Introduction of additional invertebrate species to the Water column monitoring program (with a focus on the king scallop *Pecten maximus*)

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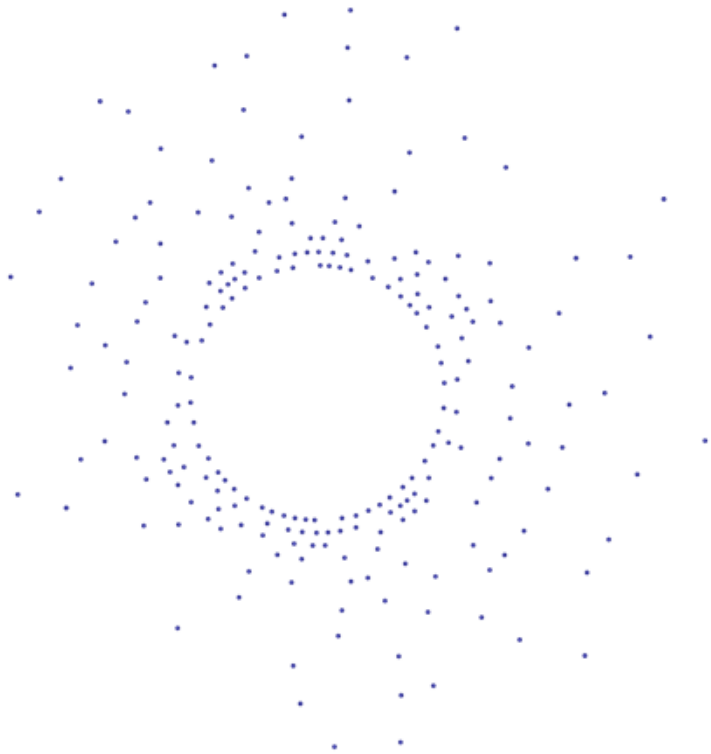

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1. Project outline

Expanding the range of invertebrate species used for WCM beyond the blue mussel (*Mytilus edulis*) will provide a broader basis for evaluating the health and well-being of marine life in areas close to oil production activities. The desk top study carried out as the first of three components of this project has investigated the potential for using invertebrate species from a range of phyla for WCM purposes. The use of caged animals deployed on site over time, as currently employed for blue mussels, is considered, along with the use of traps to capture native free living epibenthic species.

The king scallop (*Pecten maximus*) has already been recognised as a potential candidate for use in WCM. The second component of this study examined the development of protocols for use in future field monitoring campaigns. Part of this development sought to organise the handling and sampling procedures together with the choice of endpoints such that the number of animals used can be reduced to a minimum.

The third and final component of the project sought to develop haemolymph-based biomarkers using flow cytometry techniques. This research builds on previous work at NORCE where assays examining immune function and cell health in mussels were developed. Flow cytometry methods are attractive due to rapidity of analysis, low sample volume requirement, capacity for adaption to many biological models and their potential for automation. Portable flow cytometers are now available that make on-site field use possible. Immune function (phagocytosis) was the focus of the previous research at NORCE and was developed further in this short project with scallops.

The three components of the study

A. A desk top feasibility study investigating the suitability of invertebrate species from a range of phyla for inclusion within the WCM programme.

B. A laboratory and desktop study to develop and refine protocols for obtaining biological and chemical measures from scallops (*Pecten maximus*).

C. An evaluation of haemolymph based biological measures from bivalves using a field capable flow cytometer, building on previous work carried out at NORCE.

2. Searching for suitable invertebrate species for WCM

Introduction

The mussel (*Mytilus* spp) (figure 2.1) has been used globally for monitoring the presence of chemical pollutants in the marine environment and for subsequent analysis of potential biological effects for many years (Beyer et al, 2020; Beyer et al., 2017; Schøyen et al., 2017). A vast wealth of knowledge has been accumulated on the biology of this species together with method development for examining many physiological and biochemical processes and the effects of specific contaminants upon them. They have the advantage of both physiological sensitivity to certain pollutants and the ability to accumulate others – providing an indication of the availability and potential impact of specific contaminants in the marine environment. Indeed, the mussel watch program that originated in the US and adopted elsewhere uses this accumulation potential to establish annual trends for the increase or decrease of a wide range of chemical contaminants in marine waters. Furthermore, mussels are obtainable from aquaculture establishments where they are raised in clean water conditions and supplied at optimum sizes. With some care they are readily transported out of water for some hours with no long-term physiological damage. They are easy to cage and as filter feeders can typically maintain a food supply for themselves when deployed. A potential drawback with their use in deeper water monitoring is related to their natural distribution as coastal, generally shallow water animals. They are capable of survival in deeper water conditions for extended periods of time as natural populations, provided they find a hard substratum to colonise, and subtidal populations have been reported on dock pilings and offshore oil platforms, where they grow to a large size, likely due to a lack of predators (Seed and Suchanek, 1992). However, mussels used for the water column monitoring program are obtained from shallow water farming operations and so deep-water deployment may be considered suboptimal and could influence their physiological performance in assays taken following their retrieval.

Mussels are currently used as the sole permanent invertebrate test organism within the offshore water column monitoring program in the North Sea organised by the Norwegian Oil and Gas association and overseen by the Norwegian environment agency and have been used regularly within the program since its inception.

Figure 2.1
Blue mussels *(Mytilus edulis*)

Although mussels offer many good reasons for their inclusion there is a danger that as the sole invertebrate organism they may not adequately represent all relevant invertebrates in the environmental areas of interest. Furthermore, dependency on a single species can bring risks from species or genus specific diseases that could disrupt either the supply of suitable animals for use in monitoring or overtly influence the outcome of biological measures taken following their deployment. There are currently investigations into high mortality levels and disappearance of blue mussels from many European coasts, including Norway. Parasitic infestation is under suspicion as a cause for this decline, though other factors may also be contributing (Charles et al., 2020). The purpose of this present study is to review the potential for using additional invertebrate species within offshore

monitoring programs to broaden its scope. Several phyla will be investigated in the search for suitable species.

Factors influencing the choice of monitoring organisms for WCM

While there may appear to be a wide choice of organisms that could play a role in offshore monitoring programs, there are several key factors that must be considered for each candidate species to assess its suitability for the program. Some of these are influenced by physiology and ecology, while others are related to practical concerns. These factors are discussed in turn below.

Susceptibility to pollutants and ability to accumulate pollutants of interest

When considering the choice of organisms to use for monitoring specific target chemicals, such as in the WCM program, it is important to recognise how the chosen species will likely react to the expected range of concentrations to which they may be exposed. Ideally the species will exhibit measurable results when exposed to the pollutants at relevant concentrations and not be so sensitive as to suffer mortality during deployment. When bioaccumulation of contaminants is to be measured, the metabolism, transformation and depuration potential of monitoring species should be established for the contaminants of interest during the selection process.

