AtlantOS

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Deliverable title	CPR Add-on variables
Description	A report on additional and add-on enhancements for the Continuous Plankton Recorder Survey programme including add-on variables and molecular analysis.
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Work Package title	Enhancement of ship based observing networks
Lead beneficiary	IOC, GOOS, ICES
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Comments	

Stakeholder engagement <u>relating to this task</u>*

WHO are your most important stakeholders?	☐ Private company If yes, is it an SME ☐ or a large company ☐? ☐ National governmental body ◼ International organization ☐ NGO ☐ others Please give the name(s) of the stakeholder(s): IOC, GOOS, GEOBON, ICES, EMODNET, SEADATA NET, …
WHERE is/are the company(ies) or organization(s) from?	◼ Your own country ☐ Another country in the EU ◼ Another country outside the EU Please name the country(ies): The CPR survey is international funded by a consortium made up of the UK, Canada, USA and Norway
Is this deliverable a success story? If yes, why? **If not, why?**	◼ Yes, because we made excellent use of the CPR platform and proved we could get enhanced variables
Will this deliverable be used? **If yes, who will use it?** **If not, why will it not be used?**	◼ Yes, the data generated by this deliverable will be used by various stakeholders.

NOTE: This information is being collected for the following purposes:
1. To make a list of all companies/organizations with which AtlantOS partners have had contact. This is important to demonstrate the extent of industry and public-sector collaboration in the obs community. Please note that we will only publish one aggregated list of companies and not mention specific partnerships.
2. To better report success stories from the AtlantOS community on how observing delivers concrete value to society.

*For ideas about relations with stakeholders you are invited to consult D10.5 Best Practices in Stakeholder Engagement, Data Dissemination and Exploitation.

CPR Add-on Variables

Executive summary

The Continuous Plankton Recorder (CPR) survey is one of the most well established autonomous observing systems covering the North Atlantic basin-scale over multiple decades. It has 80 years of experience with working with the commercial shipping industry and is an established platform providing a global network of Ships of Opportunity for scientific research.

As part of the European project AtlantOS which aims to build a more integrated Atlantic wide observation system, the CPR survey aims to optimize and enhance its current CPR survey network. The CPR is an autonomous instrument mainly towed from ships of opportunity that has been in use for over 80 years. Currently, samples are collected covering 20,000 km per month in the major ecosystems of the North Atlantic. Recently the network has expanded to sample in the Arctic and South Atlantic and other regions globally. It has been observing over 1000 biological variables over a multi-decadal period as well as a number of physical variables.

There is an increasing need to monitor the marine environment for legislative reasons (e.g. MSDF Good Environmental Status targets) and at reduced costs using autonomous methods. Therefore, there are obviously huge cost benefits in incorporating new technologies and sensors into existing infrastructures like the CPR survey to optimize and enhance the Atlantic observing system. The CPR survey, already has good interlinkage between its observations and other SOOP activities such as Carbon VOS for example.

One of the aims of AtlantOS in a CPR perspective is to help develop this existing network and help enhance its operations. As part of the AtlantOS programme the CPR survey has been enhanced in a number of ways including additional sensors on the CPR instrument itself, new water samplers (for flow cytometry and HAB analysis), enhanced molecular techniques and also piloted optical methodologies. Here we report on a number of CPR enhancements and augmentations over the course of the AtlantOS programme:

Section 1: CPR enhanced instrumentation
Section 2: Quantitative data on HAB species and marine pathogens and Water and microplankton sampler (WaMS) operations
Section 3: Piloted optical methodology

Introduction: Enhancing North Atlantic Observations using the CPR network as an operational research platform

Cost effective physical and chemical monitoring

There is considerable scope for the further development of the CPR instrumentation programme to provide synoptic physical/biogeochemical measurements with the plankton for use in global climate change and ecological models and satellite calibration as well as to help interpret causes of plankton and fisheries variability. Variability in ocean chemistry – nutrients, pH, C02 concentration and other dissolved gas measurements – provide crucial constraints to plankton growth rates and survival as well as insight into the impact of global climate change on the ocean.   Observations of temperature and salinity can be combined to estimate ocean pH whilst waiting for maturity in the new generation of in-situ pH sensors in order to make direct measurements from the CPR platform.

Good links already exist with the physical oceanographic community and pCO$_2$ ship-of-opportunity communities and through AtlantOS these links will be further strengthened. These contacts will in addition be used to keep abreast of relevant new measurement technologies that could be fitted to CPRs and further add to the value of the autonomous survey. As part of the AtlantOS's integrated observing system the CPR survey could act as an essential regional and long-term backbone covering multiple observational scales. Currently near-real-time sensors for variables such as chlorophyll from CTD sensors are being operated on CPR transects (Section 1) across some coastal to open ocean waters and faster quantitative molecular assays of key harmful and pathogenic organisms are being investigated using new molecular techniques (Section 2).

Monitoring and collecting additional biological information

Under this area the CPR Survey has focused on continued deployment of the Water and Microplankton Sampler (WaMS) and developing quantitative molecular methods for Harmful Algal Blooms and pathogens. The water sampler opens up new opportunities to identify additional HABs as well as important smaller or delicate plankton and pathogenic species that may be missed or damaged by CPR tows. Rapid cell identification methods will continued to be explored using flow cytometry to sort cells on size and pigment for further to classify and quantify cells by size and pigment which can be isolated for later molecular analysis. The microsampler is seen as adding huge value in contributing to the the EU Marine Strategy Framework Directive and also complimenting to the molecular analysis already currently being done at SAHFOS. The main objective of the water and microplankton sampler is to enable the CPR survey to monitor the full size range of plankton in the oceans from the larger plankton (which the CPR already samples) to the nano and pico plankton size ranges. The water and microplankton sampler is also aimed at monitoring the smaller Harmful Algal Bloom (HAB) species. New automatic visual identification methods will also be continued to be developed to speed up components of the traditional taxonomic analysis (e.g. quick estimates of zooplankton biomass/size structure, Section 3).

Section 1: CPR enhanced instrumentation

The CPR Survey offers an attractive platform from which in-situ environmental measurements can be made that complement other ocean observation networks and provide validation data for remotely sensed earth observation programmes. As part of AtlantOS the CPR Survey has been exploring the use of environmental sensors on some of the established survey network (Figure 1). Since 1964 various types of instrumentation have been added to the Continuous Plankton Recorder (Glover, 1967; Aiken, 1977, 1980; Aiken and Halliday, 1980; Halliday, 1984; Aiken, 1981a,b; Aiken and Bellan, 1986a; Williams and Aiken, 1990; Williams and Lindley, 1992) to measure environmental parameters (Conductivity, Temperature or chlorophyll-a Fluorescence) or the physical performance of the towed body (flow meters, pitch and roll sensors). A review of previous instrumentation activity is given by Reid et al. (2003). Since 1996, self-logging temperature recorders (Minilog/Minilog-II, Vemco Ltd) have been attached to CPRs. These units record with an accuracy of ±0.1°C with resolution of 0.01°C. The units have built-in real-time clocks, which have proved particularly valuable as a check against hand-written log forms produced by the crew of towing ships. Deployments have largely been ad-hoc to test existing sensing technology (Batten et al., 2003). Off-the-shelf sensors have been attached to the rear end of a CPR within the box tail in the same cargo space as the Water and Microplankton Sampler is currently stowed. Deployments of low cost sensor (i.e. single channel temperature sensors) across the CPR survey network have shown qualitatively good results (Figure 2) in comparison to independent measures but do not have the stability, resolution or accuracy to be used for operational observing.

Historic CPR samples with current CPR routes and instrumentation

Figure 1. CPR tow routes and targeted instrumented routes in the North Atlantic. Red samples indicate historical samples
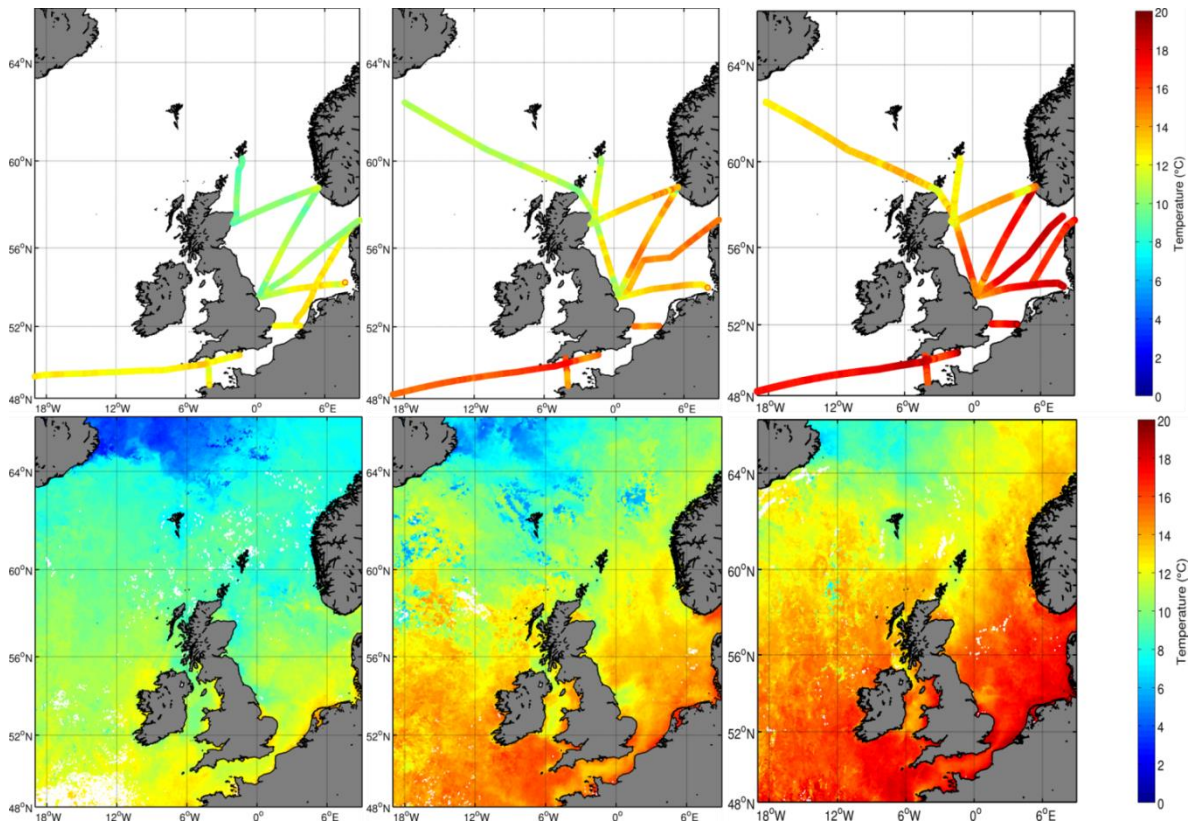
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Figure 2. Sea temperature measured by low cost Star-oddi and Minilog temperature sensors on 10 CPR tows (top) in June, July, August 2014 (left, middle and right panels) in comparison to monthly remote sensing composites of Skin Surface Temperature (bottom)
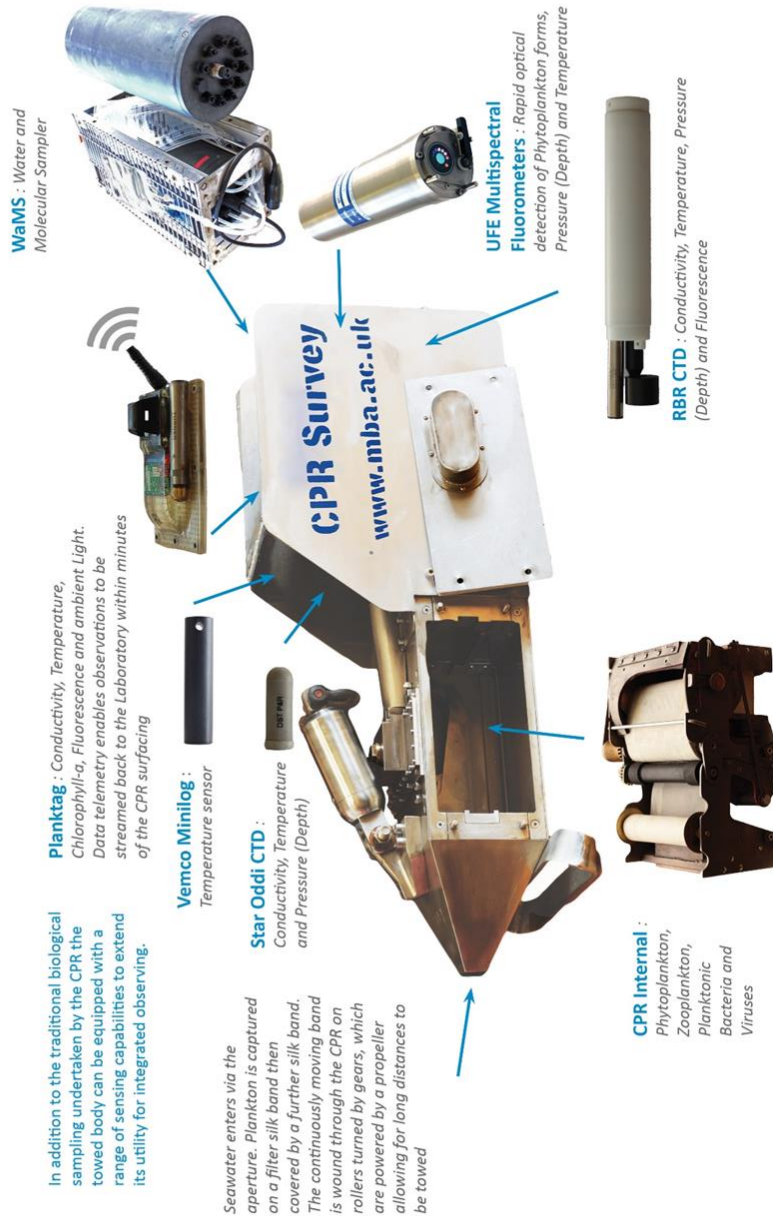
Sensor Development

