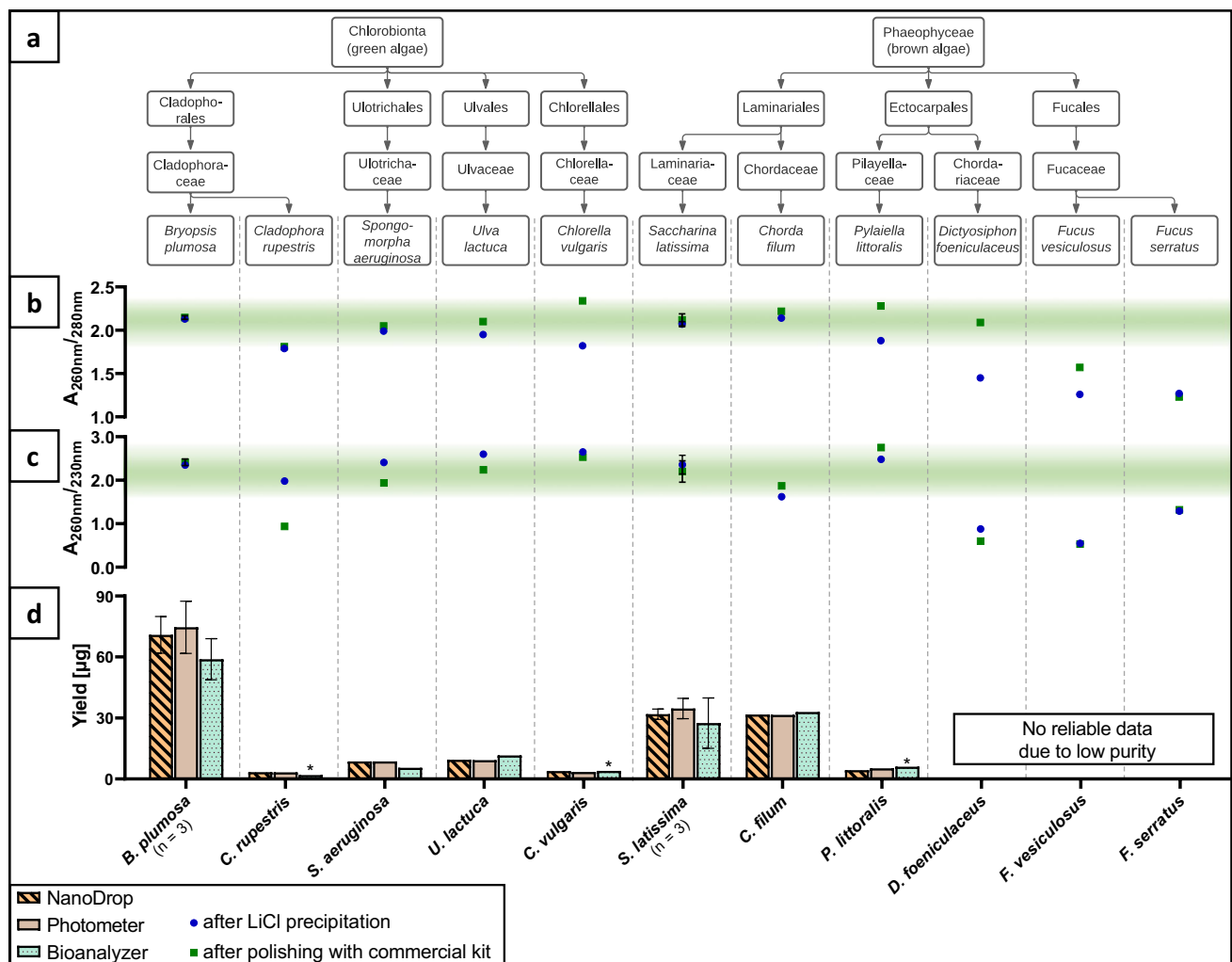
Fig. 2 RNA quantity and quality data of green and brown algae extracted by the modified CTAB method. (**a**) Taxonomic distribution/phylogenetic tree of tested samples. (**b**) A_{260nm}/A_{280nm} UV/Vis ratios. (**c**) A_{260nm}/A_{230nm} UV/Vis ratios. Samples were measured with a NanoDrop 2000 spectrophotometer after LiCl precipitation (blue dot) and polishing (green square). Green bands indicate the range of accept- able RNA purity. Bioanalyzer runs labelled with an asterisk (*) were performed with unpolished samples to fit the quantitative range of the device. (**d**) RNA quantity measured with the NanoDrop spectropho- tometer (orange), UV/Vis Eppendorf BioPhotometer (violet) and Bio- analyzer (light blue). Values for *B. plumosa* (technical triplicates) and *S. latissima* (biological triplicates) display means ± SD

