Anna Fricke*, Terue C. Kihara and Mona Hoppenrath

Studying mesoalgal structures: a non-destructive approach based on confocal laser scanning microscopy

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Abstract: Mesoalgae play a key role in shallow coastal ecosystems. Composed of small macroalgae, intermixed with filamentous cyanobacteria and colonial diatoms, these multi-specific, but minute (μm–cm) assemblages form complex three-dimensional structures, providing shelter for different unicellular (e.g. bacteria, diatoms, dinoflagellates) and multicellular (e.g. fishes, invertebrates) organisms. Characterized by a high colonization potential, these primary producers are observed to bloom and overgrow disturbed areas (e.g. damaged coral reefs, urchin barrens), and play a crucial role in terms of invasion and colonizing new habitats. Driven by anthropogenic environmental changes, mesoalgae are receiving considerable attention in current marine research. So far, most studies approach mesoalgae at the functional group level (e.g. turf algae, microphytobenthos), whereas only few studies tackle the importance of species-specific interactions, which play an important role in benthic ecology (e.g. coral-algal competition and disease spreading). To facilitate the study of not only the presence but also the composition and the structure of these habitat formers, we provide a new approach combining inexpensive fixation methodology with modern confocal laser scanning microscopy (CLSM), to study minute macroalgal structures (e.g. germlings, reproductive structures), and investigate their relation to microphytobenthic components (e.g. diatom colonies). Detailed procedures for mounting, staining and imaging phytobenthic communities are provided.

***Corresponding author: Anna Fricke,** Instituto Argentino de Oceanografía (IADO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Florida 4750, Bahía Blanca, B8000FWB, Argentina; and Leibniz-Center for Tropical Marine Ecology (ZMT), Fahrenheitstrasse 6, 28359 Bremen, Germany, e-mail: Annafricke@gmx.de
Terue C. Kihara and Mona Hoppenrath: Senckenberg am Meer, German Centre for Marine Biodiversity Research (DZMB), Südstrand 44, 26382 Wilhelmshaven, Germany

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Introduction

Growing from the splash zone down to the deeper subtidal zone (~200 m), benthic algal communities form an ubiquitous ecological key group within coastal ecosystems (Lüning 1990). As primary producers they offer nutrition, oxygen and shelter for a variety of different organisms (e.g. fishes, invertebrates, microalgae, fungi, bacteria) (Wiencke 2011). Providing the base of the benthic food web, algal communities can rapidly respond to alterations in environmental factors (Campana et al. 2009, Fricke et al. 2014) and are commonly used as indicators for water quality (Ballesteros et al. 2007, Neto et al. 2010). Driven by human activities, benthic algal communities worldwide have shown strong alterations (Campana et al. 2009), reaching dramatic proportions in the formation of intense blooms (Valiela et al. 1997, Teichberg et al. 2010) and expansion of different algal species (Fricke et al. 2013, Croce and Parodi 2014). Consequently a good knowledge of the composition and ecology of benthic algal communities is needed to understand and predict environmental changes.

According to their sizes, benthic algae are often separated into two groups, i) the macroscopically observable "macroalgae" (also known as seaweeds) and ii) the "microalgae", only detectable by microscopy. Interestingly, a major part of the benthic algal communities growing in coastal waters compose a third, intermediate group (or growth form) by forming macroscopically visible structures (e.g. tufts, turfs, mats, crusts), that cannot be identified – even at the generic level – without a light microscope (Wanders 1976, Vermeij et al. 2010). This borderline group, defined here as iii) "mesoalgae" is composed of a variety of different taxa, belonging to both macro- and microalgae. Mesoalgae include microscopic early stages (e.g. propagules and germlings), smaller forms of macroalgae (e.g. uniseriate and fine filamentous

forms), as well as macroscopic colonies of microalgae (e.g. diatoms and cyanobacteria), reaching several millimetres to decimetres in size (Lobban 1989, Mejean et al. 2010). In addition, the morphological plasticity and complex heteromorphic life cycles of many macroalgal species lead to a broad variety in thallus sizes. As seen in the tallest known macroalga itself, *Macrocystis pyrifera,* whose sporophyte can reach frond lengths of >45 m, possesses a gametophyte, which reaches sizes below 1 mm (Abbott et al. 1992), and would be, according to our definition, part of the mesoalgal community.

Size and composition of benthic algal communities can be strongly affected by environmental conditions, such as light and nutrient availability, flow rates, sedimentation or grazing pressure (Campana et al. 2009, Fricke et al. 2014, 2016). Despite mesoalgal communities providing a common growth form in shallow coastal ecosystems, the lack of a uniform definition makes it difficult to approach this specific group. Although the term "mesoalgae" (or meso-algae) has been occasionally used in a few studies (e.g. Hatcher 1989, Webster et al. 2002, Fricke et al. 2016), this borderline group has been studied under different names often related to its habitat or climate zone. So we can often find them as "turf algae" in studies of tropical (Littler et al. 2006, Titlyanov et al. 2008) or Mediterranean waters (Linares et al. 2012, Sala et al. 2012), whereas the term "turf algae" has been used in different ways, approaching dense growth forms (Hay 1981), as well as intermediate thallus sizes (Van den Hoek et al. 1978, Vermeij et al. 2010, Fricke et al. 2011a). In addition we find these communities under the terms "algal mats" (Wilhm and Long 1969) or "filamentous algae" (Potts 1977). Furthermore mesoalgae are commonly grouped under substrate-related names, like "epilithic" (Klumpp and McKinnon 1992) or "fouling communities" (Abdel Aleem 1957, Evans 1981).

Due to their sensitivity towards environmental change, mesoalgae are receiving considerable attention in current marine research (e.g. Campana et al. 2009, Littler et al. 2010, Vermeij et al. 2010, Fricke et al. 2011a, 2016). Since they are able to overgrow a variety of different substrates, mesoalgal communities can alter the structure and chemistry of benthic surfaces (Wanders 1976, Wangpraseurt et al. 2012), which may impact the whole coastal ecosystem. In addition, due to their observable high proliferation, mesoalgae play a crucial role in terms of invasion and blooms (Lovelock et al. 2008, Vermeij et al. 2010). Consequently a better knowledge of the ecological role of mesoalgae is urgently required.

As they form a borderline group, the study of mesoalgae requires a combination of different ecological and microbiological techniques, able not only to describe their macroscopic appearance (e.g. shape, color) and taxonomy, but also to study their microscopic thallus structures, investigate their interspecific relations (e.g. epiphytism) and potential ecological interplay (e.g. facilitation or inhibition; Connell and Slatyer 1977).

Developed in the mid-1950s, confocal laser scanning microscopy (CLSM) provides a powerful tool to investigate and understand complex structures and processes in a variety of scientific fields (Neu et al. 2010). Based on the examination of fluorescence emissions, CLSM is sensitive to visible (400–700 nm) to near-ultraviolet and near-infrared spectral regions (Claxton et al. 2006). Producing three-dimensional reconstructions and distinguishing different chemical compounds, CLSM has been widely applied in the field of microbiology to study structure of biofilms (Neu et al. 2010) as well as in invertebrate taxonomy to study microstructures (Michels and Buntzow 2010). In general, CLSM is used for three reasons i) visualization of multiple features, ii) analysis of structure, composition, microhabitats, activity and processes and iii) volumetric and structural measurements (Neu et al. 2010).

Showing strong auto-fluorescence signals, based on the presence of different photosynthetic pigments, algae provide an interesting group to be studied by CLSM. The work of Larson and Passy (2005) provides a milestone in phycological studies, as being one of the first quantitative approaches applying CLSM to natural biofilms for biomonitoring purposes. In later studies, CLSM has become more and more applied to different aspects of phycological research, e.g. to study the shape and structure of chloroplasts (Škaloud and Peksa 2008), to analyze microfilament organization in siphonaceous algae (Larkum et al. 2011) or to calculate biovolumes of diatom frustules (Friedrichs et al. 2012).