Representative status of the species

It is preferable to select species for monitoring that can represent a range of organisms within the region of interest. In this way it is possible extrapolate findings from the selected organisms across species that share similar biochemical and physical attributes. Consideration should also be given to the general ecology of species in this regard, with factors such as feeding style, with filter, deposit, carnivorous and carrion feeders each interacting with their environment in different ways. In many situations it is likely that several invertebrate species would ideally be used to cover this variation,

though practical limitations and cost constraints in monitoring programs will often limit the number of species used.

Potential for measurement of suitable biological effects

Protocols for biological measures have been developed for many but not all organisms that could be considered as suitable candidates for use in monitoring. Selection of new species that lack an established suite of biological effects measures will require substantial development of suitable methods prior to their adoption for use in monitoring. There are many laboratory-based measurements for a variety of physiological and behavioural endpoints in use for many invertebrate organisms. However, a key part of field monitoring within the WCM program is to sample tissues or conduct tests as soon as possible, on board the vessel, following their capture or retrieval from the deployment location. In this way factors such as transport, handling stress and depuration of accumulated pollutants, that could confound test results are minimised. This demand does remove the possibility of carrying out some more complex or demanding procedures in the field on fresh tissues, where in general the focus is more, though not exclusively, on taking and preserving tissue samples for subsequent histopathological and biochemical analyses. The possibility remains that modification of laboratory-based protocols can provide an effective means to carry out some of these analyses in the field, though these adaptations will require full validation.

Accessibility of supply

If deploying caged organisms on site it is essential to ensure they originate from clean water areas and are available in sufficient numbers to meet the demands of the deployment program. Commercially farmed or collected invertebrates provide an opportunity to meet this demand but by using this supply route the choice of organism is limited. Bivalves such as blue mussels (*Mytilus spp*) and king scallop (*Pecten maximus*) can be obtained commercially, together with various crustacean species.

Development in aquaculture of some echinoderm species for human consumption is increasing the breadth of species choice available for potential use in monitoring. Other non-commercial species must typically be collected independently, and this can be a time-consuming process and requires careful handling and transport of the animals to ensure they remain healthy and uncompromised. It also requires knowledge of the environmental condition of each collection site used. An evaluation of the endpoints to be tested in deployed animals covering both chemical accumulation and biological effects measures should be conducted from potential collection sites in freshly collected individuals.

The biological timetable of candidate species.

Cycles of reproduction and periods of increased or decreased feeding due to seasonal influences need to be considered for each species when timing the deployment/capture period. Spawning in some species, including blue mussels, has been shown to significantly alter the results of biological effect measurement techniques (Hagger et al., 2010) and storage and utilisation of tissue reserves can influence measures of chemical accumulation. The ability to compare results throughout a series of regular monitoring programs requires that the timing of sample collection recognises, minimises and accounts for, such potential influences.

Use of wild caught or caged animals at the monitoring site.

There is potential to capture invertebrates from natural populations associated with designated sampling sites within a monitoring program and to carry out similar suites of biological and chemical accumulation measurements to those already in use for deployed caged organisms. This follows the pattern of vertebrate sampling within the WCM program which uses wild caught fish of several species as a basis for assessment. Fish are typically caught using a combination of rod and line and trawling. Line caught fish are landed in better condition due to the absence of crush damage in the nets but sampling sufficient numbers from a broad range of species can present a challenge at each of the

sampling sites. The limitation presented by restrictions of trawling close to offshore oil installations does demand that rod and line fishing is used in these situations. Several species of fish are normally targeted, representing a range of feeding and activity types.

Advantages of wild caught animals include potentially longer residence times in the areas of interest and a full integration with the surrounding ecosystem and chemical/physical environment. Disadvantages include the possible inability to recover certain species at some of the test sites and obtaining sufficient numbers of comparable animals from each of the sites included within the monitoring program. Fish have the additional issue of unknown extent of migration in and out of the areas of interest. It is likely that this would be less of an issue with invertebrates, but it remains a possibility. A further potential issue is one of chosen sentinel organisms avoiding plumes generated by the outfalls from platforms. With mussel caging and deployment considerable effort is spent predicting the plume path from outfalls in order to place the cages as close to the track of the plume as possible. This approach could be considered to generate a worst-case exposure scenario, and this functions well, particularly if no overt effects are recorded in the caged animals held in association with the plume. Such results could be extrapolated to conclude that animals not in direct association with the plume are likely to receive lower exposure concentrations. If avoidance by the target sentinel species of the plume is a factor, then wild sampling is not likely to produce representative results. A further disadvantage of wild caught sampling of invertebrates lies in the time required to set and retrieve traps, together with the danger of selectively sampling only healthy individuals and leaving behind individuals potentially compromised by chemical exposure. The use of caging and deployment also exerts limitations on the number of suitable species that can be deployed continuously for the six-week period used within the WCM program. Food supply is the key issue here and why bivalve molluscs are typically favoured in monitoring with the use of cages. As filter feeders, they can capture available food from the water flowing past them with no additional feeding system provided for them. Predatory and carrion feeders will require food, or at least preconditioning prior to deployment, to

ensure that a gradual loss of their reserves is not the major factor in any deterioration of their biological condition, potentially disguising effects of environmental contamination when the tissues are analysed at the end of the deployment. Loss of energy reserves and tissue mass due to fasting will also affect measurement of body burden of contaminants. Care must also be taken in design of caging equipment to ensure that it does not create significant additional stress to the caged organisms. A potential further source of invertebrate tissue samples is the bycatch taken within fish trawls carried out within the WCM program. Accumulation studies, combined with genotoxicity assays among others, could be carried out on these animals. Inter-site variation in presence and absence among the invertebrate species taken is likely to be an issue but the trawl bycatch does offer a low effort alternative for gathering tissues for analyses. The possibility of using a shrimp trawl to specifically target epibenthic invertebrates as part of the WCM program may also be worth consideration.

Assessing candidate species for wild capture and caging.

Several surveys of epibenthic invertebrates throughout the North Sea have taken place over the last couple of decades and their results provide a useful starting point for identifying those species that occur in sufficient numbers to be considered suitable for use within the WCM program (Zuhlke et al., 2001; Jennings et al., 1999), either for wild capture or cage deployment. A further survey, focussing on biodiversity in the close vicinity of oil platforms in the North Sea, has also been reported recently (Schulter et al., 2019). A number of candidate species identified within these surveys are discussed in the following section of the report. The collection of target species from the sea floor in sufficient numbers for effective analyses is perhaps one of the major considerations when judging their suitability for inclusion within monitoring programmes. Baited traps and bottom trawling are the two primary methods that could bring target organisms to the surface. Of the two, the baited trap offers the best possibility of retrieving animals to the surface with the least damage. There is a risk, however, that the nature of the trap's operation, relying on sensory perception of the bait and active physical

mobility to reach and enter the trap, may preferentially select for healthy capable animals and leave behind those potentially compromised by exposure to contaminants. The alternative method, caging and deploying organisms, provides limitations on the type of organism than can be caged over several weeks, and generally favours filter feeding bivalves that can sustain themselves within the available current flow. The possibility to construct cages that provide food for non-filter feeding organisms exists, though this does add a level of complexity to the process. The sections that follow introduce species from three phyla, identified within the North Sea invertebrate surveys, that could potentially be used within the WCM monitoring program. Prior use of some of these species for oil/produced water exposure studies are briefly described together with practical issues associated with their use for monitoring the central North Sea regions.

