European Maritime Safety Agency - EMSA

RESEARCH STUDY

TESTING SAMPLE REPRESENTATIVENESS OF A BALLAST WATER DISCHARGE AND DEVELOPING METHODS FOR INDICATIVE ANALYSIS

REPORT No. 4

FINAL REPORT

Title:

Testing Sample Representativeness of a Ballast Water Discharge and developing methods for Indicative Analysis

Tender:

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GLOSSARY

ATP	Adenosine triphosphate
BWM	Ballast Water Management
BWTS	Ballast Water Treatment System
Chl *a*	Chlorophyll *a*
DNA	Deoxyribonucleic acid
DWT	Dead Weight Tonnage
EMSA	European Maritime Safety Agency
NM	Nautical Miles
IMO	International Maritime Organization
PAM	Pulse-Amplitude Modulated fluorometry
RNA	Ribonucleic acid

EXECUTIVE SUMMARY

The objectives of this project included to find methods to overcome one of the biggest hurdles in sampling for enforcement of the International Convention for the Control and Management of Ships' Ballast Water and Sediments2004 (BWM Convention) that is facing a Maritime Administration: the issue of how to take a sample being representative of the whole ballast water discharge. Secondly, the question was also how to conduct an indicative analysis of ballast water. The project was organised in two parts aiming for

- Part 1: To develop a sampling protocol that obtains a representative sample of the whole discharged ballast water; and
- Part 2: To develop methods for indicative analysis/sampling that provides "clear grounds" for stopping a discharge and/or enforcement action.

Two vessel voyages were undertaken to meet the objectives. Furthermore, the tenderers undertook a comprehensive Internet research on possible methods for indicative analysis of ballast water samples), and tested selected methods on-board during these voyages.

Part 1 Sample representativeness

During all the tests undertaken for this part of the contract, the water was sampled during uptake and discharge. The water flow being sampled was split into two flows. One used to take samples over the entire pumping event and the second to take three random samples.

The results show that different approaches in the sampling process influence the results regarding organism concentrations. The organisms in the discharge are affected in different ways, therefore the selection of the "wrong" sampling approach may influence the compliance control result. The organism concentrations in the ballast water discharge may therefore be underestimated, and a "faulty" ballast water treatment system (BWTS) could be recognised as compliant. Conversely organism concentrations may be overestimated, and a BWTS complying with the D-2 Standard may fail in compliance tests.

It should be noted that a certain level of pragmatism is required during on-board ballast water compliance control sampling especially when larger volumes of water need to be sampled. This is especially relevant to sample for bigger organisms, and attempts should be made to avoid negatively impairing organism survival during the sampling process. Compliance control sampling teams are unlikely to have large water collecting tanks (>1000 litres) available during the sampling event and will probably need to work with nets to concentrate the sample during the sampling procedure.

During this study it was observed that sampling duration (i.e., length of the sampling process), timing (i.e., in which point in time of the discharge the sampling is conducted), the number of samples and the sampled water quantity are the main factors that influence the results regarding organism concentrations.

Recommended sampling duration

The results show that bigger organisms are negatively affected by longer sampling times. Considering that the results show that a shorter sampling time is still representative, the recommended sampling time of a sample taken during the tests in a sequential sampling is

approximately 10 minutes. Longer sampling times result in an underestimation of the viable organism concentration in the discharge, especially for bigger organisms.

Recommended sampling timing

The results also show that organism concentrations may vary considerably if the sampling is conducted at the very beginning or at the very end of the discharge process because of the patchy distribution of organism inside ballast water tanks. It is not recommended to take a sample at the very beginning (i.e., the first 5 min) or at the very end of the discharge (i.e., the last 5 min), as an underestimation as well as an overestimation of organism concentrations may be expected. Based on this it is recommended that the sampling is conducted randomly anytime in the middle of the discharge, starting after 5 minutes from the start of discharge and ending 5 minutes before the end of the discharge.

Recommended number of samples

Organism concentrations in all organism groups vary due to the patchy distribution of organisms inside the ballast water tanks, hence a single 10 minutes sequential sample may underestimate or overestimate the concentration of organisms being discharged. The results also show that an average of organism concentrations from 2 random samples from sequential sampling provides very similar results to the average of the 3 random samples. Based on this it is recommended that sampling is conducted by undertaking at least 2 random samples, which are analysed immediately after each sampling event has ended, and that the organism concentration results are averaged.

Recommended sampled quantity

In this study sequential sampling was conducted over periods of 10 and 15 minutes with the flow rate averages ranging mainly between 30 and 45 litres per minute. To obtain the most representative results it is recommended that:

- for the bigger organisms 300 to 450 litres should be filtered and concentrated;
- for the smaller organisms a "continuous drip" sample totalling to approximately 5 litres (i.e., collect approximately 0,5 litre of sample water every minute during the entire sampling time duration, or collect about 0.5 litre of sample water every 30 to 45 litres sampled, depending on the flow rate) should be taken. The resulting 5 litres of sample water should be sub-sampled after mixing in two sets of samples, one alive and another preserved. We recommend sub-sample volumes of 60 to 100 ml;
- for the bacteria, a sample of approximately 1 litre should be taken as a sub-sample after mixing from the 5 litre "continuous drip" sample.

Other recommendations

It is also assumed that the sampling flow rates may influence the results. Lower flow rates obtained by partially closed valves of the sampling line may damage organisms, and a similar negative effect may be caused by to strong flow rates affecting mainly the filtering process of the bigger organisms. Hence, the flow rate, or “valve” effect, may cause an underestimation of the organism concentration as organisms may die during the sampling process. To avoid this negative influence it is recommended that the valve at the sampling point is opened as much as possible, however it should not exceed the flow rate of 50 litres/min, so that the water pressure is not too high during sample concentration, as this may impair organism survival.

Sampling logistics feasibility

Different types, sizes and cargo profiles of vessels trigger very different ballast water discharge profiles and times. Ballast water discharge may be conducted "at once" or "in sequence", lasting from approximately one hour (e.g., fast discharge of two tanks in parallel on e.g. container vessels), up to several days depending on the length of the cargo operation (e.g., tankers, bulk carriers and sometimes general cargo vessels load cargo during several days, hence the ballast water operation is frequently conducted in sequence over the time of cargo operation).

It is important to take this factor into account as it is difficult to imagine that the PSC officer and/or sampling team would stay on-board the vessel for several days. Considering the above recommendations on representative sampling, sampling of at least 2 random samples is feasible and is relatively easy, while sampling over the entire time of the ballast water discharge would be very difficult if long sampling times are required over several days or during night time (i.e., cargo operations are regularly conducted also in night shifts, but PSC officers may only be available at day shifts).

The challenge may become to obtain a representative sample of the whole discharge, when the vessel will be discharging ballast water from **more than one ballast water uptake location**. In such cases it is **recommended** that at least **1 sequential sample per uptake source** is taken. If a tank was filled from multiple sources this does not trigger necessity for 2 or more samples

Part 2 Methods for indicative sample analysis

Various methods for indicative analysis of the three organism groups of the Ballast Water Performance Standard of Regulation D-2 of the BWM Convention were considered. In total 8 methods for phytoplankton, 6 methods for zooplankton and 11 methods for bacteria were evaluated for their use in indicative ballast water sample analysis. The pros and cons of the methods selected are presented in Chapter 5 with a summary in Chapter 5.12.

For a ballast water sample to be analysed, certainly, as a very first step, sampling needs to be conducted. The ballast water sampling guideline does not address explicitly how indicative sampling would need to be undertaken. Implicitly, indicative analyses could be conducted on a sample, or on a part of a sample, taken during the complete D-2 compliance control sampling process, or just on a stand-alone sample.

It is important to understand that indicative sampling may be focussed only on one group of organisms (i.e., smaller and bigger organisms or bacteria). While results from each of these organism groups may give an indication that a BWTS is not performing properly, from our experience of on-board sampling, it easily can happen that, e.g., bacteria and smaller organisms would be within acceptable limits, however bigger organisms may be in too high concentrations to meet the D-2 Standard, or vice versa.

Different groups of organisms in general require different sampling approaches (e.g., in general bigger organisms require bigger water quantities to be sampled than when focussing on smaller organisms), as there are relatively lower concentrations of bigger organisms in the water than the smaller ones. Therefore, indicative sampling methods may be very different for each organism group, differing in e.g. sample duration, timing, volume, and at which sampling point it was taken.

It would be very difficult to predict in advance which group of organisms to focus on to identify possible non-compliance with the D-2 standard, as this would require a risk assessment conducted in advance. Hence, from this perspective it would be most helpful to use a sampling method which would allow conducting analyses on all organism groups. This would also offer a step-by-step process, where one analyses method may be applied first. If this shows some indication or even does not give an indication of non-compliance, another sample analysis method can be applied (e.g., start with the fastest available analysis method, and proceed with the next available method).

Nevertheless, noting all the above and after the tests and analyses conducted during this study, supported by experience and results from previous voyages, we recommend that for indicative ballast water sampling, one sequential sample is taken using the same sampling methodology as for a full D-2 compliance test (as described in subchapters 4.5.1.1, 4.5.1.2, 4.5.1.4 and 4.5.1.5).

When taking one sequential sample, the sampling time is short and the sample analysis could be conducted with a range of different methods. The results obtained from this approach can also represent very solid grounds for different actions PSC may have available in case of indicated non-compliance with the D-2 standard, e.g. (a) from an indication that more tests are needed and to proceed to complete full compliance D-2 tests, (b) to send a vessel to a designated ballast water discharge area, (c) require to discharge the ballast water in a port reception facility, or even (d) to ban a ship from further ballast water discharge, all depending on the result obtained. For instance, if the concentration of organisms identified is just above the D-2 standard, this would be an indication possibly requiring further tests. However if much higher concentrations of organisms than the D-2 standard are identified, a ship may be banned from continuing the ballast water discharge.

We also believe that in certain occasions it may be required not to take a sample from the ballast water discharge line as G2 recommends. This can only be done while the ballast water is pumped overboard. Should a vessel carry ballast water from areas known to contain outbreaks, infestations, or populations of Harmful Aquatic Organisms and Pathogens (e.g., toxic algal blooms) sampling ballast water from the discharge line while being discharged should be avoided. Should non-compliance be proven in such a case the water may have already been pumped overboard and pose a risk to the environment, human health, property or resources. Instead we recommend that in such cases an indicative ballast water sample is taken directly from the ballast water tank prior to discharge. Although such sampling methods may not be representative of the whole discharge an indicative compliance control analysis is enabled without discharging the ballast water.

1 INTRODUCTION

The results and findings presented in this report summarize the work undertaken on two vessel voyages and a desk study on ballast water sampling methods. As per the ballast water management plan of the vessel the ballast water operation is only to be started when the ballast water treatment system (BWTS) is fully operational. Therefore, even if a period of time is needed to "warm up" the BWTS to reach its most efficient working level the BWTS will only be operated when this working level is reached.

As per the tender agreement with EMSA three tests were undertaken on voyage one. One treated ballast water test was undertaken including sampling prior and after the Mahle Industriefiltration GmbH ballast water treatment system, which had been installed on this vessel, and during discharge of the treated water. Furthermore, two tests were undertaken on untreated ballast water, i.e. one test with an uptake in organism rich waters in the Port of Leixoes (Portugal) followed by a one run at sea with lower organism concentrations.

On the second voyage, in agreement with EMSA, two tests were carried out on untreated ballast water with uptakes in the Port of Karlshamn (Sweden) and Terneuzen (The Netherlands). As noted from the tests during voyage one the sampling results for the treated water tests do not permit any conclusions to be made on representative ballast water sampling (see relevant result chapter), therefore on voyage two tests were only undertaken on untreated ballast water.

During all the tests undertaken for this part of the contract, the water was sampled during uptake and discharge. The water flow being sampled was split into two flows. One used to take samples over the entire pumping event and the second to take three samples in a random sequence.

The results from our review of indicative ballast water sample processing methods are presented in a separate chapter.

2 MATERIALS AND METHODS

It was agreed that the voyages are to be undertaken on vessels which have ballast water treatment systems (BWTS) installed. **Voyage one** was undertaken on a container vessel from 24th to 29th July 2010 between the Port of Leixoes (Portugal) and Algeciras (Spain).

A test on treated water was only carried out during voyage one and the treatment system tested is described below (Mahle system). As shown by the results from the first voyage, tests involving a BWTS do not enable comparisons of ballast water sampling methods regarding the representativeness of sampling methods, because after the treatment process is completed, no living organisms where found in the ballast water discharge. Having experienced this situation EMSA was contacted and it was agreed that on the second voyage tests should be continued only on untreated water.

The tests on voyage two were also undertaken on a vessel which has a treatment system installed, but only untreated water was sampled. The reason for selecting a vessel with a treatment system for voyage two was that otherwise no suitable sampling points to enable the test would have been available.

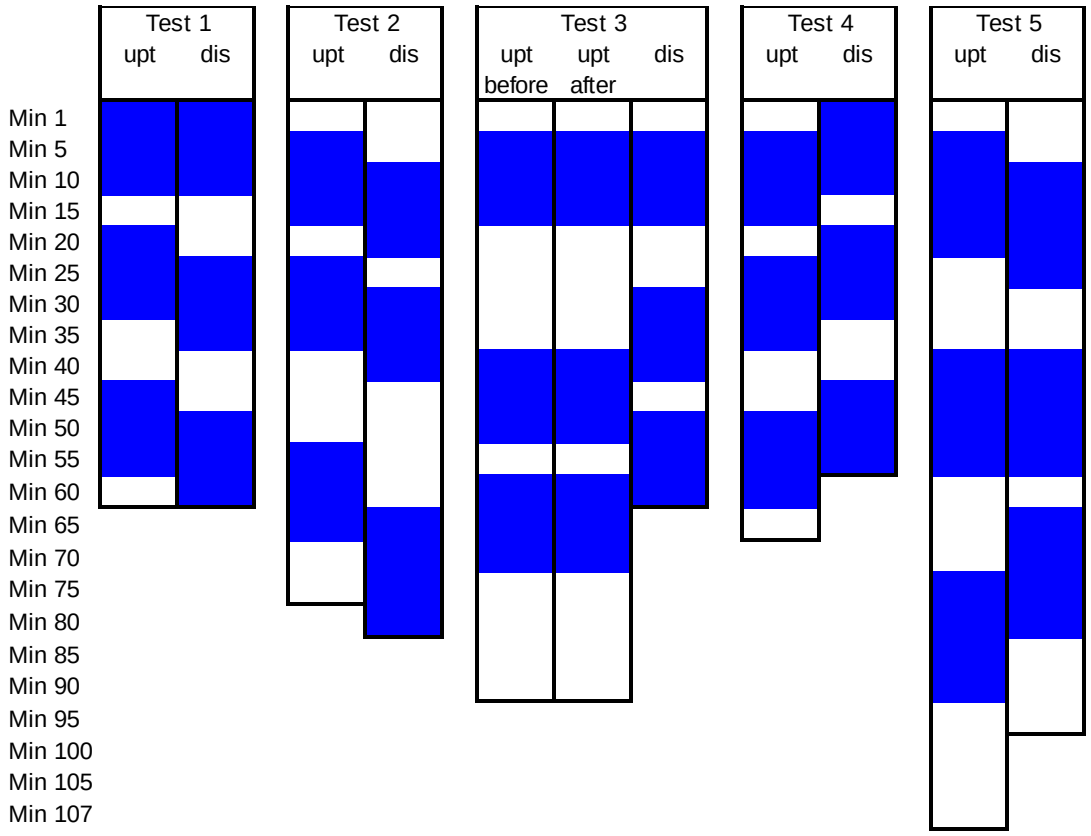
Voyage two took place on the bulker *Timbus* with the Aquaworx BWTS leaving the Port of Karlshamn (Sweden) on 26th September 2010 sailing to Terneuzen (Netherlands), and returning to the Port of Karlshamn on 2nd October 2010.

Some basic details about the sampling events during both voyages are presented in the Table 1 and Table 2.

Table 1 Samples taken during both voyages also show the start and end time of each sampling event with duration and the water quantity sampled.

Sample No.	Test No.	Date	Sample type	Start time [h:min]	End time [h:min]	Sampling time [h:min]	Time between sequences [h:min]	Quantity sampled (litre)
			VOYAGE 1					
1	1	24.07.10	uptake, untreated, sequence 1	16:05	16:15	0:10	0:00	450
2	1	24.07.10	uptake, untreated, sequence 2	16:25	16:35	0:10	0:10	450
3	1	24.07.10	uptake, untreated, sequence 3	16:50	17:00	0:10	0:15	450
4	1	24.07.10	uptake, untreated, entire uptake	16:05	17:05	1:00		2.595
5	1	25.07.10	discharge, untreated,sequence 1	16:40	16:50	0:10	0:00	450
6	1	25.07.10	discharge, untreated,sequence 2	17:05	17:15	0:10	0:15	450
7	1	25.07.10	discharge, untreated,sequence 3	17:30	17:40	0:10	0:15	450
8	1	25.07.10	discharge, untreated, entire discharge	16:40	17:40	1:00		2.869
9	2	28.07.10	uptake, untreated, sequence 1	08:50	09:00	0:10	0:05	300
10	2	28.07.10	uptake, untreated, sequence 2	09:10	09:20	0:10	0:10	300
11	2	28.07.10	uptake, untreated, sequence 3	09:40	09:50	0:10	0:20	300
12	2	28.07.10	uptake, untreated, entire uptake	08:45	10:00	1:15		2.324
13	3	28.07.10	uptake, before treatment, sequence 1	15:10	15:20	0:10	0:05	380
14	3	28.07.10	uptake, before treatment, sequence 2	15:45	15:55	0:10	0:25	380
15	3	28.07.10	uptake, before treatment, sequence 3	16:05	16:15	0:10	0:10	380
16	3	28.07.10	uptake, before treatment, entire uptake	15:05	16:35	1:30		3.287
17	3	28.07.10	uptake, after treatment, sequence 1	15:10	15:20	0:10	0:05	374
18	3	28.07.10	uptake, after treatment, sequence 2	15:45	15:55	0:10	0:25	364
19	3	28.07.10	uptake, after treatment, sequence 3	16:05	16:15	0:10	0:10	359
20	3	28.07.10	uptake, after treatment, entire uptake	15:05	16:35	1:30		2.717
21	2	29.07.10	discharge, untreated,sequence 1	08:55	09:05	0:10	0:10	380
22	2	29.07.10	discharge, untreated,sequence 2	09:12	09:22	0:10	0:07	380
23	2	29.07.10	discharge, untreated,sequence 3	09:50	10:05	0:15	0:28	540
24	2	29.07.10	discharge, untreated, entire discharge	08:45	10:05	1:20		2.562
25	3	29.07.10	discharge, after treatment, sequence 1	11:20	11:30	0:10	0:05	380
26	3	29.07.10	discharge, after treatment, sequence 2	11:45	11:55	0:10	0:15	380
27	3	29.07.10	discharge, after treatment, sequence 3	12:05	12:18	0:13	0:10	493
28	3	29.07.10	discharge, after treatment, entire discharge	11:15	12:18	1:03		1.924
			VOYAGE 2					
29	4	26.09.10	uptake, untreated, sequence 1	10:02	10:12	0:10	0:04	350
30	4	26.09.10	uptake, untreated, sequence 2	10:25	10:35	0:10	0:13	350
31	4	26.09.10	uptake, untreated, sequence 3	10:54	11:04	0:10	0:19	350
32	4	26.09.10	uptake, untreated, entire uptake	9:58	11:05	1:07		2.381
33	4	27.09.10	discharge, untreated,sequence 1	10:43	10:53	0:10	0:00	350
34	4	27.09.10	discharge, untreated,sequence 2	11:03	11:13	0:10	0:10	350
35	4	27.09.10	discharge, untreated,sequence 3	11:29	11:38	0:09	0:16	292
36	4	27.09.10	discharge, untreated, entire discharge	10:43	11:38	0:55		1.763
37	5	29.09.10	uptake, untreated, sequence 1	16:45	17:00	0:15	0:03	450
38	5	29.09.10	uptake, untreated, sequence 2	17:21	17:36	0:15	0:21	450
39	5	29.09.10	uptake, untreated, sequence 3	17:59	18:14	0:15	0:23	450
40	5	29.09.10	uptake, untreated, entire uptake	16:42	18:29	1:47		3.243
41	5	01.10.10	discharge, untreated,sequence 1	15:51	16:06	0:15	0:10	450
42	5	01.10.10	discharge, untreated,sequence 2	16:16	16:31	0:15	0:10	450
43	5	01.10.10	discharge, untreated,sequence 3	16:42	16:57	0:15	0:11	450
44	5	01.10.10	discharge, untreated, entire discharge	15:41	17:17	1:36		3.105

Table 2 Sampling events during both voyages also showing the randomness of sampling events (sequential samples in blue shading, the samples taken over the entire time with bold frame). Upt = uptake, dis = discharge, before and after indicates the samples taken before and after the Mahle treatment system during test 3.



2.1 VOYAGE 1

Voyage one was undertaken in July 2010 between Portugal and Spain. The ballast water sampling programme on this voyage is shown in Table 3.

Table 3 Location of ballast water sampling events and sample type during voyage 1.

Date	Test run number	Location	Sample type
24.07.2010	1	Port of Leixoes	uptake, untreated
25.07.2010	1	Port of Lisbon	discharge, untreated
28.07.2010	2	at sea	uptake, untreated
29.07.2010	2	Port of Algeciras	discharge, untreated
28.07.2010	3	at sea	uptake, treated
29.07.2010	3	Port of Algeciras	discharge, treated

The tests were conducted on a container vessel which has the Mahle Industriefiltration GmbH BWTS installed on-board. The vessel and test tanks specifics are shown in Table 4.

Table 4 Main dimensions of the vessel and tank details of voyage 1.

Details	Voyage 1
Vessel type	Container
Length overall	16226 m
Dead Weight Tonnage (DWT)	21825 t
Container capacity	1169 TEU
Total ballast water capacity	8527 m³
Number of ballast tanks	19
Number of ballast pumps	2
Capacity of each ballast pump	220 t/h
Number of BWTS installed	1
Capacity of BWTS	250 m³/h
Untreated test tank	No.7, side tank, starboard
Tank capacity	257 m³
Treated test tank	No. 7, side tank, portside
Tank capacity	257 m³

2.2 BALLAST WATER TREATMENT SYSTEM

2.2.1 DESCRIPTION OF THE MAHLE INDUSTRIEFILTRATION GMBH BALLAST WATER TREATMENT SYSTEM OPS (OCEAN PROTECTION SYSTEM)

The following description of the Mahle Industriefiltration GmbH BWTS Ocean Protection System (OPS) is based upon a document provided by the manufacturer Mahle (Mahle 2010).

The fully automated OPS BWTS was developed by the manufacturer Mahle Industriefiltration GmbH, Hamburg, Germany. This three-step BWTS works as an in-line system during uptake and discharge of ballast water. During uptake the first treatment step is filtration which extracts particles and organisms bigger than 200 micrometres, followed by a second filtration step to remove particles and organism bigger than 50 micrometres. The filters are self-cleaning. During ballast water intake the treatment is completed with a disinfection process using UV-light (Figure 1).

Figure 1 Visualization of a OPS-250 skid-mounted Unit for 250 m^3/h (Photo courtesy of Mahle).

Figure 2 shows the operation principle of the OPS BWTS. During discharge only the UV-disinfection component is in operation, while the other components are bypassed.

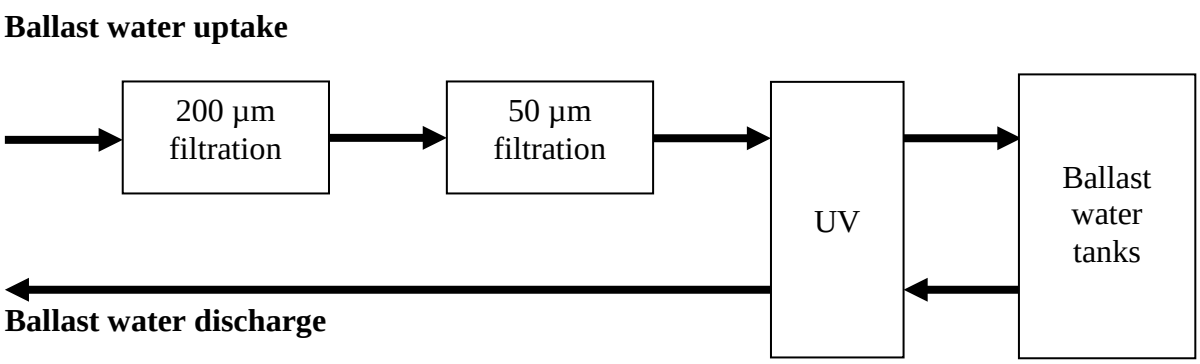


Figure 2 Simplified operation principle of the OPS.

2.2.2 DESCRIPTION OF THE SYSTEM

Filtration in combination with UV disinfection is considered a purely physical process, i.e. the OPS system does not make use of active substances.

2.2.2.1 Automatic 200 micrometre filter

Filtration takes place from the inside to the outside. The cleaned medium leaves the filter through the radially-mounted outlet flange. In the upper part of the housing, opposite from the inlet opening, is an axially slidable disc that is moved up and down during the reversible flow, with the help of a pneumatic cylinder within the filter element. The flushing mode for filter cleaning is initiated by differential pressure.

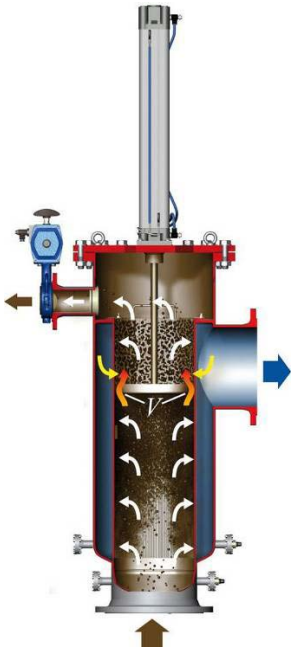


Figure 3 Filter operating principle (Photo courtesy of Mahle).

2.2.2.2 Automatic 50 micrometre filter

The filter is an automatic self-cleaning fine filter designed specifically for a ballast water treatment application. The filter is equipped with specially designed nuzzles which enables close proximity to the screen during the cleaning suction operation. The device is hydraulically operated during the cleaning cycle ensuring maximum suction forces to clean the filter screen. The filter uses minimal amounts of water for the cleaning process resulting in extremely low "wasted water" during the filtration process.

The fine screen filter is made of sintered, multi-layered stainless steel and does not require support. The filtering process creates a "cake" of sediment on the fine screen surface. As this sediment layer accumulates the filtration efficiency is enhanced. The filtration process creates a differential pressure across the screen which increases as the "cake" accumulates, until a predetermined value is reached (usually 0.5 bar) to activate the cleaning process. However, the self-cleaning process may also be activated by using a timer.

During filter cleaning the suction nozzles move across the entire screen surface while cleaning the filter screen. Should the operating pressures be low resulting in generated suction forces not reaching the required minimum level to trigger self-cleaning, a small suction pump is added to the flush line to assist the cleaning process. This is a reliable and inexpensive addition to the system, enabling the filter to operate under extremely low pressures of only 1.2 bar. The automatic self-cleaning screen filter is shown in Figure 4.

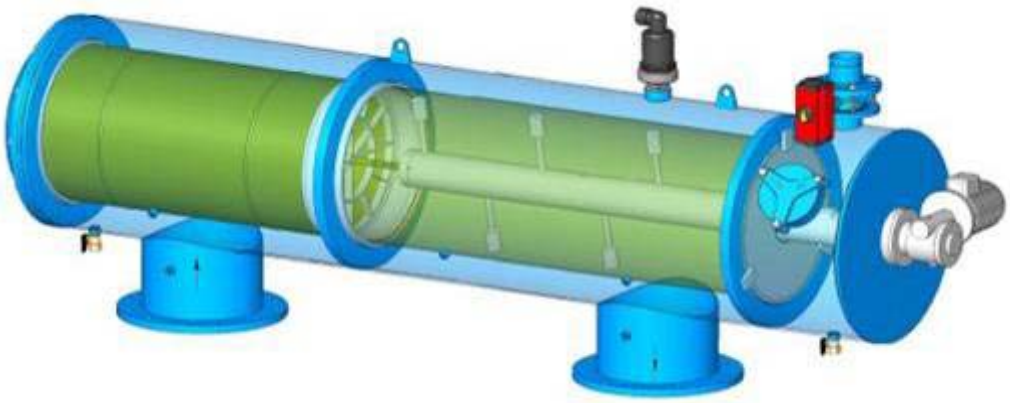


Figure 4 Automatic self-cleaning screen filter (Photo courtesy of Mahle).