In our study we applied CLSM to study mesoalgal thallus structures. The present work provides a detailed approach, allowing the investigation of material grown *in situ* by combining different ecological (settlement plates) and microbiological (light microscopy: LM, and CLSM) techniques. The main aims of our study were to i) develop a non-destructive protocol to study field grown communities, ii) provide high resolution of mesoalgal thallus structures, iii) facilitate the study of different growth forms and interspecific relations (e.g. epiphytism). Allowing insights in phytobenthic microstructures, the present study can help to understand inter-specific relations, e.g. epiphytism, within phytobenthic communities and unravel their role in functional ecology.

Materials and methods

Settlement substrate

To approach their structure and complexity, we investigated mesoalgal communities, naturally grown under field and aquarium conditions, using polyethylene terephthalate (PET, Melinex®) as a settlement substrate. This transparent material, not only provides a suitable substrate for the growth of mesoalgal communities, as observed in former studies (Fricke et al. 2016), but also allows the direct comparison of different microscopic techniques (LM vs. CLSM).

Exposure and sampling procedures

Material collected during a field study in a eutrophic tidal channel in Northern Patagonia (S40° 43′ W64° 56′; Fricke et al. 2014), served as a base for the study. At the experimental site, settlement substrates (pieces of PET, 1.5×2 cm) were exposed horizontally to the water surface on old car tires, fixed ~30 cm below low tide water level and 20 cm above sediment, and were sampled after a period of 40 days (November 2012). To avoid sampling artifacts, field grown mesoalgal communities were carefully collected with their substrates, and, still submerged, transferred to individual plastic tubes (10 ml) filled with ambient seawater (Figure 1). During the transport to laboratory (<1 h distant from the field), the plastic tubes were stored inside an opaque cool box to prevent any light and/or thermal influence on the samples. Naturally grown macroalgae were sampled in parallel next to the artificial substrate. In addition, material grown on settlement substrates in the aquarium facilities (mean temperature 27°C, 12 h light/12 h dark) of the Leibniz-Center for Tropical Marine Ecology (ZMT) was sampled after an exposure of 1 week.

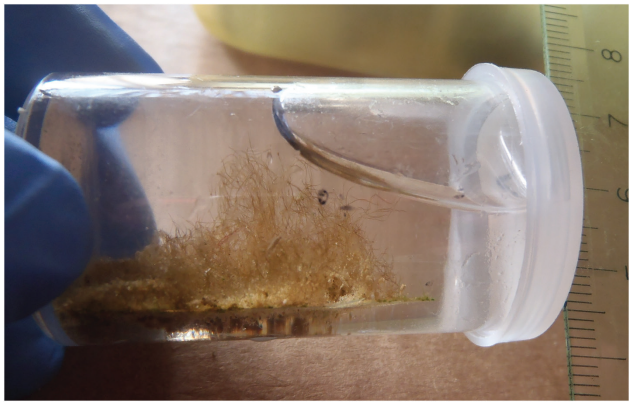

Figure 1: Mesoalgal community.
Picture shows mesoalgal community field-grown on settlement substrate (1.5×2 cm, polyethylene terephthalate plate) after sampling in an individual plastic tube (10 ml) filled with ambient seawater.

Investigated material

To demonstrate the application of CLSM on the different structures of mesoalgal communities, five different objects were chosen:

- Object 1: Mesoalgal communities grown on settlement substrates in a tropical aquarium over one week, mainly consisting of the red calcareous alga *Hydrolithon farinosum* (J.V. Lamouroux) Penrose and Y.M. Chamberlain 1993 (showing characteristic four celled initials) and the green crustose alga *Ulvella* sp.
- Object 2: Mesoalgal community sampled from a tropical aquarium, mainly composed of a variety of filamentous cyanobacteria of the genera *Lyngbya*, *Phormidium*, *Oscillatoria*.
- Object 3: Mesoalgal communities grown on settlement substrates in a temperate tidal channel over a period of 40 days dominated by the green alga *Ulva* aff. *prolifera* O.F.Müller 1778 and tube dwelling diatoms, like *Berkeleya rutilans* (Trentepohl *ex* Roth) Grunow 1880 (identified in a prior study, Fricke et al. unpublished data).
- Object 4: Field sampled thalli of the calcareous red alga *Jania rubens* (Linnaeus) J.V. Lamouroux 1816 (Corallinales, Corallinaceae), epiphytized by a variety of smaller mesoalgae (e.g. *Polysiphonia* sp.). Itself a common component of the mesoalgal community, *J. rubens* forms a heavily calcified thallus, which cannot easily be investigated under normal light microscopy.
- Object 5: Field sampled thalli of the red alga *Neosiphonia harveyi* (Bailey) M.-S. Kim, H.-G. Choi, Guiry and G.W. Saunders 2001 (Ceramiales, Rhodomelaceae). Listed as invasive species worldwide (WRIMS 2014), *N. harveyi* forms a fine filamentous thallus and can be found in mesoalgal communities. Its pronounced fertile structures (e.g. carposporophyte) provide a complex tri-dimensional structure whose inner parts are difficult to visualize under the light microscope.

Mounting and remounting techniques

When not investigated by CLSM *in situ* (e.g. Neu and Lawrence 1997), accurate preservation is crucial to

preserve natural microstructures. In this context, different mounting techniques are used in CLSM studies, e.g. embedding in agar, citifluor and cryo-preservation (Eitner 2004, Hurst and Crawford 2007). In comparison to other applied embedding media, corn is commercially available (commonly used in the food industry) and has been successfully applied for macroalgal preservation in different studies (Neto et al. 2002, Saunders 2009, Fricke et al. 2016). In the present study we used corn syrup as a mounting medium and tested its transparency and feasibility for CLSM investigations.

After sampling, algal material was mounted in semi-permanent slides using corn syrup solution. Corn syrup stock solution: 1 : 1 solution of Karo™ light corn syrup (ACH Food Companies, Inc., USA) and distilled water. For longer preservation, a few drops of 4% formaldehyde were added to the solution. The presence of formaldehyde avoids bacterial and fungal growth. For mounting, we followed the procedure listed in Table 1.

Once mounted on a glass slide, the material can be easily re-mounted at any time by carefully dissolving the corn syrup in distilled water, and the freed material can be used for further investigations or treatments. For this purpose, some drops of distilled water at the rim of the cover slip are normally enough to dissolve the mountant within a few minutes. This can help to reduce the often lengthy time delay between field sampling and later microscopic investigation, and also allows subsequent investigations, e.g. additional staining procedures.

Staining procedure

In addition to natural auto-fluorescence, the application of an artificial fluorescence stain can help to enhance or produce fluorescence signals. First described by Stanford (1885), "algic cellulose" is a common component of macroalgal cell walls, often found in filamentous and bloom forming species (Baldan et al. 2001, Mihranyan 2011). In order to increase the macroalgal fluorescence signals, we tested the suitability of the cellulose-sensitive dye Congo Red (Carder 1986) for our mesoalgal material. Commonly used for land plant investigations (Sass 1958), this stain has rarely been used in phytobenthic studies, despite its high affinity to macroalgal thalli (Vijayaraghavan and Shanthakumar 2016).

The selected algal material was stained with a saturated solution of Congo Red (modified from Michels and Buntzow 2010). The detailed staining procedure is given in Table 2. Additional information on sample treatment for individual images in Figures 2–6, with or without prior staining by Congo Red, is given in Table 3.

Table 1: Mounting procedure for mesoalgae.