In 2015, the National Environment Research Council funded a sensor development programme for the CPR Survey (under grant CC080) to develop and deploy a bespoke series of observation technologies for environmental sensing to measure Conductivity, Temperature, Fluorescence and Carbon Dioxide concentrations. Some of these additions are illustrated in Figure 3 alongside the CPR, the automated Water Sampler (see Section 2) and small low cost sensors which have been trialled in an ad-hoc manner for a number of years.

Cost Effective, Proven and Reliable Since 1931

Key Statistics

Length x width x height :
100 x 36 x 42 cm
Weight : 85kg
Tow depth : 5 - 10 metres
Tow speed : 8 - 25 knots
Aperture size : 1.27 cm^2

Collects: Phyto- and Zooplankton, planktonic bacteria and viruses.

Instruments record:
Conductivity, Temperature, Depth, Chlorophyll-a, Fluorescence, ambient Light, and three-axis accelerations.

The Continuous Plankton Recorder
A platform for Integrated Ocean Observing

WaMS : Water and Molecular Sampler

UFE Multispectral Fluorometers : Rapid optical detection of Phytoplankton forms, Pressure (Depth) and Temperature

RBR CTD : Conductivity, Temperature, Pressure (Depth) and Fluorescence

Planktag : Conductivity, Temperature, Chlorophyll-a, Fluorescence and ambient Light. Data telemetry enables observations to be streamed back to the Laboratory within minutes of the CPR surfacing

Vemco Minilog : Temperature sensor

Star Oddi CTD : Conductivity, Temperature and Pressure (Depth)

CPR Internal : Phytoplankton, Zooplankton, Planktonic Bacteria and Viruses

In addition to the traditional biological sampling undertaken by the CPR the towed body can be equipped with a range of sensing capabilities to extend its utility for integrated observing.

Seawater enters via the aperture. Plankton is captured on a filter silk band then covered by a further silk band. The continuously moving band is wound through the CPR on rollers turned by gears, which are powered by a propeller allowing for long distances to be towed

Figure 3. Summary schematic of augmented instruments and sensors deployed on the CPR to enhance its observations (CPR add-on variables).

Conductivity, Temperature, Depth and Chlorophyll-a Fluorescence

Based on existing marine mammal telemetry tags developed by the Sea Mammal Research Unit (St. Andrews University, Scotland) bespoke environmental sensors for mounting onto a CPR have been created (Figure 4) with modifications made to the housing and mounting, sampling protocol, sample frequency, power and memory. The tags use precision wet/dry and light sensors integrated alongside a Valeport Ltd temperature, conductivity and pressure unit and a Turner cyclops fluorescence sensor. Sensor performance specifications are given in Table 1. PlankTag sensor specifications (CTD)Efficient data transfer occurs with international GSM mobile phone network upon emergence of the CPR after a tow. Data is transmitted within 15 minutes of CPR surfacing (as long as tow ends within 20 miles of the coast in order to connect to GSM network).
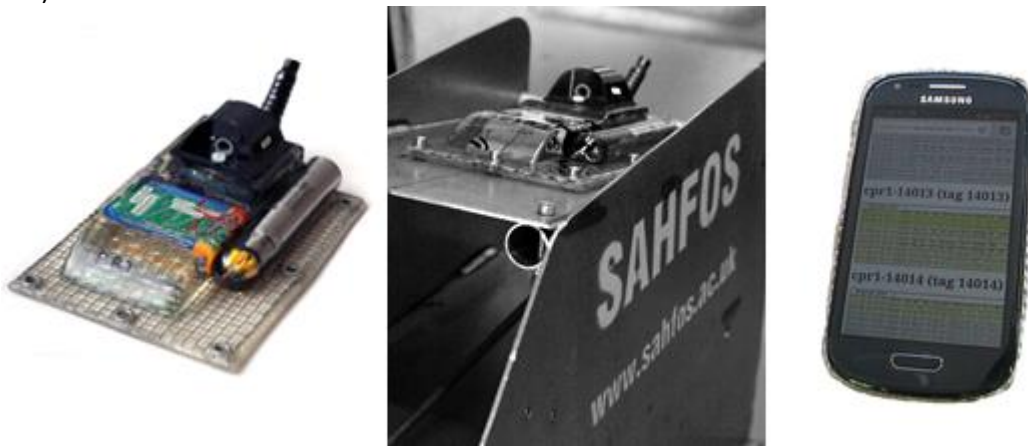

Figure 4. Planktag (left) capable of measuring temperature, salinity, depth, fluorescence and ambient light; mounted on CPR top fin (middle) for deployment; with data telemetered via GSM (right) on CPR emergence post tow.

	Temperature	Conductivity	Pressure
Range	-5° to 35°C	0 to 80mS/cm	0 to 2000 dBar
Accuracy	+/- 0.005°C	+/- 0.01mS/cm	2 dBar +/- (0.3 + 0.035%*reading)/°K
Resolution	0.001°C	0.002mS/cm	0.05 dBar

Table 1. PlankTag sensor specifications (CTD)

	Fluorescence
Excitation wavelength	465/170nm
Emission wavelength	465/170nm
Gain	X10

Table 2. PlankTag sensor specifications (Fluorescence)

The units have proved to be a reliable instrument on a number of CPR tows deployed monthly since 2016 - 2018. They have been adapted for repeat use and ruggedized for deployments at operational speeds between 10 – 25 knots) but several housings have begun to exhibit stress fatigue or have experienced physical impacts during deployment and have had to be retired from service.

Based on the experience with mark I PlankTags, a mark II sensor which uses a new CTD head from Valeport Ltd with integrated Hyperion fluorometer is on-going. The initial prototype will be available as of January 2019 but the units will not be fully operational until Q2 2019. The mark II PlankTags are designed for longer range (i.e. trans-Atlantic or trans-Pacific deployment) than the mark I versions.

Multispectal Fluorescence

For in-situ estimation of phytoplankton species composition a JFE Multi-Exciter has been trialled (Figure 5). The optical signals measured by the fluorometers have the potential to indicate promptly phytoplankton abundance and provide the capability of rapidly identifying Harmful Algal Blooms and samples of interest prior to arrival of physical samples in the lab.

Figure 5. JFE Multi-Exciter prior to laboratory validation.

The JFE MFL05W-USB multi-frequency fluorometers enable the simultaneous detection of several phytoplankton forms – (diatoms, dinoflagellates, green algae, cryptophyta and cyanobacteria). The system is capable of measuring the discrete fluorescence excitation spectra with species composition determined by solving the optimisation problem (Yoshida et al., 2011). The
Multi-Exciter measures nine wavelength excitation spectra quantifiying the total
phytoplankton biomass (chlorophyll-a) and estimating the phytoplankton group compositions using the observed excitation spectra. The instruments also include depth and temperature sensors and include a mechanical wiper to ensure the optical sensor window remains clean.

Laboratory validation with known cultures has shown potential issues with cross contamination so further development of the optimisation algorithm is required before operational deployment.

Dissolved Carbon Dioxide Concentration

A custom flow through instrument for measuring the partial pressure of carbon dioxide in-situ has been built in collaboration with Pro-Oceanus (Figure 6). This system is designed for continuous deployment on long CPR Survey transects using a combination of two sensing systems, a lower resolution and lower power system for continual operation, and a higher resolution, higher power system for spot validation. The system is currently undergoing laboratory validation prior to operational deployment.
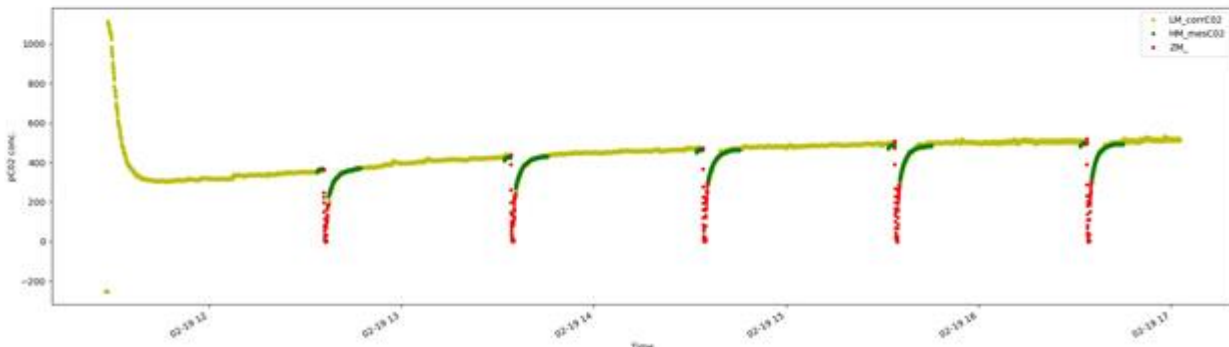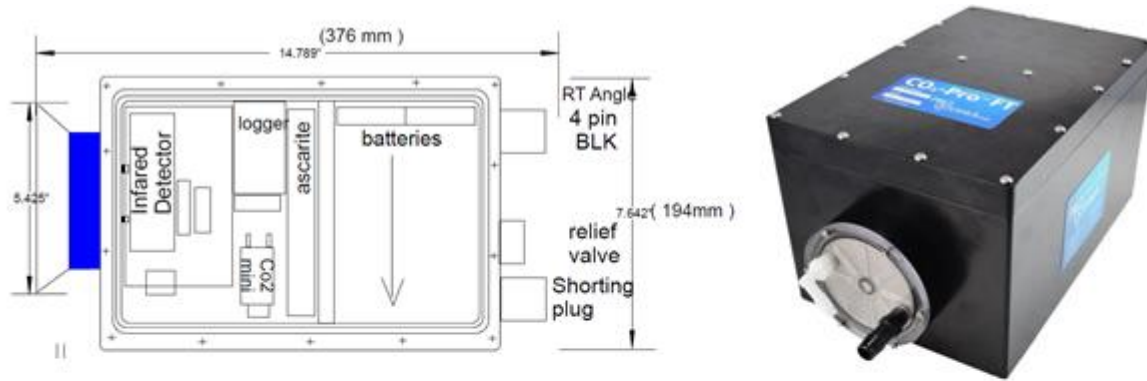
Figure 6. Prototype pC02 measurement system designed for deployment on the CPR . Initial verification of pC02 measurement system indicating operation of low resolution (light green) and high resolution (dark green) gas sensors.

