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How endo- is endo-? Surface sterilization of delicate samples: A *Bryopsis* (Bryopsidales, Chlorophyta) case study

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Abstract

In the search for endosymbiotic bacteria, elimination of ectosymbionts is a key point of attention. Commonly, the surface of the host itself or the symbiotic structures are sterilized with aggressive substances such as chlorine or mercury derivatives. Although these disinfectants are adequate to treat many species, they are not suitable for surface sterilization of delicate samples. In order to study the bacterial endosymbionts in the marine green alga *Bryopsis*, the host plant's cell wall was mechanically, chemically and enzymatically cleaned. Merely a chemical and enzymatic approach proved to be highly effective. *Bryopsis* thalli treated with cetyltrimethylammonium bromide (CTAB) lysis buffer, proteinase K and bactericidal cleanser Umonium Master showed no bacterial growth on agar plates or bacterial fluorescence when stained with a DNA fluorochrome. Moreover, the algal cells were intact after sterilization, suggesting endophytic DNA is still present within these algae. This new surface sterilization procedure opens the way to explore endosymbiotic microbial communities of other, even difficult to handle, samples.

Keywords: *Bryopsis*, endosymbionts, endosymbiotic bacteria, marine green algae, surface sterilization

1. Introduction

Numerous eukaryotes maintain close associations with bacteria, either on their surface or within their cells. To examine the latter alliance it is essential to remove the bacteria which inhabit the host's surface and form a main source of contamination. However, the ubiquity of bacterial biofilms prevents the straightforward study of these endosymbionts (Burke et al. 2009). In well established symbiosis models the surface sterilization used is in general quite aggressive. Insect eggs, larvae and adults are treated with hydrogen peroxide, formaldehyde, radiation, antibiotics or highly toxic mercury derivatives like mercuric chloride and thiomersal (Connell 1981; Meyer and Hoy 2008). Land plants or their symbiotic structures (e.g. root nodules) are mostly surface disinfected by means of beads, ethanol or sodium hypochlorite (Lodewyckx et al. 2002). Despite the use of these vigorous techniques, an effective surface sterilization remains a balancing exercise. Few surface disinfection protocols result in complete removal of ectosymbionts without penetrating interior tissues and thereby neutralizing internal bacteria; while an ineffective sterilization may result in outer surface bacteria being mistaken for endosymbionts (Lodewyckx et al. 2002). When the host is delicate, as is the case for the siphonous green alga *Bryopsis*, finding the right balance becomes even more challenging. Siphonous seaweeds are essentially single giant multinucleate cells surrounded by a xylan-cellulose cell wall, a thin parietal layer of cytoplasm and a huge central vacuole (van den Hoek 1995). Like various other macro-algae (Ashen and Goff 2000; Dawes and Lohr 1978; Turner and Friedman 1974), *Bryopsis* has long been suspected to harbor endogenous bacteria in the cytoplasm (Burr and West 1970). The identity of these endosymbionts, however, remains unknown. Further exploration of this algal-bacterial partnership requires an efficient surface sterilization of the *Bryopsis* host. After all, many seaweeds live in close association with numerous epiphytic bacteria, which control morphological development (Fries and Iwasaki 1976; Marshall et. al 2006; Provasoli and Pintner 1980; Tatewaki et al. 1983) or are linked with various metabolic functions (Chisholm et al. 1996; Croft et al. 2005; Head and Carpenter 1975; Staufenberger et al. 2008; Weinberger 2007), and *Bryopsis* seems no exception (Kan et al. 1999). Whereas the usage of axenic cultures is quite common for micro-algae, for the study of marine macro-algae this is limited. In general, axenic seaweed cultures are obtained by the addition of antibiotics to the growth medium or a combination of antibiotic use and isolation of reproductive cells (Andersen 2005; Droop 1967; Kooistra et al. 1991). Reported attempts to efficiently remove epiphytes mechanically, chemically or enzymatically from macro-algae are even scarcer. Only a few protocols has been published for the selective extraction and subsequent application of epiphytic DNA from bacteria associated with seaweeds (Burke et al. 2009; Fisher et al. 1998). In addition, siphonous macro-algae, such as *Bryopsis*, offer some extra options for the elimination of epiphytes due to their giant-cell morphology and regeneration mechanisms: the cytoplasm of these algae can be isolated by centrifugation (Berger and Kaever 1992) and the formation of protoplasts can be easily attained through