with overall little to no indications of protein, salt or (poly) phenolic contamination (as shown in Figs. 1b, c and 2b, c)Per g fresh weight, successful extractions yielded between 3.9 µg (*C. vulgaris*) and 125.9 µg (*Z. marina*) RNA based on NanoDrop measurements. The RNA quantities of each sample measured by UV/Vis spectrometry (BioPhotometer, NanoDrop) and capillary electrophoresis (Bioanalyzer) are given in Figs. 1d and 2d.

Extractions from *F. lumbricalis* (red alga) and *S. latissima* (brown alga) were performed in biological triplicates harvested between December and April and yielded 67.5 (± 40.4) µg and 31.8 (± 2.5) µg respectively. The green alga *B. plumosa* was extracted in technical triplicates and yielded 70.9 (± 9.0) µg of RNA (values

are means ± SD). Yields of particularly thin-leaved algae such as *C. rupestris*, *U. lactuca*, *P. purpurea*, *P. littoralis*, and *S. aeruginosa* were moderate, possibly due to a higher capability of retaining water and thus lower amount of biomass in 1 g of the frozen powder. After successful RNA precipitation with LiCl, $A_{260nm/280\ nm}$ ratios ranged from 1.79 (*C. rupestris*) to 2.18 (*C. corymbosum*). $A_{260nm/230\ nm}$ was above 1.98 for all samples except *C. filum* (1.62), of which the obtained RNA pellet was characterized by a viscous and rubberlike texture, indicating the co-precipitation of polysaccharides. Already good absorption ratios were hardly improved by the PCR Inhibitor Removal Kit, indicating its redundancy for most of the tested samples extracted by the CTAB method.

The quality of the *C. rupestris* RNA extract appeared to decline after polishing. It remains unclear whether this was caused by an incomplete removal of unexpected contaminants. The extractions on the brown algae *D. foeniculaceus*, *F. vesiculosus*, and *F. serratus* were considered non-successful due to low $A_{260nm/280\,nm}$ and $A_{260nm/230\,nm}$ ratios. Whereas the extraction on *F. vesiculosus* did hardly yield any pellet after LiCl precipitation, the "RNA pellet" of *F. serratus* appeared orange, indicating co-precipitation of pigments.

Since the quality of their RNA extracts were not improved by polishing, an alternative protocol using an SDS extraction buffer was established and tested with and without the addition of PVP40 (see Fig. 3, Table S2). $A_{260nm/280\,nm}$ ratios (ranging from 2.06 to 2.13) indicated no contamination of proteins regardless of the buffer composition. Low $A_{260nm/230\,nm}$ ratios (ranging from 0.48 to 1.15) indicated the presence of contaminants like polyphenols or polysaccharides that were fully or partly removed by

single or repeated polishing steps with the commercial kit. Although not tested with a high level of statistical certainty, extractions conducted with PVP40 led to higher yields. The final protocols for the modified CTAB method and the SDS method are given in Fig. 4.

RNA integrity and suitability for NGS applications

RNA samples were investigated by capillary electrophoresis. The electropherograms of a selection of analysed RNA extracts are given in Fig. 5. Overall, clearly visible 25S and 18S ribosomal peaks (23S and 16S for *A. platensis*) indicated intact RNA samples with little to no signs of degradation (RNA Integrity Number (RIN) mostly ≥ 7). Samples of the order Laminariales (*S. latissima*, *C. filum*) showed lower RINs due to additional fast migrating peaks between 40 and 45 s, which are corresponding to smaller chloroplast or mitochondrial RNA (Fig. 5g, h) (Suresh and Gassmann 2016). However, since none of these electropherograms displayed notable signals in the fast and inter regions, which are located prior and between the major ribosomal peaks, these RNA samples can be considered intact. Solely the RNA extract from the *A. platensis* (Spirulina) food supplement appeared strongly degraded (data not shown), probably caused by the extensive processing of the culture during the manufacturing process.

