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Title: Early Detection of Cyanobacterial Blooms and Associated Cyanotoxins Using Fast Detection Strategy

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **Y**

3. **Interview statements:** Considering the covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **Y, ~20 km**

Protocol Length
Number of Shots: **25**

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Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Valeria Costantino**: The fast detection strategy allows the early detection of cyanobacterial blooms and related cyanotoxins in water samples and organic matrices, such as shellfish **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

1.2. **Roberta Teta**: Cyanobacterial blooms are due to the overgrowth of cyanobacteria, which live in any kind of environment and have emerged as an ecological problem across the globe within the last 15 years **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

1.3. **Germana Esposito**: As the number of harmful cyanobacterial blooms has increased in the last several years, the need for their early detection, which is key for addressing bloom and toxin spread, has become more urgent **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

1.4. **Massimiliano Lega**: Our fast detection strategy combines remote and proximal sensing techniques **[1]** and technologies with laboratory chemical and bioinformatic analyses in a unique integrated workflow **[2]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

1.4.2. LAB MEDIA: Fig 1 new

1.5. **Valeria Costantino**: The all process is safe~~. with the remote and proximal sensing steps performed remotely and with~~ The appropriate safety measures are taken to prevent aerosol inhalation and skin contact during sampling and in-lab analysis **[1]**.

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1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. Remote and Proximal Sensing Data Retrieval and Analysis

2.1. For data retrieval, first locate the target area on a global world map **[1]** and retrieve the data from various public and private remote sensing datasets for the collection date **[2]**.

2.1.1. WIDE: Talent at computer localizing target area on global world map, with monitor visible in frame *Videographer: Important/difficult step* Authors also provided 2-1-1 screen.mov
2.1.2. SCREEN: 2-1-2 screen: 00:13-00:43 *Video Editor: please speed up*

2.2. After retrieval, process the raw data **[1A]**, calculate the multispectral indexes **[1B]**, and classify the resulting information **[1]**. Then define the sampling sites on the generated thematic map **[2]**.

2.2.1A Added shot SCREEN: 2-2-1 screen: 00:11-00:34 *Video Editor: please speed up*
2.2.1B Added shot SCREEN: 2-2-1 screen: 00:45-01:25 *Video Editor: please speed up*
2.2.1. SCREEN: 2-2-1 screen: 03:20-03:45 *Video Editor: please speed up*
2.2.2. SCREEN: 2-2-2 screen: 00:04-00:48 *Video Editor: please speed up*

2.3. For sample collection, transport the equipment to the selected sampling site in the mobile lab **[1]** and plan the drone flight path for performing a macro-area survey **[2]**.

2.3.1. WIDE: Mobile lab being driven to the sampling site
2.3.2. Talent planning the automated flight by the drone operation app with the tablet visible in frame

2.4. At the site, use several drones equipped with different payloads to perform flight missions **[1]** and use ~~analyze~~ the ~~acquired data by~~ footage acquired by the drone to validate the bloom presence and extension and to identify precise sampling points **[2]**.

2.4.1. Talent flying a drone along the planned path *Videographer: Important step*
2.4.2. SCREEN: 2-4-2 E: 00:00-00:19 *Video Editor: please speed up*

2.5. At the identified sampling point, put on the appropriate personal protective equipment **[1]** and collect three, 500-milliliter water samples from each site **[1]**.

2.5.1. Talent putting on goggles and/or mask and/or gloves
2.5.2. Talent collecting sample *Videographer: Important step*

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2.6. Measure several environmental parameters, such as the air and water temperature **[1]** and the site pH and salinity **[2]**, ~~and record the data **[3]**.~~

2.6.1. Talent measuring air and water temperature
2.6.2. Talent adding sample to refractometer to measure salinity
~~2.6.3. Talent reporting all data on a notebook~~

2.7. Then store the collected samples in the mobile lab for transport to the university lab **[1]**, prepare slides ~~**[2]**~~, and screen the samples with the mobile lab microscope equipped with a digital camera **[2]** to allow microscopic taxonomic analysis and ~~to~~ identification of the species present within the samples on the basis of their blue-green color, cell shape, and size pellets **[3]**.

