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# METHODS & TECHNIQUES

# *Symbiodinium* isolation by NaOH treatment

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## SUMMARY

**The presence of photosynthetic zooxanthellae (dinoflagellates) in the tissue of many cnidarians is the main reason for their ecological success (i.e. coral reefs). It could also be the main cause of their demise, as the worldwide bleaching of reef-building coral is nothing less than the breakdown of this symbiotic association. The stability of this relationship is the principal marker for the biomonitoring of cnidarian health. We have therefore developed a new, simple method to isolate zooxanthellae in a few steps using NaOH solution. The protocol was validated in three symbiotic cnidarian species: a sea anemone, a gorgonian and a coral. Our method allows the isolation of intact and viable zooxanthellae with better yields than classic methods, especially for species with a calcareous skeleton. Moreover, the isolated zooxanthellae were free of host nucleic contaminants, facilitating subsequent specific molecular analyses.**

Key words: zooxanthella density, symbiosis, cnidarian, nucleic acid amplification.

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## INTRODUCTION

Many cnidarian species (sea anemones, gorgonians and corals) form mutualist associations with photosynthetic dinoflagellates that belong to the genus *Symbiodinium* and that are commonly called zooxanthellae (Taylor, 1974; Trench, 1987). Most cnidarians contain extremely high densities of zooxanthellae, around 1–5 ($\times 10^6$) cells $cm^{-2}$ of host live surface tissue (Muller-Parker and Davy, 2001). They are located in vacuoles within the host endoderm cells (Glider et al., 1980). When these symbioses are subjected to environmental stress, such as an elevated seawater temperature, the zooxanthellae are lost from the host tissues, a process known as bleaching (Brown, 1997). The isolation and counting of zooxanthellae from cnidarian species is a common procedure to evaluate the severity of the bleaching.

Several techniques have been developed to isolate the symbiotic algae from the host. They include stripping coral tissues from the skeleton with a fine jet of seawater using a WaterPik (Johannes and Wiebe, 1970), grinding the whole coral (Santiago-Vázquez et al., 2006) or gorgonian tissue (Forcioli et al., 2011; Pey et al., 2011) in liquid nitrogen, manual tissue fractionation and potter homogenization of sea anemones (Richier et al., 2003). Although effective, these techniques could underestimate the zooxanthella biomass and require a large investment of time. Also, the intracellular localization of zooxanthellae complicates the molecular analyses of cnidarian species as it often leads to DNA extracts containing both cnidarian and algal genomes. We report here a new, fast and efficient method for the isolation of zooxanthella without host contaminant, validated on three cnidarian species: a sea anemone, *Anemonia viridis*; a gorgonian, *Eunicella singularis*; and a scleractinian coral, *Stylophora pistillata*.

## MATERIALS AND METHODS

### Cnidarian collection and maintenance

Mediterranean sea anemone specimens, *A. viridis* Forskål 1775, were collected in 'Baie des Croutons' (Antibes, France). Fragments of the white gorgonian *E. singularis* (Esper 1791) were collected in Banyuls (France). Sea anemones and gorgonians were maintained in aquaria as described elsewhere (Moya et al., 2012). Microcolonies of *S. pistillata* Esper 1797 collected at the Marine Science Station, Gulf of Aqaba (Jordan) were maintained at the Scientific Center of Monaco as described previously (Furla et al., 2000). Cultured zooxanthellae (CZ) were maintained in F/2 medium (Guillard and Ryther, 1962) at pH 8.2 and incubated at 26.0±0.1°C under an irradiance of 100 μmol photons $m^{-2}$ $s^{-1}$ (Sylvania Gro-Lux, Loessnitz, Germany), on a 12 h:12 h photoperiod. Stock cultures were transferred monthly.

### Zooxanthella isolation

In order to obtain freshly isolated zooxanthellae (FIZ), two extraction protocols were tested and compared for the three cnidarian species.

