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Hyperspectral imaging as a tool for assessing coral health utilising natural fluorescence

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Fluorescent proteins are a crucial visualisation tool in a myriad of research fields including cell biology, microbiology and medicine. Fluorescence is a result of the absorption of electromagnetic radiation at one wavelength and its reemission at a longer wavelength. Coral communities exhibit a natural fluorescence which can be used to distinguish between diseased and healthy specimens, however, current methods, such as the underwater visual census, are expensive and time-consuming constituting many manned dive hours. We propose the use of a remotely operated vehicle mounted with a novel hyperspectral fluorescence imaging (HyFI) “payload” for more rapid surveying and data collection. We have tested our system in a laboratory environment on common coral species including *Seriatopora* spp., *Montipora verrucosa*, *Montipora* spp., *Montipora capricornis*, *Echinopora lamellose*, *Euphyllia ancora*, *Pocillopora damicornis* and *Montipora confusa*. With the aid of hyperspectral imaging, the coral specimens’ emission wavelengths can be accurately assessed by capturing the emission spectra of the corals when excited with light emitting diodes (395–405 and 440 nm). Fluorescence can also provide an indicator of coral bleaching as shown in our bleaching experiment where we observe fluorescence reduction alongside coral bleaching.

Keywords: coral, fluorescence, fluorescent proteins (FPs), chlorophyll, hyperspectral imaging

Introduction

Corals are marine invertebrates in the class Anthozoa of the phylum Cnidaria, they often live in sessile colonies of many individual polyps. Reef-building corals are geographically distributed in tropical and subtropical waters, typically occurring between the 300° north and 300° south latitudes.¹ Corals primarily responsible for building modern reefs are Hermatypic corals, belonging

to the “Stoney corals” group or Scleractinia. Hermatypic corals contain photosynthetic algae, specifically dinoflagellates belonging to the genus *Symbiodinium* often referred to as Zooxanthellae, that live symbiotically within its cells. The algae provide the coral with energy synthesised through photosynthesis and in exchange receive protection and nutrients required to conduct

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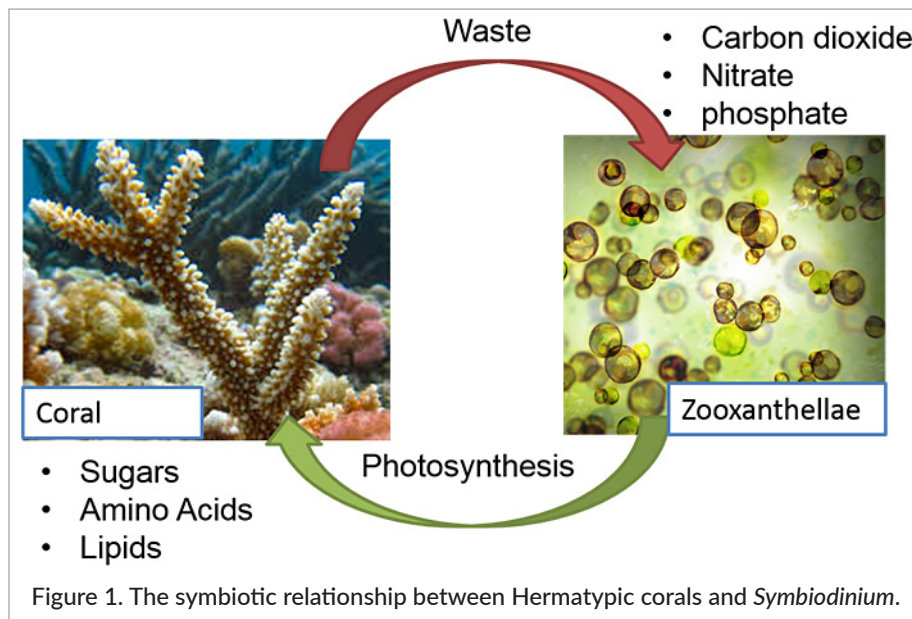
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photosynthesis (Figure 1). Ahermatypic corals do not possess this symbiosis.²

Hermatypic coral communities exhibit a natural fluorescence, both from the coral host and the *Symbiodinium*, the significance of which is yet to be determined. Previous studies have suggested many possibilities for the role that fluorescent proteins (FPs) play in corals: acting as a sunscreen by providing a photobiological system for regulating the light environment;³ or as a host stress response, through their action as antioxidants; downregulation of FPs frequently occurs in injured or compromised coral tissue;⁴ and even to attract prey.⁵ With such broad functional activity, the presence of FPs can potentially be exploited as a proxy for measuring coral health. Analysis of the natural variability in fluorescence intensity for a given species, as well as the differences between diseased and healthy specimens, enables the development of an index relating fluorescence to disease.⁶

Fluorescence is the processes in which molecules emit light from electronically excited states created by either a physical (absorption of light), mechanical (friction) or chemical mechanism. Fluorescence is observed when light is absorbed by fluorochromes, a fluorescent chemical compound that can emit light upon light excitation. Electrons are promoted from a ground state to an excited state and, as the excited molecule returns to its ground state, the relaxation process involves the emission of a photon of lower energy and hence longer wavelength than the absorbed photon.⁷

For example, if an object's emission wavelength is around 420–460 nm (blue) it can be excited with ultra-violet (UV). This means for an excitation light source we can use LEDs that emit in UV or blue which will excite across a wide range of the spectrum. This will allow us to see the whole spectrum of coral fluorescence proteins described by Alieva *et al.*,⁸ as corals mainly fluoresce in the green 478–512 nm and cyan 485–495 nm.

Green FP (GFP) are key colour determinants in reef-building corals (class Anthozoa, order Scleractinia).⁹ Reef-building corals have symbiotic relationships with dinoflagellates (Zooxanthellae) that contain chlorophyll and associated photosynthetic pigments adding further splendour to an already impressive spectral palette. Coral FPs are major determinants of the colour diversity, accounting for practically every visible coral colour other than the brown of the photosynthetic pigments of algal symbionts.¹⁰ Known coral GFP-like proteins^{11,12} can be arbitrarily subdivided into several "colour types": cyan, shortwave green, longwave green, yellow, red and a non-fluorescent purple-blue.^{8,10}

Cyan proteins typically have an emission peak between 485 nm and 495 nm, although more blue-shifted variants can occasionally be found, down to 477 nm.⁸ Green fluorescent colour is the most common in corals and is the most conspicuous of all the fluorescent colours *in situ*.¹⁰ The position of the peak excitation wavelength in the green proteins is around 478–512 nm.⁸ There are two known wild-type yellow FP with emission maxima between 525 nm and 570 nm: zoanYFP

from a Zoanthidea representative (emission maximum 538 nm) and a hydromedusan protein phiYFP (emission maximum 535 nm).⁸ Corals that excite in red can contain either the DsRed-type or Kaede-type red FP. Kaede-type proteins are mostly associated with Scleractinian corals of suborder Faviina. Red FP from all other organisms studied thus far, including other suborders of reef-building corals (Scleractinia), all sea anemones (Actiniaria) and two more Corallimorpharia representatives, possess the DsRed-like chromophore. DsRed proteins typically have an emission peak between 560 nm and 589 nm and Kaede around 574 nm.⁸