In general, no signs of genomic DNA, such as indistinct or slow migrating signals in the inter or post region, were apparent in the electropherograms. However, positive 16S and 18S rDNA PCR results with the RNA extracts showed that traces of eukaryotic and prokaryotic DNA are still present in the samples (data not shown). As mRNA is enriched by the sequencing service provider using poly(A) capturing prior to cDNA synthesis, leading to a depletion of DNA in the sample, the omission of a DNase treatment was considered acceptable for our downstream application. A selection of RNAs as were sent to the external provider for cDNA construction and Illumina sequencing, as well as a frozen tissue sample of *S. latissima* for a reference extraction. Achieved reads were trimmed, quality filtered, deduplicated and *de novo* assembled. Obtained raw data yielded between 21.1 and 49.3 million reads per species with a Q30-score above 90% and an average quality score per read of 33 for all samples, which translates to an average base call accuracy of 99.9995%. RNAs extracted by the CTAB method led to a higher percentage of sequences remaining after quality filtering and deduplication compared to the RNA sample of the external provider (see Table 2). A high duplication rate and loss of reads during quality filtering may be caused by artifacts generated during cDNA library construction due to insufficient RNA quality. Overall, our
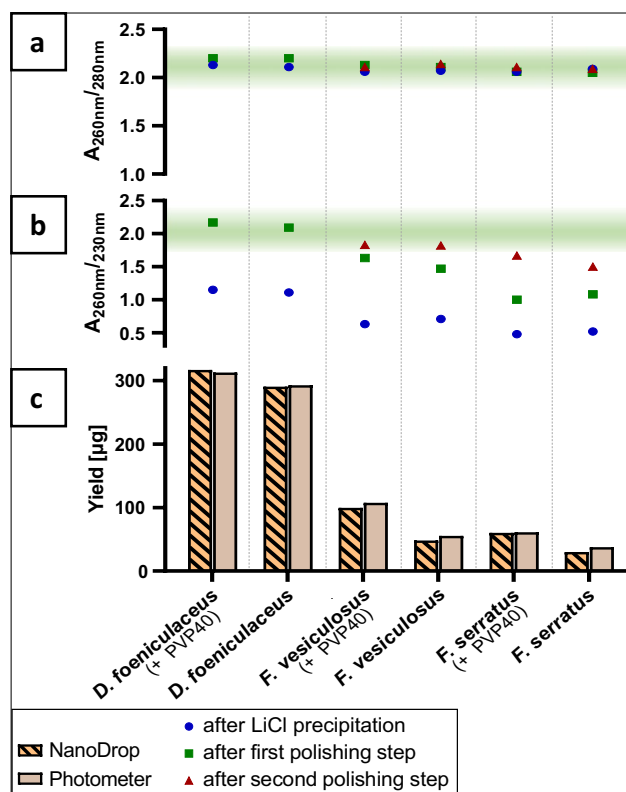