2.2.2.3 UV-disinfection

Ultraviolet light is a natural component of the electromagnetic spectrum with wavelengths between 100 and 400 nm. One of the most effectively disinfecting wavelengths and the one most often used for disinfection is at 254 nm. The spectrum is shown in Figure 5.

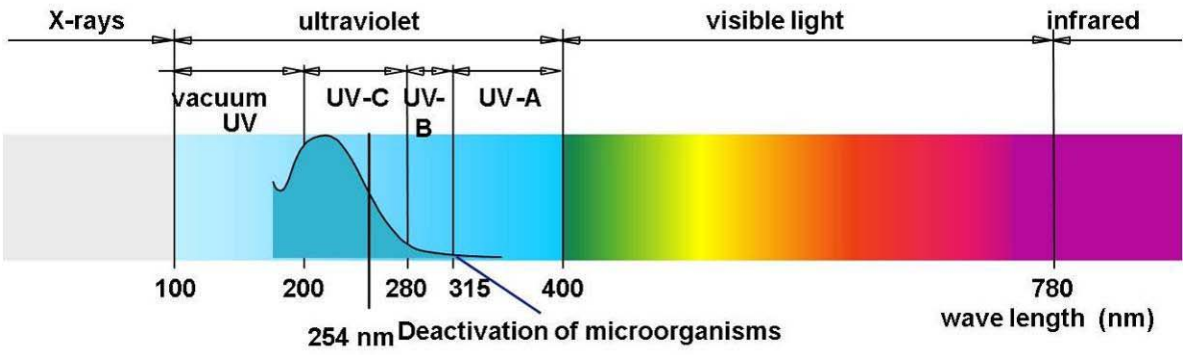


Figure 5 Electromagnetic spectrum (Drawing courtesy of Mahle).

The UV-C light necessary for disinfection is generated by low pressure, high output Amalgam lamps. Each lamp is housed and protected against the water pressure by a special quartz sleeve. A watertight quartz tube covers each lamp. Ballast water to be disinfected flows turbulently through the reactor chamber. The gas plasma generated in the lamp emits light with a primary wave length of 253.7 nm. This intensive UV light impacts by photodestruction directly on the DNA (Figure 6).

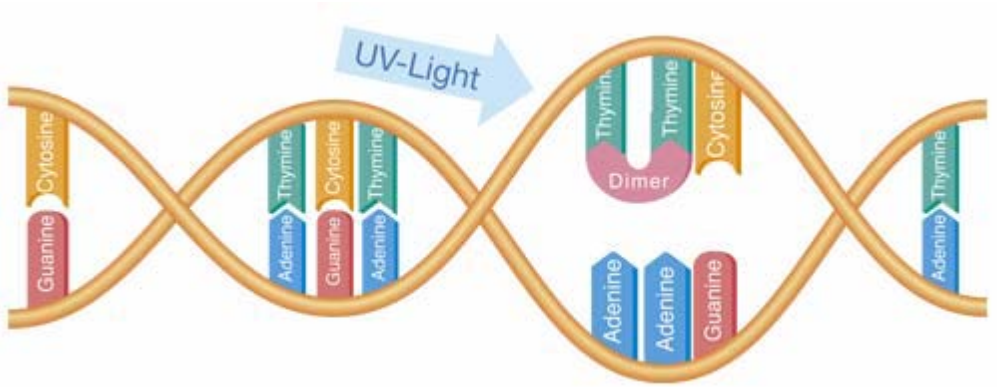


Figure 6 Impact from UV-light to DNA (Drawing courtesy of Mahle).

As a consequence DNA polymerization is inhibited which has a negative impact on cell division of affected organism – they can no longer reproduce. With this technology microorganisms in water are killed or inactivated without the addition of chemicals and thereby without harmful side effects.

The core elements of the UV systems are high performance UV lamps. Their efficiency is increased by electronic control devices specially synchronized to the lamps. In each system, calibrated UV sensors monitor and log all parameters of the disinfection process.

UV disinfection is a purely physical process, i.e. the OPS system does not make use of active substances. Microorganisms such as plankton, viruses, and bacteria etc. that are exposed to the effective UV-C radiation are inactivated within seconds.

2.2.3 INSTALLATION OF OPS ON-BOARD THE VESSEL

The tests were conducted on the container vessels, where the BWTS OPS of Mahle Industriefiltration GmbH is installed. The vessel is usually sailing on the route Algeciras – Luanda – Walvisbay – Algeciras – Leixeos – Lisbon and one voyage stretches over ca. 6 weeks. The tests reported here were conducted between Leixoes, Lisbon and Algeciras.

The system is installed in connection to the ballast pipe line on the pressure side of the ballast pump. Due to logistical and technical reasons the OPS was installed in a container. Further, this arrangement allows an easy installation of the sampling points etc. to allow performance test sampling studies. The container with the OPS was welded at the bottom of a cargo bay on a container vessel (Figure 7). It should be noted that the containerised installation was used for the performance tests of the system, but the filtration system may also be installed separately from the disinfection system. Both systems can be installed either vertically or horizontally as this has no effect on the operation or the efficiency of the system. Installations as skid-mounted or in a container are possible easing the retrofit of existing vessels.

Figure 7 OPS installed in a container which is placed at the bottom of a cargo bay on the vessel.

Figure 8 View inside the container with the OPS (Photo courtesy of Mahle).

2.3 SAMPLING PROGRAM OF THE FIRST ONBOARD TESTS

The first on-board tests were conducted on the container vessel with the OPS BWTS of the Mahle Industriefiltration GmbH (Hamburg, Germany). The system does not use active substances and treats water at the uptake **and** discharge. As planned, one test was conducted on treated ballast water, and two tests on the untreated ballast water.

In September 2009 the authors undertook a study (Gollasch and David 2009) to compare different ballast water sampling methods. The study also compared organism concentrations when sampling over the entire pumping event versus sequential samples. In the sequential tests samples of 5, 10 and 15 minute duration were taken and it was concluded that the 10 minute periods delivered good results in terms of organism concentrations (i.e., more representative than 5 minute sequential samples, but no clear difference was identified compared to 15 minute sequential samples). This also seemed to be a good compromise considering logistics during sampling, including the water volume and gear handling. Therefore, in this study we decide to take samples over time sequences of 10 to 15 minutes duration.

2.3.1 SAMPLING OF TREATED WATER

In the "treated" test, the water flow during **uptake** was split and, in parallel, one sample was taken over the entire time and three sequential samples were taken. This sampling scenario was applied before as well as after the ballast water treatment system during the uptake to

show if natural spikes in organism concentration (i.e., different concentrations of organisms in different time of taking the sample, while the vessel is sailing, or because of currents in the sea while the vessel is at berth) exist. These spikes in the uptake water may have an influence on the system performance.

At **discharge** the water flow was split and one sample was taken over the entire time and three sequential samples were taken in parallel, possibly showing if organism spikes from the ballast tank occur (i.e., different concentrations of organisms in different time and sample sequences as a result of organisms behaviour in the tank, e.g., settling on the bottom, swimming, accumulating in different areas of the tank) in the discharged water, to demonstrate the system performance in such a case, as well as to show an influence of organisms behaviour in the tank.

In summary, in 1 treated test, at the **uptake, 4 samples** were taken before and **4** are taken after the system, and at the **discharge, 4** are taken, hence all together **12 samples were taken during one treated water test**. The test was conducted at sea in navigation out of the Portuguese coast, on the way from Lisbon to Algeciras.

The treated water test process is presented in the following flow-chart diagram (Figure 9). It shows different sampling approaches to address whether or not a treatment system is operated at ballast water discharge.

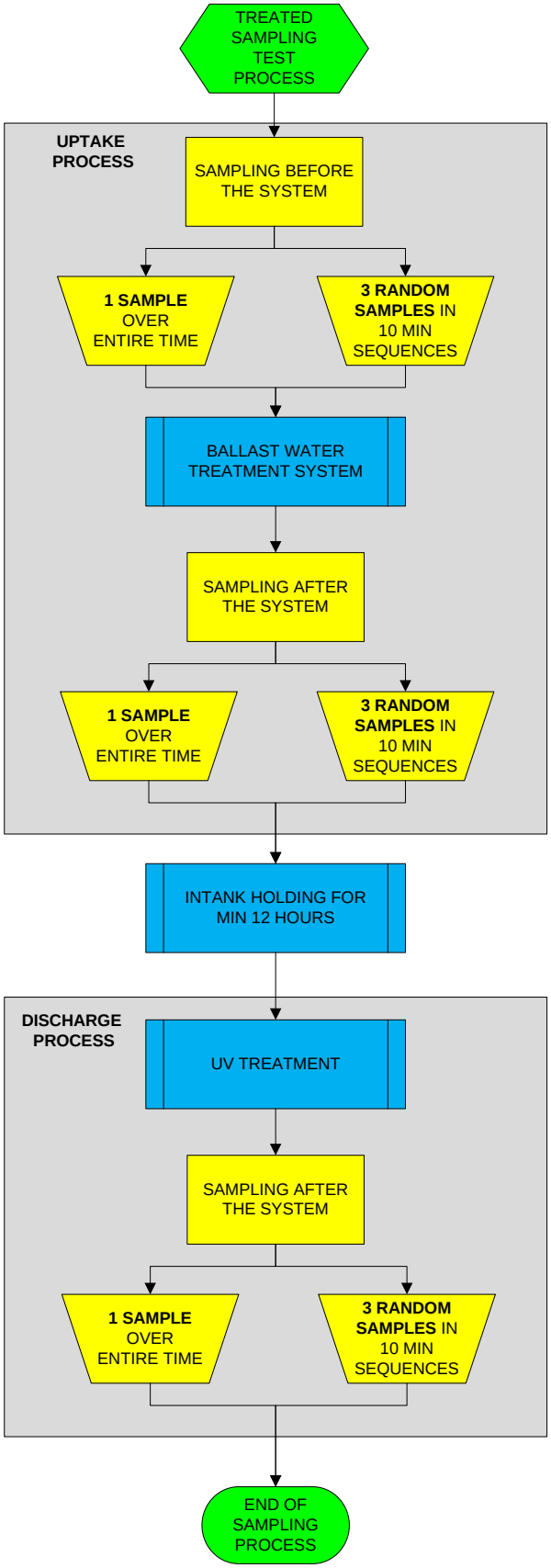


Figure 9 Description of the treated water experiment process.

2.3.2 SAMPLING OF UNTREATED WATER

Our experience from having undertaken more than 40 performance tests of different BWTS on different types of vessels showed that in most sampling events no living organisms were found at all. Therefore a ballast water sampling scenario that would include only sampling of treated ballast water would not reveal the results needed to identify the level of representativeness of different sampling methods. **In short, no organisms in the samples results in no conclusions!** Therefore, before starting the on-board sampling tests, it was agreed with EMSA that working with treated water is of very limited value to meet the objectives of the tender, and that only one test of treated water should be undertaken. This was undertaken during voyage 1. The results presented in Tables 7 and 8 confirm this view. All other tests were conducted with untreated water.

In the "untreated" tests, the water flow during **uptake** was split and, in parallel, one sample was taken over the entire time and three sequential samples were taken during the uptake. This results in documenting natural organism spikes in the uptake water and the sampling methods performance (representativeness) over longer/shorter sampling time periods. The same tests were conducted at the **discharge** to identify organism spikes from the tank in the discharge water, and differences in the sampling methods performance (representativeness) over longer/shorter time sampling periods.

In summary, in each untreated test, at the **uptake, 4 samples** were taken in-line of the water going to the tank, and at the **discharge, 4** were taken after an in-tank holding time of at least 12 hours, hence all together **8 samples were taken during one untreated water tests**. Two such tests were conducted. The uptake of the first experiment was conducted in the Port of Leixoes with discharge at sea after the vessel had left the port. The second untreated test was conducted the at sea in navigation off the Portuguese coast, on the way from Lisbon to Algeciras. This approach was chosen to address the impact of higher and lower organism concentrations on the sampling process.

The untreated water test process is presented in the following flow-chart diagram (Figure 10).

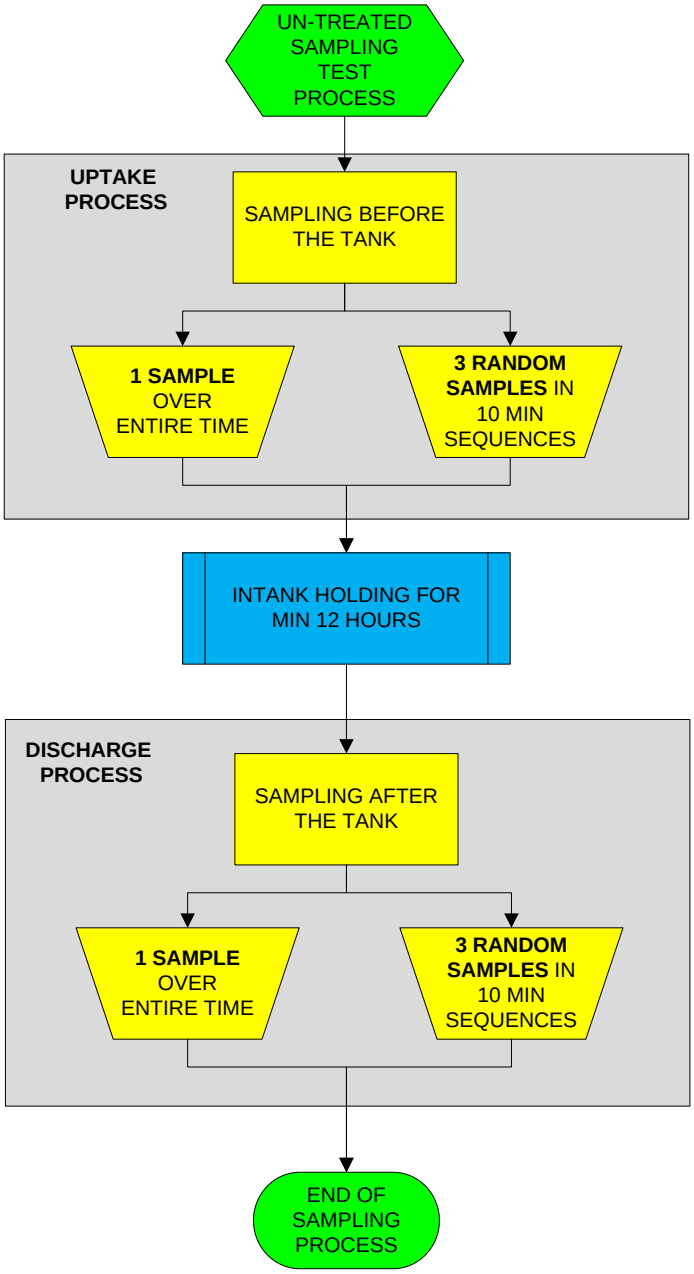


Figure 10 Description of the untreated water test process.

2.4 VOYAGE 2

Voyage two was undertaken between Sweden and The Netherlands in the end of September 2010 the bulker *Timbus* of the Rord Braren Shipping Company. The vessel and test tank specifics are given in Table 5.

Table 5 Main dimensions of the test vessel and tank details of voyage 2.

Details	Voyage 2
Vessel name	Timbus
IMO number	9198680
Vessel type	Bulker
Length overall	99.98 m
Dead Weight Tonnage (DWT)	6489 t
Total ballast water capacity	2558 m³
Number of ballast tanks	17
Number of ballast pumps	2
Capacity of each ballast pump	200 t/h
Number of BWTS installed	1
Capacity of BWTS	250 m³/h
Test tank 1	No. 3, double bottom tank, starboard
Tank capacity	120 m³
Test tank 2	No. 1, side tank, starboard
Tank capacity	215 m³

2.5 SAMPLING PROGRAM OF THE SECOND ONBOARD TESTS

The second on-board tests were conducted on the vessel *Timbus* which has the Aquaworx (Munich, Germany) BWTS installed. The system does not use active substances and treats water at the uptake **and** discharge. When installing the treatment system Aquaworx provided for inline sampling points to test for the systems performance as well as additional sampling points for control tests (sampling from the ballast water line without using the BWTS). These control test sampling points were used during this sampling study to undertake two tests of untreated ballast water.

2.5.1 SAMPLING PROCESS

The tests on this voyage were undertaken in the same way as on voyage one, i.e. the water flow was split during **uptake** and one sample was taken over the entire time and, in parallel, three samples were taken during the ballast water uptake. This results in documenting varying natural organism concentrations (spikes) from the sea. At the same time the sampling methods performance (representativeness) over longer/shorter sampling time periods was shown. The same tests were conducted at the **discharge** to identify spikes from the ballast tank in the

discharge water, and differences in the sampling methods performance (representativeness) over longer/shorter time sampling periods.

In summary, for each test, at the **uptake, 4 samples** were taken from the ballast water line going to the tank, and at the **discharge, 4** samples of the discharge were taken. In total **8 samples were taken during one test**. Two such tests were conducted. The first uptake (test run 4) was conducted in the Port of Karlshamn (Sweden) with a discharge in the next port of call, i.e. Varberg (Sweden) which resulted in a holding time of the water in the tank of ca. 24 hours. The second test (test run 5) was conducted with an uptake in Terneuzen (The Netherlands) and a discharge after ca. 48 hours holding time in the Baltic Sea during navigation after the passage through the Kiel Canal. This approach was chosen to address the impact of higher and lower organism concentrations on the sampling process in different ports.

Table 6 Location of ballast water sampling events during voyage 2.

Date	Test run number	Location	Sample type
26.09.2010	4	Port of Karlshamn	uptake, untreated
27.09.2010	4	Port of Varberg	discharge, untreated
29.09.2010	5	Port of Terneuzen	uptake, untreated
01.10.2010	5	At sea after Kiel Canal	discharge, untreated

The test process is presented in the flow-chart as shown in the previous chapter (Figure 10).

2.6 SAMPLING ARRANGEMENTS ON BOTH VOYAGES

On both voyages all samples were taken by using in-line sampling points of the ships ballast water pipework and a flow splitter was used to split the water flow equally (Figure 11). After the splitter the water was directed via flowmeters to exactly measure the amount of water sampled into plankton nets with a meshsize of 50 micrometres in diagonal dimension (Figure 12). This sampling kit was manufactured and assembled by HydroBios, Kiel, Germany (www.hydrobios.de). One split of the flow was sampled over the entire pumping operation and the second split was used to sequentially sample the discharge as described above.

Figure 11 The flow splitter arrangement (red circle).

Figure 12 Plankton nets and flow meters used during the tests.

During the sampling, the sampler ensured that the plankton net sits in water as much as possible to avoid organism damage (see green bucket in Figure 12). During the entire sampling process of both the split and unsplit samples, the continuous drip sample of approximately 5 litres was collected into a 10 l bucket. This "continuous drip sample" was used as water source for the phytoplankton and bacteriological analysis.

In the end of the sampling process the net was rinsed down with the filtered water to the cod-end at the bottom end of the plankton net, which was than unscrewed from the net and a lid was put on (Figure 14) and the closed cod-end was stored in a 10 l bucket filled with water until on-board analysis (Figure 15).

Figure 13 Cod-end with concentrated sample being unscrewed from plankton net.

Figure 14 Metal lid screwed on cod-end for storage in 10 l bucket.

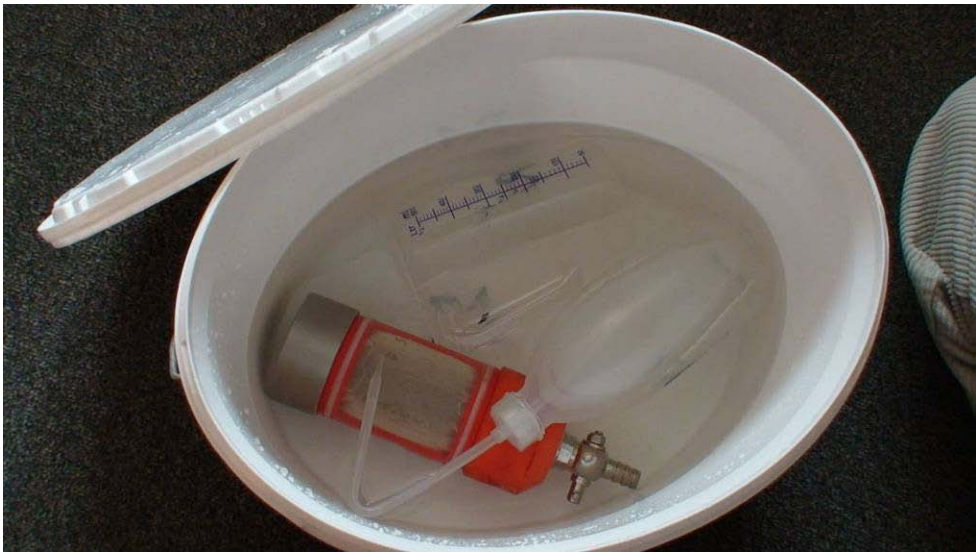


Figure 15 Unscrewed cod-end and its storage in a 10 l bucket.

2.7 BALLAST WATER SAMPLES PROCESSING

On both voyages the ballast water samples were collected and analysed as presented in the bid, and agreed with EMSA before conducting the tests on the vessel.

In contrast to the samples taken for analysis of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension, taken as 1:1 sub-samples approximately every 50 litres during the pumping event, the samples for the organisms greater than or equal to 50 micrometres in minimum dimension are concentrated while sampling.

All samples were analysed for organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension and organisms greater than or equal to 50 micrometres in minimum dimension. As agreed bacteria analyses were conducted only on the discharge samples.

Analyses of the organisms greater than or equal to 50 micrometres in minimum dimension were conducted on the vessel immediately after sampling. For the analyses of the organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension only the Pulse-Amplitude Modulated fluorometry (PAM) analyses were conducted immediately after sampling on the vessels. Further analyses were conducted by the Royal Netherlands Institute for Sea Research (NIOZ), Texel, The Netherlands. Samples were delivered to NIOZ after leaving the vessel and were processed as per the agreed protocol and as explained in section 2.7.2 addressing the PAM method further below. To measure phytoplankton viability PAM analyses were conducted on all samples on-board the vessels, as well as by NIOZ.

As a backup one set of unconcentrated samples was also taken and preserved with Lugol solution in case later analysis would have become necessary, but this was not the case.

The samples were further analysed for the presence/absence of the indicator microbes as stated in Regulation D-2 of the BWM Convention. *E. coli* and Enterococci were analysed on-board. Due to safety reasons an enrichment of toxigenic Cholera bacteria strains were not permitted on-board and such samples were delivered as soon as possible after the voyages to the laboratory IBEN, Bremerhaven, Germany.

2.7.1 SAMPLE PROCESSING FOR ORGANISMS LESS THAN 50 AND GREATER THAN OR EQUAL TO 10 MICROMETRES IN MINIMUM DIMENSION FOR ANALYSES AT NIOZ

Previous studies have shown that the organisms in this size class should not be concentrated as the concentration process damages the organisms (Veldhuis and Stehouwer pers. comm.). Samples analysed on-board the vessel were taken directly from the buckets. For analysis at NIOZ 80 ml bottles were filled with sample water taken from the 10 l bucket after mixing. Samples were properly labelled (see Figure 16).

Figure 16 Preparation of samples in 80 ml bottles for later analysis at NIOZ.

One set of samples was preserved with Lugol solution and a second set kept alive and stored in a fridge (see Figure 17).

Figure 17 Storage of samples in 80 ml bottles for the transfer to NIOZ.

2.7.2 SAMPLE PROCESSING FOR ORGANISMS LESS THAN 50 AND GREATER THAN OR EQUAL TO 10 MICROMETRES IN MINIMUM DIMENSION WITH PULSE-AMPLITUDE MODULATED FLUOROMETRY (PAM) ANALYSES ON THE VESSEL

To measure phytoplankton viability a Pulse-Amplitude Modulated fluorometry (PAM) was used on all samples on-board the vessel and the measurements were done immediately after sampling. PAM results are important for the determination of viable organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension for both the compliance monitoring process, as well as for indicative sampling methods (more in chapter on indicative methods below).

This method analysis the photochemical efficiency of photosystem II (an indicator of the 'health' condition of the cell) of phytoplankton (Schreiber et al 1993). This parameter gives a qualitative indication of the photosynthetic activity of the phytoplankton community. In addition this tool gives a bulk biomass indication.

For each analysing event the PAM was calibrated with distilled water and the result taken as the "zero sample". As a second step 3 measurements were conducted on each sample. The samples were taken from the 10 l bucket after mixing. F_0 (biomass), Fm (response), and Fv/Fm (viability) measurements were recorded for each sample, and the mean values were obtained.

2.7.3 SAMPLE PROCESSING FOR ORGANISMS GREATER THAN OR EQUAL TO 50 MICROMETRES IN MINIMUM DIMENSION

The cod-end containing the concentrated sample was emptied into a 20 micrometre filter to concentrate the sample further. The sample concentrate was transferred into a 100 ml jar and 6 ml were analysed as subsamples. Before the subsamples were taken the water in the 100 ml jar was mixed well. The subsamples of 1 or 2 ml volume were extracted from the 100 ml jar by using a pipette. The water was transferred into counting chambers and the organisms counted (Figure 18).

Figure 18 Onboard counting of organisms greater and equal to 50 micrometres in minimum dimension using a stereomicroscope.

When using Petri dishes organism counting may not be accurate as the ship movement induces water movements in the Petri dish. As a result organisms may be counted twice and some may be missed out from counting. To avoid this, a Bogorov counting chamber may be used. During minimal ship movements, this chamber proved to be efficient during on-board trials. However, with increasing ship movements the Bogorov chamber loses its advantage. HydroBios therefore designed three new counting chambers which may be used with increasing ship movements. Using these gives a much greater accuracy as here the size of the water canal width corresponds to the stereomicroscope observation field of view thereby reducing the risk to overlook organisms (Figure 19).

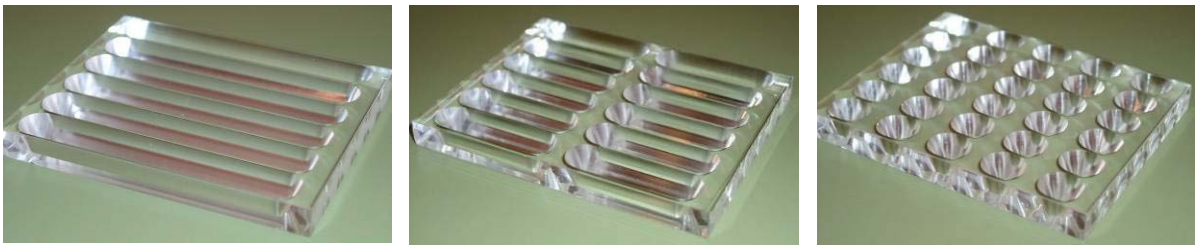


Figure 19 Newly designed counting chambers for organisms greater than or equal to 50 micrometres in minimum dimension. Photos courtesy of www.hydrobios.de.

For the size measurements a piece of the filtering mesh (50 micrometres in diagonal dimension) was placed on top of the stereomicroscope dish. This transparent mesh is used as a scale and all living organisms greater than or equal to 50 micrometres in minimum dimension were counted. The organism numbers were recorded according to broad taxonomic groups, such as copepods, decapods, polychaetes, bivalves, gastropods etc.

The organisms were counted as soon as possible after sample processing as longer waiting times may negatively impact organism survival.

2.7.4 SAMPLE PROCESSING FOR BACTERIA

Selective media (see Möller & Schmelz chapter below) were used for the *E. coli* and Enterococci analysis on-board (Figure 20). These media are watered with distilled water before incubation. Pore filters of 0.45 micrometre pore size were used to filter 10 and 100 ml of sample water for Enterococci and *E. coli* (Figure 21) and the filter was placed on the medium for incubation (Figure 22). For Cholera analysis 100 ml of sample water were filtered. Before filtration the water in the 10 l bucket was mixed well and the Cholera samples were later analysed on land by the laboratory IBEN, Bremerhaven, Germany.