Current coral diagnostic techniques

Mitigation of the impact of coral diseases and syndromes requires better diagnostic techniques to detect the early onset of disease. Ideally, these techniques would be simple, rapid and non-destructive.⁶ Many reef monitoring programmes do not prioritise coral disease assessment due to the costly and time-consuming nature of *in situ* coral health surveys.^{13–16} However, the economic drive for tourist activity associated with coral reefs has provided an array of diver friendly, simple devices for visualising coral fluorescence which employ a tight wavelength excitation via light emitting diodes (LEDs) (usually in the near UV) and an appropriate filter (usually yellow) for visualisation of the fluorescence emission. Whilst versatile, cheap and user friendly, such devices provide little, if any, meaningful and recordable data on reef fluorescence and are entirely reliant upon the human operator's attention, skill, diagnostic expertise and knowledge of coral taxonomy and disease.¹⁵

Underwater Visual Census (UVC) is one of the most commonly utilised methods for surveying coral disease levels on reefs.¹⁷ Globally standardised assessments of coral health require detailed examination of all coral colonies within the survey area, which often involves lengthy field time.¹⁸ For example, prevalence surveys within a 120-m² area¹⁶ may take two scuba divers up to 2.5 h on reefs with high coral colony densities and diversity.¹⁵ This creates a high financial cost of collecting coral health data *in situ* and a lack of skilled people with the experience to accurately and rapidly diagnose. In addition, the use of scuba is often impractical or impossible due to physical limitations (e.g. depth or currents) or the presence of unacceptable risks (e.g. the presence of dangerous animals).¹⁵ This method does not require a great deal of

specialised equipment or post-survey data processing, but it does require the observer to have a high level of diagnostic expertise in coral taxonomy and disease identification.¹⁵

Bleaching

Bleaching refers to the loss of colour in symbioses between Zooxanthellae and marine benthic animals, this is not restricted to corals but is displayed by all animals in symbiosis with Zooxanthellae. Bleaching often displays with depressed growth and increased coral mortality; always considered as a detrimental physiological response.¹⁹ The causes of coral bleaching, as generally accepted in the literature, are as a response to external factors (stressors) such as elevated water temperature usually accompanied by increased solar irradiation, change in ocean acidification or bacterial/viral infection.²⁰

Bleaching will result in a distinct change in fluorescence response of a coral to incident light. Accordingly, hyperspectral imaging of coral communities under ambient light or deliberate monochromatic illumination could provide a means of monitoring health if conducted on a routine basis.

Underwater hyperspectral imaging

Hyperspectral imaging (HI) has been shown to be able to capture fluorescence, therefore, providing spectral data outlining emission and excitation peaks of corals, however, it has been rarely used *in situ* (underwater). Studies specifically using underwater hyperspectral imaging (UHI) in association with corals are few and far between, but one such study²¹ looks at the discrimination of benthic communities of coral, algae and sand. Coral species (*Montipora capitata*, *Porite compressa*, *Porites lobata*) were identified in terms of spectral reflectance; major reflectance features were identified by the peaks at 573, 604, 652 and 674 nm. The surveys in this reported study were conducted using airborne images, using a plane-mounted HI system and use of fibre-optic cable spectroscopy for ground truthing using frags collected from the environment and using an Ocean Optics SD1000 fibre-optic spectrometer, 10 cm from the subjects (frags are coral fragmentations produced as a form of asexual reproduction or cloning in which corals are split into fragments.) The purpose of the Hochberg study²¹ was to identify and discriminate between shallow marine habitat types simply for the

purpose of mapping these areas using remote sensing. The aims were not primarily to do with individual colony health but coverage.

There is also potential for HI and UHI to be used as a bio-optical taxonomic identification tool by making an “optical fingerprint” of different marine organisms provided that the pigment composition and corresponding absorption signature of the organism is known.²² A spectral library of verified “optical fingerprints” from marine organisms would enable automated identification of the sea floor, enabling quick habitat classification and distribution surveys to be completed, improving substantially on traditional survey methods.²²

Materials and methods

Hyperspectral imaging

To characterise the subtleties of Hermatypic coral fluorescence, sample corals in both experiments in this study were imaged using a push-broom type hyperspectral camera (Nano-Hyperspec, Headwall, USA) on a motorised rotational stage (360° stepper motor), which collects and processes information from 270 spectral bands across a broad region of the electromagnetic spectrum centred on the visible (400–1000 nm) with 7.4 µm pixel size. The camera in both experiments was positioned around 30 cm from the middle of the outside tank and data was imaged through the glass. This allows us to record a spectrum from a target object and highlight any characteristic excitation/emissions. Such measurements inform the choice of filters that would be most appropriate for removing the excitation wavelengths from subsequent analysis. HI, or imaging spectroscopy, combines digital imaging and spectroscopy. For each pixel in an image, a hyperspectral camera acquires the light intensity (radiance) for a large number (typically a few tens to several hundred) of contiguous spectral bands. Every pixel in the image contains a continuous spectrum allowing for the wavelength of any fluorescence emission from the coral to be read as a peak. The recorded data was processed in ENVI (Version 5.3.1) to produce a hypercube.

The ROI (region of interest) software function was used to specify the pixels of each coral sample, the spectra of each pixel present in the ROI were used to generate an average spectrum of the characteristic coral fluorescence based on the wavelengths of the determined emission peaks.

Proof of concept experiment

Assessment of coral health was undertaken on live corals growing within a controlled aquarium setting at the London Aquarium using the in-house lighting system (Aqua Illumination/Hydra 52 Hyper Drive), consisting of coolwhite (correlated colour temperature 6200 K), UV (400 nm), royal blue (450–465 nm), blue (465–485 nm), green (520–535 nm), deep red (660 nm) LEDs, a UV (405 nm) diving torch (EMart Ltd, Seoul) and a prototype light box (blue 440 nm). This test was to confirm if relatively low power LEDs could excite live coral. The tank used is within quarantine and is propagated with coral frags and consisted of the “hard corals” *Seriatopora* spp., *Montipora verrucosa*, *Montipora* spp., *Montipora capricornis*, *Echinopora lamellose*, *Euphyllia ancora*, *Pocillopora damicornis* and *Montipora confusa* (Table 1 and Figure 2).