wounding (Kim et al. 2001). However, the objective of all techniques listed above was never to study the endophytic bacteria within these seaweeds, leaving the effect of these methods on the endophytes unaddressed.

In this study different mechanical, enzymatic and chemical procedures for the complete elimination of epiphytes from *Bryopsis* plants, in order to study the internal bacterial communities, were compared and evaluated. The aim was to develop a new, highly effective surface sterilization technique which neither lyses the algal cells nor eliminates endophytic DNA, allowing further molecular processing of the endosymbionts.

2. Materials and methods

2.1. Sampling and culturing

A *Bryopsis hypnoides* strain (BR) was collected from the lower intertidal zone in Roscoff, Brittany, France in July 2008. The plant was grown in sterile 1x modified Provasoli enriched seawater (West & McBride 1999) at 23°C under a 12:12h Light:Dark cycle with a photon flux rate of 25-30 $\mu E\ m^{-2}\ s^{-1}$. Unialgal cultures were achieved by isolating apical fragments of the vegetative thalli under a binocular dissecting microscope. The selected apical fragments were maintained under the same growth conditions as described above. To obtain more material for further applications, unialgal cultures were transferred to sterile 250 ml Erlenmeyer flasks with constant aeration.

2.2. Sterilization

Unialgal *Bryopsis* samples were submitted to a single or a combination of several mechanical, enzymatic and chemical sterilization protocols listed in Table 1. Each protocol was followed by ten washing and vortexing steps in sterile artificial seawater (ASW). Effective removal of epiphytes was tested by incubation of the washing water and sterilized algal thalli on Marine Agar plates (Becton Dickinson) for five days at 20°C. Because many bacteria are difficult to culture, the cleaned samples were stained for 15 minutes with 5 $\mu g.mL^{-1}$ 49,6-di amidino-2-phenylindole (DAPI) and subsequently viewed under a confocal and epifluorescence microscope (Zeiss) to determine whether the outer surface bacteria were effectively eliminated by the sterilization protocol applied. Also the intactness of the algal cells was microscopically verified.

2.3. Denaturing Gradient Gel Electrophoresis

To compare the effectiveness of the different sterilization procedures, the remaining bacterial diversity was examined by Denaturing Gradient Gel Electrophoresis (DGGE). Therefore the cleaned *Bryopsis* plants were placed in liquid nitrogen and ground with a sterile pestle prior to a total DNA extraction following a CTAB protocol modified from Doyle and Doyle (1987). The V3 region of the 16S rRNA gene was amplified by a PCR with the universal bacterial primers F357 (5′-CCTACGGGAGGCAGCAG-3′)

and R518 (5′-ATTACCGCGGCTGCTGG-3′) (Temmerman et al. 2003, Yu & Morrison 2004). A GC-clamp was coupled to the forward primer to improve DGGE separation. Amplifications were performed in volumes of 50µl containing 1µl of target DNA, 1x PCR buffer (GeneAmp, Applied Biosystems), 100µM dNTPs, 0.05x BSA, 0.2µM of both primers, and 1.25units AmpliTaq DNA polymerase (Applied Biosystems). After an initial denaturing step at 95°C for 1 min, 30 cycles of denaturation (95°C, 30 s), annealing (55°C, 45 s) and extension (72°C, 1 min) were completed, followed by a final amplification step at 72°C for 7 min. Successful amplification of the V3 region was verified through agarose gel electrophoresis. DGGE analysis of PCR amplicons was performed using the DCode Universal Mutation Detection System device (Bio-Rad) as described previously (Temmerman et al. 2003). Optimal electrophoretic separation was obtained using 35-70% denaturing gradient polyacrylamide gels, running for 990 minutes at 70 V in 1x TAE buffer at a constant temperature of 60°C. The gels were stained with SYBR gold (Molecular Probes, Invitrogen) for 30 min followed by visualization and digital capturing of the profiles via the Molecular Imager Gel Doc XR System (Bio-Rad). Digital images were normalized and processed by means of the BioNumerics software (version 5.1, Applied Maths).