Materials needed: vial with screw cap (e.g. 15-ml centrifuge tube), Karo™ corn syrup (ACH Food Companies, Inc., USA), distilled water, object slides, coverslip No. 1 (0.13–0.16 mm), corn syrup solution (1 : 1 in distilled water), pipette, needle

a) Preparation of mounting media (diluted corn syrup)
- Add 7 ml corn syrup (e.g. Karo™) into vial (e.g. 15-ml centrifuge tube)
- Add 7 ml distilled water
- Dilute corn syrup by frequent inversion of the closed vial
- Let liquid settle to avoid air bubbles

b) Mounting procedure
- Place wet material (ambient seawater) in the center of glass slide, gently cover it with a coverslip No. 1 (0.13–0.16 mm)
- Drop media with a pipette on the edges on the coverslip and allow to soak under it (the highly osmotic corn syrup will replace ambient seawater)
- Avoid dehydration of the material during mounting processes
- Keep the preparations drying at room temperature overnight
- If necessary, additional corn syrup solution can be added in case of evaporation
- When the mounting settles, seal the edges of the coverslip with transparent nail polish to avoid contamination
- The final preparation should be a flat hardened mounting, light in color and free of bubbles

c) Removal technique
- For subsequent removal of mounted mesoalgal material, the nail polish can be carefully removed with a needle and the corn syrup diluted using some drops of distilled water

The table provides step-wise protocol for mounting mesoalgal material on semi-permanent slides, using corn syrup solution, and describes subsequent removal technique for mounted mesoalgal material.

Table 2: Technique for staining samples of mesoalgal communities with Congo Red.

Materials needed: Congo Red (powder; Sigma-Aldrich, Steinheim, Germany), ethanol 96%, gloves, capped opaque glass bottle, syringe with filter of 0.2-μm pore size (Filtropur S 0.2 – Sarstedt AG & Co., Numbrecht, Germany), glass staining block with cover (or shallow vessel with lid to hold small quantity of stain), algal material, forceps, filtered seawater (0.2 μm)

a) Preparation of Congo Red *stock* solution:

For handling, avoid inhalation of powder and wear gloves. To guarantee stain quality, the photolytic stock solution should be prepared as freshly as possible (in small quantities) and not stored over long time periods (months)

- Place 1.5 mg of Congo Red (powder) in a capped opaque glass bottle (e.g. 5-ml opaque glass bottle)
- Add 1 ml of ethanol 96%, close the bottle and agitate the mixture
- Cover the stock bottle with aluminium foil or similar to avoid photolysis of the solution

b) Preparation of Congo Red *working* solution:

- Immediately before use, filter some stock solution through a syringe filter with 0.2-μm pore size (Filtropur S 0.2 – Sarsted AG & Co., Numbrecht, Germany) into a glass staining block

c) Staining procedure

- Place algal material carefully in the filled glass staining block
- Cover the block to avoid desiccation and allow to stain overnight
- To remove any excess stain, transfer algal material to filtered seawater (0.2 μm) for 2 h
- Mount algal material on a glass slide, following the procedure given in Table 1

Microscopic analyses and image processing

All samples were investigated by light microscopy, using a Leica DMRB light microscope with differential interference contrast optics (Leica, Wetzlar, Germany) and confocal laser scanning microscopy (CLSM), using Leica TCS SP5 (Leica, Wetzlar, Germany) equipped with a Leica DM5000 B upright microscope (Leica, Wetzlar, Germany) and three visible light lasers (DPSS 10 mW 561 nm; HeNe 10 mW 633 nm; Ar 100 mW 458 nm, 476 nm, 488 nm and 514 nm), combined with the software LAS AF 2.2.1.- Leica Application Suite Advanced Fluorescence (Leica, Wetzlar, Germany). Lasers and various settings of the experimental parameters are listed in Table 3.

To select the settings in Table 2, we took into account the autofluorescence of chlorophyll a (Chl a, excitation: 620–680 nm, emission: 650–740 nm; modified after Roshchina 2012) and of phycoerythrin (PE). Whereas Chl a is an essential pigment for every photosynthetic algal cell, PE is a photosynthetic accessory pigment, which is predominantly produced in Rhodophyta (Rossano et al. 2003) and Cyanobacteria (excitation: λmax ~ 560 nm, emission: 550–600 nm; Bordowitz and Montgomery 2010). Based on the PE presence we distinguished in the present study two crust-like growing algae, the red alga *Hydrolithon farinosum* and the green alga *Ulvella* sp. under the CLSM. In addition, the fluorescence of Congo Red (excitation: 540–560 nm, emission: > 600 nm) was taken into account (Michels and Buntzow 2010). Other settings as gain and offset were applied to obtain the cleanest image (lower noise) and the optimal pinhole aperture, to adjust the resolution of the sample and the amount of light that reaches the detectors. Modifications of these initial settings can be applied according to the material examined, always considering that each adjustment can affect the other settings. A series of stacks was obtained, collecting overlapping optical sections throughout the whole preparation. The CLSM data was presented as maximum intensity projections, a sharply focused image using the brightest value along the z-axis for each pixel.

Quantitative measurements based on fluorescence signals (Object 1)

To determine the relative abundance of different algal taxa for Object 1 (crustose algae), we calculated the area of *Hydrolithon farinosum* and *Ulvella* sp. based on their fluorescence signals. For this purpose we used the maximum projection combining Ch.1: 578–609 nm (~ PE fluorescence, red channel) and Ch.2: 659–686 nm (~ Chl a fluorescence, green channel) (Table 3, Figure 2A).

Using the image analysing software Image J, we were able to transform each channel into binary images (Green, Figure 2B–C; Red, Figure 2D–E). We applied the option "analyze particle", available in the software package, to outline the transformed image and calculate the area. We calculated the relative abundances of all photosynthetic taxa (containing Chl a, green channel) and of *H. farinosum* (containing PE, red channel). In addition, subtracting the

