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LifeWatch Belgium: FlowCam sampling and lab protocol for imaging microphytoplankton in the Belgian part of the North Sea.

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We use this protocol and it's working

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Abstract

In the framework of the Belgian Lifewatch RI, a number of fixed stations in the Belgian Part of the North Sea (BPNS) are visited during mulitdisciplinary campaigns. A grid of nine stations covers the coastal zone and are sampled monthly. Eight additional stations, located more offshore, are sampled on a seasonal basis. For the phytoplankton monitoring, samples are taken using a 55µm mesh size Apstein net, fixed in Lugol's iodine solution and preserved in dark and refrigerated conditions. In the lab, the samples are processed using a FlowCAM VS-4 benchtop model, equipped with a Sony XCD SC90 digital gray-scale camera and VisualSpreadsheet software version 4.2.52. For the monitoring, the FlowCam device is mounted with the 4X magnification and associated hardware to image a particles size range of 55-300µm. The identification of the images data is done with a CNN and followed by a manual validation step to check each predicated image. Since May 2017, this dataset provides micro- and phytoplankton observations, mainly covering diatoms, dinoflagellates and cilliates, for the Belgian Part of the North Sea (BPNS).
This published protocol descibes sampling and lab SOPs in detail.

Image Attribution

Nick Decombel

Materials

55µm Apstein net
acid Lugol iodine solution
sample falcons
marker
Pasteur pipettes
70% ethanol
MiliQ water
artificial seawater of seawater prefiltered at 0.22µm
waste recipient
lab coat
gloves
tissue paper
graduated cylinder

FlowCam VS-4 benchmodel, mounted with:
- 4x objective
- 5mL syringe
- 5mL pipette tip
- 300µm mesh prefilter
- funnel
- 300µm disposable flow cell
- flow cell holder

Troubleshooting

Safety warnings

Methodological choices are tailored to the objectives of the Belgian LifeWatch monitoring. They do not present a 'one size fits all' solution to all FlowCam applications or phytoplankton research questions. The purpose of publishing this protocol is making protocols open-access and FAIR.

Sample collection

1 9 onshore stations are visited on a monthly frequency and 8 offshore stations are visited on a seasonal frequency during the LifeWatch muldisisciplinary campaigns in the Belgian part of the Nort Sea.

Onshore stations	Longitude, Latitude	Offshore stations	Longitude, Latitude
130	2.90535, 51.27055	**LW01**	2.256, 51.568667
230	2.85035, 51.308683	**LW02**	2.556, 51.8
330	2.809083, 51.434117	**435**	2.790333,51.580667
700	3.221017, 51.377	**W07BIS**	3.012517,51.588033
710	3.138283, 51.441217	**W08**	2.35, 51.458333
780	3.057283, 51.471367	**W09**	2.7, 51.75
120	2.702483, 51.186083	**W10**	2.416667, 51.683333
215	2.61075, 51.274867	**421**	2.45, 51.4805
ZG02	2.500717, 51.33515		

Table 1: LifeWatch stations visited during the multidisciplinary campaigns.

