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Image-Based Analysis and Quantification of Biofouling in Cultures of the Red Alga *Asparagopsis taxiformis*

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Abstract

Methane is an extremely potent yet short-lived greenhouse gas and is thus recognized as a promising target for rapid climate change mitigation. About 35% of anthropogenic methane emissions are associated with livestock production, and most of these emissions are the outcome of enteric fermentation in ruminant animals. The red seaweed *Asparagopsis* is currently considered the most efficient feed additive to suppress methane emissions from enteric fermentation but is not currently available on commercial scale. The ongoing effort to successfully commercialize *Asparagopsis* requires the development of pest control frameworks which rely on the quantitative assessment of biological contamination in cultures. Here we present a low-cost readily available approach for quantifying biofouling in *Asparagopsis taxiformis* cultures based on microscopic examination and automated image analysis. The proposed methodology is demonstrated to estimate contamination associated with *Asparagopsis* biomass with error rates lower than 2% over a wide range of contamination levels and contaminating organisms, while significantly cutting down image processing time and allowing for frequent contamination quantification.

Keywords

Asparagopsis, Seaweed, Algae Contamination, Aquaculture, Contamination detection

Declarations

Funding – The study was funded by Blue Ocean Barns Inc.

Competing interests - The study was funded by Blue Ocean Barns Inc.

Availability of data and material – Data for method validation are available on Online Supplementary Information

Code availability – ImageJ macro scripts are available on Online Supplementary Information

Authors' contributions – GD conceived and wrote the manuscript, and developed the ImageJ workflow; HMR Performed the power analysis and wrote manuscript; BT adjusted ImageJ quantification and reviewed the manuscript; KB microscope photos capture and analysis; JG manual and ImageJ contamination tracing; JES secured funding, conceived project, and reviewed manuscript.

Introduction

Methane is a greenhouse gas that is 80 times more potent than carbon dioxide gas in terms of its warming potential over a 20-year time horizon (IPCC 2014). Atmospheric methane concentration has more than doubled since pre-industrial times (Dlugokencky 2022) due in large part to anthropogenic activities. However, methane has a relatively short lifespan in the atmosphere (~12 years) and is mainly produced by specific industrial and agricultural sectors, making it a high priority for global greenhouse gas mitigation. Recently 105 countries in the UN Climate Change Conference (COP 26) signed “The Global Methane Pledge” committing to a 30% reduction in methane emissions by 2030 relative to 2020 (Ming et al. 2022). About 35% of anthropogenic methane emissions are associated with livestock production, most of which are the outcome of enteric fermentation in ruminant animals (Eisen Id and Brown Id 2022). The red algal genus *Asparagopsis* when fed in small quantities has been shown to be highly effective at reducing methane emissions associated with enteric fermentation (up to 90%). Results have been consistent both in vitro and in live ruminant trials (Kinley et al. 2016, 2020; Roque et al. 2021; Min et al. 2021), yielding no apparent trade-offs in product quality, animal production or any negative environmental effects (Glasson et al. 2022). However, to date, the *Asparagopsis* used in these trials has been primarily wild-harvested and as such it is not commercially available (Nin-Pratt et al. 2022). As research with ruminant animals continues there is a need to better understand this seaweed with particular focus on developing efficient and sustainable approaches for cultivation.

As is true with the domestication of most wild organisms, developing a productive and scalable cultivation system can be challenging. Developing *Asparagopsis* production systems has some specific challenges due to the alga’s size and unique morphology. The genus is known for having

a heteromorphic triphasic alternation of generations life cycle including a macroscopic gametophyte, filamentous tetrasporophyte, and a carposporophyte. Current efforts to develop land-based cultivation systems have been largely focused on the filamentous tetrasporophyte stage. When grown in a tumble culture, the filaments, with diameter of up to 0.05 mm and length of up to several cm, usually take on a “puffball” shape with diameter of roughly 0.5-5 cm (Fig. 1). While the development of cultivation techniques for both microalgae and macroalgae has made great progress in the past decades, the intermediate size and filamentous nature of *Asparagopsis* tetrasporophytes dictates a hybrid “micro-macro” approach calling for the adoption and adaptation of tools from both industries, as well as the development of new *Asparagopsis* specific technologies. Together with cultivation optimization and scaling up of systems, overcoming or controlling biological contamination is of the utmost importance for the successful development of a commercial industry for *Asparagopsis*.

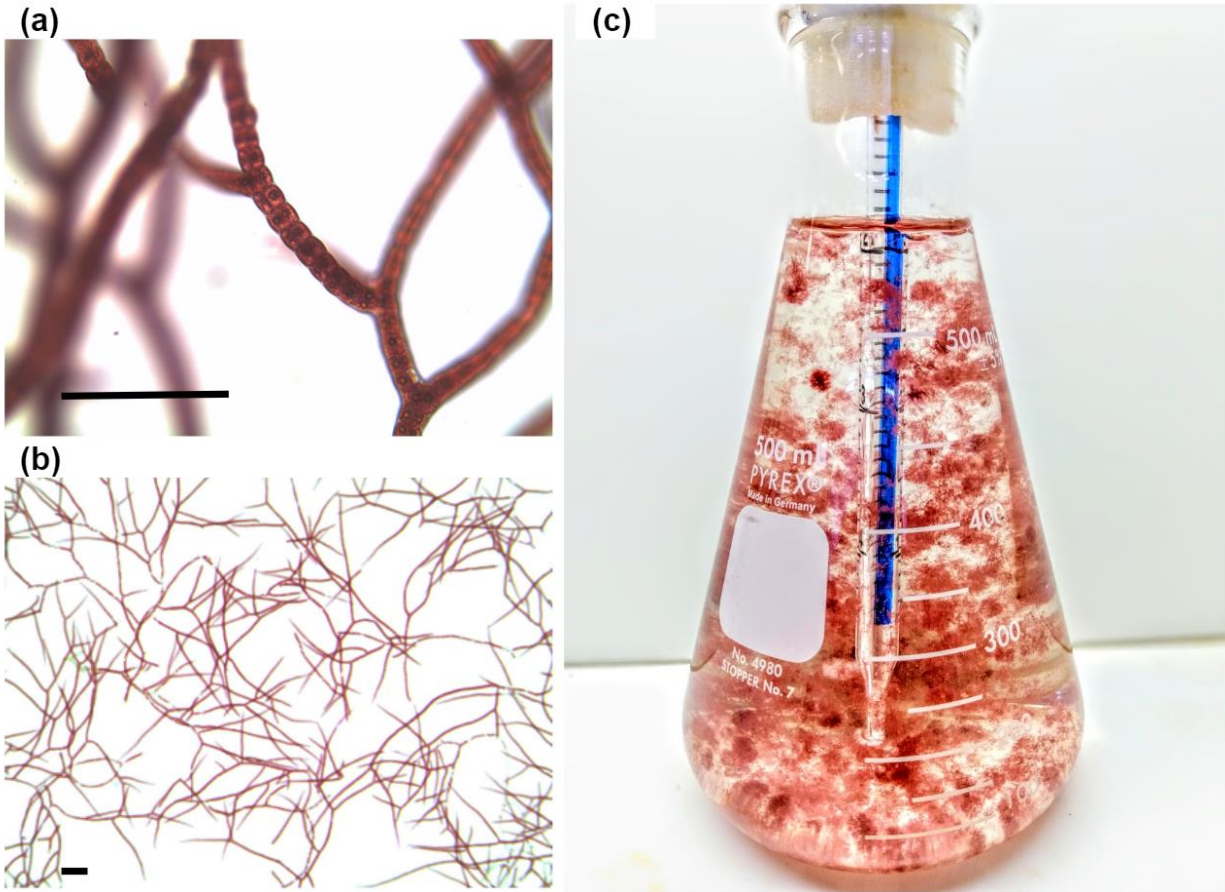


Fig. 1: *Asparagopsis taxiformis* filamentous tetrasporophyte - “Falkenbergia” phase. Representative tumble culture of *A. taxiformis* tetrasporophytes at (a) 200X and (b) 25X light microscopy, and (c) its appearance in a 500 mL flask. Branches are built of tiers of 3 pericentral cells with a total diameter of ~15-50 μm . While individual branches are quite small in diameter, branch length can exceed ~ 5 cm and weave together to form “puffballs”. Scale bars = 0.2 mm.

In aquaculture, biological contamination is defined as the invasion of cultures by any organism other than the target species. In the context of algal aquaculture, contaminating organisms may have adverse effects on the algae either by directly damaging the target species, by feeding on them, or by competing for resources (such as light and/or nutrients), subsequently impacting target species biomass/substance production. To ensure effective management of algae cultures, frequent assessments are required for the early detection of contaminants thus allowing for early action to

prevent culture collapse (Zhu et al. 2020). Further, because contamination is inevitable at some level in large-scale algae production, it is valuable to have a protocol to quantitatively assess the extent of contamination (rather than just monitoring the presence/absence of contaminants) to track trends over time and develop management strategies. Despite the enormous significance of this problem in aquaculture there are no standardized approaches for quantifying or tracking contaminating organisms in culture and as such there is a lack of quantitative information on this issue (Di Caprio 2020).

Contamination of algae cultures may be caused by many organisms, including the following:

Competing algae - Both micro and macro algae may invade cultures and compete with the target species for resources such as nutrients, light, and space. These algal contaminants may be of any algal taxon, depending on the source of contamination, growth medium composition, and environmental conditions. Common contaminant groups include diatoms (e.g. (Shea et al. 2006; Winckelmann et al. 2016), cyanobacteria (e.g. (Meseck 2007), and green algae (e.g. (Grivalský et al. 2021; Behera et al. 2022).

Epiphytes - Various organisms may colonize seaweed surfaces (including bacteria, diatoms, macro, and microalgae). Epiphytes may compete with target algae for resources or may physically settle on and grow over their surfaces, compromising light capture and water movement, subsequently leading to diminished growth and the dilution of target species. Some epiphytes may also be pathogenic (Borburema et al. 2021; Behera et al. 2022).