#### Initial protocol

FIZ extractions from the three species were performed as described elsewhere (Richier et al., 2003; Forcioli et al., 2011). *Anemonia viridis* tentacles were cut and quickly dried on paper to remove excess seawater. Each tentacle was cut lengthwise and scraped to separate the endodermal cell layer, which contains the zooxanthellae, from the epidermal tissue. The endodermal fraction was suspended in extraction buffer (0.05 mol $l^{-1}$ phosphate buffer pH 7.8, 0.4 mol $l^{-1}$ sorbitol) and disrupted by syringe homogenization. Zooxanthellae were separated from the endodermic extract by centrifugation at 3000 ***g*** for 5 min. The pellet was washed twice with extraction buffer, centrifuged at 12,000 ***g*** for 3 min and then resuspended in extraction buffer. Fragments from *E. singularis* and from *S. pistillata* were weighed and ground in a mortar with liquid nitrogen. The resulting powder was resuspended in the same extraction buffer. Samples were then filtered through a nylon mesh (100 μm) in order to eliminate as many skeleton residues as possible.

Table 1. Primer sequences used for PCR and RT-PCR

| Organism | Gene | Primer sequence (5′–3′) Forward | Reverse | $T_m$ (°C) | Elongation (s) |
|---|---|---|---|---|---|
| *Anemonia viridis* | *cop-γ* | GCCTGTTGGACACCGATGAT | TGCAAGGCTCTCTCCAGTCC | 63 | 30 |
| *Anemonia viridis* | *npc1* | GCCTGCTGTCAAGGTGTTCTC | TGCGGTTACTTTCCTGTCGTC | 63 | 15 |
| *Symbiodinium* sp. | *apx* | CGTCCAGGACCTCGAGAACT | TTGAGCTCGCCATCAGAGAA | 63 | 30 |
| | *cp23s* | TCAGTACAAATAATATGCTG | GGATAACAATTTCACACAGGTTATC-GCCCCAATTAAACAGT | 53 | 30 |
| | *ssu* | GGTTGATCCTGCCAGTAGTCATATGCTTG | AGCACTGCGTCACTCCGAATAATTCACCGG | 53 | 60 |

### Improved protocol

In this protocol, NaOH solution was used to extract FIZ from the three cnidarian species. Extracts were obtained by incubating a whole *A. viridis* tentacle, a 1.5 cm long fragment of *E. singularis* or a 500 mg sample of *S. pistillata* in 500 µl of 0.25–10 mol $l^{-1}$ NaOH solution. The combined effects of the incubation temperature (25 or 37°C) and the duration of NaOH treatment (1–4 h) were tested to evaluate the most effective extraction conditions for each species. All the samples were vigorously shaken every 15 min during the NaOH treatment.

### Zooxanthella quantification

Zooxanthella density was determined by placing 40 µl of the extracts in a Neubauer improved haemocytometer and the counts were normalized by sample wet mass. Light microscopic observations (Leica, Wetzlar, Germany) of extracts obtained from both isolation protocols were compared.

### DNA extraction

Zooxanthella extracts were centrifugated at 3000 ***g*** for 3 min at room temperature. The zooxanthella pellets were washed twice with TE buffer (0.01 mol $l^{-1}$ Tris HCl pH 8.0 and 0.001 mol $l^{-1}$ EDTA) and centrifuged at 12,000 ***g***. Pellets were kept in lysis buffer (0.02 mol $l^{-1}$ EDTA pH 8.0, 0.01 mol $l^{-1}$ Tris HCl pH 7.5, 0.4 mol $l^{-1}$ NaCl, 6% sodium dodecyl sulphate, 1 µl $ml^{-1}$ RNase and 100 µg of proteinase K) at 56°C for 2 h. Insoluble material was removed from the extract by centrifugation at 12,000 ***g*** for 5 min. Supernatant was transferred to a fresh tube and DNA was purified by phenol/chloroform/isoamyl alcohol extraction and isopropanol precipitation. DNA pellets were resuspended in TE buffer. DNA was quantified on a 150 ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

### RNA extraction

Zooxanthella extracts were centrifuged at 3000 ***g*** for 3 min at 4°C. The resulting zooxanthella pellets were washed twice with RNase-free water and 1 ml of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was added; total RNA was extracted following the manufacturer's protocol. The RNA pellets were eluted in 30 µl of RNase-free water.