Fig. 3 RNA quantity and quality data of brown algae extracted by the SDS method. (**a**) A_{260nm}/A_{280nm} UV/Vis ratios. (**b**) A_{260nm}/A_{230nm} UV/Vis ratios. Samples were measured with a NanoDrop 2000 spectrophotometer after LiCl precipitation (blue dot), a first polishing step (green square), and a second polishing step (red trangle) with a commercial kit. Green bands indicate the range of acceptable RNA purity. (**c**) RNA quantity measured with the NanoDrop spectrophotometer (orange) and the UV/Vis photometer (violet)
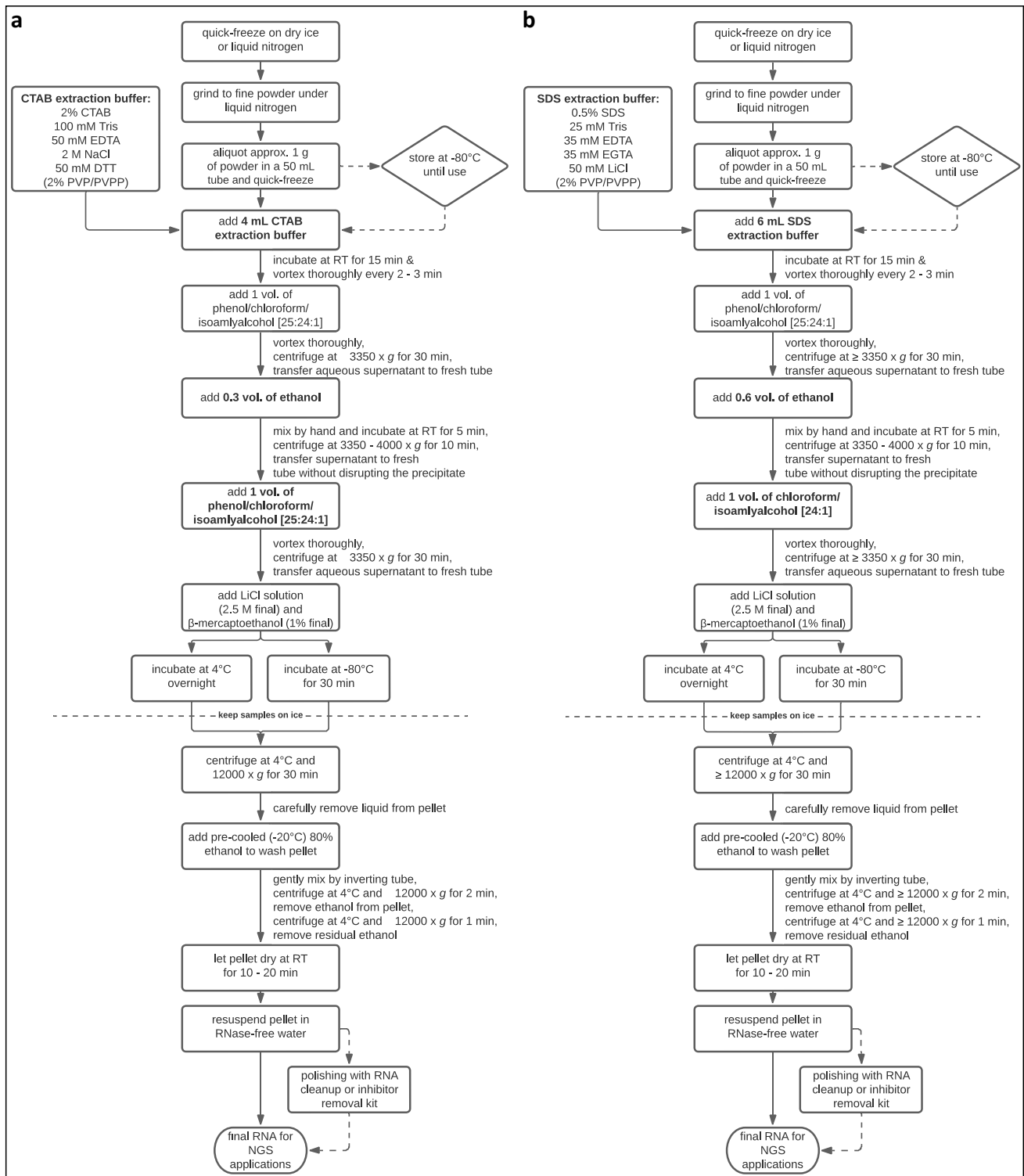
Fig. 4 Flow charts of the modified CTAB extraction protocol (**a**) and the SDS extraction protocol (**b**). Differences between both protocols are marked in bold