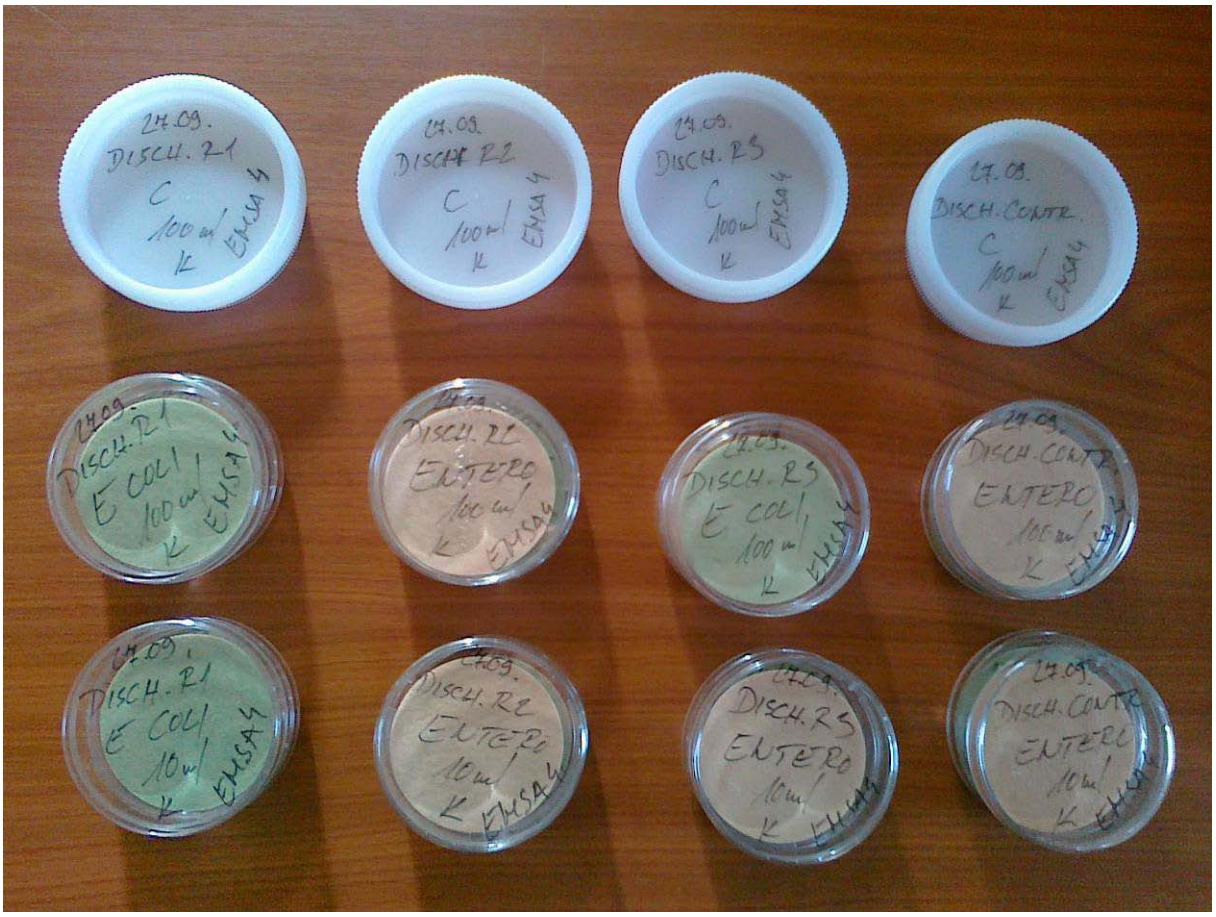


Figure 20 Labelling of dishes for bacteria analysis. Top row storage dishes for Cholera samples, middle and bottom row dishes with selective media for *E. coli* and Enterococci analysis.

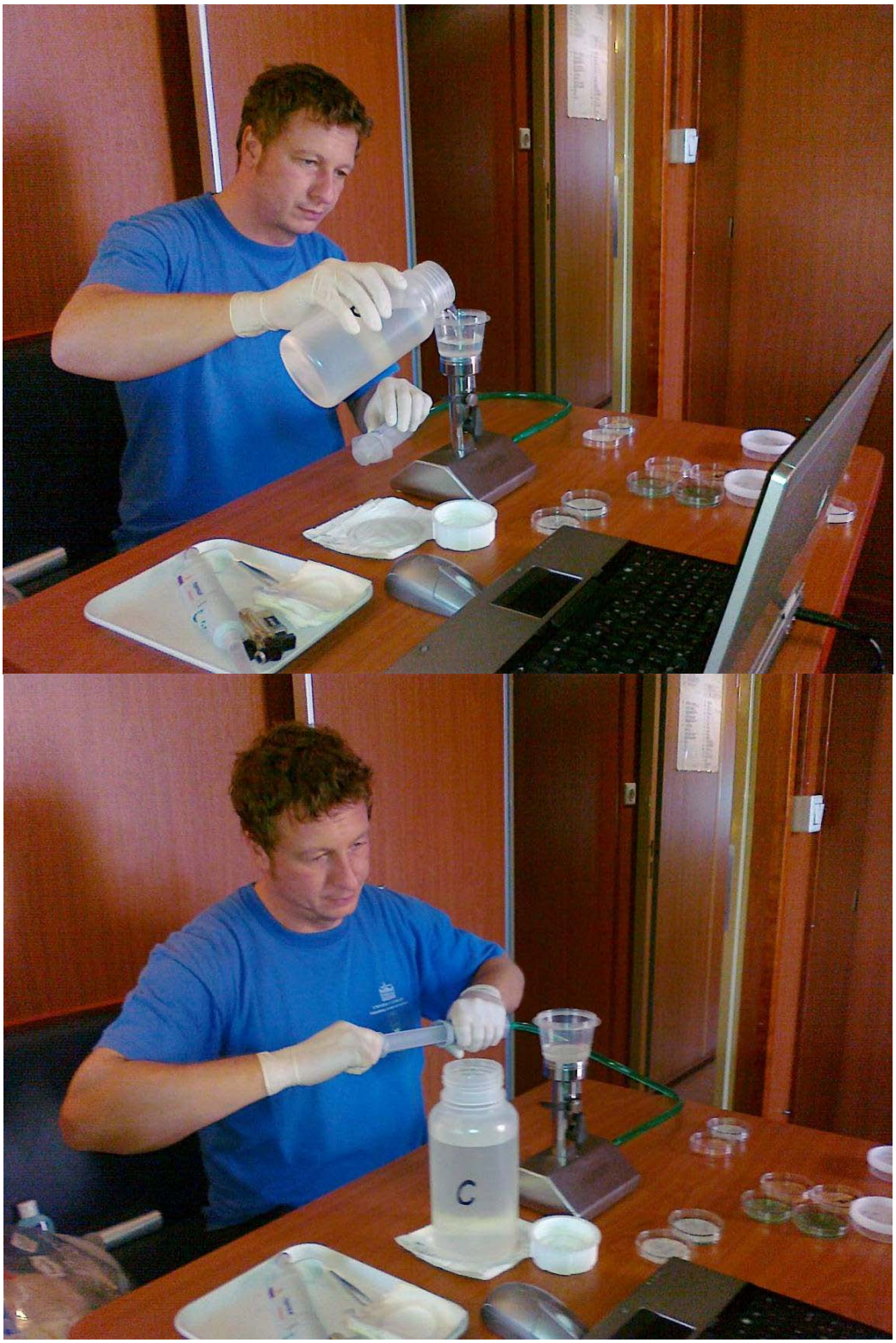


Figure 21 Onboard filtration of water for bacteria analysis using a Millipore stand.

Figure 22 Transfer of filter plates from Millipore stand to Petri dish.

Figure 23 Storage of bacteria in incubators.

Enterococci were incubated for 48 hours at ca. 37 °C. Colony forming units were identified as round dark red colonies of ca. 2 mm diameter and were counted on-board (see Möller & Schmelz method below).

Figure 24 Enterococci test plates after incubation.

E. coli were incubated for 24 hours at ca. 37 °C. This first incubation step delivers "suspect" colony forming units which look transparent and result in yellow halos visible from top and bottom of the medium. For a second incubation step to proof that the suspect colonies are *E. coli* an inoculation loop is used to transfer the suspect colonies into another medium (Tryptophane broth) which was incubated for another 24 hours at ca. 44 °C (see Möller & Schmelz method below).

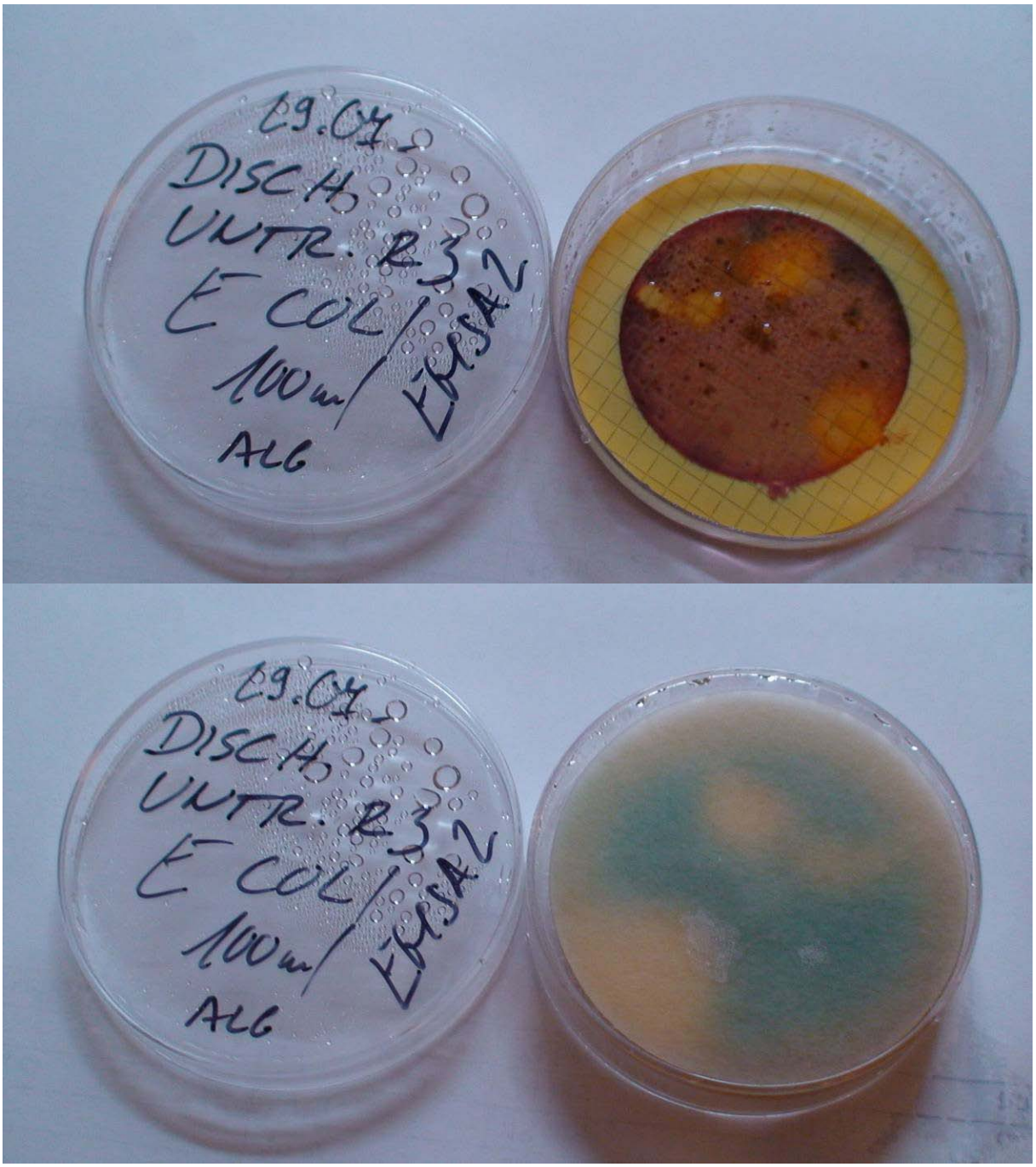


Figure 25 *E. coli* test plates after incubation. Transparent *E. coli* "suspects" (top) and yellow halos visible from underneath the Petri dish (bottom).

Figure 26 *E. coli* test plates after incubation also showing glass test tubes with Tryptophane broth (second incubation step).

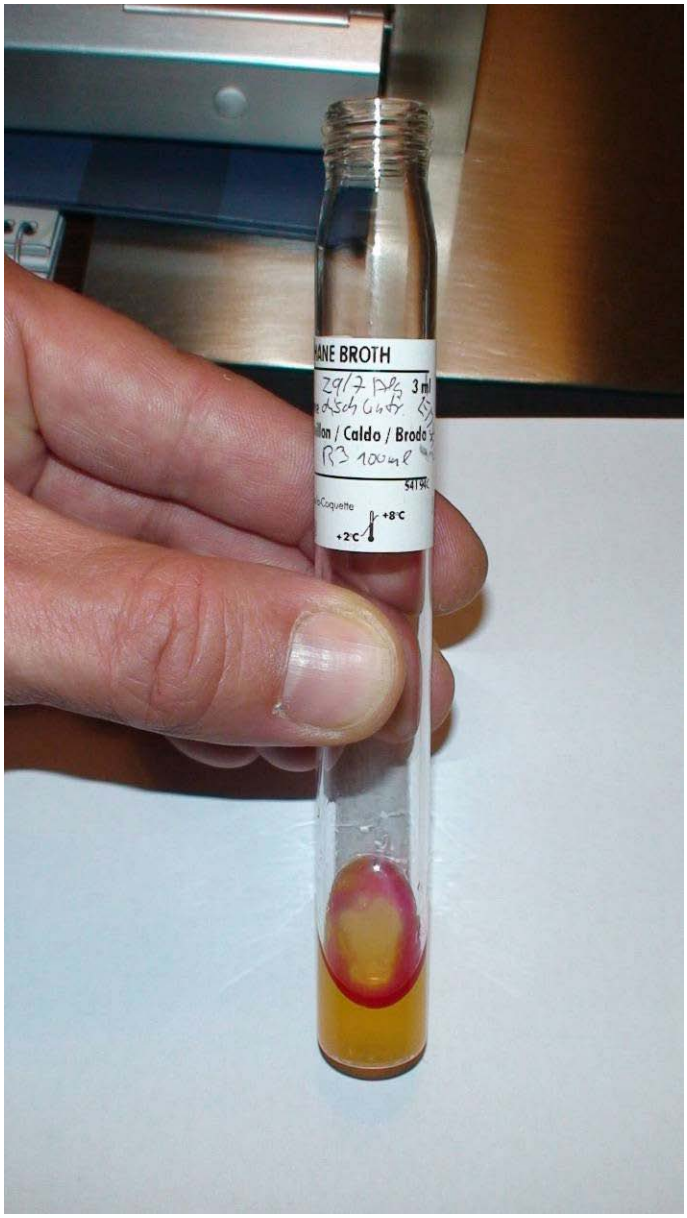


Figure 27 *E. coli* samples, second incubation step with Tryptophane broth in glass tube after addition of two drops of Kovac solution. The colour change on the surface to red confirms the presence of *E. coli*.

Cholera samples were not processed on-board. The filter plate was stored in a sealed Petri dish with filtered water to avoid the filter plate to dry.

3 RESULTS OF THE SHIPBOARD SAMPLING TESTS

3.1 ORGANISMS LESS THAN 50 AND GREATER THAN OR EQUAL TO 10 MICROMETRES IN MINIMUM DIMENSION, VOYAGE 1

3.1.1 TEST 1 UPTAKE AND DISCHARGE UNTREATED

During uptake all viable organism concentrations of all samples ranged from 60 to 77 organisms per 1 ml, with the highest number of 77 organisms per 1 ml found in the sample taken over the entire time. The highest organism concentration in this sequence was found in the first sample (75 organisms per 1 ml).

On discharge the organism numbers in the sequential samples varied from 51 to 70 organisms per 1 ml, with a slightly higher number in the first two sequential samples. The highest organism concentration in this sequence was documented in the beginning and middle of the sequence, both with 70 organisms per 1 ml. The sample over the entire time had a lower number of organisms (56 organisms per 1 ml).

3.1.2 TEST 2 UPTAKE AND DISCHARGE UNTREATED

The uptake samples organism concentrations ranged from 7 to 31 organisms per 1 ml, with the highest number found in the sample taken over the entire time (31 organisms per 1 ml). The highest organism concentration in the sequential samples was documented in the third sample (17 organisms per 1 ml).

On discharge the numbers varied from 16 to 88 organisms per 1 ml, with a clearly higher number for the last sequential sample which was taken at the very end of tank emptying. With 88 organisms per 1 ml this sample contained the highest number of all the sequential samples. The sample over the entire time resulted in 47 organisms per 1 ml.

3.1.3 TEST 3 UPTAKE BEFORE TREATMENT, UPTAKE AFTER TREATMENT, AND DISCHARGE AFTER TREATMENT

The test was carried out at sea in deeper offshore waters to accomplish the agreed tests in waters with lower organism concentrations. During this experiment the uptake samples showed organism concentrations between 9 (second sample, lowest concentration of all samples taken in this test run) and 26 (third sample, highest concentration of all samples taken in this test run) organisms per 1 ml. The sample taken over the entire time had 23 organisms per 1 ml, i.e. a slightly lower number than the sample with the highest organism concentration.

The samples taken during ballast water uptake after the system and on discharge contained no viable organisms in this size class. It is interesting to note that the number of organisms greater than or equal to 50 micrometres in minimum dimension before the treatment system (see below) were also comparatively low and that no living organisms in this size class were found after the system during discharge.

3.2 ORGANISMS GREATER THAN OR EQUAL TO 50 MICROMETRES IN MINIMUM DIMENSION, VOYAGE 1

3.2.1 TEST 1 UPTAKE AND DISCHARGE UNTREATED

On uptake the highest number of living organisms per 1000 l was found in the first sequential sample (5096 organisms / 1000 l), followed by the second (4504 organisms / 1000 l) and then the third (4237 organisms / 1000 l). The lowest organism concentration was observed in the sample taken over the entire time (2004 organisms / 1000 l) which was less than half of the lowest organism concentration of all sequential samples in this test.

On discharge the highest organisms concentrations were found in the sequential sample taken in the very end of the pumping event (2459 organisms / 1000 l), followed by the middle sample (1956 organisms / 1000 l) and the sample taken at the very beginning (1857 organisms / 1000 l). As during uptake the lowest organism concentration was shown for the sample taken over the entire time (1153 organisms / 1000 l) which is almost half of the organism number reported for the lowest organism concentration of the sequential samples in this test.

3.2.2 TEST 2 UPTAKE UNTREATED AND DISCHARGE UNTREATED

At uptake the first sequential sample provided the second highest concentrations (1689 organisms / 1000 l), the second sample had the highest organism concentration (1911 organisms / 1000 l) and the third sample the lowest organism concentrations (1067 organisms / 1000 l). The sample taken over the entire time showed by far the lowest organism number (373 organisms / 1000 l).

During the discharge sampling the organism pattern is similar to the discharge of test run 1, i.e., with the highest organism concentrations in the sample taken in the very end of the pumping event (1679 organisms / 1000 l), followed by the middle sample (1368 organisms / 1000 l) and the sample taken 10 minutes after the beginning of the pumping event (982 organisms / 1000 l). The lowest organism numbers were documented for the sample taken over the entire time (749 organisms / 1000 l).

3.2.3 TEST 3 UPTAKE BEFORE TREATMENT, UPTAKE AFTER TREATMENT, AND DISCHARGE AFTER TREATMENT

In the uptake samples before the treatment system, in the sequential samples, the lowest organisms concentration was found in the first sample (386 organisms / 1000 l), followed by the second (737 organisms / 1000 l) and the highest organism concentration was observed in the third (982 organisms / 1000 l). The sample taken over the entire time contained the lowest organism number (268 organisms / 1000 l). The organism numbers in these samples were much lower compared to all other samples most likely because the ballast water was taken on-board in deeper waters away from the coast.

During uptake after the system and upon discharge no living organisms were found.

3.2.4 ORGANISMS DISTRIBUTION, VOYAGE 1

The organisms counted in the sequential samples clearly document heterogeneity in plankton distribution in the natural environment, as well as in the ballast tanks. Table 7 shows the results during ballast water uptake, i.e. heterogeneity of organisms distribution in the sea and Table 8 the results of the discharge sampling events, i.e. heterogeneity of organisms distribution in the ballast tank.

3.2.4.1 Uptake

The difference in organism numbers in the uptake samples may refer to the patchiness of organisms in the sea while the vessel changes position during navigation, or in the port as a consequence of organism migration, changing currents or tidal differences.

For the organisms greater than or equal to 50 micrometres in minimum dimension the uptake sequential samples show no consistent trend, i.e. the highest organism number in the tests are found in different parts of the sequential sampling. For the organisms greater than or equal to 50 micrometres in minimum dimension, the results clearly document that the sample taken over the entire time contains much less organisms (1/3 to 1/2) compared to the sequential samples. The samples taken over the entire time contained the lowest organism concentrations, which may be caused by dying of organisms because they are exposed to unfavourable conditions during sampling for a longer time, e.g., spinning around in the net, more pressure in the net, stuck in the net and exposed to high water flow, or by crowding during sample processing.

This is in contrast to findings regarding the organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension where in two of the three uptakes, lower concentrations were found in the sequential samples, and the samples taken over the entire time show the highest organism concentrations (see Table 7).

Table 7 Organism concentrations indicated per test run of voyage 1 and organisms group during the **uptake** sampling events (i.e. water from the **sea**). Please refer to Table 2 for details when during the pumping process the sequential samples were taken.

	First sequential sample	Second sequential sample	Third sequential sample	Over entire time	Uptake area
Test run 1 Orgs <50 and > 10 µm [1 ml]	75	60	63	77	Port of Leixoes
Test run 2 Orgs <50 and > 10 µm [1 ml]	9	7	17	31	at sea
Test run 3, before system Orgs <50 and > 10 µm [1 ml]	16	9	26	23	at sea
Test run 3, after system Orgs <50 and > 10 µm [1 ml]	0	0	0	0	at sea
Test run 1 Orgs >50 µm [1000 l]	5.096	4.504	4.237	2.004	Port of Leixoes
Test run 2 Orgs >50 µm [1000 l]	1.689	1.911	1.067	373	at sea
Test run 3, before systems Orgs >50 µm [1000 l]	386	737	982	268	at sea

3.2.4.2 Discharge

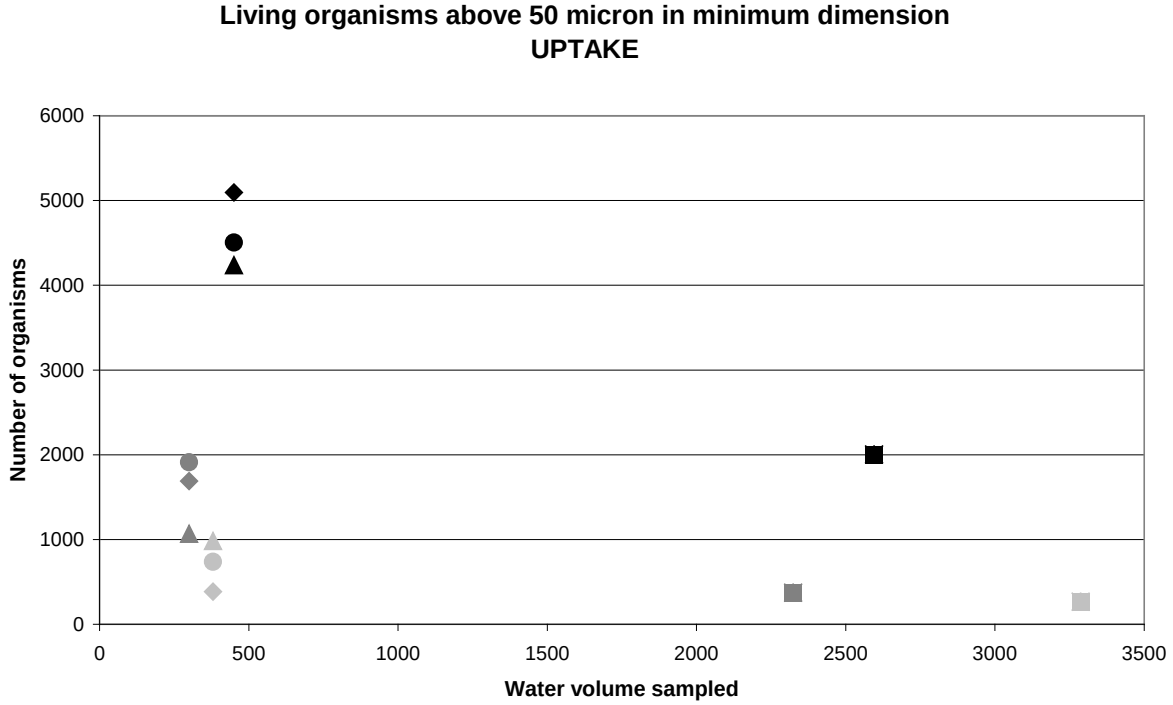
The discharge samples show the patchiness of organisms in the ballast tank after ca. 1 day holding time (Table 8).

Table 8 Organism concentrations indicated per test run of voyage 1 and organisms group during the **discharge** sampling events (i.e. water from the **tank**). Please refer to Table 2 for details when during the pumping process the sequential samples were taken.

	First sequential sample	Second sequential sample	Third sequential sample	Over entire time
Test run 1 Orgs <50 and > 10 µm [1 ml]	70	70	51	56
Test run 2 Orgs <50 and > 10 µm [1 ml]	20	16	88	47
Test run 3, treated Orgs <50 and > 10 µm [1 ml]	0	0	0	0
Test run 1 Orgs >50 µm [1000 l]	1.867	1.956	2.459	1.153
Test run 2 Orgs >50 µm [1000 l]	982	1.368	1.679	749
Test run 3, after treatment Orgs >50 µm [1000 l]	0	0	0	0

For the organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension, there was no clear trend in which of the sequential samples the highest organism concentration may be found. However, the samples taken over the entire time never show the highest organism concentrations. The beginning and the middle samples show more similar results, while opposite results are observed in the sequential samples at the very end of the sampling time, i.e., the lowest number of organisms is observed in the end sample of test run 1, and the highest was found in the end sample of the test run 2, when compared to the sample taken over the entire time.

However, for the organisms greater than or equal to 50 micrometres in minimum dimension in test run 1 and 2, the organism concentration pattern is comparable, as the highest numbers occurred in the sequential sample taken at the end of the pumping time. The samples taken over the entire time contain the lowest organism concentrations, which may be caused by dying of organisms. This may be due to the organisms being exposed to unfavourable conditions during sampling for a longer time, e.g., spinning around in the net, more pressure in the net, stuck in the net and exposed to high water flow, or by crowding during sample processing.



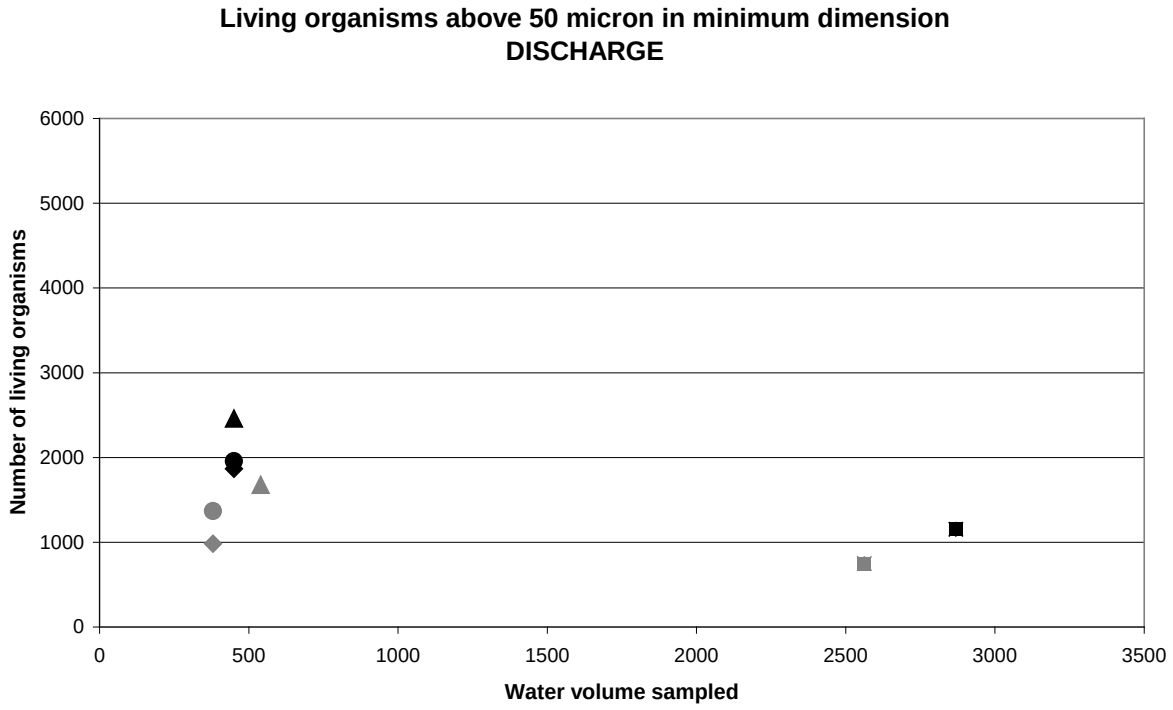


Figure 28 Number of living organisms greater than or equal to 50 micrometres in minimum dimension of voyage 1 at test run 1 (black symbols), test run 2 (dark grey symbols) and test run 3 (light grey symbols) during the uptake test (top) and during discharge (bottom) according to the water volume sampled. Diamonds indicate results of the sample in the beginning, circles the middle sample and triangles the end sample, with squares showing results from the samples taken over the entire time. Note: During discharge of the treated water (test run 3) no living organisms were found.