Candidate Phyla

Crustacea

Of the crustaceans it is the decapods that perhaps have the most to offer to the water column monitoring program. There are several crab and shrimp species whose habitat extends to the central North Sea areas that are good candidates for inclusion within a wild capture monitoring programme.

Crabs

Brown crab (*Cancer pagurus*),

Great spider crab (*Hyas Araneus)*

Toad crab (Hyas coarctatus)

Flying crab (*Liocarcinus holsatus*)

Hermit crabs (*Pagurus bernhardus, P. prodeaux and P. pubescens*).

Shrimps

Pink shrimp (*Pandalus montagui*)

Brown shrimp (*Crangon crangon*)

Commercial pot capture of crabs has long been shown to be an effective method for collection with a limited risk of physical damage to the crabs. Although deployment time of pots can vary, 1-2 days of 'soak time' is generally considered to be sufficient for many commercial operations. A deploy and recover program with this timescale could be incorporated within the cruise schedule currently operated within the water column monitoring program. There is potential for caging and deploying crabs over some weeks, though as previously mentioned, there are issues with the size of cages and feeding of animals, together with advantages gained by capturing and examining natural populations, that make wild capture of these animals more attractive. There are several crab species that could be targeted by pot fishing in the central North Sea region. The brown crab (*Cancer pagurus*) (figure 2.2), is fished commercially using pots, with the second and third, the great spider crab (*Hyas araneus*) (figure 2.3) and the toad crab (*Hyas coarctatus*) recorded as relatively abundant in the central North Sea area. There are reports of seasonal migrations in Autumn for some crab species (Hunter et al, 2013: González-Gurriarán et al, 2002), related to reproduction and moulting, that would need to be taken into account when planning sampling timetables. The hermit crabs, particularly *Pagurus bernhardus*, are recorded in surveys of the North Sea regions as quite abundant and these too can be captured within baited pots. The space required for equipment storage and deployment and retrieval equipment necessary to handle the pots would require consideration in the planning stage of sample collection. It is likely that fewer deployment sites than the number used for deployment of fixed rig animals would be targeted during the sampling cruise, perhaps dropping a string of several pots in each of a few selected locations that represent a gradient of the production water plume emerging from the selected platform.

Figure 2.2
Brown crab *Cancer pagurus.* Seen here with a heart rate sensor fitted.

Of the smaller decapods, shrimp (for example *Pandalus montagui* and *Crangon crangon*) offer the possibility of providing tissue samples for analysis. The most productive method for their collection would be short duration trawling with an adapted net to minimise stress and damage to their tissues. Tissue analyses targeting accumulation of chemicals of interest in these animals combined with histopathological examination of gill condition could yield valuable information on their condition. There is a possibility that shrimp could be collected as a bycatch from the fish trawl operations currently carried out in the WCM program, with tissues frozen or preserved on board for subsequent analyses.

Figure 2.3
Great spider crab
Hyas araneus

Decapod crustaceans have been used extensively within pollution effect studies for several decades, including those targeting the effects of oil-based contamination. Both laboratory exposures (Camus et al., 2002; Larsen et al., 2006; Sundt et al., 2006; Bechmann et al., 2010) and field collections of decapods have used a variety of biological effect measures to determine the impact of a range of oil-based pollution scenarios. A review of the use of the shore crab (*Carcinus maenas*) within ecotoxicology (Rodrigues & Pardal, 2014) provides an extensive list of biomarkers that have been measured in this decapod crab, most of which are applicable to the crustacean species listed in the present report. Careful selection of a number of these tests could provide a valuable suite of techniques to support future water column monitoring programs. These could include MN assessment

in haemolymph, PAH metabolites in urine (Nudi et al., 2010), histopathology in hepatopancreas, gill and gonad tissues (Morales-Caselles et al., 2008) and PAH accumulation in the hepatopancreas (Douglas et al., 2018).

Molluscs

Caged blue mussels (*Mytilus edulis*) have been used extensively for the water column monitoring programs and continue to act as reliable indicators of exposure and effect in the North Sea oil fields. Adding more molluscs with different trophic lifestyles could however reveal additional information. King scallops (*Pecten maximus*) were included in the most recent monitoring program with just four cages deployed. These animals were purchased from a commercial fishery that used divers to collect them, ensuring good quality, undamaged animals were gathered from unpolluted waters. A more detailed account of the king scallop is provided in the following section of the report that examines protocol development to maximise the value of including this species in the monitoring program.

There are at least four further species of epibenthic molluscs reported as widespread within North Sea invertebrate surveys that could also be suitable for the WCM program, two bivalves, the queen scallop (*Aequipecten opercularis*) and the horse mussel (*Modiolus modiolus*), together with the gastropod whelks *Buccinum undatum* and *Neptuna antiqua*.

Sampling techniques and biomarker selection described for king scallops in the section below would largely be applicable to the smaller sized queen scallop. The commercial market for queen scallop in Norway is much smaller than the king scallop and so fresh and healthy individuals will not be as available as for the king scallop. The queen scallop has appeared in reports of environmental monitoring (Bustamante & Miramand, 2004) but it has not been as widely used as the king scallop for pollution effect studies. Its use within the WCM program could either follow a cage and deploy technique or a wild capture trawling effort. The second potential bivalve candidate, the horse mussel (*Modiolus modiolus*) a large deeper water mussel species, would follow the sampling approach used

currently for blue mussels. The commercial availability of this species is limited but they could be collected by divers in sufficient numbers to support caging and deployment within the WCM program. In the latest WCM exercise two depths of blue mussels were used on each rig, at 20 and 40 m. The use of the deeper water horse mussel on the lower rig to replace the blue mussels would provide a more ecologically depth appropriate combination. Horse mussels have been used previously for field monitoring purposes, largely in accumulation studies of various metals and pollutants (Næs et al., 1998; Richardson et al., 2001; Willett et al., 2000). They often occur in large beds in deep water and provide habitat for many other invertebrates including queen scallops, whelks and crabs, with the abundance of these species often 20 times higher than in areas lacking these beds (Kent et al., 2017). Several of these species have been identified within this present report as having good potential to support WCM. If such beds occur in the vicinity of oil platforms, then a combination of baited pots and a grab may deliver sufficient numbers of these species for an effective monitoring survey with relatively little effort.

