

TITLE

Estimating biofilm biomass on objects in an aquatic environment

FIELD OF THE INVENTION

The present invention relates to a method and system for estimating the biomass of biofilm fouling on objects in an aquatic environment.

5 BACKGROUND TO THE INVENTION

Fouling is a common problem for structures that are permanently or intermittently immersed in an aquatic environment. Such structures include hulls and ballast tanks of ships, boats and yachts; fixed and floating structures for offshore oil and gas exploration, production and storage; offshore structures for
10 wind and wave energy generation; and conduits and pipelines.

Problems with fouling include increased fuel costs for boats due to increased frictional resistance. On static structures, for example on drilling rigs, it can alter the water flow around the supporting legs, risking unpredictable and increased stresses. It can also obscure defects and cracks during inspections.
15 Fouling can further reduce the cross-sectional area of pipework such as cooling water or ballast tank intakes, leading to reduced flow rates. Fouling control coatings can be used to reduce fouling growth, but such coatings do not completely prevent adhesion of organisms or biofilm formation.

Fouling can broadly be separated into two categories, namely macro-fouling (barnacles etc.) and micro-fouling (biofilms / slime). Having a quantitative
20 method that can measure how much fouling (i.e. both macrofouling and microfouling) is present on an object in an aquatic environment can help estimate the additional drag, and/or determine the need for cleaning and/or recoating of the object.

25 Biofilms responsible for microfouling generally contain pigmented microalgae and/or bacteria. These often contain photosynthetic pigments such as chlorophyll a. In-vivo chlorophyll a absorbs light very strongly at about 673nm, and biofilms tend to be mostly transparent in near infrared wavelengths. Therefore, it is possible to estimate how much chlorophyll is in a typical fouling biofilm sample by

measuring how much red light it reflects, and often by comparing this to the baseline measurement of near infrared reflection (depending on which index of biomass is used).

Although quantitative imaging of biofilms has been done before, the
5 biofilms have generally been treated mostly as homogenous materials with characteristic absorption properties. These models generally do not provide satisfactory results and/or cannot be used for many different objects. Therefore, there is a need to improve the quantitative determination of biofilm fouling.

10 SUMMARY OF THE INVENTION

An object of the invention is to provide a method and system for estimating aquatic environment-originating biofilm biomass on a coating of an object, e.g. that is immersed permanently or intermittently in an aquatic environment, that is relatively accurate, can be used on a variety of objects and is
15 easy to implement.

One aspect the invention provides a method for estimating aquatic environment-originating biofilm biomass on a coating, such as on an object that is immersed permanently or intermittently in an aquatic environment. Such an object can e.g. be a hull or ballast tank of a ship, boat or yacht; a fixed or floating structure
20 for offshore oil and gas exploration, production and storage; an offshore structure for wind or wave energy generation; or a conduit or pipeline. The aquatic environment can e.g. be a marine, fresh water or brackish water environment.

The method includes a step a) of obtaining one or more digital images of a fouled portion of the coating on the object. Each digital image of the one or
25 more digital images can be obtained in a spectral band. The spectral band can e.g. have a spectral width in the range of 0-100 nm or more. Here a spectral width of 0 nm indicates a single wavelength. Hence, a single wavelength herein is also considered to be a spectral band. The spectral band can e.g. have a spectral width in the range of 0-100 nm FWHM (full width at half maximum) or more. Mutually
30 different digital images can be obtained in the same or in different spectral bands. Each digital image can include one or more pixels.

The method includes a step b) of determining from each of the one or more images a respective reflectance value for the portion of the coating.

The method includes a step c) of determining on the basis of the one or more reflectance values a value of a spectral index representative of biomass.

5 The method includes a step d) of calculating a biomass pigment surface area density, $biomass_{SI}$, on the basis of the spectral index, SI, and one or more calibration values determined for a reference coating. The reference coating can be different from, e.g. have a different color than, the coating of the object. While this calculated biomass pigment surface area density may be a measure for biofilm
10 biomass, it has been found that the value of this determined density is affected by the reflectance of the underlying coating of the object. Therefore, this determined density is not a reliable indicator for biofilm biomass as it may overestimate or underestimate biomass depending on the reflectance of the coating of the object.

 Thereeto, the method includes a step e) of compensating the thus
15 calculated biomass pigment surface area density, $biomass_{SI}$, for the reflectance of the coating of the object by applying a compensation associated with the difference of the reflectance of the coating of the object relative to the reference coating, thus obtaining an estimated biomass density on the current coating surface, $biomass_{current}$. Hence, reflectance of the underlying coating of the object is
20 compensated for, so that biofilm biomass can be determined more accurately. Moreover, according to the present method there is no need for calibrating biomass pigment surface area density, $biomass_{SI}$, versus spectral index for each and every coating. The calibration can be done for a reference coating, and the difference between the actual coating on the object and the reference coating is then
25 compensated for. The method can be a computer implemented method.

 Optionally, the compensation is based on a comparison reflectance value representative for the portion of the coating underlying the biofilm biomass. Thus the reflectance value of the coating underlying the biomass in the one or more images can be taken into account through the comparison reflectance value. The
30 comparison reflectance value can be a reflectance value determined for a part of the coating of the object from which all biomass has been removed; a reflectance value determined from a reference object; or a reflectance value stored in a

database. The comparison reflectance value can be a minimum value of the reflectance value for the coating underlying the biofilm biomass in the one or more spectral bands in which the one or more images are obtained.

Thus, in step d) the biomass pigment surface area density, $biomass_{SI}$, can be determined as if the current coating were a reference coating having a reference reflectance spectrum. Then in step e) biomass density on the current coating surface, $biomass_{current}$, can be estimated by compensating the biomass pigment surface area density, $biomass_{SI}$, determined for the reference coating for the difference of reflectance of the current coating relative to the reference coating. Optionally, reflectance of the reference coating is determined at a predetermined wavelength, and reflectance of the current coating is determined at the same predetermined wavelength for determining the difference of reflectance of the current coating relative to the reference coating.

Optionally, the biomass estimation calculated from the Spectral Index and one or more calibration values, $biomass_{SI}$, can be adjusted by an Error Factor, $EF_{current}$, and Baseline Error, $BE_{current}$, both of which are functions of the reflectance spectrum of the current coating upon which the biomass is present. Then the estimated biomass on the current coating, $biomass_{current}$, can be determined from the equation

$$biomass_{current} = biomass_{SI} * (1 - \left(\frac{EF_{current}}{1+EF_{current}}\right)) - BE_{current}. \text{ EQ1}$$

Optionally, the spectral band is 100nm wide, FWHM, or less, such as 50 nm wide, FWHM, or less, such as 30 nm wide, FWHM, or less, such as 5 nm FWHM, or less. The spectral band can be at least 0.1 nm, for example at least 1 nm. Thus, the spectral band can be in the range of from 0.1 to 100, 0.1 to 50, 0.1 to 30 or 0.1 to 5, such as from 1 to 100, from 1 to 50 or from 1 to 30 or from 1 to 5.

Optionally, at least one of the spectral bands is chosen to encompass an absorption wavelength of chlorophyll, such as chlorophyll a. When obtaining a single digital image of the fouled portion of the coating on the object, the spectral band can be chosen to encompass an absorption wavelength of chlorophyll. When obtaining two or more digital images of the fouled portion of the coating on the object, at least one of those images can be obtained in the spectral band encompassing an absorption wavelength of chlorophyll. When obtaining two or

more digital images of the fouled portion of the coating on the object at least one of the images can be obtained in a spectral band chosen to exclude an absorption wavelength of chlorophyll. Hence, the presence of biomass can be determined from the absorption of light by chlorophyll.

5 In embodiments, the spectral band lies within the range of 350 to 900 nm, for example in the range of from 400 to 850 nm, i.e. the spectral band encompasses one or more wavelengths falling within the above ranges. Optionally at least one of the spectral bands is chosen around 433, 460, 496, 555, 584, 601, 673, or 800 nm, i.e. such that the spectral band either is or encompasses any of
10 the specified wavelengths. At least one of the spectral bands can be chosen to be within the range of 390-480 nm, 410-510 nm, 440-550 nm, 500-610 nm, 525-645 nm, 540-660 nm, 605-740 nm, or 720-900 nm.