Figure 28 shows that the samples taken over the entire time had water volumes above 2000 litres compared to the individual sequential samples with a maximum water volume sampled of 540 litres.

For both, uptake and discharge sampling events a general trend is that all samples taken over the entire time contain much fewer living organisms greater than or equal to 50 micrometres in minimum dimension compared to the sequential samples of the same test run. Such a clear trend cannot be identified for the organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension (see Table 7 and Table 8).

It is further remarkable that the number of living organisms greater than or equal to 50 micrometres in minimum dimension in the uptake water during test run 1 (untreated water) is much higher compared to the discharge test of this test run which is most likely due to the one day holding time in the tank (Figure 28). Also other ballast water sampling studies have shown a general trend that the organisms die over time inside a ballast tank (e.g. Rigby & Hallegraeff 1993, 1994, Fukuyo et al. 1995, Hamer et al. 1998, Dickmann & Zhang 1999, Zhang & Dickmann 1999, Olenin et al 2000, Gollasch et al 2000a, b, c, Carver et al. 2004, Murphy et al 2004, Mimura et al. 2005, David et al. 2007, Gray et al. 2007, McCollin et al. 2007a, b, 2008, Quilez-Badia et al. 2008, de Lafontaine et al. 2009, Klein et al. 2010, Seiden et al. 2010). However, in test run 2 the numbers of organisms at discharge of the sequential samples taken in the end and over the entire time are higher compared to the intake. This may indicate that organisms could remain in the ballast tank from the previous test run 1, during which higher organism concentrations were observed.

During discharge of the treated water in test run 3 no living organisms were found.

3.2.5 ORGANISMS LESS THAN 50 AND GREATER THAN OR EQUAL TO 10 MICROMETRES IN MINIMUM DIMENSION, VOYAGE 2

During the second voyage the organism numbers in the group less than 50 and greater than or equal to 10 micrometres in minimum dimension showed in general higher numbers in all samples. This is assumed to be because of the shorter time between the sampling and processing of the samples at NIOZ (i.e., the PAM measurements of the biomass of the same samples conducted at NIOZ were much lower than those conducted on the vessel).

3.2.5.1 Test 4 uptake and discharge untreated

During uptake all viable organism concentrations of all samples ranged from 193 to 230 organisms per 1 ml. The highest number of organisms (230 organisms per 1 ml) was found in the sample taken over the entire time and all sequential samples had different organism concentrations ranging from 193 to 217 organisms per 1 ml.

During discharge the organism numbers in the sequential samples varied from 79 to 246 organisms per 1 ml with the highest number of organisms observed in the sample at the very end of the test. The sample taken over the entire time had a concentration of 114 organisms per 1 ml. The middle sample (144 organisms per 1 ml) and the very end sequential sample (246 organisms per 1 ml) had higher concentration of organisms than the sample taken over the entire time.

3.2.5.2 Test 5 uptake and discharge untreated

The organism concentrations in the uptake sequential samples varied from 122 to 163 organisms per 1 ml, and the highest concentration of organisms was found in the sample taken over the entire time (223 organisms per 1 ml).

On discharge the concentration of organisms in the sequential samples varied from 95 to 122 organisms per 1 ml, and the highest concentration of organisms was in the sample taken over the entire time (186 organisms per 1 ml). The highest organism concentration in the sequential samples was documented in the sample at the beginning of the discharge (122 organisms per 1 ml).

3.2.6 ORGANISMS GREATER THAN OR EQUAL TO 50 MICROMETRES IN MINIMUM DIMENSION

3.2.6.1 Test 4 uptake and discharge untreated

On uptake the highest concentration of viable organisms per 1000 l was found in the second sequential sample (2762 organisms / 1000 l), followed by the first (2429 organisms / 1000 l) and then the third (2143 organisms / 1000 l). The lowest organism concentration was identified in the sample taken over the entire time (1701 organisms / 1000 l).

On discharge the highest organism concentration was found in the middlesample (1524 organisms / 1000 l), followed by the sample taken in the beginning of the discharge (1190 organisms / 1000 l). The sequential sample taken at the end contained the lowest organism concentration (970 organisms / 1000 l), which was also lower than that of the sample taken over the entire time (1068 organisms / 1000 l). The organism concentrations of the sequential samples taken at the beginning and the end of the discharge, as well as the sample taken over the entire time do not vary greatly in organism concentration, i.e. all three samples showed organism concentrations between 970 and 1190 organisms / 1000 l.

3.2.6.2 Test 5 uptake untreated and discharge untreated

On uptake the first sequential sample showed the highest organism concentration (8370 organisms / 1000 l), the third sample had the second highest organism concentrations (7444 organisms / 1000 l) and the second sample the lowest organism concentration (4593 organisms / 1000 l) of the sequential samples. The sample taken over the entire time showed the lowest organism concentration (2688 organisms / 1000 l) of samples taken during this test.

Organism concentration pattern during the discharge was different from the test run 4. The highest organism concentration was found in the third sequential sample taken 20 minutes before the end of the pumping event (2667 organisms / 1000 l), followed by the middlesample (2111 organisms / 1000 l) and then the beginning sample, taken 10 minutes after the beginning of the pumping event (1259 organisms / 1000 l). The lowest organism concentration was documented for the sample taken over the entire time (1218 organisms / 1000 l), although this value is only slightly below the concentration found in the beginning sequential sample.

3.2.7 ORGANISMS DISTRIBUTION, VOYAGE 2

The organism concentrations of the sequential samples clearly document heterogeneity in plankton distribution. Table 9 shows the results during ballast water uptake, i.e. heterogeneity of organisms in the port and Table 10 the results of the discharge sampling events, i.e. heterogeneity of organisms in the ballast tank.

3.2.7.1 Uptake

The difference in organism numbers in the uptake samples shows patchiness of organisms in the port as the water is directly pumped on-board and sampled (Table 9) over different time periods. The vessel was not in navigation during the uptake, so that the samples were taken in the port while she was alongside a pier. The organism patchiness found here refers to changing organism concentrations in the port during ballast water uptake. This may have been influenced by organism migrations, currents or tidal changes.

For the organisms greater than or equal to 50 micrometres in minimum dimension the sequential samples show different organism concentrations among different samples from both test runs. For the organisms greater than or equal to 50 micrometres in minimum dimension the results clearly document that the sample taken over the entire time shows a much lesser concentration of organisms compared to the individual sequential samples.

This is in contrast to findings regarding the organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension where, in general, lower concentrations of organisms were found in the sequential sampling, and the samples taken over the entire time show the highest organism concentrations. The same trend was observed during the uptake of the test 1 and 2 of the first voyage, only in the uptake of the test run 3 the highest concentration of organisms was observed in the third sequential sample.

Table 9 Organism concentrations indicated per test run of voyage 2 and organisms group during the **uptake** sampling events (i.e. water from the **sea**). Please refer to Table 2 for details when during the pumping process the sequential samples were taken.

	First sequential sample	Second sequential sample	Third sequential sample	Over entire time	Uptake area
Test run 4 Orgs <50 and > 10 µm [1 ml]	193	212	217	230	Port of Karlshamn
Test run 5 Orgs <50 and > 10 µm [1 ml]	122	157	163	223	Port of Terneuzen
Test run 4 Orgs >50 µm [1000 l]	2429	2762	2143	1701	Port of Karlshamn
Test run 5 Orgs >50 µm [1000 l]	8370	4593	7444	2688	Port of Terneuzen

3.2.7.2 Discharge

The discharge samples show the patchiness of organisms in the ballast tank after ca. 1 day holding time in test run 4 and ca. 2 day holding time in test run 5 (Table 10).

Table 10 Organism concentrations indicated per test run of voyage 2 and organisms group during the **discharge** sampling events (i.e. water from the **tank**). Please refer to Table 2 for details when during the pumping process the sequential samples were taken.

	First sequential sample	Second sequential sample	Third sequential sample	Over entire time
Test run 4 Orgs <50 and > 10 µm [1 ml]	79	144	246	114
Test run 5 Orgs <50 and > 10 µm [1 ml]	122	95	112	186
Test run 4 Orgs >50 µm [1000 l]	1190	1524	970	1068
Test run 5 Orgs >50 µm [1000 l]	1259	2111	2667	1218

For the organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension during discharge of test run 4 an increasing concentration of organisms during the sampling event was found, i.e. the lowest concentration in the beginning sequential sample and the highest in the very end sample. In the test run 5 none of sequential samples were taken at the very beginning or the very end of the discharge (i.e., all could be described as random middle samples), and the highest concentrations were found in the first and the third sample. The samples taken over the entire time in test run 4 showed a lower concentration of organisms than the middle and the very end sequential samples, while in the test run 5 the organism concentration of the sample taken over the entire time was higher than the organism concentrations in the sequential samples.

For the organisms greater than or equal to 50 micrometres in minimum dimension in test run 4, the highest organism concentration was observed in the middle sample, while in test run 5 this occurred in the very end sample. In test run 4, the lowest organism concentration was found in the very end sequential sample, while in test run 5, the sample taken over the entire time contained the lowest organism concentration.

Figure 29 shows the numbers of living organisms in the sequential samples and the sample taken over the entire time per water volume during both test runs.

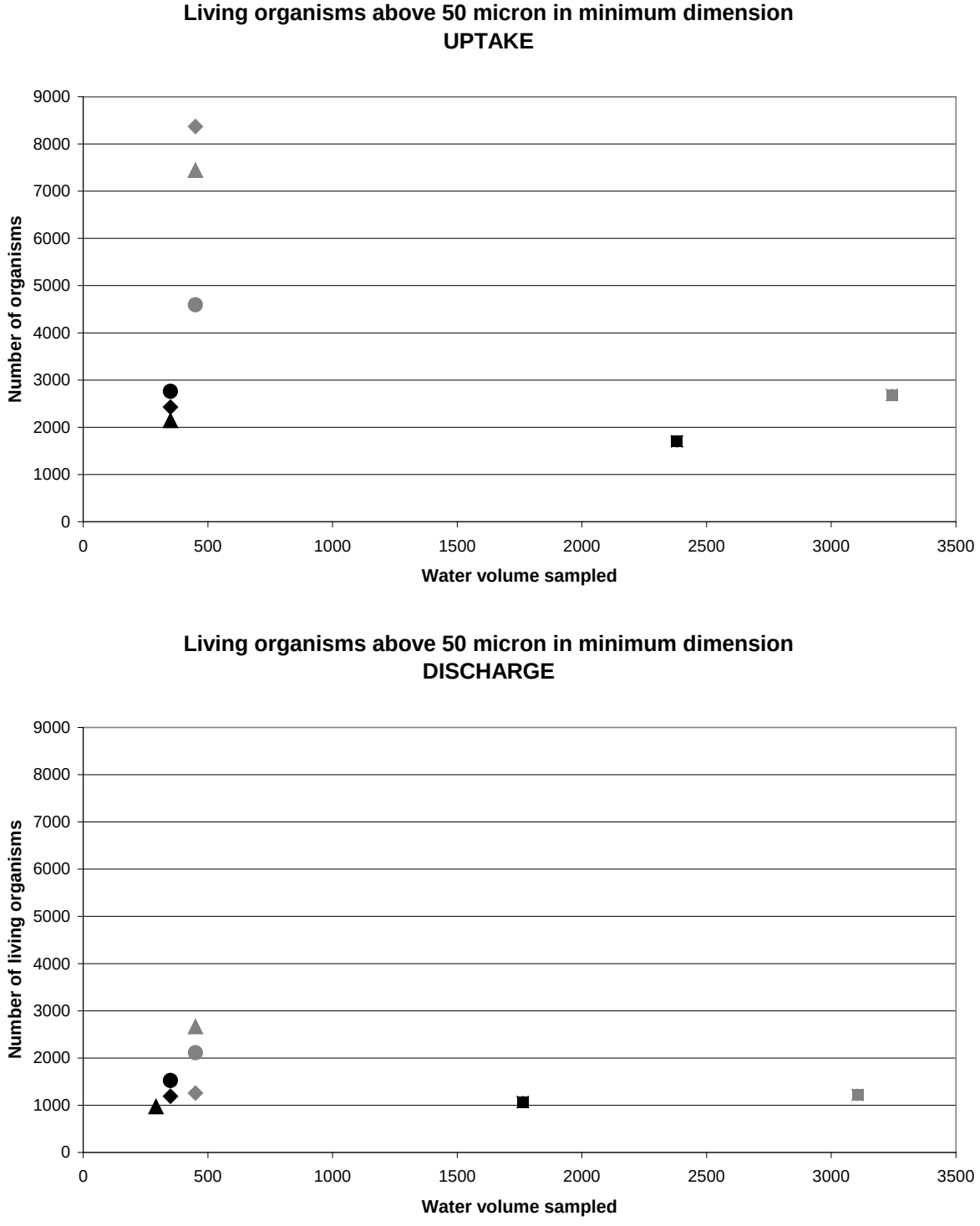


Figure 29 Number of living organisms greater than or equal to 50 micrometres in minimum dimension of voyage 2 at test run 4 (black symbols), test run 4 (grey symbols) during the uptake test (top) and during discharge (bottom) according to the water volume sampled. Diamonds indicate results of the sequential sample taken at in the beginning of the discharge, circles the middle sample, andtriangles the end, with squares showing results from the samples taken over the entire time.

Figure 29 shows that all samples taken over the entire time resulted in water volumes above 1500 litres (smallest sample 1763, largest sample 3243) compared to the individual samples with a maximum water volume sampled of 450 litres.

3.3 BACTERIA

3.3.1 CHOLERA, BOTH VOYAGES

All samples taken during both voyages were negative, i.e. the absence of *Vibrio cholera* was documented in all samples.

3.3.2 *E. COLI*, VOYAGE 1

Test 1 discharge untreated :
20 cfu were found per 100 ml in the discharge of the untreated water in the sample collected over the entire discharge time. All sequential samples were negative.

Test 2 discharge untreated:
E. coli were found in sequential sample 3 (4 cfu per 100 ml), which was taken in the end of the pumping time and in the sample taken over the entire pumping event (6 cfu per 100 ml).

Test 3 discharge after treatment:
The presence of *E. coli* in the discharge water could not be shown.

3.3.3 *E. COLI*, VOYAGE 2

Test 4 discharge untreated:
13 cfu were found per 100 ml in the discharge of the untreated water in the sample collected over the entire discharge time and all sequential samples also contained *E. coli* cfu, i.e. beginning sample = 18 cfu, middle sample = 21 cfu and end sample = 70 cfu per 100 ml.

Test 5 discharge untreated:
The presence of *E. coli* was confirmed in all sequences, but not in the sample taken over the entire pumping event. The beginning sample contained 90 cfu, the middle sample 22 cfu and the end sample 2 cfu per 100 ml.

3.3.4 ENTEROCOCCI, VOYAGE 1

Test 1 discharge untreated:
The only sample containing Enterococci was the sequential sample taken in the middle of the discharge event. Here 10 cfu per 100 ml were located.

Test 2 discharge untreated:
Enterococci were only found in sequential sample 1, taken in the beginning of the discharge event, in a density of 2 cfu per 100 ml.

Test 3 discharge after treatment:
Enterococci were found in sequential sample 1 (5 cfu per 100 ml), which was taken in the beginning of the pumping time and in the sample taken over the entire pumping event (9 cfu per 100 ml).

3.3.5 ENTEROCOCCI, VOYAGE 2

Test 4 discharge untreated:
The sample taken over the entire time contained 2 Enterococci cfu, the beginning sample showed the highest number of all samples taken from all tests with 100 cfu per 100 ml. In the middle sample no cfu could be found and the sample taken in the end contained 5 Enterococci cfu per 100 ml.

Test 5 discharge untreated:
No Enterococci were found in the sample taken over the entire time. In the beginning sample 7 cfu per 100 ml were found, 4 were found in the middle sample and 3 were found in the end sample.

3.3.6 SUMMARY BACTERIA

All Cholera samples were analysed after the voyage in a certified laboratory and no colony forming units were found. The results of *E. coli* and Enterococci show very low numbers of colony forming units. Patchiness was identified, as the colony forming units of these bacteria were found in different samples , without a clear pattern as to which sample shows higher bacteria densities. This is also influenced by the low number of samples.

The same conclusion may be drawn when comparing the representativeness of the sequential vs. the entire time sampling events (no trend could be identified). It could be assumed that if there are bacteria in the water the entire time sample should show this, but in the individual samples they may have been missed. However the results do not confirm this hypothesis.

On voyage 1 the *E. coli* and Enterococci samples had to be prepared on-board in the so called Suez Cabin and on voyage 2 in an office cabin. Although greatest care was undertaken to minimize bacteriological contamination this cannot fully be avoided. It is already not easy to prepare uncontaminated clean bacteria analysis plates in a microbiological laboratory and this situation is even more difficult on a commercial vessel when working in a cabin with a rest room nearby. This situation creates a contamination risk with human bacteria such as *E. coli* and Enterococci.

It is therefore assumed that the bacteria found in the discharge samples either originate from the uptake in the port, or from contamination during sample taking and/or sample processing. This is especially the case for the discharge of the treated water (voyage 1, test 3) as the treatment system has shown very high bacteria treatment efficacy during the land-based and shipboard tests undertaken according to G8, for the certification tests of this treatment system (i.e., was always 0). On voyage 2 the presence of bacteria was shown in most samples, but usually in very low numbers. An exception was the high *E. coli* concentration recorded with 90 cfu per 100 ml in the beginning sample of test run 5, and 100 Enterococci cfu per 100 ml in the beginning sample of test run 4. These are the highest numbers we ever observed in a sample during the 40+ voyages we have undertaken. In this test no treatment system was applied and therefore the bacteria numbers in the discharge samples may either originate from the uptake water in the ports or may have been a result of a contamination during sample processing.

4 RECOMMENDATIONS FOR A BALLAST WATER SAMPLING PROTOCOL

The main goal of Part 1 of the study was to provide recommendations for a ballast water sampling protocol that is representative of the entire discharge. The observations and conclusions presented in this chapter are based on the ballast water sampling tests conducted during two vessel voyages of this study, and where possible, are supported by experience from other ballast water sampling tests.

The bacteriological results were not processed further as, in comparison to the other organism groups, the presence of the bacteria was too scattered to allow firm conclusions.

Some further questions to be answered were:

- Is there a **homogeneity**, or lack of, **of organism concentrations in the uptake?**
- Is there a **homogeneity**, or lack of, **of organism concentrations in the discharge?**
- How representative are the **results observed during the entire discharge** of ballast water?, and
- Whether using an "**instantaneous**" or "**average**" testing sampling protocol will have an influence on the procedure used to obtain a representative sample?

To ease the reading in some paragraphs hereafter the terms bigger and smaller organisms refer to the organism size groups as stated in the D-2 Standard, i.e. organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension are the *smaller organisms* and organisms greater than or equal to 50 micrometres in minimum dimension are the *bigger organisms*.

4.1 HOMOGENEITY OF ORGANISM CONCENTRATIONS IN THE UPTAKE

The homogeneity or lack of, of organism concentrations in the uptake may exist as a result of organism patchiness in the natural environment (e.g., different concentrations of organisms in different areas, migration of organisms to the upper levels of water during darkness, changes of organism concentrations during tidal changes currents or upwelling situations).

To observe any changes in the uptake organism concentrations over time, which are thought to possibly affect the BWTS efficiency, three samples taken randomly, one at the beginning, one in the middle and one at the end of the ballast water uptake have been compared. The difference in organism concentrations among the sequential samples taken during one uptake would show non-homogeneity or unequal distributions of organisms, resulting in a variation in organism concentrations during the uptake.

4.1.1 UPTAKE ORGANISMS CONCENTRATIONS

Uptake of the tests 1, 4 and 5 were conducted in the port, while tests 2 and 3 were undertaken in navigation off the Portuguese coast (42 NM from the nearest shore in water depths from ~1000 – 3000 m). In test 3 ballast water was treated, hence samples were taken during uptake and after the treatment system before the water was pumped into the ballast tank.

4.1.1.1 Organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension

In general it was observed that uptake samples taken outside the ports in navigation at the open sea contained lower organism concentrations compared to those from the ports. Furthermore, it was observed that organism concentrations were always different among the three sequential samples of each uptake, hence showing variability in organism concentrations during uptake in the ports as well as in uptake during navigation at the open sea (see Figure 30).

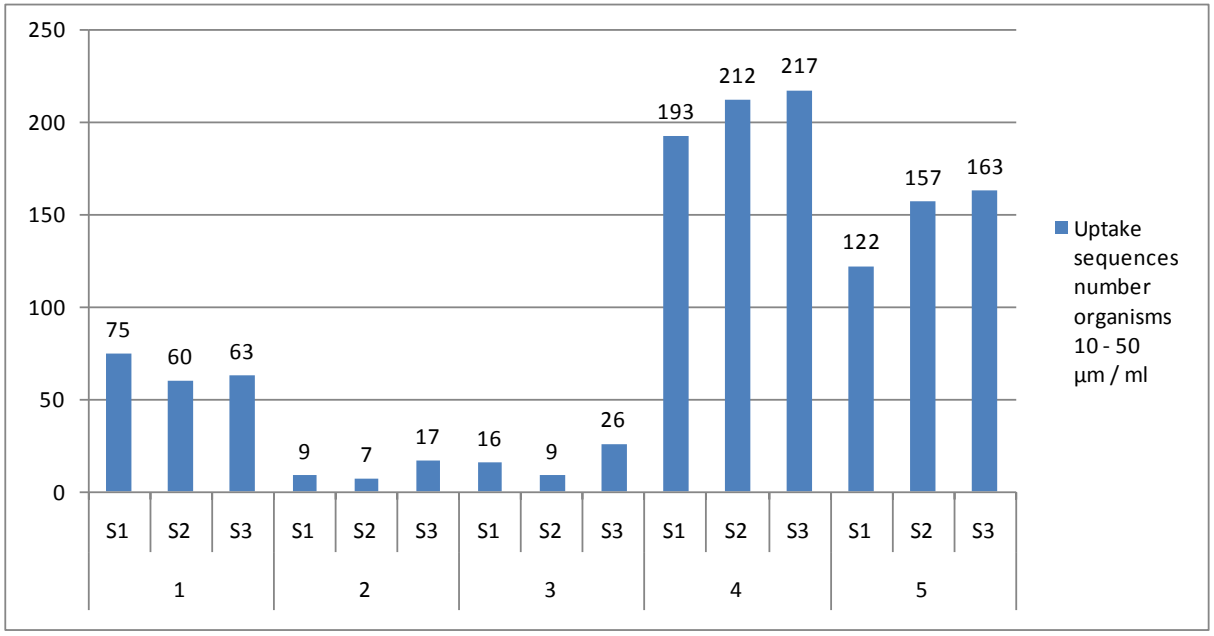


Figure 30 Concentrations of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension in the sequential sample of five uptake tests. S1, S2 and S3 mean sample 1, 2 and 3. Numbers 1 - 5 at the bottom refers to test numbers.

This confirms that BWTS are being exposed to ballast water where organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension are varying in concentration throughout the treatment process. During the uptake of test 3, ballast water was treated and sequential samples were taken immediately after the BWTS in a sample sequence that mirrored the sequential sampling undertaken before the BWTS.

Considering our experience from previous tests, where uptake samples and samples immediately after the BWTS have been undertaken, several tests had much higher influent

organism concentrations compared to this study, however the results after treatment by the BWTS were still zero living organisms.

4.1.1.2 Organisms greater than or equal to 50 micrometres in minimum dimension

In general it was observed that uptake samples taken outside the ports, i.e. in navigation at the open sea, contained lower organism concentrations compared to those from the ports. Furthermore, it was observed that organism concentrations were always different among the three sequential samples of each uptake, hence showing variability in organism concentrations during uptakes in the ports or during navigation in the open sea (see Figure 31).

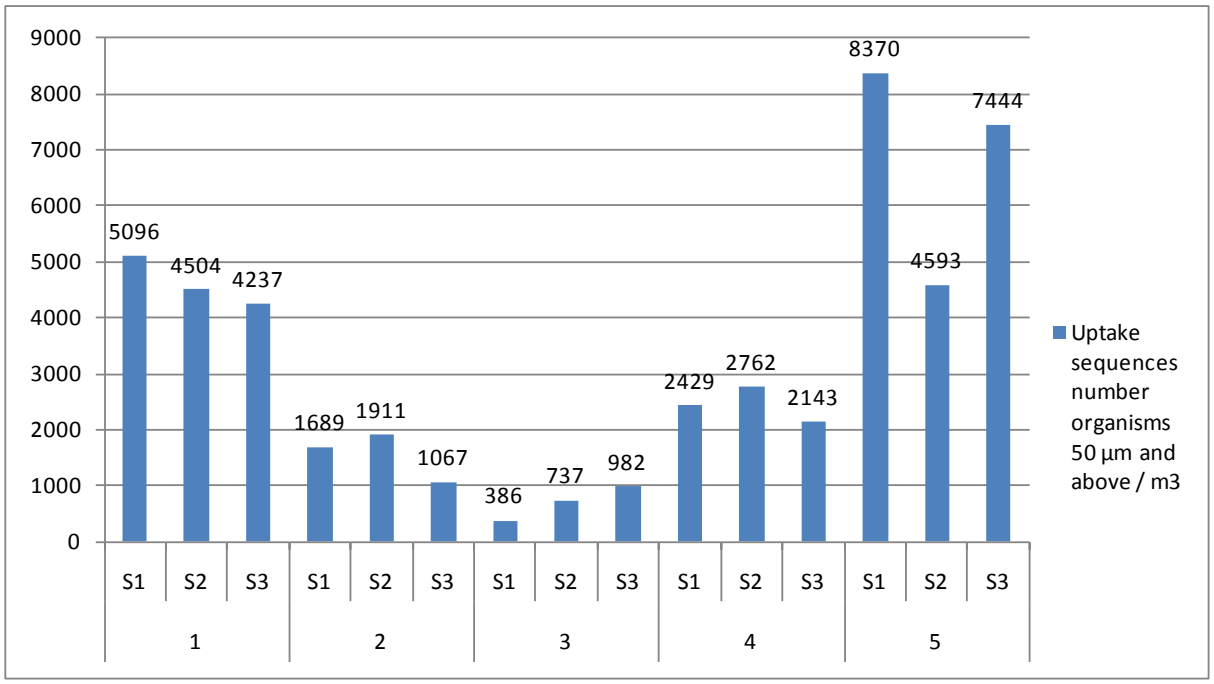


Figure 31 Concentrations of organisms greater than or equal to 50 micrometres in minimum dimension in the sequential samples of five uptake tests. S1, S2 and S3 mean sample 1, 2 and 3. Numbers 1 - 5 at the bottom refer to test numbers.

This confirms that BWTS are exposed to ballast water where organisms greater than or equal to 50 micrometres in minimum dimension are varying in concentration throughout the treatment process. During the uptake of test 3, ballast water was treated and sequential sample were taken immediately after the BWTS in a sample sequence that mirrored the sequential sampling undertaken before the BWTS.

Considering our experience from previously undertaken tests where uptake samples were taken also immediately after the BWTS, several tests had much higher influent organism concentrations compared to this study. In some tests few viable organisms were detected after the BWTS, however no correlation between organism concentrations before and after the BWTS has been recognised.

4.1.2 SUMMARY OF ORGANISM CONCENTRATIONS IN THE UPTAKE

In general it was observed that the uptake samples taken outside the ports in navigation at the open sea contained much lower organism concentrations than those from the ports. Further it was observed that organism concentrations were always different among the three sequential samples of each uptake, hence showing a variability in organism concentrations in different natural environments and conditions (i.e., during uptakes in the ports or in navigation at the open sea).

When comparing concentrations of the two main groups of organisms (i.e., concentrations of smaller and bigger organisms) in different samples between among the five tests , a slightly negative correlation (correlation coefficient 0,07) was observed, and no clear trend could be identified (e.g., the same trend regarding higher or lower organism concentrations of bigger and smaller organisms could not be identified in any of the tests) (see Figure 32).

