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Parallelisable non-invasive biomass, fitness and growth measurement of macroalgae and other protists with nephelometry

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3 **Short title: Nephelometry for algal and protistan phenomics**
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121 **Abstract**
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124 With the exponential development of algal aquaculture and blue biotechnology, there is a strong
125 demand for simple, inexpensive, high-throughput, quantitative phenotyping assays to measure the
126 biomass, growth and fertility of algae and other marine protists. Here, we validate nephelometry, a
127 method that relies on measuring the scattering of light by particles in suspension, as a non-invasive
128 tool to measure in real-time the biomass of aquatic micro-organisms, such as microalgae, filamentous
129 algae, as well as non-photosynthetic protists. Nephelometry is equally applicable to optic density and
130 chlorophyll fluorescence measurements for the quantification of some microalgae, but outperforms
131 other spectroscopy methods to quantify the biomass of biofilm-forming and filamentous algae, highly
132 pigmented species and non-photosynthetic eukaryotes. Thanks to its insensitivity to the sample's
133 pigmentation, nephelometry is also the method of choice when chlorophyll content varies between
134 samples or time points, for example due to abiotic stress or pathogen infection. As examples, we
135 illustrate how nephelometry can be combined with fluorometry or image analysis to monitor the
136 quantity and time-course of spore release in fertile kelps or the progression of symptoms in diseased
137 algal cultures.
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155 **Keywords**
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159 algal cultivation; nephelometry; biomass; phenotyping; biotechnology
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1. Introduction

Underpinned by strong economic and political drivers such as food safety, blue biotechnology and transition to a low carbon economy, macroalgal cultivation is the fastest growing of all aquaculture sectors worldwide, with a sustained exponential growth attaining almost 10% in value annually. Beyond their traditional use as sea vegetables, macroalgae are increasingly used as animal feed and for hydrocolloids, biofuels, bioplastics and pharmaceuticals [1, 2]. Over the last few years, genomic resources for marine macroalgae have been established, especially for the filamentous brown alga *Ectocarpus siliculosus* [3], the carrageenophyte *Chondrus crispus* [4] and more recently, the kelp *Saccharina japonica* [5]. First initiated in China in the 1950s, industrial macroalgal breeding led to the development of kelp cultivars with increased yield and iodine content ([6] and references included). Over the last decades, seaweed aquaculture has rapidly spread to most continents, leading to the ongoing rapid domestication of several dozen species [7]. Cultivar development however remains largely empirical [8]. For the vast majority of species, wild genetic resources are at best poorly characterised, and their exploitation is therefore very limited [9]. Thanks to second generation sequencing technologies, characterising this diversity is now technically within reach and accordingly, demand for marker-assisted breeding tools is booming [e.g. 10]. However, the implementation of quantitative trait loci (QTL) or genome-wide association studies (GWAS) is slowed down by the limited availability of effective high throughput, quantitative phenotyping methods for traits of interest.

In land agriculture, phenomics is now a well-established field that underpins selection and breeding; high throughput phenotyping facilities have become mainstream and typically combine, parallelise and automate various hyperspectral or temporally-resolved imaging techniques. In aquatic sciences, lab-based high-throughput quantitative phenotyping is to some extent available thanks to sizing and sorting technologies such as Coulter counters and flow cytometers [11], coupled to image analysis (e.g. FlowCam). Thus far, unicellular microalgae have typically been models of choice whenever large-scale phenotyping efforts are required, such as for conducting genetic screens [e.g. 12]. In sharp contrast, high-throughput phenotyping tools for multicellular aquatic organisms, including

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238
239 macroalgae, are very much lagging behind, including in the submillimetric to centrimetric size range.
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241 In particular, due to the highly hygroscopic nature of macroalgal cell walls, there is no simple way to
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243 accurately measure the fresh weight, let alone to follow the growth of seaweed non-invasively. As a
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245 result, total chlorophyll fluorescence (or parameters such as F_0 , the minimal chlorophyll fluorescence
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247 under non-actinic light) and spectrophotometry are often used as proxies for measuring the biomass
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249 of algae (Fig. 1A-B). However, fluorescence measurements are inherently sensitive to variations in
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251 chlorophyll content linked to environmental factors such as stress, nutrient supply or light conditions,
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253 i.e. the very variables that are typically most relevant to breeders and ecologists alike.
254
255 Spectrophotometry is equally unsatisfactory for measuring thick, opaque samples such as seaweed
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257 tissue and is sensitive to the sample pigmentation, a limitation particularly relevant when biomass
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259 measurements are needed in the context of disease monitoring.
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262 In flow cytometers, the measurement of the light scattered by particles in suspension is widely used
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264 to assess the size and biomass of live cells [e.g. 13], yet such measurements can only be conducted on
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266 single cells with a maximum diameter of ca. 30 μm . Nephelometers (Fig. 1C) also rely on the
267
268 measurement of scattered light and are commonly used in aquatic ecology to measure water turbidity
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270 (i.e. combined plankton and sediment content). In chemistry, another application is to follow in real
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272 time the solubilisation of fine particles or analyse ligand-binding responses, for example in
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274 immunoassays. More anecdotally, the radiation properties of microalgae have been explored with
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276 nephelometry, within the context of optimising photobioreactor design [14]. Recently, we
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278 demonstrated the applicability of nephelometry to monitor the growth of filamentous
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280 phytopathogenic fungi such as *Botrytis*, *Aspergillus* and *Alternaria* [15-17]. The potential of this
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282 technique for quantifying algal biomass was already recognised over forty years ago [18]. However,
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284 the instruments available at that time only allowed to measurement of one sample at a time and with
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286 a fairly low sensitivity, hence the technique was never widely adopted. Nowadays, significant design
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288 improvements have increased the sensitivity of nephelometers and reduced the variability of
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290 measurements and the availability of microplate readers allows for the necessary the automation and
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298 replication required for medium- to high-throughput analyses. Here, we assessed the applicability of
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300 using a microplate-format nephelometer to monitor in real time and non-invasively the biomass of
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302 algae and other aquatic protists.
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306 307 **2. Materials & Methods**

308 309 **2.1. Strains and cultivation media**

310 Clonal partheno-sporophytes of the fully sequenced, male *Ectocarpus siliculosus* strain CCAP 1310/4,
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312 clonal female gametophytes of the *Macrocystis pyrifera* strain CCAP 1323/1 and the obligate biotroph
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314 oomycete *Anisolpidium ectocarpii* strain CCAP 4001/1 were maintained as previously described at
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316 15°C, under a 12:12 light:dark photoperiod and low white light intensity of 2 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ [19,
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318 20]. These low light conditions are optimised to facilitate the maintenance of the pathogen. Whereas
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320 healthy *E. siliculosus* and *M. pyrifera* both grow at such low light levels, their development is much
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322 faster under an irradiance of 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Cultures were transferred into fresh half-strength
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324 Provasoli enriched seawater medium every 3 weeks and monitored on a weekly basis by microscopy.
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326 Whenever homogeneity of the biological material was paramount (e.g. for serial dilutions), the algal
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328 filaments (*E. siliculosus*, *M. pyrifera*) were finely disrupted with a sterile Potter tissue grinder, and
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330 allowed to regenerate for 2-3 weeks before being measured.
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334 The microalgae *Haematococcus pluvialis*, *Micromonas* sp. (RCC3510), *Emiliania huxleyi* (RCC3553),
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336 *Phaeocystis* sp., *Alexandrium minutum* (RCC1490), *Guinardia flaccida* (RCC3088) were kindly gifted by
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338 the Roscoff Culture Collection. The axenic *Paraphysoderma sedebokerense* strain PS1 was sub-
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340 cultured every 3-4 weeks to fresh liquid chytrid growth medium as detailed in Strittmatter *et al.* [21].
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342 A monoekaryotic chytrid strain of *Zygorhizidium effluens* isolated during a bloom of its freshwater
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344 diatom host, *Asterionella formosa*, was grown in diatom medium, at 20°C under a 12h photoperiod
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346 and irradiance of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ [22]. A suspension of chytrid zoospores was obtained by filtration of
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348 an infected culture on a 10 μm pore size nylon membrane. The calibration curves obtained by serial
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350 dilution in fresh or seawater, as appropriate (biological replicate, n=3).
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357 Two *Saccharina latissima* unfertile sporophytes (named A and B) were sampled in the port of Roscoff
358 (France) and 28x14cm pieces of the blades were cultivated under controlled conditions (8:16
359 light:dark photoperiod; flowing filtered seawater at 13°C) until the appearance of fertile sori occurred.
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361 To trigger spores release, two fragments (0.5x3cm) of sorus were cut with a scalpel from each of the
362 two sporophytes and allowed to air-dry for 8 hours in the dark at 13°C. The fragments were then
363 carefully bent and disposed vertically against the inner side of individual wells in a 24-well plate,
364 outside the central area measured by the nephelometer and submerged in 2 mL of filtered and
365 sterilised seawater (inset on Fig. 8D).
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376 **2.2. Nephelometric measurements and data analysis**