Optionally, the spectral index determined in step c) includes a ratio of two reflectance values. The ratio of reflectance values allows normalization of the
15 reflectance value, e.g. relative to reflectance of the object coating. Optionally, the spectral index determined in step c) includes a sum and/or difference of two reflectance values. One of the reflectance values can be for a spectral band encompassing an absorption wavelength of chlorophyll. The other of the reflectance values can be for a spectral band not encompassing an absorption
20 wavelength of chlorophyll. Hence, the relative effect of the absorption of light by chlorophyll can be accounted for.

Optionally, the spectral index, SI, determined in step c) is one of:

- coating normalized red reflectance defined as

$$SI = rNorm673 = \frac{R673}{R673_{clean}}; \text{ or}$$

25 - Normalized Difference Vegetation Index defined as

$$SI = NDVI = \frac{R800+R673}{R800-R673}.$$

It will be appreciated, however, that other spectral indices may be used, such as

(but not limited to) Microphytobenthos Index, defined as $SI = MPBI =$

$$\frac{2 \cdot R584}{R496+R673} - 1, \text{ Ocean Color Index, defined as } SI = OC = \frac{R443}{R555}, \text{ or Diatom}$$

30 Index, defined as $SI = I_{diatom} = \frac{2 \cdot R601}{R460+R673} - 1.$

Herein R_{433} is the determined reflectance value in the range of 390-480 nm, such as at 433 nm; R_{460} is the determined reflectance value in the range of 410-510 nm, such as at 460 nm; R_{496} is the determined reflectance value in the range of 440-550 nm, such as at 496 nm; R_{555} is the determined reflectance value in the range of 500-610 nm, such as at 555 nm; R_{584} is the determined reflectance value in the range of 525-645 nm, such as at 584 nm; R_{601} is the determined reflectance value in the range of 540-660 nm, such as at 601 nm; R_{673} is the determined reflectance value in the range of 605-740 nm, such as at 673 nm; R_{800} is the determined reflectance value in the range of 720-900 nm, such as at 800 nm; $R_{673_{clean}}$ is the reflectance value in the range of 605-740 nm, such as at 673 nm at the coating of the object without biomass, such as a determined reflectance value at a cleaned portion of the object, a reflectance value of a reference having the same or similar coating as the object or a stored reference reflectance value. The spectral indices can be used for estimating the biomass pigment surface area density in step d). It has been found that the spectral indices coating normalized red reflectance and Normalized Difference Vegetation Index provide a good estimate of biomass pigment surface area density.

From the above it can be seen that the coating normalized red reflectance can be determined from a single digital image of the fouled portion of the coating on the object in a spectral band, in combination with a stored value for $R_{673_{clean}}$. In the other instances the spectral index is determined from two or more digital images of the fouled portion of the coating on the object in mutually different spectral bands. The two or more images could be separate layers of a hyperspectral image.

The calculating of the biomass pigment surface area density in step d) is based on one or more calibration values. The calibration values can be determined for a reference coating having a predetermined reflectance. According to the invention, the reference coating need not be the current coating on the object. The step e) is provided for compensating the biomass pigment surface area density calculated on the basis of the current coating for the reflectance of the coating of the object by applying a compensation associated with the reflectance of the coating of the object relative to the reference coating.

Optionally, in step d) the pigment surface area density is determined from

$$\text{pigment surface area density} = \left(\frac{SI-c}{a} \right)^{\frac{1}{b}} \quad \text{EQ2}$$

wherein SI is the spectral index value and a , b and c are calibration values. The calibration values a , b and c can be determined for the reference coating having a predetermined reflectance.

In an example, the calibration values a , b and c have been determined for a reference coating having a grey color and a reflectance value of 24%. In that case, the following calibration values have been found.

- 10 - if the spectral index is the coating normalized red reflectance, $a = -1.921$, $b = 0.093$, $c = 2.477$; and
- if the spectral index is the Normalized Difference Vegetation, $a = 0.618$, $b = 0.240$, $c = -0.321$.

Since these calibration values result in step d) in an estimated biomass pigment surface area density, biomass_{SI} , applying if the current coating were the reference coating, in step e) the estimated biomass pigment surface area density, biomass_{SI} , can be compensated for the reflectance difference of the current coating of the object relative to the reference coating by applying a compensation associated with the reflectance of the coating of the object relative to the reference coating. As indicated above, the estimated biomass on the current coating, $\text{biomass}_{current}$, can be determined from the equation EQ1. Thus, when combining equations EQ1 and EQ2 the biomass pigment surface area density for the current coating, $\text{biomass}_{current}$, can be determined from:

$$\text{biomass}_{current} = \left(\frac{SI-c}{a} \right)^{\frac{1}{b}} \cdot \left(1 - \left(\frac{EF_{current}}{1+EF_{current}} \right) \right) - BE_{current} \quad \text{EQ3}$$

25 It will be appreciated that the steps d) and e) can be performed as separate steps, or as a combined step. For example, equations EQ1 and EQ2 can be used when performing the steps d) and e) separately. The equation EQ3 can for instance be used when performing the steps d) and e) as a combined step.

The following exemplary Error Factor, $EF_{current}$, and Baseline Error, $BE_{current}$, values have been found:

30 $BE_{current}$, values have been found:

- if the spectral index is the coating normalized red reflectance,
 $EF_{current} = 0.26 \ln (R_{current673}) - 1.00$; $BE_{current} = 0.0862 \ln (R_{current673}) + 0.0164$;
- if the spectral index is the Normalized Difference Vegetation,
 $EF_{current} = 0.24 \ln (R_{current673}) - 0.83$; $BE_{current} = 0.025 \ln (R_{current673}) + 0.1025$;

5 wherein $R_{current673}$ is the reflectance value of the current coating at 673 nm.

Using these calibration curves the Error Factor, $EF_{current}$, and Baseline Error, $BE_{current}$, values for a particular coating on the object can be determined. Thus, it is possible to determine an estimate for the biomass pigment surface area density for the current coating, $biomass_{current}$, using the calibration values a , b and c determined for a reference coating and the Error Factor, $EF_{current}$, and Baseline Error, $BE_{current}$, values compensating for the difference of reflectance of the current coating relative to the reference coating.

15 Optionally, the one or more digital images are obtained using a submarine, such as an unmanned submarine. The submarine can include a digital camera, such as a hyperspectral camera. Alternatively, or additionally, the one or more digital images can be obtained by a diver, hull crawler, imaging unit lowerable along the object, a (dry dock) photo booth, or the like.

According to another aspect of the invention, there is provided a system for estimating aquatic environment-originating biofilm biomass on a coating on an object, that is e.g. immersed permanently or intermittently in an aquatic environment. The system includes a processor. The processor is configured for obtaining one or more digital images of a fouled portion of the coating on the object. The processor is configured for determining from each of the one or more images a respective reflectance value for the portion of the coating. The processor is configured for determining on the basis of the one or more reflectance values a value of a spectral index representative of biomass. The processor is configured for calculating a biomass pigment surface area density on the basis of the spectral index, SI, and one or more calibration values determined for a reference coating. The processor is configured for compensating the calculated biomass pigment surface area density for the reflectance of the coating of the object by applying a compensation associated with the reflectance of the coating of the object.

Optionally, the system includes a digital camera,. The digital camera can be used for obtaining the one or more images. An image obtained by the camera can be obtained a spectral band. The camera can e.g. be a general purpose digital camera, wherein one or more of the red, green and blue channels, and optionally an infra-red channel, of the camera can be used as the spectral band. The digital camera can include a spectral band filter, e.g. having a band width of 0-100 nm FWHM. The camera can be a multispectral camera. The multispectral camera can e.g. have a red spectral band from about 600 nm to about 700 nm. The multispectral camera can have an infra-red spectral band from about 750 nm to about 950 nm or higher. The multispectral camera can e.g. have a blue spectral band from about 400 nm to about 500 nm. The multispectral camera can e.g. have a green spectral band from about 500 nm to about 600 nm. The camera can be a hyperspectral camera.

The digital camera can be held by a diver. Optionally, the system includes a submarine, such as an unmanned submarine, for obtaining the images of the object below the water line. Alternatively, or additionally, the system includes a hull crawler, an imaging unit lowerable along the object, a (dry dock) photo booth, or the like.