Fungi and bacteria - Fungi and heterotrophic bacteria are functionally differentiated from algae by the absence of photosynthetic capacity and therefore do not directly compete with algae for nutrients or light. Nevertheless, some fungal and bacterial species may become pathogenic in algal cultures and have been documented to cause cultures to crash (e.g. (Fott 1967; Berland et al. 1972; Gutman et al. 2009; Carney and Lane 2014; Fulbright et al. 2018). The majority of fungal and bacterial pathogens are still uncharacterized (Deore et al. 2020).

Zooplankton grazers – As algae form the basis of aquatic food webs, they may be grazed by organisms of various sizes, from unicellular zooplankton to large multicellular organisms such as fish and invertebrates. While large grazers can be easily excluded from on-land algae cultivation systems and are only relevant to open off-shore operations, micrograzers make up a significant portion of the biological contamination in algae cultures (Di Caprio 2020). The main representatives of this group documented to affect algae cultures are ciliates, rotifers, copepods, amoeba, and dinoflagellates (Deore et al. 2020).

Generally, micro and macro algae differ in their sensitivity to contamination type mostly based on size differences. While microalgae are smaller than or of a comparable size as common micrograzers and are therefore prone to intense grazing (Day et al. 2017; Di Caprio 2020; Zhu et al. 2020), macroalgae are usually much bigger and are less prone to direct consumption by microorganisms. On the other hand, macroalgae are often a good substrate for microorganisms to settle on and are susceptible to various types and levels of epiphytism. Both micro and macroalgae are prone to pathogenic microbial and viral contamination (Zhu et al. 2020; Behera et al. 2022). It

should be noted, however, that in some cases, "contaminating" microorganisms have been shown to function as symbionts that can improve algal growth and performance (Amaral et al. 2013; Wichard 2022), and have even been shown to be “farmed” by seaweed to some extent (Saha et al. 2020).

Biological contamination of both micro and macroalgal cultures is one of the greatest challenges for efficient, reliable and scalable production of algal biomass (Fernandes et al. 2011; Zhu et al. 2020; Deore et al. 2020; Behera et al. 2022). Addressing this challenge requires the development of standardized protocols for quantifying and analyzing contamination in algal cultures (Day et al. 2017; Di Caprio 2020). Specifically, in order to develop and operate a reliable *Asparagopsis* cultivation system, biological contamination must be prevented or controlled, requiring adequate contamination assessment tools.

Current approaches for assessing the level of biological contamination in algal cultures have mostly focused on microalgae and include the following methods:

Plate counting is used to quantify bacterial populations by counting Colony Forming Units (CFU) based on their growth on agar plates. This approach is labor intensive and is biased toward bacterial species that can grow on the specific medium chosen. Furthermore, this method is dependent on culturing bacteria for a few days, during which contamination may take over the culture and make contamination mitigation more difficult.

Microscopic inspection is the most basic and direct contamination assessment. Usually this involves counting contaminants in a given water volume using a Hemocytometer or Sedgwick Rafter counting chamber. Counting can be done manually or can be automated with image analysis software in the case of well-defined contaminants (e.g., spherical cells with a fairly uniform size and color). Contaminant identification can be further assisted by using various selective staining techniques (Carney and Lane 2014). Generally, microscopy is laborious and reliant on the observer's skill and experience, and it has been shown to underestimate the number of algae grazers found in cultures (Day et al. 2012; Deore et al. 2020). The time intensive nature of this approach limits the intervals of sampling for screening efforts, which may hamper proper early detection of contamination. However, a significant decrease in labor and processing time may be achieved with the automation of image analysis. Microscopic examination may be focused either on water suspended particles or on particles associated with the algae biomass. Specifically for *A. taxiformis* tetrasporophyte cultures, the focus of contamination quantification should be on epiphytic communities rather than on suspended contaminants, dictating the quantification of contamination *per unit of algae* rather than *per water volume*. Furthermore, because of the diversity in shape and size of contaminating organisms, counting individuals is not an appropriate metric. Rather, quantification of area or the proportion of biomass that is fouled is more valuable.

Flow-through microscopy may be used to count and identify microorganisms continuously. Assuming both algae and contaminants are small enough to flow through a “measurement zone” where they are microscopically photographed or optically measured

(e.g. by measuring fluorescence, absorption, or reflectance), automated systems (e.g. the FlowCam, Yokogawa Fluid Imaging Technologies, USA), are available to characterize microplankton (by their size, shape, or fluorescence) in the size range of 20–200µM. This approach has also been shown to be effective in quantifying grazers in microalgae cultures (Sieracki et al. 1998; Day et al. 2012; Havlik et al. 2013). While being capable of continuously monitoring various contaminants, flow-through microscopy is relatively costly and is not applicable for larger macroalgal cultures that are too large for the measurement zone. Orenstein et al. (2020) demonstrated the capability of an *in situ* free-space imaging system (the Scripps Plankton Camera) to identify and monitor plankton passively. This approach may be a promising solution to overcome the limitations of measurement zone size of current systems, but is not yet adapted nor tested for macroalgae cultivation needs.

Molecular-based methods are applicable and being used to identify contaminating organisms in aquaculture systems across scales. These methods are usually based on the PCR amplification of specific markers (mainly 16S rRNA and/or 18S rRNA for bacteria and eukaryotes, respectively) and are sensitive and becoming increasingly available. However, these tools rely to some extent on prior knowledge of contaminating organisms and are limited to identifying organisms that have previously been sequenced (Deore et al. 2020). In addition, basic PCR amplification can only reveal the presence of contamination but not its quantity. And while Quantitative or real-time PCR is becoming increasingly available, qPCR can only detect and quantify a limited number of known contaminants.

Detection of specific metabolites may be used to monitor the onset and progress of specific biochemical processes within cultures. Assuming a metabolite is unique to the contaminant and does not vary in concentration (grams metabolite per gram of contaminant), taxon-specific metabolites, such as pigments or toxins, can be measured in cultures as a proxy for the level of contamination (Di Caprio 2020). However, careful attention is needed when picking these metabolites as their concentrations can be significantly affected by changes in growth conditions (e.g. pigment contents in response to light or nutrients variations (Grossman et al. 1993)). A slightly different approach uses the effect of contaminant metabolism on the cultivation medium. Some contaminants may alter the chemical environment in cultures, allowing the use of specific changes in environmental variables as proxies for contamination (Zhu et al. 2017). A “Sniffer” for biomass-dependent volatile gas production was developed to monitor various processes taking place in microalgae ponds. Real-time measurements have been performed using chemical ionization mass spectrometry (CIMS) to monitor the impact of grazer infections on cyanobacterial cultures (Sauer et al. 2021). This novel technology may have important applications, mostly for large scale algae ponds, but is currently expensive and is not commercially available especially for macroalgal cultures.

Optical characteristics of a culture can also be used as indicators of contamination. This approach is especially promising since optical sensors are non-invasive, fast, robust with no moving parts, and rather inexpensive compared to some non-optical methods (Havlik et al. 2022). Optical sensing may be based on **spectral absorption** (e.g. used with artificial neural network (ANN) to differentiate microalgal species in suspension (Franco et al.

2019)), **reflectance** (e.g. for the detection of diatoms in open ponds, (Reichardt et al. 2020)), **chlorophyll fluorescence** (e.g. NPQ measured by PAM to detect grazing (Deore et al. 2020)), or **Fourier Transform Infrared (FTIR) spectroscopy** (e.g. for detection of grazers (Deore et al. 2021)), and has the potential to monitor aspects of contamination in a real-time, continuous manner. However, spectrometers (at their various kinds and specificities) are still not available in most aquaculture facilities, and their use may face some challenges due the heterogeneity of macroalgae cultures (as opposed to fairly homogeneous microalgae suspensions)

Contamination in *A. taxiformis* cultures has been noted in previous studies (e.g. (Schuenhoff et al. 2006; Mata 2008)) but has not been effectively quantified. The quantification of biological contamination in *Asparagopsis* tetrasporophyte cultures may use tools and techniques from the above descriptions but will require some fundamental adaptations due to the filamentous nature of this alga.

Quantification of microorganisms suspended in the water can be achieved with any of the above-mentioned techniques. However, due to the larger size of the tetrasporophytes, microorganisms suspended in the water can also be washed away by sieving the algae and replacing the water. Hence, the more problematic part of contamination is the contamination associated with the algae itself, either truly epiphytic or merely tangled in tetrasporophyte filaments. Furthermore, due to the heterogeneity of *Asparagopsis* cultures (in comparison to fairly homogeneous microalgae cultures), the amount of *Asparagopsis* per volume sampled is highly variable, and since most contaminants occur in association with *Asparagopsis*, random sampling of small volumes may not capture contaminants and thus may underestimate the degree of contamination. For these reasons,

sampling of *Asparagopsis* biomass (rather than culture volume) is preferred, and quantifying the amount of contaminants per unit of *Asparagopsis* biomass is more appropriate than quantifying the contamination per culture volume. The large size and fragility of tetrasporophytes (relative to microalgae) also prevents the use of flow-through microscopy and flow cytometry. On the other hand, being much smaller than other cultivated macroalgae, *Asparagopsis* tetrasporophytes are difficult to remove epiphytes from and can often get covered by epiphytes of comparable size.

In this paper, we describe a low-cost contamination quantification methodology specific for *Asparagopsis* tetrasporophyte cultures, based on an automated image analysis of microscope slide photos. While these techniques were developed specifically for *Asparagopsis* tetrasporophyte cultures they could be applied to any filamentous algal cultivation setting.