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378 Sterile 96 UV-star, 48 and 24 cell culture Greiner microplates were filled with 300 µL, 1 mL and 2 mL
379 samples, respectively. Nephelometric measurements were recorded with a NEPHELOstar Omega
380 (BMG Labtech, Offenburg) reader equipped with a 635 nm laser. Each well was measured for 0.1 s
381 with a laser beam focus of 2.5 mm and 80% laser intensity.
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386 Filaments and clumps of *E. siliculosus*, *M. pyrifera*, *H. pluvialis*, *P. sedebokerense*, *P. littoralis* data were
387 measured using a well-scan protocol and a 5x5 pixel grid covering 5 mm in diameter (48-well
388 microplates) or a 7x7 pixel grid covering 10 mm diameter (24-well microplates). Spores of *S. latissima*
389 and other microalgae were measured using an endpoint protocol. The time-course of *S. latissima*
390 spore release was recorded using a time-course of endpoint (plate mode) protocol, during which the
391 24-well plates were subjected to shaking at 175 rpm for 5 min every 10 min.
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398 To check for the possible presence and subsequently control for any artefact caused by a meniscus in
399 the nephelometric well scans, the data analysis was conducted on the average measurement recorded
400 in a centred disc of 3.5 mm diameter using the corresponding function in the Mars software (BMG
401 Labtech, Offenburg). CSV files were exported and further processed in Microsoft Excel and R [23].
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416 corresponding mean values were subtracted from all measurements. For time courses, an initial
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418 relative nephelometric unit (RNU) value was calculated as the mean of the initial three measurements
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420 and then subtracted from each curve value as described by Joubert *et al.* [[15](#)].
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423 424 425 **2.3. Spectrophotometry and Fluorometry**

426 For comparative purposes, absorbance and fluorescence data were recorded on the samples used in
427
428 nephelometry using a SPECTROstar Omega (BMG Labtech, Offenburg). The recording wavelength (450
429
430 nm or rarely, 250 nm) was selected to give the most sensitive measurements, depending on the
431
432 species used. Chlorophyll fluorescence was recorded using a 12 nm band pass excitation filter, centred
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434 on 485 nm, and a 50 nm band pass emission filter, centred on 655 nm. Unless specified otherwise, all
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436 experiments were performed on at least three (and up to six) biological replicates, each composed of
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438 3 technical replicates.
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441 442 443 **2.4. Dry weight measurements**

444 A serial dilution of *E. siliculosus* tufts (three technical replicates per concentration) was analysed using
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446 nephelometry, spectrophotometry and fluorometry before dry weight was determined. Due to the
447
448 small amounts of biomass used, the *E. siliculosus* fragments contained in the three technical replicates
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450 were pooled, oven-dried overnight and then weighed three times with a precision scale (Sartorius,
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452 Secura 225D-1S, d=0.00001g).
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456 457 458 **2.5. Image analysis**

459 The reproductive effort of kelp sporophytes was evaluated as a function of the percent blade area
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461 occupied by sori (De Wreede & Klinger, 1988). The blade fragments were photographed; the fertile
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463 area (slightly swollen dark spots) showed the highest contrast with the vegetative part of the thallus
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465 in the red frame of the RGB image, which was thus used for analysis. The red frame was segmented
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467 as described in Rousseau *et al.* [[24](#)]: briefly, the distribution of pixel intensity was plotted for a
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473 manually-identified unfertile area, in order to determine the signal intensity threshold under which
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475 the red channel intensity could be assigned to a sorus; we determined that setting the threshold to
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477 the maximum red channel intensity of the lower 2nd percentile of vegetative pixels gave best results.
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479 All images were then processed and split using this threshold, with all pixels automatically split
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481 between the dark (fertile) and bright (vegetative) areas.
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488 **2.6. Microscopy**

489 Algal material was observed directly in the multiwell plates with an AxioObserver (Zeiss) microscope,
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491 using differential interference contrast and phase contrast. For epifluorescence, culture aliquots were
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493 mounted on microscope slides. Photographs were taken using an AxioCam HRC (Zeiss) and processed
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495 with Axiovision software (Zeiss, version 4.7 or 4.8).
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502 **3. Results**

503 **3.1. Comparison of fluorometry, spectrophotometry and nephelometry for non-invasive** 504 505 **quantification of algal and non-photosynthetic eukaryote biomass.** 506 507

508 The performance of nephelometry, fluorometry and optical density to measure the biomass of micro-
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510 and macroalgae and non-photosynthetic eukaryotes was benchmarked against dry weight
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512 measurement. The excitation (12 nm band pass, centred on 485 nm) and emission (50 nm band pass,
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514 centred on 655 nm) wavelengths of the fluorimeter were chosen to capture a proxy of the total
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516 chlorophyll content.
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519 A sample of *E. siliculosus* filaments was measured ten times with each of the three methods in order
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521 to assess the reproducibility of measurements. The standard deviation was around 5% for the
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523 fluorimeter and nephelometer but as high as 22% with spectrophotometry, despite the fact that the
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525 recorded wavelength (440 nm) had been chosen to maximise the signal/noise ratio. Therefore, optical
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534 density (OD) appears less adapted to estimate algal biomass, at least on filamentous algae such as *E.*
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536 *siliculosus* (Fig. S1).

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538 Using a serial dilution of *E. siliculosus* filaments, we then measured the degree of agreement between
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540 dry weight, nephelometry, fluorometry and spectrophotometry measurements. As expected, dry
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542 weight measurements were highly correlated to the dilution factor applied ($r^2 = 0.9786$, Fig. 2A) and
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544 the slope of the linear regression curve had a negligible variation compared to the expected one
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546 (1.0%). However, some variability was apparent for the lowest biomass containing samples.
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548 Additionally, the length and destructiveness of the dry weight protocol make it unsuitable for
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550 phenomic applications. As judged from the linearity ($r^2 = 0.9933$ and 0.9817 , respectively) and slope
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552 deviation (2.8% and 1.0%, respectively), nephelometry and spectrophotometry performed best
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554 amongst the three light-based methods tested (Fig. 2B). Fluorometry measurements betrayed a lower
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556 sensitivity for low quantities of biological material, and thus showed some deviation from linearity (r^2
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558 = 0.9711) and proportionality (5.5%, Fig. 2C).

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560 To test the generality of the above findings, the linearity of the nephelometric, fluorometric and
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562 spectrophotometric readings was further tested on different algal and protistan species,
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564 encompassing a broad range of morphologies, mobile or biofilm-forming properties and pigment
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566 content. For each species, calibration curves were built as described in Fig. 2 and the r^2 of the resulting
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568 linear fit was plotted (Fig. 3). With r^2 values equaling at least 0.97, all three methods yielded
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570 satisfactory standard curves for a variety of microalgae (*Micromonas* sp., *Emiliana huxleyi*,
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572 *Haematococcus pluvialis*, *Alexandrium minutum* and *Guinardia flaccida*). However, the calibration
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574 curve obtained with spectrophotometry was poor for highly pigmented (*Macrocystis pyrifera*,
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576 $r^2=0.739$) and biofilm-forming (*Phaeocystis* sp., $r^2=0.927$) biological material; for these two species,
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578 fluorometry also gave slightly lower r^2 values compared to nephelometry (0.929 vs. 0.989 for *M.*
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580 *pyrifera* and 0.900 vs. 0.981 for *Phaeocystis* sp. respectively). Predictably, fluorometry was inadequate
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582 to measure the biomass of non-chlorophyllous species ($r^2 = 0.026$ and 0.076 for *Paraphysoderma*
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584 *sedebokerense* and *Zygorhizidium effluens*, respectively). In comparison, OD measurements led to a
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593 much better r^2 of 0.943 for *P. sedebokerense*, yet the spectrophotometer struggled to pick up a
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595 meaningful signal with low concentrations the *Z. effluens* spore suspensions, resulting in a poor r^2 of
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597 0.386. For both chytrid species, the best calibration curves were obtained with nephelometry, as
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599 shown by r^2 values of 0.974 (*P. sedebokerense*) and 0.963 (*Z. effluens*).
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602 603 **3.2. Non-invasive monitoring of algal growth**