### cDNA synthesis

Reverse transcription of 1 µg of total RNA per reaction was carried out using SuperScript II reverse transcriptase, following manufacturer's procedure (Invitrogen). The obtained cDNA was used as the template in PCR reactions with the specific primers.

### PCR amplification

Two *A. viridis* nuclear genes, *cop-γ* (coatomer subunit gamma) and NPC1 (Niemann pick type C1); two *Symbiodinium* nuclear genes, *apx* (ascorbate peroxidase) and *ssu* rDNA (small ribosomal subunit); and one *Symbiodinium* chloroplastic gene (*cp23s*) were amplified. Specific primers were synthesized as described elsewhere (Ganot et al., 2011; McNally et al., 1994; Santos et al., 2003). The primer sequences used in this study are listed in Table 1. PCR was performed in a total volume of 20 µl with 10× PCR buffer, 0.2 mmol $l^{-1}$ each dNTP, 1.5 mmol $l^{-1}$ $MgCl_2$, 0.2 µmol $l^{-1}$ each of the two primers and 1 U Platinum *Taq* DNA polymerase (Invitrogen). The amplification profile consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at a temperature dependent on primer melting temperature ($T_m$) and extension at 72°C for 30 s to 1 min depending on amplicon length (see Table 1). A final extension step was carried out at 72°C for 7 min. Amplicon specificity for either *A. viridis* or *Symbiodinium* sp. was tested respectively against epidermal tissue extracts (tissue without zooxanthellae) or CZ extracts. PCR products were electrophoretically analysed on 1% agarose gel stained with gelRed (Interchim, Montluçon, France) and compared with a 1.5 kb plus DNA ladder (Invitrogen). PCR and RT-PCR were performed from at least three independent RNA and DNA extractions, respectively.

### Viability assay

FIZ viability was assessed after the initial and improved extraction protocols. All extracts were centrifuged at 5000 ***g*** for 3 min. The zooxanthella pellets were washed twice and resuspended in 1 ml of F/2 medium. Zooxanthella suspensions were neutralized with 1 mol $l^{-1}$ HCl at pH 8.5 and incubated at 25°C for 4 days. The effect of NaOH treatment on CZ was assessed by comparing 1 or 4 mol $l^{-1}$ NaOH incubation with F/2 incubation (considered as the control) for 1 h at 25°C, followed by centrifugation and a washing step as described for FIZ. Cell viability was determined by the vital stain Evans Blue method (Morera and Villanueva, 2009). Cells were counted under a phase-contrast microscope using a Neubauer improved haemocytometer. The number of viable cells was expressed as a percentage of the total population (a minimum of 300 cells per sample).

### Statistical analysis

All experiments were performed at least in triplicate. Results are expressed as means ± s.e.m. and normalized according to the wet mass of each fraction. Statistical analyses were performed using Kruskal–Wallis non-parametric ANOVA followed by Tukey HSD *post hoc* tests. Results were considered significant when $P<0.05$.

## RESULTS

### Zooxanthella extraction by the improved protocol on three cnidarian species

After NaOH incubation, intact and clean zooxanthellae were successfully isolated from the three species (Fig. 1). Variable yields