Methods

Biological material:

A number of individual cultures of *A. taxiformis* tetrasporophytes were used to develop a contamination quantification protocol. Specifically, samples were collected from a number of southern California locations, and standard approaches were used to isolate and purify samples as best as possible (Based on Kawai et al. (2005)). Despite these efforts, occasional contaminants would find their way into cultures resulting in various contamination levels in the cultures used for quantification purposes.

Cultures were grown in various environmental conditions and contained a wide range of contaminants. Indoor lab cultures were grown in natural seawater filtered to 0.2 μm and autoclaved and were supplemented with commercially available F/2 solutions (Proline F/2 Algae Food) for

major and minor nutrients. Cultures were constantly stirred and aerated by bubbling air, were held at ~21°C, and were illuminated with 50-100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ PAR at 12:12 hours light: dark cycles. Additional samples were taken from cultivation experiments to include algae originally collected at various Southern California locations and cultivated under different light conditions. In order to further increase the range of environmental conditions and contaminants, outdoor cultures, grown at the Scripps outdoor aquarium facility, fed by natural seawater, and exposed to natural variation in environmental conditions, were sampled as well.

Sample preparation for microscopic examination:

A small amount of the tetrasporophyte stage of *A. taxiformis* was placed in a petri dish in sterile seawater, and a small amount was then transferred using fine-tipped forceps to a standard glass microscope slide. The sample was then viewed under a stereomicroscope and was spread out to ensure that there were very few overlapping branches and no prominent clumps. The amount of *A. taxiformis* on the slide was adjusted until it covered slightly less than the area of a coverslip. The cover slip was gently placed on top of the algae with a drop of seawater, and gently depressed to remove excess water and push out any bubbles. Photographs were taken with Olympus SZX16 microscope at 25x magnification and bright field background using standardized capture settings (in Infinity Capture software: R: 1.85, G: 1.65, B: 2.10, Gain: 1.24, Exposure: 13). 4 to 5 photos were taken per slide, making sure that there was no overlapping area between the photos.

Image analysis to quantify contamination:

In order to quantify the contamination associated with *A. taxiformis* biomass, two parameters were measured per microscope slide photo: (1) the total area of all algal biomass, including both *A.*

taxiformis and contaminants, and (2) contamination area. These two parameters were used to calculate the contamination percentage per photo (Fig. 2) as $\text{Contamination \%} = \text{Contamination Area} / \text{Total Area} \times 100$. All image analyses were conducted with ImageJ (version 1.53b) primarily through an automated procedure, based on color threshold selections for the areas of interest. The automation of this procedure was achieved using a macro script consisting of three adjustable elements: color space, individual color channels, and particle size. The total area of *A. taxiformis* and associated contaminants in each photo required the use of the HSB color space. To quantify contamination alone, either RGB or LAB was selected as the color space, depending on the type of contaminants present in a sample. The RGB color space was used primarily to detect green contaminants, while the LAB color space was used primarily to detect contaminants with dark green or dark brown hues. The values for the individual color channels (R, G, and B for the RGB color space; L, A, and B for the LAB color space), each ranging between 0 and 255, could be adjusted to selectively target the contaminants while disregarding the *A. taxiformis* branches. Lastly, we adjusted the minimal particle size according to the nature and size of the contaminants, to avoid the unintended selection of small non-contaminant particles. Individual images could be analyzed by opening an image in the ImageJ program and running the macro script of interest (Plugins > Macros > Edit). Alternatively, an automated process, hereafter referred to as the batch process, could be used to run the macro script on all images within a single dataset (Process > Batch > Macro), so long as the macro script could be used to consistently and accurately quantify the contamination in all the images of a given dataset (Full description can be found in [Online Resource 1](#)).

Quantification of the total area of *A. taxiformis* and associated contaminants in each photo was achieved through a single macro script (based mostly on brightness differences between biomass and background) across all datasets, given that the sample preparation and microscope settings for each dataset were consistent enough to yield similar color tones among all images. To quantify contamination, however, each dataset (i.e. set of photos prepared and photographed using similar settings and containing similar contamination patterns) typically required some adaptations to the macro script to accurately highlight the contaminants. A full description of the macro scripts as well as the specific thresholds used in this study can be found in [Online Resource 1](#).

To tune the macro script for a specific dataset before running the automated batch process, around 5 representative images, ranging in different contamination levels and occasionally different contamination types, were used to determine the most optimal quantification settings. Ideal settings aim to maximize the selection of the contamination present while minimizing selection of areas consisting of non-contamination components. Based on these representative images, the elements of the macro script were optimally adjusted. After testing the macro script on these representative images, the batch process was used to quantify contamination in all the images from a single dataset (e.g., contained in a single folder). Output images of the selected area for each sample were stored in a separate folder. After running the batch process, each output image was compared to its original image to evaluate whether the contamination was accurately selected. This last step was taken while developing the protocol, to verify the success of the batch process in accurately capturing contamination and gaining confidence in its reliability, and is not necessary for all future analyses.

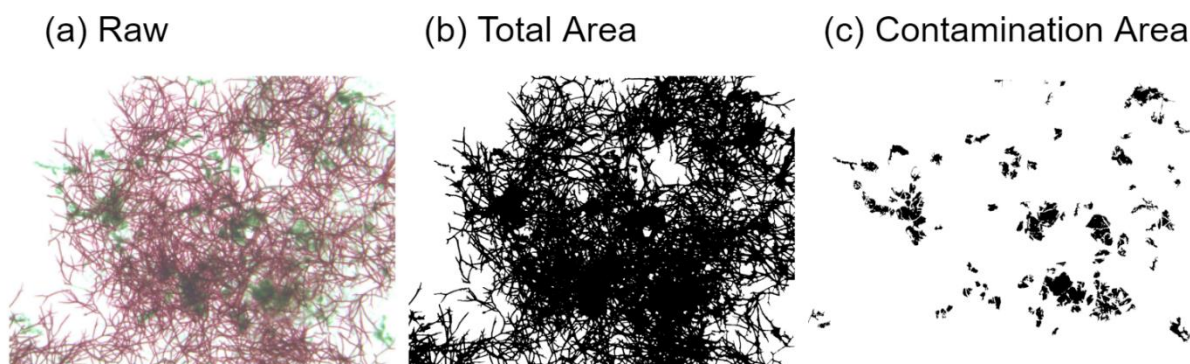


Fig. 2: Illustration of the image analysis procedure. (a) Samples were taken from *A. taxiformis* sporophyte cultures and photographed on a microscope slide. (b) The total biomass area (including *A. taxiformis* as well as contaminant area) was selected and quantified based on brightness differences from the background. (c) Contamination area was selected and quantified based on RGB differences from *A. taxiformis*. Contamination % = contamination area / Total area X 100.

Image analysis validation

In order to quantitatively validate the above workflow, contamination areas were traced manually using ImageJ selection tools in 100 microscope photos taken at various occasions. Contaminants were mostly green microalgae and filamentous cyanobacteria. The image analysis based quantification was then applied to those 100 photos and correlated with the manual tracing results (Fig. 3). The prediction error was calculated as the Standard Error of Estimates (SEE).

Assessing sample size required for contamination quantification from microscope photos

A power analysis was run to determine the minimum number of microscope photographs needed to accurately predict the concentration of contamination within a culture of *A. taxiformis*. To run the analysis, four cultures, with varying types and amounts of contamination, were analyzed. These cultures included (I) one with a minimal amount of filamentous cyanobacteria, (II) one with a competitive green alga, (III) one with an encrusting unicellular green alga, and (IV) a final culture

with a high concentration of diatoms. For each culture, 10 slides were prepared, and 5 microscope photos were taken per slide for a total of 50 microscopic photos per culture. The image capture and contamination quantification analysis were completed using the protocol described above.

The contamination quantification of the 50 samples from each culture were used to determine the accuracy of the sample mean as it compares to the culture mean for sample sizes ranging from 3 to 49 measurements. The average percent contamination of the 50 samples was used to represent the population mean of the culture. For each sample size, samples were randomly selected using the sample() function in R and the average % contamination of the samples was calculated. Each sample size was re-sampled 10,000 times and the standard deviation of the sample means were calculated to show the variability in the sample mean from the culture mean (table 1).

Table 1: Calculation of power parameters to estimate the required sample size for contamination quantification from microscope slide photos at 25X magnification

<i>Standard Deviation of Sample Means</i> $\sqrt{\frac{\sum_{i=1}^r (\mu_n - \mu)^2}{r - 1}}$	μ_n = sample mean of sample size n μ = culture mean n = sample size, 3-49 r= number of repeated samplings, 10000
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Results

Overall, 280 microscope slide photos were analyzed to quantify contamination levels associated with *A. taxiformis* tetrasporophyte tissue. These samples, collected from cultures of various origins and cultivated under a wide variety of culture conditions, contained mostly three groups of contaminants: (1) Cyanobacteria, mostly filamentous, forming dark tangled mats within *A. taxiformis* branches, (2) Green microalgae, mostly unicellular, growing attached (encrusting) on *A. taxiformis* branches to various levels, and (3) Diatoms, mainly attached to *A. taxiformis* branches as individuals or colonies. While sampling for this analysis, red algae contaminants (rarely present but in some cultures) were avoided due to difficulties in color-based separation. Batch processing of microscope photos for contamination was efficient and required only minor changes in macro script parameters ([Online Resource 2](#)) to be adjusted between different contaminants.

100 samples were used to validate the automated contamination quantification results in comparison to manual tracing in microscope slide photos. Due to the small size and complex shape of many diatom species, it was not feasible to manually trace them accurately, so validation was only conducted for green algae and cyanobacteria. There was a strong correlation between the automated contamination quantification and manual tracing ($R^2=0.93$) with an error of $\pm 1.43\%$ (Standard Error of Estimation) over a wide range of contamination levels ranging from 0-22%, including photos taken across various cultures and at various occasions (Fig. 3).