Linking bleaching and fluorescence experiment

Following preliminary experiments (proof of concept experiment), a further experiment looking at whether coral health could be determined based on fluorescence was established. The experiment set out to deliberately bleach coral samples in a laboratory setting by slowly increasing the water temperature until Zooxanthellae expulsion occurred (bleaching). The laboratory system was designed to provide reproducible temperature treatments under recirculating conditions for a diversity of species of Scleractinian corals. The system consists of two tanks (Aqua One NanoReef 35 L Aquarium L33 × W33 × H33 cm) fitted with integrated life systems (55 watt heater, protein skimmer, water filter). The water used to populate the tanks was tap water treated with (Seachem, Prime) to remove ammonia and chlorine. This was then combined with Reef Salt (Aqua One Synthetic Reef Salt) at a ratio of 1 kg of salt to 25 L water, creating a sea water analogue at 35 parts per thousand (ppt) salt content. One tank was a “control” with optimal temperature conditions (26 °C) constantly maintained; with the second tank as an “Experiment” tank where temperature was incrementally varied. The coral sample was imaged in three light conditions, under white light (Aquarium light bar, 6200 K), deep blue light (Aquarium light bar, 440 nm) and UV light (BlueRobotics UV light pod, 405 nm) The spectra, taken on an Ocean Optics Flame spectrometer, are outlined in Figure 3. The temperature of the

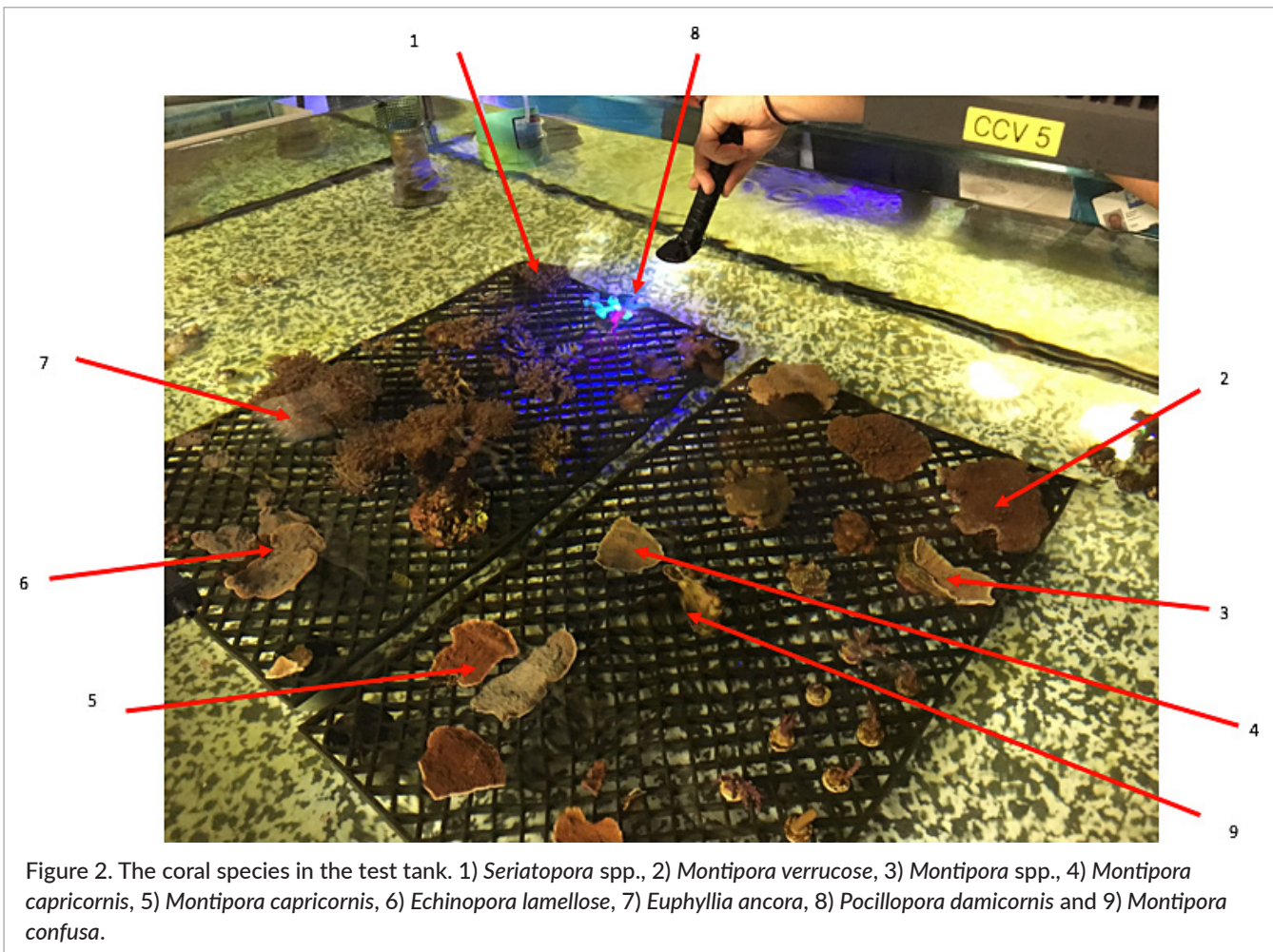
Table 1. The coral specimens used in the London Aquarium for testing. Latin names, common names and their global wild distribution. Numbers relate to annotated Figure 2.²³

No.	Coral species	Common name	Distribution
1	<i>Seriatopora</i> spp.	Needle corals, birds-nest corals or finger corals	Red Sea, the Indo-Pacific region and some parts of the Central Pacific Ocean.
2	<i>Montipora verrucosa</i>	Pore coral	Indo-West Pacific, the Red Sea and the Gulf of Aden, the south-west and north-west Indian Ocean, the Arabian/Iranian Gulf, the northern Indian Ocean, the central Indo-Pacific, Australia, south-east Asia, Japan and the East China Sea, the oceanic West Pacific, the Central Pacific, the Hawaiian Islands and Johnston Atoll.
3	<i>Montipora</i> spp.	<i>Montipora</i>	Red Sea, the western Indian Ocean and the southern Pacific Ocean.
4/5	<i>Montipora capricornis</i>	Leaf plate <i>Montipora</i> , vase coral, cap coral or <i>Montipora</i>	Indian and Pacific oceans, as well as the Red Sea.
6	<i>Echinopora lamellose</i>	Blue chalice coral, hedgehog coral	Persian Gulf, Gulf of Aden, Red Sea, Gulf of Aden, eastern Africa, Madagascar, Seychelles, Mauritius, Chagos Archipelago, Maldives, western India, Sri Lanka, Myanmar, Thailand, south-east Asia, Vietnam, South China Sea, Japan, Papua New Guinea, western to eastern Australia, Solomon Islands, Vanuatu, New Caledonia, Palau, Micronesia, Marianas Islands, Ogasawara Island (Japan), Marshall Islands, Fiji, Samoa, Cook Islands and French Polynesia.
7	<i>Euphyllia ancora</i>	Anchor coral, sausage coral, hammer coral, ridge coral and bubble honeycomb coral	Tropical waters of the Indo-West Pacific area from the Maldives to the Salomon Islands with a large presence in Indonesia.
8	<i>Pocillopora damicornis</i>	Cauliflower or lace coral	Tropical and subtropical parts of the Indian and Pacific Oceans
9	<i>Montipora confusa</i>	Velvet coral	South-east Asia, Vietnam, South China Sea, Papua New Guinea, north-east Australia, Solomon Islands and Fiji.

heating unit was increased +2 °C each week starting at 24 °C and rising to 32 °C. The coral was then imaged using the Headwall hyperspectral camera every 48 h of each week where temperatures were between 24 °C and 28 °C as these represent the temperature range of coral. At 30 °C, where we expect to see bleaching, imaging was undertaken every 24 h to try to observe the expelling of the Zooxanthellae from

the coral cells. The hyperspectral data collected by the Headwall camera was then compiled into a hypercube and loaded into image analysis software, ENVI (v5.4), where it could be viewed and spectra obtained.