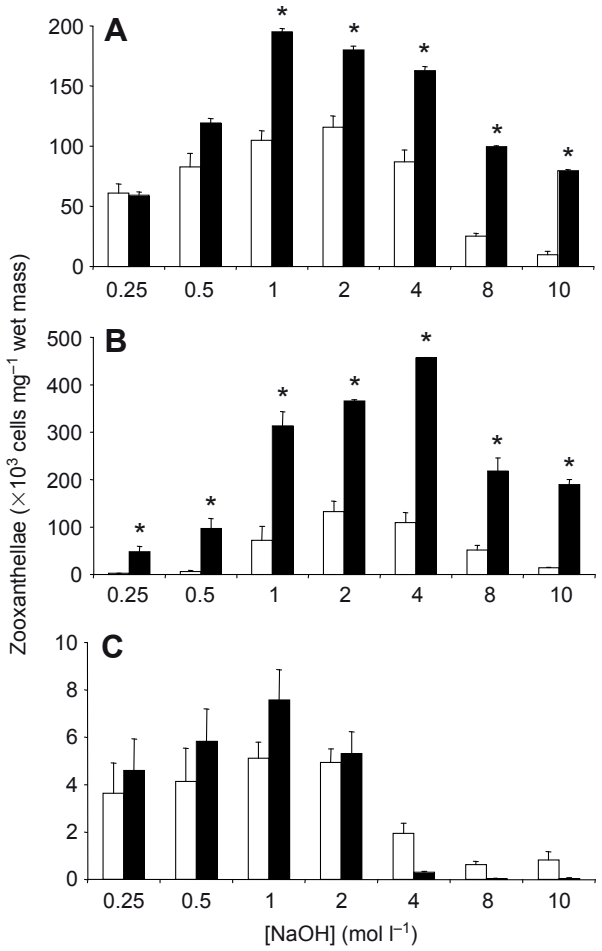


Fig. 1. Zooxanthella extraction by the improved protocol from three cnidarian species: *Anemonia viridis* (A), *Eunicella singularis* (B) and *Stylophora pistillata* (C). The samples were incubated in different NaOH concentrations at 25°C (white bars) and 37°C (black bars). Results are expressed as means ± s.e.m. of nine independent samples. Asterisks indicate significant differences between the extraction conditions ($P$<0.05; Kruskal–Wallis).

for FIZ extraction were calculated with respect to NaOH concentration and temperature incubation. Within the same species, the combined effect of these two parameters led to a variable success of FIZ extraction and allowed us to determine the optimal condition. For *A. viridis* (Fig. 1A), the highest zooxanthellae yields were obtained with 1, 2 and 4 mol $l^{-1}$ NaOH combined with incubation at 37°C. For *E. singularis* (Fig. 1B), the number of FIZ measured after incubation at 37°C was 3- to 20-fold higher than after incubation at 25°C, depending on NaOH concentration. The optimal condition was incubation at 37°C in 4 mol $l^{-1}$ NaOH ($P$<0.001). For *S. pistillata* (Fig. 1C), no significant effect of the temperature of incubation was observed on the FIZ yield, whatever the NaOH concentration. These results are probably related to the thinness and the fragility of *S. pistillata* tissues, making them more easily degradable with low NaOH concentration and low temperature. The effect of treatment time (from 1 to 4 h) was then tested on the extraction yield in *A. viridis* (data not shown). No significant effect of incubation time on the extraction yield was measured. In the light of these results, a 1 h incubation was chosen for all further analyses.

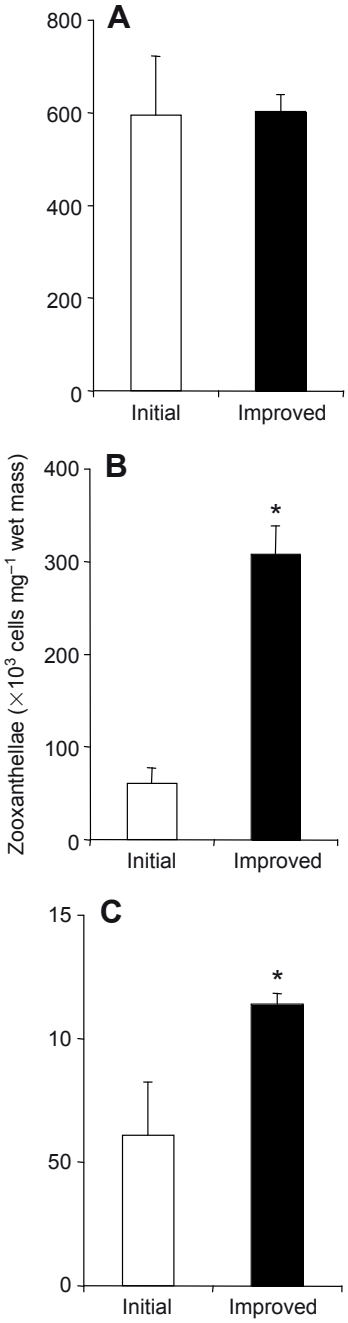


Fig. 2. Comparison of initial and improved zooxanthella extraction protocols. The amount of zooxanthellae extracted after the initial (black bars) or improved method (white bars) was compared for *A. viridis* (A), *E. singularis* (B) and *S. pistillata* (C). In the improved extraction protocol, samples were incubated for 4 h at 37°C (*A. viridis*, *E. singularis*) or 25°C (*S. pistillata*). Results are expressed as means ± s.e.m. of three independent samples. Asterisks indicate significant differences ($P$<0.05; Kruskal–Wallis).