The number of microscope photos required to accurately determine contamination levels per culture was assessed for four cultures using a power analysis. The accuracy of the sample size, as it relates to variability from the culture mean, was found to be dependent on the concentration and patchiness of the contamination, with patchiness referring to the extent to which the contamination

was homogenous throughout the culture. Level of patchiness was determined by taking the standard deviation of the 50 samples. The contamination pattern in these four cultures differed in both level of concentration and patchiness from less than 1% contamination and inherently low patchiness (indicated by low STDV levels; 0.38% STDV) for culture (I), to almost 40% contamination concentrated in diatom clumps causing high patchiness (8.6% STDV) for culture (IV). Thus, the accuracy of each sample size varied between the cultures.(Fig. 4). If an accuracy of 1% (calculated as standard deviation from the mean) is sought, a sample size of 3 is required for a culture with low concentration, low patchiness (II), while a sample size of 10 is required for a culture with high contamination low patchiness (III) and a sample size of 30 is required for a culture with high contamination, high patchiness (IV) (Fig. 4).

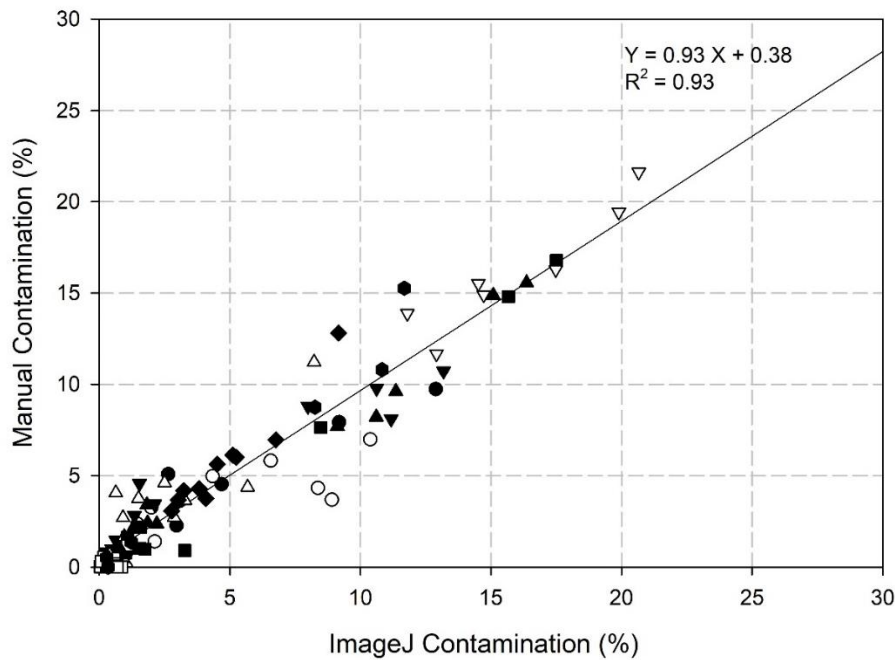


Fig. 3: Manual validation of ImageJ contamination quantification. Contamination percentage results were plotted versus manual contamination tracing for 100 microscope slide photos taken by various users across various culture conditions and contamination types. Symbols represent different datasets (including mostly green algae and cyanobacteria contamination). N=100, Standard Error of Estimates (SEE) = 1.43%

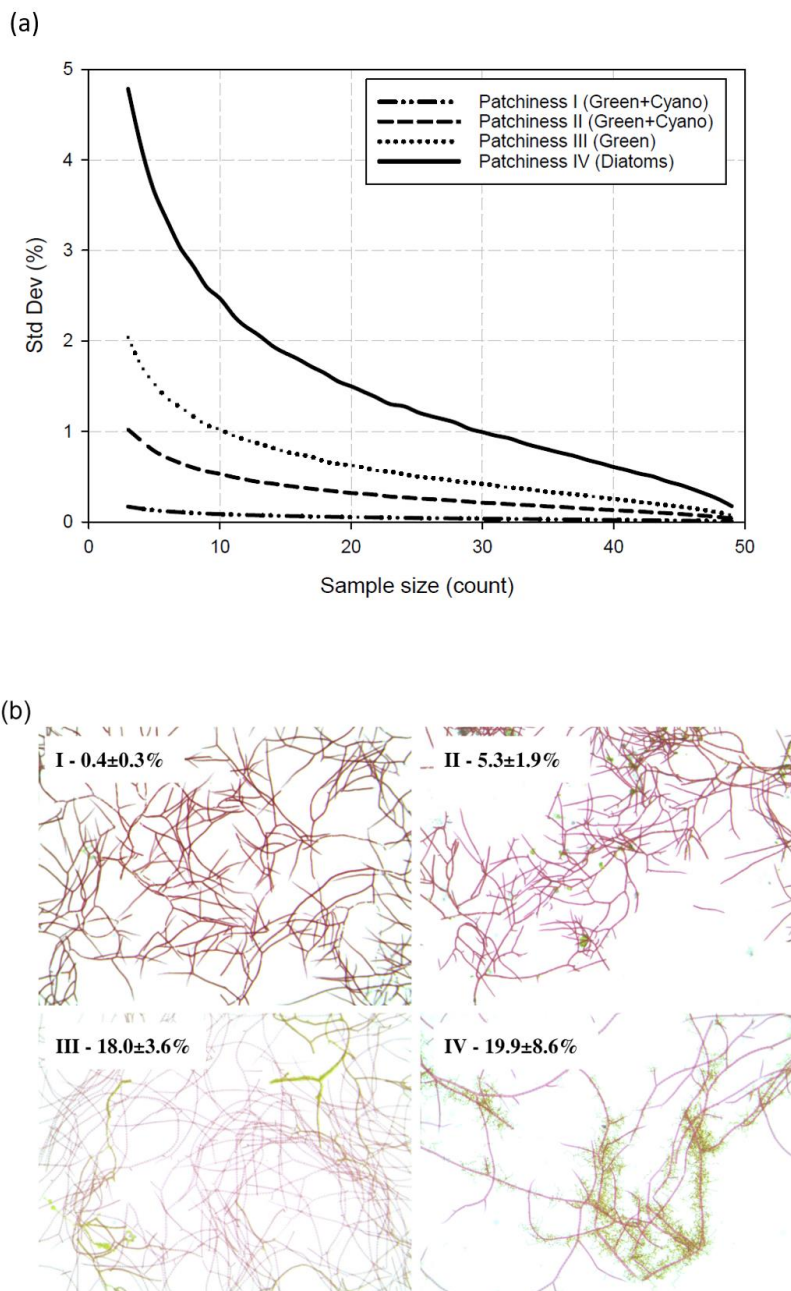


Fig. 4: (a) Sample size power assessment of standard deviations associated with the number of photos analyzed for contamination quantification in various *A. taxiformis* cultures. The different lines represent the types of contamination found in the cultures and represent different contamination and patchiness levels (I to IV from low to high patchiness). (b) Microscope photos (25X magnification) on the bottom demonstrate the appearance of slides for each dataset assigned with contamination-patchiness categories (I-IV), the average contamination percentage, and standard deviation of the 50 samples.

Discussion:

In aquaculture, most conventional contamination assessments have been developed for microalgae cultures and have largely focused on contaminants suspended in culture media. With a growing interest in macroalgal cultivation, especially for the red alga *Asparagopsis taxiformis* as a methane mitigating supplement for livestock, there is an urgent need to develop approaches that specifically target contaminating organisms associated with this species. The biological contamination of *Asparagopsis* poses a significant challenge in achieving reliable large-scale production and should not be overlooked. Realizing that at full scale production contamination needs to be controlled rather than eliminated, quantitative assessments of contamination are a prerequisite to developing mitigation tools and pest control frameworks. The microscopic examination procedure shown here has been demonstrated to be applicable for reliably quantifying contaminants associated with *A. taxiformis* tetrasporophyte biomass, yielding error rates lower than 2%. While sample preparation for microscopic examination is still time consuming and may not be carried out as part of continuous in-line monitoring of cultures, the automated image analysis described here allows for batch processing of hundreds of microscope photos in minutes, and may be used to quantitatively assess contamination in small scale experiments as well as in commercial scale systems.

Overall, we identify three main limitations for our proposed technique, namely (1) the need for discrete sampling (rather than automated sampling) and the time required for microscope slide preparation, (2) the limited success in quantifying non-target red algae (when their color is too similar to *Asparagopsis* in culture), and (3) the possible error due to heterogeneity in contamination through cultures (relative to homogenous microalgae cultures) which requires increased sampling efforts.

Despite the limitations, our approach allows for a consistent and comprehensive quantitative contamination characterization of algal cultures across scales, from wild-collected specimens, to lab cultures, to full-scale production. Such data are needed for the development of a data-driven approach for contamination control. This low-cost contamination quantification tool requires merely a microscope with camera and a computer running the ImageJ software (accessible for free at <https://imagej.nih.gov/ij/>) and can therefore be instantly implemented at most algae research and farming facilities. We urge researchers and cultivators to further investigate and develop more efficient and accurate tools, either within the ImageJ framework described here, or using any other approach to improve contamination quantification tools in *Asparagopsis* cultivation. Such tools will be critically important for informing the successful development of commercial scale *Asparagopsis* farming in order to be available as cattle feed for methane mitigation worldwide.

Ultimately, a complete assessment of contamination in *Asparagopsis* culture should include the identification and quantification of contaminants in a continuous or frequent manner. An accurate identification of contaminants might be especially important in the case of attempts to control microbial populations towards specific beneficial microbial assemblages. The highest level of **Identification of contaminants** may be achieved with PCR-based techniques. These techniques are currently out of reach for many cultivators but are constantly improving and expected to become more readily available, both for qualitative and quantitative assessment of contaminants. A particularly promising technology is the relatively fast and low-cost *long-read nanopore sequencing*, demonstrated by van der Loos et al. (2021) to efficiently characterize algal microbiomes. Alternatively, outsourcing these analyses will remove the need for farm-scale

molecular equipment and expertise as the price and turnover time for molecular tests decreases in the future. Nevertheless, this approach will probably not provide continuous monitoring of contamination state.