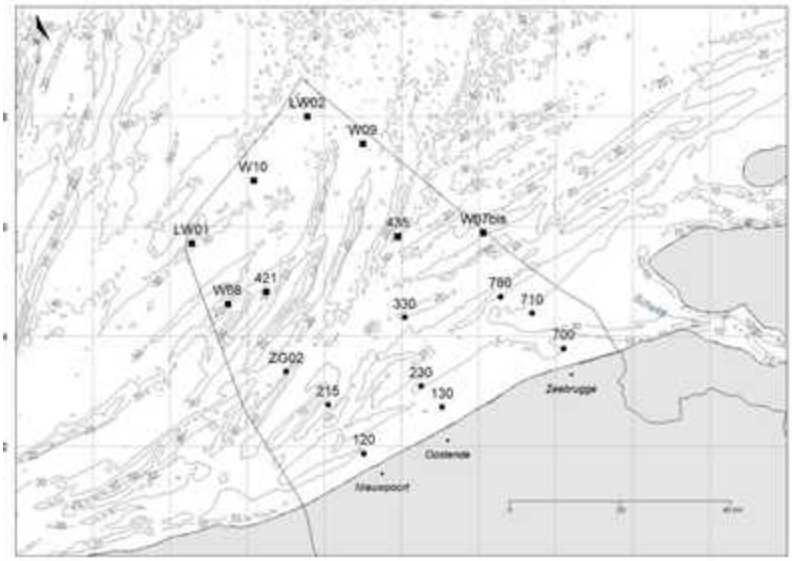

Figure 1: Map of LifeWatch stations in the Belgian part of the North Sea (BPNS). From Mortelmans et al., 2019.

2 1 replicate sample per station is taken while logging the action in the Research Vessels' information system (MIDAS). Choose actiontype 'Planktonnet Apstein', fill in reporter, select the correct station and press start and stop before and after taking the sample.

3 Collect 50L of surface water with a stainless-steel bucket and rope from the back of the starboard side of the ship.

4 Filter the 50L through a 55µm Apstein net (1.2 m long, 0.5 m diameter).

5 Transfer the sample from the cod-end of the net into a sample recipient.

6 Note down station, date, project and PI on the sample recipient.

7 Fix the phytoplankton sample at max 1% final concentration of acid Lugol Iodine's solution.

8 Store at 4°C in the dark until analysis in the lab (as fast as possible).

FlowCAM hardware set up

9 Wear lab coat and gloves, Lugol can stain.

10 Turn on FlowCAM using the power switches located on the rear and front (UPS system should never be turned off in order to ensure a continuous power supply).

11 Clean and screw in 4X objective.

12 Attach sample introduction stand and 5mL pipette tip. Put funnel on top of pipette tip and put 300µm prefilter in place.

13 Clean the 300µm disposable flow cell (FC) and place in the correct FC holder with the short tube end at the top. Screw the holder ring to attach the FC in to the holder, don't put too much pressure to avoid breaking the glass photo chamber. The tubes of FCs are cut to 20cm (bottom, direction of syringe) and 10cm (top, direction of pipette tip) length, these measurements are saved in the context file and important to calculate the dead volume.

14 Place FC with FC holder onto the FC holder mount on the rail of the FlowCam. Centre the flow cell in the holder if necessary using the screws on the FC holder. Make sure the flow cell is vertical (straight edges) and the edges are not in the field of view of the camera.

15 Attach the short tube end (10cm) to the pipette tip and the longer tube end (20cm) to the inlet of the syringe pump. Ensure that the tubing from the FC to the pipette tip is as straight as possible.

16 Attach an extra tube at the outlet of the syringe and put the end in a small beaker to collect the waste outflow.

17 Open VisualSpreadsheet on the computer.

18 Select the correct objective. Ensure that the status bar colour (if displayed) at the bottom of the main window matches the coloured stripe on the installed objective.

19 Select Setup > Pump > Change syringe and follow the appropriate steps to install the 5mL syringe. Select the correct syringe size mounted when you're done.

Load sample and focus

20 Select Setup and Focus.

21 Adjust flow rate: Choose a flow rate within the range of the mounted syringe. We use a max. flow rate of 5mL/min for the 4X magnification.

22 Rinse FC thoroughly:
Setup and focus > Start pump.
Alternate rinsing with 5mL Milli-Q water and 5mL 70% ethanol and leave air bubbles in between, do this 3 times each and finish by rinsing with Milli-Q water.

23 Measure the volume of the sample sample and note the measurement on the sample recipient cap.

24 Load some sample into the flow cell using a Pasteur pipette, gently mix the sample before pipetting to avoid sedimentation of cells. Leave some air between the sample and Milli-Q water from the last flush. Go to the Setup and Focus menu and press Start Pump, pause the pump when the sample is visible in the field of view, pinch the flow cell tubing if air bubbles would be present in the tubing.