The carnivorous/scavenging gastropod whelks would bring a different trophic lifestyle than the filter feeding bivalves to the survey. They are fished commercially by using pots baited with crab and fish waste (Fahy, 2001) and this technique could be employed within the WCM program. As with some of the other species listed above their use within biomonitoring is rather limited but they have been used for investigations into imposex related to tributyl tin (TBT) exposure (Poloczanska & Ansell, 1999; Strand et al., 2006), together with bioaccumulation studies (Beach et al., 2010; Hellou et al., 1993). Reproduction timing and gonad histology have also been investigated in *Buccinum undatum* (Borsetti, et al., 2020; Couillard & Brulotte, 2020) and these investigations would have direct relevance if this species was to be incorporated into the WCM program.

Figure 2.4
Images of the whelk *Buccinum undatum*.

Echinoderms

There are two main candidates for inclusion within WCM among the echinoderms, the starfish (*Asterias rubens)* and the urchin (*Strongylocentrotus droebachiensis*). *Asterias rubens* is widely distributed in the central North Sea and represents the best opportunity for wild sampling of an echinoderm. Several studies have examined aspects of their behaviour and physiology following their exposure to a variety of treatments. Behavioural tests include recording time taken to right themselves after being turned onto their dorsal surface (Canty et al., 2009) and food location performance (Georgiades et al., 2003) following timed exposures to treatments. Biomarkers used on this species include neutral red retention time, micronuclei assessment and DNA strand breaks (Canty et al., 2009), cytochrome P450 (Den Besten et al., 2001; Everaarts et al., 1994; Georgiades et al., 2003), acetylcholinesterase activity and benzo (A) pyrene hydroxylase (BPH) activity (Den Besten et al., 2001). Bioaccumulation studies that included PAHs have also been conducted on Asterias in the North Sea region (Den Besten et al., 2001). Starfish can be collected in baited traps or as a bycatch during trawling operations. There is also the possibility of deploying cages of starfish over time, with the inclusion of live mussels within the cage as a source of food and perhaps even using this combination as a study of biomagnification potential. The rather more delicate structure of the urchin (*Strongylocentrotus*

droebachiensis) suggests that deployment in cages would better suit them than wild collection. With land-based farming operations underway for this species, within Norway and elsewhere, access to well-conditioned animals to deploy should not pose a problem. Consideration will need to be given on how to best feed them during deployment. It is the gonad tissue in the urchins that is the primary focus for biomarker measurement, with antioxidant responses (Amri et al., 2017), oxidative stress measures (Quetglas-Llabrés et al., 2020) and EROD activity (Osse et al., 2018) among measures reported in the scientific literature.

3. Protocols for field monitoring with King scallops.

King scallops (*Pecten maximus*) (fig 3.1) are large bivalve molluscs that are ecologically depth appropriate for biomonitoring programs in the North Sea and can be considered as substitutes for the queen scallop (*Aequipecten opercularis*) which has a distribution throughout the central North Sea region. They are self-sustaining filter feeders, passing large volumes of water over their gills (Laing, 2004) aiding accumulation studies. Adult scallops contain large quantities of tissue when in pre-spawning condition which can provide material for multiple assays. *Pecten maximus* are hermaphrodites, producing both sperm and egg gametes simultaneously and have a spawning season that runs from April to September (Marshall and Wilson, 2008). The relative abundance of tissues in these scallops could serve to reduce the number of animals required to fulfill the requirements of monitoring surveys. They are tolerant of non-natural conditions for many months in the laboratory (pers obs) and are commercially farmed within net structures as juveniles, suggesting that deployment of adult scallops in the field for the duration of the WCM monitoring program should not pose them any problems. They are a commercially important species with a widespread distribution and can be purchased in good condition from fisheries that employ divers to carry out hand collection.

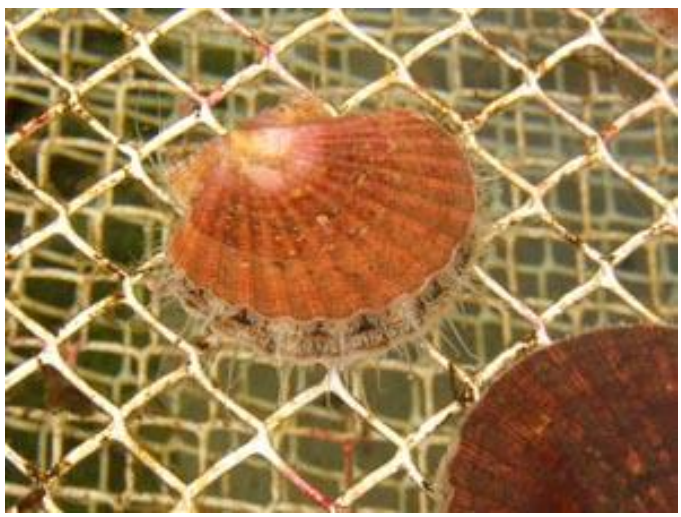

Figure 3.1

Adult king scallop (*Pecten maximus*) With open valves and tentacles extended.

As well as bringing positive aspects to promote their use in field monitoring, the size and permanent subtidal lifestyle of scallops also brings some challenges. The minimum permitted commercial landing size for scallops is between 10 and 11 cm shell width and market size individuals are typically closer to 15 cm. The primary challenges related to field work concern their transportation to field sites and their caging during deployment. Transport to offshore deployment sites requires careful planning to maintain the animals in a healthy condition prior to deployment, with clean oxygenated water available to supply containers suitable for housing the animals. There are several commercial live transport systems for scallops on the market that could be considered for future field deployments. Some scallop species, including *Pecten maximus* can swim short distances. This is typically triggered by an escape response following disturbance or an approach by a predator, such as starfish (Barbeau & Scheibling, 1994). When deploying cages containing scallops it is desirable to house them individually to avoid damage from interlocking shells as they swim or adjust their position within the confined space of a cage. Such occurrences could lead to damaged tissues and interfere with normal feeding and respiration activity. Individual compartments also provide stable support and protection for the animals while they are handled on deck during deployment and recovery operations. The separation of individuals does however demand large cages when housing 40 scallops (figure 3.2) and this requires additional consideration when designing deployment rigs, in terms of ease of handling and buoyancy calculations. Finally, holding of scallops prior to deployment requires relatively large-scale holding facilities with continuous flow seawater systems.

Based on the factors set out above it is perhaps the role of scallops to add selective support to the wider monitoring effort provided by blue mussels in the WCM program, with fewer scallop cages deployed in areas of specific interest rather than full coverage of all sites that is provided by the mussels. With careful selection of biological measures and some adaptation to individual protocols to allow multiple endpoint measurement from each individual scallop, it should be possible to lower the number of animals required at each site and reduce the size of cage required. These changes would

make the regular use of scallops in field deployments in the WCM program more feasible. There was a trial deployment run included in the latest WCM program (2021) using four cages, each containing 40 scallops. Figure 3.2 shows one of the cages used. The space is divided internally to provide individual compartments. The deployment, recovery and sampling of the scallops proceeded well and the results from the analyses are now awaited.

Figure 3.2
Cage used in the deployment of 40 scallops during the 2021 WCM program (4 cages used in total).

Biological measures taken during the recent WCM survey.