2. Results

2.1. Sterilization

Attempts to efficiently eliminate the epiphytes by means of vortexing, ultrasonic bath sonication, beads, lysozyme, proteinase K or ethanol were unsuccessful. Incubation of the washing water on Marine Agar plates indicated that the former techniques were able to reduce the amount of epiphytes (data not shown), but bacteria were still visible when the sterilized algae themselves were plated or stained with DAPI (Fig. 1b and c, Fig. 2d to i). In addition, the application of ultrasonic probe sonication appeared to be too rough, the *Bryopsis* thalli were totally fragmented in a fraction of a second. Also the use of sodium hypochlorite was too aggressive, causing elimination of the endosymbionts due to instantly bleaching of the algae. When *Bryopsis* thalli treated with different lysis buffers or the bactericidal cleanser Umonium Master were cultivated on agar plates, no bacterial growth was noticeable (Fig. 1d and e); although, some bacterial fluorescence remained on the plants after DNA staining (Fig. 2j to o). The results mentioned above show that no single sterilization procedure was able to completely remove the epiphytes. Consequently, several combinations of two or more protocols were tested and evaluated (data not shown). Only a combination of CTAB buffer, proteinase K and the bactericidal cleanser Umonium Master proved to be highly effective. Unialgal *Bryopsis* plants were directly placed into CTAB buffer (2g CTAB, 1g PEG 8000, 1.5M NaCl, 0.02M EDTA and 0.1M Tris-HCl) with 20 mg.mL^{-1} proteinase K for 30 minutes at 60°C. Subsequently, the thalli were washed three times with sterile ASW and incubated overnight in a 1:1 mixture of 0.2µm filtered Umonium Master and sterile ASW. After lysis of the epiphytes, *Bryopsis* samples were washed ten times in sterile ASW with vigorous vortexing in between the washing steps to

remove the lysed bacterial DNA (step-by-step protocol of the combined approach, Table 1). The absence of cultivable epiphytes and bacterial DNA on the sterilized samples was verified as described above. Plating of these sterilized *Bryopsis* thalli on Marine agar showed no bacterial growth (Fig. 1f). More significant, however, was the staining of the sterilized *Bryopsis* samples with the DNA fluorochrome DAPI, revealing the absence of bacterial fluorescence on the surface of the algae (Fig. 2p to r). The algal cells themselves, on the other hand, were not lysed by the sterilization procedure as confirmed by light, epifluorescence and confocal microscopy, suggesting endophytic DNA is still present within the algae after the used chemical and enzymatic surface sterilization.

2.2. Denaturing Gradient Gel Electrophoresis

Total DNA, of both algal and bacterial origin, was extracted from *Bryopsis* thalli using the CTAB approach. This DNA mixture appeared to contain an excess of plant enzymes which interfere with PCR amplification. In order to decrease the algal inhibitors in the DGGE PCR, a 1:10 dilution of template DNA was used. Following electrophoresis, all samples, except the one treated with bleach, displayed an expected band of approximately 200bp on the agarose gel. Each band on the agarose gel represents a mixture of fragments of 16S rRNA genes from potential remaining epiphytes, endophytes and chloroplasts. Hence, DGGE was used to separate these fragments and examine the bacterial diversity surviving the various sterilization protocols applied. Figure 3 depicts a decrease in bacterial diversity in proportion to the vigorousness of the used sterilization. Mechanically cleaned samples show more individual DGGE bands, indicating an unsuccessful removal of epiphytes, compared to enzymatically and chemically sterilized plants. The newly presented combined sterilization protocol displayed the strongest reduction in bands and thus the most effective elimination of outer surface bacteria. Taking together evidence from the fluorescence imaging along with these molecular results strongly suggests that the remaining 16S rRNA gene diversity, including the chloroplast 16S rDNA, is of endophytic origin.