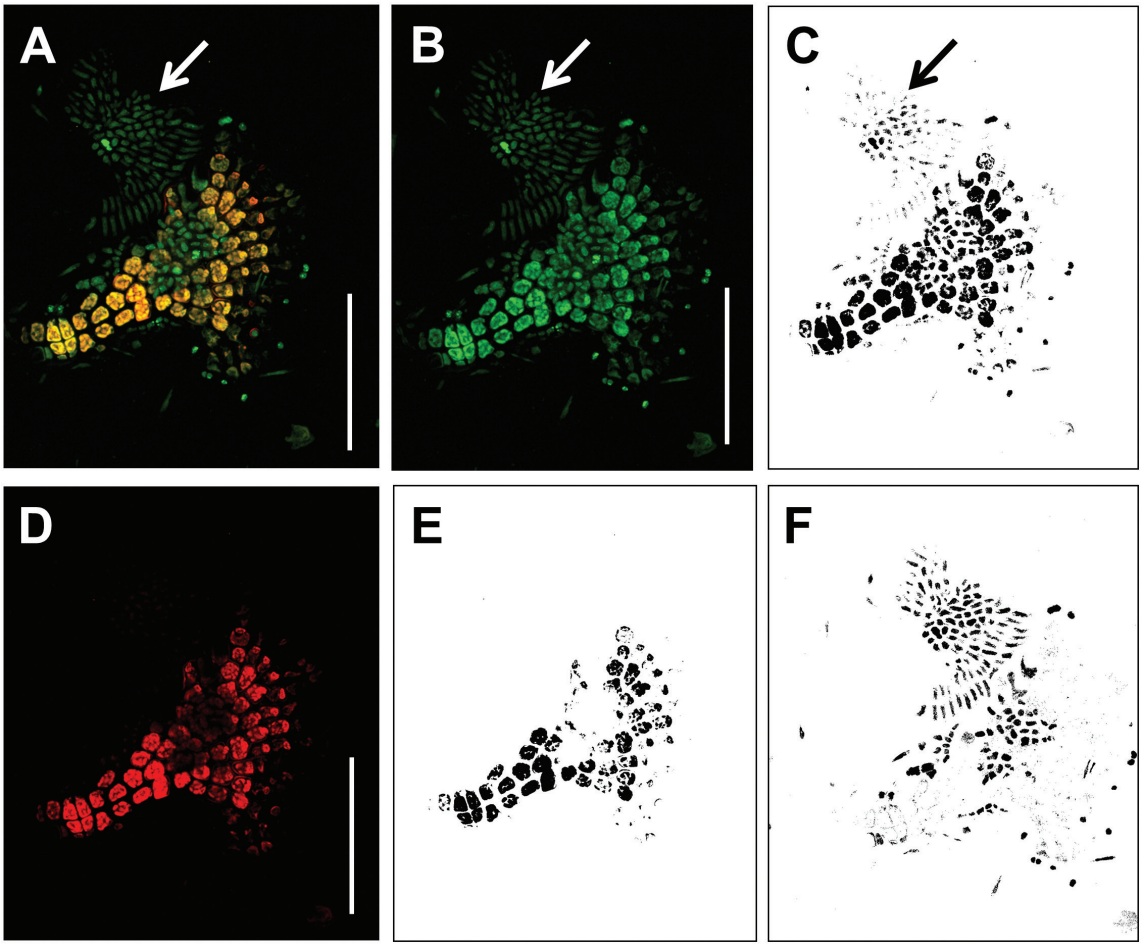

Figure 2: Spectral differentiation between algal thalli, based on the presence of phycoerythrin (PE) in red algae.
Material for confocal laser scanning microscopy was excited at 561 nm and 633 nm (for details see Materials and methods section). (A) shows emissions detected at both 578–609 nm (~ PE fluorescence, Ch.1) and 659–686 nm (~ Chl a fluorescence, Ch.2). (B–C) show emissions detected for Chl a (Ch.2) only and (D–E) show emissions detected for PE (Ch.1) only, resulting in the spectral disappearance of the green alga *Ulvella* sp. (indicated by arrows in A–C). (C) and (E) show binary projections applied in IMAGE J of (B) and (D), respectively, used to calculate relative abundances of different taxa. (F) shows the result of subtracting the optical binary information in E (i.e. PE fluorescence) from that in C (i.e. Chl a fluorescence), resulting in the area (relative abundance) of *Ulvella* sp. and other (non-PE containing) photosynthesizing taxa.

optical binary information of Figure 2E (PE fluorescence) from that of Figure 2C (Chl a fluorescence), we obtained the relative abundance of *Ulvella* sp. and other (non-PE containing) photosynthesizing taxa (Figure 2F).

Optical sections for emphasizing structural details

For a better understanding of internal structures, optical sections of two objects were performed: i) a cut through a mature pericarp of *Neosiphonia* aff. *harveyi*, and ii) the dissection of an epiphytized filament of *Lyngbya* sp. hidden within a dense cyanobacterial mat. For this purpose, series of stacks were obtained for the whole material but the optical sections at the uppermost part of the material were cropped showing the interior of the pericarp with mature carposporangia, as well as the epiphytized filament.

Results

Mounting procedure (all objects)

The fixation with corn syrup allowed an instant preservation of the mesoalgal community (see below). Storage

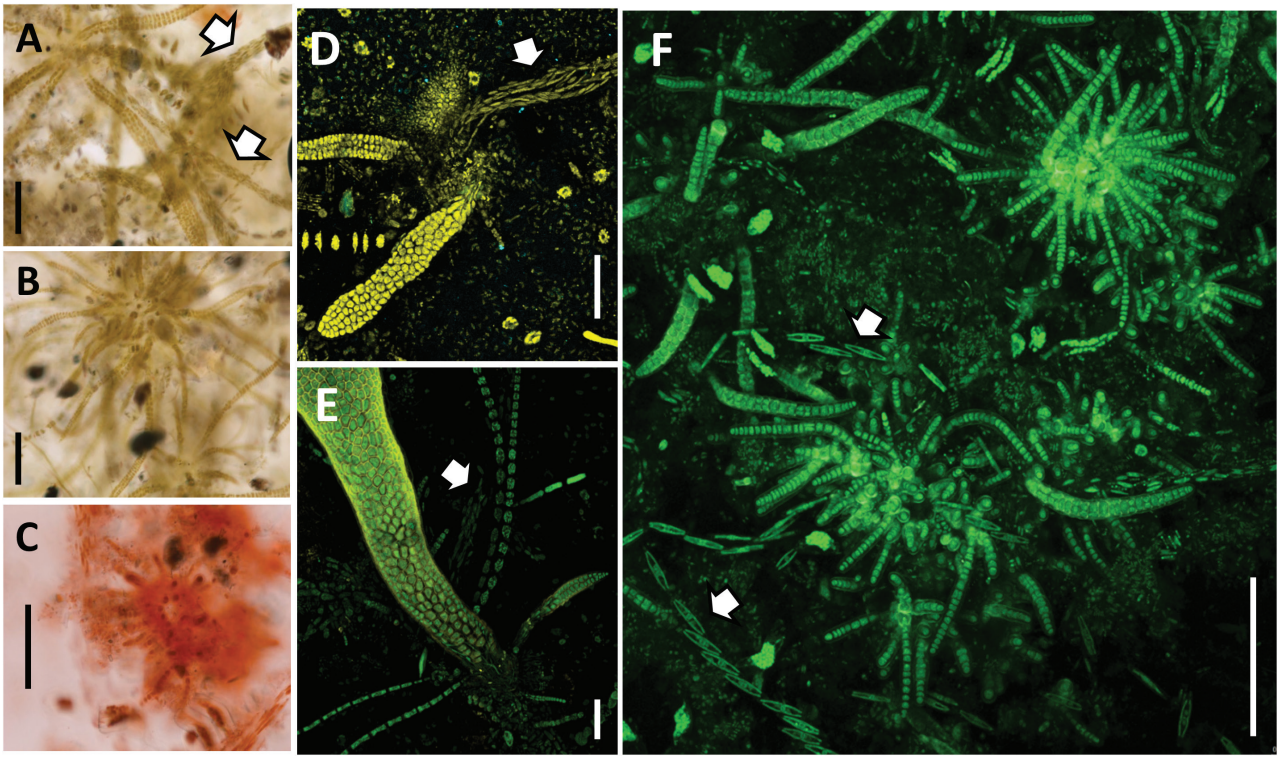

Figure 3: Mesoalgal community structure.
Mesoalgal communities naturally grown on settlement substrates (1.5 × 2 cm, polyethylene terephthalate plates) exposed for 40 days in the field; mainly composed of germlings of *Ulva* aff. *prolifera* O.F. Müller 1778 and *Ulva* aff. *flexuosa* Wulfen 1803. Images show non-stained (A, B, D) and Congo Red stained (C, E, F) mesoalgal communities, investigated under light microscopy (A–C) and under confocal laser scanning microscopy (D–F) excited at 561 nm and 633 nm (for details see Materials and methods section). White arrows in A and D–F indicate positions of colony-forming tube-dwelling diatoms. Scale bars = 100 μm.

and transport of the mesoalgal communities was possible at ambient temperatures. This is a crucial point for many field studies, taking account of storing capabilities, handling costs, and often extended time frames between field collection and laboratory analyses.

Corn syrup mounting medium provided high transparency allowing the investigation with light microscopy (Figures 3A–C; 4A–C; 5A–C; and 6A) and CLSM (Figures 2; 3D–F; 4D–E; 5D–F; and 6B–E) at different magnifications (10×, 20×) with high resolution and no observable quality loss.

Another feature of this mounting technique, compared to other mounting reagents (e.g. the hydrophobic resin Euparal), is the possibility to easily remove and remount the material without risking additional dehydration (Brandham 1970). This allowed additional treatments and analyses, such as reposition of the sample or application of subsequent staining techniques. In addition to stepwise microscopic investigations, sequential molecular analyses are potentially applicable (in this case the use of any additional fixative, like formaldehyde, has to be avoided).

For longer time preservation, the additional use of formaldehyde as component within the mounting medium seemed to be suitable, e.g. by avoiding fungal growth inside the samples. We observed, that that samples preserved with formaldehyde, and stored in the dark, maintained their natural coloration over a long time (months to years).