According to a further aspect of the invention, there is provided a computer program product for estimating biofilm biomass on a coating on an object that is immersed permanently or intermittently in an aquatic environment including computer implementable instructions which when implemented by a programmable computer cause the computer to:

- obtain one or more digital images of a fouled portion of the coating on the object;
- determine from each of the one or more images a respective reflectance value for the portion of the coating;
- determine on the basis of the one or more reflectance values a value of a spectral index representative of biomass;
- calculate a biomass pigment surface area density on the basis of the spectral index, SI, and one or more calibration values determined for a reference coating; and

- compensate the calculated biomass pigment surface area density for the reflectance of the coating of the object by applying a compensation associated with the difference of the reflectance of the coating of the object relative to the reference coating.

5 The computer program product can be stored on a non-volatile memory device.

 It will be appreciated that all features and options mentioned in view of the method apply equally to the system and computer program product, and vice versa. It will also be clear that any one or more of the above aspects, features
10 and options can be combined.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the present invention will now be described in detail with reference to the accompanying drawings in which:

15 Figure 1 shows an example of a system;
 Figures 2A and 2B show examples of an experimental setup;
 Figure 3 shows reflectance spectra of exemplary coatings;
 Figure 4 shows reflectance spectra;
 Figures 5A and 5B show examples of determined values of spectral
20 indices as a function of measured chl a density;
 Figures 6A-6F show examples of measured spectra;
 Figures 7A-7F show examples of normalized spectra;
 Figure 8 shows NDVI estimation errors;
 Figures 9A and 9B show estimation of error factors and baseline errors;
25 Figure 10 shows spectral band widths;
 Figures 11A and 11B show estimation errors; and
 Figure 12 shows a schematic representation of a method.

DETAILED DESCRIPTION

Objects immersed permanently or intermittently in an aquatic environment are prone to fouling by biofilms. Such fouling biofilms are generally comprised of diverse communities of microbial organisms embedded in an extracellular matrix that the cells exude. The matrix provides adhesion, cohesion and protection. Example microbes generally responsible for biofilm matrix formation include bacteria, archaea, and microalgae such as cyanobacteria and protozoa. Because these types of microbes are key constituents, aquatic fouling biofilms are interchangeably referred to as microfouling. It is possible, however, for fouling biofilms to include other passengers incorporated into the matrix, such as detritus or non-living sediment, embedded cells which are usually categorized as planktonic (e.g. phytoplankton), or small multi-cellular organisms such as fungi or minor filamentous algae.

To reduce fouling growth, immersed surfaces, (e.g. ship hulls, static oil rigs, marine renewable devices) can be coated in marine fouling control coatings which deter, although not completely prevent, biological growth.

Quantitative characterisation of the marine biofilms found on fouling control coatings provides insight as to the coating modes of action and efficacy. Microscopy, phospholipid analysis, microelectrodes, and optical coherence tomography are among the many techniques that have been used to explore biofilm processes. A commonality across these methods is they hinge on collection and/or analysis of small biofilm samples, but this has limited practicality to capture variability in biofouling across a large object, such as a vessel. It has been found that hyperspectral imaging is highly suitable to map fouling by pigmented organisms such as algae, microalgae and/or bacteria, such as photosynthetic algae, microalgae and/or bacteria.

Biofilms that foul immersed objects, such as ships, are generally phototrophic. Diatoms are usually reported as the primary microalgal constituent taxon in fouling biofilms. Other microalgal phototrophs reported in fouling biofilms include cyanobacteria and additional eukaryotes. Pigmented bacteria and/or algal biomass can be characterised by spectral reflectance data, as algal taxa have characteristic pigments (e.g. photosynthetic and accessory pigments) which result in characteristic reflectance spectral features. Chlorophyll a, herein also denoted

as Chl *a*, the pigment common to all photosynthetic groups, for example, absorbs strongly at about 670nm and this feature generally does not overlap with any other pigment peaks.

Spectral imaging captures quantitative spectral reflectance data and so
5 is well suited to examining large spatial processes relating to algal biomass as can be determined from algal spectral characteristics. Spectral imaging readily scales, in contrast to sample-derived biofilm characterisation methods, and thus has high potential for characterising immersed object biofilm fouling processes. The image obtained by spectral imaging can include one or more pixels.

10 An advantage of the invention is that it is possible to map fouling over large areas to give an overall picture of the fouling on the structure, and hence can help to identify any problem areas that may need particular attention, e.g. cleaning. However, it can also be adapted to be used over smaller areas, even individual pixels, such that more rapid information on specific areas of interest can be
15 obtained.

Hyperspectral imaging can quantify and map biomass using hyperspectral biomass indices calibrated to algal pigment concentrations. Chlorophyll *a* is an established proxy of primary productivity and biomass, and so can be adopted for hyperspectral calibration. Chl *a* can be indexed by comparing
20 the absorbance feature depth at about 670nm with reflectance at a reference wavelength in near infrared or blue which are less influenced by other pigments. Examples are the Normalised Difference Vegetation Index, NDVI, or Microphytobenthos Index, MPBI. Additional indices which quantify euglenid (green algae) and diatom (brown algae) biomass have also been developed.

25 Biofilm reflectance spectra are modified by the spectra of the underlying substrata. In marine shipping, colourful marine fouling control coatings are the substrata, so accounting for their spectral signatures is necessary when taking spectral measurements of biofilm fouling. Biocidal antifouling coatings are very commonly red or reddish-brown due to higher copper oxide content, for example,
30 and coatings applied to floating marine renewable platforms are required to be yellow for high visibility, while yacht antifouling coatings are formulated in a rainbow of colours. These coloured coatings all have non-flat reflectance spectra, so

methods can be used to ensure the spectral reflectance of the background coating does not skew experimental results.

Figure 12 illustrates a method 100 for estimating aquatic environment-originating biofilm biomass on a coating of an object, that is e.g. immersed permanently or intermittently in an aquatic environment. The method includes:

- a) obtaining 102 one or more digital images of a fouled portion of the coating on the object in a spectral band;
- b) determining 104 from each of the one or more images a respective reflectance value for the portion of the coating;
- 10 c) determining 106 on the basis of the one or more reflectance values a value of a spectral index representative of biomass;
- d) calculating 108 on the basis of the spectral index, SI, and one or more calibration values a biomass pigment surface area density; and
- e) compensating 110 the calculated biomass pigment surface area density for the reflectance of the coating of the object by applying a compensation associated with the reflectance of the coating of the object.

15 Examples of the steps a) through e) will be described in more detail below.

Figure 1 shows an example of a system 10 for estimating aquatic environment-originating biofilm biomass on a coating 12 on an object 14, that is e.g. immersed permanently or intermittently in an aquatic environment. In this example, the object 14 is a vessel, partially submerged below water line 16. In this example, the system 10 includes a digital camera 18, such as a hyperspectral camera, arranged for obtaining one or more digital images of a fouled portion 20 of the coating on the object 14 in a spectral band. The spectral band can e.g. have a spectral width in the range of 0-100 nm FWHM (full width at half maximum). The digital camera can include one or more pixels. The digital camera can include a 1D sensor array, sometimes referred to as line-scan camera. The digital camera can include a 2D sensor array.

30 The system 10 includes a processor 22 connected or connectable to the camera 18. The processor can be part of an under-water portion of the system. It is also possible that the processor is part of an above-water portion of the system.

The processor 22 is arranged for obtaining one or more digital images of a fouled portion of the coating on the object from the camera. The processor 22 is arranged for determining from each of the one or more images a respective reflectance value for the portion of the coating. The processor is arranged for determining on the basis of the one or more reflectance values a value of a spectral index representative of biomass. The processor 22 is arranged for calculating a biomass pigment surface area density on the basis of the spectral index, SI, and one or more calibration values determined for a reference coating. The processor 22 is arranged for compensating the calculated biomass pigment surface area density for the reflectance of the coating of the object by applying a compensation associated with the difference of the reflectance of the coating of the object relative to the reference coating. This will be explained in more detail below.

The following experiments demonstrate that a good estimate for microfouling biomass can be achieved by compensating the calculated biomass pigment surface area density for the reflectance of the coating of the object by applying a compensation associated with the reflectance of the coating of the object.