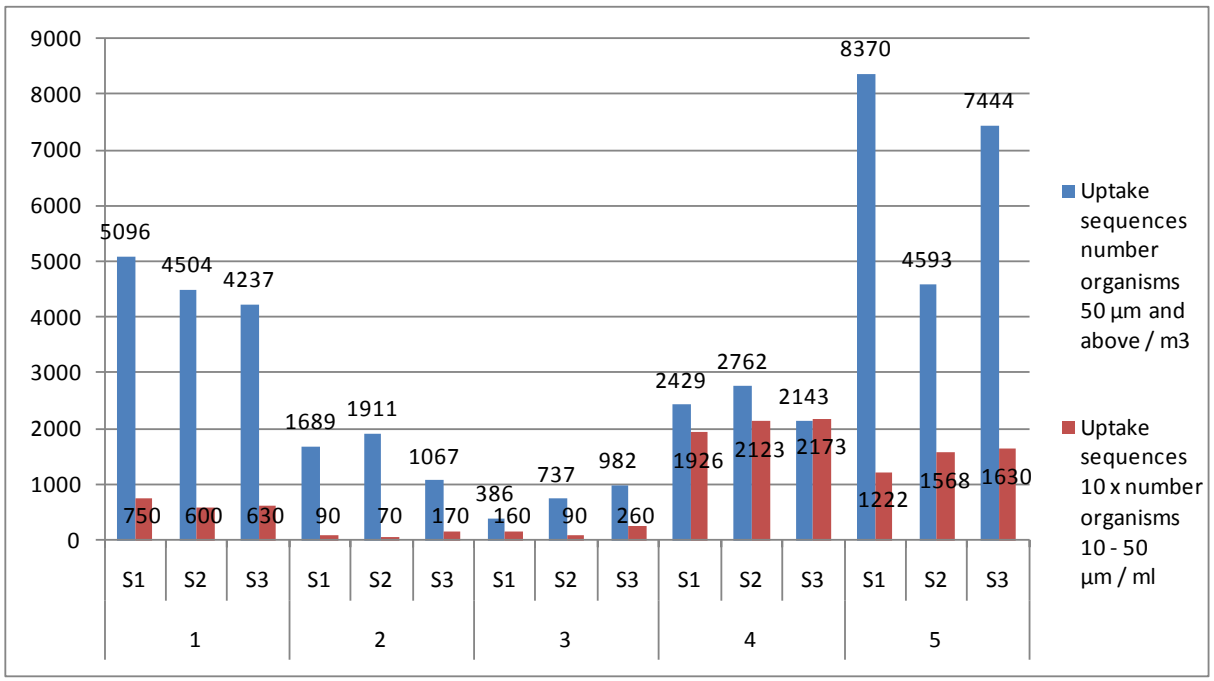


Figure 32 Concentrations of the two main groups of organisms greater than or equal to 10 micrometres in minimum dimension in the sequential sampling of five uptake tests. The number of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension was multiplied by 10 to ease the graphical comparison. S1, S2 and S3 mean sample 1, 2 and 3. Numbers 1 - 5 at the bottom indicate the test numbers.

As a conclusion, the tests showed that BWTS are exposed to ballast water where organisms in different size groups vary in concentration (e.g., when a higher concentration of bigger organisms was observed a lower concentration of smaller organisms occurred and vice versa). With this, and the fact that no living organisms were found in all samples immediately after the BWTS or on discharge from the tank, it could be concluded that the BWTS performance might not be influenced by the influent concentration of organisms. At least such an effect has not been detectable at this stage. This statement is based upon experience gained by the authors on more than 40 on-board tests of BWTS, including BWTS that make use of active

substances, and it seems that this can be attributed mainly to the efficient operation of the BWTS.

Further, it should be noted that there may also be other factors to influence the BWTS system efficiency, which may vary during ballast water uptake operations (e.g., possible difficulty of Chlorine generation in BWTS using electrolysis from low salinity water, possible reduced UV efficiency in waters with very high sediment load).

4.2 HOMOGENEITY OF ORGANISM CONCENTRATION IN THE DISCHARGE

The homogeneity, or lack of, of organism concentrations in the discharge may exist as a result of organism behaviour inside the ballast tanks (e.g., organism migration to a certain part of the tank, settling at the tank bottom). Understanding organisms behaviour or patterns in the ballast tanks is very important, as this might directly influence the results of a compliance control test, i.e., higher or lower concentrations of organisms sampled in a certain period of discharge may be a result of a non-homogeneous distribution of surviving organisms inside the tank after the treatment.

Previous studies as discussed below have shown that organism migration and its vertical concentration is relatively well investigated in natural environments, but the conditions inside ballast tanks are very different compared to natural waters. The main differences include:

- The absence of light which may confuse the orientation of organisms. It further results in un-advantageous conditions of organisms whose metabolism is based upon light, e.g. phytoplankton which may also negatively impact zooplankton by reducing its food source,
- changes of water parameters which may occur in short time intervals as e.g. the water temperature may change according to the vessel route.

To our knowledge three previous ship sampling studies have been undertaken to investigate possible plankton patchiness in ballast tanks with a focus on zooplankton. All three studies were undertaken on commercial voyages (Gollasch et al.2000, Murphy et al. 2002, Taylor et al. 2007). Gollasch et al. (2000) installed hoses at 5, 10 and 15 m depth inside a 20 m high ballast tank prior to tank filling with water to evaluate whether or not zooplankton accumulates at certain depths inside a ballast tank. In daily sampling events during this 4 day voyage, a hand-pump was used to extract water from the tank by connecting it to the pre-installed hoses. During the first two sampling days the zooplankton numbers (mainly copepods) were much higher in the sample taken at the tank surface (more than 7500 organisms per 1000 L in the surface sample versus less than 500 organisms per 1000 L in the samples taken from 5, 10 and 15 m depth). On day two, the surface sampling revealed more than 2000 organisms and the samples taken from other depths contained less than 250 organisms per 1000 L. On day three and four no significant difference in organism concentration between all sampling points could be identified. It was concluded that the reduced number of organisms in the samples over time is a consequence of die-offs inside the tank, which was also observed in many other ballast water sampling studies (e.g. Rigby & Hallegraeff 1993, 1994, Fukuyo et al. 1995, Hamer et al. 1998, Dickmann & Zhang 1999, Zhang & Dickmann 1999, Olenin et al 2000, Gollasch et al 2000a, b, c, Carver et al. 2004, Murphy et al 2004, Mimura et al. 2005, David et al. 2007, Gray et al. 2007, McCollin et al. 2007a, b, 2008, Quilez-Badia et al. 2008, de Lafontaine et al. 2009, Klein et al. 2010, Seiden

et al. 2010). It was further concluded that the difference in organism numbers from the different sampling depths was possibly due to organism migrations inside the tank. It is important to note that the samples were taken via an opened manhole and, although this was covered as much as possible during the sampling event, light penetrated into the tank which may have attracted the organisms to migrate to the surface thereby possibly contributing to the much higher number of organisms in the surface layer compared to the deeper sampling points. Further, a gale with wind forces up to 8 Bf occurred on this voyage after the second day and after the gale, on day three and four, the organisms were more similar in concentration at all the sampling points. The wind-induced water movements inside the tank may have contributed to the mixing of the organisms throughout the entire tank. However, the authors concluded that this experiment showed that organisms are not always homogenously distributed inside ballast tanks.

Murphy et al. (2002) did a similar study also pumping up ballast water from different depths (0.5, 2 and 6 m) by using a pump and a suction hose lowered to certain depths via opened manholes. The tank height was 13,6 m. The authors looked at all zooplankton, but considered bivalve larvae and crab zoea larvae separately. For bivalves the distribution inside the tank at the three depths was more homogeneous compared to the crab zoea, especially in the beginning of the voyage. As observed during the study performed by Gollasch et al. (2000) a dramatic zooplankton mortality occurred over time.

The third study was undertaken by Taylor et al. (2007). Water was sampled from 0.5, 7 and 14 m of a 15 m deep tank. The study was primarily undertaken to prove the biological efficiency of ballast water exchange at sea during a trans-Pacific voyage. At the same time the heterogeneity of organism distribution in ballast tanks was shown in the control tank experiments.

All three studies showed that crustacean concentrations (mainly copepods in Gollasch et al 2000 and zoea larvae in Murphy et al 2002) generally decreased with depth. Murphy et al. 2002 speculated that the lack of light in ballast tanks may result in exposing vertically migrating species to continued “night mode”, which for many zooplankton taxa triggers migration to the top water layers. The results of this study confirm this view.

In September 2009 the authors undertook a study (Gollasch and David 2009) to evaluate different ballast water sampling methods. It was shown that the organisms were not homogenously distributed in a ballast tank. The study also compared organism concentrations when sampling over the entire pumping event versus sequential samples. In the sequential samples, it was shown that the organism concentrations were different when sampling in the beginning, middle and end and no trend could be observed, i.e. phytoplankton and zooplankton samples show the contrary results. It was further found that the samples taken over the entire pumping event contained less organism numbers compared to the sequential samples and this effect was stronger for zooplankton compared to phytoplankton organisms. In the sequential tests sequences of 5, 10 and 15 minute duration were taken and it was concluded that the 10 minute sequences seemed to be a good compromise considering logistics during sampling, including the water volume and gear handling. It was further concluded that future studies of similar objectives may be undertaken to test for sample representativeness on a wider range of vessel types.

Considering this, three basic types of samples were recognised:

1. the very beginning sample (samples sediments around the suction point in the tank and the lowest water layer in the tank);
2. the middle sample (samples all water layers other than very beginning and very end with few sediment content);
3. the very end sample (samples upper tank water layer and most sediment washed out at the very end of suction of the ballast pump).

These differences imply that depending on where in the tank different types of organisms may "accumulate" (i.e., in different water layers or sediment), different organism and sediment concentrations may be expected when sampling in that period of discharge.

As a result, a BWTS may treat water satisfactorily to the D-2 standard during ballast water uptake (Figure 33, A), but the organisms surviving the treatment process and the holding time before discharge may have similar behaviour, e.g., settle down or migrate to certain areas in the tank (Figure 33, B). This means that the organism concentration which results of sampling in that period when ballast water is being discharged from a certain tank area would be influenced possibly resulting in an overestimation of organism concentrations. Consequently, a BWTS might fail to be compliant, while sampling from any other tank area would be an underestimation. Hence the consideration of possible in-tank organism patchiness patterns in compliance monitoring tests is very important.

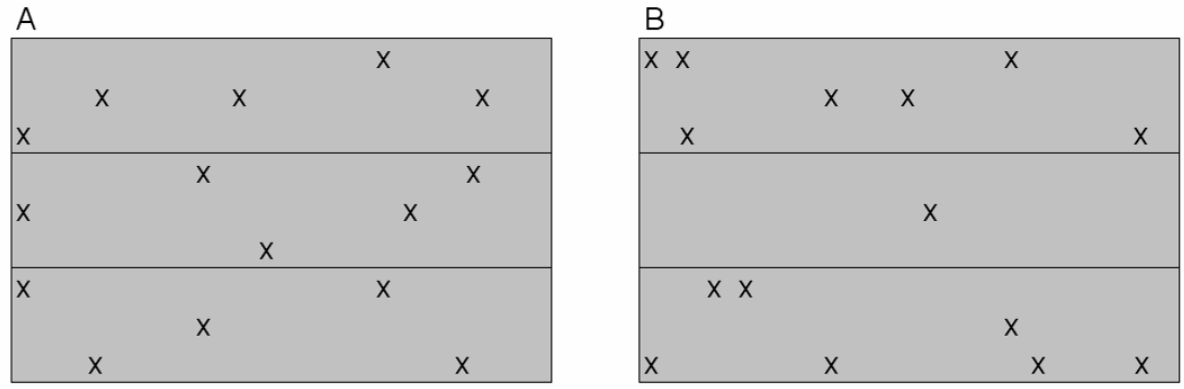


Figure 33 Hypothetical organism concentrations inside a ballast tank. A = organism distribution inside a ballast tank without organisms concentration in certain ballast tank parts, B organisms distribution inside a ballast tank showing an organism concentration at the top and bottom part of the tank. Should only the organism rich parts be sampled non-compliance may be shown due to the higher concentration of the organisms in the samples (overestimation) although when including other tank parts into the sample, compliance may have been shown.

4.2.1 DISCHARGE ORGANISMS CONCENTRATIONS IN SEQUENTIAL SAMPLING

There were 5 discharge tests conducted, one of these with treated ballast water. The treated test (test 3) resulted in zero living organisms in all discharge samples, hence this test is not to be considered in the analyses of this section.

Sequential samples were taken randomly, at the very beginning, randomly in the middle, and at the very end (see Table 2), as follows:

- **two very beginning** samples were taken in the discharge of the tests 1 and 4;
- **three very end samples** were taken in the discharge of the tests 1, 2 and 4;
- **all other** samples were taken **randomly in the middle of the discharge**, with random timings between the beginning sample, and the end sample.

4.2.1.1 Organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension

It was observed that organism concentrations were, other than in the very beginning and the middle sequential samples of test 1, always different among the three sequential samples of each discharge. There was no pattern recognised between the beginning, middle or end sequential samples Actually the highest, as well as the lowest organism concentrations were observed in each of the samples at least once during all the tests. The very beginning sample showed the highest organism concentration (along with the middle sample) in test 1, while this sample contained the lowest organism concentration in test 4. The very end sequential sample was the highest in organism concentration in test 2 and 4, while this sample showed the lowest organism concentration in test 1. The second sequential sample (compared to the very beginning or very end sample in the same test) had high organism concentration in the test 1 (and the same organism concentration as the very beginning sample), while the lowest concentration in the middle sample was observed in test 2 (see Figure 34).

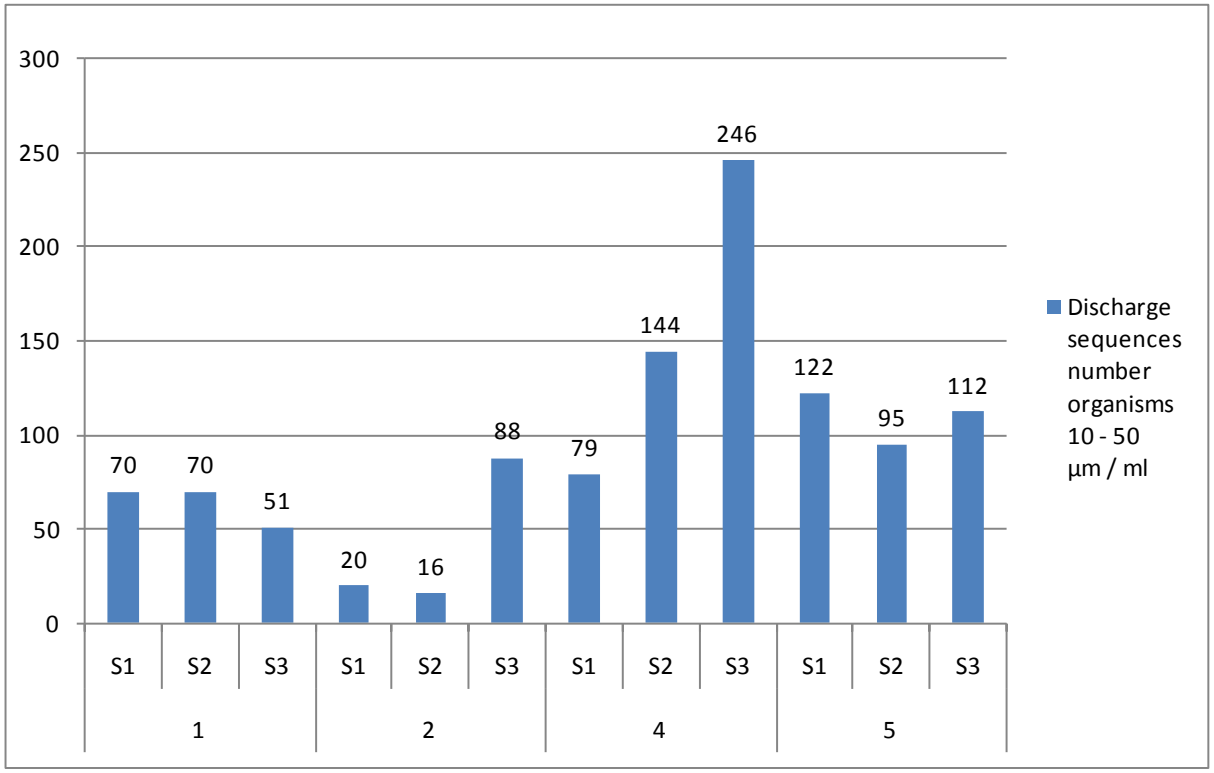


Figure 34 Concentrations of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension in the sequential samples of the four discharge tests. S1, S2 and S3 mean sample 1, 2 and 3. Numbers 1 - 5 at the bottom refer to test numbers.

When comparing the "stability" of the results among the three sequential samples, the lowest differences in organism concentrations compared among them and compared to the average of the samples in each test, were observed in the middlesample and in the very beginning sequential sample (other than in test 4), and the highest organism concentration was observed in the very end sample (up to 113 % in test 2, and ~ 4 x more than the other sequential samples) (see Table 11).

Table 11 Discharge organisms concentrations for organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension during sequential sampling, highlighting the differences among these and the mean organism concentration per test in numbers and percentage. Yellow coloured squares are the very beginning sequential sample, orange coloured are very end sequential sample. S1, S2 and S3 mean sample 1, 2 and 3.

Test No.	Sample type	Discharge sequences number organisms 10 - 50 µm / ml	Discharge sequences mean values	Difference between sequences and mean values (No. org)	Difference between sequences and mean values (%)
	S1	70		6	10
	S2	70		6	10
1	S3	51	64	-13	-20
	S1	20		-21	-52
	S2	16		-25	-61
2	S3	88	41	47	113
	S1	79		-77	-49
	S2	144		-12	-8
4	S3	246	156	89	57
	S1	122		12	11
	S2	95		-15	-13
5	S3	112	110	2	2

From these results it can be concluded that in compliance monitoring, which would be based on organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension, the most stable results could be expected to be obtained from random samples in the middle of the sampling sequence. Tests based on, or including, the very beginning, and especially the very end sequential samples would not be recommended, as these show highest variation in results

4.2.1.2 Organisms greater than or equal to 50 micrometres in minimum dimension

It was observed that organism concentrations were always different among the three sequential samples of each discharge, however a general pattern was recognised. The concentrations of organisms increased in all tests from the beginning to the end sample and did not show much difference between the sequential samples at the very beginning of the sampling procedure and:

- those taken some 10 min after the start of the pumping event;
- the very end of the sequential sampling procedure; or,

- those taken some 10 min before the very end of the pumping event.

The exception from this "rule" was in the very end sequential sample of test 4 which had the lowest organism concentration among that test sequence. Considering the sample specifics of the end sample of test 4 it was recognised that the lower organism concentration may have also been influenced by a very high concentration of sediment in that sample, and also the smaller sample water quantity compared to the other two samples - the tank was empty earlier than expected and the sampling had to stop. This pattern would also confirm that bigger organisms might be present in higher concentrations in the upper water levels of the tank as a consequence of organism migration, and are therefore found in higher concentrations in the end sample (see Figure 35).

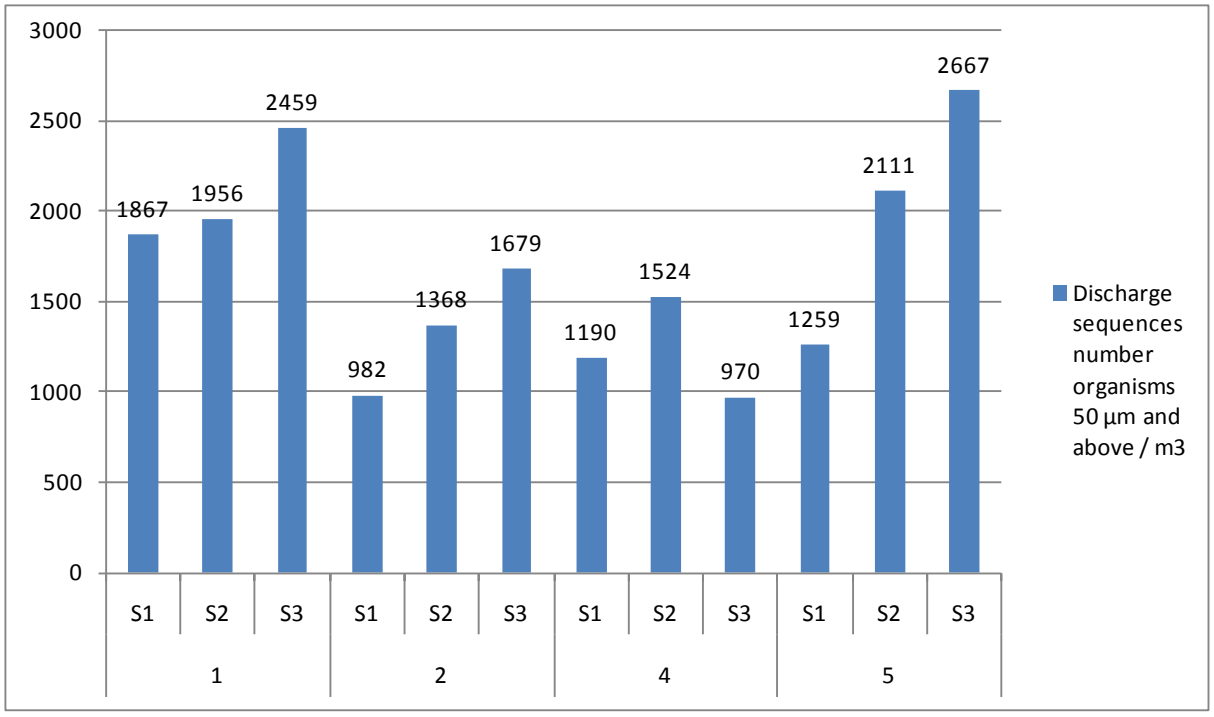


Figure 35 Concentrations of organisms greater than or equal to 50 micrometres in minimum dimension in the samples of the four discharge tests. S1, S2 and S3 mean sample 1, 2 and 3. Numbers 1 - 5 at the bottom refer to test numbers.

When comparing the "stability" of the results among the three samples taken during sequential sampling , the lowest organism concentrations may be expected in the beginning sample, and the highest in the end sample. The organism concentrations in the beginning sample, when compared to the average of the sequence, in each test (with the exception of test 4) show that there is an 11 to 37% lower organism concentration, whilst during the end sample there is a 17 to 33 % higher organism concentration. (see Table 12).

Table 12 Discharge organisms concentrations for organisms greater than or equal to 50 micrometres in minimum dimension highlighting the differences among these and the mean organism concentration per test in numbers and percentage. Yellow coloured squares are the very beginning sequential sample, orange coloured are very end sequential sample..

Test No.	Sample type	Discharge sequences number organisms 50 µm and above / m3	Discharge sequences mean values	Difference between sequences and mean values (No. org)	Difference between sequences and mean values (%)
	S1	1867		-227	-11
	S2	1956		-138	-7
1	S3	2459	2094	365	17
	S1	982		-361	-27
	S2	1368		25	2
2	S3	1679	1343	336	25
	S1	1190		-38	-3
	S2	1524		296	24
4	S3	970	1228	-258	-21
	S1	1259		-753	-37
	S2	2111		99	5
5	S3	2667	2012	654	33

From these results it can be concluded that for compliance monitoring based on organisms greater than or equal to 50 micrometres in minimum dimension, the most stable results could be expected to be obtained from the random middle samples. The sequential samples sampled from the first third of the ballast water discharge from a tank could be expected to be an underestimation, and an overestimated organism concentration may be expected when sampling from the last third of the discharge from a tank.

4.2.2 SUMMARY OF ORGANISM CONCENTRATIONS IN THE DISCHARGE

In general it was observed that the organism concentrations in the discharge samples from a ballast water tank were always different among the three sequential samples of each test, therefore indicating a non-homogenous distribution of organisms in the tank.

When comparing concentrations of the two main groups of organisms (i.e., concentrations of smaller and bigger organisms) between different sequential samples, a negative correlation (correlation coefficient -0.29) was observed, showing that the organism concentrations of these two groups frequently tend to have opposite trends (see Figure 36).

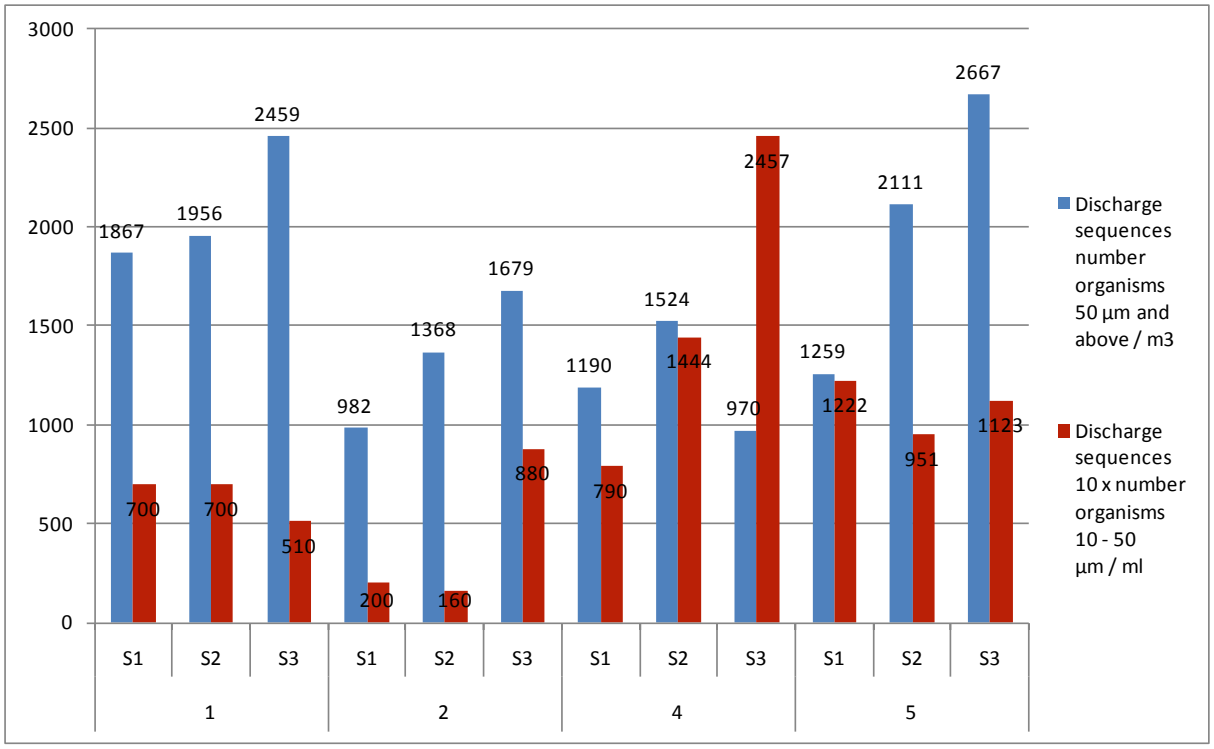


Figure 36 Concentrations of the two main groups of organisms greater than or equal to 10 micrometres in minimum dimension in the sequential samples of the four discharge tests. The number of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension was multiplied by 10 to ease the graphical comparison. S1, S2 and S3 mean sample 1, 2 and 3. Numbers 1 - 5 at the bottom refer to test numbers.

As a conclusion, the tests showed that there is heterogeneity in plankton distribution in ballast water tanks, and organisms in different size groups vary in concentration (e.g., when a higher concentration of bigger organisms was observed a lower concentration of smaller organisms occurred and vice versa). Hence, sampling at discharge is biased by tank patchiness of organisms.

4.3 REPRESENTATIVENESS OF SAMPLES OVER ENTIRE TIME VERSUS SEQUENTIAL SAMPLING

To test the representativeness of sampling over entire time, continuous sampling from the very beginning to the very end of the pumping time was conducted, and results compared to the samples taken in the random sequences. Samples over the entire time were taken for sampling durations of approximately one to two hours sampling time.

The main concept was to identify if there are, and what are the differences of longer sampling time and bigger sampled water quantities vs. shorter sampling times and lower sampled water quantities. In general the sampling process is thought to be stressful for organisms hence longer time sampling may negatively affect organism survival, while on the other hand shorter sampling times are thought not be statistically representative.

To obtain samples over the entire time and during sequential samples after the sampling point, the flow was split in two equal flows and subjected to identical conditions of sampling (e.g., the same length of the hose from the split, identical hoses, flow meters and other sampling elements), to obtain, as much as possible, identical conditions in the parallel samples.