Preservation of mesoalgal structures and study of mesoalgal complexity (all objects)

The forms and shapes of different mesoalgae were perfectly preserved and became clearly visible under the microscope. In addition to fine filamentous macroalgal thalli and minute germlings (Figure 3), fragile fertile parts (e.g. carposporophytes; Figure 6), were well conserved, which allowed subsequent species identification. For the microalgae, diatom frustules (Figures 4D, 5F), as well as trichomes and sheaths of cyanobacteria became clearly visible, allowing the study of single filaments within the tangled algal mass (Figure 5). Although

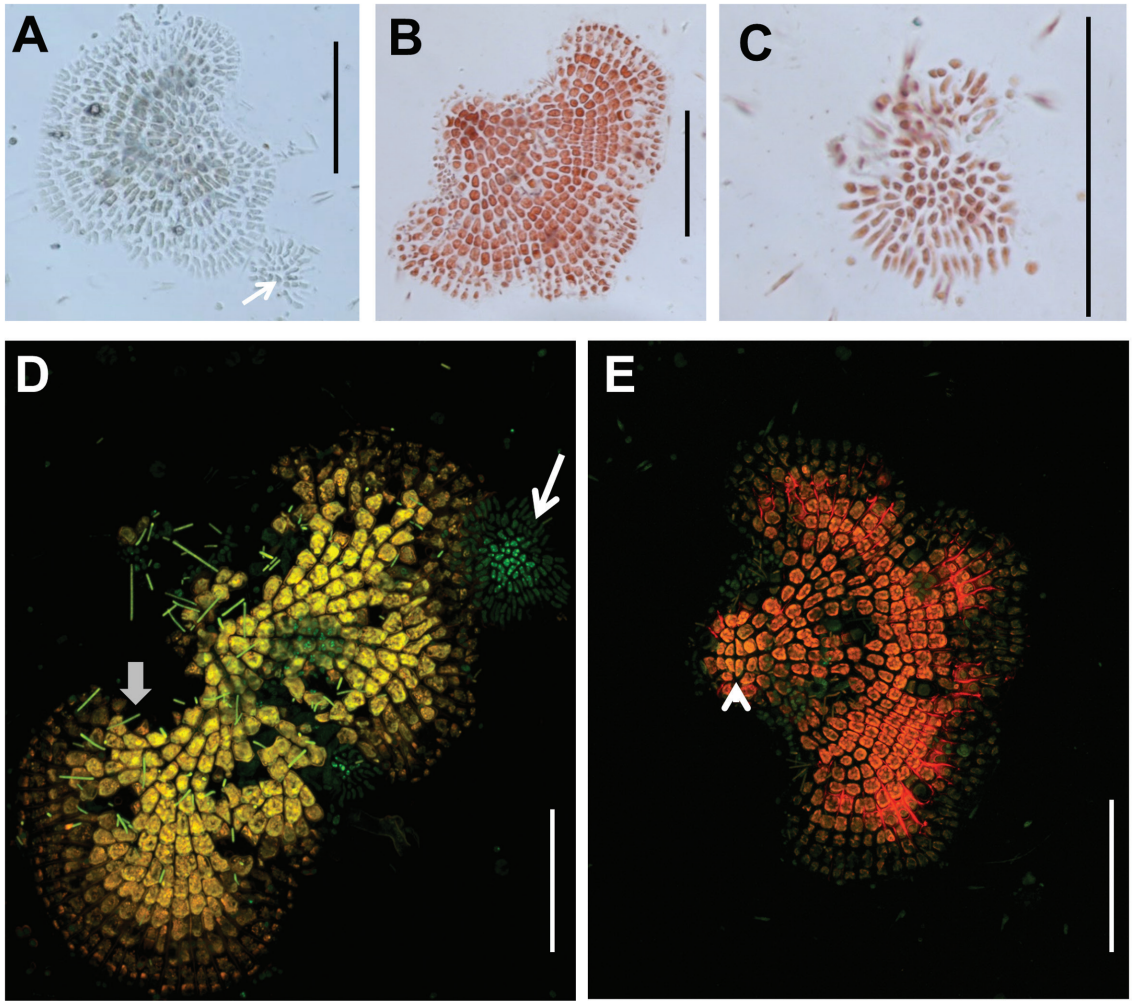

Figure 4: Crustose mesoalgae: *Hydrolithon farinosum* (J.V. Lamouroux) Penrose and Y.M. Chamberlain 1993 and *Ulvella* sp. Images show thalli of *H. farinosum* (A–E, arrowhead in E indicates position of characteristic four-celled initials), and *Ulvella* sp. (A, C, D, thallus positions in A and D marked with white arrows in mixed samples). Algal thalli are partly stained with Congo Red (B, C, E) and were investigated under light (A–C) and confocal laser scanning microscopy (D–E), excited at 561 nm and 633nm (for details see Materials and methods section), with emission detected at 578–609 nm (~ PE fluorescence) and 659–686 nm (~ Chl a fluorescence). Gray arrow in D indicates position of stick-like epiphytic diatoms. Scale bars = 100 µm.

the mounting procedure may slightly affect the community structure, e.g. bending long upright filaments, the viscous liquid not only preserved the shape of different mesoalgal components (e.g. macroalgal thalli), but also conserved interspecific relationships, e.g. positions of epiphytic attachments (Figures 4D, 5C) and the shape of microalgal colonies (Figure 3D–F). Since extracellular polymeric substances remained transparent in this study, the shape of tube-dwelling diatom colonies became clearly visible (Figure 3A, D–F), allowing an investigation of their direct relationship to other mesoalgal components. Consequently the applied mounting technique allowed the investigation of micro- and macroalgal growth forms and thus of the microstructure of the whole phytobenthic community (Figures 2–4).

Staining procedure (all objects)

Staining with Congo Red increased the resolution, by providing additional information on cell walls and frustule structures in most of the observed cases (Figures 3C, E–F; 4B–C E; and 5C–E). As well as macroalgal cell walls, cellulose-free diatom colonies appeared more clearly after the staining procedure. Interestingly, cyanobacteria-dominated communities showed lower fluorescence