BIOFILM EXPERIMENTS

The experiments were based on cultured microalgal and bacterial biofilms approximating what has been observed in real world marine microbial ship fouling. Monoculture biofilms of various densities were prepared. The monoculture species were selected from algal groups that have been reported in literature as present in microfouling assemblages and/or observed in fouling biofilm samples. Mixed population microalgal and bacterial biofilms seeded from sampled marine fouling were also prepared and tested to provide insight as to the effect of taxonomic heterogeneity.

The biofilms were grown on glass and the fouled glass positioned over each of six coating colours for spectral imaging, so that the same biofilms could be measured with different colour backgrounds.

Biofilm cultures

Monocultures: Monocultures of two diatom species (*Achnanthes* sp, CCAP1095/1, *Amphora* sp, CCAP1001/3), a green algae (*Chlorella* sp,

CCAP211/53), and a cyanobacterium (*Nodularia* sp, CCAP1452/6) were obtained from the Scottish Association of Marine Science Culture Collection of Algae and Protozoa (SAMS CCAP). For each species, the original seed cultures (20mL) were scaled up to 3 x 125mL cell suspensions at 18°C under a 12 hour on/off light cycle
5 (Sylvania T8 fluorescent white cool 840 + white warm 840 (Lorenz et al., 2005, Maintenance of Actively Metabolizing Microalgal Cultures. Algal culturing techniques, 145). The microalgae were cultured as per the CCAP specifications in nutrient-enriched artificial sea water microalgal growth media: Guillard's f2 + Si – a general marine microalgal growth medium with an addition of silica to support
10 diatom growth (*Achnanthes*, *Amphora*), Guillard's f2 – without the silica (*Chlorella*), (Guillard and Ryther, 1962, Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. Canadian journal of microbiology, 8, 229-239), or Blue Green Media – developed for culture of blue-green algae (*Nodularia*) (Stanier et al., 1971, Purification and properties of
15 unicellular blue-green algae (order Chroococcales). Bacteriological reviews, 35, 171).

Mixed population culture HP (Hartlepool): The source populations for the HP culture were biofilms collected from non-toxic panels immersed approximately 0.5m below the surface for 3-6 months in Hartlepool Marina on the
20 northeastern English coast. The biofilm samples were blended in Guillard's f2 + Si media to create a 500mL cell suspension and filtered through a 125 µm filter to remove large particles.

Mixed population culture IP (International Paint): The source populations for the IP culture were biofilms collected from non-toxic panels
25 immersed approximately 0.5m below the surface for 1 month in Raffles Marina, Singapore. The biofilm samples were blended in seawater to create a cell suspension and filtered through a 125 µm filter to remove large particles. The 1L cell suspension culture was scaled up at 25°C under a 12 hour on/off light cycle (58W Marine White, Actinic Blue, Arcadia) in International Paint's 250L
30 recirculating mixed population autotrophic marine biofilm culturing system (Longyear, 2014, Section 2 Mixed population fermentor. Biofouling Methods, 214) containing artificial seawater enriched with Guillard's f2 + Si nutrient medium.

Biofilm formation

All biofilms were grown on glass cover slips (circular 19mm diameter, Fisher Scientific). As they are fragile, the cover slips were placed on glass microscope slides (25x75mm, Fisher, 2-3 per slide) for support and secured with microplate sealing film. The sealing film was pre-punctured with circular holes (9mm diameter) created with a crafting hole punch and centred on the underlying cover slips. This configuration exposed only the central area of each coverslip to fouling. When the masking films were removed after the growth period, the resulting biofilms had well defined perimeters and were surrounded by contrasting areas of clean glass.

For the monocultures and mixed culture HP, the biofilms were grown in 4-well plates (Fisher Scientific) (2-3 plates per population). In order to culture a range of biofilm densities, the ratio of inoculate (the parent cultures) to nutrient media added to each 10mL well was increased sequentially, with the final well containing exclusively inoculate. The plates were sealed, and biofilms cultured at 18°C under a 12 hour on/off light cycle (Sylvania T8 fluorescent white cool 840 + white warm 840; (Lorenz et al., 2005, Maintenance of Actively Metabolizing Microalgal Cultures. Algal culturing techniques, 145). Every two weeks, 5mL of nutrient media per well was replaced by serological pipetting, careful not to disrupt the biofilms, and the plates were resealed, until biofilms of visible varying densities had developed across the masking films and exposed glass. After the growth period (circa 2 months) the masking was removed and 8-12 slips with biofilms from each population were prepared for imaging.

For mixed culture IP, the slides were placed horizontally into illuminated, 8cm deep channels integrated into the recirculating culture system. Over the course of several weeks, spatially heterogeneous biofilms colonised all surfaces of the channel, including the slides. The slides were removed from the channel and the masking removed. Coverslips fouled by a range of biofilm densities and compositions were prepared for imaging.

Instrument Configuration

Biofilms were imaged with a hyperspectral line scanning system (Resonon benchtop pikaXC, 377-1029nm, 3.3nm wavebands, 200 channels,

17mm focal length Schneider objective lens, 30.8° field of view). With this instrument the sample is placed on an automated translation stage beneath the lens, the imager collects spectra for a single line of 1600 pixels in width, and the image is created by continuous capture of data at a user-defined frame rate as the
5 sample stage is moved across the field of view (FOV). The spatial width dimension of each pixel is determined by the lens FOV and distance from the lens to the imaging surface. The length dimension of each pixel is determined by the speed of the linear translational stage and frame rate. The imaging system was set to generate approximately square aspect ratio pixels approximately 0.06mm x
10 0.06mm, which allowed for the full length of a 75mm microscope slide to be imaged within a single scan.

The translation stage was illuminated across the visible and near-infrared spectrum (in the range of about 380nm to about 980nm) by a four-light assembly of wide spectrum quartz halogen lamps (Resonon). The dynamic range
15 of the images was maximised by setting the instrument exposure level manually using the lens iris so that the reflectance spectrum measured from a piece of white Teflon (Resonon) spanned, but did not saturate, the 14-bit sensitivity of the instrument.

Instrument electrical noise spectra were collected with the lens cap in place and accounted for using the instrument software (Spectranon) dark correction as specified by the manufacturer. Variable illumination across the imaging field due to the positioning of the lamps was measured by imaging a plain white Teflon panel and accounted for in the software using the standard response correction function as recommended by the manufacturer.
20

25 A Spectralon reflectance standard (50% flat spectral response from 360-1100nm, NIST-certified) was included centrally in each experimental image field of view to allow for reflectance calibration during image processing.

Biofilm Imaging

Each biofilm was repeatedly imaged with different colour underlying
30 backgrounds through a process of gentle repositioning of the glass coverslips on coated microscope slides.

Non-toxic backgrounds: Grey-scale (white, grey, black) and primary colour (red, yellow and blue) variants of the fouling release coating Intersleek™ 900 (International Paint, Ltd) were chosen as they represented a range of brightness levels and spectral characteristics and were also non-toxic. The coatings were applied by roller to glass microscope slides over an anticorrosive primer and fouling release tie coat (Intershield™ 300 and Intersleek™ 757), following the coating's specified application scheme. The slides were immersed in seawater in the Hartlepool Marina for several months, retrieved, and cleaned thoroughly with a sponge.

Biofilm transfers and repeat imaging: The first colour microscope slides were placed in an inverted well plate cover and filled to a shallow depth (<2mm) with artificial seawater. Three or four cover slips with biofilms were gently placed atop each slide with forceps, taking care not to dislodge the biofilms. When all biofilms per culture (8 to 12 biofilm for each) were loaded in to the dish, the image was collected. The next set of colour slides were then placed adjacent to the first, and the cover slips were gently slid with forceps across from one colour to another, after which the first colour slides were removed. The second set of slides were centered in the dish and the second image was collected, and so on. Approximately every 2 images the water in the dish was replaced because it warmed under the halogen lamps. The order in which the colours were used was arbitrarily chosen to minimise any order effects if any small portions of biomass were dislodged during transfer (although this was rarely observed). By using this strategy spectra could be acquired from the exact same biofilms but with different colour backgrounds thus limiting background colour as the only variable.

Biofilm pigment analysis

Pigment analysis provided the traditional, quantitative, but destructive metric of microalgal biomass and generated the foundation dataset for post-processing spectral index calibrations.