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605 The growth of *E. siliculosus* filaments was followed in 48-well plates over 13 days (Fig. 4). As expected
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607 from Fig. 2, all three techniques gave measurements roughly consistent with each other, and
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609 spectrophotometry measurements were noisier. Note also that the initial fluorometric measurement
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611 was below the value of a blank sample (i.e. below the detection threshold of the machine), whereas
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613 the last time point was saturated. This illustrates how the narrower dynamic range of this technique
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615 hampers long-term monitoring of a culture, a limitation that was not encountered when
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617 nephelometry or spectrophotometry were used. Additionally, we evaluated how the three techniques
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619 discriminated between mild growth variations during hyposaline stress, while also testing the impact
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621 of physiologically-significant variations in chlorophyll content by exposing cultures to increased light
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623 levels (20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ vs. 2 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in the control; Fig. 5). At five days, nephelometry and
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625 spectrophotometry detected a significant increase in biomass in response to increased illumination
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627 compared to the standard illumination regime, with fold increases of 1.3 and 1.6. However, a strikingly
628
629 different pattern was observed with fluorometry: between the first and second day of the time course,
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631 the total fluorescence soared from below 1kRFU to 32 kRFU. Daily observation of the microplates by
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633 the experimenters ruled out the possibility that such a sudden overnight increase corresponded to a
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635 commensurate biomass increase, and was therefore attributed mainly the induction of chlorophyll
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637 synthesis due to photoacclimation [see [25](#), [26](#)]. Subsequently, the fluorescence readings remained at
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639 least one order of magnitude higher in the 20 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ condition compared to the low light control
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641 until the end of the experiment (Fig. 5B). For example, we recorded 1.8kRFU for the control and
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643 47kRFU under increased light at 5 days, suggesting far higher fold changes than those recorded by
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652 nephelometry and OD. Therefore, we conclude that the meaningful biomass changes detected with
653 spectrophotometry and nephelometry were entirely obscured in fluorometry by the co-occurring
654 photoacclimation. Taking into account the general agreement between nephelometry and
655 spectrophotometry data, the complete insensitivity of nephelometry to pigmentation (see next
656 section) and the noisiness of OD measurements (Fig. S1), we conclude that nephelometry is the most
657 accurate technique to follow non-invasively *E. siliculosus* growth over time, across different culture
658 conditions. Similarly, nephelometry proved useful to follow the growth of several macro and
659 microalgae species as well as the non-photosynthetic *P. sedebokerense*, over time periods ranging
660 from 3 days to 2 weeks (not shown).
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673 **3.3. Diagnosis and monitoring of the progression of symptoms in diseased algal cultures.**

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675 As light scattering by particles is insensitive to pigmentation, we reasoned that nephelometry should
676 be blind to the accumulation of dead algal cells in diseased cultures. To confirm this hypothesis,
677 calibration curves were obtained with *E. siliculosus* filaments before and after being killed, with 1%
678 sodium hypochlorite (i.e. a five-fold dilution of a commercial 5% bleach solution, Fig. 6). The
679 nephelometric standard curve for the dead biomass remained indistinguishable from the one
680 obtained before treatment ($p=0.62$), despite the complete loss cell content of the algal filaments (Fig.
681 6D-E); the fluorescence of bleach-treated biomass flat-lined across the whole concentration range
682 tested. With spectrophotometry, the slope of the calibration curve for the bleached samples was
683 roughly halved compared to the live control, illustrating again that OD measurements co-vary with
684 both sample biomass and pigmentation.
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696 We exploited the insensitivity of nephelometry to the physiological status of the algae to monitor
697 symptom progression in algal cultures infected by pathogens. As an example, Fig. 7A-C shows the
698 evolution of the nephelometry, fluorometry and spectrophotometry values recorded for
699 gametophytes of the Pacific kelp *M. pyrifera* inoculated with the intracellular oomycete pathogen
700 *Anisolpidium ectocarpii* CCAP 4001/1 [[see details in 20](#)]. Microscopic observation of symptoms
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711 revealed that most algal filaments were dead within three days of inoculation, which was visibly
712 accompanied by loss of chlorophyll fluorescence in infected and dead cells (Fig. 7D-E).
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714
715 Macroscopically, the infected algae exhibited a depigmentation similar to the one observed with
716 sodium hypochlorite. The arrest of algal growth in infected cultures was detected in the nephelometric
717
718 measurements over the time course, whereas the uninfected control grew steadily up until the end
719
720 of the experiment (Fig. 7A). In contrast, total chlorophyll fluorescence values went down in the
721
722 infected samples after two days (Fig. 7B). Consistent with our earlier observations, OD measurements
723
724 were noisy and difficult to interpret, because the conjugated effects of depigmentation and growth
725
726 arrest could not be disentangled (Fig. 7C). Therefore, and provided all other factors likely to affect
727
728 intracellular chlorophyll content are controlled for (e.g. light regime), normalising chlorophyll
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730 fluorescence over nephelometric measurements would be a cost-effective and non-invasive way to
731
732 monitor disease progress in defined algal pathosystems, thus providing a high-throughput alternative
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734 to scoring symptoms with microscopy.
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739 740 **3.4. Quantification of macroalgal fertility** 741

742 The life cycle of kelps alternates a diploid macroscopic sporophyte with haploid dioecious
743 gametophytes. The sporophytes produce meiospores in one specialised part of the blade called the
744 sorus. After release, the spores differentiate in either male or female gametophytes, by which
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746 fertilization give a new sporophyte. One main challenge in the study of the fertility for the diploid
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748 phase, is the estimation of the total area occupied by the sori and the average number of spores to be
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750 produced and released. To demonstrate the feasibility of fertility measurements, we evaluated the
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752 number of spores released by kelp sporophytes through the combined measurement of their fertile
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754 area with image analysis, and of the spore release by unit of surface and time, using nephelometry.
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757 Firstly, fertile sporophytes of *Saccharina latissima* were photographed in rudimentary lighting
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759 conditions, close to those that might be encountered in the field (Fig. 8A). The sori have a darker
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761 pigmentation than the vegetative parts of the thallus, allowing for a threshold to be set in order to
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770 distinguish between fertile and vegetative tissue. The picture shown in Fig. 8A was thus segmented to
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772 determine the percentage of pixels corresponding to the totality of the sporulation area (Fig. 8B); in
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774 this particular example the sorus covered 45.36% of the photographed area. Visual inspection showed
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776 that the proportion of pixels misclassified as a result of the shadows projected on the uneven surface
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778 of the kelp was negligible. Therefore, image analysis could easily be automated to measure the fertile
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780 areas of kelps, both in the laboratory and in the field.