4.3.1 SAMPLING TIMES AND QUANTITIES

The sampling duration over the entire time ranged from 55 min to 1h 47 min, with sampling flow rates from 38 to 48 litres/min and sampled quantities from 1763 to 3287 litres. In tests 1 to 4 the sequential sampling was conducted in 10 min periods with flow rates ranging from 30 to 45 litres/min, and sampled quantities between 300 to 450 litres. However in test 5 samples were collected over 15 min periods with flow rates of 30 litres/min, hence collecting 450 litre samples. The very end samples of test 2 and 4 were respectively shorter and longer than planned because of the difficulty to predict the exact end time of the sampling event.

Sampling times and water quantities of sampling over the entire time and in sequences are presented in the Table 13. Samples where zero organisms were detected (test 3 after the BWTS and at discharge) are excluded.

Table 13 Sampling times and water quantities of samples during the entire time and sequential samples. . S1, S2 and S3 mean sample, OET = samples taken over the entire time, UPT = uptake samples, DISCH = discharge samples.

Sample		Time				Quantity		
Test No.	Sample type	Start time [h:min]	End time [h:min]	Sampling time [h:min]	Time between sequences [h:min]	Quantity sampled [litre]	Sampling flowrate [litre/min]	Quantity of sequence vs. Entire sample [%]
1	UPT, S1	16:05	16:15	0:10	0:00	450	45	17
1	UPT, S2	16:25	16:35	0:10	0:10	450	45	17
1	UPT, S3	16:50	17:00	0:10	0:15	450	45	17
1	UPT, OET	16:05	17:05	1:00	0:05	2595	43	
1	DISCH, S1	16:40	16:50	0:10	0:00	450	45	16
1	DISCH, S2	17:05	17:15	0:10	0:15	450	45	16
1	DISCH, S3	17:30	17:40	0:10	0:15	450	45	16
1	DISCH, OET	16:40	17:40	1:00	0:00	2869	48	
2	UPT, S1	08:50	09:00	0:10	0:05	300	30	13
2	UPT, S2	09:10	09:20	0:10	0:10	300	30	13
2	UPT, S3	09:40	09:50	0:10	0:20	300	30	13
2	UPT, OET	08:45	10:00	1:15	0:10	2324	31	
2	DISCH, S1	08:55	09:05	0:10	0:10	380	38	15
2	DISCH, S2	09:12	09:22	0:10	0:07	380	38	15
2	DISCH, S3	09:50	10:05	0:15	0:28	540	36	21
2	DISCH, OET	08:45	10:05	1:20	0:00	2562	32	
3	UPT, S1	15:10	15:20	0:10	0:05	380	38	12
3	UPT, S2	15:45	15:55	0:10	0:25	380	38	12
3	UPT, S3	16:05	16:15	0:10	0:10	380	38	12
3	UPT, OET	15:05	16:35	1:30	0:20	3287	37	
4	UPT, S1	10:02	10:12	0:10	0:04	350	35	15
4	UPT, S2	10:25	10:35	0:10	0:13	350	35	15
4	UPT, S3	10:54	11:04	0:10	0:19	350	35	15
4	UPT, OET	9:58	11:05	1:07	0:01	2381	36	
4	DISCH, S1	10:43	10:53	0:10	0:00	350	35	20
4	DISCH, S2	11:03	11:13	0:10	0:10	350	35	20
4	DISCH, S3	11:29	11:38	0:09	0:16	292	32	17
4	DISCH, OET	10:43	11:38	0:55	0:00	1763	32	
5	UPT, S1	16:45	17:00	0:15	0:03	450	30	14
5	UPT, S2	17:21	17:36	0:15	0:21	450	30	14
5	UPT, S3	17:59	18:14	0:15	0:23	450	30	14
5	UPT, OET	16:42	18:29	1:47	0:15	3243	30	
5	DISCH, S1	15:51	16:06	0:15	0:10	450	30	14
5	DISCH, S2	16:16	16:31	0:15	0:10	450	30	14
5	DISCH, S3	16:42	16:57	0:15	0:11	450	30	14
5	DISCH, OET	15:41	17:17	1:36	0:20	3105	32	

4.3.2 ORGANISM CONCENTRATIONS

To assess the representativeness of samples over entire time, concentrations of organisms in these samples were compared to the sequential samples. In this comparison, uptake and discharge samples are taken into account equally, resulting in 9 tests being observed (5 uptake and 4 discharge tests).

4.3.2.1 Organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension

When comparing samples over the entire time to the individual sequential samples it was observed that in 5 tests (55%) the highest concentration of organisms was found in samples over entire time, and in 4 tests (45%) the highest concentrations were observed in the sequential sampling tests. In 2 tests (22%) (test 2 discharge and test 3 uptake) one sequential sample had the highest concentration and in the other 2 tests (22%) (test 1 discharge and test 4 discharge), 2 samples had higher concentration than the sample over entire time. There was no test where the lowest organism concentration was found in the sample over the entire time (see Figure 37).

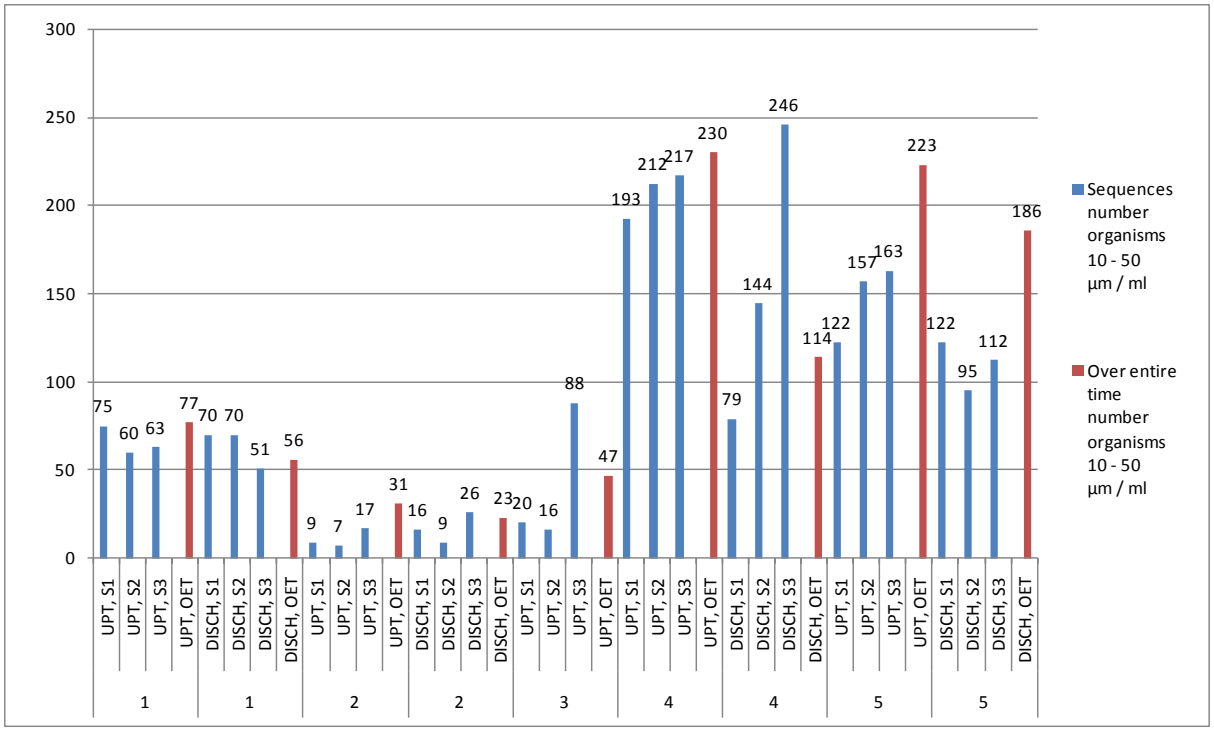


Figure 37 Concentrations of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension. UPT = uptake; DISCH = discharge; OET = over entire time; S1, S2 and S3 mean sample 1, 2 and 3. Numbers 1 - 5 at the bottom refer to test numbers.

Based on these results no clear organism concentration trend could be observed. When comparing samples over the entire time with single samples, a small majority of tests (55%) showed higher organism concentrations in the samples over the entire time.

When comparing the "stability" of the results it was observed that the concentration of organisms in the sequential sampling compared to concentrations in samples over the entire time of the same tests, ranged from being 77% lower (less than 1/3) in sample 2 of the uptake in test 2, to being 116% higher (more than double) in sample 3 of the uptake in test 2 . On average, sequential sampling contained lower concentrations of organisms in 7 of 9 tests (78%), ranging from being 12% to 65% lower. In two tests (22%) (discharge tests 1 and 4), the average organism concentrations in the sequential sampling were 14% and 38% higher than the concentrations in the samples over entire time (see Table 14).

Table 14 Concentrations of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension, and the differences in numbers and percentage between the sequential and over entire time samples. UPT = uptake; DISCH = discharge; OET = over entire time; SEQ AV = sample average; S1, S2 and S3 mean sample 1, 2 and 3.

Test No.	Sample type	Total number organisms 10 - 50 µm / ml	Difference between sequence and OET (No. org)	Difference between sequence and OET (%)
	UPT, S1	75	-2	-3
	UPT, S2	60	-17	-22
	UPT, S3	63	-14	-18
	UPT, SEQ AV	66	-11	-14
1	UPT, OET	77		
	DISCH, S1	70	14	25
	DISCH, S2	70	14	25
	DISCH, S3	51	-5	-9
	DISCH, SEQ AV	64	8	14
1	DISCH, OET	56		
	UPT, S1	9	-22	-71
	UPT, S2	7	-24	-77
	UPT, S3	17	-14	-45
	UPT, SEQ AV	11	-20	-65
2	UPT, OET	31		
	DISCH, S1	20	-27	-57
	DISCH, S2	16	-31	-66
	DISCH, S3	88	41	87
	DISCH, SEQ AV	41	-6	-12
2	DISCH, OET	47		
	UPT, S1	16	-7	-30
	UPT, S2	9	-14	-61
	UPT, S3	26	3	13
	UPT, SEQ AV	17	-6	-26
3	UPT, OET	23		
	UPT, S1	193	-37	-16
	UPT, S2	212	-17	-8
	UPT, S3	217	-12	-5
	UPT, SEQ AV	207	-22	-10
4	UPT, OET	230		
	DISCH, S1	79	-35	-30
	DISCH, S2	144	31	27
	DISCH, S3	246	132	116
	DISCH, SEQ AV	156	43	38
4	DISCH, OET	114		
	UPT, S1	122	-101	-45
	UPT, S2	157	-67	-30
	UPT, S3	163	-60	-27
	UPT, SEQ AV	147	-76	-34
5	UPT, OET	223		
	DISCH, S1	122	-64	-34
	DISCH, S2	95	-91	-49
	DISCH, S3	112	-74	-40
	DISCH, SEQ AV	110	-77	-41
5	DISCH, OET	186		

In general higher concentrations of organisms were observed in samples taken over the entire time, leading to the conclusion that short sampling times may, for the size group of small organisms, result in the underestimation of the real concentration of viable organisms.

Attempting to understand what leads to these results, some main parameters were recognised. Firstly, the sample processing in sequential and over the entire time samples is identical, i.e., the quantity of the sequential and over entire time samples is approximately the same, about 5 - 6 litres of unconcentrated sample were collected constantly over the sampling time, and after mixing and unconcentrated sample was put in 80 ml bottles. The sample collection is conducted with a jar from the sample flow and emptying the jar into a bucket, which seems not to be very stressful for these organisms.

Secondly, in this size group of organisms, almost all organisms counted are phytoplankton organisms which were analysed with flowcytometry and verified by PAM measurements (i.e., more than 99% of the organisms were phytoplankton). The remaining part of counted organisms are those found in concentrated samples analysed under a stereomicroscope (i.e., zooplankton), where very few organisms below 50 micrometres were recognised with viability determined. Their influence on the concentration of organisms in a sample was very low, as these are counted in concentrated samples of several hundred litres, while the concentration of organisms in this size group is calculated in number of organisms/ml according to the D-2 standard of the BWM Convention. Through our previous studies, and especially a test undertaken in the framework of the Interreg IVB Project "Ballast Water Opportunity", we noticed that phytoplankton can also survive longer times (even more than one month) in an enclosed space like a sample bottle, hence it is assumed that the sampling time difference (~50min to 1h 30min) does not significantly influence the result (Figure 38).

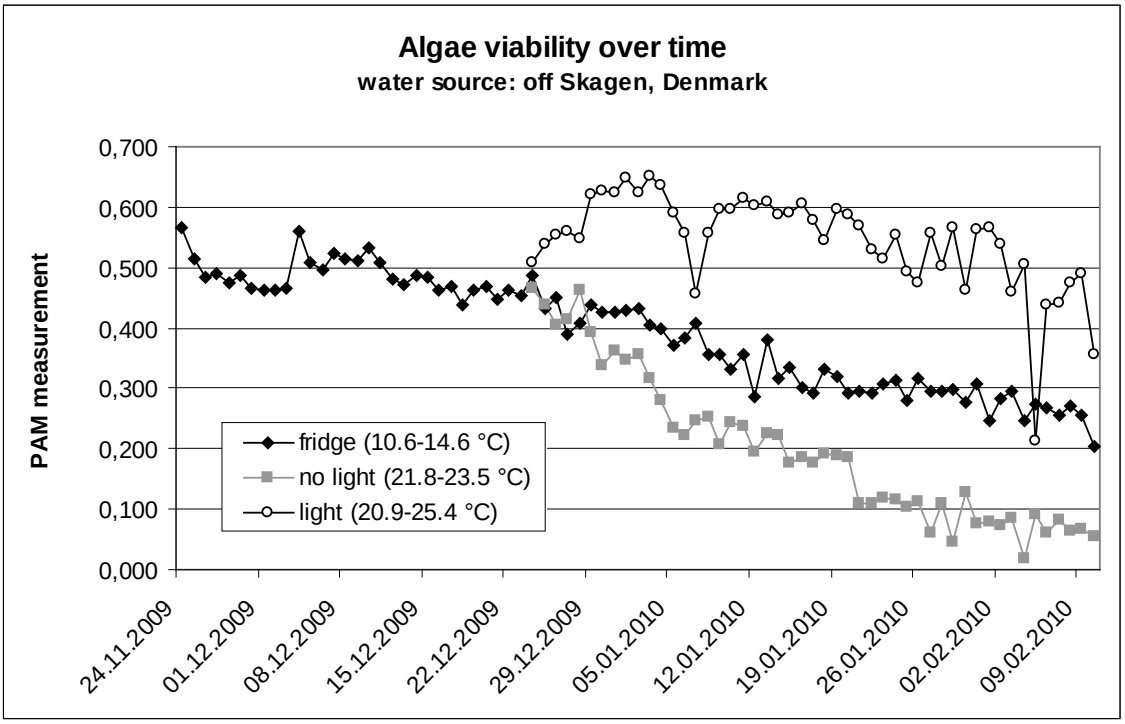


Figure 38 Long-term algae viability of a ballast water sample. The samples were stored under different conditions in 1 litre sample bottles and analysed daily (PAM viability measurement) (Modified after Gollasch 2010).

Figure 38 shows the viability measurements over the entire observation time during this experiment. Algae survival was documented to last up to 79 days. It seems that during the first 4 weeks of the experiment the viability remains almost unchanged with a slight downwards trend. In the continuing period the sample was split and organisms in all three sub-samples showed a stronger downwards viability trend. The strongest viability reduction was measured for the sample stored without light at room temperature. It was concluded that a sample storage time of up to two weeks in a larger volume bottle stored in a dark and cool environment has little influence on the organism viability (Gollasch 2010).

As a result, it can be concluded that in this size group of organisms the sampling process and sample storage does not much influence the organism concentration difference between sequential (shorter sampling) and over the entire time (longer sampling) samples. However, factors influencing the differences between these could not be clearly identified.

4.3.2.2 Organisms greater than or equal to 50 micrometres in minimum dimension

When comparing samples over the entire time to the individual sequential samples it was observed that in all 9 tests, much lower concentrations of organisms were identified in samples taken over the entire time. Actually, in all the tests, all but one of the sequential samples (sample 3 of test 4 discharge) contained much higher concentrations of organisms than samples over the entire time (see Figure 39).

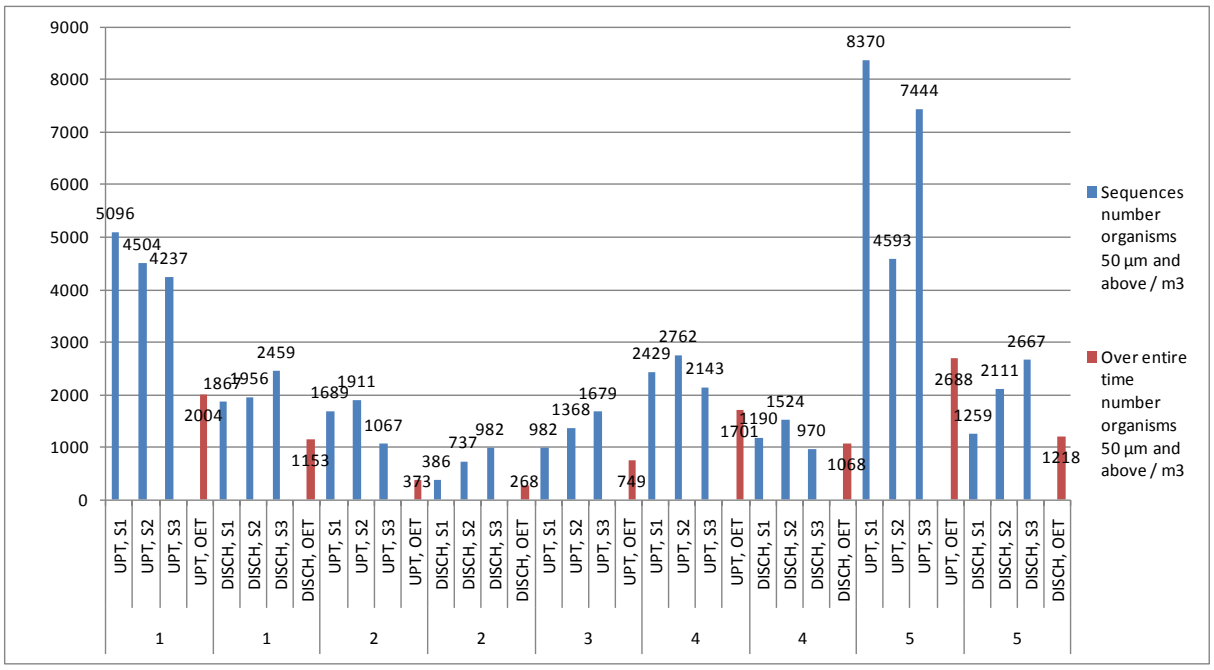


Figure 39 Concentrations of organisms greater than or equal to 50 micrometres in minimum dimension. UPT = uptake; DISCH = discharge; OET = over entire time; S1, S2 and S3 mean sample 1, 2 and 3. Numbers 1 - 5 at the bottom refer to test numbers.

Based on these results it was recognized that much higher concentrations of organisms were observed in almost all sequential samples.

When comparing the "stability" of the results it was noted that the concentration of organisms in the sequential samples compared to the concentration in samples over the entire time of the same tests, range from being 3% to 412% (more than four times) higher, only sample 3 of test 4 discharge had 9% lower organism concentration compared to the over the entire time sample of this test. In average of all 9 tests, the sequential samples contained higher concentrations of organisms ranging from 15 % to 315% (more than three times) (see Table 15).

Table 15 Concentrations of organisms greater than or equal to 50 micrometres in minimum dimension, and the differences in numbers and percentage between the sequential and over entire time samples. UPT = uptake; DISCH = discharge; OET = over entire time; SEQ AV = average of sequential sampling; S1, S2 and S3 mean sample 1, 2 and 3. Numbers 1 - 5 at the bottom refer to test numbers.

Test No.	Sample type	Total number organisms 50 µm and above / m3	Difference between sequence and OET (No. org)	Difference between sequence and OET (%)
	UPT, S1	5096	3092	154
	UPT, S2	4504	2500	125
	UPT, S3	4237	2233	111
	UPT, SEQ AV	4612	2608	130
1	UPT, OET	2004		
	DISCH, S1	1867	714	62
	DISCH, S2	1956	803	70
	DISCH, S3	2459	1307	113
	DISCH, SEQ AV	2094	941	82
1	DISCH, OET	1153		
	UPT, S1	1689	1316	353
	UPT, S2	1911	1538	412
	UPT, S3	1067	694	186
	UPT, SEQ AV	1556	1183	317
2	UPT, OET	373		
	DISCH, S1	386	118	44
	DISCH, S2	737	469	175
	DISCH, S3	982	715	267
	DISCH, SEQ AV	702	434	162
2	DISCH, OET	268		
	UPT, S1	982	233	31
	UPT, S2	1368	619	83
	UPT, S3	1679	930	124
	UPT, SEQ AV	1343	594	79
3	UPT, OET	749		
	UPT, S1	2429	728	43
	UPT, S2	2762	1061	62
	UPT, S3	2143	442	26
	UPT, SEQ AV	2444	743	44
4	UPT, OET	1701		
	DISCH, S1	1190	122	11
	DISCH, S2	1524	456	43
	DISCH, S3	970	-98	-9
	DISCH, SEQ AV	1228	160	15
4	DISCH, OET	1068		
	UPT, S1	8370	5683	211
	UPT, S2	4593	1905	71
	UPT, S3	7444	4757	177
	UPT, SEQ AV	6802	4115	153
5	UPT, OET	2688		
	DISCH, S1	1259	41	3
	DISCH, S2	2111	893	73
	DISCH, S3	2667	1448	119
	DISCH, SEQ AV	2012	794	65
5	DISCH, OET	1218		

As a result, much higher concentrations of organisms were observed in the sequential samples, leading to a conclusion that longer sampling times may in most cases result in the underestimation of the real concentration of viable organisms being discharged.

When trying to understand what causes this, some main parameters were recognised. The predominant type of organisms in this group are zooplankton, of which many are fragile species. The sampling process may have an impact on such organisms as concentrating a sample may result in (much) higher organism concentrations than in nature. Consequently the organisms will likely hit each other when moving. Also, during longer sampling durations, the organisms are exposed to the sampling process which may result in unnatural movements (spinning around in the sample bottle) and pressure effects may occur due to the on going sampling process. This is amplified in cases when larger volumes of water are concentrated and during longer sampling events. The high concentration of organisms may cause organism stress and mortality during the sampling process and during sample storage before analysis (crowding effect).

Based on these findings it can be concluded that in this size group of organisms the main factor influencing the results is the sampling process and sampling duration. The longer the sampling process is, the more organisms will die, resulting in a strong underestimation of organism concentration in the discharge.

4.4 "INSTANTANEOUS" OR "AVERAGE" TESTING SAMPLING PROTOCOL

The main question here is to identify which sampling approach may be more representative of the whole discharge. The first important element to be considered here is representativeness of "one-point-in-time" short time sampling, i.e., "instantaneous", compared to an "average" sampling. In average sampling two different basic approaches are to be considered, i.e., sampling over entire time and the average of sequential sampling results. Please note that test 3 was excluded in the following analysis as no living organisms were found in the discharge of the treated water (see above).

4.4.1 REPRESENTATIVENESS OF AN INSTANTANEOUS SAMPLE

Following the results of the sequential samples tests, when considering the results in single sequential samples for concentrations of smaller and bigger organisms, relatively high variations in organism concentrations are shown ranging from underestimations to overestimations.

Based on this it can be concluded that an instantaneous sample cannot be representative of the whole ballast water discharge. However, instantaneous samples in some terms can give a very solid indication, i.e., clear grounds, that a BWTS is not compliant (more in chapter on indicative sampling).

4.4.2 REPRESENTATIVENESS OF SEQUENTIAL SAMPLES AVERAGE

The sequential samples average result means the average concentration of organisms from two or more sequential samples. This analysis is focused on representativeness of sampling for compliance control, hence only discharge tests are considered. The average concentration of sequential samples is compared with the organism concentrations in samples taken over the entire time.

4.4.2.1 Organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension

The analyses of average concentrations of **3 random** samples compared to samples over the entire time in the discharge tests of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension, show lower organism concentrations, ranging from 12% to 41%, in 50% of the tests, and higher organism concentrations, ranging from 14% to 38%, in the other 50% (see Table 16).

Table 16 Concentrations of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension in over entire time, the average of 3 sequential samples, and the differences in percentage between these. DISCH = discharge; 3 SEQ AV = average of sequential sampling; OET = over entire time; S1, S2 and S3 mean sample 1, 2 and 3.

Test No.	Sample type	Total number organisms 10 - 50 µm / ml	Difference between sequence and OET (%)
1	DISCH, 3 SEQ AV	64	14
	DISCH, OET	56	
2	DISCH, 3 SEQ AV	41	-12
	DISCH, OET	47	
4	DISCH, 3 SEQ AV	156	38
	DISCH, OET	114	
5	DISCH, 3 SEQ AV	110	-41
	DISCH, OET	186	

The results show that there is almost the same probability to sample lower or higher concentrations of organisms in the **average of 3 sequential samples** or in **the sample over entire time**, as the results are similar. Based on this it can be concluded that in the size group of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension the average organism concentration of 3 samples can be representative of the whole discharge in the same way as the sample over entire time. A further statistical analysis of the 3 random sequences was impossible due to the low number of test runs. However, the following chapters address the basic statistical analysis when comparing organism concentrations of 2 random sequential samples and the sample taken over the entire time.

The average organism concentrations of **2 random sequential samples** was also calculated to document possible representativeness. To obtain the average values of 2 of the samples in each sequential sample, two different simulations were conducted:

1. the sample with the **second highest organism concentration was excluded** from the average calculations to follow the logic of averaging random samples with the highest and the lowest value (see Table 17); and
2. following the findings of the sequential samples tests and to possibly avoid **tests with samples at the very beginning and end of the discharge**, these were excluded if possible (i.e., in test 1 and 2, while in test 4 which included both samples at the very beginning and end , the very beginning sample is excluded, and in test 5 which had no very beginning and end samples, the end sample was chosen to obtain a balance between beginning and end samples (see Table 18)).

The analyses of average organism concentrations of **2 random sequential samples** (with the second highest concentration sample excluded) compared to samples over entire time of the discharge tests of smaller organisms show a 42% lower concentration in 1 test, while higher concentration ranging from 8% to 43% were identified in the other 3 discharge tests (see Table 17).

Table 17 Concentrations of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension in over entire time and the average of 2 sequential samples, and the differences in percentage between these. DISCH = discharge; 2 SEQ AV = average of 2 samples from the sequential sampling OET = over entire time; S1, S2 and S3 mean sample 1, 2 and 3. Samples with the second highest organism concentrations (red colour shading) were excluded from average calculation.

Test No.	Sample type	Total number organisms 10 - 50 µm / ml	Difference between sequence and OET (%)
	DISCH, S1	70	25
	DISCH, S2	70	25
	DISCH, S3	51	-9
	DISCH, 2 SEQ AV	61	8
1	DISCH, OET	56	
	DISCH, S1	20	-57
	DISCH, S2	16	-66
	DISCH, S3	88	87
	DISCH, 2 SEQ AV	52	11
2	DISCH, OET	47	
	DISCH, S1	79	-30
	DISCH, S2	144	27
	DISCH, S3	246	116
	DISCH, 2 SEQ AV	162	43
4	DISCH, OET	114	
	DISCH, S1	122	-34
	DISCH, S2	95	-49
	DISCH, S3	112	-40
	DISCH, 2 SEQ AV	109	-42
5	DISCH, OET	186	

Furthermore, the analyses of the average organism concentrations of **2 random** sequential samples` (with the very beginning or very end sample excluded) compared to samples over the entire time of the discharge tests, from tests on the group of smaller organisms, show lower organism concentrations ranging from 42% to 62% in 50% of tests, while higher organism concentration ranging from 8% to 72% were identified in the other 50% of discharge tests (see Table 18).

Table 18 Concentrations of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension in over entire time, the sequential samples and the average of 2 of the sequential samples, and the differences in percentage between these. DISCH = discharge; 2 SEQ AV = average of 2 samples from the sequential sampling; OET = over entire time; S1, S2 and S3 mean sample 1, 2 and 3. Samples with red colour shading (very beginning or very end) were excluded from average calculation. Yellow coloured are samples from the very beginning of the discharge, orange coloured are from the very end.