Coral samples used in this experiment were obtained from Sealife London Aquarium, where they were cultured and fragged from larger colonies and mounted on plugs. The coral species used in this experiment was *Montipora*



digitata with a total of six frags. These frags were then split into two groups of three; the first group to remain in the control tank and the second group to undergo the bleaching treatment. The control and bleaching treatments lasted three weeks in total.

Results and analysis

Proof of concept experiment

As Figure 4 shows, the spectra of the corals selected from the hypercube image produced from the hyperspectral camera (Headwall Nano) indicate that the corals tested mostly all fluoresced in the cyan or green wavelengths. Samples were present representing at least one of every species, including corals emitting fluorescence and those that do not. The primary peaks were used to correlate to the colour corals are emitting. The coral samples' identification correlate to Figures 5 and

6, showing an image of the hypercube and the same coral tank stitched from a series of top-down images taken on traditional RGB camera (GoPro Hero 5). Corals displaying cyan fluorescence, as classed by Alieva *et al.*,⁸ are as follows: C1 (459 nm), C2 (445 nm). Corals emitting in the green are: C3 (481–501 nm), C4 (503 nm), C6 (481 nm), C9 (481–494 nm), C10 (490 nm), C11 (481 nm), C14 (481 nm), C15 (496 nm), C16 (469 nm), C17 (519 nm), C18 (492 nm), C19 (492–496 nm), C21 (496 nm), C23 (496–516 nm). Some of the corals did not produce any fluorescence such as C5, C7, C12, C13, C20 and C22. These make a suitable control as non-fluorescence coral spectra vs fluorescence coral spectra. Some of the coral samples exhibited other weaker emission peaks displaying the presence of dual colour fluorescence, such as C2, which displays a main peak at 445 nm (cyan) and weaker one at 510 nm (green). Many of the corals exhibited wide emission peaks indicating a spectrum of colour emitted. Many of the spectra profiles

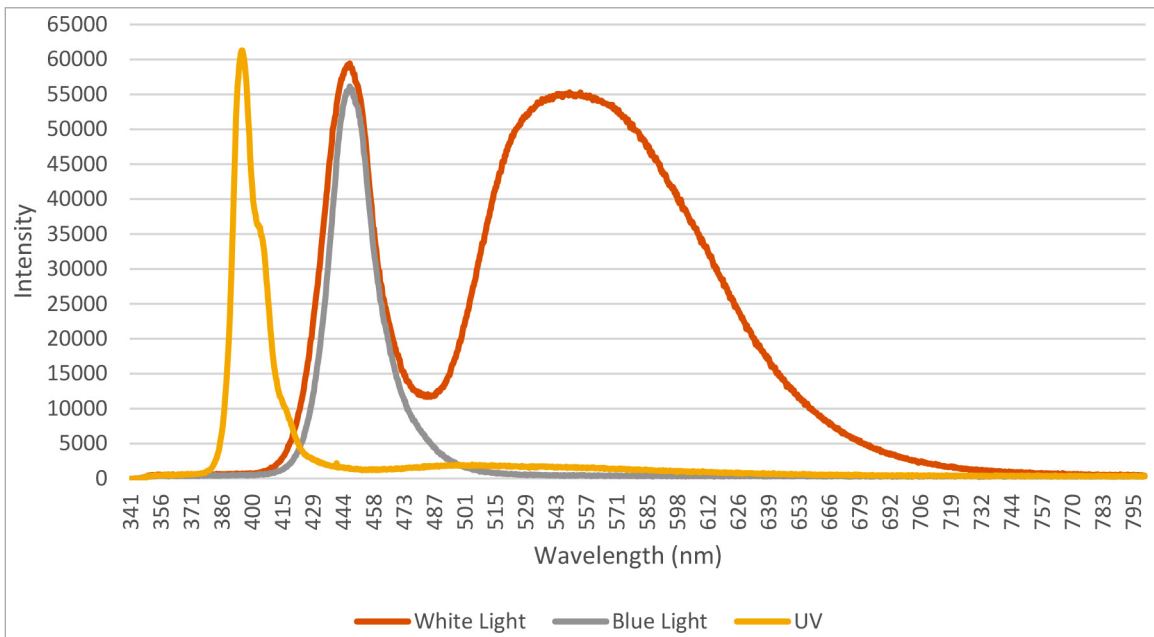


Figure 3. A combined spectrum of the LED emission wavelengths, (UV peak 395 nm, blue light 448 nm), spectrum taken on an Ocean Optics Flame spectrometer.

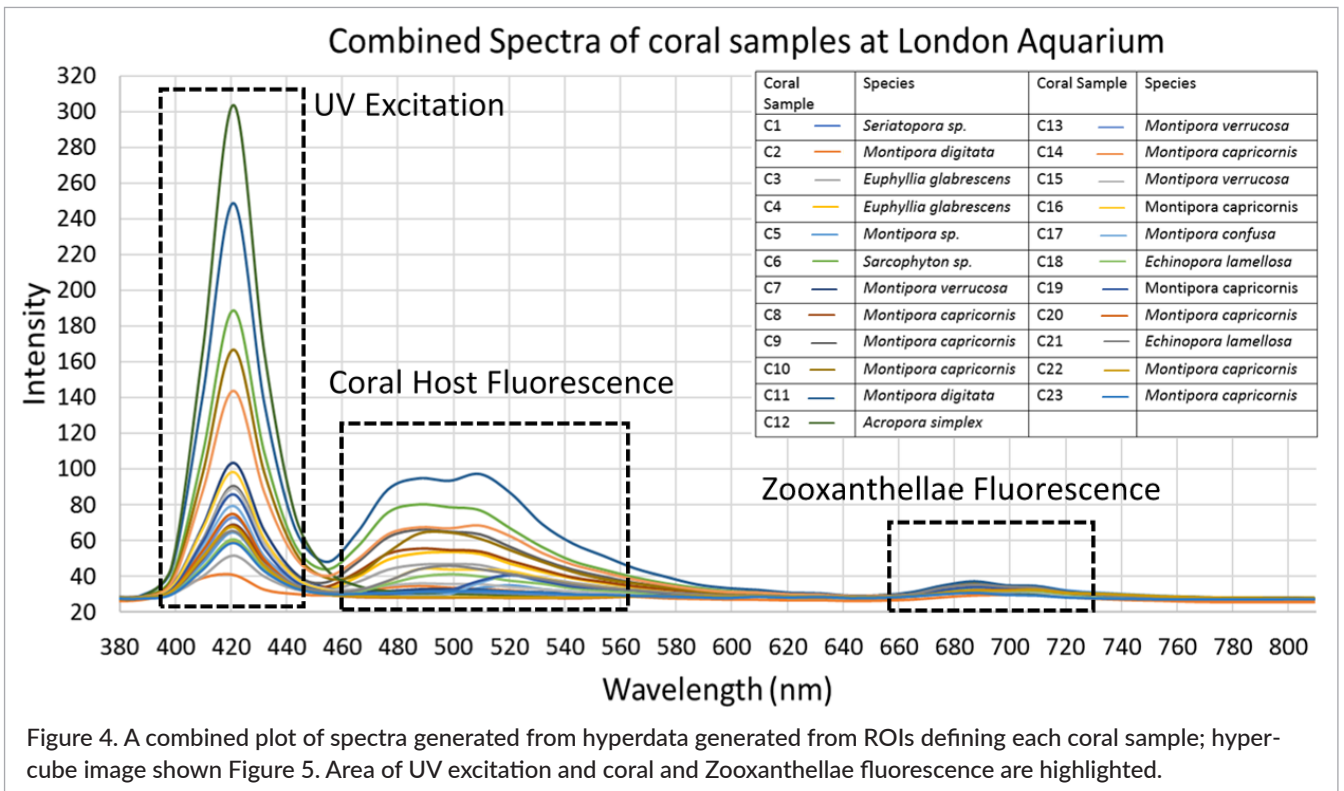


Figure 4. A combined plot of spectra generated from hyperdata generated from ROIs defining each coral sample; hypercube image shown Figure 5. Area of UV excitation and coral and Zooxanthellae fluorescence are highlighted.