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782 Secondly, spores were collected from fragments of *S. latissima* sori incubated overnight in the dark at
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784 200 rpm in sterile seawater. The spore suspension was calibrated with a Malassez cell, serially diluted
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786 in triplicates, and measured by nephelometry and spectrophotometry (Fig. 8C). A linear relationship
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788 between the number of *S. latissima* spores and the nephelometric units ($r^2= 0.997$) was found for
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790 concentrations ranging from 10^4 to 10^6 spores mL^{-1} . Finally, spore release was monitored in real-time
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792 using nephelometry (Fig. 8D). Fertile areas were cut with a scalpel (0.5x3cm) in duplicates, from two
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794 individuals named A and B. At the end of the 150 min measurement period, we determined that, on
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796 average, the sori of individuals A and B released $9.76 \cdot 10^5$ and $5.13 \cdot 10^5$ spores cm^{-2} , respectively.
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801 **4. Discussion**

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803 Here, we piloted nephelometry as a precise, non-invasive method to carry out biomass and growth
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805 measurements with algae and other aquatic eukaryotes. In all experiments, nephelometry always
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807 performed at least equally well and often outperformed spectrophotometry and/or fluorometry, in
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809 terms of reproducibility, response linearity, sensitivity, and dynamic range. Among the three non-
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811 invasive light-based methods tested, nephelometry and spectrophotometry came closest to
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813 destructive, end-point dry weight measurements in terms of accuracy. Our results are consistent with
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815 the findings of Chioccioli *et al.* [27], who found that spectrophotometry was less reliable and noisier
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817 than light scattering measurement in a flow cytometry assay designed to measure microalgal biomass;
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819 note however that our approach is considerably simpler than that of Chioccioli *et al.* [27]. For non-
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821 invasive monitoring of microalgae and non-photosynthetic protists alike, nephelometry offers a fast
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829 and sensitive alternative to painstaking cell counts [e.g. 28] or flow cytometry, at a fraction of the cost.
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831 In addition, the possibility to use 6-well microplates makes the technique applicable to aquatic
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833 macroorganisms up to a centrimetric size, the growth of which could thus far not easily be monitored.
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835 Using a treatment with sodium hypochlorite, we experimentally confirmed that nephelometry
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837 measurements are insensitive to sample discolouration. Therefore, we conclude that nephelometry
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839 delivers true, rapid, inexpensive and non-invasive measurements of total biomass, irrespective of the
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841 organism's pigmentation or physiological status. Note however that a potential drawback of the
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843 apparatus blindness to pigmentation is that measurements are sensitive to the presence of unwanted
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845 particles, such as contaminating microorganisms, any precipitate or sediment. Whereas the technique
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847 is suitable to monitor pure laboratory cultures, caution with data interpretation should be applied
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849 when working with species mixes or environmental samples.
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852 Nephelometry therefore offers an attractive alternative to the widely-used assays that rely on OD,
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854 total chlorophyll or F_0 measurements as a proxy for biomass [e.g. 29]. As shown here, physiologically-
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856 relevant variations in chlorophyll content, such as photoacclimation or pathogen-induced cell death,
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858 may result in fluorescence readings not being strictly proportional to biomass, in addition to being
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860 sensitive to cell aggregation [30]. Whereas fluorometry appears adequate to compare biomass
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862 variations on the short term, or to compare differences between dilutions of a single sample, our data
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864 beg into question the validity of using F_0 or chlorophyll fluorescence to follow biomass variations over
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866 several days.
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869 The microplate format of the Nephelostar reader allows easy combination with other plate readers
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871 (e.g. fluorometer), to relate sample biomass to physiological parameters (e.g. total chlorophyll
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873 fluorescence). Combined with fluorescence (total chlorophyll, or any other parameter measurable
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875 with pulse amplitude modulated fluorometry), nephelometry is therefore the method of choice to
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877 quantitatively assess the effects of abiotic stress (e.g. light regime, temperature, nutrient limitation,
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879 exposure to a pollutant) or biotic stress on micro- and macroalgae. Additionally, the microplate format
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881 also allows for the parallelisation and automation required for phenomics experiments. This opens
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888 the perspective of developing fast, medium-throughput, quantitative assays to support the
889 development of QTL and genome wide association studies on models such as *Ectocarpus* [31], and
890 breeding initiatives on commercially important species such as kelps [10, 32]. The statistical power of
891 QTL or GWAS studies increase with the experimenter's ability to simultaneously analyse multiple traits
892 of interest [e.g. 33]. For example, disease monitoring in *E. siliculosus* or other filamentous brown algae
893 currently relies on time-consuming microscopy, which requires highly trained staff, or end-point
894 measurement of relative pathogen and algal abundance with real-time PCR [19]. We are currently
895 piloting disease monitoring assays, that combine nephelometric measurements with pathogen
896 detection using cell-wall stains like Calcofluor white [for the detection of chytrids or of some
897 oomycetes, 20, 34] or specific, fluorescently-labelled lectins [for some oomycetes, 35]. In parallel,
898 chlorophyll fluorescence and quantum yield may be monitored for the simultaneous, real-time
899 measurements of growth, pathogen prevalence, and algal disease tolerance, respectively.

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901 As different type of possible application, we show that combining image analysis with nephelometry
902 provides a rapid and simple estimation of the reproductive effort of kelp sporophytes, with minimal
903 damage to the plants. Evaluating of the fitness in kelps species is a main challenge because fitness is
904 related to the growth and the fertility of both the sporophytes and gametophytes. While
905 morphological traits are easily measured on macroscopic sporophytes, our growth data *E. siliculosus*
906 open interesting perspectives to monitor the growth of gametophytes and young sporophytes.
907 Second, our real-time quantification of spore release shows that nephelometry is applicable to
908 characterize the reproductive traits of propagules (i.e. spores or gametes), release, opening the
909 prospect of exploring both sporogenesis and gametogenesis. Although we did not explore this feature
910 here, the time-course of propagule release could easily be monitored, for example depending on
911 environmental factors.

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913 This new approach would have immediate applications for crop improvement, or could be also used
914 to address outstanding questions in evolutionary genetics: for example, seaweeds are characterized
915 by a unique variability of life cycles, ranging from diploid through haploid-diploid to haploid life cycles

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947 [36, 37]. Despite considerable theoretical work, there is still no unequivocal explanation for the
948 existence and stability of such a large diversity of life history traits [38]. To address this question,
949 reliable estimates of individual fitness in the field and/or in the lab are required [39]. Such experiments
950 can now be planned to compare the fitness of the haploid and diploid life stages using nephelometry.
951 In the long term, the versatility and potential applications of microplate-format nephelometers for
952 macroalgal and protistan biomass monitoring is probably best illustrated by the current diversity of
953 flow cytometry assays that rely on light-scattering measurements [e.g. 40]. Novel specific applications
954 of nephelometry might encompass the investigation of phototrophic biofilms, which are typically
955 made of heterogeneous mix of bacteria and microalgae. Also, combining fluorometry and
956 nephelometry might enable to parallelise grazing assays, e.g. between ciliates and algae. Finally, the
957 theoretical possibility to measure particles of different sizes with a microplate nephelometer should
958 be noted, a feature that has already been successfully exploited in flow cytometry [41].
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980 outstanding technical support.
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1000 **Author contributions**

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BC, MS, MMP and BJ performed most experiments; CR contributed her expertise in image analysis;
BC, MV, YB and CMMG conceived the original research plan, with the assistance of JMC and CD. MV
and CMMG supervised the experiments; BC, MMP and CMMG wrote the manuscript, with
contributions of all other authors. All authors approved the final manuscript.

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1065 **Figure Legends**
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1070 **Fig. 1. Principles of fluorometry (A), spectrophotometry (B) and nephelometry (C), with an overview**
1071 **of their respective pros and cons.**

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1075 **Fig. 2. Assessment of linearity and conservation of proportionality for dry weight measurement,**
1076 **nephelometry, fluorometry and spectrophotometry.**

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1080 Comparison of calibration curves obtained using a serial dilution of *E. siliculosus* tufts with dry weight
1081 measurement (A), nephelometry (B), fluorometry (C) and spectrophotometry (D). Three biological
1082 replicate wells were measured for each dilution of the *E. siliculosus* material (arbitrary unit on x axis)
1083 using the spectrometry methods (B-D, data plotted are average and SD). The algal tufts were then
1084 transferred into Eppendorf tubes, the medium was removed by pipetting, followed by overnight
1085 drying at 60°C. Due to the low amount of material present, the three replicates of each dilution had
1086 to be pooled into one tube. Tubes (one replicate per dilution) were then weighed three times with a
1087 precision scale, leading to the measurements (average and SD) shown in A. Note therefore that the
1088 standard deviations for the dry weight measurements (in A) are not directly comparable with those
1089 for the light-based measurements (B-D). A linear regression was calculated for each calibration curve.
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1091 Furthermore, the deviation (D) between the observed slope and the dilution factor effectively applied
1092 was calculated with this formula: “(expected slope[x1, x2] - linear regression slope)/ expected
1093 slope[x1, x2]*100”, where x1 and x2 are the two extreme points of the dilution series.