### Direct comparison of the initial and the improved extraction protocols

We compared the number of FIZ obtained from three cnidarian species with the initial and the improved protocols (Fig. 2). For *E. singularis* and *S. pistillata*, the improved protocol using NaOH solution was more efficient than the initial protocol; the extraction rates of FIZ were significantly increased 5- and 1.9-fold, respectively ($P$<0.05). For *A. viridis*, no significant difference was observed between the

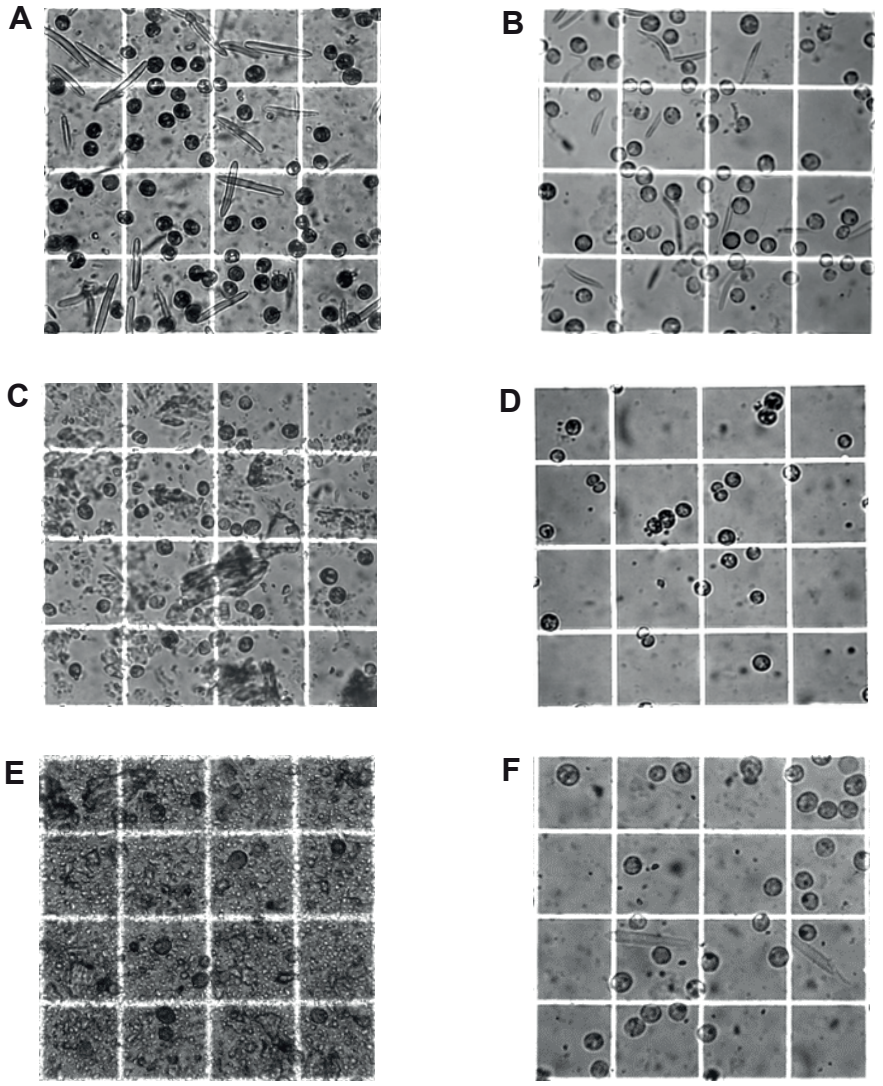


Fig. 3. Light microscopy images of zooxanthella extracts obtained after the initial or improved protocol from three cnidarian species: *A. viridis* (A,B), *E. singularis* (C,D) and *S. pistillata* (E,F). In the improved extraction protocol, samples were incubated for 4 h at 37°C (*A. viridis*, *E. singularis*) or 25°C (*S. pistillata*). Each square side has a length of 50 μm.