exhibited a low intensity emission band in the red region (after 643 nm) towards the end of the spectrum. This is likely the emission peak of chlorophyll contained within the *Symbiodinium*.

In order to gain the best excitation wavelength for a broad spectrum of coral emissions, it is best to use UV LEDs in the final fluorescence imaging payload. Laboratory experiments tested different LED arrays of varying wave-

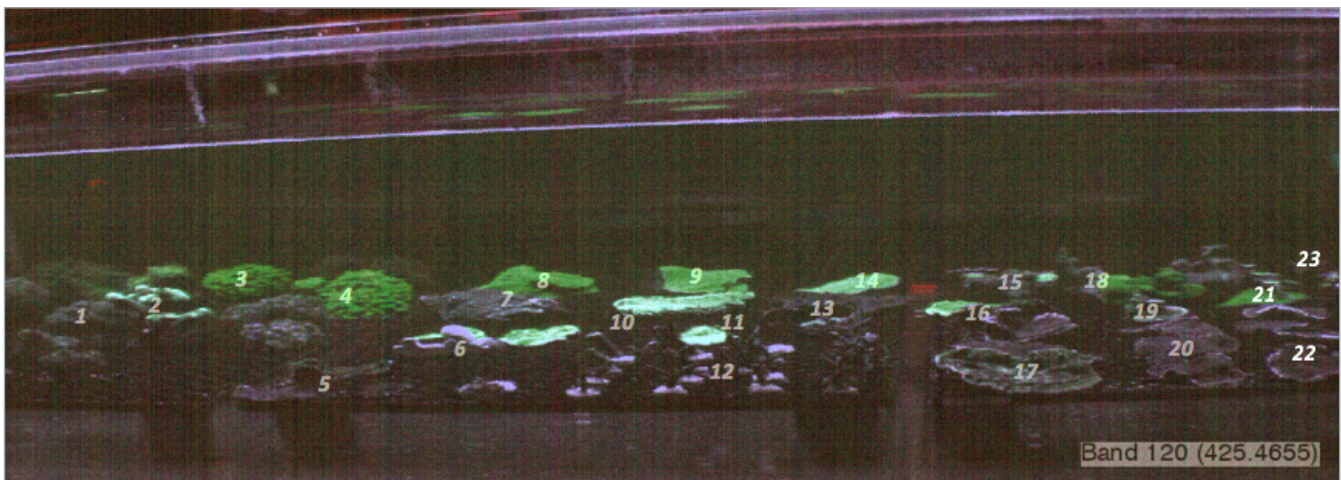


Figure 5. An image generated from a hypercube (hyperspectral data loaded in ENVI) of the front of the coral test tank (Sealife London), corals' numbering corresponds to spectra graphs. Colours set at R: 627 nm, G: 503 nm B: 436 nm.

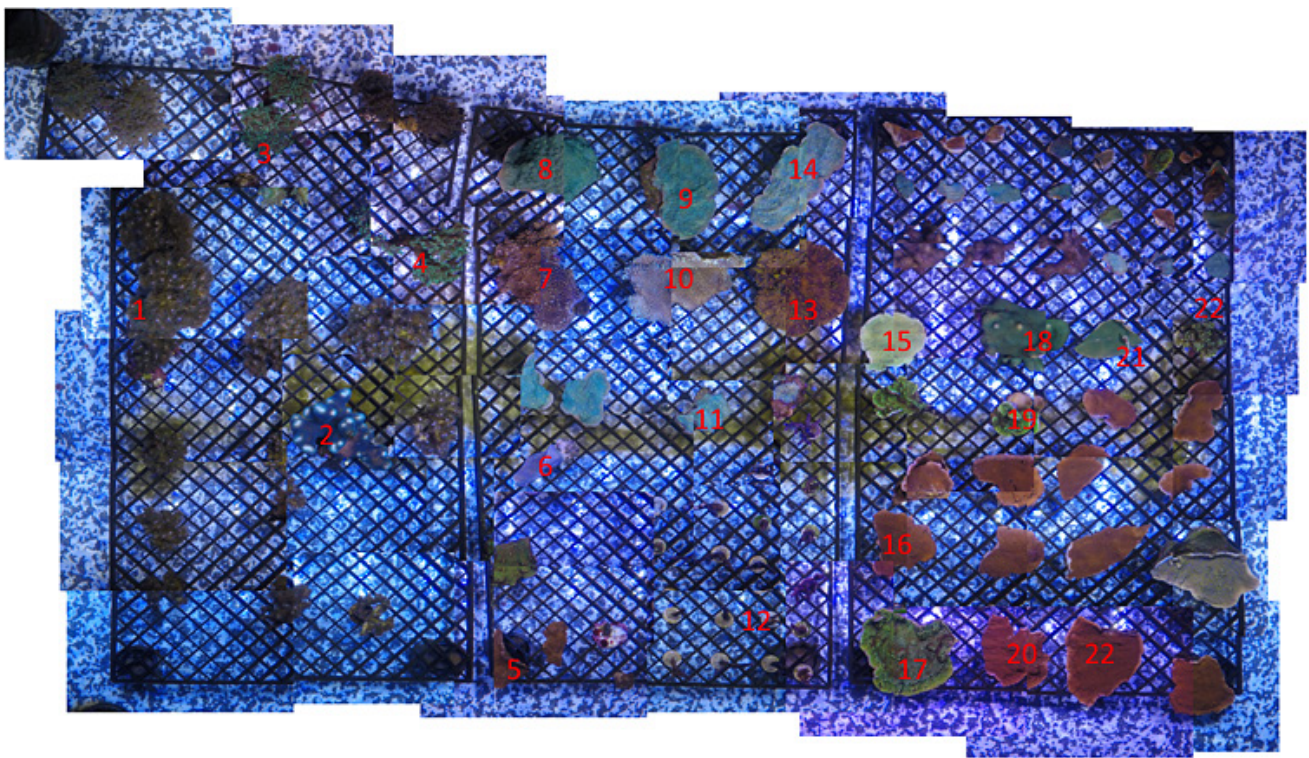


Figure 6. A top-down photomosaic (image stitching using Image Composite Editor, Microsoft) of the same coral tank used in the hyperspectral image (Figure 5).

lengths (405, 440, 500 and 600nm) providing relatively monochromatic illumination. Spectral analysis confirmed the theory that UV illumination yielded the strongest fluorescence response from across the whole array of samples. Despite some coral samples displaying more

visible excitation under “blue” light (465–485 nm), UV LEDs will be more appropriate for the final payload as they provide more power and so these will be able to excite the entire visible light spectrum, which will cover a wider range of fluorescence characteristics.