Continuous monitoring of contamination can probably be achieved, at various levels of accuracy, using optical measurements and Artificial Intelligence (AI) data processing. The digital output acquired directly from an optical sensor may be used for inline monitoring of contamination and automatic response by regulation of culture medium or environmental conditions. Recent developments in this field include the promising use of Low-Resolution Raman Spectroscopy (supported by AI algorithms) for the quantification and identification of contaminants (Adejimi et al. 2022), the assessment of contamination of large-scale ponds by reflectance measurements (Reichardt et al. 2020), and the use of spectral absorption together with artificial neural network (ANN) for data processing (Franco et al. 2019). Continuous data acquisition may be also achieved using the “sniffer” approach based on CIMS sensing of airborne metabolites (Sauer et al. 2021), or in situ microscopic imaging systems complemented with AI-based algorithms (Orenstein et al. 2020).

While we expect to see significant technological advancements in contamination quantification, making pest management possible for the emerging *Asparagopsis* industry, these innovative technologies are not expected to become fully operational in the next couple of years, leaving an urgent need for the interim and readily replicable approach described here. The image analysis-based approach detailed here is demonstrated to cut down processing time while retaining ~ 1% error for most contaminated cultures and may fill the gap until more robust technologies are available.

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Supplementary Online Material

Online Resource #1

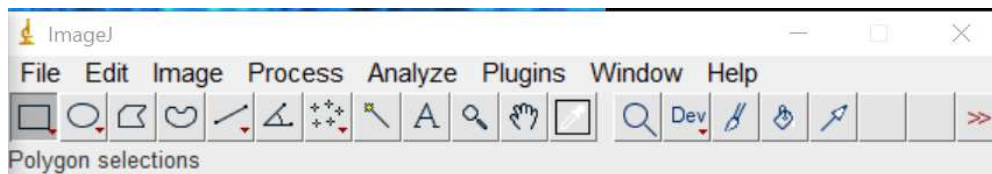
Image analysis protocol

ImageJ installation -

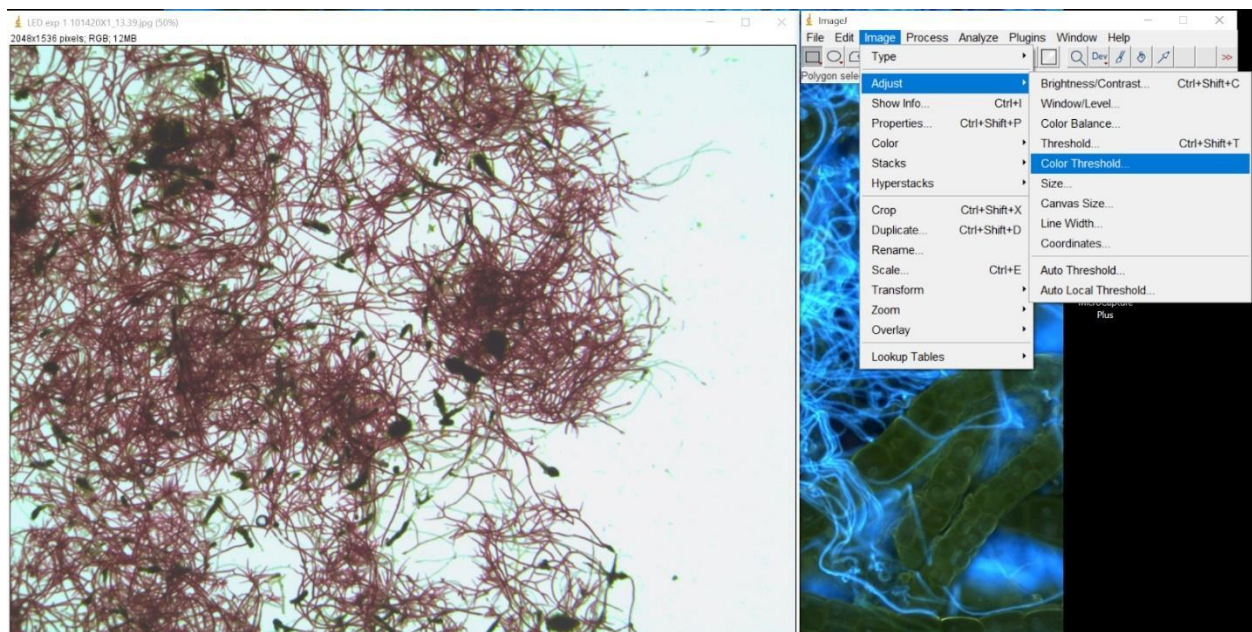
1. Download ImageJ from <https://imagej.nih.gov/ij/download.html>
2. Download the appropriate file (Windows/Mac)

Non-automated quantification using color threshold settings -

1. Open the ImageJ application file
2. Drag your image file and drop it on the ImageJ tab

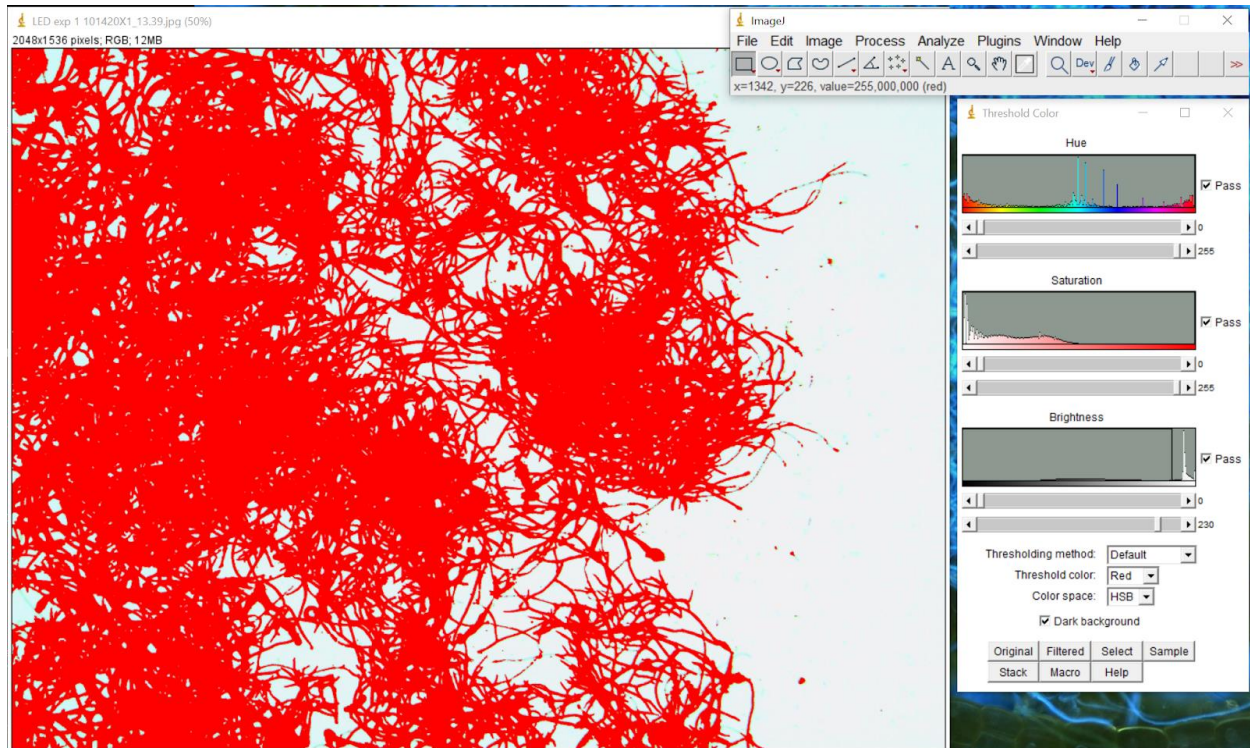


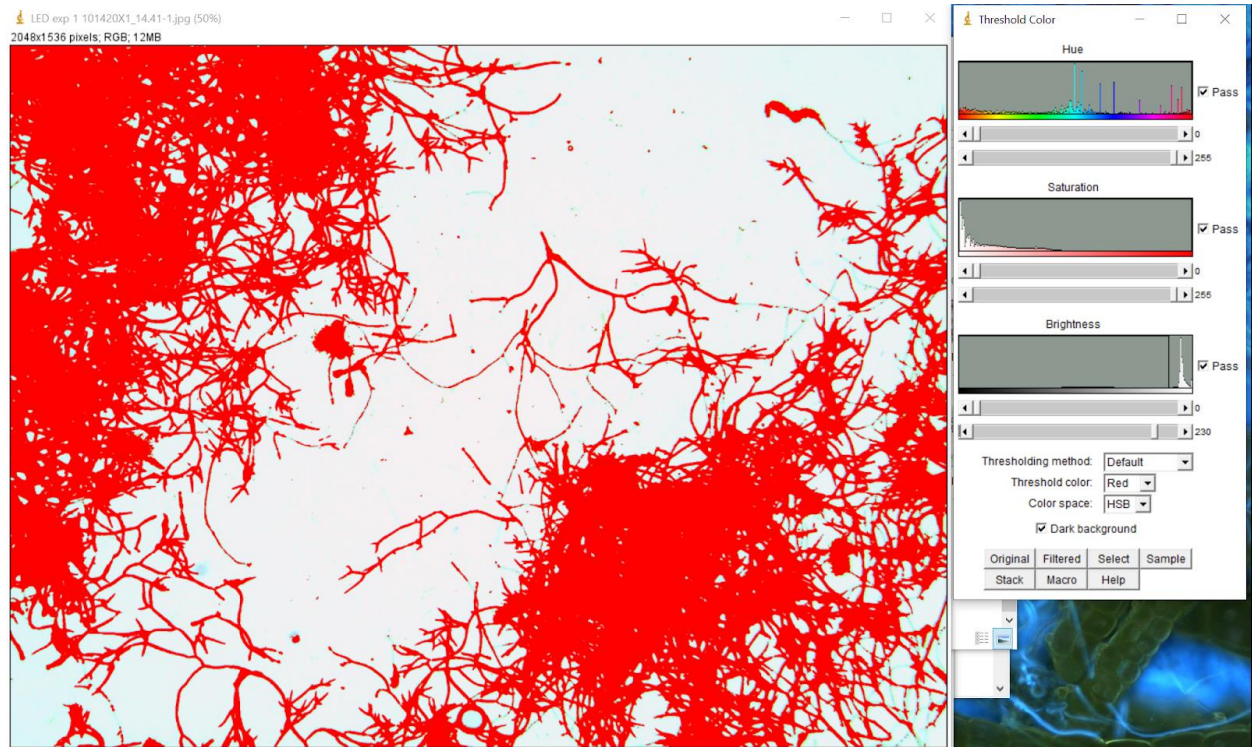
1. On the IMAGE menu, open [adjust] > [color threshold]