In-situ Deployment of Sensors

NERC funded PlankTag sensors are deployed on 10 CPR Survey routes. As part of AtlantOS, four routes (

	A			B			PR			LR/V		
	'16	'17	'18	'16	'17	'18	'16	'17	'18	'16	'17	'18
January												
February												
March												
April												
May												
June												
July												
August												
September												
October												
November												
December												

Table 3

	A '16	A '17	A '18	B '16	B '17	B '18	PR '16	PR '17	PR '18	LR/V '16	LR/V '17	LR/V '18
January	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩		🟩	🟩	
February	🟩	🟩				🟩		🟩	🟩	🟩		🟩
March	🟩	🟩	🟩			🟩		🟩	🟩	🟩	🟩	🟩
April	🟩	🟩	🟩		🟩	🟩		🟩		🟩		
May	🟩		🟩	🟩	🟩	🟩		🟩		🟩	🟩	🟩
June	🟩	🟩	🟩	🟩		🟩		🟩	🟩	🟩	🟩	🟩
July	🟩	🟩	🟩	🟩	🟩	🟩		🟩	🟩	🟩	🟩	🟩
August	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	
September		🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	
October	🟩	🟩	🟩		🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
November	🟩	🟩	🟩	🟩		🟩	🟩		🟩	🟩	🟩	
December	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩

Table 3. Sensor deployments on the A,B,PR and LR/V CPR Survey routes since 2016.) have been instrumented to ascertain feasibility of near-real time reporting .

	A '16	A '17	A '18	B '16	B '17	B '18	PR '16	PR '17	PR '18	LR/V '16	LR/V '17	LR/V '18
January	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩		🟩	🟩	
February	🟩	🟩				🟩		🟩	🟩	🟩		🟩
March	🟩	🟩	🟩			🟩		🟩	🟩	🟩		🟩
April	🟩	🟩	🟩		🟩	🟩		🟩			🟩	🟩
May	🟩		🟩	🟩	🟩	🟩		🟩		🟩	🟩	🟩
June	🟩	🟩	🟩	🟩		🟩		🟩	🟩	🟩	🟩	🟩
July	🟩	🟩	🟩		🟩	🟩		🟩	🟩	🟩	🟩	🟩
August	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	
September	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	
October	🟩	🟩	🟩		🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
November	🟩	🟩	🟩	🟩		🟩	🟩		🟩	🟩	🟩	🟩
December	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩		🟩	🟩	🟩

Table 3. Sensor deployments on the A,B,PR and LR/V CPR Survey routes since 2016. Green colours indicate successful monthly tow on a particular route. Routes A and B are equipped with a Minilog (temperature only), routes PR and LR/V are equipped with a PlankTag (CTD+F).

Sensor data is transmitted by GSM to the data centre at SMRU before being pushed to the CPR Survey via FTP. Data is typically transmitted within 15 minutes of the CPR surfacing and arrives for processing within 30 minutes. Once received, sensor data undergoes merger with AIS vessel positioning data (retrieved from Marine Traffic) and automated quality control. Calculation of averaged quantities within discrete geospatial areas to match CPR Samples (estimated as 10 nautical mile sections of tow) requires matching sensor (with AIS positioning data) and CPR sample mid-points. Errors in reported CPR positions (in comparison to positions measured by AIS), which presumably arise during manual data entry, make this match-up a non-trivial task. As a result, only correctly positioned sensor data is considered in this report rather than derived spatial averages.

Intercomparison with independent observations

The PR route is towed by Brittany Ferries vessel Armorique or Pont Aven. The Armorique has a FerryBox system installed making intercomparison between the CPR PlankTag and inde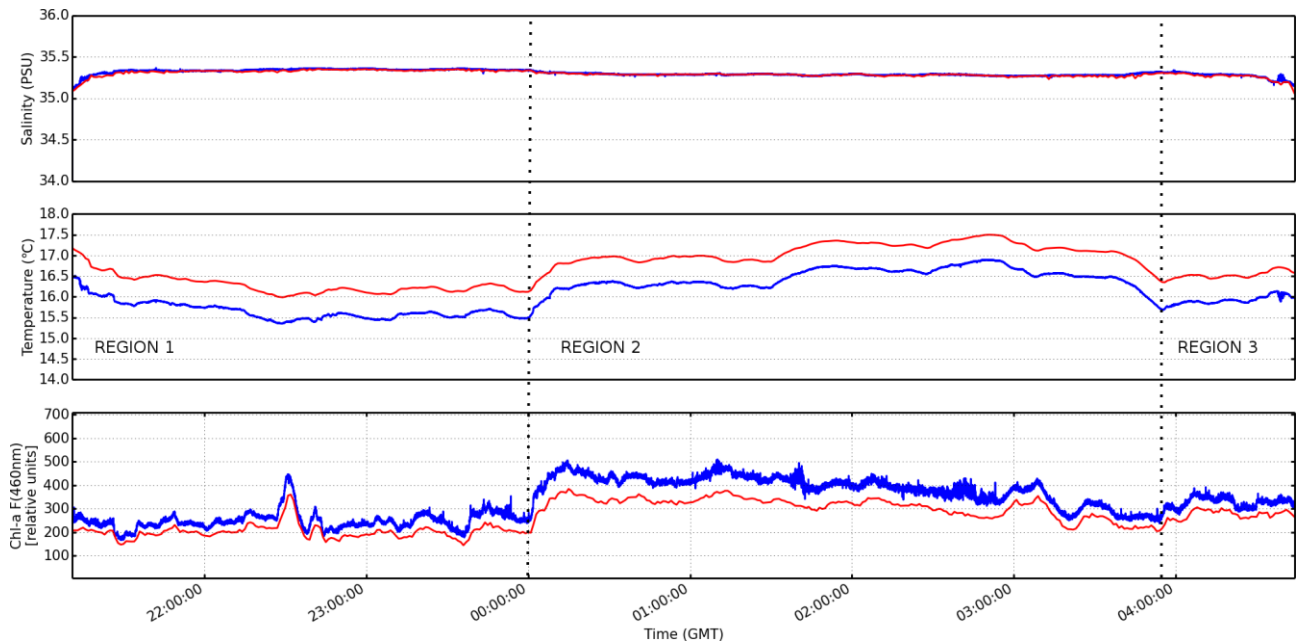pendent observations possible (Figure 7. Time series (5 min rolling average) of Ferrybox observations (red) and PlankTag observations (blue) for Salinity (top panel), Temperature (middle) and fluorescence (bottom).).

Figure 7. Time series (5 min rolling average) of Ferrybox observations (red) and PlankTag observations (blue) for Salinity (top panel), Temperature (middle) and fluorescence (bottom).

Generally, agreement is good (Figure 8) with fixed offsets in temperature (approximately 0.4°C) and small conductivity offsets (0.25 mS/cm) which therefore result in salinity offsets (approximately 0.1PSU). We think the temperature offsets are associated with warming by the FerryBox system but this is currently under investigation. Uncalibrated fluorescence units are also offset due to the different gain settings of the two observation systems.
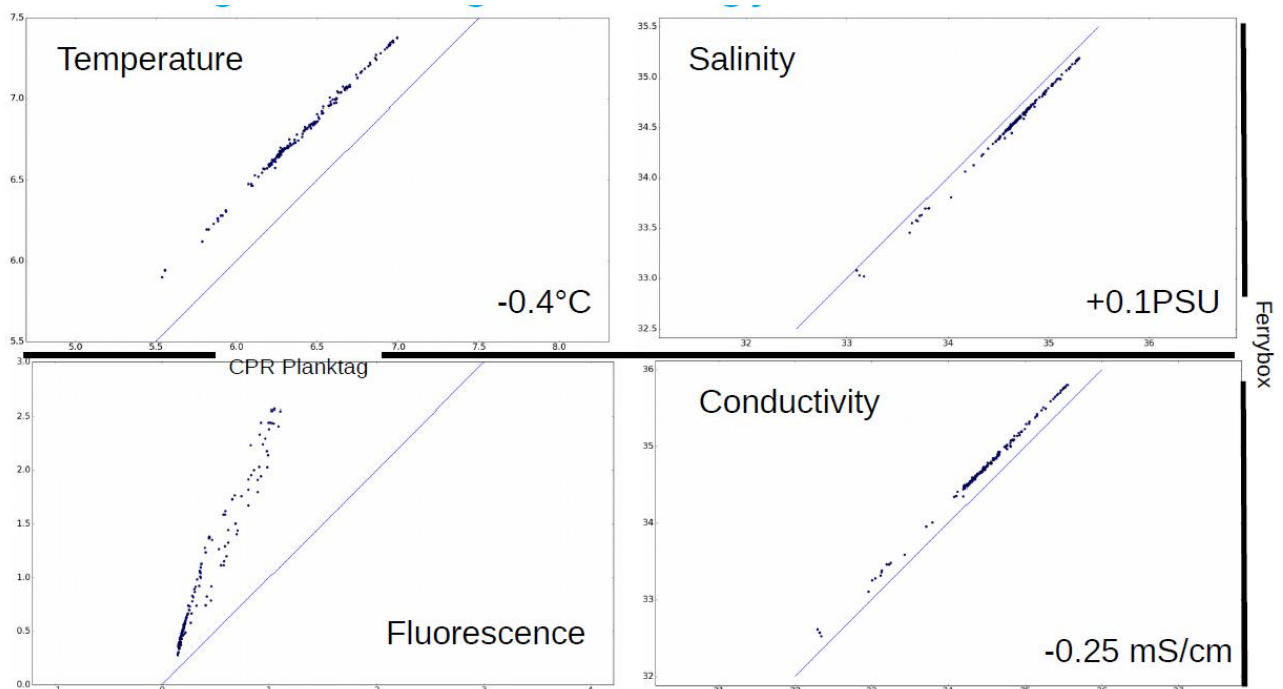
Figure 8. 1:1 plots of Temperature, Salinity, Conductivity and Fluorescence between FerryBox and PlankTag observations.

As to be expected, the fluorescence signals can vary in agreement between the two observation systems and their appears to be a seasonal component to the variability (Figure 9).
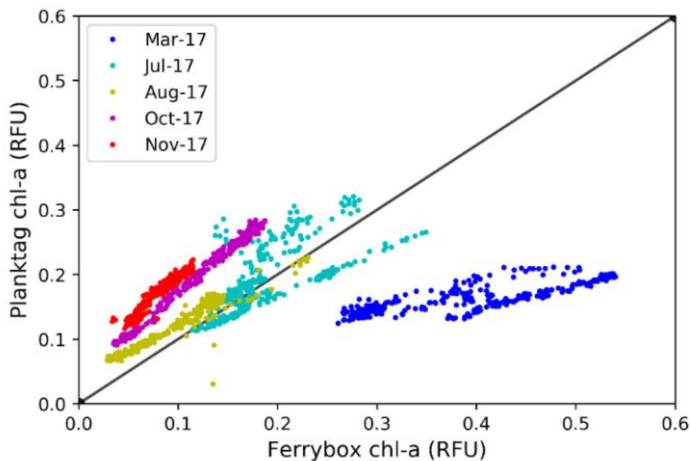
Figure 9. Monthly varaiblilty in raw fluorescence observations during 2017 between PlankTag and FerryBox.

Abrupt changes in sea surface temperature and fluorescence can be used to identify frontal structures which mark the transition from coastal water masses (Figure 7 regions 1 and 3) from mid-channel water masses (Figure 7 region 2).

