Flow cytometry and imaging in flow methods facilitate automated observations and monitoring of algal blooms and phytoplankton abundance and diversity in automated platforms

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Use of automated multi-spectral fluorometer and/or automated flow cytometry in JERICO-NEXT

Note: not all ferries, cruises and fixed systems used are included on map.
# Essential Ocean Variables

<table>
<thead>
<tr>
<th>PHYSICS</th>
<th>BIOGEOCHEMISTRY</th>
<th>BIOLOGY AND ECOSYSTEMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea state</td>
<td>Oxygen</td>
<td>Phytoplankton biomass and diversity</td>
</tr>
<tr>
<td>Ocean surface stress</td>
<td>Nutrients</td>
<td>Zooplankton biomass and diversity</td>
</tr>
<tr>
<td>Sea ice</td>
<td>Inorganic carbon</td>
<td>Fish abundance and distribution</td>
</tr>
<tr>
<td>Sea surface height</td>
<td>Transient tracers</td>
<td>Marine turtles, birds, mammals abundance and</td>
</tr>
<tr>
<td>Sea surface temperature</td>
<td>Particulate matter</td>
<td>distribution</td>
</tr>
<tr>
<td>Subsurface temperature</td>
<td>Nitrous oxide</td>
<td>Live coral</td>
</tr>
<tr>
<td>Surface currents</td>
<td>Stable carbon isotopes</td>
<td>Seagrass cover</td>
</tr>
<tr>
<td>Subsurface currents</td>
<td>Dissolved organic carbon</td>
<td>Macroalgal canopy</td>
</tr>
<tr>
<td>Sea surface salinity</td>
<td>Ocean colour</td>
<td>Mangrove cover</td>
</tr>
<tr>
<td>Subsurface salinity</td>
<td>(Spec Sheet under development)</td>
<td>Microbe biomass and diversity (*emerging)</td>
</tr>
<tr>
<td>Ocean surface heat flux</td>
<td></td>
<td>Benthic invertebrate abundance and distribution</td>
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<tr>
<td></td>
<td></td>
<td>(*emerging)</td>
</tr>
</tbody>
</table>

The Essential Ocean Variables as defined by UNESCO Global Ocean Observing System

[http://www.goosocean.org](http://www.goosocean.org)
The Marine Strategy Framework Directive was updated in May 2017

<table>
<thead>
<tr>
<th>Habitats</th>
<th>Broad habitat types of the water column (pelagic) and seabed (benthic) (Note 5), or other habitat types, including their associated biological communities throughout the marine region or sub-region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per habitat type:</td>
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<tr>
<td></td>
<td>— habitat distribution and extent (and volume, if appropriate)</td>
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<tr>
<td></td>
<td>— species composition, abundance and/or biomass (spatial and temporal variation)</td>
</tr>
<tr>
<td></td>
<td>— size and age structure of species (if appropriate)</td>
</tr>
<tr>
<td></td>
<td>— physical, hydrological and chemical characteristics</td>
</tr>
<tr>
<td></td>
<td>Additionally for pelagic habitats:</td>
</tr>
<tr>
<td></td>
<td>— chlorophyll a concentration</td>
</tr>
<tr>
<td></td>
<td>— plankton bloom frequencies and spatial extent</td>
</tr>
</tbody>
</table>
What are harmful algae?

Main types

- Fish killers
- Toxin producers – affecting human health through fish, shellfish, aerosols etc.
- Nuisance blooms – affecting tourism etc.
- High biomass blooms connected to eutrophication – results in low oxygen conditions

Shellfish may transfer algal toxins to humans
Examples of harmful algal bloom species

Note: Approximately 2000 species of phytoplankton are found in samples analysed using microscopy, meta barcoding of rDNA indicates that this is an underestimate by a factor of 20

- **Dinophysis spp.**
- **Alexandrium tamarense**
- **Nodularia spumigena**
- **Prymnesium polylepis**
- **Karenia mikimotoi**
- **cf. Azadinium spinosum**
- **Protoceratium reticulatum**
- **Pseudo-nitzschia sp.**
- **Chaetoceros concavicornis**

Photos: Bengt Karlson, Ann-Turi Skjevik, Lars Edler, Jahn Throndsen and Wenche Eikrem

Photo: Ann-Turi Skjevik

Pseudochattonella farcimen
Observing the phytoplankton - ongoing methods

Traditional phytoplankton sampling and analysis

- Sampling devices
  - Niskin bottles
  - Tube sampling
  - ISCO-samplers
  - Etc.