data suggest RNA extracts obtained by the modified CTAB method to be suitable for NGS applications, generating results equally good or better to those of the RNA extraction service provider.
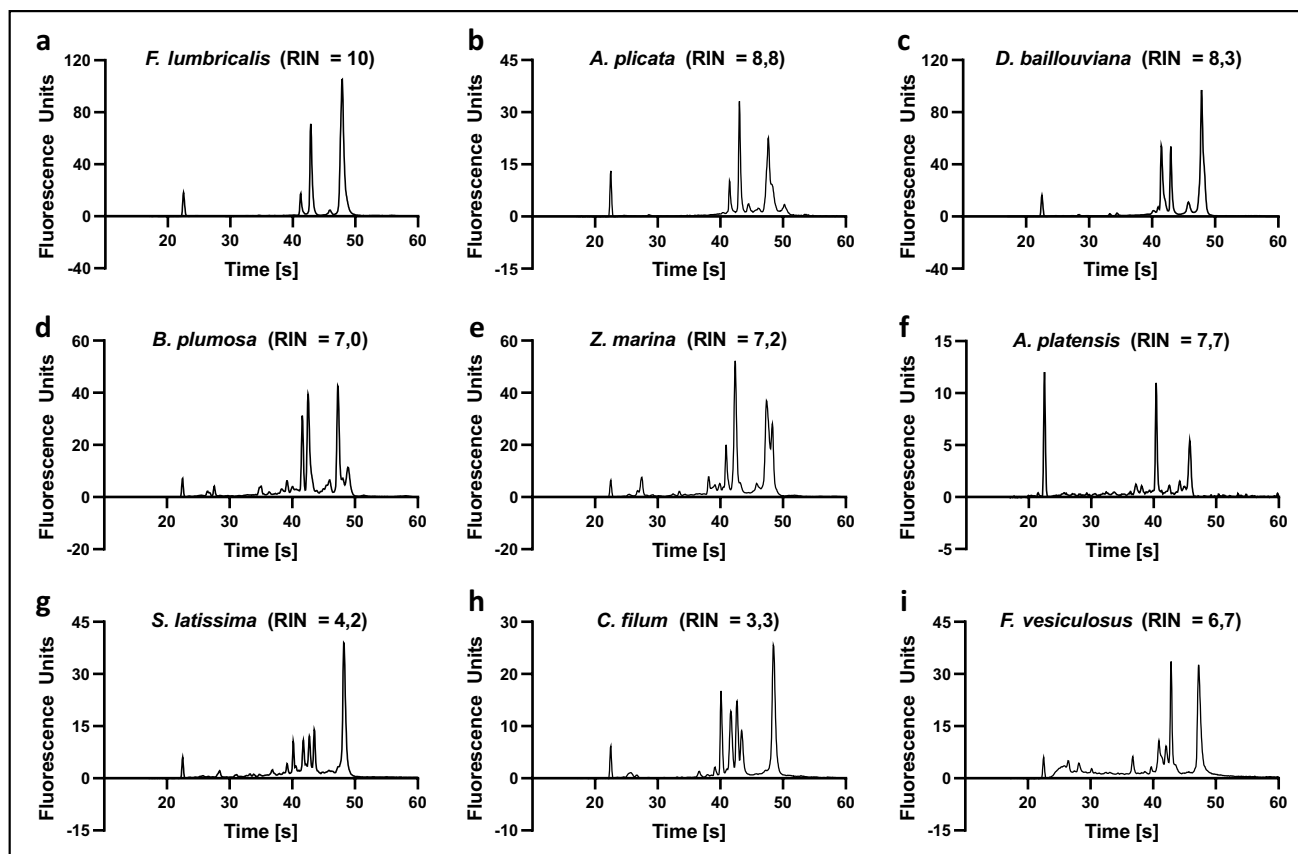
Fig. 5 Electropherograms of RNA samples from *F. lumbricalis* (**a**), *A. plicata* (**b**), *D. baillouviana* (**c**), *B. plumosa* (**d**), *Z. marina* (**e**), *A. platensis*, fresh culture (**f**), *S. latissima* (**g**) and *C. filum* (**h**) extracted by the CTAB method as well as *F. vesiculosus* (**i**) extracted by the SDS method and polished two times with the OneStep PCR Inhibitor Removal Kit. The marker signal is displayed at approx. 23 s. Major peaks at approx. 48 s and 43 s correspond to the 25S and 18S ribosomal subunits in eukaryotic RNA samples. Major peaks at approx. 46 s and 40 s correspond to the 23S and 16S ribosomal subunits in the *A. platensis* sample

Table 2 Number of raw and filtered reads obtained by sequencing as well as percentage of reads remaining after quality filtering and deduplication

	Modified CTAB method, this work				Reference extraction done by external laboratory
	F. lumbricalis	*A. plicata*	*C. filum*	*S. latissima* (Sample 1)	*S. latissima* (Sample 2)
Total raw reads (unfiltered) in M	21,0.1	24,0.8	23,0.6	49,0.3	32,0.8
Total reads (filtered) in M	19,0.3	22,6	20,5	42,3	21,2
Reads remaining after quality filtering	91.3%	91.0%	87.1%	85.9%	64.5%
Reads remaining after deduplication according to FastQC	59.8%	51.4%	49.9%	42.8%	32.6%

Discussion

The modified CTAB method

This work provides a strategic approach for RNA extraction from a broad range of algae based on the CTAB extraction method published by Pearson et al. (2006).