The recent trial deployment of scallops included the sampling of haemolymph and tissues from retrieved animals to provide material for a range of biological measures. The selection of measures was based on those used for the blue mussels deployed as part of the regular WCM program. A notable exception was the Stress on Stress test used on mussels to establish how long they could remain alive when removed from seawater. This is considered by some to be a measure of physiological condition

in blue mussels and that compromised individuals die significantly sooner than healthier animals. The length of this test often extends beyond 15 days. Scallops, as permanently submerged organisms, lack any physiological or behavioural process to resist this stress, with repeated 'clapping' of valves once removed from water typically leading to the rapid loss of their pallial water. All other tests carried out on blue mussels could be applied to the scallops. The sampling plan for scallops within the 2021 survey is set out in figure 3.3.

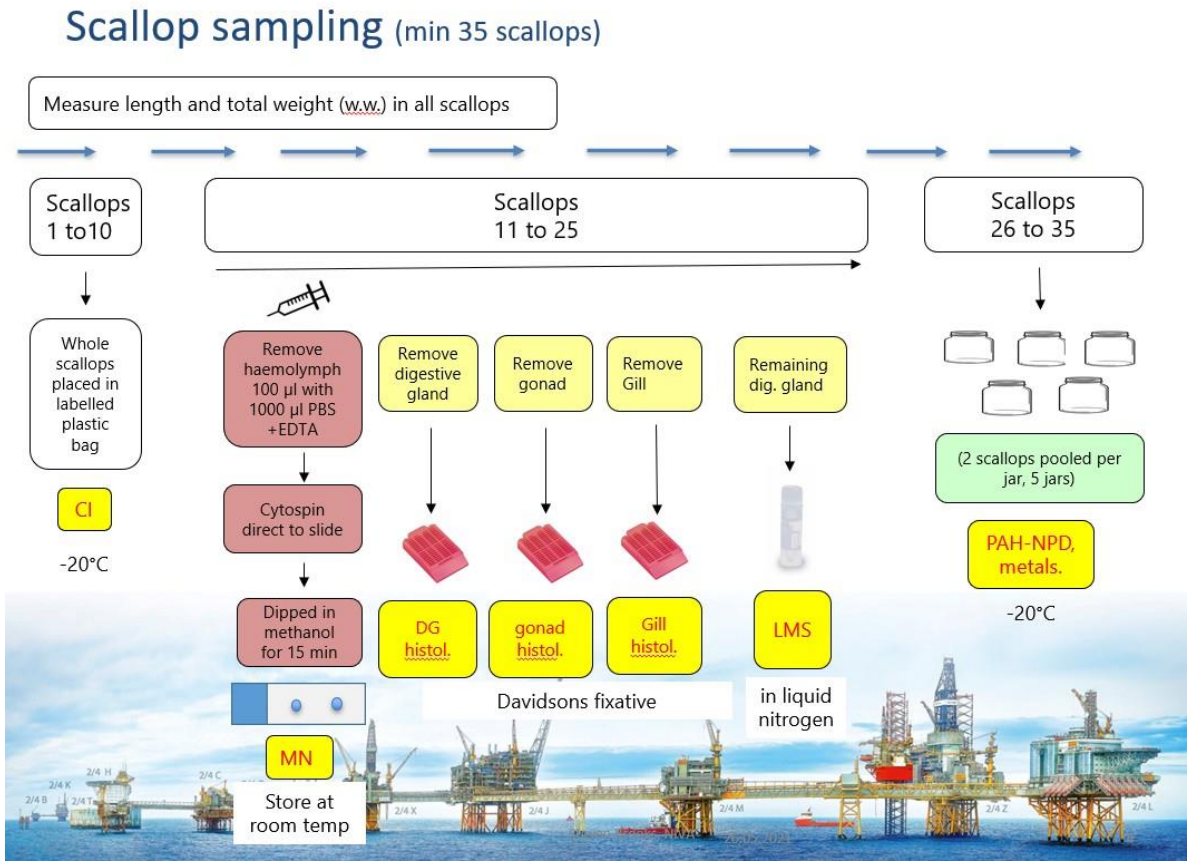

Fig 3.3
Schematic of samples taken from scallops during WCM program 2021
(Courtesy of Steven Brooks, NIVA)

Reducing the number of deployed scallops

Thirty-five scallops were used to complete the sampling program as set out in figure 3.3. (with 5 additional scallops deployed to cover for any mortality during the 6-week deployment). If this number could be reduced by adapting existing protocols without diminishing the quality of the information derived from these assessments, then the logistical challenges associated with sourcing, transporting and caging the scallops could also be reduced. This not only follows the 3 Rs principle adopted by many animal research authorities – Replacement, Reduction and refinement (information on the three Rs can be found at: https://norecopa.no) but also generates multiple strands of information from single individuals that taken together will allow greater insight into the interrelationships among its physiological processes in the event of a response to chemical exposure.

An advantage with large bivalves like the king scallop is that one individual can provide ample tissue for a variety of biological measures. Examining the sampling plan for scallops presented in figure 3.3 we see three distinct groups of animals. There is a group of 10 scallops used for condition index analysis, 15 scallops for haemolymph and histopathology samples and a final group of 10 for chemical accumulation analysis. Starting with this final group, with the volume of tissue available the accumulation analyses could manage with a single scallop rather than pooling two for each of the five replicates. Furthermore, the results will reflect the accumulation in each individual animal rather than a pool. This immediately reduces the number of scallops by 5. For condition index, which currently uses 10 dedicated scallops for this single assessment, there is an option that would further reduce the overall number of scallops required. The second group of 15 scallops used for haemolymph and tissue samples could provide several wet weight measures of various tissues to allow a condition index to be generated and remove the need for the additional scallops currently used for this purpose. In its simplest form, measurement of total weight of shell and contents after pallial water has been drained combined with weight of shell with all tissue removed, will provide a basis for a condition index comparison between sites. In addition, the large adductor muscle, not used for any other purpose in

the current sampling plan, could be carefully dissected out once all the remaining tissues have been sampled for histological analyses and either wet or dry weight (frozen on site and dried on return to the laboratory) used to establish a condition comparison among these animals. This would reduce the number of scallops required for the bio-measures to 20, plus perhaps 3 additional scallops to cover for mortality during deployment.

Suggested protocol for onboard sampling of scallops based on existing biomarker list

For each of the first 15 of the 20 scallops

1. *Measure and weigh scallop (wet weight with pallial water drained)*
2. *Withdraw haemolymph from adductor muscle/ pericardial sinus for MN analysis*
3. *Separate valves by carefully cutting adductor muscle*
4. *Remove gonads, record weight of entire tissue mass and process for histopathology*
5. *Remove digestive gland and divide to provide a section for histological sectioning and freeze the remainder in liquid nitrogen for subsequent LMS analysis*
6. *Remove gills for histopathological analysis*
7. *Dissect out all adductor muscle tissue and record wet weight and then store at -20°C*
8. *Place shell valves from each scallop into a labelled bag, seal it and store at -20°C*

For each of the 5 remaining scallops

1. *Measure and weigh scallop (wet weight with pallial water drained)*
2. *Separate valves by carefully cutting adductor muscle*
3. *Dissect out all adductor muscle tissue and record wet weight*
4. *Remove and then add all tissues, including adductor muscle, to glass sample jar and store at -20°C for later chemical analyses.*
5. *Place shell valves from each scallop into a labelled bag, seal it and store at -20°C*

Figure 3.4
Withdrawing haemolymph from a king scallop (*Pecten maximus*)

Additional biomarker assays using scallops that could be considered within the WCM program.