4. Discussion

Obtaining axenic macro-algal cultures while maintaining endophytic bacteria is challenging. Established culture techniques relying on the usage of antibiotics are inadequate as antibiotics may penetrate through the algal cell wall and eliminate the endophytes. Observations that antibiotics clearly affect the growth of algae or even kill them indicate such diffusions (Andersen 2005). Also the special features of siphonous seaweeds, e.g. the formation of protoplasts and the ability to isolate their cytoplasm, are insufficient to generate epiphytic-free algal material. Since these extraction techniques both depend on cutting or squeezing the algae, the outer xenic membranes become damaged and give rise to contaminations (personal observations). It has to be mentioned that the development of protoplasts in some larger and therefore easier to manipulate siphonocladous algae, like for example *Boergesenia* and

Ventricaria, shows potential for the formation of epiphytic-free algal material (personal observations, Mine et al. 2008). Hence, traditional algal cultivation and manipulation methods appear inadequate for the removal of bacterial epiphytes from *Bryopsis* plants without affecting the endophytes. Consequently, different mechanical, enzymatic and chemical surface sterilization protocols were tested and compared. None of these techniques seems on its own able to effectively eliminate the outer surface bacteria. The mechanical and enzymatic methods are highly insufficient, in accordance with observations by Burke and coworkers (2009). Also the use of various lysis buffers and disinfectants appears to be ineffective, in contrast with previous published studies (Burke et al. 2009, Fisher et al. 1998). In these studies Fisher and coworkers (1998) successfully sterilized filamentous green algae by placing them directly in UNSET buffer (Table 1), and Burke and colleagues (2009) fruitfully treated the green and red seaweeds, *Ulva australis* and *Delisea pulchra*, with calcium- and magnesium-free artificial seawater (CMFSW) supplemented with EDTA and a rapid multi-enzyme cleaner. These protocols, which were designed for the selective extraction of epiphytic DNA from algae associated bacteria, seem inefficient to completely sterilize *Bryopsis* externally with the aim of studying the bacterial endophytes. This objective is achieved by a combined chemical and enzymatic approach as presented here. *Bryopsis* thalli treated with CTAB lysis buffer, proteinase K and the bactericidal cleanser Umonium Master are highly effectively sterilized. They show no bacterial growth on agar plates (Fig. 1f) and no bacterial fluorescence on their cell wall when observed with confocal and epifluorescence microscopy after DAPI staining (Fig. 2p to r). Although often neglected by conventional surface sterilization protocols, the latter verification is essential since only 1% of all known bacteria suspected to be culturable (Amann et al. 1995). For example, the untreated sample and the ethanol and enzymatically cleaned plants all show growth when plated (Fig. 1a to c). On the other hand, fluorescence images allow a more detailed assessment of the outer surface community: the untreated, ethanol-sterilized and enzymatically cleaned samples show, respectively, bacterial biofilms (Fig. 2a to c), reduction of surface biofilms (Fig. 2d to f) and destruction of the biofilms into unattached bacteria (Fig. 2g to i). Much less expected were the results of the DAPI staining of *Bryopsis* plants sterilized with lysis buffer or Umonium Master. While both samples indicate no bacterial existence on agar (Fig. 1d and e), fluorescence images prove the presence of DNA on the surface of the algae (Fig. 2j to o). Even after vigorously vortexing during the several washing steps, bacterial DNA remains trapped in the degraded algal cell wall which is still clearly outlined by the blue DAPI stain. Despite the fact that these cell walls are gradually more damaged as surface sterilization becomes more effective, they were never fully lysed after the different disinfectant treatments. As shown in Figure 2 (internal fluorescent foci from nuclei and chloroplasts, a to r) the degraded but intact algal cell walls become permeable for the DAPI fluorochrome after chemical and enzymatic surface sterilization. Not only does this indicate internal algal and bacterial DNA is still present, it also seems suitable for additional molecular processing like