Linking bleaching and fluorescence experiment

Hypercubes are loaded and displayed in user-defined RGB bands, here we use R: 649 nm, G: 500 nm B: 460 nm for white light and blue lights. The UV data was loaded in greyscale at band 440 nm, allowing the coral to be seen (as towards the end of the experiment the fluorescence signal was weak) and used in the ROI software function (Figure 7). The data illuminated under white light provides a baseline of what can be seen without the use of fluorescence (Figure 9). It clearly shows the intensity of colour decreasing as the temperature increases. This colour change is not necessarily indicative of loss of Zooxanthellae which is why using fluorescence provides an advantage, as we can characterise Zooxanthellae by its unique spectral profile.

The data collected using deep blue (440 nm) (Figure 10) revealed the broad fluorescence peak (489–516 nm) of the coral occurring on the shoulder of the excitation peak. The true nature of the whole peak is masked by the overwhelmingly broad signal from the excitation source. To improve the spectral capture and differentiation of the induced fluorescence, we adopted a UV excitation source with a narrower emission peak shifted further along the spectrum from the fluorescence peak. This UV spectrum reveals the true nature of the fluorescence emission peak. There is also a secondary peak in the red which corresponds with chlorophyll fluorescence present in the *Symbiodinium* (687 nm peak centre).

Figure 8 shows the simplified spectral profile (full data set shown in Figure 11) of the coral, *Montipora digitate*, before and after an induced bleaching event, stressed by temperature rise. The reflection signal of the excitation source (385–450 nm) has been removed, with the two peaks after this representing the fluorescence emitted by the coral's FPs and chlorophyll. The data depict the difference in the presence/absence of Zooxanthellae. As the coral is stressed by the temperature increase, the coral exhibited typical bleaching behaviour and expelled the Zooxanthellae, thus reducing the overall measured fluorescence from beginning to end. However, during the experiment, fluorescence was shown to increase despite bleaching occurring; this is likely to be due to the loss of dinoflagellates noted in previous studies²⁴ This could potentially allow for more of the excitation source to reach the GFP and/or resulting emitted fluorescence signal is reduced attenuation is reduced.²⁴ As the temperature stressor continues and increases, this fluorescence decreases markedly as the coral tissue becomes comprised and undergoes necrosis (cell death).

Conclusion and future work

In this study we used temperature as a stressor to induce coral bleaching in a laboratory setting, where time-resolved spectral measurements of fluorescence could

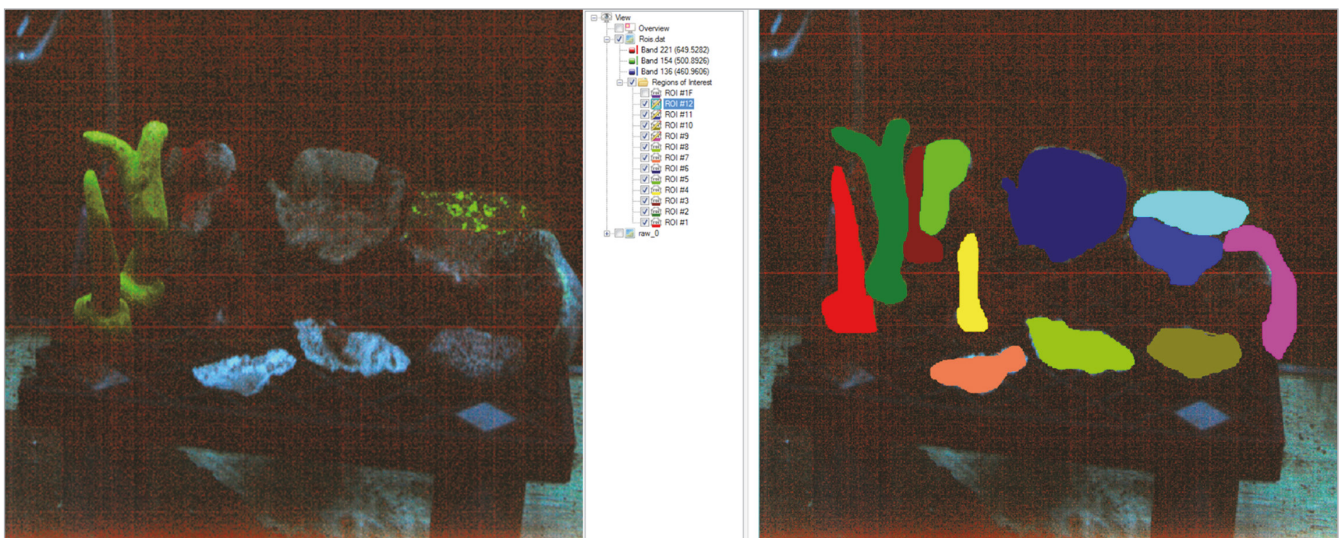


Figure 7. Region of interest/ROI tool, highlighting the area in pixels of each coral sample. Image of 440-nm excitation hypercube loaded in ENVI. Colours set at R: 627 nm, G: 503 nm, B: 436 nm.