two extraction methods. However, the standard errors of the mean for the improved protocol indicate a better reproducibility. Light microscopy pictures of zooxanthella extracts obtained with the improved extraction protocol showed intact zooxanthellae and very low background in the three species, compared with the extracts obtained from the initial protocol (Fig. 3)

### DNA purification assay after zooxanthella extraction by the improved protocol

After zooxanthella isolation with the initial or the improved protocol (1 or 4 mol $l^{-1}$ NaOH for 1 h at 25 or 37°C), genomic DNA was extracted and the *A. viridis* (animal) nuclear gene *cop-γ* and the *Symbiodinium* nuclear gene *apx* were amplified by PCR. Fig. 4A shows that only PCR reactions with samples obtained from incubations at 37°C in 1 or 4 mol $l^{-1}$ NaOH amplified a zooxanthella DNA amplicon of the expected size (i.e. 100 bp), although the greatest yield was observed with 4 mol $l^{-1}$ NaOH. No animal contamination from the host tissue was detected in this condition, although the animal nuclear gene was still amplified in the other tested conditions (Fig. 4B). Thus, incubation at 37°C with 4 mol $l^{-1}$ NaOH was the optimal condition to amplify zooxanthella DNA free from animal DNA contamination. This condition was then tested to determine the detection limit and the specificity of the assay. Fig. 5 shows the successful amplification of a specific *ssu Symbiodinium* sequence of 1800 bp and of the *Symbiodinium* chloroplastic gene *cp23s*, validating the NaOH extraction method for taxonomy analyses.

### RNA purification assay after zooxanthella extraction by the improved protocol

mRNA isolation and RT-PCR were performed on zooxanthellae extracted by the improved and the initial protocols. Fig. 6A shows an enhanced amplification efficiency of zooxanthella cDNA with increasing durations of treatment in 4 mol $l^{-1}$ NaOH, reaching a level equivalent to that obtained with mRNA isolated after the initial protocol. Contamination with mRNA from animal tissue was weak and decreased with increasing treatment duration, as shown by tissue amplifications obtained with *cop-γ* (Fig. 6B).

### Zooxanthella viability after extraction by the improved protocol

The effect of NaOH treatment on FIZ and CZ viability was assessed by cell viability assays. During the 48 h following the extraction of FIZ with 1 or 4 mol $l^{-1}$ NaOH, measurements revealed viability rates equivalent to those obtained with FIZ extracted with the initial protocol (more than 96% viability, Fig. 7A). Four days after