PCR amplification and DGGE (Fig.3). This molecular approach is of great value in further research on the identity and functionality of the – possibly unculturable – endosymbiotic bacteria in *Bryopsis* algae. Future investigation will probably reveal these bacteria have significant functions within their host. Moreover, some of the compounds produced by these bacterial symbionts may have important applications like for example the production of the anticancer drug kahalalide as suggested by Kan et al. (1999).

5. Conclusion

Although surface sterilization is a critical step in endosymbiosis research, it remains challenging, especially in delicate organisms such as algae. Certainly in new symbiosis systems, it is worthwhile to test and evaluate conventional sterilization techniques. This study demonstrates that small alterations or combinations of established disinfection protocols permit an efficient sterilization. The protocol presented here will likely be useful in studies of new and difficult to handle hosts, allowing exploration of novel symbiosis systems.

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Table and Figure legends

Table 1 List of protocols applied for the surface sterilization of *Bryopsis* plants

Fig.1. Incubation of untreated (a) and sterilized (b-f) *Bryopsis* thalli on Marine Agar plates. Like the untreated sample (a), the ethanol (b) or enzymatically (c) cleaned samples still show growth of epiphytic bacteria after five days incubation, indicating an unsuccessful surface sterilization. In contrast, *Bryopsis* plants treated with lysis buffers (d), Umonium Master (e) or the new combined approach (CTAB buffer, proteinase K and Umonium Master; f) showed no bacterial growth after plating, suggesting that (culturable) epiphytes are effectively eliminated. Scale bars: 5mm (a, b, and c), 2.5mm (d), and 1mm (e and f). Arrowheads: *Bryopsis* thalli.

Fig.2. Fluorescence microscopy images of untreated (a-c) and sterilized (d-r) *Bryopsis* thalli stained with DAPI . Columns display from left to right the result of progressively more aggressive treatments, rows show increasing magnification from top to bottom. When surface sterilization is more aggressive and consequently more effective (left-right), DAPI staining of the outer surface bacteria becomes less profuse. Images of the untreated sample (a-c), the ethanol (d-f) and enzymatically (g-i) sterilized samples show an intense staining of epiphytic DNA on their cell walls. This DAPI staining becomes gradually weaker on the images of plants treated with lysis buffer (j-l) or Umonium Master (m-o), and is missing on fluorescence pictures of *Bryopsis* thalli sterilized with the new combined approach (CTAB buffer, proteinase K and Umonium Master; p-r). Only this latter signifies an effective surface sterilization. In addition, algal cell walls become more permeable for the DAPI stain (e.g. more fluorescent foci from nuclei and chloroplasts at the inside of the algal cells) as surface sterilization is more aggressive, but they were never fully lysed. Scale bars: 100µm (a, d, g, j, m, and p; confocal microscopy images), 50µm (b, e, h, k, n, and q; confocal microscopy images), and 10µm (c, f, i, l, o, and r; epifluorescence microscopy images).

Fig.3. Normalized DGGE profiles of 16S rDNA fragments amplified from untreated and sterilized *Bryopsis* plants. DGGE profiles represent the bacterial diversity of the untreated (lane 2) and sterilized (lane 3 till 11) samples. As the disinfection protocols applied are more effective (up-down), DGGE profiles become less complex, reflecting a more successful surface sterilization. With exception of the DGGE profile of the bleach-sterilized sample (lane 8) which

shows no remaining bacterial diversity. Furthermore, *Bryopsis* plants treated with the new combined approach (CTAB buffer, proteinase K and Umonium Master; lane 11) still show a clear occurrence of bands, indicating endophytic DNA is still present within these sterilized plants and suitable for further molecular processing. In addition, lane 1 and 12 contain a molecular marker used for normalization. The white box indicates chloroplasts 16S rDNA fragments as verified by DNA sequencing.