- Microscopy
  - Utermöhl method
  - Fluorescence microscopy
  - Etc.

Bulk measurements based on pigment content

- HPLC
- Fluorescence
  - Chlorophyll
  - Phycocyanin
  - Phycoerythrin
  - Multi spectral

- Absorbtion
  - Single wavelength
  - Multi spectral
Beyond the impediment of discrete sampling

Observation of phytoplankton in near real time

Imaging flow cytometry
Single cells – size and morphology of organisms

Flow cytometry
Single cells – fluorescence – pigment content and scattering (size, shape)

Fluorescence and absorption (multi-spectral)
Pigment based methods – bulk properties
Instruments for imaging in flow

- Imaging FlowCytoBot
  - McLane Inc., USA
- CytoSense and CytoPro
  - CytoBuoy, the Netherlands
- FlowCAM
  - Fluid Imaging Tech., USA
- FastCAM (prototype)
  - Ifremer-LDCM
- ImageStream

Some of the images from Dashkova et al 2017
Imaging FlowCytoBot (IFCB) principle

- Images of all organisms ~10-150 µm
- Sampling every 20 min.
- Several thousand images per sample of 5 mL
- Fluorescence and scattering mainly used for triggering camera
- Morphology-based
- > 200 parameters measured on each organism
- Random forest based automated classifiers
Continuous Filtered Sheath Flow

Detector/Filter

Flash/Image Acquisition

B. Karlson & M. Brosnahan

http://mclanelabs.com/
Acquisition of plankton images during field work

Manual identification of organisms in training set

Images of identified organisms used to produce classifiers

Re-analyses of training set

Quality estimate
Number of correct identifications
False positives
False negatives

Step 1
In situ instrument (IFCB) used

Step 2
Phytoplankton identification specialist needed

Step 3
Analysis of the whole data set – final results produced

Classification of plankton images

Training set of images

> 1,000,000 images of plankton

Step 3
Classifiers used to analyse the whole set of images

Results
Species composition and cell abundance
The raft at the Tångesund observatory
A simplified view of the Tångesund coastal ocean observatory
IFCB results Tångesund 28 Sep. 1313 UTC

Total number
1412 in 5 mL
282400 targets per Litre
Examples of *Dinophysis* spp. images from IFCB producer of Diarrhetic Shellfish Toxins (DST)
Examples of dinoflagellates
Examples of diatoms

*Pseudo-nitzschia* sp. producer of domoic acid
Example of results – *Lingulodinium polyedrum*

SEM photo by: Mats Kuylenstierna
Source: http://nordicmicroalgae.org
Imaging FlowCytoBot at SYKE

- Imaging FlowCytoBot purchased 2016
- primed, tested and run in the SYKE lab in winter 16/17; team trained by experienced user, Sílvia Anglès from the US, using test samples from Alg@line ferrybox
- part of team travelled to the US (McLane and WHOI) for further training
- deployed successfully at Utö 03/2017, connected to flow through system inside research hut
At Utö IFCB samples every 20 min and transfers raw images to Helsinki in real time.

Automated cleaning cycle, but due to clogging issues (due to large cells) this is now supplemented by remotely operated one.

Some issues with pump, electronics and camera recently; although a lot can be done by operator, device will be send for the first service late 2017.

Creation of image library for the further training of the automated image classifier is in progress and funding seeked to compare IFCB data with trad. cell counts, optical data and phys-chem data collected at Utö.
Phytoplankton community in Utö, northern Baltic proper on 20.7.2017
Sirpa Lehman, Marine Research Centre of the Finnish Environment Institute (SYKE)

Phytoplankton community in Utö, northern Baltic proper, is dominated by cyanobacteria Aphanizomenon flos-aquae and Dolichospermum sp. Only some filaments of the hepatoprotein producing cyanobacterium Nodularia spumigena have been observed. These three species are able to N-fixing, which may give them competitive advantage when there is plenty of phosphorus available in the sea water.