Test No.	Sample type	Total number organisms 10 - 50 µm / ml	Difference between sequence and OET (%)
	DISCH, S1	70	25
	DISCH, S2	70	25
	DISCH, S3	51	-9
	DISCH, 2 SEQ AV	61	8
1	DISCH, OET	56	
	DISCH, S1	20	-57
	DISCH, S2	16	-66
	DISCH, S3	88	87
	DISCH, 2 SEQ AV	18	-62
2	DISCH, OET	47	
	DISCH, S1	79	-30
	DISCH, S2	144	27
	DISCH, S3	246	116
	DISCH, 2 SEQ AV	195	72
4	DISCH, OET	114	
	DISCH, S1	122	-34
	DISCH, S2	95	-49
	DISCH, S3	112	-40
	DISCH, 2 SEQ AV	109	-42
5	DISCH, OET	186	

Results show that there is almost the same probability to have lower or higher concentration of organisms in the **average of 2 sequential samples** or in the sample over entire time, as well as a very similar difference between the results. Based on this it can be concluded that in the size group of smaller organisms the average concentration of 2 of the samples in the sequential samples can be representative of the whole discharge the same way as the sample over entire time.

4.4.2.2 Organisms greater than or equal to 50 micrometres in minimum dimension

The analyses of average organism concentrations of **3 random** sequential samples compared to samples over the entire time of the discharge tests for the group of bigger organisms always show a higher concentration, ranging from 15% to 82% (see Table 19).

Table 19 Concentrations of organisms greater than or equal to 50 micrometres in minimum dimension over the entire time and the average of sequential samples, and the differences in percentage between these. DISCH = discharge; 3 SEQ AV = average of 3 samples; OET = over entire time.

Test No.	Sample type	Discharge sequences number organisms 50 µm and above / m3	Difference between sequence and OET (%)
1	DISCH, 3 SEQ AV	2094	82
	DISCH, OET	1153	
2	DISCH, 3 SEQ AV	1343	79
	DISCH, OET	749	
4	DISCH, 3 SEQ AV	1228	15
	DISCH, OET	1068	
5	DISCH, 3 SEQ AV	2012	65
	DISCH, OET	1218	

The results show that there were always higher concentrations of organisms in the **average of 3 sequential samples** than in the sample over entire time, leading to the assumption that samples over the entire time are underestimating the quantity of organisms discharged. Based on this it can be concluded that in the size group of bigger organisms the average concentration of the 3 samples in the sequential samples are representative of the whole discharge.

The analyses of average organism concentrations of **2 random** sequential samples (with the sample with the second highest concentration excluded) compared to samples over the entire time of the discharge tests in the group of bigger organisms always show higher organism concentrations in the averaged samples, ranging from 17% to 88% (see Table 20).

Table 20 Concentrations of organisms greater than or equal to 50 micrometres in minimum dimension over entire time, from sequential sampling and the average of the 3 sequential samples, and the differences in percentage between these. DISCH = discharge; 3 SEQ AV = average of 3 samples; OET = over entire time; S1, S2 and S3 mean sample 1, 2 and 3. Samples with the second highest organism concentrations (red colour shading) were excluded from average calculation.

Test No.	Sample type	Total number organisms 10 - 50 µm / ml	Difference between sequence and OET (%)
	DISCH, S1	1867	62
	DISCH, S2	1956	70
	DISCH, S3	2459	113
	DISCH, 2 SEQ AV	2163	88
1	DISCH, OET	1153	
	DISCH, S1	982	31
	DISCH, S2	1368	83
	DISCH, S3	1679	124
	DISCH, 2 SEQ AV	1331	78
2	DISCH, OET	749	
	DISCH, S1	1190	11
	DISCH, S2	1524	43
	DISCH, S3	970	-9
	DISCH, 2 SEQ AV	1247	17
4	DISCH, OET	1068	
	DISCH, S1	1259	3
	DISCH, S2	2111	73
	DISCH, S3	2667	119
	DISCH, 2 SEQ AV	1685	38
5	DISCH, OET	1218	

Furthermore, the analyses of average organism concentrations of **2 random** sequential samples (with the very beginning or very end sample excluded as for the analysis with the smaller organisms) compared to samples over the entire time of the discharge tests in the group of bigger organisms always show higher organism concentrations in the averaged samples from sequential samples , ranging from 17% to 92% (see Table 21).

Table 21 Concentrations of organisms greater than or equal to 50 micrometres in minimum dimension in over the entire time, from sequential sampling and the average of 2 sequential samples, and the differences in percentage between these. DISCH = discharge; 2 SEQ AV = average of 2 samples; OET = over entire time; S1, S2 and S3 mean sample 1, 2 and 3. Samples with red colour shading (very beginning or very end) were excluded from average calculation. Yellow coloured are samples from the very beginning of the discharge, orange coloured are from the very end.

Test No.	Sample type	Total number organisms 10 - 50 µm / ml	Difference between sequence and OET (%)
1	DISCH, S1	1867	62
	DISCH, S2	1956	70
	DISCH, S3	2459	113
	DISCH, 2 SEQ AV	2207	92
	DISCH, OET	1153	
2	DISCH, S1	982	31
	DISCH, S2	1368	83
	DISCH, S3	1679	124
	DISCH, 2 SEQ AV	1175	57
	DISCH, OET	749	
4	DISCH, S1	1190	11
	DISCH, S2	1524	43
	DISCH, S3	970	-9
	DISCH, 2 SEQ AV	1247	17
	DISCH, OET	1068	
5	DISCH, S1	1259	3
	DISCH, S2	2111	73
	DISCH, S3	2667	119
	DISCH, 2 SEQ AV	1685	38
	DISCH, OET	1218	

The results show almost same pattern as those from the test with the average of the 2 samples . It was also observed that there are always higher concentrations of organisms in the **average of 2 sequential samples** than in the sample over the entire time, leading to the assumption that samples over the entire time are underestimating the quantity of organisms discharged. Based on this it can be concluded that in the size group of bigger organisms the average concentration of 2 samples from the sequential sampling are representative of the whole discharge at the same level as the average of 3 samples.

4.4.3 REPRESENTATIVENESS OF A SAMPLE OVER ENTIRE TIME

On the basis of this study a sample over the entire time means a sample that was taken over the entire pumping time, i.e. the entire discharge time for approximately 1 up to 2 hours.

The results have shown that samples over entire time were identified to be relatively representative for the smaller organisms. In contrast the size group of bigger organisms show a relatively large underestimation of the organism concentration in the samples over the entire time.

4.5 RECOMMENDATIONS FOR A BALLAST WATER SAMPLING PROTOCOL THAT IS REPRESENTATIVE OF THE WHOLE DISCHARGE

4.5.1 SAMPLES REPRESENTATIVENESS

The results of this study show that different approaches in the sampling process influence the results regarding organism concentrations. The organisms in the discharge are affected in different ways, therefore the selection of the "wrong" sampling approach may influence the compliance control result. The organism concentrations in the ballast water discharge may therefore be underestimated, and a "faulty" BWTS could be recognised as compliant. Conversely organism concentrations may be overestimated, and a BWTS complying with the D-2 Standard may fail in compliance.

Certainly, sampling results may also be influenced by the sampling and analyses methodology and gear. It is recommended that to obtain the most representative results, the same methodology and gear should be used as in this study.

It should be noted that a certain level of pragmatism is required during on-board ballast water compliance control sampling especially when larger volumes of water need to be sampled. This is especially relevant to sampling for bigger organisms, and attempts should be made to avoid negatively impairing organism survival during the sampling process. Compliance control sampling teams are unlikely to have larger water collecting tanks (>1000 litres) available during the sampling event and will probably need to work with nets to concentrate during the sampling procedure. The Ballast Water Sampling Guideline (G2) also addresses this: *sampling should be undertaken in a safe and practical manner; and samples should be concentrated to a manageable size.*

In cases where other sampling equipment than described here is used, comparison tests would be needed to ensure representativeness.

During this study it was observed that sampling duration (i.e., length of the sampling process), timing (i.e., in which point in time of the discharge the sampling is conducted), the number of samples and the sampled water quantity are the main factors that influence the results regarding organism concentrations.

4.5.1.1 Recommended sampling duration

The results show that bigger organisms are negatively affected by longer sampling times. Considering that the results show that a shorter sampling time is still representative, the **recommended** sampling time of a sample taken during the tests in a sequential **sampling** is **approximately 10 minutes**. Longer sampling times result in an underestimation of the viable organism concentration in the discharge, especially for bigger organisms.

4.5.1.2 Recommended sampling timing

The results show that organism concentrations may vary considerably if the sampling is conducted at the very beginning or at the very end of the discharge process because of the patchy distribution of organism inside ballast water tanks. It is not recommended to take a sample at the very beginning (i.e., the first 5 min) or at the very end of discharge (i.e., the last

5 min), as an underestimation as well as an overestimation of organism concentrations may be expected. Based on this it is **recommended** that the **sampling** is conducted **randomly anytime in the middle of the discharge, starting after 5 minutes from the start of discharge and ending 5 minutes before the end of the discharge.**

4.5.1.3 Recommended number of samples

The results show that organism concentrations in all organism groups vary due to the patchy distribution of organisms inside the ballast water tanks, hence a single 10 minutes sequential sample may underestimate or overestimate the concentration of organisms being discharged. The results also show that an average of organism concwbtrations of 2 random samples in a sequential sampling procedure provide very similar results to the average of the 3 random samples. Based on this it is **recommended** that sampling is conducted by undertaking at least **2 random samples**, which are **analysed immediately after each sampling event has ended**, and that the **organism concentration** results are **averaged**.

4.5.1.4 Recommended sampled quantity

In this study sequential sampling was conducted over periods of 10 and 15 minutes, with the flow rate averages ranging mainly between 30 and 45 litres per minute. To obtain most representative results it is **recommended** that:

- for the **bigger organisms 300 to 450 litres** should be filtered and concentrated;
- for the **smaller organisms** a "**continuous drip**" sample totalling to approximately **5 litres** (i.e., collect about 0,5 litre of sample water every minute during the entire sampling time duration or collect about 0.5 litre of sample water every 30 to 45 litres depending on the flow rate) should be taken. The resulting 5 litres of sample water should be **sub-sampled after mixing** in two sets of samples, one alive and another preserved. We recommend sub-sample volumes of **60 to 100 ml**;
- for the **bacteria**, a sample of approximately **1 litre** should be taken as a sub-**sample after mixing** from the 5 litre "continuous drip" sample.

4.5.1.5 Other recommendations

It is also assumed that the sampling flow rates may influence the results. Lower flow rates obtained by partially closed valves of the sampling line may damage organisms, and a similar negative effect may be caused by to strong flow rates affecting mainly the filtering process of the bigger organisms. Hence, the flow rate, or “valve” effect, may cause an underestimation of the organism concentration as organisms may die during the sampling process. To avoid this negative influence it is recommended that the valve at the sampling point is opened as much as possible, however it should not exceed the flow rate of 50 litres/min so that the water pressure is not too high during sample concentration as this may impair organism survival.

4.5.2 SAMPLING LOGISTICS FEASIBILITY

Different types, sizes and cargo profiles of vessels trigger very different ballast water discharge profiles and times. Ballast water discharge may be conducted “at once” or “in sequences”, lasting from approximately one hour (e.g., fast discharge of two tanks in parallel on container vessels), up to several days depending on the length of the cargo operation (e.g.,

tankers, bulk carriers and sometimes general cargo vessels load cargo during several days, hence is the ballast water operation frequently conducted in sequence over the time of cargo operation).

It is important to take this factor into account as it is difficult to imagine that the PSC officer and/or sampling team would stay on-board the vessel for several days. Considering the above recommendations on representative sampling, sampling of at least 2 random samples from a sequential sampling strategy is feasible and is relatively easy, while sampling over the entire time of the ballast water discharge would be very difficult if long sampling times are required (e.g., 2 to 12 hours), over several days or during night time (i.e., cargo operations are regularly conducted also in night shifts, but PSC officers may only be available at day shifts).

The challenge may become to obtain a representative sample of the whole discharge, when the vessel will be discharging ballast water from **more than one ballast water uptake location**. In such cases it is **recommended** that at least **1 sequencial sample per uptake source** is taken. If a tank was filled from multiple sources this does not trigger necessity for 2 or more samples

5 INDICATIVE SAMPLE ANALYSIS

The tenderers undertook a comprehensive Internet research on possible methods for indicative analysis of ballast water samples, and tested some of these on-board. However, this summary is not meant to deliver a fully comprehensive inventory of all methods available world-wide.

The main premises for identification of appropriate indicative sample analysis methods and tools were that it:

- should provide reliable results as grounds/proof of possible non-compliance;
- needs to deliver prompt results (in maximum 30 minutes for obtaining a result);
- should be relatively simple to apply (possibly no very specific background required for the person using it, e.g., no diploma in biology, chemistry);
- should be portable equipment for one person that the analysis can be undertaken "on the spot", i.e., at or near the sampling point possibly on the vessel;
- should not be too expensive (capital costs); and
- should be cost effective (running costs).

Nevertheless, the search was not limited to the above premises to obtain a broader perspective of "all" currently possible methods, hence hereafter we present a wider range of methods, with the identified "pros" and "cons" for each specific application.

Fundamentally different methods were identified. The section begins with generic organism detection tools to proof the presence of organisms in ballast water by DNA, RNA (DNA and RNA are both nucleic acids, but differ from each other), ATP or Chl *a* detection as well as measurements of increased oxygen content which is followed by more specific methods to assess the three organism groups as referred to in Regulation D-2 of the BWM Convention. In the end of this chapter we provide summary tables of the methods considered here.

Among all described methods, we were able to test the PAM in a greater level of detail for the application of indicative sample analysis for phytoplankton.

5.1 DNA METHODS

Deoxyribonucleic acid (DNA) is a nucleic acid which contains the genetic information needed for the development and functioning of all known living organisms. The presence of DNA may be taken as an indication of life and organisms are considered to be viable if they are capable of DNA replication.

Besides that DNA is present in all living organisms it should be noted that so called free-DNA is persisting in water and also in dead organisms. Should DNA become released from an organism after disintegration of cells, e.g. after a passage through a ballast water treatment system, this DNA may be persistent in water. Therefore, the DNA content in water cannot be taken as an indicator for living organisms at the moment of investigation, it can only indicate that life must have existed in this body of water. However, when no DNA is detected it can be assumed that no organisms are in the water, i.e. the treatment process was successful and the organism standards in Regulation D-2 are likely met.

No direct organism enumeration can be undertaken with DNA detection methods, but good correlations are known between DNA content measurements and quantitative organism culturing. Qualitative validation studies have shown that a > 0.950 correlation exists when comparing DNA contents with classical culturing methods (Jansen, AquaExplorer, pers. comm.). Further, DNA detection can be done on genus or species level thereby proving the DNA presence of selected (target) species or indicator microbes as stated in the Regulation D-2 (see below).

Manufacturers have developed a variety of DNA detection systems and at present fully autonomous systems are available which are capable of quantitative DNA measurement, i.e. the methods can show if there is a high or low DNA content in the water analysed. In addition the presence of microorganism species, including indicator microbes, can be shown, but the presence or even quantification of colony forming units cannot be proven.

Flow cytometric immunophenotyping (i.e. analysis and characterization of heterogeneous organism groups) may also be used in combination with real-time (quantitative) Polymerase Chain Reaction (PCR) methods to document the presence/absence of species specific DNA in water as developed by Zebra Bioscience (http://www.zebrabioscience.nl). In immunophenotyping antibodies are used to identify cells by detecting specific antigens of these cells (markers). In combination with staining the presence/absence of certain organisms can be shown. PCR is used to amplify a piece of DNA generating thousands to millions of copies of a target DNA sequence. Primers (short DNA fragments) containing sequences complementary to the target DNA region together with a DNA polymerase allow selective and repeated DNA amplification. Suitable detectors may thereafter be used to show the presence of DNA and this process can also be used as species specific identification. However, although PCR can also be used to quantitatively show the presence of DNA colony forming units cannot be confirmed with this method as PCR detection tools cannot distinguish between living and dead organisms.

5.2 RNA METHODS

Ribonucleic acid (RNA) is a biologically important molecule which is very similar to DNA, but differs in a few important structural details, e.g. in the cell, RNA is usually single-stranded, while DNA is usually double-stranded. RNA is transcribed from DNA by enzymes (RNA polymerase). RNA is a central component for protein synthesis.

Similar to DNA detection in water (see above), should RNA be detected from water this cannot be taken as an indicator of living organisms but it can indicate that life must have existed in this water. Zero RNA measurements may indicate that no organisms are in the water.

One of the tools to identify RNA is Aquascope, developed by AquaExplorer. The principal functioning of Aquascope is the use of cytometry in conjunction with species- or genus specific fluorescently labelled RNA-probes to detect certain sequences of ribosomal RNA which is specific to certain target species (Jansen, AquaExplorer, pers. comm.). The tool utilizes autonomous in situ hybridization as a working principle. It has shown to be able to quantify and determine bacterial species. Probes specific for IMO-pathogens are available and validated (Jansen, AquaExplorer, pers. comm.). As in DNA detection technologies colony forming units cannot be confirmed with this method.

5.3 ATP METHODS

Adenosine triphosphate (ATP) is a coenzyme acting as the main energy storage and carrier molecule in the cells of all known (prokaryotic as well as eukaryotic) organisms. ATP is an indicator for organism viability because it is present in a high concentration in cells with an active metabolism. The ATP concentration declines very rapidly when the cells are dead. Therefore the presence of ATP may be taken as a proxy for the presence of viable organisms. As only free-ATP can be measured and not ATP inside the cells or organisms, no organism numbers can be assessed with the ATP detection methods and it will further be impossible to undertake species specific analysis to e.g. proof the presence of some target species or indicator microbes as stated in the Regulation D-2.

The foremost method to detect ATP is based on the production of light caused by the reaction of ATP with added luciferase (the active enzyme) and luciferin (substrate), i.e. in the presence of luciferase and lucerferin and energy from ATP light is emitted. The emitted light is proportional to the ATP concentration. In this chemical reaction one photon emission corresponds to a consumption of one ATP and sufficiently sensitive photon detection technology, e.g. a luminometer, which are available in many configurations, may be used for detection.

The common luciferase ATP test is a complex method and is time consuming. The reagents need to have a shelf-life of 6 months when kept frozen at least -20 °C. However, other ATP detection instruments were developed (e.g. the LIGHTNING Multi-Variable Platform instrument of BioControl Systems, Inc., USA) and are easier to operate with hand-held tools (Figure 40).

Figure 40 The LIGHTNING Multi-Variable Platform instrument of BioControl Systems, Inc., USA (Photo modified from www.rapidmethods.com).

Especially in lower ATP concentrations a noise ratio may negatively impact the results. A contamination risk also exists as all organisms, including those that may be blown into the

sample during the sampling process, e.g. terrestrial insects, contain ATP what highlights the need for a clean and unexposed working environment when preparing for the ATP measurement. Therefore the practicability and precision of this method for indicative sample analysis needs to be evaluated.

5.4 CHL *A* METHODS

Chlorophyll *a* (Chl *a*) is a photosynthetic pigment which is essential for photosynthesis, but is only found in phytoplankton. It is a specific form of chlorophyll used in oxygenic photosynthesis. It absorbs most energy from light.

The presence of Chl *a* in water indicates the presence of phytoplankton. A wealth of tools exist to document the Chl *a* content of water, but a cell count cannot be measured directly on the basis of this parameter. In general a high Chl *a* concentration indicates a proportional higher phytoplankton abundance in the water body analysed. However, phytoplankton are of different sizes (varying over five orders of magnitude) and many small cells may result in a similar Chl *a* signal as few bigger cells.

Tools to measure Chl *a* in water are "standard" Fluorometers, which are available from different manufacturers. Fluorometers may be used to measure the Chl *a* content in a cell or in water. Both measurements are quick and the tools are easy to use. Fluorometers are also available in hand-held format, not bigger than a salinity meter (see below). However, standard Chl *a* measurement tools cannot be used to determine the viability of phytoplankton cells. One exception are active fluorometers, such as the Pulse-Amplitude Modulated fluorometer (Water-PAM) of Walz GmbH (www.walz.com) which is described further below, the PhytoFlasch of Turner Designs (www.turnerdesigns.com) and FASTtracka of Chelsea Technologies Group (www.chelsea.co.uk). These tools measure Chl *a* content in living cells by triggering the phytoplankton electron chain to respond. Such a response is only existing in living cells thereby assessing photosynthesis activity by utilizing the relationship of chlorophyll fluorescence and photosynthesis to describe phytoplankton 'health'.

It should be noted that Chl *a*, after being released from damaged cells during e.g. ballast water treatment, may be persistent in water. As a consequence, Chl *a* concentration measurements from ballast water cannot be taken as a direct indicator of living algae. However, zero Chl a concentrations may indicate that no algae are in the water, i.e. the treatment process was successful.

5.5 OXYGEN MEASUREMENT

For the identification of living phytoplankton organisms an oxygen measurement may be undertaken as living phytoplankton cells, when exposed to light, will produce oxygen as one by-product of their photosynthetic activity. A ballast water sample may simply be stored in a sealed container with an appropriate oxygen detection sensor and be exposed to light for one hour and the oxygen content be monitored during the light exposure. An increasing oxygen level indicates living phytoplankton organisms in the sample (Ole Larson, pers. comm.).

This method does not enable an enumeration of phytoplankton organisms. However, zero oxygen production may indicate that no living algae are in the water. Key uncertainties with this method are the sensitivity of the oxygen sensor and the number of active phytoplankton organisms needed to enable oxygen measurements in shorter time periods as suggested here.

5.6 PULSE-AMPLITUDE MODULATED FLUOROMETRY (WATER-PAM)

To measure phytoplankton viability a PAM was used on all samples on-board the vessel and the measurements were done immediately after sampling (Figure 41). In addition samples were also analysed later by the Royal Netherlands Institute for Sea Research (NIOZ) also to determine the effect of transport and storage of ship board samples.

This method analysis the photochemical efficiency of photosystem II (F_v/F_m: an indicator of the 'health' condition of the cell) of phytoplankton (Schreiber et al 1993). This parameter gives a qualitative indication of the photosynthetic activity of the phytoplankton community. In addition this tool gives a bulk biomass indication in terms of total chlorophyll fluorescence (F_0).

For each analysing event the PAM-fluorometer was calibrated with distilled water and the result taken as the "zero sample". As a second step 3 replicate measurements were conducted on each sample. The samples were taken from the 10 l bucket after mixing. F_0 (chlorophyll fluorescence acting as a estimate of phytoplankton biomass), F_m (response), and F_v/F_m (viability) measurements were recorded for each sample, and the mean values were obtained.

The method works fast and delivers results in less than 10 minutes and is easy to operate. However, this instrument cannot report the number of viable cells.

Figure 41 Onboard set-up for PAM analysis.

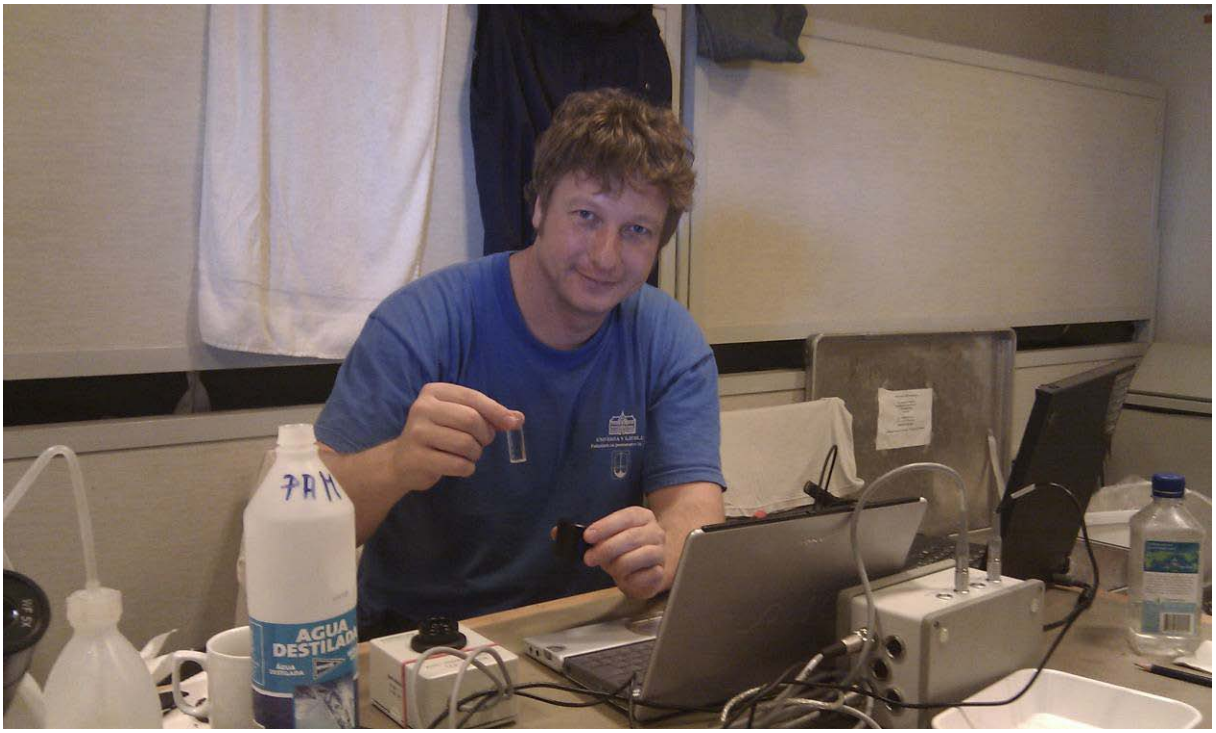


Figure 42 Handling the PAM analysis sample cuvette.

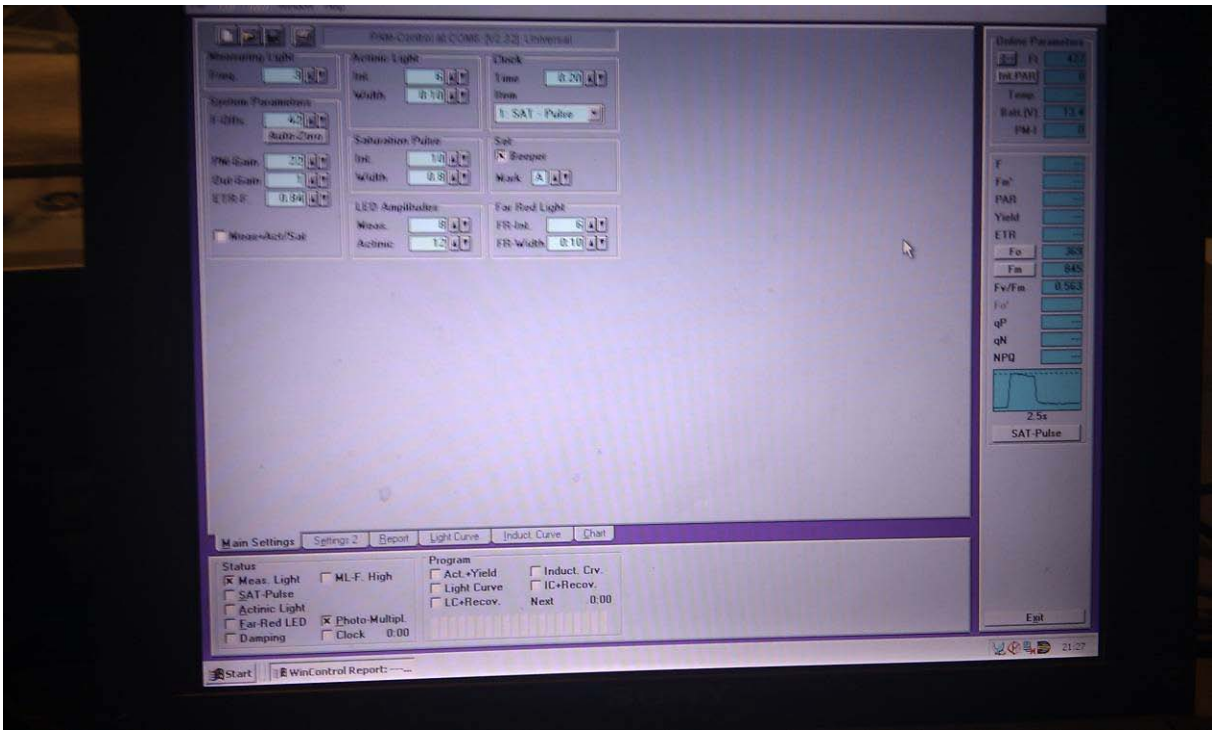


Figure 43 PAM fluorometry; a fast method to determine (bulk) phytoplankton biomass and the physiological condition of the photosynthetic system of a cell (viability).