Table and Figure sizes

Table 1: full page width (174 mm width) Colour for electronic version, B&W for printed version

Fig. 1: one and a half column width (129 mm width) Colour for electronic version, B&W for printed version

Fig. 2: full page width (174 mm width) Colour for both electronic and printed version

Fig. 3: one and a half column width (129 mm width)

Figure 1

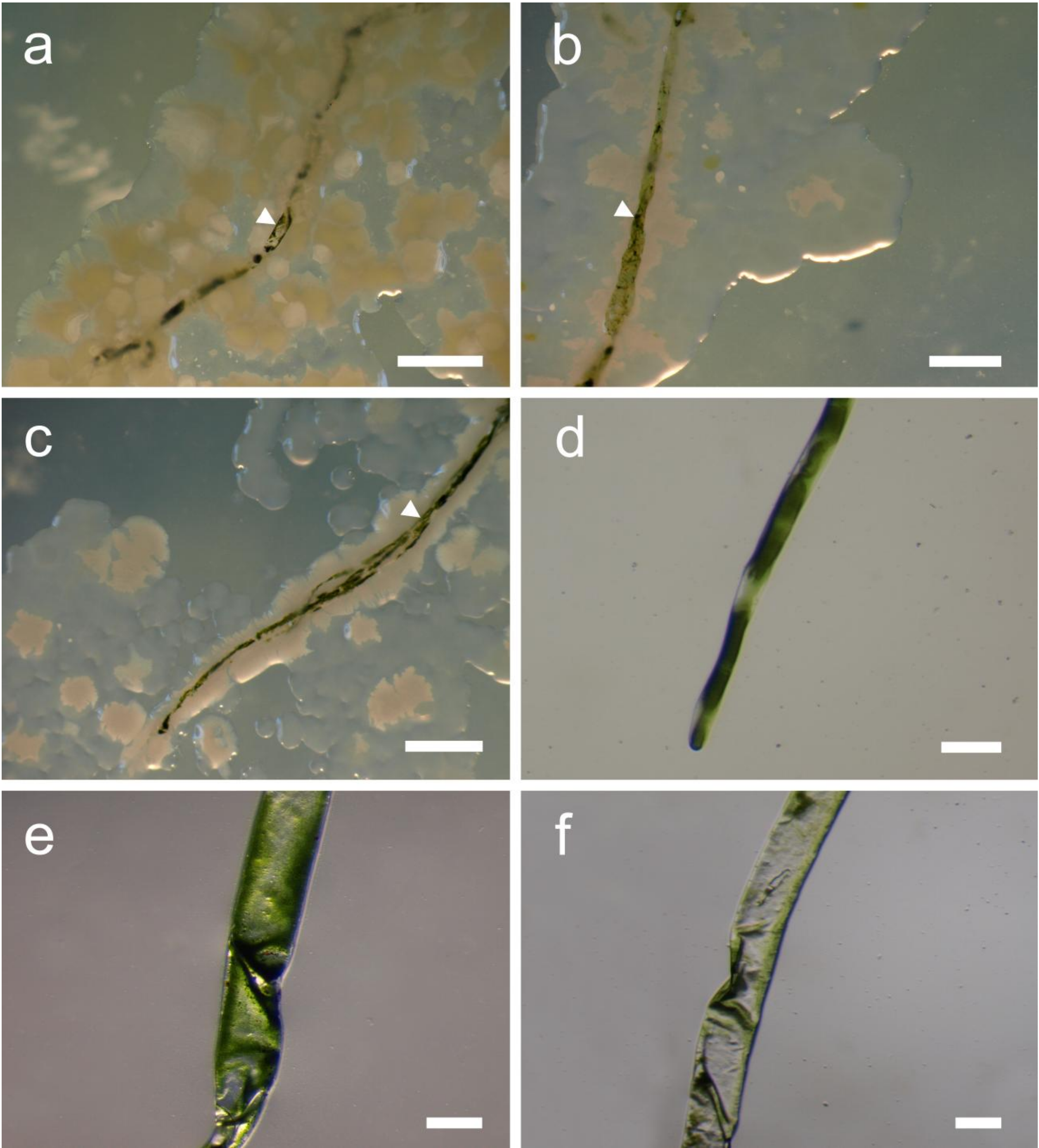


Figure 2

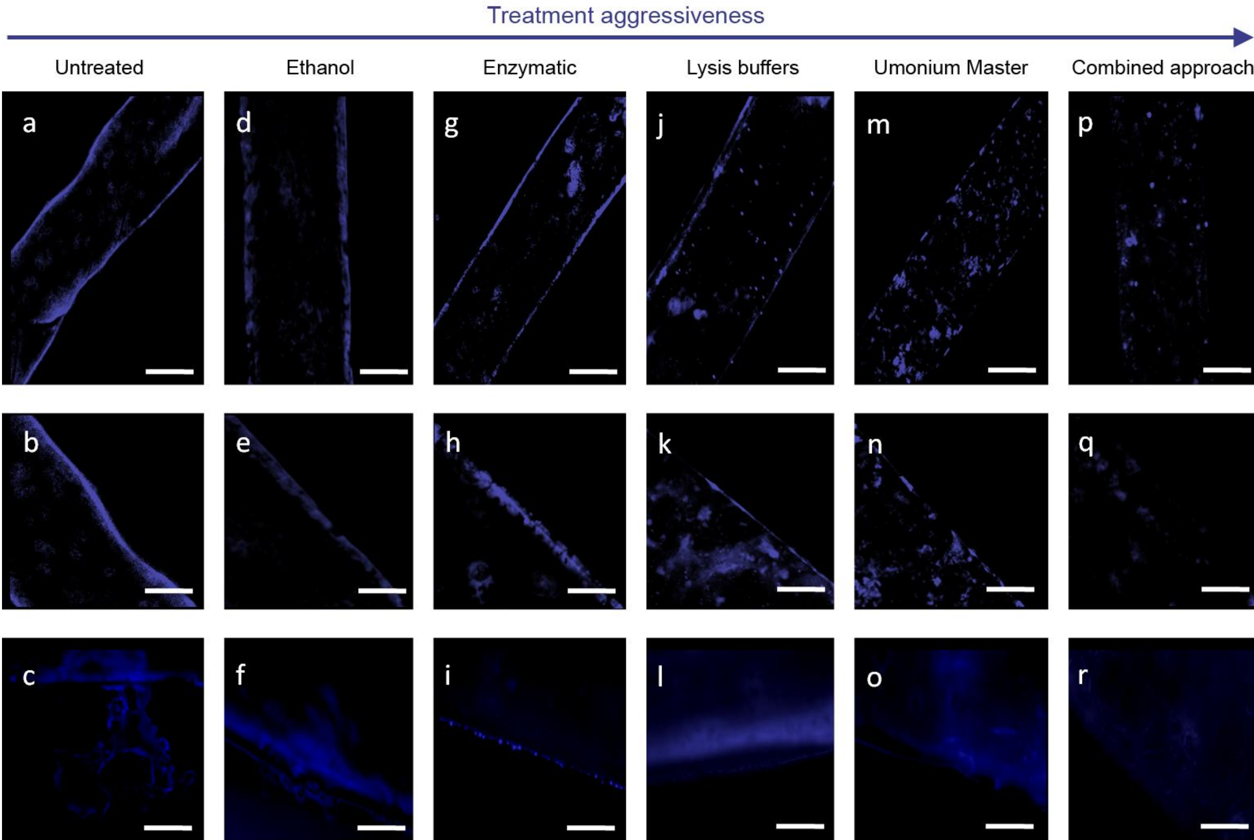
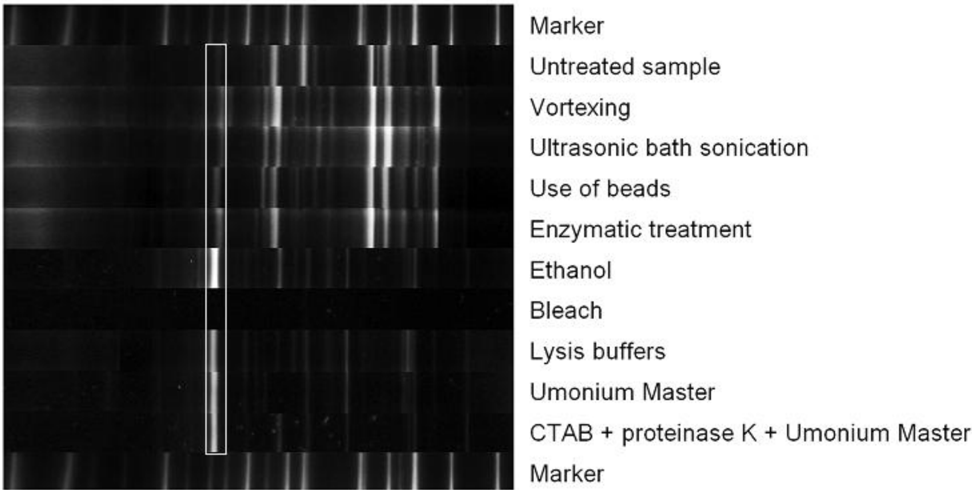