Previous research on these scallops, including exposures to PAHs and dispersed oil, provides a solid foundation for further development of biological effect measures. A review covering scallops and marine contaminants (Marsden & Cranford, 2016) includes reference to exposure to oil and gas operations. Laboratory studies using scallops have included various well established biomarkers associated with oxidative stress, acetylcholinesterase, immune function and other cellular responses (Baussant et al., 2009; Bocquene, 1997; Hannam et al., 2010 A; Hannam et al., 2010 B). Several of these biomarkers could readily be added to those already identified in the suite of measures carried out as part of the WCM for bivalves, though relevance to exposure to low concentrations of produced water much be a prerequisite for selection. The opportunity to develop new biomarkers is important, particularly when it is possible to utilize available sample material that does not demand additional animals. As an example, haemolymph from bivalves is currently used only for assessment of micronuclei in haemocytes and what remains could be used for additional measures such as phagocytosis potential or simple haemocyte cell diameter distribution and total haemocyte counts.

The selection of biomarkers to use on WCM surveys is regularly reviewed and a combination of the assessment of the performance of those assays already on the list combined with an awareness of new developments in the field of relevant and field feasible biomarkers (and the potential use of additional or replacement organisms) worthy of a trial, will serve to keep the WCM survey as an effective and worthwhile undertaking.

4. Flow cytometry methods for field investigations.

Introduction

Flow cytometer techniques are widely used to analysis cellular form and function in bivalve molluscs (Kim et al., 2020; Le Guernic et al., 2020; Lu et al., 2021). NORCE (then known as IRIS) investigated the use of this technique to support high throughput analysis of biomarker endpoints in blue mussels (*Mytilus edulis*). Part of this research program was to examine phagocytosis capacity in the mussels that included the development of a new protocol which ultimately proved to be reliable and reproducible. As part of this present project this assay was evaluated for its suitability for inclusion within the suite of biological measures in the Water Column Monitoring program.

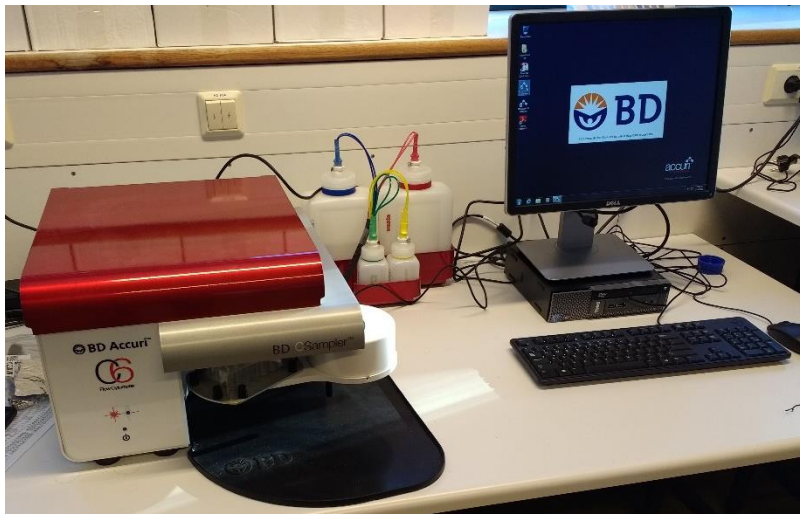

Figure 4.1
The Accuri C6
flow cytometer.

The flow cytometer used by NORCE, the Accuri C6 supplied by BD, is relatively small in size and unlike many larger systems it's various components are manufactured and fitted to allow portability of the unit for movement between laboratories or possibly into the field (figure 4.1).

The protocol for analysing phagocytosis in bivalve haemolymph cells developed at NORCE includes several stages and these are described in brief below. A full description of the method can be found in

(Bamber, 2018). The first stage allows cells to adhere to the walls of the sample tube. A fixed volume of haemolymph is mixed with physiological saline within a tube and kept chilled. After the incubation is complete the tube is rinsed with saline to remove unattached cells. The next step incubates these attached cells with a solution of fluorescent beads, allowing a fixed period of time to allow phagocytosis of these beads to take place. Beads not taken up by cells are then removed by gentle rinsing of the tube with saline. The final treatment occurs immediately prior to analysis on the flow cytometer where a trypsin solution is added to the tube to release the adhered cells. These cells are then processed through the flow cytometer where intensity of fluorescence from beads contained within each individual cell is detected and recorded. Cells that contain multiple beads produce greater fluorescence and it is the pattern of fluorescence intensity produced by each sample that indicates the degree of phagocytosis that has taken place within each sample. A representative plot is shown in figure 4.2 which shows fluorescence intensity from a sample containing mussel haemocytes showing five clear peaks that indicate cells with 1, 2, 3, 4 and five beads contained within. Phagocytosis is recorded both in terms of capacity and efficiency, with Capacity indicating the percentage of cells within the sample that contain 1 or more beads, and efficiency indicating the percentage of cells with 3 or more beads phagocytised.

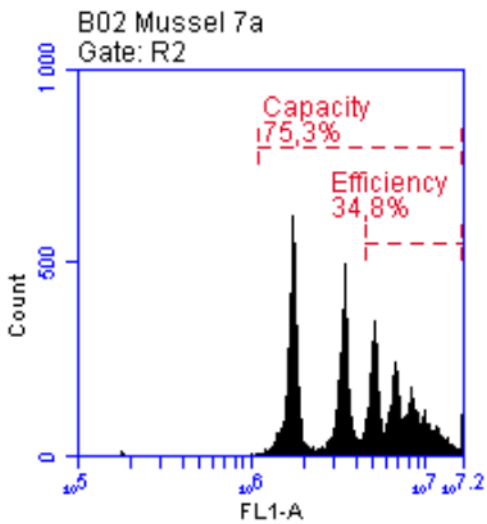

Figure 4.2
Plot showing phagocytosis activity within a sample of mussel haemocytes.

Phagocytosis activity

>=1 bead engulfed – *Capacity*
>=3 beads engulfed - E*fficiency*

Although the protocol has previously been shown to be effective with mussel haemocytes, the first element of the present study was to determine whether it would function with scallop haemocytes. The second element of the study was to consider how best to conduct flow cytometry for phagocytosis analyses on samples collected in the field in support of Water Column Monitoring activities.

Performance of scallop haemocytes within the existing phagocytosis flow cytometry protocol.