Although we generally agree on the effectiveness of this protocol on algae rich in polysaccharides and polyphenols, in our hands, the success of this protocol varied depending on the extracted species. Yields obtained at this scale often appeared to be smaller to those reported by the author. Also, weighing in such small amounts of frozen material without thawing the sample can be laborious, and, in

some instances, the volumes of algae biomass and buffers required for this protocol exceeded the maximal possible volume of 2 mL when working with non-lyophilized material. Therefore, we included a variety of modifications and tested the modified protocol on a broad range of algae, seagrass, and cyanobacteria samples.

First, the amounts of biomass, buffer and solvents were scaled up four times in order to yield higher amounts of RNA. While other protocols recommend higher centrifugation speeds up to $12000 \times g$ during the organic separation steps, we decreased the centrifugation speed to $3350 \times g$ since high-speed centrifugation of organic solvents might become impracticable in some laboratories at this scale. However, if an ultracentrifuge is available, we recommend using higher centrifugation speeds to shorten centrifugation time, improve phase separation und reduce putative carryover from the organic phase. During polysaccharide precipitation, the centrifugation speed may be increased to $4000 \times g$.

Second, we used PCI instead of CI as organic phase. Although the effectiveness of CI as a substitute of PCI for nucleic acid extraction from algae has been well established by Pearson et al. (2006) and others, PCI has shown a higher capability of removing proteins (Wang et al. 2005) and, in our experience, often leads to higher yields. In addition, the UV/Vis absorbance spectra of our samples did not display any shifted maxima, which would indicate the presence of phenolic impurities (Lee et al. 2014), nor did we observe nicking of RNA by phenolic oxidation. However, if phenolic carryover or insufficient phase separation is observed during RNA extraction, using CI in the second separation step is a suitable option as it is substantially denser than water and capable of removing residual phenol from aqueous solutions.

Third, PVP40 was included into the CTAB extraction buffer as an optional component as it was shown to effectively bind and separate phenolic inhibitors and helps to prevent oxidation of the sample during RNA extraction (Porebski et al. 1997; Koonjul et al. 1999). Interestingly, despite the reports of higher efficiency of soluble PVP (Porebski et al. 1997), several authors preferred using insoluble polyvinylpolypyrrolidone (PVPP) as soluble PVP is said to be incompatible with phenol or to obstruct RNA precipitation (Claros and Cánovas 1998; Salzman et al. 1999; Ghangal et al. 2009), although experimental evidence for these hypotheses is sparse. In contrast, we experienced yields and purity to be equally good or better, when PVP40 was added to the extraction buffers used in this study, although we did not systematically assess the effectiveness of PVP40 on multiple species or with high statistical certainty. It should also be noted that different plant tissues might contain polyphenols with different chemical properties. Therefore, their removal may be more or less efficient with PVP or PVPP of different molecular weights. Also, adding PVP or PVPP can strongly alter the density and viscosity of the extraction buffer with further putative implications for the centrifugation procedure used during extraction.

Fourth, we incorporated a one-step PCR Inhibitor Removal Kit to remove putative contaminants remaining after the extraction procedure. However, this step appeared redundant for most samples extracted by the CTAB method and may be omitted.

By incorporating the above-discussed changes into our protocol, we were able to produce high quality RNA extracts from a variety of algal samples. The modified CTAB method had its limitations with respect to certain brown algae species. Interestingly, while we struggled to obtain clean RNA extracts from *F. vesiculosus* with the modified method, this species was successfully extracted by the CTAB method reported by Pearson et al. (2006). The reasons for this can only be speculated, with one possible explanation being the altered buffer composition containing PVP40 and reduced centrifugation speeds that may be insufficient for some algae samples rich in viscous polysaccharides, although this needs to be verified in future experiments.