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1108 RNU: relative nephelometric unit. RFU: relative fluorescence unit. OD: optical density.
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1111 **Fig. 3. Comparison of nephelometry (purple), spectrophotometry (pink), and fluorometry (green)**
1112 **for the quantification of aquatic eukaryotes.** Calibration curves were obtained using the same
1113 approach as that illustrated in Fig. S2, i.e. using a serial dilution of different groups of algae and
1114 zoosporic fungi (“chytrids” *sensu lato*). Three biological replicate wells were measured for each
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1124 dilution. A linear regression was calculated for all the calibration curve for each optical methods
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1126 (relative unit). The radar plot depicts the r^2 of the resulting linear fits.
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1130 **Fig. 4. Non-invasive monitoring of *E. siliculosus* growth with spectrophotometry (black bars,**
1131 **nephelometry (grey bars), and fluorometry (white bars).** The photographs illustrate algal growth in
1132 one representative well. "Sat." indicates data saturation. For comparison purposes, all measurements
1133 are scaled in arbitrary units (AU) on the same Y axis as follows: RNU / 100 for nephelometry, $OD * 10^3$
1134 for spectrophotometry, and RFU *10 for fluorometry. Bars represent the mean and SD of three
1135 biological replicates.
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1145 **Fig. 5. Detection of changes in *E. siliculosus* growth induced by abiotic factors.** Time-course in control
1146 conditions (full salinity and $2 \mu\text{mol m}^{-2} \text{s}^{-1}$), and under hyposaline stress (10, 20 and 50% of normal
1147 salinity) or increased light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$), as measured with nephelometry (A), fluorometry (B) and
1148 spectrophotometry (C). Data points represent the average and SD of three biological replicates.
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1155 **Fig. 6. Quantification of dead algal tissue.** A-C. Calibration curves obtained with serial dilutions of *E.*
1156 *siliculosus* using nephelometry (A), fluorometry (B) and spectrophotometry (C) after treatment with
1157 1% sodium hypochlorite (white circles) or not (black circles). The unit on the x axis is arbitrary, with
1158 the highest quantity of *E. siliculosus* set to 1. D-E. Exemplary wells containing the same amount of algal
1159 biomass (three biological replicate), treated (D), or not (E), with 1% sodium hypochlorite. Scale bars:
1160 200 μm .
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1170 **Fig. 7. Non-invasive monitoring of disease progression in infected algal cultures.** A-C. Growth curves
1171 of *Macrocystis pyrifera* gametophytes infected by the intracellular biotrophic oomycete *Anisolpidium*
1172 *ectocarpii* as measured with nephelometry (A), fluorometry (B) and spectrophotometry (C). D-E.
1173 Representative microscopic field of views of a control (D) and an infected (E) *M. pyrifera* culture under
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1183 differential interference contrast (left hand side) and epifluorescence (right hand side), illustrating the
1184 phenotypic changes of infected cultures. Infected algal cells contain a parasitic thallus (E, arrowheads),
1185 surrounded by chestnut brown cell debris. At maturity, the oomycete releases zoospores through an
1186 exit tube (double arrowheads). Chlorophyll fluorescence collapses in infected cells from the early
1187 stages of the infection onwards. Scale bars: 20 μm .
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1195 **Fig. 8. Quantification of spore release in kelp sporophytes, as a proxy for fertility measurement.**

1196 **A-B.** The dark brown fertile area (sorus) of the kelp *S. latissima* (A) was photographed in field
1197 conditions and measured using image analysis (B). Scale bars: 1 cm. **C.** In parallel, a serial dilution of a
1198 calibrated spore suspension (with a Malassez cell) was measured with spectrophotometry (white
1199 circles, y axis on the right) and nephelometry (black circles, y axis on the left). For each concentration
1200 tested, three biological replicates were measured. **D.** Finally, the time-course of spore release was
1201 measured with nephelometry (D); the inset shows how kelp fragments (0.5x3 cm each) were disposed
1202 against the plate wall, in order to be fully immersed in the medium without interfering with the
1203 nephelometer's laser beam. The curves show two biological replicates (suffixed .1 and.2) for two
1204 individuals (A and B). The final RNU values at 150 min have be use to estimate the number of spores
1205 released per unit of fertile surface during the experiment. This value is multiplied by the total fertile
1206 surface to obtain a proxy for the individual's fertility.
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1220 RNU: relative nephelometric unit. OD: optical density at $\lambda=230$ nm.
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1225 **Supplementary Figures**
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1229 **Fig. S1: Comparison of nephelometer, spectrophotometer and fluorometer accuracy.** The same
1230 sample of *E. siliculosus* filaments was measured ten times with each protocol (experimental
1231 replication, n=10). Whisker plots were drawn to visualise the data dispersion: the central thick line
1232 represents the median, the box contours represent the upper and lower quartiles, and dashed lines
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show the minimum and maximum values measured. The standard deviation was calculated and normalised over the mean, for each method.

RNU: relative nephelometric unit. RFU: relative fluorescence unit. OD: optical density.

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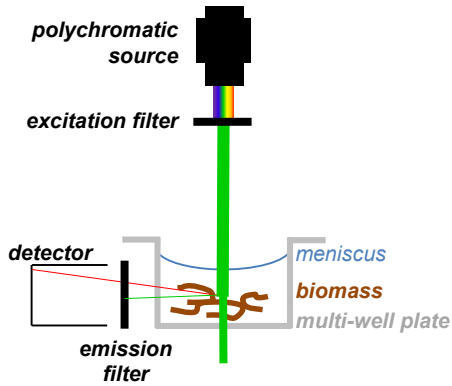
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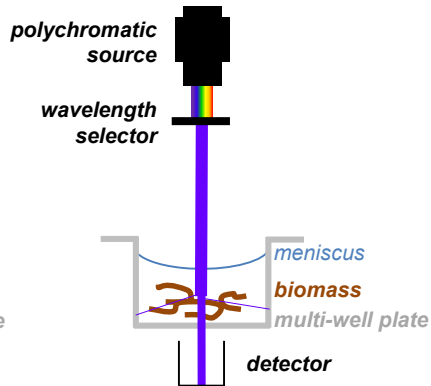
Fig. 1

A. Fluorometry



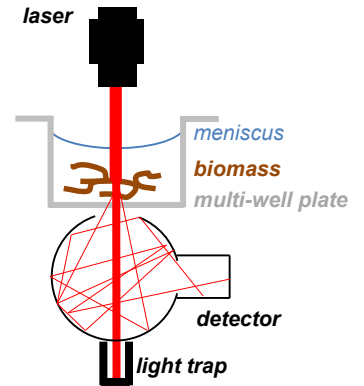
colour multiplexing possible
insensitive to meniscus
dynamic range ++

B. Spectrophotometry (optical density)



wavelength selectable
insensitive to meniscus
dynamic range +++

C. Nephelometry

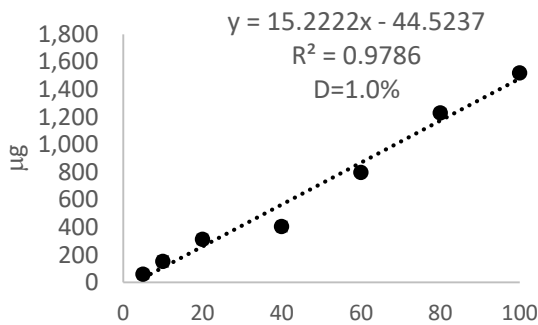


colour-independent
sensitive to meniscus
dynamic range +++

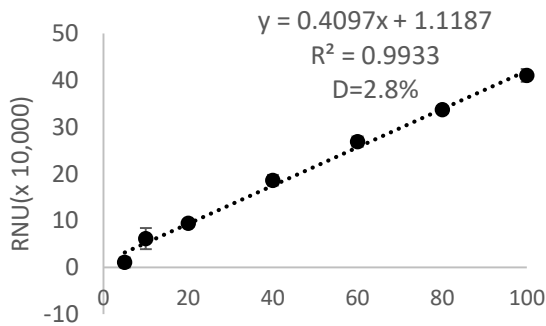
Fig. 1. Principles of fluorometry (A), spectrophotometry (B) and nephelometry (C), with an overview of their respective pros and cons.