Pigment extraction: After biofilm imaging, the biofilms and cover slips were sandwiched in glass fibre filters (0.45µm), flash frozen, and placed in cryostorage (LN₂ vapour phase) until extraction (8-10 months). Algal pigments were extracted in 1mL chilled methanol (MeOH, 100%, analytical grade) with 1

minute sonication (55watt QSonica sonicator) to rupture cell membranes and disrupt the biofilm matrix, then syringed through an in-line 0.45µm filter (Watman) to remove remaining sediment.

High Performance Liquid Chromatography (HPLC): HPLC analysis of the biofilm extracts followed the method outlined by Van Heukelem and Thomas (Van Heukelem and Thomas, 2001, Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. Journal of Chromatography A, 910, 31-49), adjusted to use with an Agilent HP 1100 instrument (Agilent Technologies, CA) with a diode array detector (UV signal/wavelength = 450nm/4, wavelength range 350-750nm) and Zorbax Eclipse XDB-C8 (RP, 3.5µm, 4.6x150mm) column. On the day of analysis, a maximum of 18 samples were first mixed with a buffer (28mM aqueous tetrabutyl ammonium acetate (TBAA), pH 6.5) in a 1:1 preparation prior to the run, kept in amber vials, held queued at 5°C in the chilled auto-sampler, and run overnight. Mobile phases A (70:30 (v/v) methanol, 28mM aqueous TBAA, pH 6.5) and B (methanol) were introduced by a 1.1ml/min flow following the profile [0 min: 95% A, 5% B; 22 min: 5% A, 95% B; 31min, 95%A, 5%B], with a column temperature of 60°C.

Determination of pigment area densities: The HPLC instrument and method were calibrated for peak height for sixteen microalgal pigments that are found in microalgal groups such as diatoms, green algae, cyanobacteria, dinoflagellates, and cryptophytes that are often found on microfouled ship and immersion board samples. The pigments were chlorophylls *a*, *b* and *c2*, peridinin, prasinoxanthin, violaxanthin, lutein, fucoxanthin, zeaxanthin, beta-carotene, diadinoxanthin; alloxanthin; myxoxanthophyll; divinyl chlorophyll *a*, 19-hex-fucoxanthin; and neoxanthin; from commercial supplier DHI. Experimental sample chromatograms were analysed, and peak identities confirmed by absolute and relative retention times and UV absorbance spectra for each peak related to a calibrated pigment. Concentration of the sixteen calibrated pigments [ng/mL MeOH] in each biofilm were determined from chromatogram peak height. The concentrations were converted to pigment surface area density [µg/cm²] by dividing

each pigment concentration by biofilm area, measured with the manual pixel selection tool after scaling the experimental images in ImageJ (NIH. 1.51d).

BIOMASS ESTIMATION FROM HYPERSPECTRAL INDICES

5 Non-destructive hyperspectral measurements and destructive pigment analysis measurements were linked through the development of calibrated formulae to convert spectral index values into estimates of biomass.

Background coating reflectance spectra

Figure 2A shows how coating reflectance spectra were measured from
 10 clean areas of coverslip glass (solid line, 1); biofilm spectra were measured from regions of interest, ROIs, including all biomass (dotted line, 2) and paired local coating spectra were measured from ROIs of clean glass surrounding the biofilm (shaded region, 3). The mean reflectance spectra of the coatings, $R_{coating}$, were measured from ROIs drawn around clean areas of the glass coverslips in the
 15 hyperspectral images (see Figure 2A at 1) using the manual selection tool of the imager's software package (Spectranon, version 2.68, Resonon, MT, USA). Mean reflectance spectra of a 50% reflectance standard, R_{ref} , (see Figure 2B at 4) were also measured from the hyperspectral images. The measured coating spectra were converted from brightness digital number measurements [DN] to percentage
 20 reflectance of incident light [0-100%] by scaling to the spectra of the reflectance standard, where

$$R_{coating} [\%] = R_{coating} [DN] \times 0.5 [\%] / R_{ref} [DN].$$

Figure 3 shows that the reflectance spectra of the white (W), grey (G) and black (Bk) coatings were relatively flat (64%, 24%, and 1% mean reflectance,
 25 respectively), while the spectra of the primary-coloured coatings yellow (Y), red (R) and blue (Bl) had colour-specific reflectance and absorbance features.

In this experiment the grey (G) coating subset of the hyperspectral images was selected as reference coating for calibrating the hyperspectral indices, as the coating was found to have a flat spectral response and overall reflectivity
 30 closely matching the previously published spectra of natural sediments. It will be appreciated, however, that any other coating or substrate could be selected as reference.

Biofilm reflectance spectra

For each biofilm (n) of each culture (c), the mean spectrum was measured for the ROI tracing the perimeter of the biofilm, $R_{bio_{m(c,n)}}$, (Figure 2A, arrow 2). A grey coating ROI local to each biofilm, $R_{grey_{m(c,n)}}$, (Figure 2A, arrow 3) was also defined, enclosing an area of clean glass coverslip surrounding the biofilm. Local coating spectra were measured because the surfaces were not perfectly flat, causing local variation in illumination.

Estimating Biomass by Spectral Indexing

Two hyperspectral indices drawn from microalgae remote sensing literature were assessed for their suitability to estimate the biomass of the spectrally variant experimental biofilms. The calibrations of the indices to pigment spatial densities were ranked by their estimation errors.

Spectral indices: The various indices combine wavelengths across the visible and near-infrared spectrum. Table 1 below provides Microalgal biomass hyperspectral index details.

Herein R_{673} is the determined reflectance value in the range around 673 nm, such as in the range of 605-740 nm, such as at 673 nm; R_{800} is the determined reflectance value in the range around 800 nm, such as in the range of 720-900 nm, such as at 800 nm; $R_{673_{clean}}$ is the reflectance value in the range around 673 nm, such as in the range of 605-740 nm, such as at 673 nm at the coating of the object without biomass.

Table 1

Index	Equation	General (G) / Specific (S) Biomass Estimator
Coating-normalised red reflectance	$rNorm_{673} = \frac{R_{673}}{R_{673_{clean}}}$	G: a simple measure of the chlorophyll a absorbance feature at about 673nm
Normalised Difference Vegetation Index	$NDVI = \frac{R_{800} - R_{673}}{R_{800} + R_{673}}$	G: Compares chlorophyll a absorbance feature at 673nm with an NIR baseline that is theoretically invariant with biomass (single wavelengths adopted instead of broad waveband averages)

Index Calibrations to Biomass: Power models, fit by nonlinear least squares estimation, were defined to describe the relationships between the spectral indices calculated from the biofilm datasets, $Rbio_{norm(g)}$, and the spatial densities of select biofilm pigments, where

$$index = a * pigment\ density^b + c$$

From this fit the calibration values a , b and c are determined.

Index values for the two indices rNorm and NDVI were calculated for all the normalised biofilm spectra and were compared to biofilm densities of the universal photosynthetic pigment chlorophyll a , a widely accepted biomass proxy.

Comparison of biomass estimation errors across indices: To make a quantitative assessment of how well the indices estimated biomass (measured as pigment surface area density), the power models were inverted to conversion formulae, where

$$pigment\ density = \left(\frac{index - c}{a} \right)^{\frac{1}{b}}$$

For every biofilm, the index values calculated from $Rbio_{norm(g)}$ were converted to biomass estimates and the residual sum of squares and mean estimation error (estimated – measured pigment density) were calculated for all indices.

BIOMASS ESTIMATION ON ANY COLOUR BACKGROUND

Fouling control coatings are pigmented to a range of standard and custom colours, each with characteristic spectral reflectance and absorption features of potentially higher magnitude than the spectral features of any fouling microalgae. The index calibrations derived above were tested for universal applicability to microfouling across the six coloured coatings.

Colour-specific coating-normalised biofilm reflectance spectra

The coating and biofilm spectra for the white, black, red, blue and yellow coating hyperspectral images were processed following the procedures outlined for the grey coating dataset: measurement of biofilm and local coating spectra ($Rbio_m$, $Rcoating_m$), conversion to percent reflectance, and normalisation of all biofilm spectra to the local coating measurement, ($Rbio_{norm(w)}$, $Rbio_{norm(bk)}$,

$R_{bio_{norm}(r)}$, $R_{bio_{norm}(bl)}$, $R_{bio_{norm}(y)}$). The original grey-normalised data ($R_{bio_{norm}(g)}$) were also included in the analyses.