Selecting the whole biomass area:

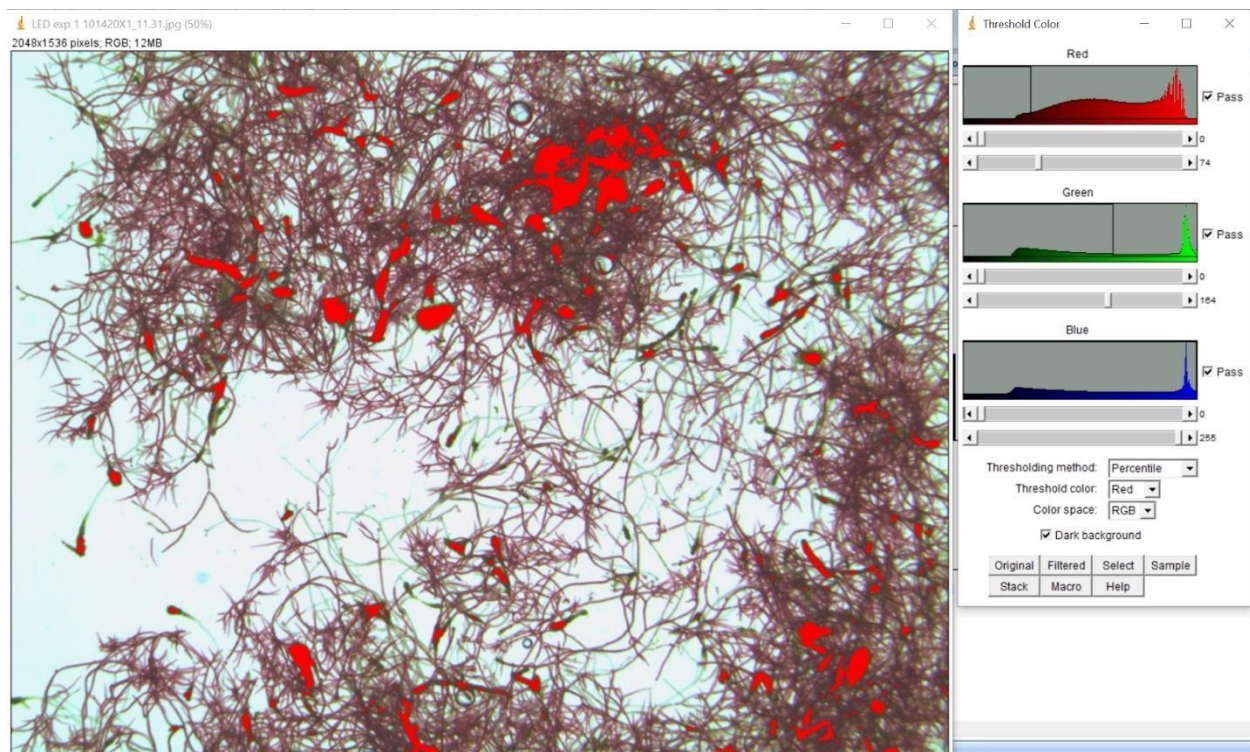
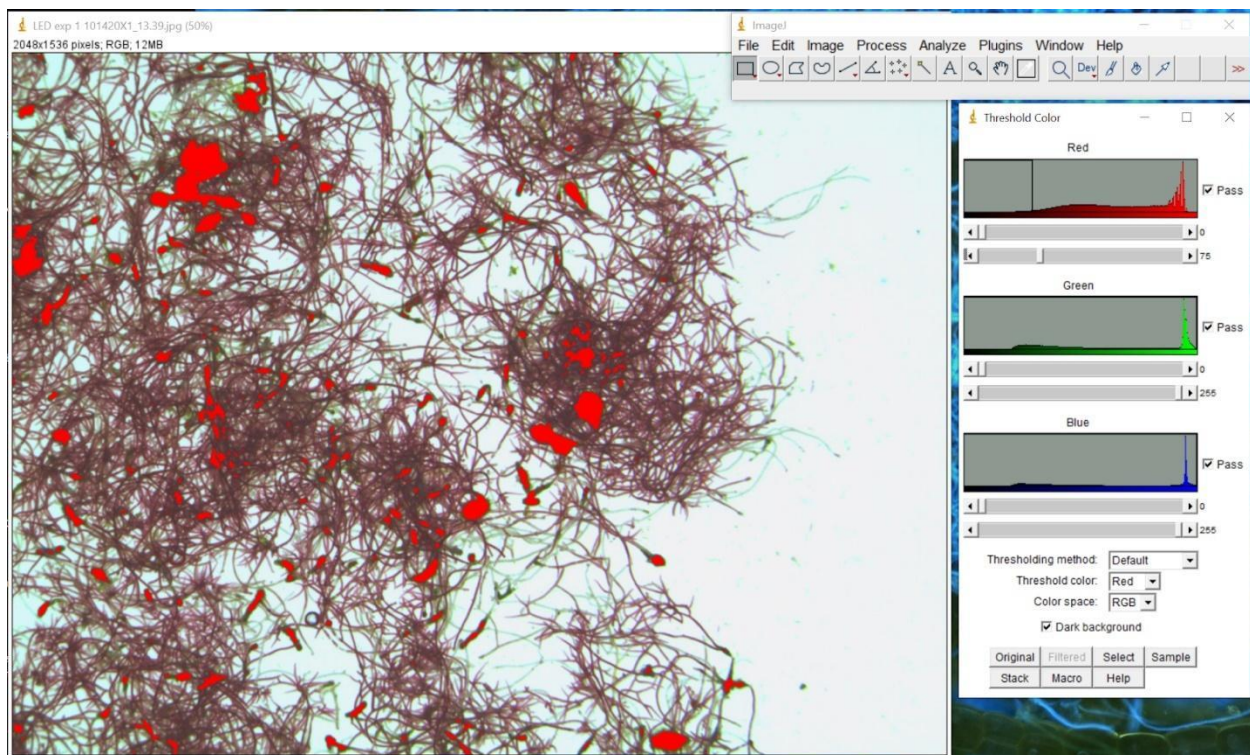
- On HSB color space
 - Brightness 0-230
- Press [Select] and take measurement with [ctrl + M]





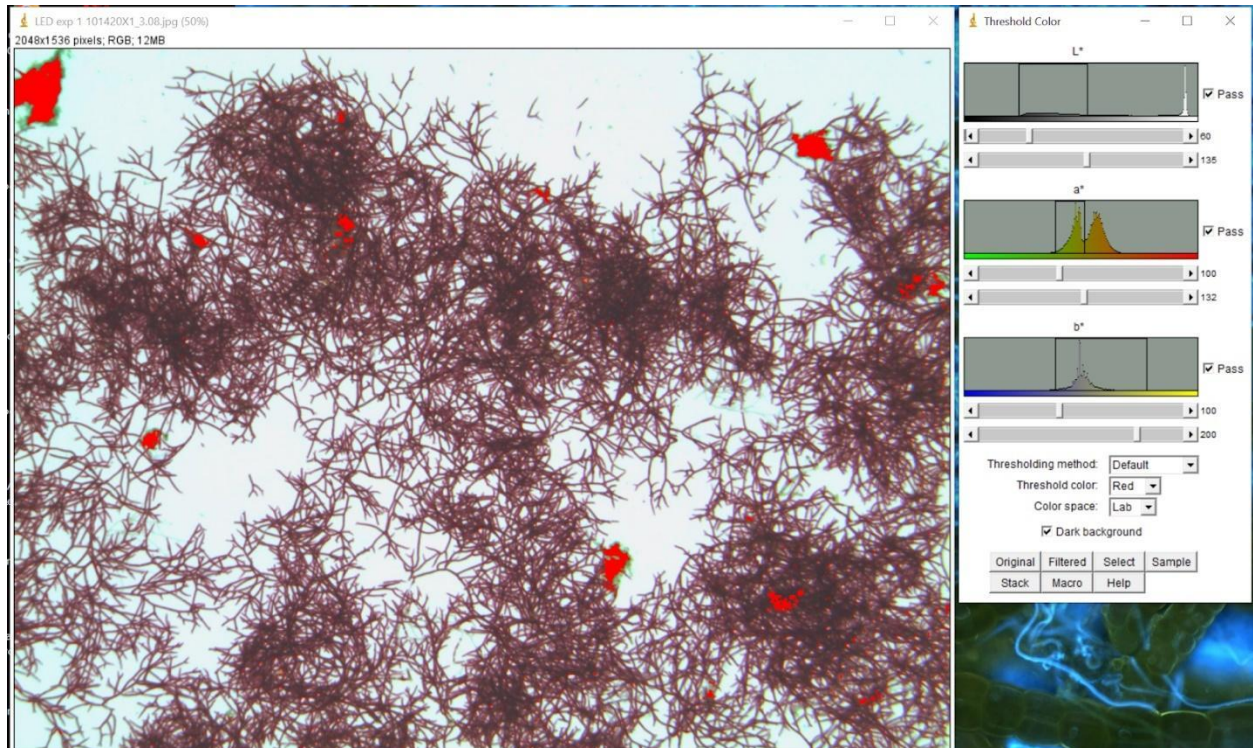
Green algae:

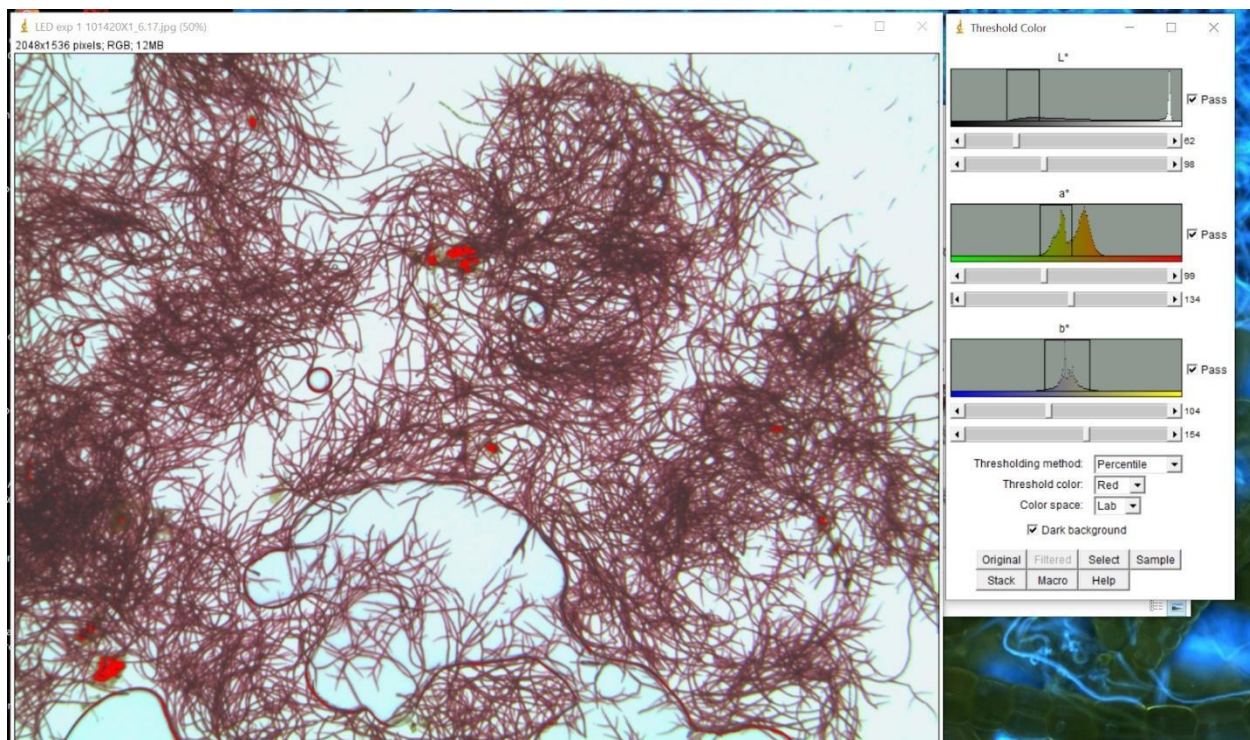
- On RGB color space
 - Green 0-250
 - Red 0-75



Cyano chunks:

- On LAB color space
 - L 60-135
 - A 100-130
 - B 100-200





Automated procedure:

Preparing the files

1. Download the images (if they are on Google Drive) and extract them if they are downloaded in a zipped folder. Gather all the images for a given dataset (e.g. “LED3 050721”) into one folder.
2. In this folder, add two sub folders for output images. Name them “Output total area [dataset]” and “Output contamination [dataset]”

Adjusting the macro script

While the total area macro has been observed to accurately highlight the total area for all datasets without the need for adjustments, the contamination area macro scripts will typically need to be adjusted for each unique dataset and occasionally for individual pictures within a dataset. For the RGB color space, which is most useful for capturing green contaminants, the R, G, and B values refer to the red, green, and blue hues respectively. Thus, based on the images in the dataset, these three values can be adjusted to selectively target the hues associated with the contamination. Typically, the G setting will be set to a larger range while the R setting will be set to a smaller range, to highlight more of the green hues associated with the contamination and less of the red hues associated with A.tax. The LAB color space utilizes a different method of characterizing color and is most useful for capturing dark-colored contaminants. Based on informal observations, the LAB settings can vary largely for different datasets. For the L setting, the minimum value can be changed, but the maximum value can typically stay at 255. For the A setting, the minimum value is usually adjusted to target the contamination area, while the maximum value stays

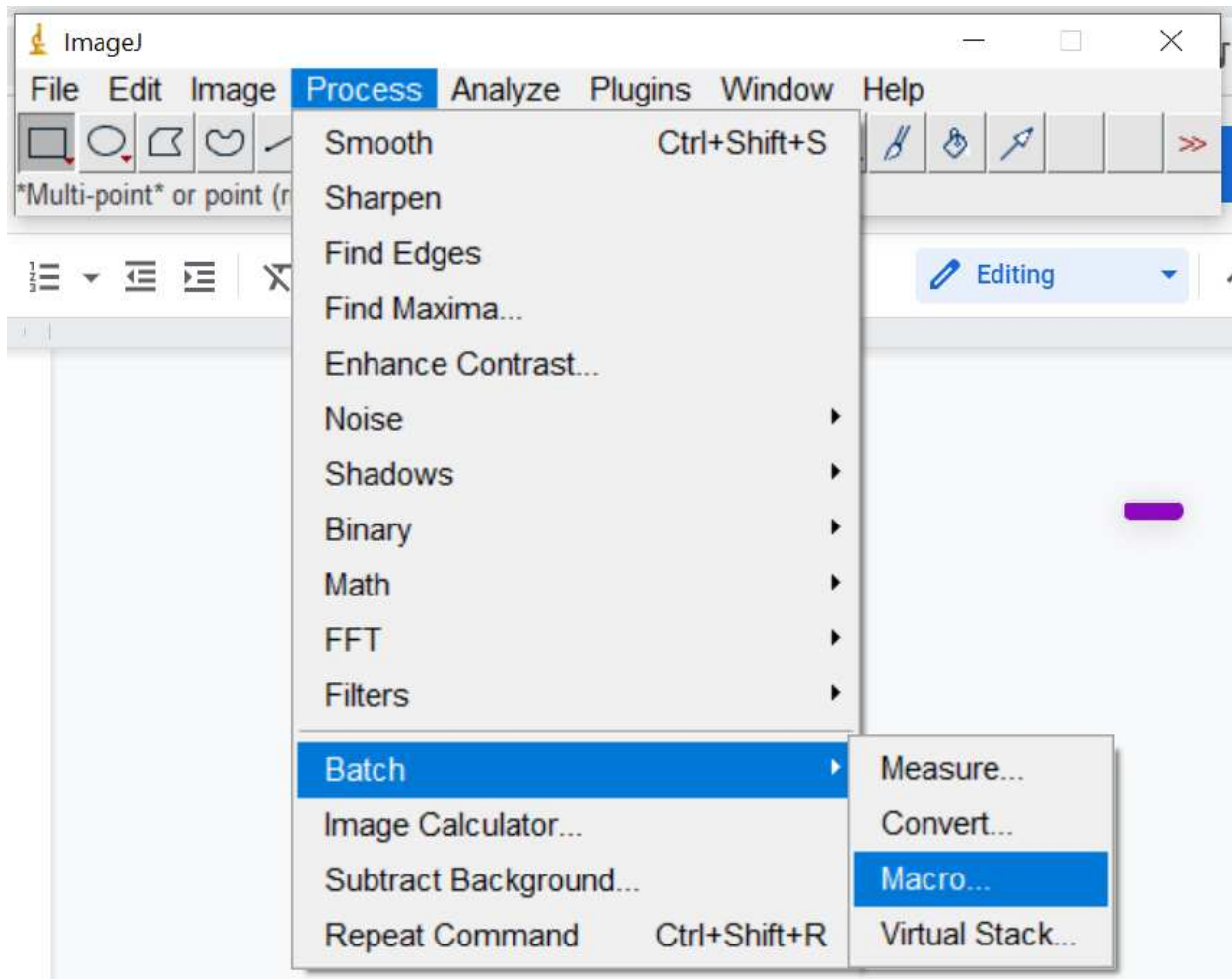
consistent around 250. The B setting is typically the most sensitive setting and can dramatically alter the selected area when adjusted in small increments. Lastly, adjust the minimum particle size by aiming to maximize the selected contamination area while minimizing the selection of small non-contaminant particles. Often, with a low particle size, the RGB and LAB settings tend to highlight the outlines of the A.tax branches as they appear more similar to the contamination color due to the background lighting of the microscope pictures. The minimum particle sizes for the macros used in this study ranged from 50 to 500.