The SDS method

Due to the limitations of the modified CTAB method with respect to the three brown algae *D. foeniculaceus*, *F. vesiculosus*, and *F. serratus*, an alternative SDS-based RNA extraction protocol was established and shown to be more effective on polysaccharide-rich brown algae. As a cationic surfactant, CTAB is usually applied in nucleic acid extractions from plants as it not only interacts with proteins but also helps to remove polyphenols and polysaccharides (Lever et al. 2015; Barbier et al. 2019). SDS, in contrast, is an anionic surfactant, which is also commonly used in nucleic acid extractions due to its efficiency in interacting with proteins, disrupting cell membranes and facilitating the release of nucleic acids from the cell (Lever et al. 2015; Xia et al. 2019; Vennapusa et al. 2020). Similar to other authors, we experienced the SDS-based method to yield higher nucleic acid quantities (Xia et al. 2019; Vennapusa et al. 2020), although the presence of contaminants absorbing at 230 nm required subsequent polishing. Therefore, using a PCR Inhibitor Removal Kit was critical for the crude extracts achieved by this method. Since some RNA pellets from brown algae obtained by the CTAB method (*e. g. C. filum*) exhibited a viscous, rubberlike texture, we also increased the amount of EtOH added in this protocol to precipitate polysaccharides more thoroughly. Using high EtOH concentrations, however, is not generally recommended for all samples as EtOH decreases the solubility of nucleic acids and thus might cause a loss of RNA. If the removal of polysaccharides remains insufficient, alternative or additional precipitation methods such as the addition of 0.2 M potassium acetate may be included to improve polysaccharide precipitation (Dos Reis Falcão et al. 2008).

Further optimisation potential

Although our RNA extraction procedures were shown to produce good results on a broad taxonomic range of species, we would like to stress that our protocols still have room for improvement. For instance, we experienced comparably low yields from algal species with thin leaves which are capable of absorbing and retaining a lot of additional water, thus likely resulting in a lower amount of algal biomass in 1 g of ground algae powder. Apart from a putative impact of varying environmental conditions, varying water contents may also be a possible explanation for the yield fluctuations between the replicates of *F. lumbricalis*. Thus, freeze-drying of the tissue such as performed by Pearson et al. (2006) can improve stability and compensate yield fluctuations caused by varying water contents of the samples. A proper tissue disruption is crucial to obtain high quality RNA and more efficient tissue homogenization methods (*e. g.* cryogenic mills or tissue homogenizers) may strongly improve yields and reproducibility of the extractions. Alternatively, incorporating sanitized sand can improve tissue disruption while grinding (Chan et al. 2002).

Like the group around Pearson, we recommend downscaling the extraction procedure depending on the quantities needed to lower chemical consumption and increase sample throughput. This is especially recommended for the SDS method, where RNA yields from 1 g algal fresh weight often appear to be considerably higher than needed, even for extensive analysis and multiple downstream applications. By running the protocols on a smaller scale, higher centrifugation speeds can easily be applied, further improving phase separation during organic separation and reducing putative phenolic carryover.

According to the product description of the PCI used in this study, at acidic pH, DNA becomes uncharged and is separated into the interphase whereas RNA stays in the aqueous phase during organic separation. Also, LiCl is said to specifically precipitate RNA (Barlow et al. 1963). Since traces of DNA are nonetheless present in the final products, incorporating a DNase treatment before using the RNA products for RT-PCR or other downstream applications is recommended. The observed DNA contamination is not only of eukaryotic, but also prokaryotic origin. Therefore, it can be assumed that RNA is also co-extracted from microbial inhabitants and cleaning the leaf surface with EtOH and RNase-free water prior to freezing might reduce the microbial influence on the RNA products.

Conclusion

With this study we strived to provide optimised and adaptable RNA extraction strategies for algae modified from previously published protocols. The modified CTAB extraction method led to sufficient yields of RNA from a variety of green, red, and brown algae as well as seaweeds and cyanobacteria. We also demonstrated that the extracted RNAs are suitable for NGS applications. The extraction of RNA from certain brown algae such as *Fucus* spp. proved difficult with this method and an alternative extraction protocol using an SDS-based extraction buffer combined with subsequent polishing steps with commercial kits seemed to be more effective on some polysaccharide and polyphenol rich brown algae. Our protocols are easily scalable, adaptable, and can be performed with basic laboratory equipment on a broad range of algae and other species rich in polyphenols and polysaccharides without lengthy optimisation procedures.

Declarations

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