25 Focusing is done manually on the sample before the run as this allows to focus on the main cell shape in a sample as opposed to spherical manufacturers beads. Focus on the cells in the field of view by first manually moving the FC holder on the rail using the screws, and in a second step by fine scale manual focus using Focus > Tools > Enable manual focus and choose + or - to finetune focus. Close the setup and focus menu when done.

Presample run

26 For each sample we perform a small presample run to determine particle load and appropriate dilution of the sample to avoid particles overlapping in a single frame.

27 Load the sample context file and check the settings: context > load >load context file: 'VLIZ_FC300_Focus_sample.ctx'. This context file will process 1.5mL sample volume in order to estimate cell load in the sample.

27.1

VLIZ_FC300_Focus_sample.ctx		
Capture	Distance to nearest neighbor	0
	Close hole iterations	1
	Particles defined by: dark pixels, segmentation threshold	20
	Collage image border padding (pixels)	5
	Save image collages	yes
	Save binary image collages	no
	Save raw camera images	no
	Acceptable region left	1
	Acceptable region right	1291
	Acceptable region top	1
	Acceptable region bottom	963
	Rolling calibration enable	no
Flow cell	FC type	FC300
	Flowcell depth (µm)	300
	Flowcell width (µm)	3000
	tubing inner diameter (cm)	0.16
	tubing length above flow cell (cm)	10
	tubing length below flow cell (cm)	20
	valve to rinse (cm)	0
Fluidics	Sample volume (mL)	1.5
	Flow rate (mL/min)	1.7
	AutomImage Rate (fps)	20
	Estimated Efficiency (%)	41.8
	Estimated run time (min)	0.88
	priming	Manual prime with sample
	Sample dilution/concentration enable	no
Filter	Basic acquisition filter	Use ESD
	Diameter minimum (µm)	50
	Diameter maximum (µm)	300
	Use advanced acquisition filter	no
Stop	Stop after 1.5 mL of fluid imaged	enable
	Recalibration interval (min)	30

Table 2: VLIZ_FC300_Focus_sample.ctx content.

28 Press AutoImage Mode.

- Nomenclature for LifeWatch sample run:
  YYYYMMDD_station_samplevolume_dilution_sample
  e.g. 20220929_230_SV113_sub1_3

29 Press start.

30 After the presample run, navigate to the run output in file explorer, open the run_summary.txt file and check the Particles Per Used Image (PPUI). Ideally, the PPUI should be between 1,00 and 1,20. If the PPUI exceeds a value of 1.20, the sample should be diluted according to;

- $Dilution_{PPUI1.20}$ =(0.87055*ln(1.20) - 0.03325)/(0.87055*ln(PPUI) - 0.03325)

- $Dilution_{PPUI1.10}$ =(0.87055*ln(1.10) - 0.03325)/(0.87055*ln(PPUI) - 0.03325)
- Choose a dilution between these two values, the formulas have been derived from an in-house dilution experiment.

31 Flush the sample out via Setup and Focus > Start pump. Alternate rinsing the flow cell with 5mL Milli-Q water and 5mL 70% ethanol and leave air bubbles in between, do this 3 times each and finish by rinsing with Milli-Q water. Keep flushing until all cells are gone.

32 Make appropriate dilution of sample using artificial seawater of 0.22 prefiltered seawater.

Sample runs

33 Take some diluted sample with a Pasteur pipette. Do not shake the sample, but gently mix to avoid sedimentation of cells in the sample. Insert the sample in the pipette tip. Leave some air between the sample and Milli-Q water from the last flush.

34 Use Setup and focus > Start pump to load the sample into the field of view again. Stop pump when the sample comes into view. Make sure no air bubbles are present in the tube, if so remove by pinching.