Concluding Remarks

A range of new sensing technologies have been introduced across the CPR Survey network from simple, low cost temperature loggers to bespoke sensing systems for precision temperature, conductivity and fluorescence measurement. Further systems for multispectral fluorescence or in-situ gas sensing are still in laboratory validation stage. Near real time observations of sea water temperature, salinity and plankton fluorescence have been achieved using PlankTags with a lag time of approximately 30 minutes between CPR emergence post tow and data transmission to the MBA. PlankTags have been robust during operational deployment, with a service lifetime of about 2 years (barring impact incidents or entanglement). The sensors exhibit good performance whilst underway and compare very favourably with independent underway systems. The sensor systems show promise for physical oceanographic observing from the CPR Survey network with, for example, frontal systems readily identifiable from the towed platform and datasets able to be cross calibrated and validated against independent observations. Further work is on-going to instigate full automation in data processing and geospatial match up with CPR samples, in order that this new environmental data can be used in conjunction with traditional CPR plankton observations, and to develop a fully automated data feed to make the environmental datasets available to third parties.

Section 2: Quantitative data on HAB species and marine pathogens and Water and microplankton sampler (WaMS) operations

One of the lesser-known biologically measured variables are smaller plankton and harmful species. Other than a few pathogens and bacteria that need to be monitored by law, little is known of harmful planktonic organisms. We develop genetic assays for twelve harmful organisms: seven harmful algae and six pathogens and tested their presence in the English Channel using traditional Continuous Plankton Recorder (CPR) and an automated water sampler (WaMS) that collected small volumes of water alongside the CPR and obtained quantification data from four harmful algae. Additionally we tested for harmful algae in the North Sea that is oceanographically distinct from the English Channel.

Water samples proved more amenable to genetic assays as they could be preserved in DNA-preserving agents. The CPR samples were fixed in formalin (a historic choice), a DNA-damaging chemical, which limited our assay selection. However, WaMS design at the time limited water volume and therefore detection of low abundance organisms. The CPR sampling device is designed to collect larger plankton with a 200µm mesh and one harmful algae that failed to be detected in CPR samples was detected in water samples, showing the utility of a smaller-sized plankton collection device. Overall in the English Channel, levels of five harmful algae, *Aureococcus anafferagens, Pseudo-nitzschia fraudulenta, Pseudo-nitzschia delicatissima, Pseudo-nitzschia multiseries* and *Alexandrium minutum* showed distinct spatio-temporal patterns. *A. anafferagens* was a surprising new finding to this region, being detected in offshore waters where it usually prefers estuarine shallow water. Our tests of CPR samples revealed the presence and seasonality of *Alexandrium tamarense* group III in the North Sea, known to be present coastal North Sea waters. However, our findings show the first open water distribution of this species. Such information shows the potential to map biogeographical boundaries of harmful organisms.

Although quantification of pathogens was not possible, we did detect unusual pathogens, Legionella and its host, *Acanthamoeba*, plus another potentially harmful amoeba, *Hartmanella* spp. from water samples in the English Channel. Normally reported from freshwater regions we show these pathogens can survive in marine open waters in the UK that potentially should be monitored.

Finally our water sampler in the English Channel were subjected to flow cytometry and also yielded five years of size-separated cell classes (under 20µm in size). These showed spatial variability of size-structured cells within the English Channel, reflecting water sources. Seasonal trends were observed for the photosynthetic bacteria *Synechococcus* that sometimes matched phytoplankton greenness indicator (PCI), a rough proxy for phytoplankton biomass, indicating the potential to delineate and compare smaller phytoplankton classes.

Introduction

Plankton monitoring traditionally is performed using microscopic counts that are known to be size-biased towards larger and morphologically distinct organisms. However, in terms of biodiversity and biomass, the majority of planktonic organisms are below 20µm ((de Vargas *et al.* 2015)), representing viruses, bacteria and so-called pico- and nano-eukaryotes. Most of these organisms are not counted as they are too small and indistinct to identify or their morphology is destroyed by preservatives. Most harmful organisms are below 20µm, especially pathogens that attach to or live within other organisms. Our aim was to determine if we could measure these unseen harmful organism using genetic methods that do not require morphology to be identified. Part of the development of automated collection of biological variables is to collect samples automatically, saving time and money. The next stages would be the development of rapid chemical tests that can be miniaturised and deployed on an *in situ* marine testing platform.

To this end we developed an automated water and microplankton sampler (WaMS) that could be deployed within the CPR platform to capture organisms smaller than 20µm (Stern *et al.* 2015), as the CPR sampling device is designed to collected larger plankton on a net with a 270µm mesh size ((Batten 2003)). Our aim was to determine whether harmful algae data can be reliably detected and quantified on the WaMS platform from two selected targeted ecoregions, chosen to be the English Channel and the North Sea.

Water Sampler

An automated water and microplankton sampler (WaMS) was deployed on the CPR platform, previously developed to capture planktonic organisms under 50 µm – especially the pico (<2µm and nano (<20µm) sized fractions (Fig. 10). This device is a pump connected to sampling bags that is deployed inside the CPR's cargo bay and in this instance can take up to 10 sample bags of up to 100-150ml each, due to space

limitation on the CPR platform. Autonomous software control triggers sample acquisition where sampling volume, location, timing and peristaltic tube flushing can be controlled.
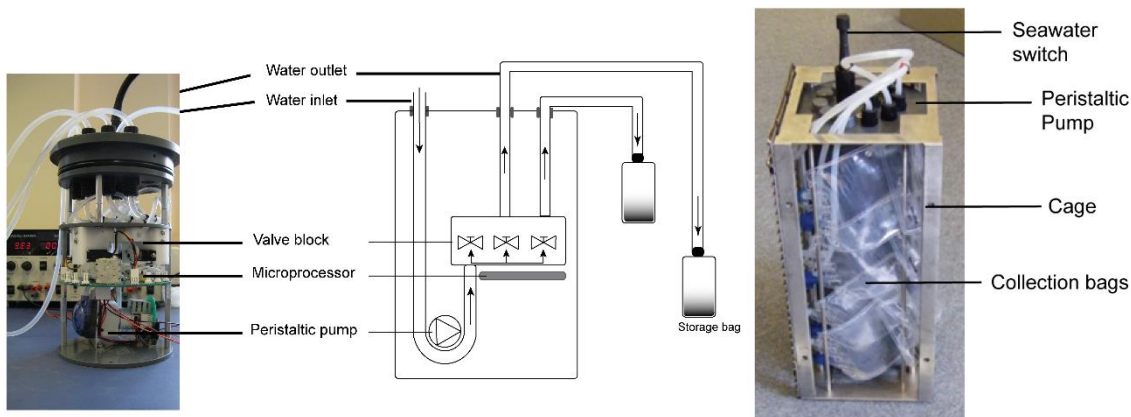

Fig. 10: shows the internal (right) and external (left) components of the WaMS.

Quantification of Harmful organisms from Water Samples

We selected a range of harmful algae and pathogens known to be present in North Atlantic temperate waters from a range of size classes (2-200μm). Existing published assays were validated and new assays developed to test for their presence in the English Channel (see Table S1 in Appendix) from total DNA extracted from WaMS samples, as described previously (Stern *et al.* 2015). Only three pathogens could be detected but at such low levels that we focused on harmful algae to develop quantification tests. High-resolution Melt Curve (HRM) quantitative PCR (qPCR) assays (reviewed by (Wittwer 2017) were used to provides specific identification (Fig. 11) and quantification of the target species using a fluorescent dye that can track the quantity of DNA compared to a set of standards (Fig. 12). Appendix 1 summarises all of the assays that were developed and validated. Quantification of DNA can be used on its own (as we have used in Fig. 134) or can be related to cell numbers to provide cell abundances.
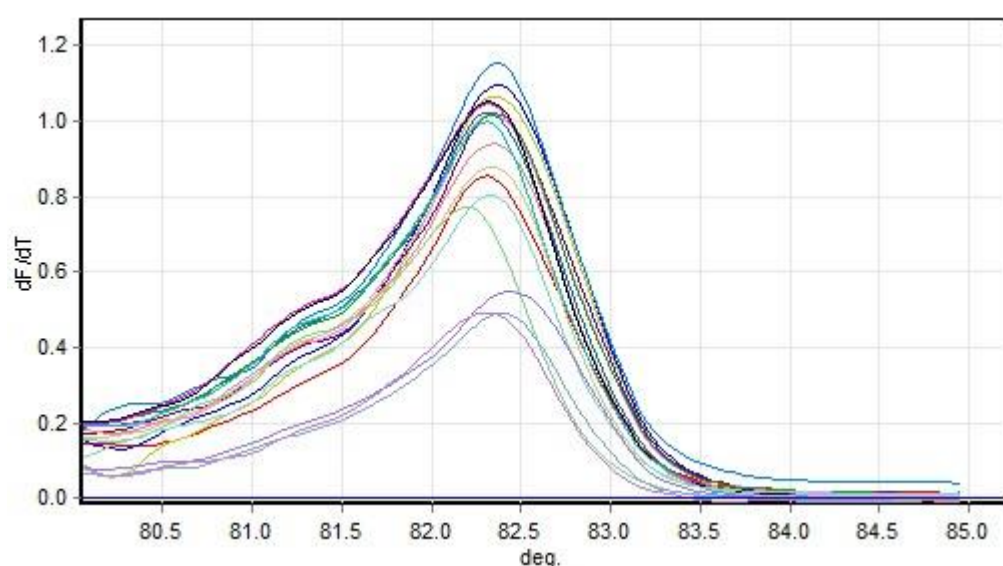

Fig. 11: Example of HRM curve of *Aureococcus anophagefferans* fluorescently labelled PCR product, that has a specific melting temperature where DNA strands separate, shown as a peak, unique to a targeted DNA region of that species.
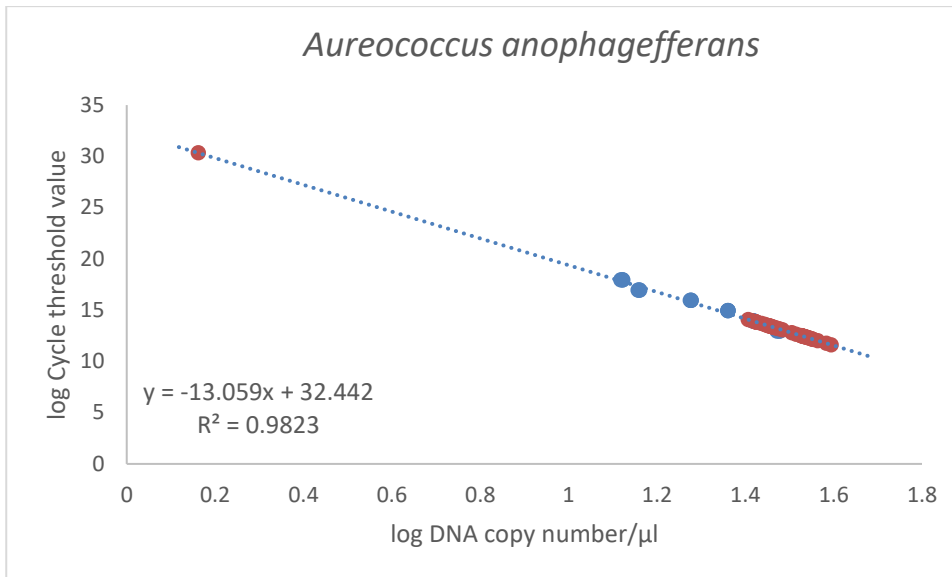
Fig. 12: Example of relationship between DNA from cultured standards of *A. anophagefferans* and environmental samples to determine copy numbers using the equation of a straight line.