Figure 3



	Sterilization technique	Extended protocol
Mechanical	**Vortexing**	Repeatedly vortex the plants in 0.2µm filtered ASW with five changes of washing water
	Ultrasonic probe sonication	Ultrasonic probe sonication of the samples in sterile ASW for 15 seconds at 30 kHz
	Ultrasonic bath sonication	Ultrasonic bath sonication of the samples in sterile ASW for 15 minutes at 47 kHz
	Use of beads	Add glass beads (0.5 mm, BioSpec Products) to the algal tissue and bead beat the mixture at 30kHz for 3 x 85 seconds
Enzymatic	**Lysozyme**	Add 10µl lysozyme (1 $mg.mL^{-1}$ in 10mM Tris HCl) and 190µl sterile ASW to the specimens and incubate for 5 minutes at room temperature
	Proteinase K	Incubate the algal thalli in a mixture of 1µl 20$mg.mL^{-1}$ proteinase K and 99µl ASW for 30 minutes at 60°C
Chemical	**Ethanol**	Rinse plants in 80% ethanol for 5 minutes
	Bleaching	Sterilize algae in 3% sodium hypochlorite for 30 seconds
	Alkaline lysis buffer	Place thalli in 80µl sterile ASW with 20µl alkaline lysis buffer (1M NaOH and 10% sodium dodecyl sulfate) for 15 minutes at 95°C
	CTAB buffer	Put plants directly into 100µl CTAB buffer (2g CTAB, 1g PEG 8000, 1.5M NaCl, 0.02M EDTA and 0.1M Tris-HCl) for 30 minutes at 60°C
	UNSET buffer	Place samples in 100µl UNSET Lysis Buffer (8M urea, 2% sodium dodecyl sulfate, 0.15M NaCl, 0.001M EDTA, 0.1M Tris pH 7.5) for 15 minutes at 55°C (Fisher et al. 1998)
	Bactericidal cleanser	Sterilize plants overnight in a 1:1 mixture of 0.2µm filtered Umonium Master (Huckert's International) and sterile ASW
	Combined approach	1. Place unialgal *Bryopsis* plants directly into CTAB buffer with 20 $mg.mL^{-1}$ proteinase K for 30 minutes at 60°C 2. Wash the *Bryopsis* thalli with sterile ASW 3. Repeat step 2 two times 4. Incubate overnight the washed thalli in a 1:1 mixture of 0.2µm filtered Umonium Master and sterile ASW 5. Wash thalli in sterile ASW 6. Repeat step 5 ten times with vigorous vortexing in between the washing steps