35 Go to Setup > Context > Load > Load a Context file: VLIZ_FC300_standard_context_file.ctx

35.1

VLIZ_FC300_standard_context_file.ctx		
Capture	Distance to nearest neighbor	0
	Close hole iterations	1
	Particles defined by: dark pixels, segmentation threshold	20
	Collage image border padding (pixels)	5
	Save image collages	yes
	Save binary image collages	no
	Save raw camera images	no
	Acceptable region left	1
	Acceptable region right	1291
	Acceptable region top	1
	Acceptable region bottom	963
	Rolling calibration enable	no
Flow cell	FC type	FC300
	Flowcell depth (µm)	300
	Flowcell width (µm)	3000
	tubing inner diameter (cm)	0.16
	tubing length above flow cell (cm)	10
	tubing length below flow cell (cm)	20
	valve to rinse (cm)	0
Fluidics	Sample volume (mL)	15
	Flow rate (mL/min)	1.7
	AutomImage Rate (fps)	20
	Estimated Efficiency (%)	41.8
	Estimated run time (min)	8.82
	priming	Manual prime with sample
	Sample dilution/concentration enable	no
Filter	Basic acquisition filter	Use ESD
	Diameter minimum (µm)	50
	Diameter maximum (µm)	300
	Use advanced acquisition filter	no
Stop	Stop when particle count reaches 1500 particles	enable
	Stop after 15 mL of fluid imaged	enable
	Recalibration interval (min)	30

Table 3: VLIZ_FC300_standard_context_file.ctx content.

36 Select AutoImage Mode. Name the sample run appropriately, this is crucial for data pipelines!

- Nomenclature for LW: YYYYMMDD_station_SVsamplevolume_subdilution_replicaterun Eg. 20220716_120_SV278_sub0.01_1 → change the sub value to the dilution you performed, and put 1, 2 or 3 at the end for the replicate run you're at

37 Press start and keep pipetting sample into the pipette for the duration of the run, keep the sample gently mixed.

38 Repeat the steps in the sample run three times to perform three technical replicate runs for each sample. Flush between each replicate run by alternate rinsing with 5mL

Milli-Q water and 5mL 70% ethanol and leave air bubbles in between, do this 3 times each and finish by rinsing with Milli-Q water.

39 After performing 3 replicate tuns, rinse FC and filter thoroughly inbetween samples. Alternate rinsing with 5mL Milli-Q water and 5mL 70% ethanol and leave air bubbles in between, do this 3 times each and finish by rinsing with Milli-Q water. Keep flushing until all cells are gone.

Post analysis

40 Store samples in the fridge in the dark to slow down degradation of the sample.

41 Rinse FC thoroughly. Alternate rinsing with 5mL Milli-Q water and 5mL 70% ethanol and leave air bubbles in between, dothis 3 times each and finish by rinsing with Milli-Q water. Keep flushing until all cells are gone. Dirty FCs need to be replaced.

42 Leave Milli-Q water in the flow cell, never let it dry.

43 Robocopy data to the VLIZ archiving server in the folder of your project.

44 Shut down the FlowCam.

45 Raw FlowCam data is processed using in-house Python packages. Single images are cut from the collages using image coordinates, height and width pulled from the .lst files, background is not removed. Desired metadata is pulled from the run summary, .ctx file and .lst file. Sample directory name is parsed, and the associated sampling metadata is pulled from MIDAS. All data is formatted and uploaded to the in-house BioSense MongoDB data, hosting images, metadata, trained classifiers, classifier metadata and training data splits. Images are predicted using Convolutional Neural Networks (CNNs), trained on the Belgian LifeWatch data. All predictions are checked and corrected if necessary. Data is aggregated tot cell densities and taxon abundances and made available through **https://rshiny.lifewatch.be/flowcam-data/**. For a full description of data processing and publication we refer you to the associated data paper.

Protocol references

Methodological choices were made based on the manufacturers guide, relevant literature and in-house conducted lab experiments.

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