Key findings from the WaMS

Five harmful algae species were successfully identified in the English Channel. *Pseudo-nitzschia fraudulenta* and *Pseudo-nitzschia delicatissima* species have been shown to produce the neurological toxin, Domoic acid that damage wildlife (Work *et al.* 1993). Both species are larger phytoplankton (40-199µm long) but thin (<5µm wide). Both were present over the entire channel transect (Fig. 13, top panels). Through a relatively short temporal window-both species showed different temporal patterns, for example *P. delicatissima* was present all year round including the winter in 2011-12 whilst *P. fraudulenta* was not detected in the winter. *Pseudo-nitzschia multiseries* was present at low levels (below standard range) in 2011-2012. Previous research on morphological taxa groups of Pseudo-nitszschia in the English Channel report different seasonal patterns for different taxa groups, although unfortunately not to species level (Downes-Tettmar 2012). Thus these species assays will be helpful in delineating and modelling spatio-temporal patterns of *Pseudo-nitzschia* species. *Alexandrium minutum* is 15-30µm and showed a near-coastal distribution near fresh water riverine outlet currents only appearing in summer of 2011 and 2013 (Fig. 13, bottom left panel), in keeping with its preference for near coastal protected sites with a freshwater influence ((Vila *et al.* 2005)), such as the river Tamar near site 5. *Aureococcus anophagefferens* is only 2µm, the size of a bacteria, and has never been quantified from this region. This species tends grow in very high numbers to cause brown tides, that deplete oxygen from the water and suffocate fish. This species was present year round and in 2011 was much more abundant in summer. *A. anophagefferens* is reported to prefer shallow estuarine water with low light and inorganic nutrient levels and high levels of organic carbon and nitrogen sources ((Gobler *et al.* 2011)). Its presence in offshore English Channel may not be an obvious habitat, and why it does not appear in bloom-forming numbers, but our findings show this species can grow in marine oceanic waters.

The water sampler proved to be useful in the detection of harmful algae, providing novel information and species-specific abundance of *Pseudo-nitzschia* and *Alexandrium* that are difficult or impossible to differentiate into species by light microscopy. Such assays, when fully worked-up, can produce data in days. Additionally spatial-temporal patterns can be related to physical parameters to better understand their habitat preferences. However, our observation showed that the volume of water from the WaMS was often insufficient to determine the abundance of species, often falling below the range of standards. Additionally preservation of the samples proved difficult to achieve due to health and safety concerns for ship crew who were non-experts that were handling the WaMS. A platform that collets water on a filter that is instantly

preserved would be a great improvement. Volumes would not be limited and samples could be preserved on site to avoid changes caused by microbial activity.
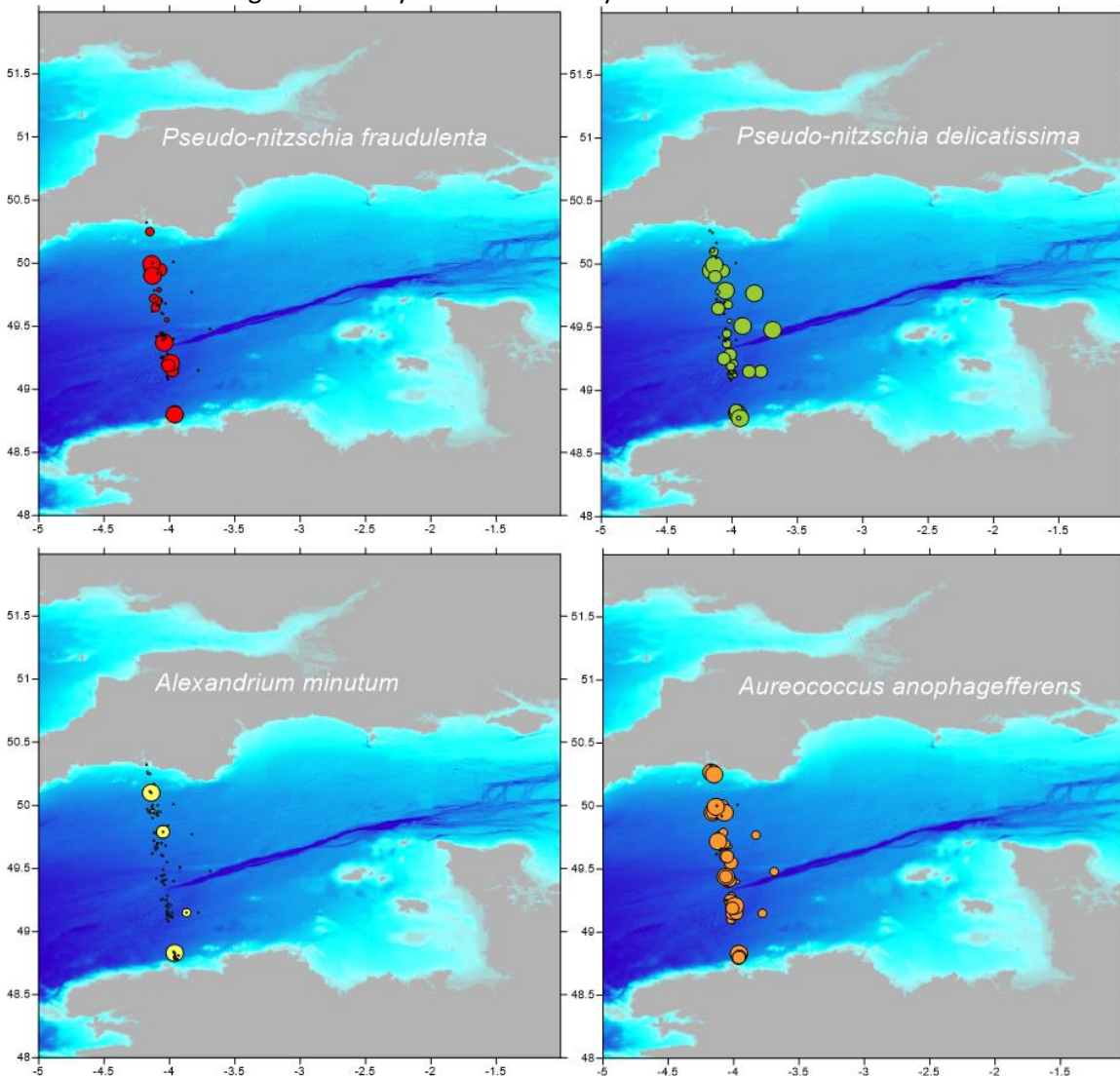

Fig. 13: Distribution and abundance of four harmful algae in the English Channel over 2 years for *P. fraudulenta* and 3 years (remaining).

Pathogen detection from WaMS samples

Three out of six pathogens were detected. *Legionella* is a pathogenic bacteria causing *Legionellosis* disease in humans (Newton *et al.* 2010), normally from inhalation of contaminated aerosols. *Legionella* bacteria shown to be obligate parasites within with free-living, freshwater or soil Amoebae such as *Acanthameoba* or *Hartmanella* (Fields 1993). We tested for both these organisms and found their presence in 10 out of 90 WaMS samples from all sampling locations. Four were identified as *Legionella massiliensis,* and the rest could only be narrowed down to groups of Legionella that were very similar to human pathogenic varieties. Two species were not similar to anything in the existing database. All samples containing Legionella also contained host species, *Acanthamoeba* and *Hartmanella* amoebae. Legionella has been reported in pelagic marine habitats (Palmer 1993; Gast 2011). Metagenetic data (sequencing a target gene using high-throughput sequencing methods) over one year of water samples has also revealed marine fungal pathogens from the *Rozellomycota* lineage (Stern *et al.* 2015), in winter and spring, 2011. A large array of microbial lineages were found, most from Alveolata (52%), which are often over-represented in molecular

sequencing surveys because of the large number of target genes copies, Chlorophytes (11%) and Stramenopiles (11%) and Haptophytes (6%).

Quantification of Harmful Organisms from CPR samples

The CPR sample collects water from $3m^3$ of water and therefore capable of collecting large biomass of plankton. Despite being preserved in formalin, that damages DNA, previous studies have shown that DNA-based identification is possible from formalin-preserved CPR samples including harmful algae (Stern R.F. *et al.* 2018), coccolithophores (Ripley *et al.* 2008) and *Vibrio* bacteria (Vezzulli *et al.* 2012).

DNA was extracted from Spring, Summer and Autumn from North Sea and English Channel CPR samples (in duplicate) over a 3 year period and the abundance of two harmful algae, *A. tamarense* group III and *P. delicatissima*. Unfortunately we could only achieve presence data for *P. delicatissima*, which was absent in CPR samples from the English Channel, in contrast to WaMS samples, indicating CPR sampling for this species may be inconsistent and confirms the need for sampling for smaller (>50µm) plankton.

A full three years of seasonal data was achieved for *A. tamarense*, group III (Fig. 14). A clear seasonal pattern was observed where abundance peaked in the summer and continued in Autumn. Our sampling site was in Scottish waters and this species has independently been reported from various coastal regions in the North Sea, both as cysts in the sediment and in pelagic samples (Brown et al. 2010), confirming our reports here. However this is the first open water report of this species in Scottish waters. This species was absent in the English Channel over the same period, suggesting a preference for the North Sea, or that these species re-grow every year from long-term cysts populations in the North Sea.

This is the first quantitative detection of harmful algae from CPR samples and shows how archival samples can be a useful resource for baseline data which can inform planning of future biological data collection.
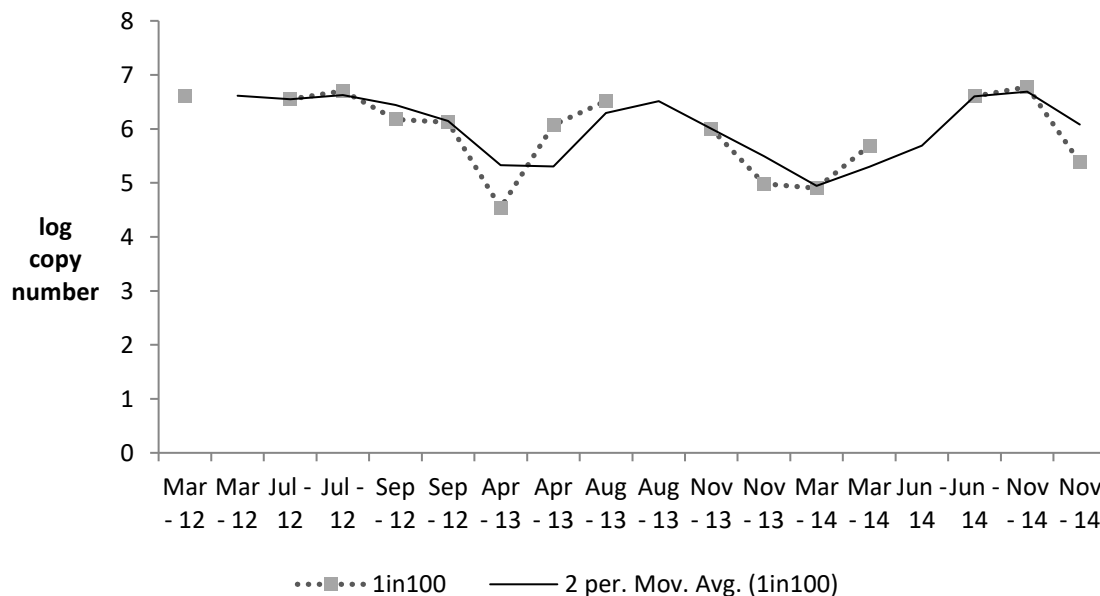
Fig. 14: Distribution of *Alexandrium tamarense*, Gp. III in the North Sea from CPR samples using quantitative PCR. Dotted lines are real data and solid line is moving average.