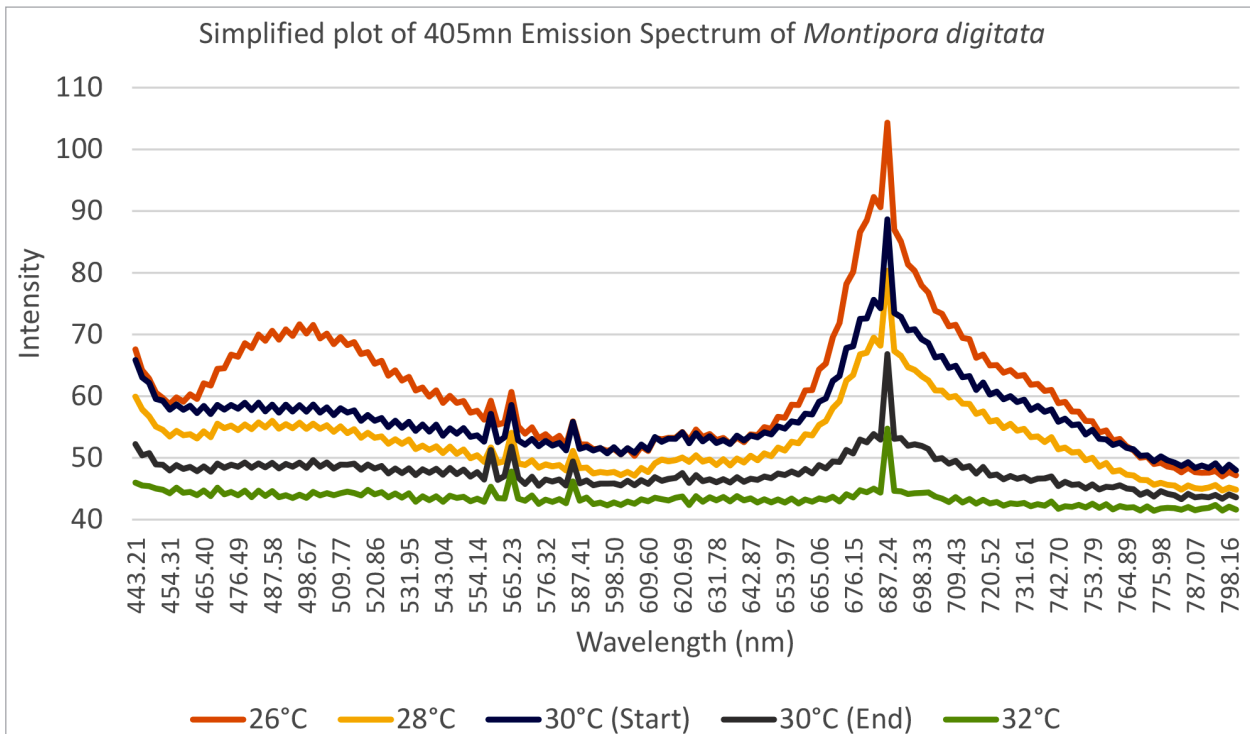


Figure 8. A simplified spectrum (of Figure 11) highlighting fluorescence emission [excited by UV (405 nm)] of a *Montipora digitata* sample using the ROI tool during the bleaching experiment and showing the beginning of each temperature week 26–32°C with 30°C start and end referring to the temperature week.

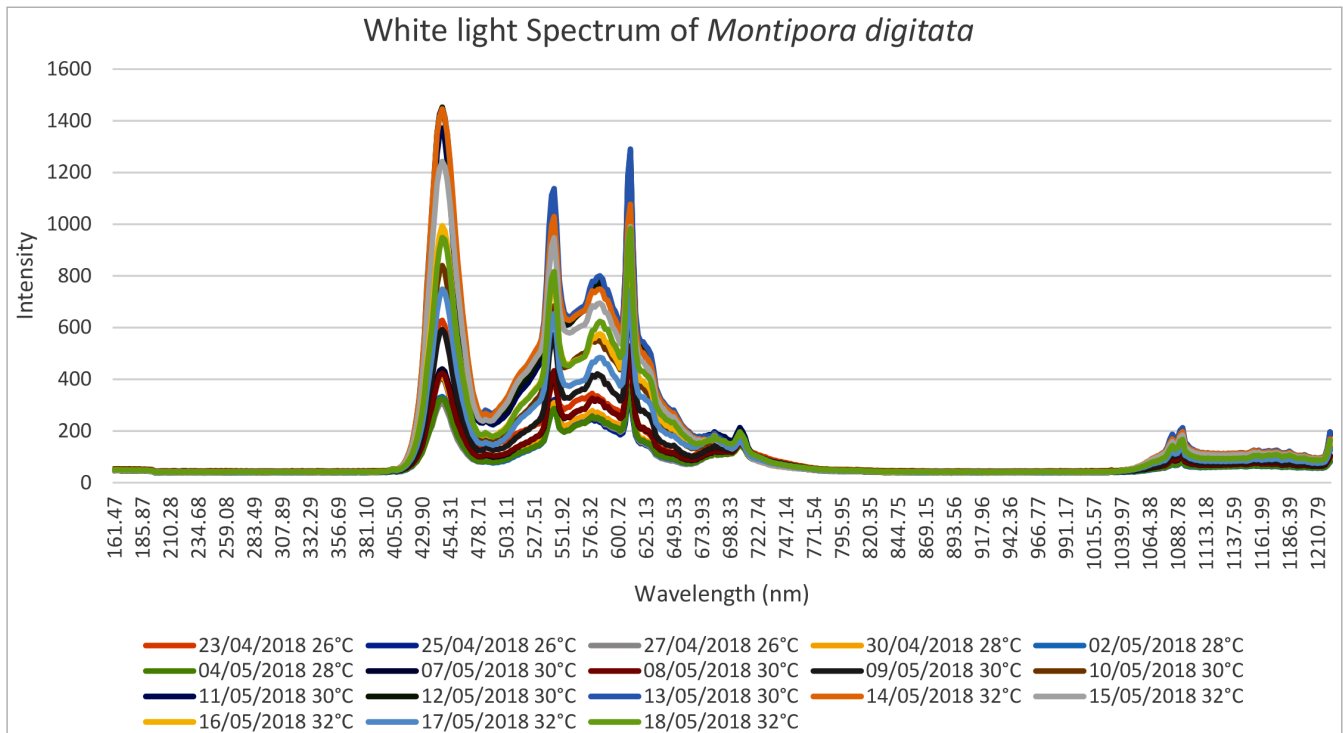
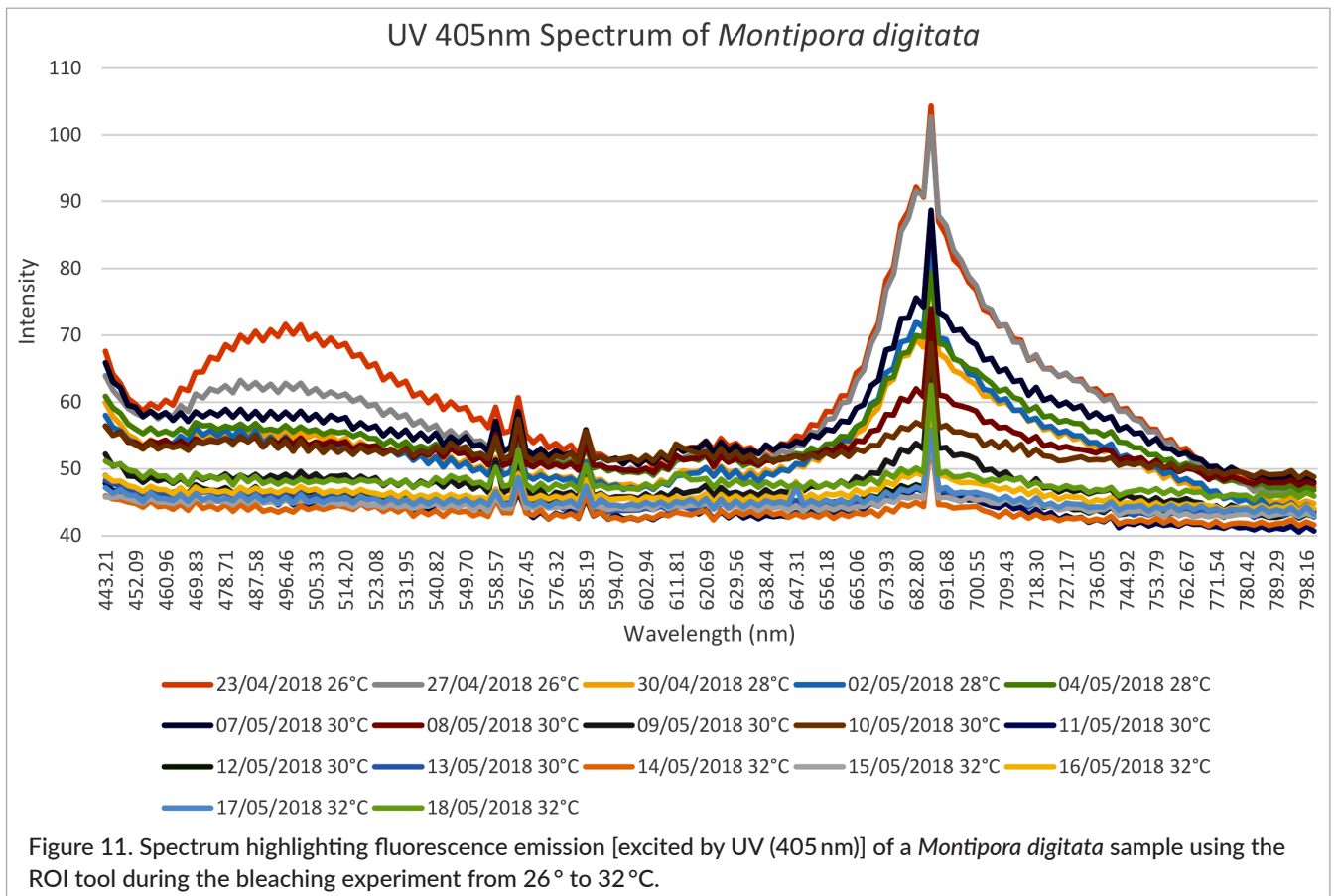
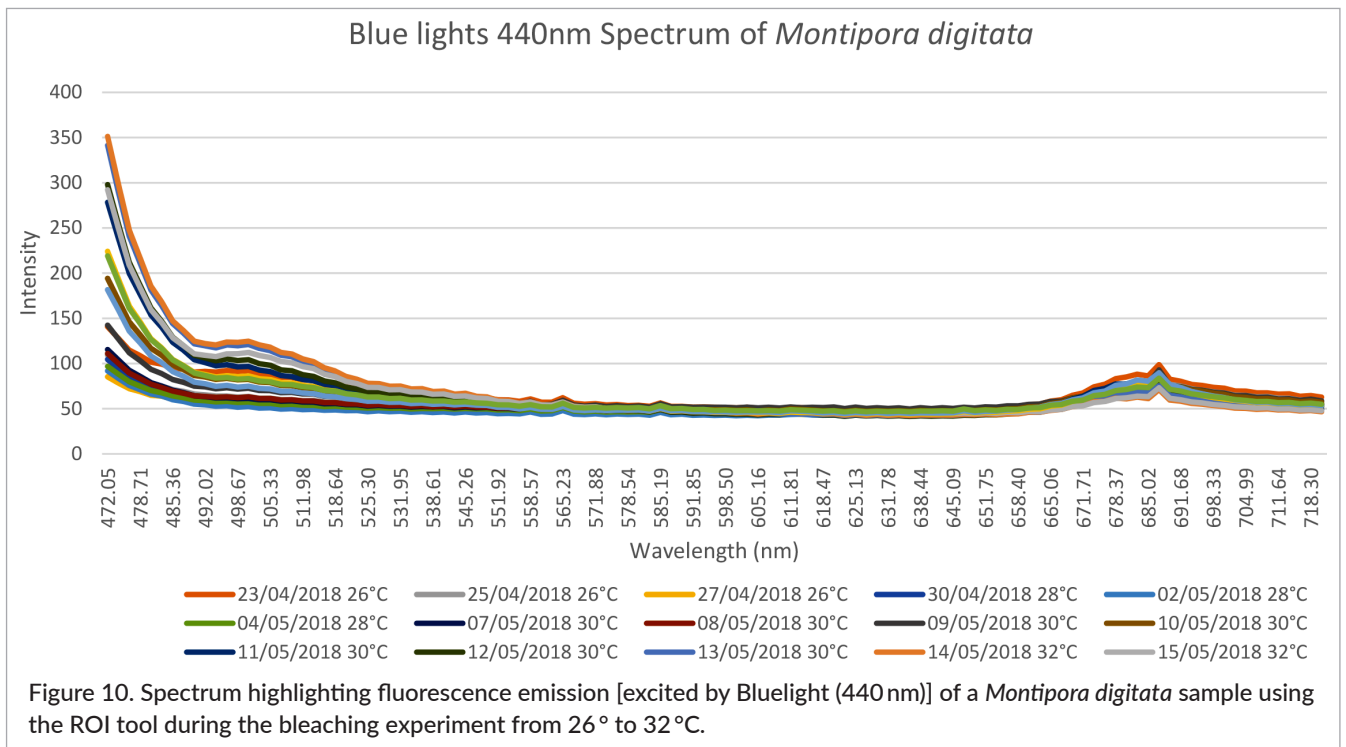