Dinoflagellates Dinophysis spp and Heterocapsa triquetra, diatom Chaetoceros spp., and nanoflagellates including e.g. cryptos., prasinos., and prymnesiophytes were the other most common phytoplankton taxa (Fig. 1).

Surface temperature is ca. 15°C and chlorophyll a concentration ca. 5-6 μg/l in the northern Baltic proper, based on the AlgalFline FerryBox data collected from the route of M/S Finnmaid.

Data sources:
Phytoplankton community is observed continuously using the Imaging FlowCytobot (IFCB), [https://www.finnish-marine-infrastructure.fi/1028108956], owned by the SYKE Marine Research Centre. IFCB is situated in the Utö Atmospheric and Marine Research Station of the Finnish Meteorological Institute [DP4-4958N, 21°22'36,33' Utö Island is located at the outermost edge of the Archipelago Sea, facing the Baltic proper (Fig. 2).]

IFCB, Utö Atmospheric and Marine Research Station, and the AlgalFline FerryBox network are parts of the Finnish Marine Research Infrastructure FINMAR [https://www.finnishmarine-infrastructure.fi].

Fig. 1. Selected images taken by the Imaging FlowCytobot (IFCB) on 20.7.2017 at Utö. Images from left to right: Aphanizomenon flos-aquae (upper), Dinophysis norvegica, Dolichospermum sp., Chaetoceros cf. magnusii, Chaetoceros cf. similis.

Fig. 2. Phytoplankton cells passing the flow-through system of the Imaging FlowCytobot (IFCB) can be seen in real time in the Kumpula laboratory in Helsinki (left). IFCB is owned by the Marine Research Centre of the Finnish Environment Institute (SYKE), and it is situated in the Utö Atmospheric and Marine Research Station of the Finnish Meteorological Institute (FMI). Utö Island is located at the outermost edge of the Archipelago Sea, facing the Baltic proper (right).
CytoSense/CytoPro principle

- Similar to IFCB but different
- Fluorescence and scattering are the main parameters
- Optical pulse-shape profiles are recorded as signatures
- A limited number of organisms can be imaged

Standardized output:
- *Synechococcus* (pico-cyanobacteria)
- Eukaryotic picoplankton
- Nanoplankton
- Microplankton
Optical signatures

Pictures: Machteld Rijkeboer
Subgroups can be discriminated based on similar optical properties.

- **Manual clustering software:** CytoClus (CytoBuoy)
- **Automated clustering softwares:**
  - **EasyClus**/EasyClus LIVE (Thomas Rutten projects): supervised, unsupervised analysis
  - **RclusTool** (LISIC, CNRS-LOG ULCO): supervised, unsupervised, semi-supervised analysis
The open Mediterranean sea is dominated by pico-nanoeukaryotes, even during spring blooms

- Oligotrophic sea, max ~ 1 µg/L Chla a
- Spring bloom is often dominated by <20 µm cells and mostly nanoeukaryotes size classes

Cytosense flow cytometer was improved to cover <1µm and > 500 µm: Cytogrammes depicting recorded cells

Vocabulary standardisation/database under progress within seadatacloud

https://chrome.mio.univ-amu.fr/chrome-cytobase/
http://www.mio.univ-amu.fr/cytobase/
Images from CytoPro


Photos: G. Gregori and M. Thyssen
Relationship between total Fluorescence by Flow cytometry and FerryBox

\[
y = 1 \times 10^{-9}x + 0.012
\]

\[R^2 = 0.6663\]

Total FLred (FCM) vs Total Fluorescence (FB)
Phytoplankton functional types

Abundance

Red fluorescence as a proxy for biomass

- orange_pico - Sum FlRed
- red_pico - Sum FLRed
- orange_nano - Sum FLRed
- red_nano - Sum FLRed
- red_micro - N/mL

Red picoplankton abundance

Orange picoplankton abundance
Innovative technologies on board Research Vessels
Lefebvre A., Wacquet G., Colas F., Louchart A., Artigas L.F.