Flow cytometry data

Water samples were classified into five different size classes of phytoplankton and total bacteria over a five year period from 2011-2016, although consistent results were obtained from 2012 onwards. We present

summary of the most abundant class. The photosynthetic cyanobacteria was the most abundant. Plots of its abundance (Fig. 15) showed a repeated seasonal pattern where abundance was maximal in summer months, possibly influence by temperature. Spatial separation was also apparent, with mid-channel stations showing highest abundance of *Synechococcus*, which are influenced by the Atlantic current. Coastal stations also differed, the stratified French coastal waters showed little variation in abundance, whilst mixed waters of English coast showed moderate peaks in abundance in the summer months. This dataset fills an obvious gap in phytoplankton and chlorophyll observation in this region, providing a breakdown of size classes and some taxa groups as reported by AtlantOS report 633211 (Akpinar 2018).
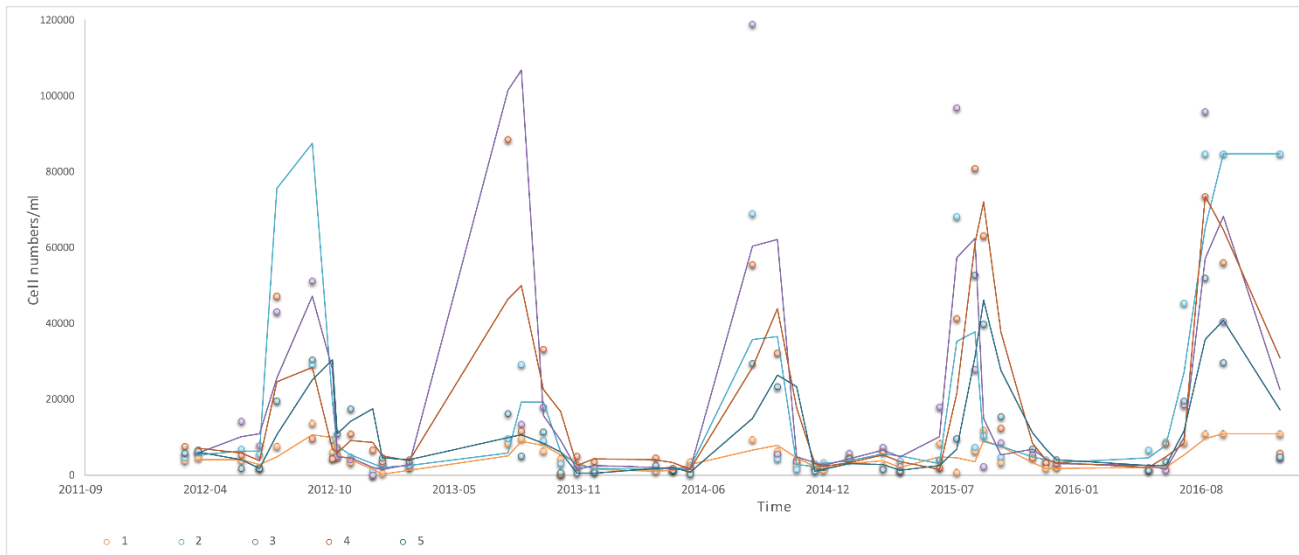

Fig. 15: Temporal patterns of the cyanobacteria, *Synechococcus*, along five stations in the English Channel (1: French coast, 2-4: Mid channel, 5: English coast) from 2012-2016.

The proportion of *Synechococcus* to total bacteria was also explored as heterotrophic bacteria have a tight association with marine cyanobacteria (Zheng et al. 2017), attached to the bacteria. Fig. 16 shows temporal patterns of bacteria are very similar, following each other, indicating co-dependence that relates to successional carbon utilisation. Nutrient data would be a useful addition to this dataset, but unfortunately could not be acquired for these water samples as they require immediate freezing upon collection.
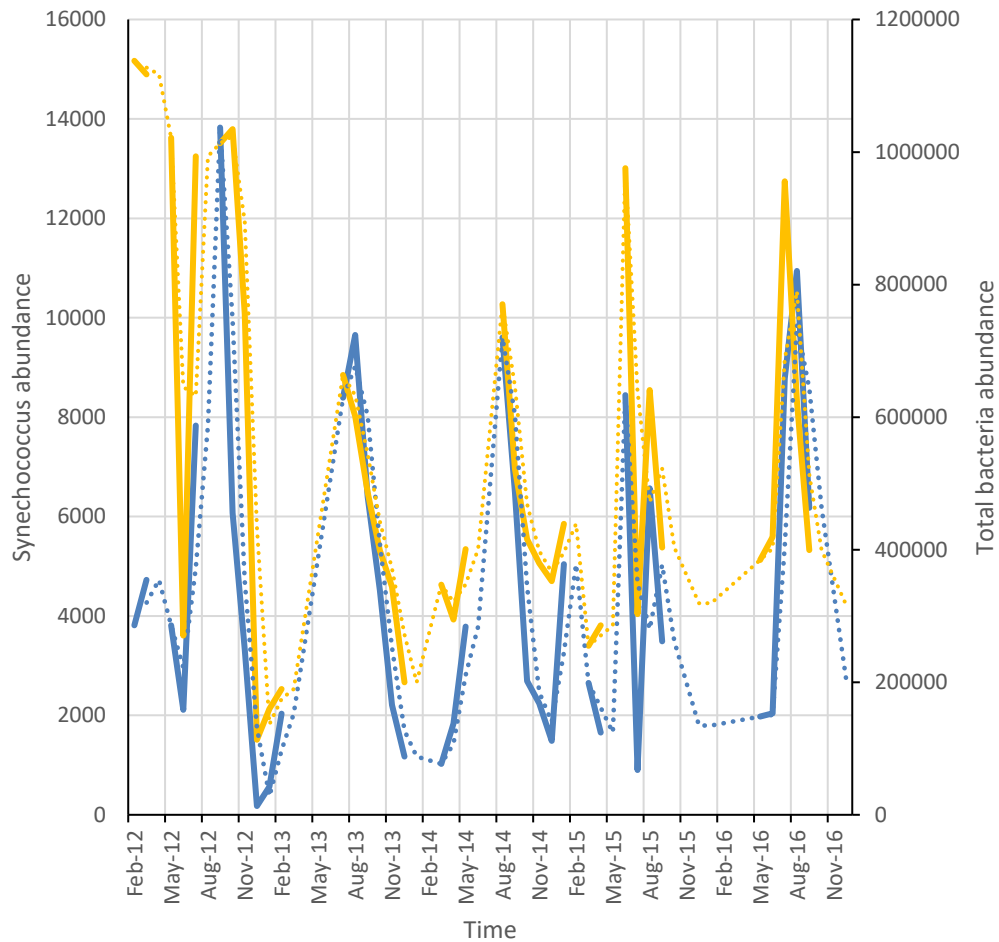
Fig. 16: Total bacterial abundance (yellow line) versus that of the cyanobacteria, *Synechococcus* (blue line) over 4 years from station 1.

The CPR survey records Phytoplankton Greenness Index (PCI), an indicator of total phytoplankton biomass that is related to total chlorophyll a (Batten *et al.* 2003). Patterns of PCI has previously been related to Satellite chlorophyll a successfully (Raitsos 2005). Previous research has shown a disparity between PCI and total diatoms and dinoflagellates, the most abundant phytoplankton measured by CPR survey. Plots of *Synechoccocus* against PCI (Fig. 17) show there is a close relationship, especially in 2012, 2013 and 2016, where peak PCI abundance relates to that of *Synechococcus* and other periods in 2014 and 2015 where PCI abundance is distinct from *Synecochoccus.* Further work relating multiple environmental parameters will provide a
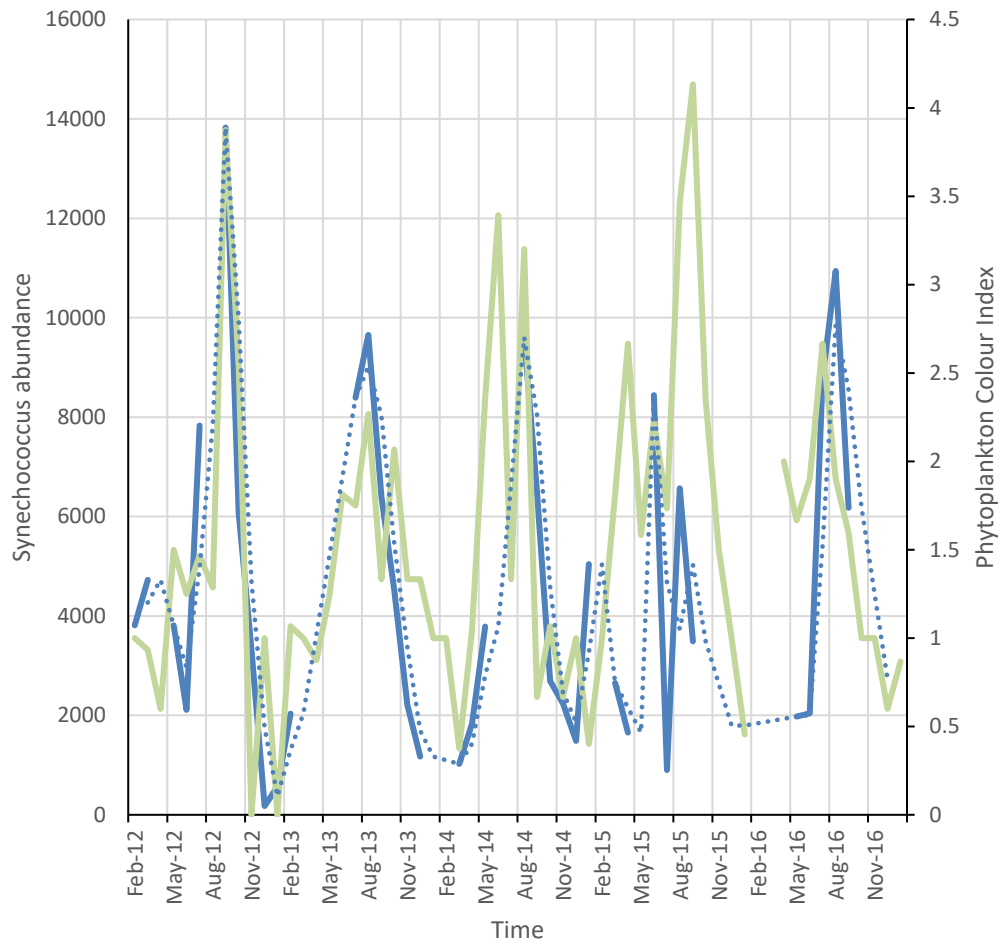
Fig. 17: Abundance of *Synechococcus* (blue line) from station 1 versus Phytoplankton Colour Index (green line) measured from CPR survey nearest location to station 1.

Research Products

1. Five validated assays to quantify a range of harmful algae and the capability to quantify other assays for which we only have presence detected.
2. Five year flow cytometry data shows useful correlations that can be used to develop indicators and modelling products amongst the smallest phytoplankton, in which there is currently a knowledge gap.
3. Validated Pathogen assays capable of use for quantification

Concluding remarks

Quantitative genetic data on harmful organisms was obtained from both autonomously collected water and CPR samples. Low volumes were an issue with water samples, due to limations on water bag sizes that can be addressed by redesigning to collect on filters. There is further scope to develop an automated collection process for biological variables by using digital droplet Qpcr that has increased sensitivity and possibly an in situ testing platform using miniaturised lab on a chip. Such developments require substantial funds for research and development over the next 5 years.

Section 3: Piloted optical methodology

To further enhance its observational capabilities, SAHFOS is also exploring the latest in autonomous technology for rapid particle counting (abundance estimation) and discrimination (identification and speciation) in order to improve monitoring and reporting speed of zooplankton observations. As part of the AtlantOS project, SAHFOS are investigating the feasibility of using the new Fluid Imaging Inc. FlowCam Macro for the rapid determination of zooplankton abundance to complement the manual taxonomic analysis using conventional microscopes that the organisation traditionally undertakes. In this section we explore some of the initial development work that is being carried out to ascertain how the Flow Cam might be used for rapid zooplankton monitoring in order to complement traditional SAHFOS analysis.