Figure 9. Spectrum highlighting fluorescence emission [excited by white light (6200 K)] of a *Montipora digitata* sample using the ROI tool during the bleaching experiment from 26° to 32°C.



be used to chart the bleaching process. Other possible stressors may include the water parameters shifting, such as salinity, phosphates and nitrates. However, the response is largely consistent across most stressors resulting in the same outcome of Zooxanthellae expulsion from the host coral.

The results provide compelling evidence to prove that fluorescence may be used to indicate coral reef health. The ongoing technological challenge is, therefore, related to (1) building a spectral library for many different coral species and (2) developing a field deployable system, sufficiently cheap and semi-automated such that reef systems can be automatically and rapidly assessed on a repeated regular basis.

This latter challenge would necessitate developing a portable, fully waterproof, low-cost HI system (payload) that can be mounted on an ROV (remotely operated vehicle). The low-cost element is largely due to the high associated costs of commercially available hyperspectral systems and the risks operating within the underwater environment pose. A ROV, which is essentially a tethered underwater robot, would provide a stable, highly manoeuvrable platform for characterising the marine environment as well as the necessary movement required for push-broom techniques. This method draws parallels from unmanned aerial vehicles or drones used in the agricultural sector.

The final payload will consist of UV and blue LEDs of 395–405 nm and 440 nm to provide sufficient energy to excite corals at a reasonable distance (approximately 1 m) and high enough power to mitigate against the effects of light absorption by seawater. The LEDs will be mounted in series powered from the ROV's (BlueROV2) control board, coupled with a BlueRobotics watertight enclosure and custom frame.

Previous work⁹ has been conducted testing a UV light "payload" over a live reef in Aitutaki, Cook Islands, where natural coral fluorescence was successfully observed. The surveys revealed that intensities of coral fluorescence were often unable to be quantified due to the ROV moving too fast over the coral and not allowing enough time for the camera to focus. The project looked at gathering intensity measurements relative to brightness on the images. We intend to develop and demonstrate an improved fluorescence imaging payload over a live reef in the next six months.

For the former challenge we will conduct further bleaching experiments to quantifying Zooxanthellae

concentrations as a function of surface area. This will give the number of Zooxanthellae per unit of area as the samples undergo heat-induced bleaching to link levels of bleaching with symbiont density. This will enable a more quantitative measurement of the bleaching process.

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