Haemolymph samples were taken from scallops and processed in an identical fashion as mussels. Clear fluorescent signals were detected in the five scallops sampled indicating that the haemocytes had adhered to the tube walls and were not lost during the wash procedures. They successfully phagocytised fluorescent beads and recorded expected patterns of fluorescence intensity following analysis on the flow cytometer (fig 4.3). It was possible to establish that scallops provided a generally comparable performance to mussels within the phagocytosis test. However, some differences were noted between the species. Cell count and dimension analyses using a Coulter counter showed that the cell count in the scallops was much higher than that typically seen in blue mussels and the average cell diameter within the main peak of the cells was smaller (Table 1).

Scallop	Cells per ml x 10^6	Mean diameter of largest peak µm	Mussel	Cells per ml x 10^6	Mean diameter of largest peak µm
1	5.7	9.03	1	1.9	11.08
2	20.3	9.14	2	6.0	11.73
3	14.7	9.03	3	1.8	11.21
4	8.0	8.9	4	1.6	10.71
5	2.7	9.14	5	1.9	10.47
Average	10.3	9.05	**Average**	2.6	11.04
StDev	7.2	0.1	**StDev**	1.8	0.48

Table 1. Total haemocyte counts (6-20 µm particles) and average diameters of haemocytes in the largest peak within each sample of scallop and mussel haemolymph.

Even though the cell count was much higher in the scallops there appeared to be fewer cells actively phagocytosing the fluorescent beads and those that did, engulfed fewer beads than the level typically seen in mussels (figure 4.3)

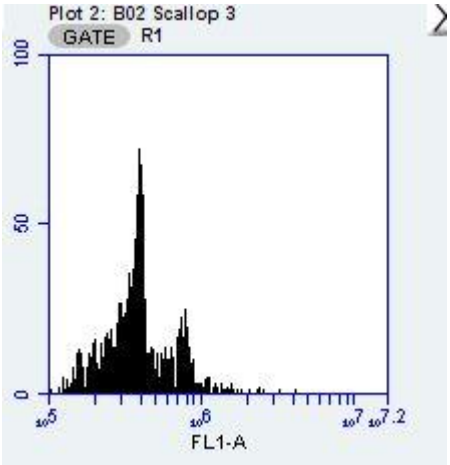

Figure 4.3
Plot of scallop haemocyte sample showing peaks of fluorescence corresponding to bead uptake.

There was also greater variation observed in total cell count in the scallops. The limited scope of the present study prevented further examination of these findings but future work in this area would improve our knowledge on the observed discrepancies between the species. Sampling haemolymph from scallops is a little more challenging than in mussels. Their valves are quite active when taken from water, so exerting control whilst extracting haemolymph from the large adductor muscle does take some practice. Blue mussels seek to close their valves when taken from water and a gap between them can be created by carefully inserting a wedge between them (a sharp, thick, bevelled knife blade works well), and this provides a stable platform for inserting the needle and extracting haemolymph in a controlled manner. The random movements from the scallops can be controlled to some extent by inserting a wedge between the valves, as with the mussels, with the addition of an elastic band to supress gaping activity. Haemolymph is taken from the large adductor muscle from the frontal dorsal area. Care must be taken to avoid piercing surrounding tissues as this contaminates the sample. This is particularly the case when sperm from gonad tissue is accidentally taken – with large numbers of

small cells appearing during cell counts. An example of this is shown in figure 4.4 where a plot of cell count and cell size distribution from five scallop haemolymph samples is shown. The main peaks of interest shows haemocytes at around 9 µm diameter, but two of the samples shown here contain large numbers of small particles. It is best to discard haemolymph samples that appear cloudy and try again with a fresh syringe and needle.

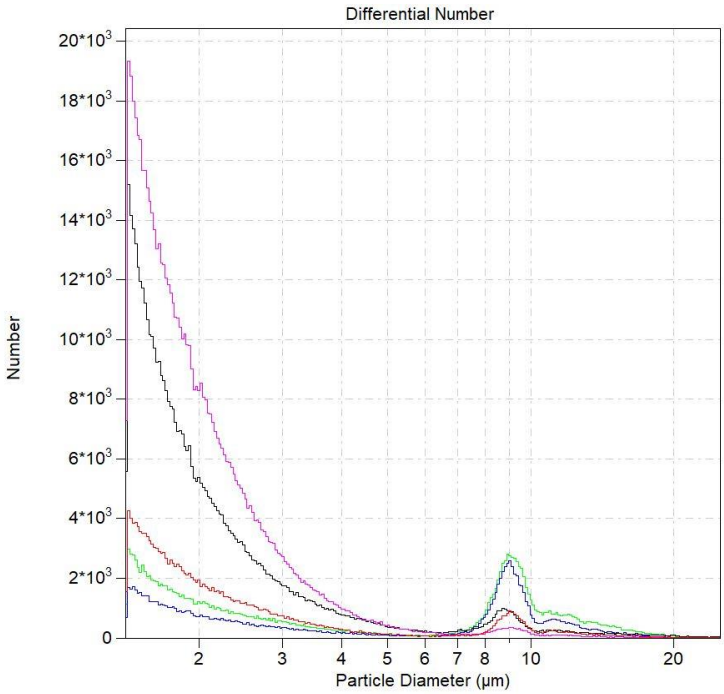

Figure 4.4
Plot of particle size distribution in 5 scallop haemolymph samples.

Conducting sampling and sample preparation for phagocytosis analyses in the field.

There are several considerations to be made concerning how best to measure biological endpoints using flow cytometry within the water column programme.

1. How many samples will require processing within a given time window? Are all sites sampled or just a selected few?
2. How secure is the operational area for the flow cytometer and its workstation? Although built for mobility the marine atmosphere and a potentially unstable foundation platform may cause damage to the machine.

3. Is there a way to securely partially process samples on board and preserve them for later analyses in the laboratory?

While the portable nature of the flow cytometer does allow for its use in the field and onboard ship, the time required to complete the current protocol limits the number of animals that could be processed over 12 hours to approximately 36 (mussels or scallops). With the recent increase in the number of deployed bivalves (two depths) and the possibility of additional sites added to the WCM program, a more feasible approach may be to adapt and carry out those steps in the protocol that precede the flow cytometer and then preserve the cells for later analyses on the flow cytometer on their return to the laboratory. This allows greater flexibility in sample preparation that can offset potential disruptive elements during research cruises such as an uneven timeline in recovery of sample organisms or poor weather conditions. An important goal for any new method for WCM procedures is to make it as feasible and reliable as possible and to enable its incorporation into the process flow of existing sampling techniques. The protocol for phagocytosis analysis follows a sequence of incubations of the haemolymph cells as described above. It should be possible for this procedure to be followed to the stage of post bead incubation washing, with the attached cells then killed, preserved and stored, prior to the completion of the final steps of the protocol back in the laboratory. The key process for this to be successful is the preservation of the adhered cells.