Combining high speed imaging, flow cytometry and microscopy in a single unit, the FlowCam Macro is designed to automatically detect individual particles in an aqueous sample, take high resolution digital images of particles and derive more than 30 different types of measurements per particle. The main difference between the traditional flowcam used in phytoplankton analysis and the FlowCam Macro (FCM) is the targeted size range, with the FCM aimed at the range between 50 micros and 5 mm which fits the size-range of the mesozooplankton. Parameters include count, size and volume and advanced, morphological measurements such as circle fit, perimeter and roughness. The system is capable of imaging and characterising thousands of particles per second in real-time and of differentiating particle types in a heterogeneous sample (Figure 18). Utilising image libraries containing similar particles types, the FlowCam can automatically identify and classify the particles as they are imaged.
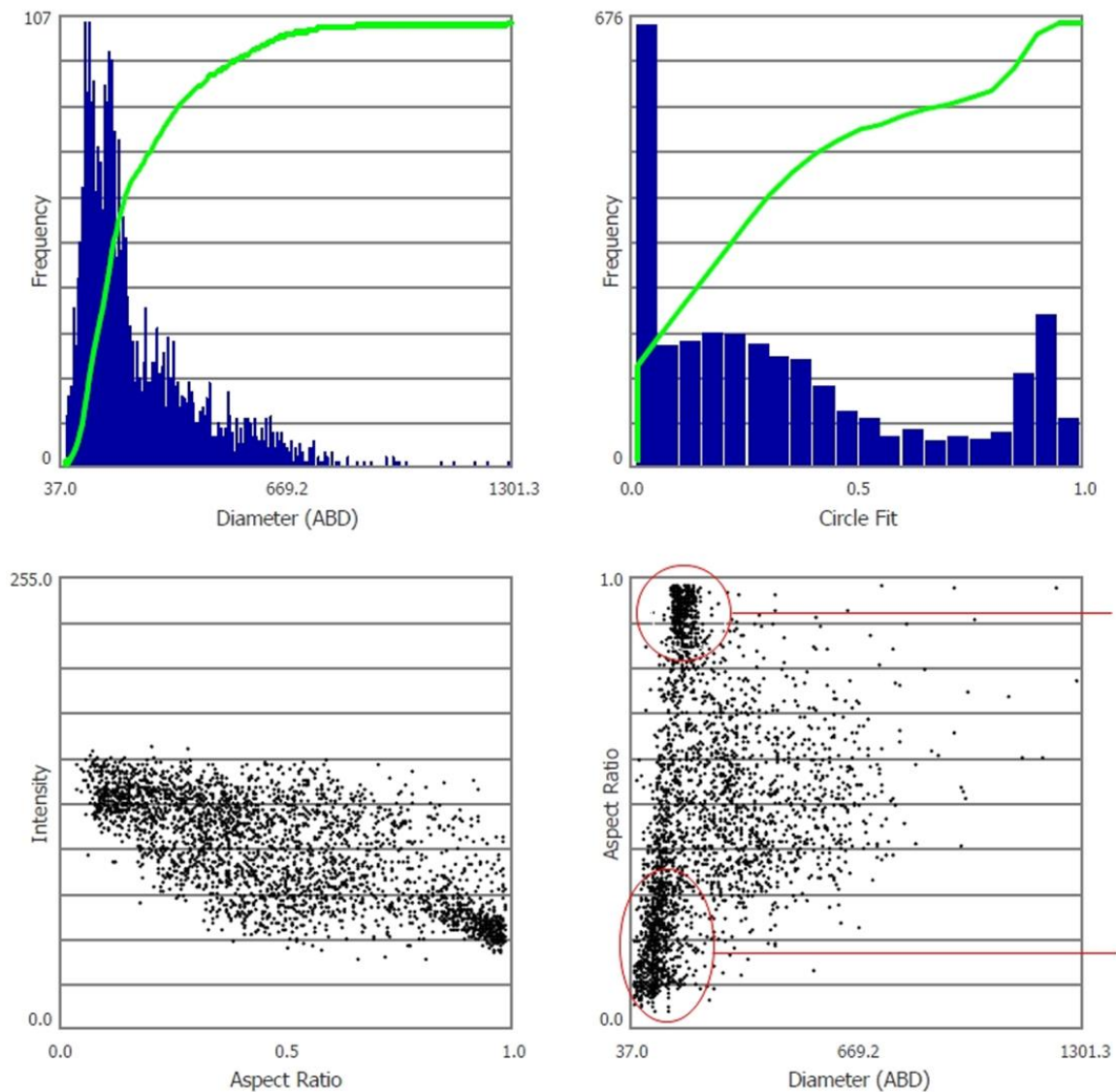
Fig 18: An example of bar and scatter plots of plankton sample particle properties. Aspect-Ratio versus diameter scatterplot shows the partitioning and clustering of bubbles and fibres. This is used to differentiate plankton from extraneous particles.

Traditional CPR sample analysis is conducted in two stages to examine phytoplankton and zooplankton. For the zooplankton eyecount stage of traditional CPR analysis, identification and quantification is performed 'off-silk', all material ≥≈2 mm is removed from the filtering and covering silks, transferred to a Bogarov tray or watchglass and analysed using different microscopes than used for the phytoplankton and traverse zooplankton analysis stages. Both the very small sized phytoplankton and some of the very small microzooplankton stages of traditional CPR sample analysis could be considered semi-quantitative making direct comparison with FlowCam Macro counts problematic. Because the material identified and quantified for the zooplankton eyecount stage is removed from the silk, it presents a perfect opportunity to interpret the traditional process (where, once analysed, the eyecount material would be returned to the silk and the sample labelled, wrapped and stored) and analyse this material using FlowCam Macro. A proportion of the traverse zooplankton analysed using traditional methods fall below the 250μm lower recommended operational limit stated by the manufacturer. SAHFOS have investigated this lower detection limit and found acceptable particle identification down to 150μm for some CPR species / groups). For these reasons the decision was taken to focus on the zooplankton eyecount stage of traditional CPR analysis, where counts are fully-quantitative and the minimum particle size counted is ≈2 mm, therefore direct comparison is potentially achievable.

System Setup

A funnel is attached to the inlet tubing, which runs vertically down to the flow cell, held in place by the flow cell holder. Positioned to the right of the flow cell is the light source, and to the left is the fast repetition rate (FRR) camera. The outlet tubing then runs vertically downwards and turns 90° to exit the FlowCam Macro. The outlet tubing then passes through the peristaltic pump, and attached to the end is an inline 63μm mesh filter. The end of the tubing, including filter is placed inside a collection vessel to catch the sample in case of filter failure. Prior to running CPR samples through FlowCam Macro, a number of performance tests were undertaken to determine the most suitable hardware and software configurations, balancing ease of use, quality of image capture and reproducibility of results.

A sample of 63 adult stage VI *Calanus helgolandicus* (firstly counted and speciated by SAHFOS analysts) were analysed to investigate particle capture consistency. The sample was passed through the FlowCam Macro 10 times. Despite efforts to pass all particles through the FlowCam, filter the effluent and recapture all the particles, a discrepancy between input and re-captured particle numbers was observed between runs. The discrepancies were not consistent, indicating that the FlowCam was randomly retaining some particles within the fluidics system. On investigation, there appeared to be a number of reasons why this particle loss was occurring. Some were adhering to the tubing either on the line in or line out, making those particles unavailable for the next run, or released during a later run to further skew the data. Additionally, if this occurred on the line in, the result was no image capture for that run. Some particles were missed during input, and some were lost due to errors in post-run filtration before the next run began.
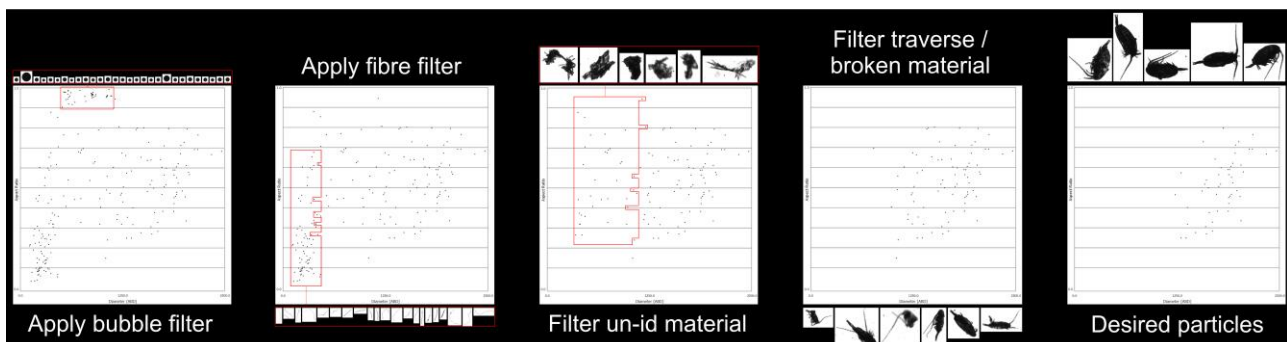

Fig. 19: An example of a CPR sample processed using the FlowCam Macro to first remove extraneous/unwanted particles by step 1 applying bubble and step 2 fibre filters. Step 3: removing unidenfied material. Step 4: splitting traverse and broken biological material. Step 5: Quantification and identification of desired zooplankton component.

In an attempt to minimise these problems the setup described above was chosen. The line in and line out tubing was reduced to an absolute minimum to avoid particle adherence. A funnel delivery system allowed the line in to be vertical and of minimal length prior to the flowcell, adding gravity assist to particle flow and reducing turbulence, and ensuring all particles entered the system. Placing the peristaltic pump on the line out rather than the line in allowed the funnel delivery system to be used, and an inline filter captured all particles onto a small filter mesh allowing complete capture and ease of handling for the next sample run.

In a second experiment, a sample of 70 *Calanus* spp. were analysed to investigate the use of a sample injection system to ensure all available particles were passed through the system. Whilst this setup improved the time efficiency of sample handling prior to a sample run, loss of particles was still encountered and particle recovery post-analysis was cumbersome. Reproducibility of results was improved but ultimately use of this system was rejected because of the increase in post-analysis sample handling time. In a third test, a sample of 50 *Calanus* spp. were analysed to investigate the use of a funnel delivery

system to ensure all available particles were passed through the system. An additional benefit of this setup was a significant reduction in fluidics path length between the point of particle introduction and the FlowCam Macro imaging flow cell. Whilst this setup improved sample handling prior to a sample run, loss of particles was still encountered and particle recovery remained cumbersome. This test illustrated that without a robust method of particle recovery, reproducibility of results could remain problematic. The funnel delivery system was accepted as the preferred method of sample introduction.

In further tests, a sample of 50 *Calanus spp*. were analysed to investigate the use of an inline post-analysis filtering system using 63 micron mesh. Loss of particles was virtually eliminated and particle recovery greatly improved. Reproducibility of results fell within acceptable limits. To achieve the closest possible correlation between number of particles introduced and number of particles imaged, a range of flow rates and camera frame rates were investigated. A high flow rate can be used with a high frame rate but the speed of sample throughput makes the processing of small volume samples problematic. To overcome this, flow rate can be reduced but keeping the same frame rate can lead to the generation of duplicate images as particles are imaged multiple times as the pass through the flow cell. Reducing the frame rate to overcome this can lead to particles passing through the flow cell without being captured. Flow rates between 26-200 ml/min were investigated combined with frame rates between 1-40 FPS. A sample of 10 *Calanus* spp. were passed through the system multiple times whilst changing the flow rate and frame rate until consistency of particle counts was achieved and missed / duplicate particles were reduced as far as possible during imaging. It is difficult to completely remove all risk of an underestimation of particle abundance (missed particles) or an overestimation (duplicate imaging of particles) using the FlowCam system. The decision was taken that it is favourable to generate some duplicate images which can potentially be removed from the data in post-processing than to miss particles completely.

Data Processing Methodology

Once the particle capture is completed, the first step is to remove unwanted particles such as air bubbles and fibres to leave a cleaner subset of images. Bubbles can be isolated using a number of particle properties – their aspect ratio, circularity and circularity (Hu) is close to 1.00 therefore within a sample they can be ranked accordingly using any of these properties and removed. With fibres, in regards to particle properties, the reverse is true – their aspect ratio, circularity and circularity (Hu) are usually in the range 0.01-0.10 and again, within a sample they can be ranked accordingly using any of these properties and removed.

For the benefits of this comparison of analysis methods, all particles significantly smaller (<1000μm) than the zooplankton eyecount minimum size value of 2mm can then be separated from the dataset, leaving a subset containing only the larger zooplankton traverse and the desired zooplankton eyecount images. Duplicate images are generally easy to identify and remove by using a combination of the particle I.D. number (sequential numbering of captured particles) and comparing particle properties, with a visual check to confirm. The remaining cleaner subset of images can then be ranked using any number of available particle properties in an attempt to show differences between taxonomic groups, genera and species. A combination of this ranking and expert taxonomic analysis can then be used to identify and count the particles. As the different sections of the training samples are classified, the resulting images and their particle properties can then be used to create reference libraries with which to interrogate other datasets. Once the above steps are observed to be robust, they can be employed in advance to automatically remove, group or identify particles as desired. Circularity / aspect ratio / image library filters can be pre-selected to remove bubbles and fibres, and a minimum particle size limit set so that all particles below a threshold are not captured. The result is a subset of all potential particles, containing only those particles with a realistic chance of identification and classification. This subset can automatically interrogate any pre-selected image libraries in an attempt to best-fit the remaining particles into taxonomic groups / genera or species. This workflow is illustrated graphically in Figure 19.