Estimations of biomass on all coatings

Values for the two spectral indices were calculated from the coating-normalised biofilm reflectance spectra for all six coating colours and all biofilms. The calibrated conversion formulae were then used to estimate biomass from every index value for all biofilms on all coatings.

Biomass estimation errors (estimated minus measured pigment density) were calculated for each index and each colour coating. To examine any biomass-linked estimation error patterns, errors for each coating colour dataset were compared to measured biomass and fit with linear regressions to determine baseline error (intercept) and error factor (slope). The error factor and baseline estimation error for each coating colour were compared to the minimum of the coating reflectance values at the index wavelengths. For NDVI, for example, error factor and baseline error for each colour coating were compared to the minimum of coating reflectance at 673 and 800nm. Logarithmic equations were calculated to describe the relationships between the baseline estimation errors and error factors and coating reflectance at the index wavelengths.

RESULTS

Biofilm Formation

Overall, the experimental culturing methods successfully resulted in six sets of spatially defined biofilms of varying densities.

Biofilm Pigments as determined by HPLC

Monocultures: Across all species, chlorophyll *a* was the primary pigment and was present at the highest densities. For the diatom species, fucoxanthin was also present at high levels, and notable minor pigments included chl *c2*, beta carotene, and, for *Amphora* *sp.*, diadinoxanthin. The minor pigment suite for *Nodularia* *sp.* was limited solely to beta carotene, but additional phycobilin pigments not measurable by HPLC are likely also to have been present and contributed to structuring the biofilm reflectance spectra. The *Chlorella* *sp.* biofilms contained high levels of chlorophyll *b*, and detectable levels of the minor pigments violaxanthin, alloxanthin, zeaxanthin, lutein, and beta carotene.

Mixed cultures: For the HP biofilms, chl *a* was the primary pigment, with a maximum density of 1.6 $\mu\text{g}/\text{cm}^2$, giving a range of densities similar to most of the monocultures. Fucoxanthin was the next major pigment, confirming the observation that diatoms were abundant in these biofilms. Of the suite of remaining pigments, chl *b* indicated presence of green algae, alloxanthin indicated presence of cryptophytes, while high values of beta carotene were not readily attributable to any taxon.

The range of chl *a* densities for the IP biofilms was much greater (maximum density 6 $\mu\text{g}/\text{cm}^2$) than for all other cultures barring the *Achnanthes sp* monoculture. Fucoxanthin was present only in very minor amounts, indicating diatoms were not prevalent in these samples, which agreed with the microscopy. Alloxanthin, indicative of cryptophytes, was highly concentrated in the redder biofilms, suggesting an identity for the small red-brown flagellated single cell algae observed by microscopy. Relatively high levels of chl *b* indicated presence of green algae, matching the bright lime green visual appearance of some of the biofilms. The diverse additional pigments, including those unidentifiable, underscored the diversity and heterogeneity of the IP biofilms.

BIOMASS ESTIMATION FROM HYPERSPECTRAL INDICES

20 *Microalgal biofilms reflectance spectra*

The measured reflectance spectra from the six sets of laboratory biofilms had varied characteristic absorbance features, which were highly visible against the background flat spectral reflectance of the grey coating as can be seen in the absorbance spectra shown in Figure 4. Figure 4 shows reflectance spectra of the six laboratory biofilm sets, where the y axis gives the observed reflectance range. Deepening spectral features indicate denser biomass. Panel (a) shows *Achnanthes sp.* biofilms (b) *Amphora sp.* (c) HP mixed culture biofilms (d) *Nodularia sp.* biofilms (e) *Chlorella sp.* biofilms and (f) IP mixed culture biofilms. There was significant noise in the measurements below 400nm, as well as minimal illumination, and so the wavelengths below 400nm were not considered further in the experiment. In the infrared there was little observable change in the reflectance

spectra from 850nm to 1100nm apart from increasing noise, and so wavelengths above 850nm also were not considered further in the experiment.

The spectra of all six cultures were marked by an absorbance feature at 673nm, which is attributable to chlorophyll *a* and deepened as the density of the biofilms increased. The diatom biofilms displayed absorption features at about 630nm, typical of absorbance in brown algae due to presence of chlorophyll *c*. The green algae biofilm reflectance spectra were marked by strong absorbance from 400-500nm, but little absorbance around 500-600nm. The cyanobacteria biofilms showed spectral absorbance at about 625nm, possibly attributable to phycocyanin. The spectra of the HP mixed population biofilms closely resembled the *Amphora sp* spectra, suggesting diatoms are an important component of these biofilms. The IP biofilms had inconsistent spectra that did not map closely to any monocultures. The spectra of the greener of the biofilms were similar to the *Chlorella sp* biofilm spectra, while the spectra of the red-brown biofilms differed.

15 *Estimating Biomass by Spectral Indexing*

Figures 5A and 5B show the determined value of the spectral index rNorm and NDVI, respectively, as a function of measured chl *a* density in $\mu\text{g}/\text{cm}^2$ from coating-normalised reflectance spectra of biofilms on grey coatings. The filled circle, square, and triangle represent the diatomaceous samples (*Amphora sp.*, *Achnanthes sp.*, Hartlepool biofilm) and the open circles, squares and triangle represent the green algae, blue-green algae, and Singapore slime samples.

Strong alignment across the rNorm and NDVI index data for the visually highest density bright green IP mixed culture biofilms (Figures 5A, 5B, open triangles) and the highest density brown monoculture *Achnanthes sp.* biofilms (filled squares) underscored that the two indices truly are broad tools for quantifying microalgal biomass.

Table 2 shows power model coefficients for the calibration curves (solid lines) as shown in Figures 5A and 5B, calculated from the grey coating-normalised biofilm reflectance spectra, and mean absolute error of the biomass estimates from these indices as compared to extracted pigment density measurements.

Table 2

Index	a	b	c	residual sum of squares	Mean absolute estimation error
rNorm	-1.921	0.093	2.477	0.57	0.422
NDVI	0.618	0.240	-0.321	0.36	0.407

*BIOMASS ESTIMATION ON ANY COLOUR BACKGROUND*5 *Biofilm reflectance spectra*

The experimentally measured spectra of the biofilms, R_{bio_m} , were strongly shaped by the spectra of the underlying coatings (Figures 6A-6F), and the relative reflectances of the biofilms and coatings were more clearly revealed in the normalised spectra (Figures 7A-7F), wherein each spectrum is normalised against the spectrum of the respective coating without biofilm present.

The spectra of the background coatings (see Figure 3) clearly structured the reflectance spectra measured from the experimental biofilms. The normalised spectra of biofilms on red, blue, and black coatings (Figures 7B, 7D and 7E) indicated the biofilms were brighter than the coatings (normalised reflectance >1) across colour-specific wavelength ranges. In contrast, the biofilms were all darker than the white, grey and yellow coatings (normalised reflectance <1, Figures 7A, 7C and 7F), and the coating-normalised spectra for biofilms on these colours were very similar.

Although the intensity varied with coating colour, the strong chl *a* absorbance feature around 673nm was clearly discernible in the spectra.

The clean (i.e. no biofilm) grey, white, and black coatings had very similar flat reflectance spectra differing only slightly apart from brightness levels (Figure 3). The spectra of the biofilms on the black coating showed differences to the spectra of the biofilms on grey or white. For the biofilms on the grey coating – the dataset which was the basis of the calibrations in this experiment – the features of the measured spectra were closely matched by the features of the normalised spectra, see Figures 6C and 7C. All the spectral values were approximately equal or to or less than 1 following normalisation, indicating the biofilms were darker than the clean grey coating. The same general spectral features were observable in the

normalised spectra of the biofilms on white coatings as on grey. The spectra for the black coating before (Figure 6E, mean reflectance 0-4%) and after normalisation (Figure 7E, reflectance 0.48-2.15) indicated that the biofilm was more reflective than the black coating itself, except for those wavelengths where the microalgae had strong absorbance features (e.g. around 673nm).