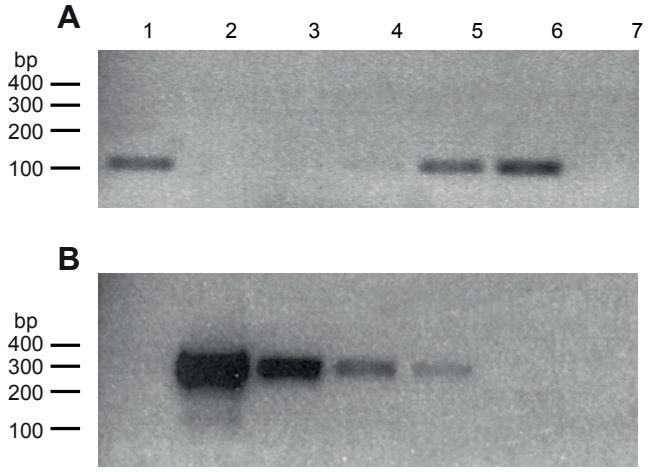


Fig. 4. Optimal zooxanthella extraction conditions from *A. viridis* by the improved protocol for genomic DNA isolation and PCR amplification. The effect of NaOH concentration and incubation temperature on genomic DNA amplification efficiency was tested after zooxanthella extraction. *Symbiodinium* nuclear gene *apx* (A) and *A. viridis* nuclear gene *cop-γ* (B) were amplified by PCR using the following DNA templates: genomic DNA from cultured zooxanthellae obtained with the initial protocol and used as zooxanthella reference (lane 1); genomic DNA from *A. viridis* epidermis obtained with the initial protocol and used as animal reference (lane 2); genomic DNA extracted from zooxanthellae obtained after 1 mol $l^{-1}$ NaOH treatment and incubation at 25°C (lane 3); genomic DNA extracted from zooxanthellae obtained after 4 mol $l^{-1}$ NaOH treatment and 25°C incubation (lane 4); genomic DNA extracted from zooxanthellae obtained after 1 mol $l^{-1}$ NaOH treatment and 37°C incubation (lane 5); genomic DNA extracted from zooxanthellae obtained after 4 mol $l^{-1}$ NaOH treatment and 37°C incubation (lane 6); no DNA template (lane 7).

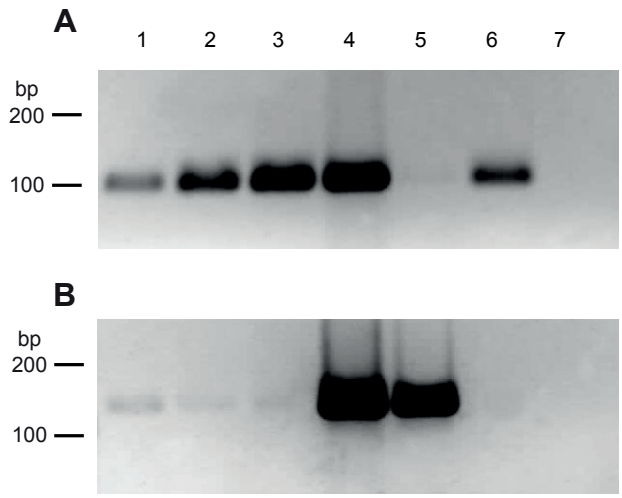


Fig. 6. Optimal conditions for zooxanthella extraction from *A. viridis* by the improved method for mRNA isolation followed by RT-PCR amplification. mRNA isolation was performed after zooxanthella extraction by 1, 2 or 4 h of incubation with 4 mol $l^{-1}$ NaOH at 25°C. RT-PCR amplification was performed using *Symbiodinium* nuclear gene *apx* (A) or *A. viridis* nuclear gene *cop-γ* (B) primers and the following cDNA templates: cDNA extracted from zooxanthellae obtained with 1, 2 or 4 h of NaOH incubation (lanes 1, 2 and 3, respectively); cDNA from zooxanthellae obtained using the initial method (lane 4); cDNA from *A. viridis* epidermis extracted with the initial method and used as the animal reference (lane 5); cDNA extracted from cultured zooxanthellae (CZ) with the initial method and used as zooxanthella reference (lane 6); no DNA template (lane 7).

extraction, a loss of viability was measured in FIZ extracted with both methods. This result validates the NaOH extraction method for further studies on free-living zooxanthellae. Surprisingly, CZ treated with 1 or 4 mol $l^{-1}$ NaOH lost about 70% viability as early as 24 h after treatment and more than 80% after 4 days (Fig. 7B).

## DISCUSSION

By this new NaOH extraction method, we were able to successfully isolate a large number of intact symbiotic FIZ from three cnidarian species with different structures, *A. viridis* (only collagenous tissues), *E. singularis* (proteinaceous and calcareous skeleton) and *S. pistillata* (calcareous skeleton). We have defined the optimum conditions to extract the highest number of FIZ, which, for the three species, was a combination of incubation at 37°C and a NaOH concentration of 1–4 mol $l^{-1}$. In addition, compared with initial methods, our protocol offers advantages in terms of cost, speed and effectiveness. Indeed, isolation of zooxanthellae from many samples can be processed simultaneously and requires less than 2 h. Furthermore, as our protocol involves few experimental steps, the variability between samples, mainly due to loss of tissue caused by