At present FlowCam Macro is not a complete replacement for traditional CPR analysis which currently identifies ~1000 taxonomic entities many to species level. For example, subtle morphological differences between important indicator species such as *Calanus helgolandicus* and *Calanus finmarchicus* are unlikely to be visible on imaged particles. On occasion when these features are visible, they will not produce a difference in particle statistics that allows for these species to be separated. Figure 20 illustrates the hierarchical match up currently possible between analysis using FCM and traditional CPR methodologies.

A combination of traditional microscopic analysis to determine species ratios within a sample, combined with rapid assessment of abundance/biomass using FlowCam Macro could be used to reach a more satisfactory result.
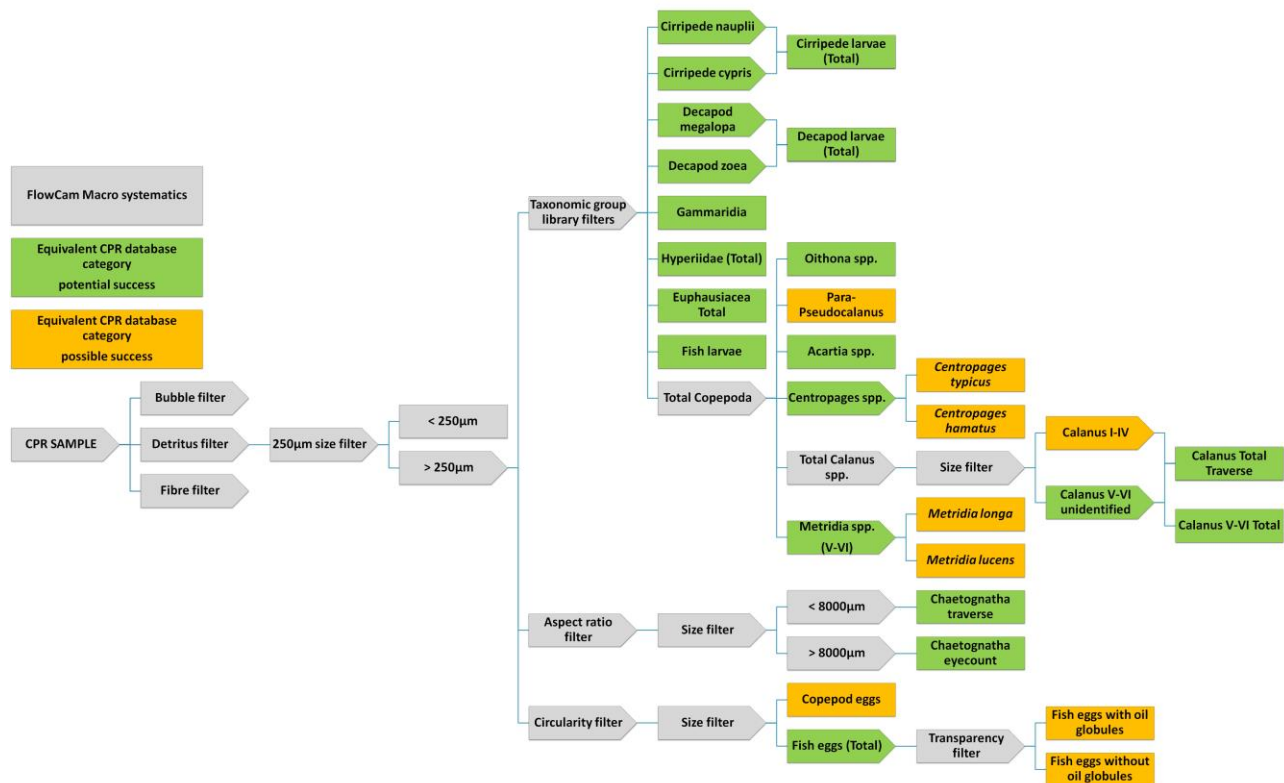

Fig 20: A schematic of how to process a CPR sample using the Flowcam Macro based on a hierarchical approach and employing both a taxonomic library filter and a hard property filters.

The bulk categories that exist within the CPR database present an opportunity to provide meaningful data from FlowCam Macro that could be incorporated into CPR datasets and time series. Where particle identification cannot be taken to species, genus level or higher taxonomic groups can provide a directly comparable category between the two analysis methods, for example:

- For a number of taxonomic groups, such as the Euphausidae and Hyperidae, traditional CPR sample analysis usually does not attempt to speciate observed organisms. Automatic particle classification to this higher level should be possible, although confirmation of this should be achieved by the processing of a larger number of CPR samples.

- For a number of organisms identified to species-level by the CPR survey (particularly within the Copepoda), categories exist within the database that sit above the species-level (i.e. *Calanus* V-VI Total) which should allow direct comparison between traditional analysis counts and FlowCam

Macro counts. Automatic particle classification to these categories should be possible, although again, confirmation of this should be achieved by the processing of a larger number of CPR samples.

- With further development and testing FlowCam Macro should be able to provide a number of zooplankton metrics/indices in a more rapid manner including estimates of biomass/biovolume, size-ranges of community; higher taxonomic level biodiversity data and course functional type based information.

In summary, the FlowCam Macro has proven to consistently produce high quality images of the main components of the mesozooplankton including euphausiids, decapods, copepods and hyperiids (Figure 21). The information obtained from samples run on the FlowCam could compliment and contribute to the marine observation work carried out by SAHFOS and the collection of bulk zooplankton data needed to support the AtlantOS project in answering challenging questions about the impact of climate change on marine ecosystems. Rapidly and automatically determining the abundance and bio-volume of different zooplankton improves calculations of total carbon concentrations and estimates of carbon transport from the surface to the deep sea. The speed, efficiency and reliability of data acquisition are paramount and automated systems such as the FlowCam are helping to accelerate the pace of research into the health of fundamental components of the marine ecosystem. Ongoing tests and research at SAHFOS will further investigate the potential of the FlowCam to obtain fast and reliable estimates of zooplankton biomass and other plankton metrics.
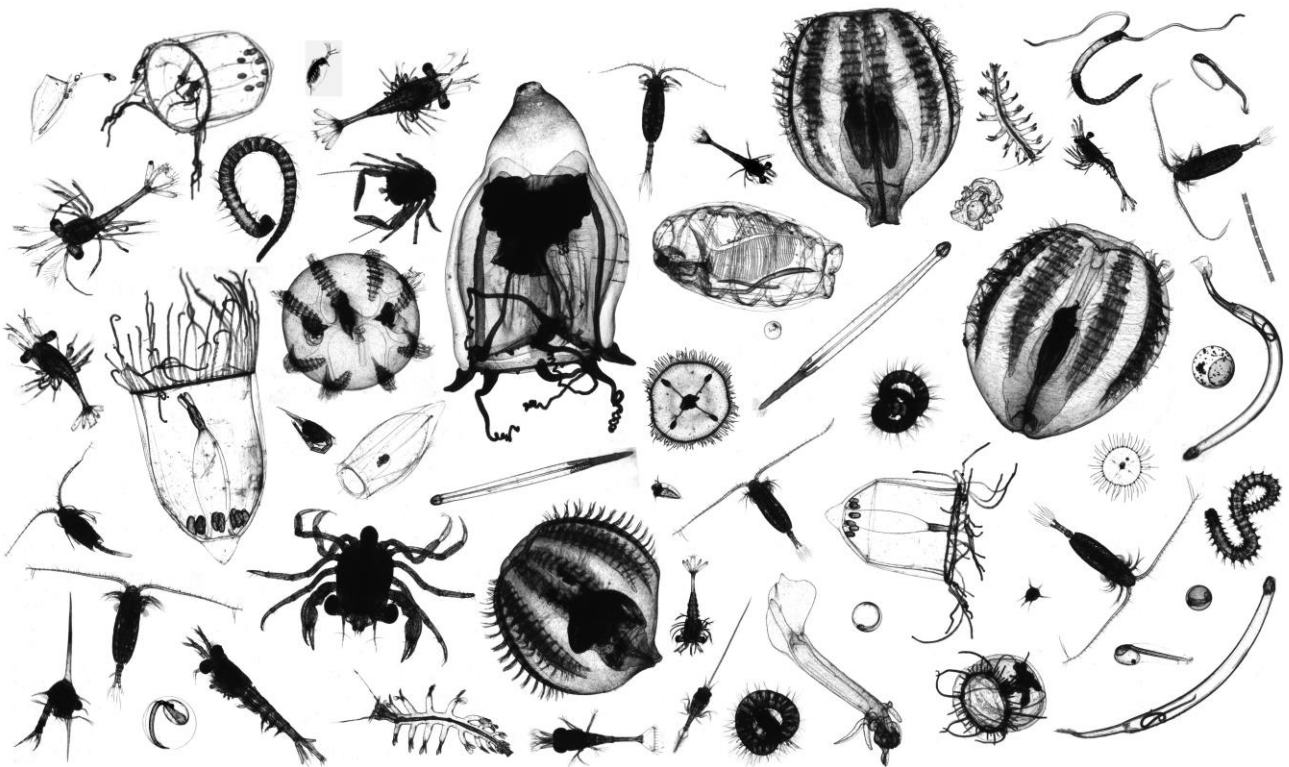

Fig 21: A montage of taxonomic entities from samples in the English Channel used to create the Flowcam macro libraries.

Concluding remarks

As of November 2018, 54 samples have been analysed, 60 taxonomic entities (libraries) have been created and over 50,000 particles have been classified into libraries allowing for the rapid production of biomass and other biological metrics. As an example of the utility of the Flowcam Macro in terms of speed of analysis and identification; in September 2018, high numbers of *Penilia avirostris* (a warm-water cladoceran) were identified using the Flowcam Macro from the western English Channel for the first time, possibly due to the combination of high temperatures and calm conditions in the region, and an influx of subtropical waters into the English Channel. The adults are unlikely to survive the winter, but if sexual reproduction has occurred then resting eggs may hatch when conditions become more favourable in 2019. By utilising Flowcam libraries of *P. avirostris* built from 2018 data, the potential re-appearance of the species in 2019 and in the future can be flagged immediately.

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Appendix

Table S1: Summary of quantitative PCR assays developed and validated and environmental results. WS: Water sample, CPR: CPR sample, D: Detected, Q: Quantified, EC: English Channel, NS: North Sea. Grey: no data, blue: human pathogen, pink: animal pathogen, brown: deoxygenator, red: toxin producer

Assay from	Species	WS	CPR	Dates	Detected EC	Detected NS	Harmful effect
Andree et al. 2012	*Pseudo-nitzschia fraudulenta*	1	0	2011-2012	D, Q		
CPR survey	*Pseudo-nitzschia delicatissima*	1	1	2011-2013	D, Q	D	
Andree et al. 2012	*Pseudo-nitzschia multiseries*	1		2011-2012	D.Q		
Toebe et al. 2013	*Alexandrium tamarense, type III*	1	1	2011-2013	D, Q	D, Q	
Penna et al. 2007	*Alexandrium minutum*	1	0	2011-2013	D,Q		
Popel et al. 2003	*Aureococcus anophagefferans*	1	0	2011-2012	D,Q		
Cloud et al. 2000	*Legionella spp.*	1	0	2011-2012	D		
Schroeder et al. 2001	*Acanthamoeba*	1	0	2011-2012	D		
Chern et al. 2009	*Escherischia coli*	0	0	2011-2012	D		
Marsh et al 1995, Ulrich et al. 2007	*Perkinsus marinus*	0	0	2011-2012	D		
Kuiper et al. 2006	*Hartmanella vermiformis**	1	0	2011-2012	D		
White et al. 2013	*Icthyophonida*	0	0	2011-2012	D		