The majority of effort in this section of the project therefore was to identify a process sequence to allow field collected samples of scallop/mussel haemolymph to be prepared and preserved so that phagocytosis and other cell parameters can be measured on return to the laboratory. Starting with practical considerations it was decided to use mussel haemolymph in the development steps as there was a plentiful supply of these in the laboratory. The prior observation that scallop haemocytes behave in a similar manner to those taken from mussels allowed the use of the later as a surrogate for the development of these subsequent stages.

The initial tasks involved the choice of test vessel suitable for ship-board handling, together with the type of fixative to preserve cells in good physical condition prior to their subsequent analysis. The current spotlight on the health risks of formalin, a component of preservatives used for many years, has driven the search for an effective replacement. A fixative is essential to hold the partially processed samples in stasis until they can be transported back to laboratory. The demands on the fixative are to maintain cellular form and to allow continued adhesion of the cells to the tube wall. The formulation of this fixative and the effects on treated cells was an important focus for the research presented here. Standard cryovials with screw top lids were tested and proved to be effective for cell adhesion and general handling of samples. The choice of fixative created more of a challenge and further work beyond this project will be required to address this issue fully. FineFix is a commercial histological fixative which is combined with ethanol to generate a working solution. Several trials were run with varying concentrations of this working solution with storage periods of up to 10 days. When samples from an initial trial using a range of relatively high concentrations of the fixative solution were assessed on a multisizer (Coulter counter) it was evident that the cells had been destroyed. Further trials using lower concentrations of the FineFix working solution in combination with various carriers such as physiological saline and Alsever's solution were then attempted. Alsever's solution is a commercial isotonic balanced salt solution routinely used as an anticoagulant and blood preservative for mammalian blood that permits the storage of whole blood at specified storage conditions under refrigeration for up to 10 weeks. The basis for all the preservation tests conducted was to compare cell counts and dimensions in a series of haemolymph preparations based on single samples taken from individual mussels. In this manner it was possible to compare the haemocyte preparations against a benchmark sample processed immediately after haemolymph was taken. A series of preliminary assessments of fixative solutions culminated in a final trial where the preserved samples were stored for 7 days prior to processing through the Multisizer.

The following treatments were tested in this final trial:

Approximately 500 µl of haemolymph was taken from each of five mussels. The haemolymph from each sample was divided among four treatments in the manner set out below (FF = FineFix working solution).

A. Immediate processing - 100 µl haemolymph in 300 µl mussel physiological saline.
B. 100 µl haemolymph in 300 µl Alsever's solution with 1% FF/ethanol working solution.
C. 100 µl haemolymph in 300 µl mussel physiological saline with 1% FF/ethanol working solution.
D. 100 µl haemolymph in 300 µl Alsever's solution alone.

300 µl of each of these treatments was then added to 20 ml of filtered seawater prior to the analysis of a 1 ml volume of these treatments on the multisizer.

Treatments B, C, and D were processed 7 days after preparation, during which time they were kept under refrigeration at 8 °C.

The results from the multisizer analyses are shown in figure 4.5.

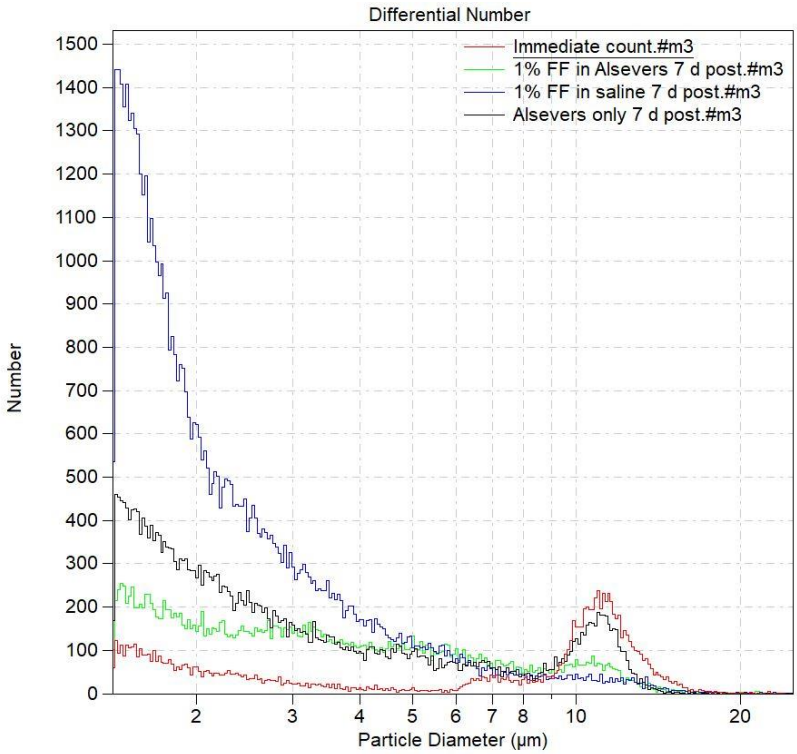

Figure 4.5
Plot taken from the multisizer showing cell counts and dimensions following the treatments described in the legend.

The immediate assessment of the haemolymph sample (red line) shows a typical plot shape, with the presence of some smaller particles, with a subsequent peak in particle counts at around 11 µm, the expected haemocyte diameter. The blue line plots the result from treatment with 1% Finefix working

solution combined with physiological saline. It shows a large increase in the proportion of small particles and a marked reduction in particles at the diameter where one would expect to see cells. This likely indicates the breakdown of the majority of the cells over the 7-day period. The green line plots the result of combining Finefix with Alsever's solution and indicates a more moderate increase in small particles together with an increase in particle count around the 11µm region, though still significantly lower than the benchmark count. The final treatment (black line) plots the effect of storing the cells in Alsever's solution alone. Here we once again see an increase in smaller particles compared with the haemolymph processed immediately following sampling, but we also see a defined peak in the 11µm size range indicating that cellular integrity has been maintained in many of the cells. It is certainly clear that Alsever's solution provides the best preservation within the set of treatments tested. It is also clear that Finefix, typically used for tissue preservation, is not effective at preserving haemocytes.

Conclusions from the flow cytometer study

- The onboard use of the flow cytometer and associated equipment during water column monitoring operations cannot currently be considered as feasible. This is based both on the sensitivity of equipment to a marine atmosphere and potentially unstable conditions, together with space and time demands for its use within a very busy sampling schedule.
- Processing of samples through the steps of the protocol prior to the flow cytometry stage in the field and preserving the cells for transport back to the laboratory for the final stages of analysis provides an alternative route.
- Cryovials proved to be a secure substrate for cell adhesion and subsequent steps in the phagocytosis protocol.
- Progress was made in identifying a suitable effective preserving solution that maintains cellular structure and contents over some days, but further development work is required.

- The possibility remains that adjustment to various steps within the phagocytosis protocol to better suit high intensity field work, such as shortening or possibly combining incubation stages, can be made to ease its incorporation into the already busy sampling schedule of the WCM program.

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