Individual automated quantification

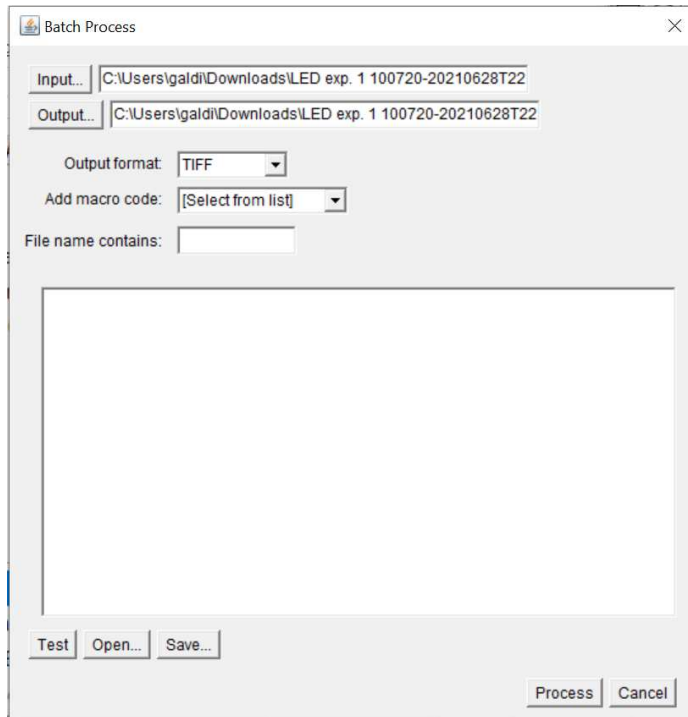
1. Drag your image file and drop it on the ImageJ tab
2. Go to [Plugins]>[Macros]>[Edit]
3. Copy and paste the Macro script of interest (see below) to the macro window. Use *Total Area Macro* to get the total biomass area, and *LAB Contamination Area Macro* or *RGB Contamination Macro* for the contamination area. Run the selected macro for the image.

Batch processing of contamination pictures

1. Go to [Process]>[Batch]>[Macro]



1. Browse the [input] and [output] to choose the folder with your images (input) and where you want to save the analyzed pictures (output). Output photos for the total area should be saved in the “Output total area [dataset]” folder, and likewise output photos for the contamination area should be saved in the “Output contamination [dataset]”.



1. Copy and paste the Macro script of interest (see below) to the macro window. Use *Total Area Macro* to get the total biomass area, and *LAB Contamination Area Macro* or *RGB Contamination Macro* for the contamination area. Run the total area and the contamination macros separately.

Total Area Macro

```
// Color Thresholder 1.52a
// Autogenerated macro, single images only!
min=newArray(3);
max=newArray(3);
filter=newArray(3);
a=getTitle();
run("HSB Stack");
run("Convert Stack to Images");
selectWindow("Hue");
rename("0");
selectWindow("Saturation");
rename("1");
selectWindow("Brightness");
rename("2");
min[0]=0;
max[0]=255;
filter[0]="pass";
min[1]=0;
max[1]=255;
filter[1]="pass";
min[2]=0;
max[2]=230;
filter[2]="pass";
for (i=0;i<3;i++){
    selectWindow(""+i);
    setThreshold(min[i], max[i]);
    run("Convert to Mask");
    if (filter[i]=="stop") run("Invert");
}
imageCalculator("AND create", "0","1");
imageCalculator("AND create", "Result of 0","2");
for (i=0;i<3;i++){
    selectWindow(""+i);
    close();
}
selectWindow("Result of 0");
close();
selectWindow("Result of Result of 0");
rename(a);
// Colour Thresholding-----
run("Analyze Particles...", "size=500-Infinity show=Masks display clear summarize in_situ");
```

LAB Contamination Macro

```
// Color Thresholder 1.52a
// Autogenerated macro, single images only!
min=newArray(3);
max=newArray(3);
filter=newArray(3);
a=getTitle();
call("ij.plugin.frame.ColorThresholder.RGBtoLab");
run("RGB Stack");
run("Convert Stack to Images");
selectWindow("Red");
rename("0");
selectWindow("Green");
rename("1");
selectWindow("Blue");
rename("2");
min[0]=173;
max[0]=255;
filter[0]="stop";
min[1]=143;
max[1]=255;
filter[1]="stop";
min[2]=95;
max[2]=179;
filter[2]="pass";
for (i=0;i<3;i++){
    selectWindow(""+i);
    setThreshold(min[i], max[i]);
    run("Convert to Mask");
    if (filter[i]=="stop") run("Invert");
}
imageCalculator("AND create", "0","1");
imageCalculator("AND create", "Result of 0","2");
for (i=0;i<3;i++){
    selectWindow(""+i);
    close();
}
selectWindow("Result of 0");
close();
selectWindow("Result of Result of 0");
rename(a);
// Colour Thresholding-----
run("Analyze Particles...", "size=2000-Infinity show=Masks display clear summarize in_situ");
```

RGB Contamination Macro

```
run("Color Threshold...");
// Color Thresholder 1.52a
// Autogenerated macro, single images only!
min=newArray(3);
max=newArray(3);
filter=newArray(3);
a=getTitle();
run("RGB Stack");
run("Convert Stack to Images");
selectWindow("Red");
rename("0");
selectWindow("Green");
rename("1");
selectWindow("Blue");
rename("2");
min[0]=0;
max[0]=75;
filter[0]="pass";
min[1]=0;
max[1]=255;
filter[1]="pass";
min[2]=0;
max[2]=255;
filter[2]="pass";
for (i=0;i<3;i++){
    selectWindow(""+i);
    setThreshold(min[i], max[i]);
    run("Convert to Mask");
    if (filter[i]=="stop") run("Invert");
}
imageCalculator("AND create", "0","1");
imageCalculator("AND create", "Result of 0","2");
for (i=0;i<3;i++){
    selectWindow(""+i);
    close();
}
selectWindow("Result of 0");
close();
selectWindow("Result of Result of 0");
rename(a);
// Colour Thresholding-----
run("Analyze Particles...", "size=100-Infinity show=Masks display clear summarize in_situ");
```

Online Resource #2:

Macro Parameters Used for the Quantification of Various contamination types

Contamination Type	Macro Parameter	Color space (RGB/LAB)		
			LAB	RGB
Cyanobacteria	Min[0]	Min	120	100
		Max	200	210
	Max[0]	Min	255	160
		Max	255	240
	Min[1]	Min	137	75
		Max	160	105
	Max[1]	Min	250	110
		Max	255	200
	Min[2]	Min	60	0
		Max	95	130
	Max[2]	Min	133	145
		Max	195	255
	Particle size	Min	50	100
		Max	500	150
Diatoms	Min[0]	Min		165
		Max		165
	Max[0]	Min		200
		Max		200
	Min[1]	Min		110
		Max		110
	Max[1]	Min		140
		Max		140
	Min[2]	Min		120
		Max		120
	Max[2]	Min		165
		Max		165
	Particle size	Min		50
		Max		50
Green algae	Min[0]	Min	160	0
		Max	200	165
	Max[0]	Min	255	90
		Max	255	235
	Min[1]	Min	137	0
		Max	160	105
	Max[1]	Min	250	125
		Max	255	170
	Min[2]	Min	95	0
		Max	95	130
	Max[2]	Min	135	165
		Max	185	255
	Particle size	Min	300	100
		Max	500	100

Online Resource #3:

Tabulated data - Manual Vs. ImageJ macro tracing of contamination

Contamination Type	Manual Contamination Area (%)	ImageJ Contamination Area (%)
Cyano	0.0	0.1
Cyano	0.0	0.0
Cyano	0.0	0.0
Cyano	0.1	0.3
Cyano	0.5	0.2
Cyano	0.3	0.1
Cyano	0.0	0.6
Cyano	1.2	1.1
Cyano	0.0	0.9
Cyano	0.0	0.7
Cyano	3.8	4.1
Cyano	3.1	2.8
Cyano	4.2	3.2
Cyano	4.3	3.8
Cyano	6.0	5.2
Cyano	3.7	3.0
Cyano	5.6	4.5
Cyano	12.8	9.2
Cyano	6.1	5.1
Cyano	7.0	6.8
Cyano	1.0	0.5
Cyano	0.4	0.3
Cyano	0.3	0.4
Cyano	0.3	0.1
Cyano	2.8	1.3
Cyano	9.8	10.6
Cyano	8.1	11.2
Cyano	8.8	8.0
Green	10.7	13.2
Green	0.3	0.5
Green	0.7	1.1
Green	3.4	2.1
Green	4.6	1.5
Green	0.8	0.3
Green	0.2	0.5
Green	1.5	0.6

Cyano	1.0	0.5
Cyano	0.2	1.0
Cyano	0.0	0.7
Cyano	0.7	0.4
Green	11.2	8.2
Green	2.7	2.9
Green	4.4	5.7
Green	3.6	3.3
Green	4.6	2.5
Green	0.5	0.9
Green	0.6	0.6
Green	0.7	0.6
Green	3.8	1.5
Green	1.6	1.0
Green	2.7	0.9
Green	4.1	0.6
Cyano	0.6	0.3
Cyano	5.1	2.6
Cyano	2.3	3.0
Cyano	9.7	12.9
Cyano	4.5	4.7
Cyano	7.9	9.2
Cyano	5.8	6.6
Cyano	1.4	2.1
Cyano	3.3	2.0
Cyano	2.3	1.5
Cyano	5.0	4.3
Green	3.6	3.1
Green	4.3	8.4
Green	3.7	8.9
Green	7.0	10.4
Cyano	0.0	0.3
Cyano	0.5	0.3
Cyano	10.8	10.8
Cyano	15.3	11.7
Cyano	8.7	8.3
Green	1.4	1.2
Green	13.9	11.8
Green	15.5	14.5
Green	16.3	17.5
Green	11.7	12.9
Green	14.9	14.7
Green	21.6	20.7

Green	19.4	19.9
Cyano	3.4	1.8
Cyano	1.0	0.8
Cyano	15.6	16.4
Cyano	2.4	1.9
Cyano	2.4	2.2
Cyano	2.3	1.4
Green	8.2	10.6
Green	9.6	11.4
Green	7.7	9.1
Green		1.5
Green	14.9	15.1
Cyano	7.6	8.5
Cyano	2.2	1.6
Cyano	1.0	1.6
Cyano	0.8	1.0
Cyano	1.6	1.1
Cyano	1.0	1.8
Cyano	0.9	3.3
Cyano	14.8	15.7
Cyano	16.8	17.5