2.7.1. Talent storing samples
2.7.2. Talent spreading sample on a slide under the hood
2.7.3. Talent analyzing samples at microscope with monitor visible in frame
Videographer: Important step

3. Cyanobacteria Species Identification

3.1. Once the species of ~~any~~ cyanobacteria collected within the samples has been identified, at the university lab, centrifuge the samples **[1-TXT]** and transfer each supernatant to a new container without disturbing the sample pellets **[2]**.

3.1.1. WIDE: Talent placing samples into centrifuge **TEXT: 5 min, 11,200 x *g*, RT**
3.1.2. Talent transferring supernatant

~~3.2. Spread a drop from each sample pellet onto individual glass microscope slides **[1]** and use an optical microscope equipped with a digital camera and 400x and 1000x objectives to analyze the sample pellets for the presence of cyanobacteria **[2]**.~~

~~3.2.1. Talent spreading sample on a glass slide and placing onto microscope stage~~
~~3.2.2. Talent at microscope, imaging slide~~

~~3.3. Then perform microscopic taxonomic analysis of the pellets to identify the species present within the samples on the basis of their blue-green color, cell shape, and size pellets **[1-TXT]**.~~

~~3.3.1. LAB MEDIA: To be provided by Authors: Image of cyanobacteria in a representative pellet sample **TEXT: See text for taxonomic analysis details**~~

3.4. ~~Next,~~ Add 500 milliliters of butanol to each sample supernatant **[1]** and transfer each solution to be extracted into a separatory funnel **[2]**.

3.4.1. Talent adding solution to funnel *Videographer: Important step*
3.4.2. Talent adding butanol to supernatant, with butanol container visible in frame *Videographer: Important step* Note: Authors switched these shot descriptions but now they don't match the VO. Please make sure that the action in the videos matches the text for these steps, even if the authors notes are wrong.

3.5. After shaking and placing the funnels upright in individual ring clamps, ~~and~~ allow the aqueous phases to drain into individual Erlenmeyer flasks **[1]**.

3.5.1. Water draining into Erlenmeyer flask

3.6. After repeating the layer separation three times, concentrate the organic phases **[1]** under vacuum and weigh them **[2]**.

3.6.1. Talent concentrating phase under vacuum
3.6.2. Talent showing dried sample

4. Cyanotoxin Identification and Bioinformatic Analysis and Molecular Networking

4.1. For sample extraction using organic solvents, add 50 milliliters of fresh methanol to ~~transfer~~ each sample pellet ~~into a flask~~ **[1]** and sonicate the samples in an ice bath **[2]**.

4.1.1. WIDE: Talent adding sample to flask
4.1.2. Talent sonicating sample

4.2. After 5 minutes, add 50 milliliters of fresh methanol to each sample **[1]** and gently shake the flask **[2]** before filtering the solutions through individual pieces of filter paper **[3]** and collecting the filtrates in round bottom flasks **[4]**.

4.2.1. Talent adding methanol to sample, with methanol container visible in frame
4.2.2. Talent shaking flask
4.2.3. Talent adding sample to filter
4.2.4. Filtrate being collected in flask bottom

4.3. After filtering each sample two more times as just demonstrated, analyze the sample extracts by liquid chromatography and high-resolution tandem mass spectrometry according to standard protocols **[1]**.

4.3.1. Talent injecting sample into instrument

4.4. Then generate a molecular network using the Global Natural Products Social platform **[1]** and use the appropriate tools to analyze the resulting network and tandem mass

spectrometry data to identify any toxins determined to be present within the collected samples **[2]**.