The spectral reflectance features of the biofilms on the red, blue and yellow coatings were comparable to their white, grey or black counterparts over wavelength ranges specific to each coating. The red datasets were split into dark and bright spectral ranges, below and above approximately 550nm, respectively. In the bluer wavelengths (<550nm), the region of the spectrum in which the red coating itself was only weakly reflective (see Figure 3), the biofilm was brighter than the coating, and the normalised spectral values were greater than 1.0 (maximum 1.98, Figure 7B), similar to the black dataset. In the redder wavelengths (>550nm), the wavelength range in which the red coating was brightest, the normalised spectral values ranged from 0.18 – 1 and resembled the white and grey datasets. For the blue coating datasets (Figure 7D), spectral reflectance decreased as biomass increased in the 400-500nm wavelength range as well as in the near infrared (generally), where the blue coating was brightest and similar to the grey and white datasets. The blue coating reflected little light between 500 and 700 nm (about 5%, see Figure 3). Over this wavelength range the biofilm was typically brighter than the coating (normalised values 1-1.25), with the exception that the chl a absorbance feature at 673nm was still present in the normalised spectra, though shallower relative to the white and grey datasets. The yellow coating normalised spectra were similar in profile to the white and grey datasets, and had similar spectral values (0.15-1).

Biomass estimation errors when applying the grey calibrations to other colours:

Density-specific goodness of fit: Figure 8 shows the biomass estimation errors (estimated minus measured $\mu\text{g}/\text{cm}^2$ chl a) that resulted from applying the NDVI calibration derived from the grey data to the other five colours were directional and specific to each coating color. The color-specific estimation errors were directional and linearly related to biomass. For example, the blue dataset

errors (the biomass estimation errors arising from applying the index calibrations determined from biofilms on the grey coating to biofilms on blue) consistently underestimated biomass, and the underestimation error grew linearly as biomass increased ($R^2 = 0.84$). For the blue coating dataset, both the baseline biomass estimation error and the error factor (intercept and slope of the linear model, respectively) were significant ($p < 0.05$, $p < 0.001$). A similar pattern of biomass underestimation was also measured for the black coating NDVI dataset (baseline error $p < 0.1$, error factor $p < 0.001$, $R^2 = 0.853$). In contrast, white and yellow coating dataset NDVI values resulted in linearly increasing overestimates of biomass, though only the slope of the white linear model was significant ($P < 0.05$).

Spectral index biomass estimation errors as a function of the coating spectra

The range of marine coating colours far exceeds the six included in the experiment, and so the individual color patterns of density-specific biomass estimation errors were considered relative to the coating spectra to infer general relationships between coating colour and hyperspectral index biomass estimation errors. The baseline estimation errors, BE, and error factors, EF, for each colour were compared to the minimum reflectance of the coatings at the wavelengths incorporated in each index (e.g. 673 or 800nm for NDVI). Non-linear relationships between coating spectral properties and estimation error are apparent.

Figures 9A and 9B show estimation of error factors, EF, and baseline errors, BE, for rNorm, and NDVI, respectively, plotted against minimum of coating reflectance at the wavelengths incorporated in the index (673nm for both).

Table 3 shows the models for estimating the error factors, EF, and baseline errors, BE, as determined from Figures 9A and 9B.

Table 3

Index	BE	EF
rNorm	$0.0862 \ln(R_{673}) + 0.0164$	$0.26 \ln(R_{673}) - 1.00$
NDVI	$0.025 \ln(R_{673/800}) + 0.1025$	$0.24 \ln(R_{673/800}) - 0.83$

Baseline error and error factors for rNorm and NDVI biomass estimates across the six coating datasets varied with the log of coating minimum reflectance (see Figure 9A, 9B). The error factor relationship parameters were very similar for the two indices (for rNorm, NDVI respectively: $R^2 = 0.99, 0.95$, coefficient = 0.26, 0.24). The rNorm baseline errors followed a logarithmic increase with coating minimum reflectance (Figure 9A, $R^2 = 0.91$, coef=0.086). The NDVI errors were less prominently logarithmic (Figure 9B, $R^2 = 0.43$, coef = 0.025), and could, if desired, be described by a linear model.

Figure 10 shows an example of possible different spectral ranges for use for the digital images, in this example relative to reflectance spectra of Hartlepool (HP) biofilms on a grey substrate (cf, Fig. 4, panel (e)). In this example a first spectral range is chosen around 673 nm, e.g. centered around 673 nm. A second spectral range in this example is chosen around 800 nm, e.g. centered around 800 nm. In Figure 10 w1a denotes a first spectral range of 3.3 nm FWHM and w2a denotes a second spectral range of 3.3 nm FWHM. In Figure 10 w1b denotes a first spectral range of 9.9 nm FWHM and w2b denotes a second spectral range of 9.9 nm FWHM. In Figure 10 w1c denotes a first spectral range of 16.5 nm FWHM and w2c denotes a second spectral range of 16.5 nm FWHM. In Figure 10 w1d denotes a first spectral range of 29.7 nm FWHM and w2d denotes a second spectral range of 29.7 nm FWHM. In Figure 10 w1e denotes a first spectral range of 62.7 nm FWHM and w2e denotes a second spectral range of 62.7 nm FWHM. In Figure 10 w1f denotes a first spectral range of 100 nm FWHM and w2f denotes a second spectral range of 100 nm FWHM. In this example the spectral band w1f is not centered around 673 nm. It will be appreciated that the spectral band w1f approximately corresponds to a red channel spectral, R, sensitivity of commercially available digital cameras. The spectral band w2f approximately corresponds to an infrared channel, IR, spectral sensitivity attainable by modified commercially available digital cameras (e.g. after removal of IR-cut filter).

Figures 11A and 11B show biomass estimation errors (in $\mu\text{g}/\text{cm}^2$) as a function of bandwidth of the spectral band used. Figure 11A relates to NDVI. Figure 11B relates to rNorm. Each panel indicates the respective spectral band width in nm. In each panel the vertical axis denotes the mean estimation error (for each of

the six colors) after applying the compensation associated with the difference of the reflectance of the coating of the object relative to the reference coating. In each panel the horizontal axis denotes the mean estimation error (for each of the six colors) without applying the compensation associated with the difference of the reflectance of the coating of the object relative to the reference coating. Thus, when an indicated point lies closer to the horizontal axis than it lies to the vertical axis, the compensation associated with the difference of the reflectance of the coating of the object relative to the reference coating reduced the biomass estimation error. As can be seen from Figure 11A, for NDVI, the compensation associated with the difference of the reflectance of the coating of the object relative to the reference coating reduced the biomass estimation error for all colors, except for black. As can be seen from Figure 11B, rNorm, the compensation associated with the difference of the reflectance of the coating of the object relative to the reference coating reduced the biomass estimation error for all colors except for black at the larger band width values.

Although the method can be applied in respect of any color of the current coating of the object, they are sometimes less accurate where the coating is black. Therefore, the method can be applied with improved accuracy where the current coating of the object is not black, for instance where it has a reflectance of at least 5% for at least one wavelength, and preferably more than one wavelength, in the range 500 to 700 nm, such as at the wavelength(s) or in the spectral band(s) used in determining the spectral index.

Results for black coatings may be improved by increasing the number of data points used to calculate the Error Factor, $EF_{current}$, and Baseline Error, $BE_{current}$, for rNorm and NDVI.

From the above, it follows that the biomass estimation calculated from the reference Spectral Index calibration, $biomass_{SI}$, can be adjusted by the Error Factor, $EF_{current}$, and Baseline Error, $BE_{current}$, both of which are functions of the reflectance spectrum of the current coating upon which the biomass is present. Then the estimated biomass on the current coating, $biomass_{current}$, can be determined from the equation

$$biomass_{current} = biomass_{SI} * (1 - \left(\frac{EF_{current}}{1 + EF_{current}}\right)) - BE_{current},$$

And using the error factor, EF, and baseline error, BE, e.g. as found in table 3.

This can be combined with the equation for determining the pigment surface area density from the spectral index and using the and the calibration values a , b and c , e.g. as found in table 2

$$5 \quad \text{pigment surface area density} = \left(\frac{SI-c}{a} \right)^{\frac{1}{b}}.$$

The two equations can be combined into the equation $\text{biomass}_{\text{current}} =$

$$\left(\frac{SI-c}{a} \right)^{\frac{1}{b}} \cdot \left(1 - \left(\frac{EF_{\text{current}}}{1+EF_{\text{current}}} \right) \right) - BE_{\text{current}}.$$

Hence, it is possible to estimate the aquatic environment-originating biofilm biomass on a coating on an object, e.g. that is immersed permanently or
 10 intermittently in an aquatic environment, on the basis of the spectral index, SI, and one or more calibration values while compensating the calculated biomass pigment surface area density for the reflectance of the coating of the object by applying a compensation associated with the reflectance of the coating of the object. Thus, biofilm biomass can be estimated on any color object, such as a vessel, using the
 15 presented equations and calibrated values.