Fig. 2

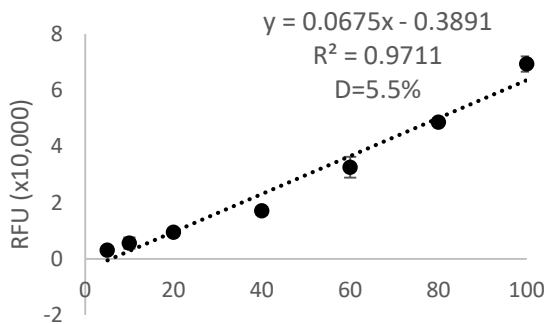
A. Dry weight



B. Nephelometry



C. Fluorometry



D. Spectrophotometry

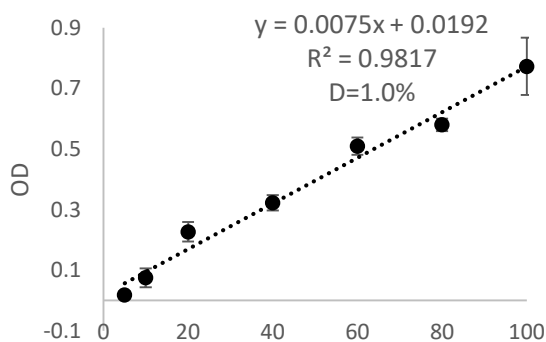


Fig. 2. Assessment of linearity and conservation of proportionality for dry weight measurement, nephelometry, fluorometry and spectrophotometry.

Comparison of calibration curves obtained using a serial dilution of *E. siliculosus* tufts with dry weight measurement (A), nephelometry (B), fluorometry (C) and spectrophotometry (D). Three biological replicate wells were measured for each dilution of the *E. siliculosus* material (arbitrary unit on x axis) using the spectrometry methods (B-D, data plotted are average and SD). The algal tufts were then transferred into Eppendorf tubes, the medium was removed by pipetting, followed by overnight drying at 60°C. Due to the low amount of material present, the three replicates of each dilution had to be pooled into one tube. Tubes (one replicate per dilution) were then weighed three times with a precision scale, leading to the measurements (average and SD) shown in A. Note therefore that the standard deviations for the dry weight measurements (in A) are not directly comparable with those for the light-based measurements (B-D). A linear regression was calculated for each calibration curve. Furthermore, the deviation (D) between the observed slope and the dilution factor effectively applied was calculated with this formula: $\{(\text{expected slope}[x_1, x_2] - \text{linear regression slope}) / \text{expected slope}[x_1, x_2] * 100\}$, where x_1 and x_2 are the two extreme points of the dilution series.

RNU: relative nephelometric unit. RFU: relative fluorescence unit. OD: optical density.

Fig. 3

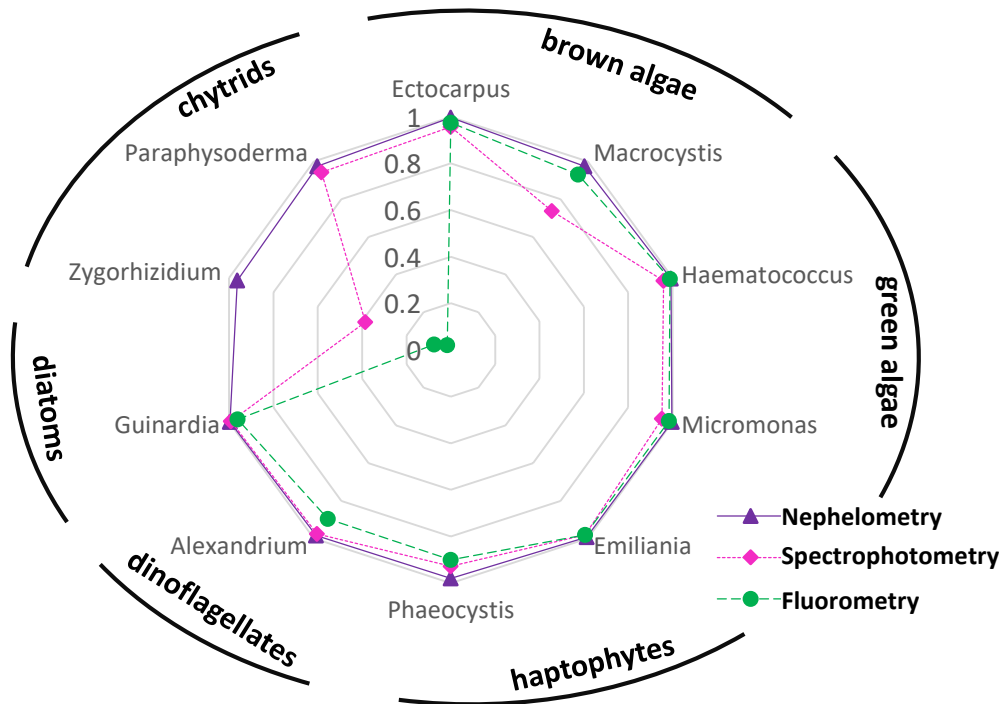


Fig. 3. Comparison of nephelometry (purple), spectrophotometry (pink), and fluorometry (green) for the quantification of aquatic eukaryotes. Calibration curves were obtained using the same approach as that illustrated in Fig. 2, i.e. using a serial dilution of different groups of algae and zoosporic fungi (“chytrids” *sensu lato*). Three biological replicate wells were measured for each dilution. A linear regression was calculated for all the calibration curve for each optical methods (relative unit). The radar plot depicts the r^2 of the resulting linear fits.

Fig. 4

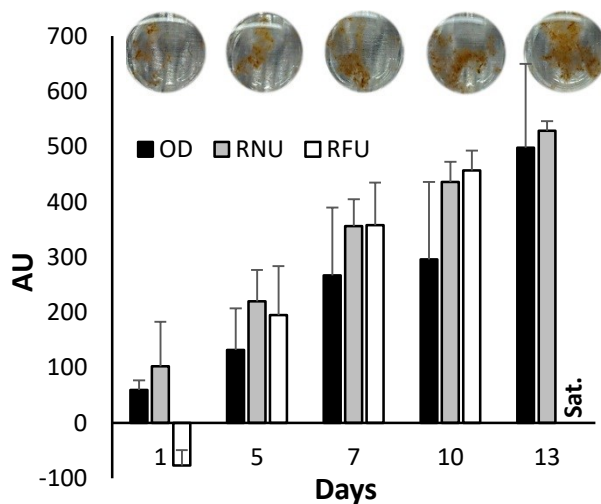


Fig. 4. Non-invasive monitoring of *E. siliculosus* growth with spectrophotometry (black bars, nephelometry (grey bars), and fluorometry (white bars). The photographs illustrate algal growth in one representative well. "Sat." indicates data saturation. For comparison purposes, all measurements are scaled in arbitrary units (AU) on the same Y axis as follows: RNU / 100 for nephelometry, OD * 10³ for spectrophotometry, and RFU *10 for fluorometry. Bars represent the mean and SD of three biological replicates.

Fig. 5

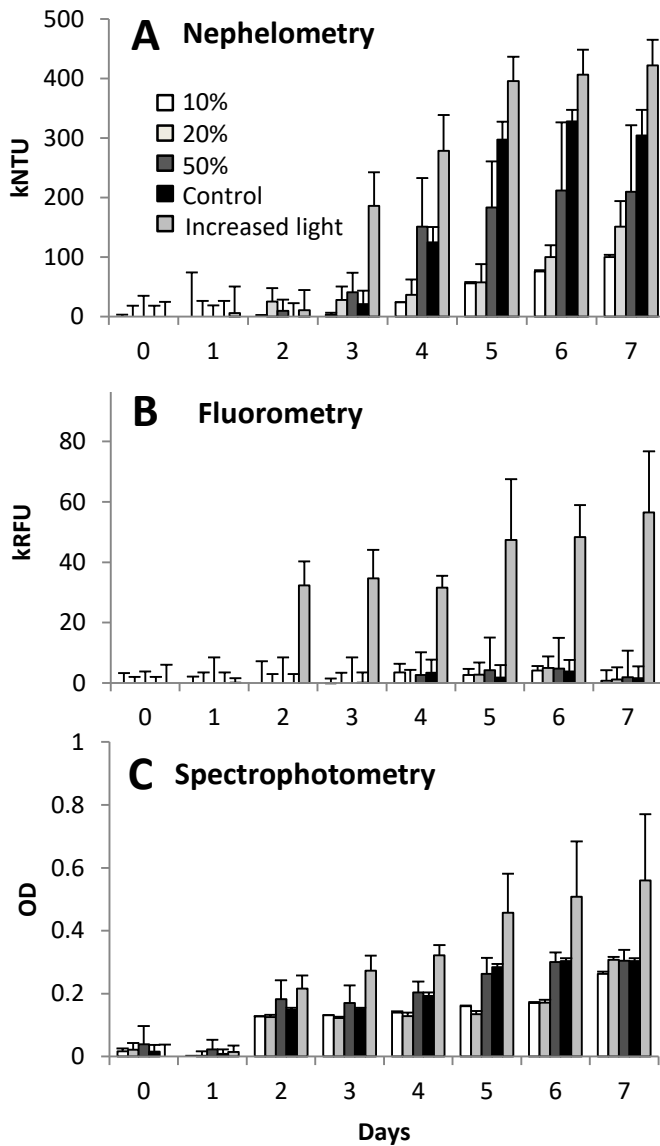


Fig. 5. Detection of changes in *E. siliculosus* growth induced by abiotic factors. Time-course in control conditions (full salinity and $2 \mu\text{mol m}^{-2} \text{s}^{-1}$), and under hyposaline stress (10, 20 and 50% of normal salinity) or increased light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$), as measured with nephelometry (A), fluorometry (B) and spectrophotometry (C). Data points represent the average and SD of three biological replicates.