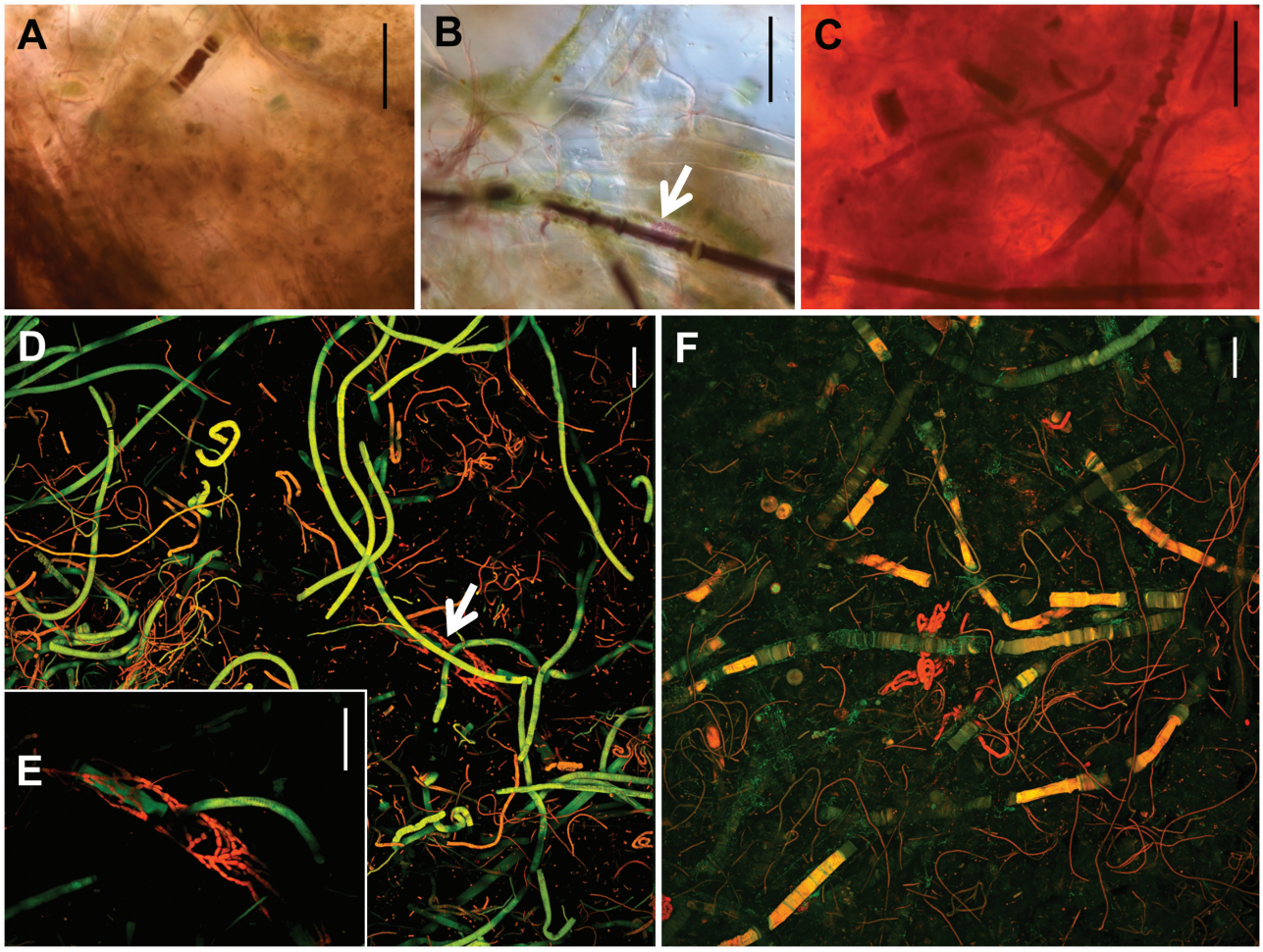

Figure 5: Cyanobacterial mat.
A mesoalgal community dominated by cyanobacteria of the genera *Lyngbya*, *Phormidium* and *Oscillatoria*, investigated under light microscopy (A–C), partly stained with Congo Red (C), and under confocal laser scanning microscopy, excited at 561 nm and 633 nm, showing natural autofluorescence (D, E) and combined Congo Red fluorescence signals (F). E. Detail of epiphytized sheath of *Lyngbya* sp., emphasized by optical cut, removing superimposed filaments. Positions of epiphytes in B and D are indicated by arrows. Scale bars = 100 µm.

intensities after the staining procedure (Figure 5D versus 5F). This bleaching might be correlated with a leakage of the dominant water soluble phycobiliproteins during the remounting and staining procedure, and seemed to affect the quality of analyses of stained material by losing natural fluorescence signals.

Study of shaded surfaces (Object 4)

The shaded surface of the calcareous thallus of *Jania rubens* (Object 4; Figure 6A) became clearly observable under CLSM (Figure 6B–C). Shapes of the surface cells, as well as structures and attachment positions of epiphytic algae, like the filamentous *Polysiphonia* sp., became visible (Figure 6C). Furthermore the three-dimensional character of the CLSM images increased the resolution of the fluorescent surfaces, which allowed the study of different mesoalgal components under different angles.

Optical separation and enhancement via pigment composition (Object 1)

Combining different emission ranges (Ch.1: 578–609 nm ~PE fluorescence, and Ch.2: 659–686 nm ~Chl a fluorescence), a detailed image of both green and red algal thalli was achieved (Figure 2A). The spectral differentiation between the algal thalli, based on the presence of PE was also successful. This optical separation was obtained considering each emission range separately; signals measured only with Ch.1 (PE fluorescence) resulted in the

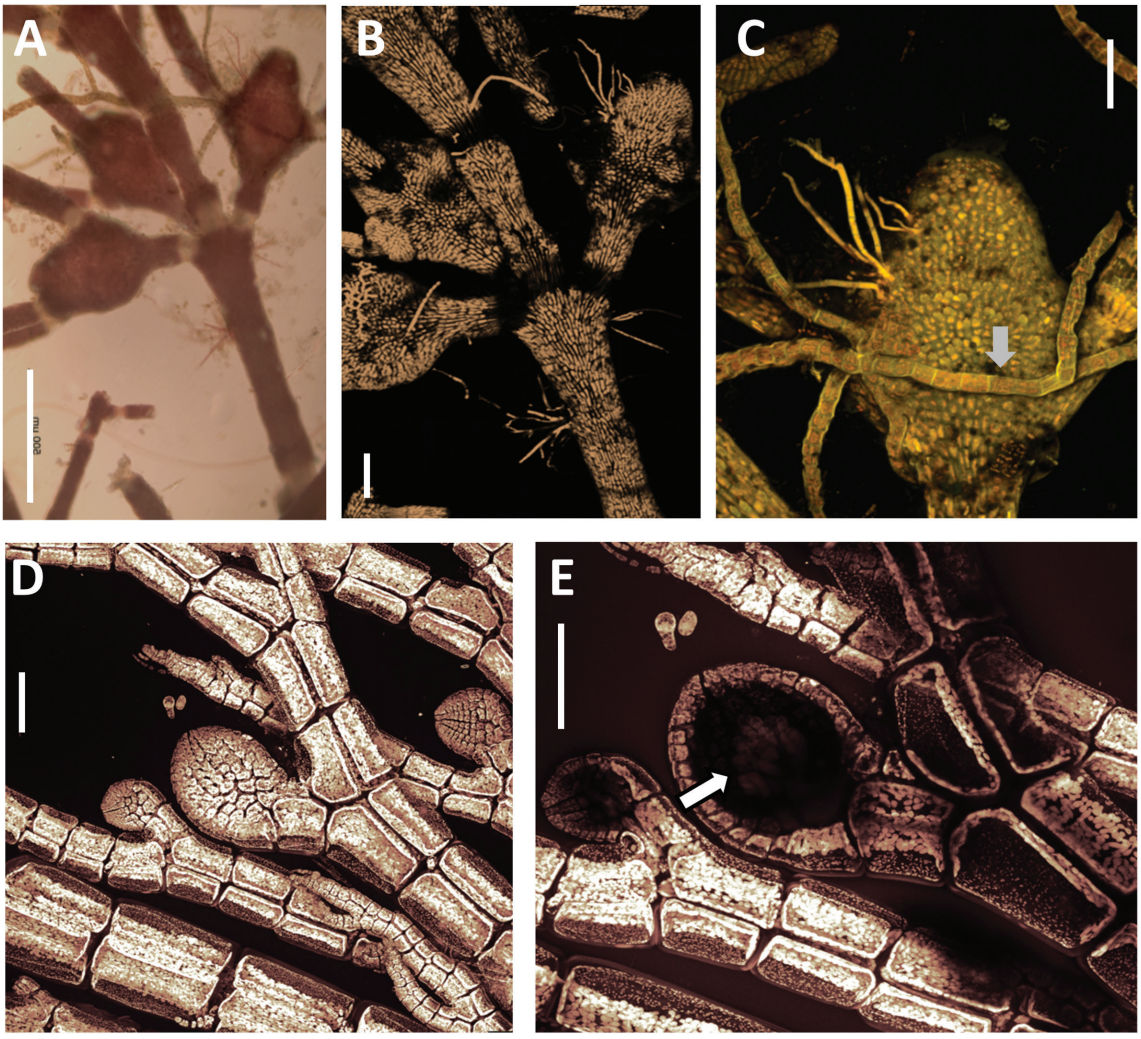

Figure 6: Macroalgal thallus structures of *Jania rubens* (Linnaeus) J.V. Lamouroux 1816 and *Neosiphonia* aff. *harveyi* (Bailey) M.-S. Kim, H.-G. Choi, Guiry and G.W. Saunders 2001.
(A–C) *Jania rubens* under light microscopy (A) and confocal laser scanning microscopy (CLSM), excited by 561 nm and 633 nm lasers, showing natural autofluorescence (B) and combined Congo Red fluorescence signals (C); gray arrow indicates position of an epiphytic *Polysiphonia* sp. (D–E) Fertile branch of female gametophyte of *Neosiphonia* aff. *harveyi* investigated by CLSM showing combined natural auto-fluorescence and Congo Red fluorescence signals. (E) Optical cut through carposporangium (removing pericarp) with position of carpospores indicated by white arrow. Scale bars = 100 µm.

spectral disappearance of the PE-free green alga *Ulvella* sp. (Figure 2D–E).