Objective: Towards the operational implementation of HF resolution techniques during ecosystemic cruises (fish stock assessment, research, MSFD purposes)

Example of combination of conventional low resolution monitoring strategy with high resolution Analysis by in vivo recording innovative technologies:
- Ferry-Box
- Spectral fluorometry
- Flow cytometry
- FlowCAM
Example of water masses discrimination based on phytoplankton abundance/discrimination using a new training set for the English Channel

High variability of phytoplankton abundance between water masses on a relatively short time period (< 1 month). For each area, automated discrimination of up to 28 (image analysis) and 8-10 phytoplankton groups (automated flow cytometry).

Now available (English Channel):
- Automated Classification
- New variables
- High resolution strategy
- Early warning systems
- Quantifiable errors
- Data base to secure raw data

FlowCAM
in vivo analysis

Microphytoplankton (cell.ml⁻¹)
Synechococcus (cell.ml⁻¹)
FlowCAM principle

- Similar to IFCB but different
- No sheath fluid - not a flow cytometer
- Images (in colour or black & white) of all organisms
- Fluorescence and scattering mainly used for triggering camera
- Morphology-based
- Automated classifiers (as ZooImage package in R)
  - Recognition tools build from training sets
  - Development of analytical modules like active learning, partial validation of predictions

- Dynamic imaging-in-flow system
- Camera: 8 to 22 frames per second
FlowCAM software: Visual Spreadsheet

- 1 frame can contain multiple particles
- Pattern recognition software:
  1. Segregate particle from background
     - Grayscale pixel ≠ grayscale background → particle pixel
     - Binary image created
     - Each particle = tiff file
     - .lst created = collage of particles

Frame | Binary image collage | Particle collage = .lst file
1. Monthly sampling campaigns

2. 9 Samples (2% lugol)

3. Monthly after campaigns

4. Digital copy of samples (.lst files)

5. Semiautomatically identify plankton
   Visual spreadsheet

6. Offer validated data via online interface

Klaas Deneudt et al. VLIZ
FastCAM: a prototype

Main objectives:
- Speeding up digital images acquisition (340 vs 22 images/sec)
- Use of a high resolution Camera (1024 x 2048)
- Use of an autofocus mode

⇒ **13 min.** for 1 sample (10x / 100 µm) vs **143 min.** with the FlowCAM

FlowCAM performances

<table>
<thead>
<tr>
<th></th>
<th>4X</th>
<th>10X</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\phi_{\text{min}} (\text{nL/µm})$</td>
<td>1,2</td>
<td>0,07</td>
</tr>
<tr>
<td>t_{16mL} (min)</td>
<td>8,3</td>
<td>143</td>
</tr>
</tbody>
</table>
About image analysis data

1. Transfer and storage of millions of small images (3 gigabyte per 3 months)
2. Automated analysis of images
3. Results:
   1. At the species/genus level
      1. Abundance per litre
      2. Cell volume per litre
   2. Harmful taxa
   3. Aggregating data to higher taxonomic levels
      1. Class level
         1. Bacillariophyceae (diatoms)
         2. Dinophyceae (dinoflagellates)
         3. Cyanophyceae (cyanobacteria)
         4. Haptophyceae
         5. Etc.

Example: FlowCAM
*in vivo* analysis
EcoTaxa a system for storage of millions of images and automated classification (species identification)

http://ecotaxa.obs-vlfr.fr/explore/

Picheral M., Stemman L., MIW 2017
Conclusions

• Imaging flow cytometry is a reliable method for sustained, automated observation of phytoplankton biodiversity and biomass, complementing manual methods for sampling and microscope analyses.

• Development of classifiers for automated identification/discrimination of organisms is time consuming and requires specific skills on signal analysis and on taxonomy.

• Automated flow cytometry has proven to be a useful tool for counting phytoplankton and for describing the phytoplankton community as size based classes and functional groups, four main functional groups were selected for inter-comparison exercises:
  • *Synechococcus* (pico-cyanobacteria)
  • Eukaryotic picoplankton
  • Nanoplankton
  • Microplankton.

• New classification tools are being defined and tested which should allow improved discrimination of phytoplankton functional groups.
This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 654410.