4.4.1. Talent at computer/instrument, analyzing molecular network, with monitor visible in frame *Videographer: Important/difficult step*

4.4.2. LAB MEDIA: 4-4-2 screen: 00:03-00:37 *Video Editor: please speed up*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.1., 2.4., 2.5., ~~3.2.,~~ 3.4., 4.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
2.1. Informative data can be acquired using satellites, aircrafts, helicopters, UAVs, covering all layers in the hierarchical monitoring approach. The information layers that derive from missions performed by platforms that fly at lower altitudes than satellites (e.g. aircrafts, helicopters, UAVs) restitute information with great resolution but these are very expensive and also require more time to complete the full acquisition process that also includes flight plan defining and approval. The solution that matches the minimum needs required by the fast detection strategy and can guarantee the success in guiding sampling spot choice is the use of the only satellite products.

4.4. Once that spots to be samples have been selected, analytical/bioinformatics analyses (Molecular networking of LC-MS data) is the tool for fast dereplication of the water samples and fast detection of cyanotoxins. 16S metagenomic analysis takes at least two weeks of work. Moreover, even when cyanobacterial species that are generically toxic are identified, their toxins production is not demonstrated. For the same reason, microscopic observation is not itself sufficient to reveal the presence of toxic cyanobacteria. Of course, MS analysis and molecular networking have some limitations, e.g. they are really effective if compounds of interest (e.g. toxins) are well ionized in the applied conditions, if they are in sufficient amount to be detected. For the purpose of the known cyanobacterial toxins detection and monitoring the MS-based molecular networking actually represents one of the more robust and reliable technologies.

Results

5. Results: Representative Fast Detection Strategy Analysis

5.1. The proposed strategy was validated by the results obtained in the coastal monitoring program active on the Campania region in southern Italy from 2015-2021 **[1]**.

5.1.1. LAB MEDIA: Figure 4 new

5.2. A visual workflow that links the techniques with the produced results was generated. During the subsequent monitoring campaigns, each step was optimized with the aim of fast detection **[1]**.

5.2.1. LAB MEDIA: Figure 4 new *Video Editor: please emphasize workflow in oval on left side of image*

5.3. Optimization of the remote sensing workflow permitted a reduction in the number of platforms and missions while improving the generated product level **[1]**.

5.3.1. LAB MEDIA: Figure 2 new *Video Editor: please emphasize satellite, plane, and drone images and corresponding cones*

5.4. For example, this fast detection strategy facilitates a transition from the use of several different aerial platforms ~~(e.g. satellites, aircraft, and drones)~~ to satellite and drone platforms only **[1]** and from the use of several different multispectral specialized indexes to the more informative chlorophyll-a and normalized difference vegetation index thematic maps ~~to the more informative chlorophyll-a map~~ **[2]**.

5.4.1. LAB MEDIA: Figure 4 new *Video Editor: please emphasize satellite and drone and related cones*

5.4.2. LAB MEDIA: Figure 4 new *Video Editor: please emphasize aerial platforms at and multispectral thematic maps at top right of Figure*

5.5. In parallel, the chemical workflow was reduced from requiring a 16S (sixteen-S) metagenomic analysis to using microscopic observation only for determining the cyanobacterial community **[1]** and the new biological workflow utilizes LCMS-based molecular networking for a quick and accurate cyanotoxin detection **[2]**.

5.5.1. LAB MEDIA: Figure 3 new *Video Editor: please emphasize top row of flow chart*

5.5.2. LAB MEDIA: Figure 3 new *Video Editor: please emphasize bottom row of flow chart*

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Conclusion

6. Conclusion Interview Statements

6.1. **Roberta Teta**: This strategy allows the study of cyanobacterial blooms as bioindicators of pollution, particularly within areas in which the presence of blooms is related to eutrophication processes induced by anthropogenic pressure **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

6.2. **Massimiliano Lega**: This multidisciplinary strategy requires the combination and integration of different techniques, technologies, and expertise in a unique workflow for its successful implementation **[1]**.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

6.3. **Valeria Costantino**: ~~This~~ Fast detection strategy is useful for preventing health community problems due to harmful cyanobacterial blooms and allows to monitor large areas in short time ~~the quantification of both cyanobacterial bloom and toxin spread over space and time~~ **[1]**.

6.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

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