Measurement of relative abundances (Object 1)

The application of the Image J software to the maximum projections taken by CLSM allowed the calculation of relative abundances, measured as surface area of the different taxonomic groups, red algae and other photosynthetic taxa (Object 1). Based on the pigments we calculated total areas (relative abundances) for *Hydrolithon farinosum* as 1769 µm^2 compared with 1778 µm^2 for *Ulvella* sp. and other (non-PE containing) taxa.

Study of hidden structures via optical dissection (Object 2 and Object 5)

Using the optical dissection option, cropping out selected areas from the scanned sections, the investigation of inner morphological structures became possible, e.g. position and size of carposporangia within the mature pericarp of *Neosiphonia harveyi* (Figure 6E, Object 5). Also hidden taxa, like an unknown epiphyte covering a sheath of

Table 3: Material and settings for investigations of mesoalgal material with confocal scanning laser microscopy (CLSM).

Figure no.	Material	Congo Red as stain	Objective/numerical aperture	Immersed in	Microscopy	Excitation wavelength (nm)/laser power (%)	Excitation beam splitter	Detected emission wavelength (nm)	Detector gain (V)/ amplitude offset (%)	Electronic zoom	Pinhole aperture (µm)
2A	*Hydrolithon farinosum* (J.V. Lamouroux) Penrose and Y.M. Chamberlain 1993, *Ulvella* sp.	Yes	40×/0.75	Air	CLSM	561/69 633/50	TD 488/561/633	Ch1: 578–609 Ch2: 659–686	Ch1: 675/– 4.9 Ch2: 661/ – 2.0	1.0×	113.2
2B	*H. farinosum*, *Ulvella* sp.	Yes	40×/0.75	Air	CLSM	633/50	TD 488/561/633	Ch: 659–686	Ch:661/– 2.0	1.0×	113.2
2D	*H. farinosum*	Yes	40×/0.75	Air	CLSM	561/69	TD 488/561/633	Ch: 578–609	Ch: 675/– 4.9	1.0×	113.2
3A	Mesoalgae	No	10×	Air	LM						
3B	Mesoalgae	No	10×	Air	LM						
3C	Mesoalgae	Yes	20×	Air	LM						
3D	Mesoalgae	No	10×/0.4	Air	CLSM	488/87 514/91 561/85	DD 488/561	Ch1: 528–553 Ch2: 665–717	Ch1: 859/– 10.1 Ch2: 657/– 5.1	2.0×	53.0
3E	Mesoalgae	Yes	20×/0.7	Oil	CLSM	561/68 633/10	TD 488/561/633	Ch1: 578–609 Ch2: 659–686	Ch1: 647/– 4.2 Ch2: 604/ 0.0	1.0×	60.6
3F	Mesoalgae	Yes	20×/0.7	Oil	CLSM	561/68 633/45	TD 488/561/633	Ch1: 575–609 Ch2: 665–712	Ch1: 667/– 4.2 Ch2: 487/– 0.9	1.0×	59.9
4A	*H. farinosum*, *Ulvella* sp.	No	20×	Air	LM						
4B	*H. farinosum*	Yes	20×	Air	LM						
4C	*Ulvella* sp.	Yes	20×	Air	LM						
4D	*H. farinosum*, *Ulvella* sp.	No	40×/0.75	Air	CLSM	561/90 633/10	TD 488/561/633	Ch1: 578–609 Ch2: 659–686	Ch1: 494/– 0.7 Ch2: 586/– 0.5	1.0×	113.1
4E	*H. farinosum*	Yes	40×/0.75	Air	CLSM	561/69 633/50	TD 488/561/633	Ch1: 578–609 Ch2: 659–686	Ch1: 714/– 4.9 Ch2: 650/– 0.7	1.0×	113.2
5A	Cyanobacterial mat	No	10×	Air	LM						
5B	Cyanobacterial mat	No	10×	Air	LM						
5C	Cyanobacterial mat	Yes	10×	Air	LM						
5D	Cyanobacterial mat	No	10×/0.4	Air	CLSM	561/68 633/40	TD 488/561/633	Ch1: 578–609 Ch2: 659–686	Ch1: 451/– 5.9 Ch2: 449/– 1.7	1.0×	53.0
5E	Epiphyte on *Lyngbya* sp.	No	10×/0.4	Air	CLSM	561/68 633/40	TD 488/561/633	Ch1: 578–609 Ch2: 659–686	Ch1: 451/– 5.9 Ch2: 449/– 1.7	1.0×	53.0
5F	Cyanobacterial mat	Yes	10×/0.4	Air	CLSM	561/68 633/40	TD 488/561/633	Ch1: 578–609 Ch2: 659–686	Ch1: 670/– 4.2 Ch2: 574/0.0	1.0×	53.0
6A	*Jania rubens* (Linnaeus) J.V. Lamouroux 1816	No	10×	Air	LM						
6B	*J. rubens*	No	10×/0.4	Air	CLSM	514/35 633/33	TD 488/561/633	Ch1: 527–600 Ch2: 650–750	Ch1: 815/– 2.5 Ch2: 505/– 2.5	1.0×	53.0
6C	*J. rubens*	Yes	20×/0.7	Oil	CLSM	561/80 633/30	TD 488/561/633	Ch1: 578–609 Ch2: 659–712	Ch1: 733/– 4.2 Ch2: 681/– 0.9	2.0×	53.0
6D	*Neosiphonia* aff. *harveyi* (Bailey) M.-S. Kim, H.-G. Choi, Guiry and G.W. Saunders 2001	Yes	20×/0.7	Air	CLSM	561/68 633/45	TD 488/561/633	Ch1: 578–609 Ch2: 659–890	Ch1: 676/– 4.2 Ch2: 6301/– 0.9	1.0×	64.2
6E	*N.* aff. *harveyi*	Yes	20×/0.7	Oil	CLSM	561/68 633/49	TD 488/561/633	Ch1: 578–609 Ch2: 659–890	Ch1: 733/– 4.2 Ch2: 681/– 0.9	1.6×	61.5

The table lists different types of algal material (material) illustrated in different figures (figure numbers), and whether stained with Congo Red before investigation (Congo Red as stain), as well as the magnification (objective/numerical aperture), type of objective (immersed in) and microscopy (CLSM and light microscopy, LM). Information on CLSM settings, including excitation beam splitter, detected emission wavelength, detector gain/amplitude offset, electronic zoom and pinhole aperture are given for each object investigated.

Lyngbya sp. was easily emphasized by optical removal of the covering filaments (Figure 5D–E, Object 2).