Fig. 6

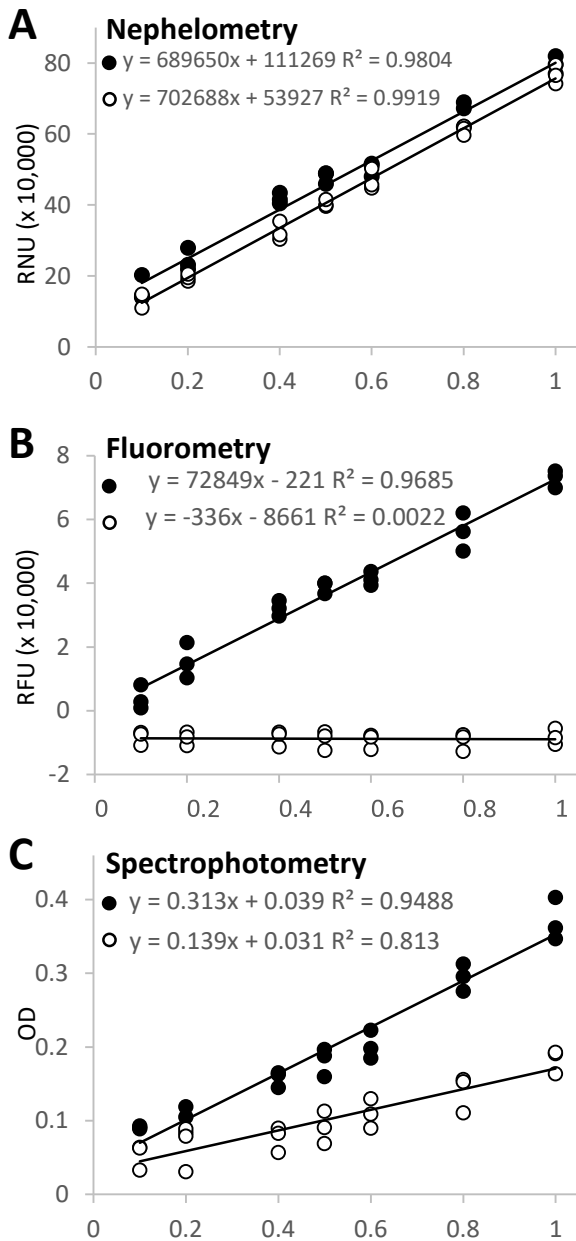


Fig. 6. Quantification of dead algal tissue. A-C. Calibration curves obtained with serial dilutions of *E. siliculosus* using nephelometry (A), fluorometry (B) and spectrophotometry (C) before (black circles) and after treatment with 1% sodium hypochlorite (white circles) or not (black circles). The unit on the x axis is arbitrary, with the highest quantity of *E. siliculosus* set to 1. D-E. Exemplary wells containing the same amount of algal biomass (three biological replicate), treated (D), or not (E), with 1% sodium hypochlorite. Scale bars: 200 μm .

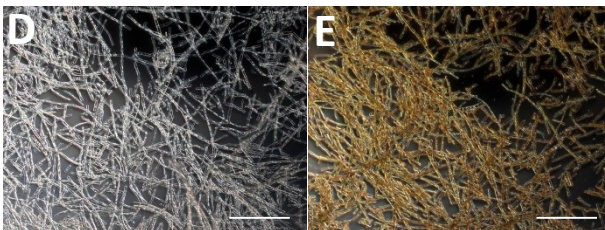


Fig. 7

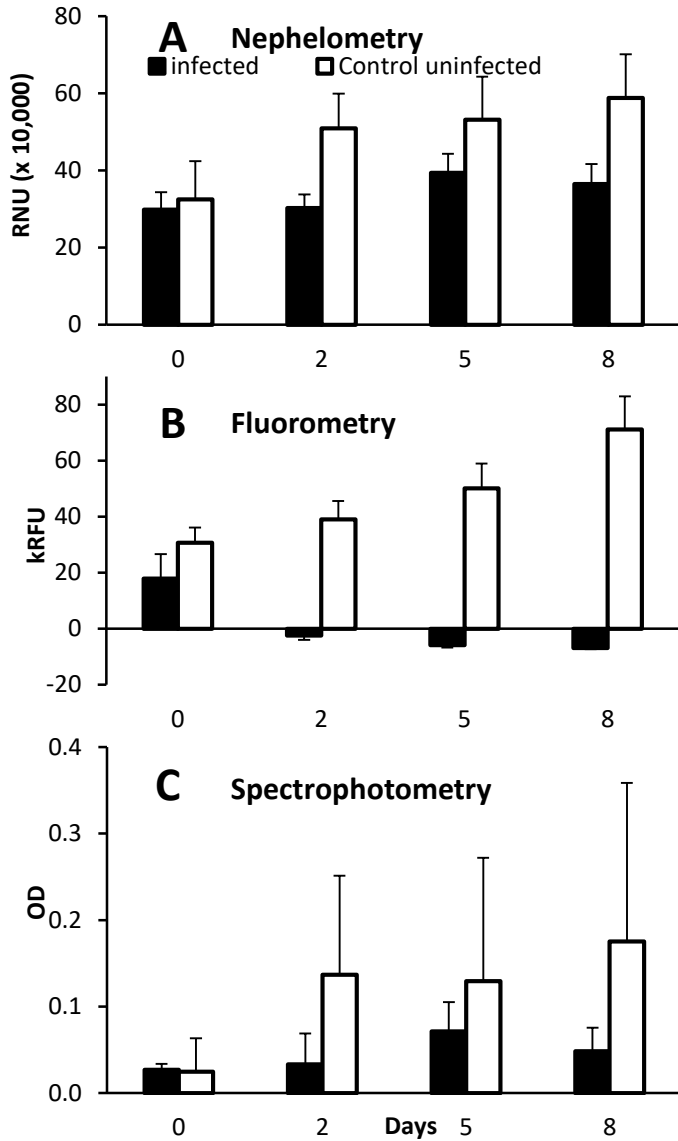


Fig. 7. Non-invasive monitoring of disease progression in infected algal cultures. A-C. Biomass of *Macrocystis pyrifera* gametophytes infected by the intracellular biotrophic oomycete *Anisopidium ectocarpii* as measured with nephelometry (A), fluorometry (B) and spectrophotometry (C). D-E. Representative microscopic field of views of a control (D) and an infected (E) *M. pyrifera* culture under differential interference contrast (left hand side) and epifluorescence (right hand side), illustrating the phenotypic changes of infected cultures. Infected algal cells contain a parasitic thallus (E, arrowheads), surrounded by chestnut brown cell debris. At maturity, the oomycete releases zoospores through an exit tube (double arrowheads). The Chlorophyll fluorescence collapses in infected cells, from the early stages of the infection onwards. Scale bars: 20 μm .