It will be appreciated that herein the grey coating, having a reflectance of 24% was chosen as the reference point for determining the calibration values a , b and c for estimating the biomass pigment surface area density on the basis of the spectral indices, but that another reference coating, e.g. having a different
 20 reflectance could have been chosen, which would have changed the numerical values of the calibration values a , b and c . This, however, does not affect the inventive concept behind the present method.

It will also be appreciated that herein the grey coating, having a reflectance of 24% was chosen as the reference point for determining the error factor, EF, and baseline error, BE, for correcting for different color coatings underneath the microfouling layer, but that another reference coating, e.g. having a different reflectance could have been chosen, which would have changed the numerical values of the error factor, EF, and baseline error, BE. However, this also
 25 does not affect the inventive concept behind the present method.

30 Using the present method, regardless of the color of the coating underneath a biofilm layer, a spectral index can be determined from one or more

digital images, on the basis of which a biomass pigment surface area density can be estimated as if the coating color corresponded to the reference coating color used in determining the calibration values, and the thus estimated biomass pigment surface area density can be compensated for reflectance of the current
5 coating of the object by applying the compensation associated with the reflectance of the coating of the object, relative to the reference coating color as described above.

The present method allows for obtaining one or more digital images of a fouled portion of the coating of the object, such as under water, e.g. using an
10 (unmanned) submarine vessel. The one or more images can include images at different spectral ranges, such as spectral ranges, e.g. having a FWHM of 100 nm or less, such as 50 nm or less, such as 20 nm or less, such as 3 nm or less. The one or more images can be obtained using a hyperspectral camera. It is also possible to obtain the one or more images using a general purpose digital camera.

15 Diver and remotely operated vehicle, ROV, inspections of ships can use commercial underwater cameras and lighting rigs. The cameras can be adapted with band pass filters specifically tuned to the chl a red light absorption feature at 673nm. If paired with suitable calibration and imaging protocols, it is possible to incorporate rNorm biomass estimation into underwater inspection. NDVI imaging
20 at a depth of about 2m or more below the water line. In embodiments, NIR reflectance from a surface can be measured, optionally using equipment such as IR lamp assemblies and an IR-sensitive imaging system.

Herein, the invention is described with reference to specific examples of embodiments of the invention, which should not be considered limiting on the
25 scope of the invention claimed. For the purpose of clarity and conciseness, features are described herein as part of the same or separate embodiments, however, alternative embodiments having combinations of all or some of the features described in these separate embodiments are also envisaged.

In the examples, a hyperspectral camera is used. It will be clear that it
30 is also possible to use a broadband digital camera in combination with one or more bandpass filters. It is also possible to use a broadband digital camera in combination spectral band illumination. It is also possible to use a broadband digital

camera, e.g. using a red channel and optionally an infrared channel. The red channel can be approximately from 600 to 700 nm. The infrared channel can be approximately from 750 to 850 nm.

However, other modifications, variations, and alternatives are also possible. The specifications, drawings and examples are, accordingly, to be regarded in an illustrative sense rather than in a restrictive sense.

In the claims, any reference sign placed between parentheses shall not be construed as limiting the claim. The word 'comprising' does not exclude the presence of other features or steps than those listed in a claim. Furthermore, the words 'a' and 'an' shall not be construed as limited to 'only one', but instead are used to mean 'at least one', and do not exclude a plurality.

Claims

1. A method for estimating aquatic environment-originating biofilm biomass on a coating of an object, including:
 - 5 a) obtaining one or more digital images of a fouled portion of the coating on the object;
 - b) determining from each of the one or more images a respective reflectance value for the portion of the coating;
 - c) determining on the basis of the one or more reflectance values a value of
10 a spectral index representative of biomass;
 - d) calculating a biomass pigment surface area density on the basis of the spectral index, SI, and one or more calibration values determined for a reference coating; and
 - 15 e) compensating the calculated biomass pigment surface area density for the reflectance of the coating of the object by applying a compensation associated with the difference of the reflectance of the coating of the object relative to the reference coating.
2. The method of claim 1, wherein the compensation is based on a comparison
20 reflectance value representative for the coating underlying the biofilm biomass.
3. The method of claim 2, wherein the comparison reflectance value is:
 - 25 - a reflectance value determined for a part of the coating of the object from which all biomass had been removed;
 - a reflectance value determined from a reference object; or
 - a reflectance value stored in a database.
4. The method of any of claims 1-3, wherein each of the one or more images is
30 obtained in a spectral band, of e.g. 0-100 nm.

5. The method of claim 4, wherein the comparison reflectance value is a minimum value of the reflectance value for the coating underlying the biofilm biomass in the one or more spectral bands.
- 5 6. The method of any of claims 1-5, wherein in step e) the estimated biomass pigment surface area density on the current coating, $biomass_{current}$, can be determined from the biomass calculated from the Spectral Index, $biomass_{SI}$, determined in step d) by an Error Factor, $EF_{current}$, and Baseline Error $BE_{current}$, from the equation
- 10
$$biomass_{current} = biomass_{SI} * (1 - \left(\frac{EF_{current}}{1+EF_{current}}\right)) - BE_{current}.$$
7. The method of any of claims 1-6, wherein at least one of the spectral bands is chosen to encompass an absorption wavelength of chlorophyll, and/or at
- 15 least one of the spectral bands is chosen to exclude an absorption wavelength of chlorophyll.
8. The method of any of claims 1-7, wherein at least one of the spectral bands is chosen around 433, 460, 496, 555, 584, 601, 673, or 800 nm.
- 20 9. The method of any of claims 1-8 wherein the spectral index includes a ratio of two reflectance values.
10. The method of any of claims 1-9, wherein the spectral index, SI, is one of:
- 25 - coating normalized red reflectance defined as
- $$SI = rNorm673 = \frac{R673}{R673_{clean}};$$
- Normalized Difference Vegetation Index defined as
- $$SI = NDVI = \frac{R800+R673}{R800-R673},$$
- wherein $R673$ is the determined reflectance value in the range of 605-740 nm, such as at 673 nm; $R800$ is the determined reflectance value in
- 30 the range of 720-900 nm, such as at 800 nm; $R673_{clean}$ is the reflectance

value in the range of 605-740 nm, such as at 673 nm at the coating of the object without biomass.

11. The method of claim 10, wherein
 - 5 - if the spectral index is the coating normalized red reflectance,

$$EF_{current} = 0.26 \ln (R_{current673}) - 1.00; BE_{current} = 0.0862 \ln (R_{current673}) + 0.0164;$$
 and
 - if the spectral index is the Normalized Difference Vegetation,

$$EF_{current} = 0.24 \ln (R_{current673}) - 0.83; BE_{current} = 0.025 \ln (R_{current673}) + 0.1025;$$
 - 10 wherein $R_{current673}$ is the reflectance value of the current coating at 673 nm.
12. The method of any of claims 1-11, wherein in step d) the pigment surface area density is determined from
 - 15
$$pigment\ surface\ area\ density = \left(\frac{SI - c}{a} \right)^{\frac{1}{b}},$$
 wherein a , b and c are calibration values.
13. The method of claim 12, wherein
 - if the spectral index is the coating normalized red reflectance,
 20 $a = -1.921, b = 0.093, c = 2.477;$ and
 - if the spectral index is the Normalized Difference Vegetation,
 $a = 0.618, b = 0.240, c = -0.321.$
14. The method of any of claims 1-13, wherein the one or more digital images
 - 25 are obtained using a submarine, such as an unmanned submarine.
15. A system for estimating aquatic environment-originating biofilm biomass on a coating of an object, including a processor configured for:
 - obtaining one or more digital images of a fouled portion of the coating on
 - 30 the object;
 - determining from each of the one or more images a respective reflectance value for the portion of the coating;

- determining on the basis of the one or more reflectance values a value of a spectral index representative of biomass;
- calculating a biomass pigment surface area density on the basis of the spectral index, SI, and one or more calibration values determined for a reference coating; and
- 5 - compensating the calculated biomass pigment surface area density for the reflectance of the coating of the object by applying a compensation associated with the difference of the reflectance of the coating of the object relative to the reference coating.