Discussion

The present study provides a powerful, easy-to-handle protocol to study different aspects of mesoalgal communities. The combination of the use of artificial settlement substrates, LM and CLSM provides a non-destructive way to study the structure and composition of naturally grown, entire communities. The use of CLSM allowed the study of structural details of different substrates, e.g. transparent PET as well as shaded calcareous thalli. Consequently, CLSM can be used to study mesoalgae independently of the type of natural growth substrata. This is a big advantage, as the traditional approach based on light microscopy, often requires the removal (e.g. by scraping) and thus the structural destruction of the targeted algal community (Filho et al. 2006, Cetz-Navarro et al. 2015)

In general the separation of different taxa, according to their pigment composition is a common approach used in different ecological studies. For example the HPLC based Chemtax approach, developed by Mackey et al. (1996) with the aim to calculate algal class abundances based on photosynthetic pigment concentrations, has been applied to study planktonic, and more recently also epibenthic, algal communities (e.g. Irigoien et al. 2004, Lauridsen et al. 2011). Also the use of CLSM allows "spectral fingerprinting", as shown for different microalga taxa (i.e. cyanobacteria, green algae, and diatoms) (Larson and Passy 2005) and for mesoalgae in the present study. Here, *Hydrolithon farinosum* was easily separated from the similarly shaped *Ulvella* sp. due to the fluorometric signal of its PE content. As shown in our study, a laser based fluorometric approach can help not only to rapidly distinguish minute mesoalgal taxa, but also to measure the abundance of the algal taxa present, allowing quick identification and quantification of the algal biomass. A more applied approach based on pigment markers might become of interest for the growing aquaculture industry, where the minute (1–5 mm) green alga *Ulvella lens* has been grown in mass cultures since the 1980s to stimulate the settlement of commercially cultivated abalone and sea urchins (Hannon et al. 2014). A pigment based identification and separation might help to control the monoculture and avoid contamination. Pigments other than PE could be investigated to serve as taxa-specific spectral markers.

In addition to natural autofluorescence signals, the use of artificial stains can provide additional information. In our study, the polysaccharide-sensitive, negatively charged Congo Red (Langille et al. 2000) not only stained the cellulose cell walls of macroalgae, but also colored parts of the extracellular polymeric substances (EPS) produced by biofilm microorganisms, e.g. diatoms. The possible presence of chitin fibers, recently observed in diatom frustules (Brunner et al. 2009), might increase the fluorescence signal. With regard to the observed high taxonomic diversity within mesoalgal assemblages (e.g. Fricke et al. 2011a,b), the application of additional and complementary dyes could increase the specific study of mesoalgal communities in the future.

As shown in the present study, our approach allows a detailed investigation of macroalgal thallus structures and microalgal colony forms. It provides insights into the organization of individual taxa, as well as the functional relations between the different mesoalgal components. For example, we observed potential epiphytic relations (e.g. attachment points and positions of epiphytes) and were able to investigate the shape and structure of shaded surfaces. Furthermore, as shown in two examples, optical sections through algal material using CLSM provide a powerful tool, which might be of interest not only in further studies of epiphytes but also for endophytic or parasitic studies. To date these kind of relations are mainly studied by transmission electron microscopy, a microscope technique requiring intensive sample preparation (including different dehydration steps, embedding and sectioning; Mukhopadhyay 2003). A less time- and cost-intensive CLSM approach might therefore provide a valuable complement in this field. Furthermore the non-destructive CLSM approach might even be applicable to living algal material. This could open up new perspectives and allow more interdisciplinary research, e.g. observing changes in endophytic parasite structures during algal morphogenesis and growth.

Overall, algal interactions play a crucial role in benthic succession processes, since early settlers may facilitate or inhibit the recruitment of other colonizers and consequently shape the whole benthic ecosystem (Connell and Slatyer 1977). Knowledge of species composition and ecological relations is crucial to identify disturbances in the environment at an early stage. For example, the influx of elevated nutrient levels can cause drastic changes in the coastal system (Valiela et al. 1997, Teichberg et al. 2010), including the formation of oxygen minima, the so-called dead zones, driven by bacterial degradation of organic material originating from algal blooms (Diaz and Rosenberg 2008). Benthic algae have been proven to be highly sensitive to alterations in nutrient levels and can serve as valuable early indicators for environmental changes (Neto

et al. 2010, Fricke et al. 2016). The detection of indicative and/or potential bloom forming taxa at an early stage might therefore play a crucial role in developing an appropriate management plan for the affected area. As shown in our study a CLSM supported approach can meet the early detection needs by overcoming substrate related shading issues and providing detailed three-dimensional analyses of macro- and microalgal composition and structures. Samples of rapidly settling mesoalgae can be randomly taken from the investigated area, including from a wide variety of different substrates, to investigate and monitor the current and developing environmental situation. Furthermore, as shown in our study, CLSM can help not only to investigate early successional stages, but also to unravel the composition of conspicuous mesoalgal communities (algal mats), often formed in response to eutrophication events (Littler et al. 2010). These dense communities can be composed of a variety of different taxa (e.g. filamentous algae, cyanobacteria, diatoms, dinoflagellates), entangled with their filamentous thalli or colony structures (Lobban 1989, Mangialajo et al. 2008, Mejean et al. 2010, Fricke et al. 2016). Our approach may not only help to quickly unravel their taxonomic components, but also provide insights into the structural complexity of the entangled mass and emphasize hidden structures, like inconspicuous epiphytic taxa, as shown here with the optical crop method.

Overall, by defining mesoalgae as an independent ecological group and providing a methodological approach, that allows a detailed observation of their structure and composition, the present study establishes a baseline to facilitate the study of these complex communities in the future.

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Bionotes

Anna Fricke
Instituto Argentino de Oceanografía (IADO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Florida 4750, Bahía Blanca, B8000FWB, Argentina; and Leibniz-Center for Tropical Marine Ecology (ZMT), Fahrenheitstrasse 6, 28359 Bremen, Germany,
Annafricke@gmx.de

Anna Fricke is a researcher at the Instituto Argentino de Oceanografía (IADO, Argentina) and guest researcher at the Leibniz Center for Tropical Marine Ecology (Germany). She was awarded a PhD in Natural Sciences jointly by the University of Bremen and the Leibniz Center for Tropical Marine Ecology (Germany) for her work on succession patterns of tropical turf algae, and was enrolled in International Graduate School for Marine Sciences "Global Change in the Marine Realm" (GLOMAR, MARUM Bremen). In her research she addresses the biodiversity and ecophysiology of benthic algal communities at different latitudes, ranging from descriptive to experimental studies in relation to environmental changes.

Terue C. Kihara
Senckenberg am Meer, German Centre for Marine Biodiversity Research (DZMB), Südstrand 44, 26382 Wilhelmshaven, Germany

Terue C. Kihara is a researcher at the German Center for Marine Biodiversity Research, Senckenberg am Meer. She holds a PhD in Bioscience at Department of Zoology at Universidade de São Paulo and has research background is in scanning electron and confocal laser scanning microscopy. She is currently working in many projects involving meio- and macrofauna imaging combined to the investigation of the fauna from the Indian Ocean hydrothermal vents.

Mona Hoppenrath
Senckenberg am Meer, German Centre for Marine Biodiversity Research (DZMB), Südstrand 44, 26382 Wilhelmshaven, Germany

Mona Hoppenrath is head of the Division of Marine Botany at the German Center for Marine Biodiversity Research and a private lecturer at the Carl von Ossietzky University Oldenburg. She studied Biology in Göttingen and received her doctorate in Hamburg in 2000 (on a thesis about the taxonomy and ecology of marine sand-dwelling flagellates). As a post-doc she worked on phytoplankton taxonomy at the Biological Institute at Helgoland and the Wadden Sea Station Sylt of the Alfred Wegener Institute, Germany until transferring to the University of British Columbia, Canada in 2004. Her research focusses on the taxonomy, systematics and phylogeny of dinoflagellates.

Graphical abstract

Anna Fricke, Terue C. Kihara and Mona Hoppenrath

Studying mesoalgal structures: a non-destructive approach based on confocal laser scanning microscopy

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Research article: Mesoalgae are an intermediate sized highly diverse algae group forming macroscopically visible structures that cannot be identified at generic level without microscopy and their investigation requires combined ecological and microbiological techniques, as demonstrated in the present study.

Keywords: coralline crustose algae; filamentous cyanobacteria; macroalgal germlings; tube-dwelling diatoms; turf algae.