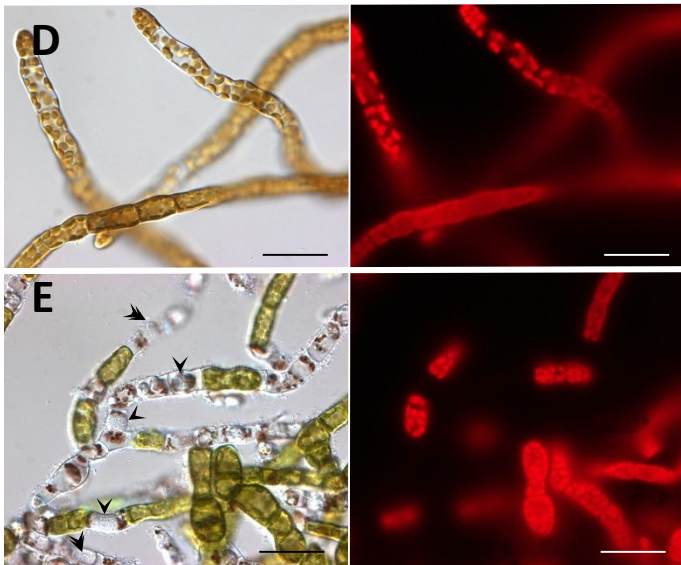


Fig. 8

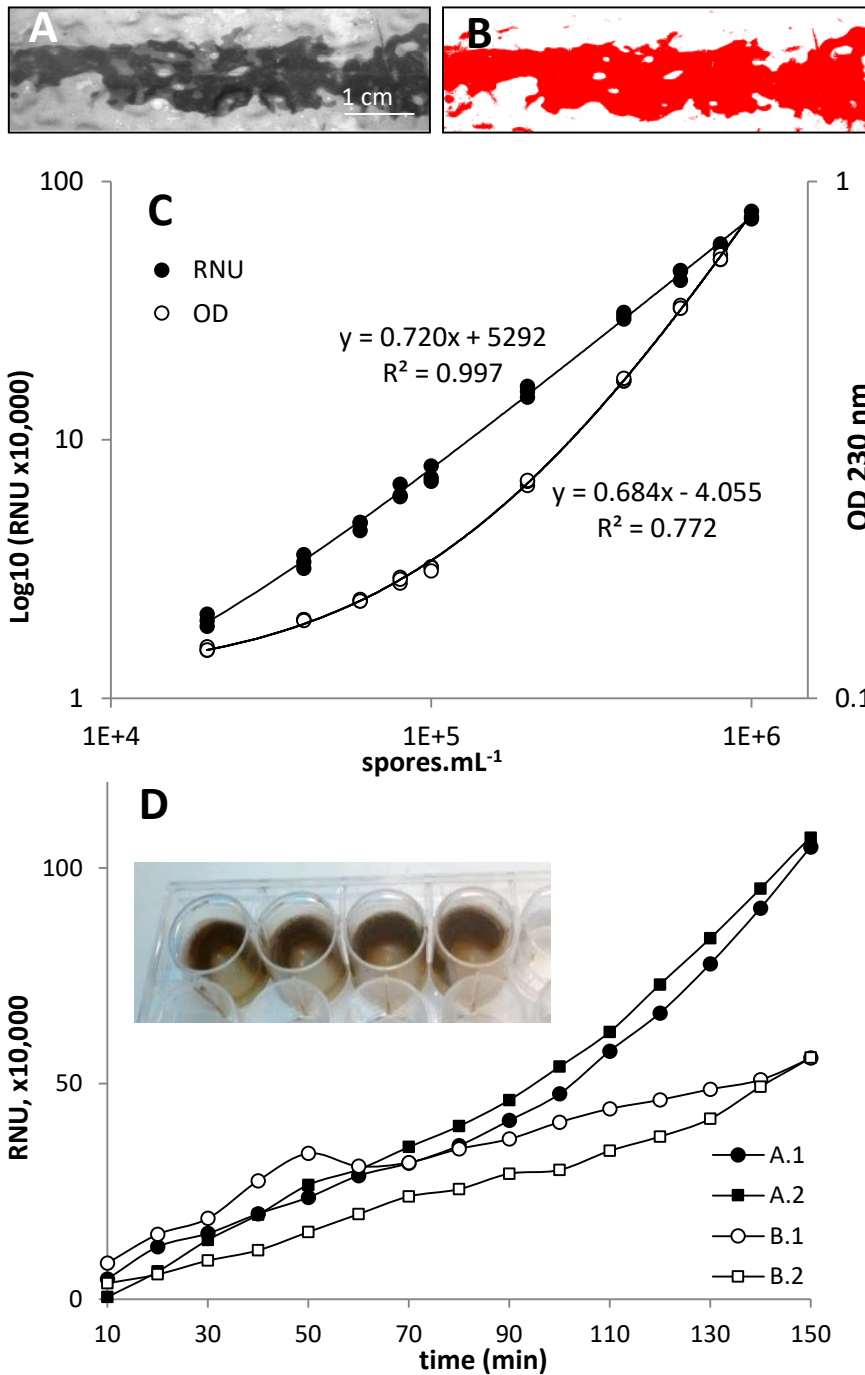


Figure 8: Quantification of spore release in kelps, as a proxy for fertility measurement.

A-B. The dark brown fertile area (sorus) of the kelp *S. latissima* (A) was photographed in field conditions, and measured using image analysis (B). Scale bars: 1 cm. **C.** In parallel, a serial dilution of a calibrated spore suspension (with a Malassez cell) was measured with spectrophotometry (white circles, y axis on the right) and nephelometry (black circles, y axis on the left). For each concentration tested, three biological replicates were measured. **D.** Finally, the time-course of spore release was measured with nephelometry (D); the inset shows how kelp fragments (0.5x3 cm each) were disposed against the plate wall, in order to be fully immersed in the medium without interfering with the nephelometer's laser beam. The curves show two biological replicates (suffixed .1 and .2) for two individuals (A and B). The final RNU values at 150 min have been used to estimate the number of spores released per unit of fertile surface during the experiment. This value is multiplied by the total fertile surface to obtain a proxy for the individual's fertility.

RNU: relative nephelometric unit. OD: optical density at $\lambda=230$ nm.

Fig. S1

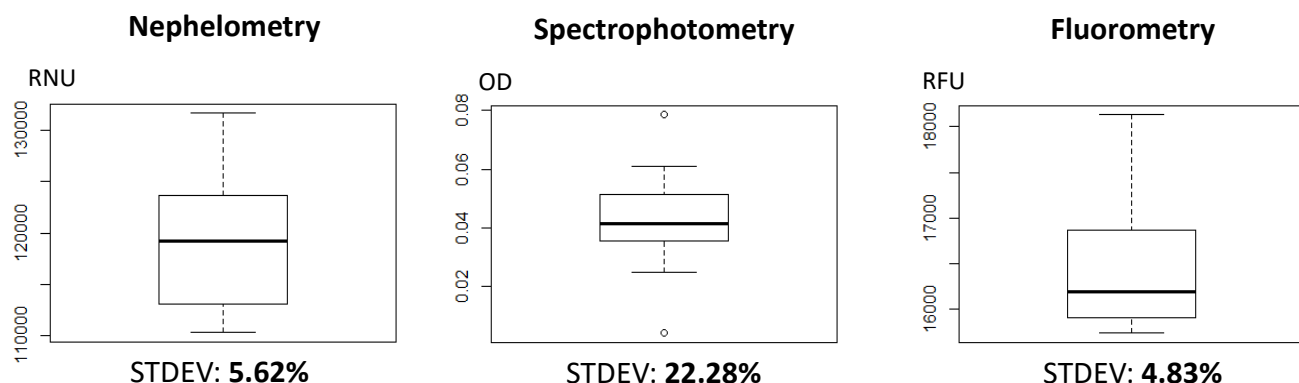


Figure S1: Comparison of nephelometer, spectrophotometer and fluorometer accuracy. The same sample of *E. siliculosus* filaments was measured ten times with each protocol (experimental replication, n=10). Whisker plots were drawn to visualise the data dispersion: the central thick line represents the median, the box contours represent the upper and lower quartiles, and dashed lines show the minimum and maximum values measured. The standard deviation (STDEV) was calculated and normalised over the mean, for each method.

RNU: relative nephelometric unit. RFU: relative fluorescence unit. OD: optical density.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Author contributions

BC, MS, MMP and BJ performed most experiments; CR contributed her expertise in image analysis; BC, MV, YB and CMMG conceived the original research plan, with the assistance of JMC and CD. MV and CMMG supervised the experiments; BC, MMP and CMMG wrote the manuscript, with contributions of all other authors. All authors approved the final manuscript.