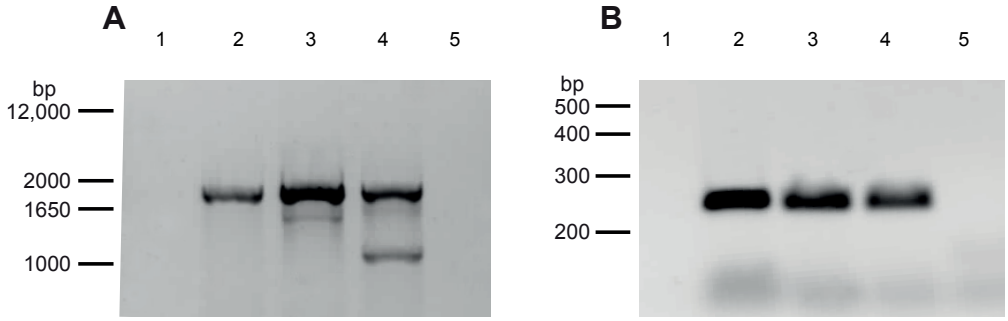


Fig. 5. PCR amplification of zooxanthella nuclear and chloroplastic DNA after zooxanthella extraction from *A. viridis* by the improved protocol. The experimental conditions of the improved extraction method were 1 h treatment in 4 mol $l^{-1}$ NaOH at 37°C. PCR was performed using zooxanthella-specific *ssu* rDNA primers (A) or chloroplastic *cp23s* primers (B) and the following DNA templates: genomic DNA from *A. viridis* epidermis obtained with the initial protocol and used as animal reference (lane 1); genomic DNA extracted from zooxanthellae obtained with the improved protocol (lane 2); genomic DNA extracted from zooxanthellae obtained with the initial protocol (lane 3); genomic DNA from cultured zooxanthellae obtained with the initial protocol and used as zooxanthella reference (lane 4); no DNA template (lane 5).

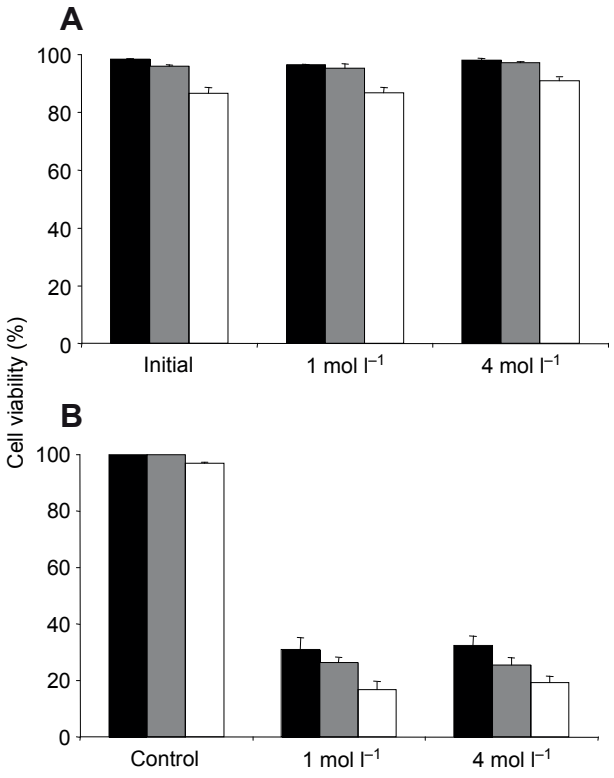


Fig. 7. Cell viability determined by Evans Blue assay on freshly isolated zooxanthellae (FIZ) (A) and CZ (B). Zooxanthella viability was measured 1 day (black bars), 2 days (grey bars) and 4 days (white bars) after their isolation from *A. viridis*. FIZ extraction was performed with the improved method (using 1 or 4 mol $l^{-1}$ NaOH for 1 h at 25°C) and compared with the viability of zooxanthellae extracted with the initial method. CZ viability was measured after incubation with 1 or 4 mol $l^{-1}$ NaOH or in culture medium (control condition).

the classic extraction protocols, is strongly reduced. This new method allows a significant increase in extraction yields and enables zooxanthella extracts to be obtained free of animal host tissue, including cnidocytes and spicules. This is a strong asset of our NaOH method for molecular analysis of zooxanthellae, as it allows amplification of zooxanthella genomic DNA and cDNA uncontaminated by host animal DNA, a feat not ensured by the other extractions techniques. Finally, we showed that the viability of FIZ extracted with our protocol is not affected, validating this procedure of zooxanthella isolation for culture purposes.

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