As the PAM method is a well developed and straightforward "technical tool" (does not require biological or other specific background for a person conducting the test) and the measurements may be conducted in very short time (calibration and processing with three test trials on one sample can be done in 10 minutes), and for different other reasons (e.g., light to carry, running costs not expensive, not much maintenance) the tool seems promising as stand alone tool for indicative analysis. Having in mind that the PAM does not measure organism numbers, the focus of our investigation was to find out if any of the results obtained with the PAM may have some correlation with the number of smaller phytoplankton organisms.

In short it was recognised, that there is a clear correlation between the F_0 value (Chlorophyll content / biomass) and the number of viable smaller phytoplankton organisms. It should be noted that also smaller and bigger phytoplankton organisms may occur in the sample and are measured by the PAM at the same time, and in nature the number of these certainly varies, nevertheless, the tests showed a clear correlation of F_0 with the smaller organisms (see Table 22 and the graph in Figure 44).

Table 22 PAM biomass and viability measurement results on the vessels and at NIOZ, and flow-cytometry counts of organisms also conducted at NIOZ. Red marked are the results where, because of a low biomass, the viability results of NIOZ had high variation, probably because of the lower-end precision of the PAM used. In these cases response curves and results from the tests conducted on-board were used as additional determinants (F_0 = biomass, Fv/Fm = viability). The table also indicates the number of phytoplankton organisms below 50 and equal to or greater than 10 micrometres in minimum dimension.

Test No.	Date	Sample type	Mean F_0 Vessel	Mean F_0 NIOZ	Mean Fv/Fm Vessel	Mean Fv/Fm NIOZ	Total number phyto 10 - 50 µm / ml
		VOYAGE 1					
1	24.07.10	uptake, untreated, sequence 1	337	51	0,451	0,537	75
1	24.07.10	uptake, untreated, sequence 2	319	48	0,488	0,558	60
1	24.07.10	uptake, untreated, sequence 3	290	57	0,486	0,495	63
1	24.07.10	uptake, untreated, entire uptake	429	60	0,506	0,557	77
1	25.07.10	discharge, untreated,sequence 1	217	25	0,426	0,533	70
1	25.07.10	discharge, untreated,sequence 2	168	29	0,356	0,457	70
1	25.07.10	discharge, untreated,sequence 3	164	21	0,385	0,485	51
1	25.07.10	discharge, untreated, entire discharge	218	29	0,369	0,397	56
2	28.07.10	uptake, untreated, sequence 1	221	3	0,225	0,790	9
2	28.07.10	uptake, untreated, sequence 2	160	14	0,164	0,545	7
2	28.07.10	uptake, untreated, sequence 3	200	14	0,156	0,467	17
2	28.07.10	uptake, untreated, entire uptake	225	10	0,289	0,605	31
3	28.07.10	uptake, before treatment, sequence 1	147	1	0,088	0,722	16
3	28.07.10	uptake, before treatment, sequence 2	148	2	0,099	0,762	9
3	28.07.10	uptake, before treatment, sequence 3	166	4	0,077	0,568	26
3	28.07.10	uptake, before treatment, entire uptake	190	3	0,100	0,733	23
3	28.07.10	uptake, after treatment, sequence 1	148	3	0,012	0,000	0
3	28.07.10	uptake, after treatment, sequence 2	159	1	0,015	0,333	0
3	28.07.10	uptake, after treatment, sequence 3	148	4	0,029	0,500	0
3	28.07.10	uptake, after treatment, entire uptake	161	4	0,020	0,417	0
2	29.07.10	discharge, untreated,sequence 1	187	26	0,344	0,600	20
2	29.07.10	discharge, untreated,sequence 2	193	15	0,346	0,588	16
2	29.07.10	discharge, untreated,sequence 3	348	88	0,579	0,716	88
2	29.07.10	discharge, untreated, entire discharge	306	77	0,525	0,711	47
3	29.07.10	discharge, after treatment, sequence 1	179	1	0,052	0,476	0
3	29.07.10	discharge, after treatment, sequence 2	191	3	0,023	0,433	0
3	29.07.10	discharge, after treatment, sequence 3	189	2	0,031	0,667	0
3	29.07.10	discharge, after treatment, entire discharge	192	2	0,039	0,000	0
		VOYAGE 2					
4	26.09.10	uptake, untreated, sequence 1	261	336	0,666	0,617	193
4	26.09.10	uptake, untreated, sequence 2	250	392	0,675	0,658	212
4	26.09.10	uptake, untreated, sequence 3	237	356	0,690	0,654	217
4	26.09.10	uptake, untreated, entire uptake	260	316	0,681	0,622	230
4	27.09.10	discharge, untreated,sequence 1	195	140	0,557	0,553	79
4	27.09.10	discharge, untreated,sequence 2	278	191	0,533	0,552	144
4	27.09.10	discharge, untreated,sequence 3	262	208	0,547	0,538	246
4	27.09.10	discharge, untreated, entire discharge	247	172	0,574	0,467	114
5	29.09.10	uptake, untreated, sequence 1	237	208	0,603	0,613	122
5	29.09.10	uptake, untreated, sequence 2	218	201	0,588	0,603	157
5	29.09.10	uptake, untreated, sequence 3	221	214	0,612	0,627	163
5	29.09.10	uptake, untreated, entire uptake	228	238	0,599	0,624	223
5	01.10.10	discharge, untreated,sequence 1	202	125	0,533	0,591	122
5	01.10.10	discharge, untreated,sequence 2	210	137	0,557	0,587	95
5	01.10.10	discharge, untreated,sequence 3	207	143	0,546	0,576	112
5	01.10.10	discharge, untreated, entire discharge	216	189	0,547	0,531	186

The general explanation/interpretation by NIOZ for the Fv/Fm (viability) value is:
>0,600 to 0,800 organisms in sample are very healthy;
>0,300 to 0,800 organisms in sample are healthy;
<0,300 organisms are moderately affected and may die over time;
<0,200 organisms are affected/dead, no recovery potential;
<0,100 all organisms dead (modified from Stehouwer et al. 2010),
.

Nevertheless, the response curve shown next to numbers on the screen may indicate intensive response even when numbers are below 0.300. As suggested by the NIOZ experts, in such case the phytoplankton should be considered still as viable (see Figure 44).

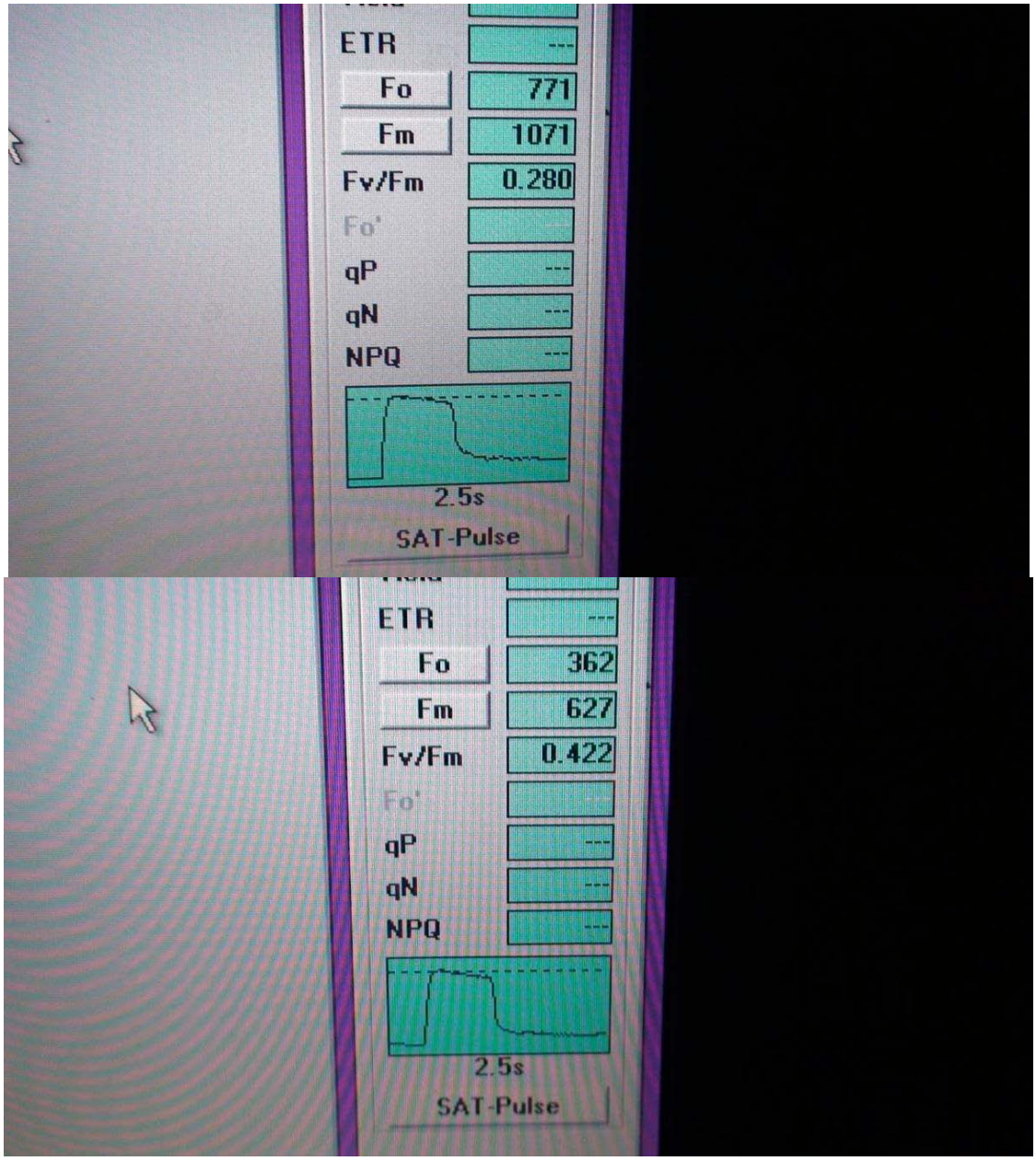


Figure 44 PAM values and response curves of two measurements one below and one above the 0.300 viability threshold, however very similar in response curve.

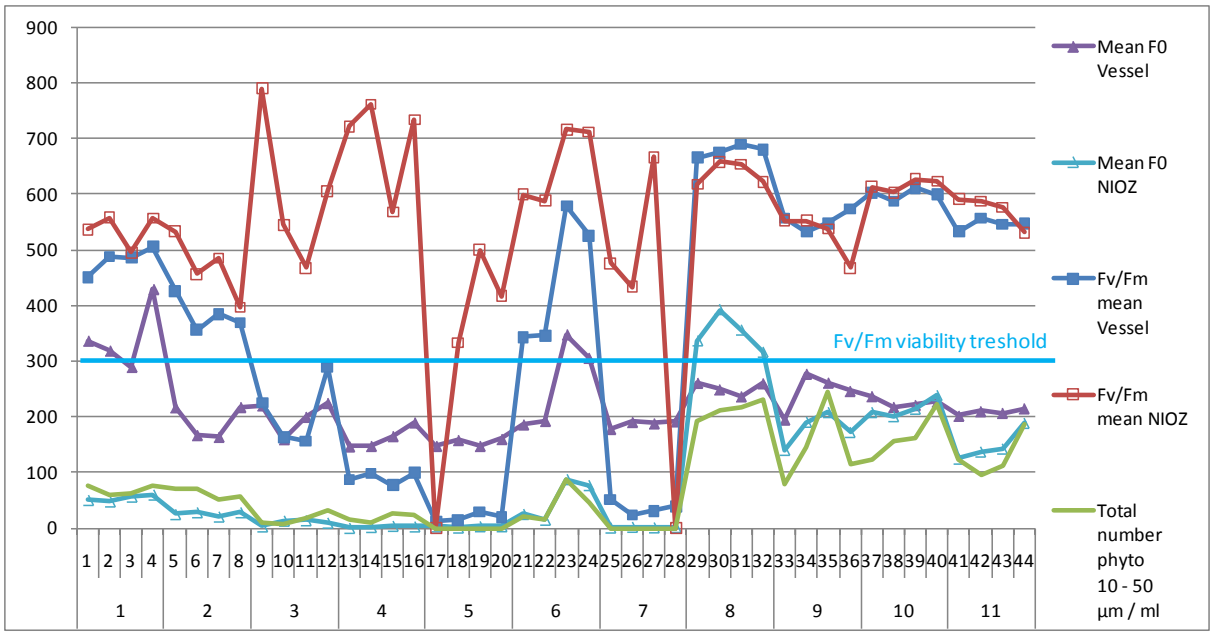


Figure 45 PAM results; Fv/Fm values are multiplied by 1000 for clearer presentation.

Table 23 Correlation coefficients (CC) between the F_0 (Chlorophyll content / biomass) and the number of phytoplankton organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension; in the first part all measurements are considered, and than separately for the first and second voyage; The correlation coefficient is a measure of the extent to which two measurement variables "vary together".

Tests	Variables	CC
ALL	CORREL VESSEL/NR=	0,40
	CORREL NIOZ/NR=	0,93
VOYAGE 1		
	CORREL VESSEL/NR=	0,74
	CORREL NIOZ/NR=	0,83
VOYAGE 2		
	CORREL VESSEL/NR=	0,56
	CORREL NIOZ/NR=	0,74

It was noted that a difference between the PAM measurements conducted on the vessel and those at NIOZ occurred, what was mainly due to up to 10 days delayed processing of the samples at NIOZ due to sample transport. We suspect that this most probably influenced the lower correlation (0.4 overall, 0.74 for voyage 1 and 0.56 for voyage 2) between the biomass measurement on the vessel and number of viable organisms measured at NIOZ. The difference is greater in the measurements of the tests conducted on the first vessel, as at the

second voyage it was possible to transport the samples with shorter delay due to the support of Aquaworx.

As the main result it was observed that whenever the F_0 chlorophyll biomass value was above 20 (with this excluding results with higher variations because of PAM precision level) only considering viable organisms (the Fv/Fm was above 0.300), the number of organisms was 20 and above (actually just in one case were 20 and than 47 and above) (see Table 24, Figure 46 and Figure 47). The Gain for these measurements was 20. It should be noted that lowering the organism number in this analysis to 10 (see D-2 Standard) was intentionally avoided as this would likely increase the level of inaccuracy because this value gets closer to the detection limit of the PAM.

Table 24 Number of phytoplankton organisms below 50 and greater or equal to 10 micrometres in minimum dimension in relation to PAM biomass (F0) and viability measurement (Fv/Fm) comparing the on-board measurements with the NIOZ results.

Mean F_0 Vessel	Mean F_0 NIOZ	Mean Fv/Fm Vessel	Mean Fv/Fm NIOZ	Total number phyto 10 - 50 µm / ml
337	51	0,451	0,537	75
319	48	0,488	0,558	60
290	57	0,486	0,495	63
429	60	0,506	0,557	77
217	25	0,426	0,533	70
168	29	0,356	0,457	70
164	21	0,385	0,485	51
218	29	0,369	0,397	56
187	26	0,344	0,600	20
348	88	0,579	0,716	88
306	77	0,525	0,711	47
261	336	0,666	0,617	193
250	392	0,675	0,658	212
237	356	0,690	0,654	217
260	316	0,681	0,622	230
195	140	0,557	0,553	79
278	191	0,533	0,552	144
262	208	0,547	0,538	246
247	172	0,574	0,467	114
237	208	0,603	0,613	122
218	201	0,588	0,603	157
221	214	0,612	0,627	163
228	238	0,599	0,624	223
202	125	0,533	0,591	122
210	137	0,557	0,587	95
207	143	0,546	0,576	112
216	189	0,547	0,531	186

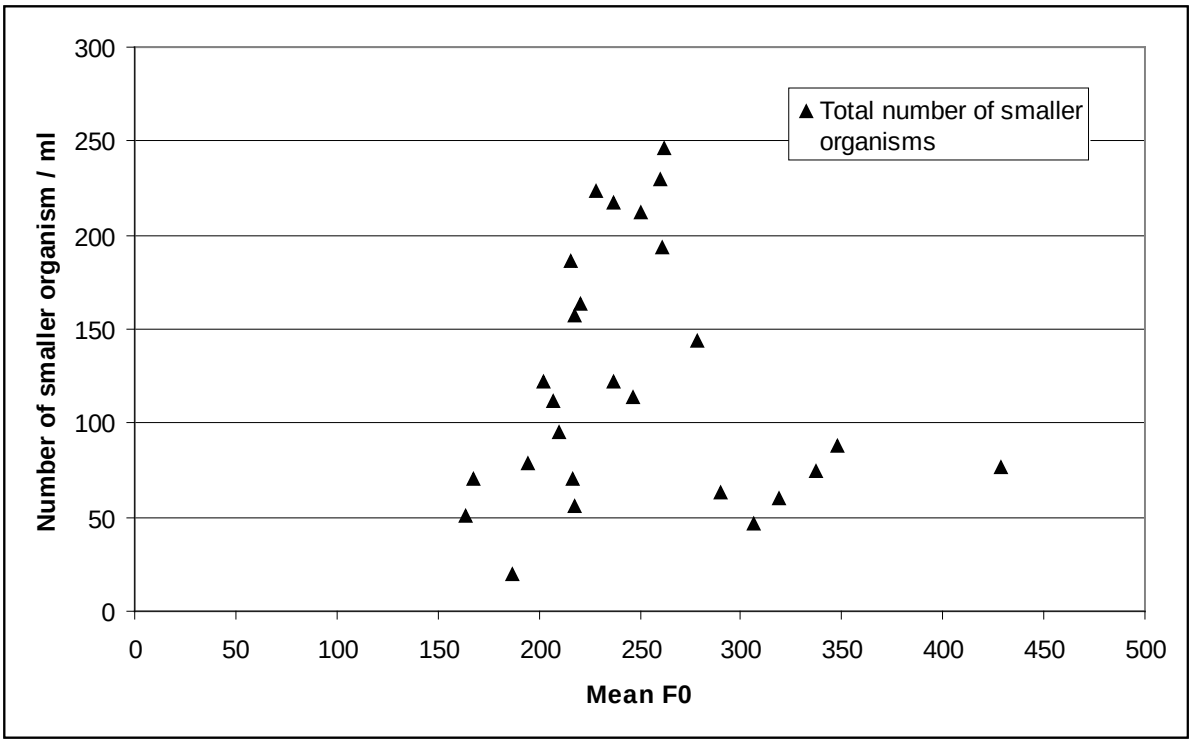


Figure 48 Number of smaller organisms according to mean PAM F_0 values measured on-board.

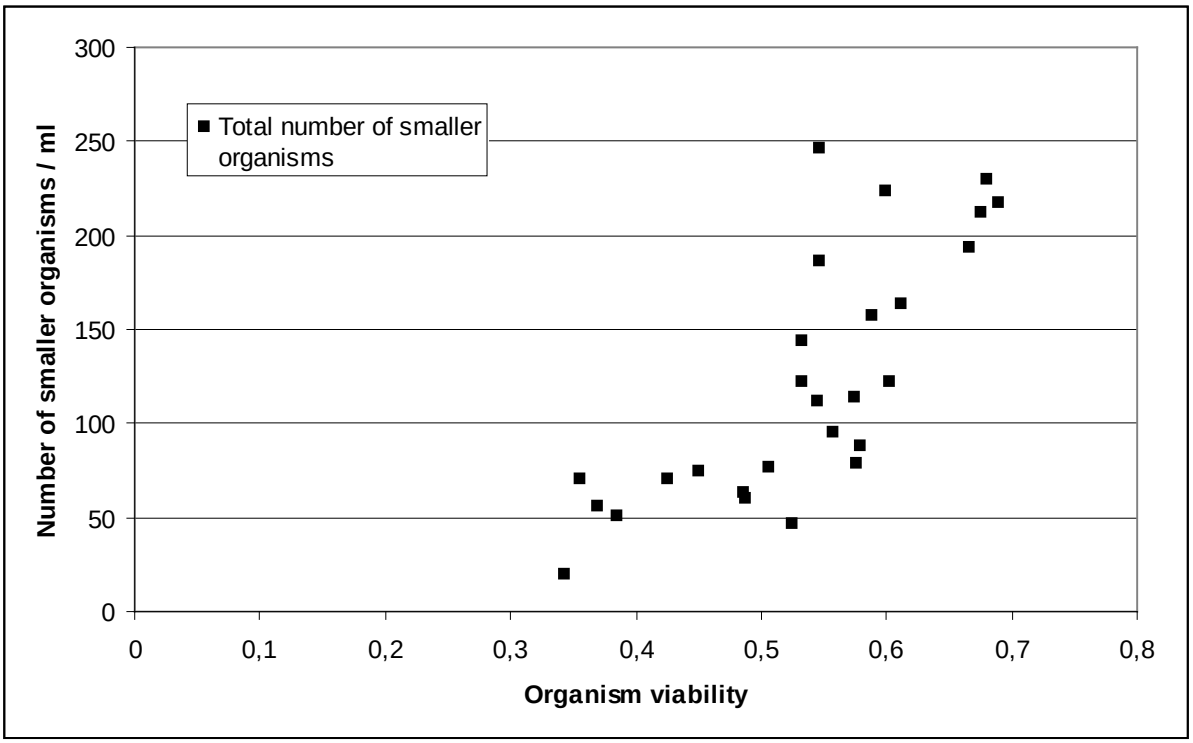


Figure 49 Number of smaller organisms according to mean viability (Fv/Fm) measured on-board.

In the 27 samples analysed, the organisms numbers ranged from 20 to 246, the mean value is 122 organisms, the peak of distribution tends to be around 112 organisms (median) and the standard deviation is 65,99 (see Table 25).

Table 25 Statistical analysis of phytoplankton organisms below 50 and greater or equal to 10 micrometres in minimum dimension.

Number of phytos 10 - 50 µm / ml when F_0 >20	
Mean	121,96
Median	112,35
Mode	70,00
Standard Deviation	65,99
Range	225,68
Minimum	20,00
Maximum	245,68
Count	27,00
Confidence Level(95,0%)	26,11

5.7 FLOWCYTOMETRY

For analysis of organisms above 10 micrometres in minimum dimension an image inflow camera may be used. Several manufacturers of such systems were identified. All systems have in common that the camera automatically counts particles, including organisms, per size class in a fluid. The more simplified systems cannot separate organisms from sediment etc and neither can separate living and dead organisms. More sophisticated systems can also assess organism viability for phytoplankton by using organism stains together with flowcytometry. The separation of living phytoplankton from detrital material and zooplankton is based on the presence of auto chlorophyll fluorescence of phytoplankton cells.

Flow camera systems deliver results promptly. They can be used to assess the number of viable phytoplankton cells in a sample which may be done in combination with a specific stain. However, concerns were raised by users that the viability in smaller algae may not always be categorised correctly as the viability signal may be too low for detection. The sample preparation in this approach may take 20 minutes and the sample analysis by an expert user in minimum another 20 minutes.

At least two image inflow camera manufacturers work towards the development of simplified and portable flow cameras which may be used for the indicative analysis of ballast water on-board vessels.

Fluid Imaging Technologies, Inc., USA produces two organism detection technologies which were considered for ballast water sample processing. One system is a comprehensive FlowCAM®. The second system is a hand-held tool with a simplified technology (Figure 50).

The FlowCAM® is an integrated system which enables a rapid analysis of particles in a moving fluid. This tool combines capabilities of flowcytometry, microscopy and fluorescence detection. It automatically counts objects, takes images and analyses particles also on the basis of fluorescence properties.

Figure 50 The hand-held flow camera of Fluid Imaging Technologies, Inc, USA.

The FlowCAM®-HF is an imaging particle analyzer which was specifically designed for applications where only an indicative analysis of particles is required. As all objects detected by the machine are photographed a separation of organisms and sediment etc may be undertaken by photo inspection of an expert biologist. In addition, data gathered, including minimum dimension, can be plotted in a distribution graph indicating organism counts and the concentration of organism size distribution. The pictures may further be used to identify if the organisms are intact. However, when the organisms are identified as intact it cannot be assessed whether or not this organisms was viable at the moment the picture was taken.

Another inflow camera manufacturer considered in this overview is Cytobuoy, The Netherlands (www.cytobuoy.com). Cytobuoy´s CytoSense is a portable flowmeter which works as an image analyser. Viable phytoplankton can be distinguished from other particles by and expert using the tool. The manufacturer currently works on a more simple system with the aim to use it for indicative analysis of ballast water samples. Although a phytoplankton viability assessment is possible, it seems difficult to enable a viability assessment of zooplankton with this tool (see above).

5.8 HOLOGRAPHIC MICROSCOPY

Digital holographic microscopy provides quantitative imaging which is suitable for high resolution analysis of objects in water.

As flow cameras, this method is also suitable to analyse organisms above 10 micrometres in minimum dimension. For the viability assessment of the organisms detected no stain needs to be used and the data are provided in real-time organism counts. The viability assessment (based on movement of organisms) is based upon the images provided by the tool which need to be analysed by an expert (Dr. Mathuis, Ovizio, Holoflow, Belgium, pers. comm.). A portable system directed to the use of ballast water sample analysis is currently under development.

5.9 VISUAL INSPECTION

The visual inspection of a small subsample may show living organisms in the sample Figure 51 shows such a sample inspection. It should be noted that without magnification a visual inspection will result only in bigger organisms being detected in a sample. It is assumed that organisms bigger than 1000 micrometres in minimum dimension may be determined in such way.

The viability in such an inspection is limited to complete body movements of the organisms as organ activity or antenna movements can not likely be seen.

Figure 51 Inspection of an unconcentrated ballast water sample at the sampling point.

5.10 STEREOMICROSCOPE

To analyse for viable bigger organisms a stereomicroscope may be used for analysis under magnifications of at least 10 x. The viable organism numbers may be recorded according to broad taxonomic groups, such as copepods, decapods, polychaetes, bivalves, gastropods,

phytoplankton etc, however these are not required for to ascertain compliance to the D-2 Standard.

After sampling the preparation of the sample for a stereomicroscope analysis may take 5 minutes during which the sample will be concentrated. A subsample will be analysed and the process recommended is identical with the method description of analysis of bigger organisms in the section on representative sampling (see above).

For the analysis up to 40 minutes would be needed (3 subsamples to be analysed, approx. 10 min per each subsample), and the processing needs to be done by a trained expert. The viability assessment should be based on movements of intact organisms. This movement may be stimulated by light exposure (under the stereomicroscope) or by careful poking with a fine needle. In addition organ activity should be observed and non-moving organisms which show organ activity should be counted as living.

Figure 52 A stereo-microscope in use for on-board analysis of organisms greater than or equal to 50 micrometres in minimum dimension. A Bogorov counting chamber is shown on the white tray in the foreground.

Hand-held microscopes such as Eyeclops (www.bionicam.com) were also considered, but the only tools identified are used to analyse surfaces, i.e. cannot be used to investigate organisms in water.

5.11 METHODS FOR IDENTIFICATION OF BACTERIA

There is a wealth of methods available, but all lack some of the requirements needed for indicative analysis as stated above. In common, all methods to deliver numbers of colony forming units (cfu), require a certain incubation time of the samples, which is never shorter than 4 hours. During our on-board experiments to test bacteria methods, lightweight, portable and robust incubators were used (Figure 53). For reasons of comparison we have also added a few methods which show only the presence/absence of indicator microbes, but do not show

cfu, as this may be a sufficient result for an indicative analysis and at least one tool exists to deliver such results promptly (See Fluoroquick below).

Figure 53 Lightweight bacteria incubator.

To identify bacteria which may occur in lower numbers, such as the D-2 standard indicator microbes in marine waters (as shown in our experience from previous tests), a concentration of water may be needed to reach the detection limit of the analytical methods. Such concentration technologies to support the detection of microorganisms from liquid samples are available, e.g. the Cell Trap of Harvey-Coleman Ltd, UK (www.harvey-coleman.com). This system uses inter-capillary capture of organisms via hollow fibre membranes and can filter up to 15 l of water. It was used to prepare for microbiological DNA analysis during another experiment. Concentrated organisms need to be recovered from the hollow fibres by using syringes with a buffer (Figure 54).

Figure 54 The Cell Trap of Harvey-Coleman Ltd, UK (Photo modified from www.harvey-coleman.com).

A similar such device is Planktontrap, UK (www.ballast-water.co.uk). It concentrates plankton in ballast water samples and captures species above 0.2 micrometre without causing damage. According to the manufacturer, dependent on the size of the device, it can filter up to 20 m³ of water. However, the flowrates this tool can handle were not identified. Whether or not this method can also be used to identify (viable) phytoplankton and/or zooplankton needs further consideration (Figure 55).

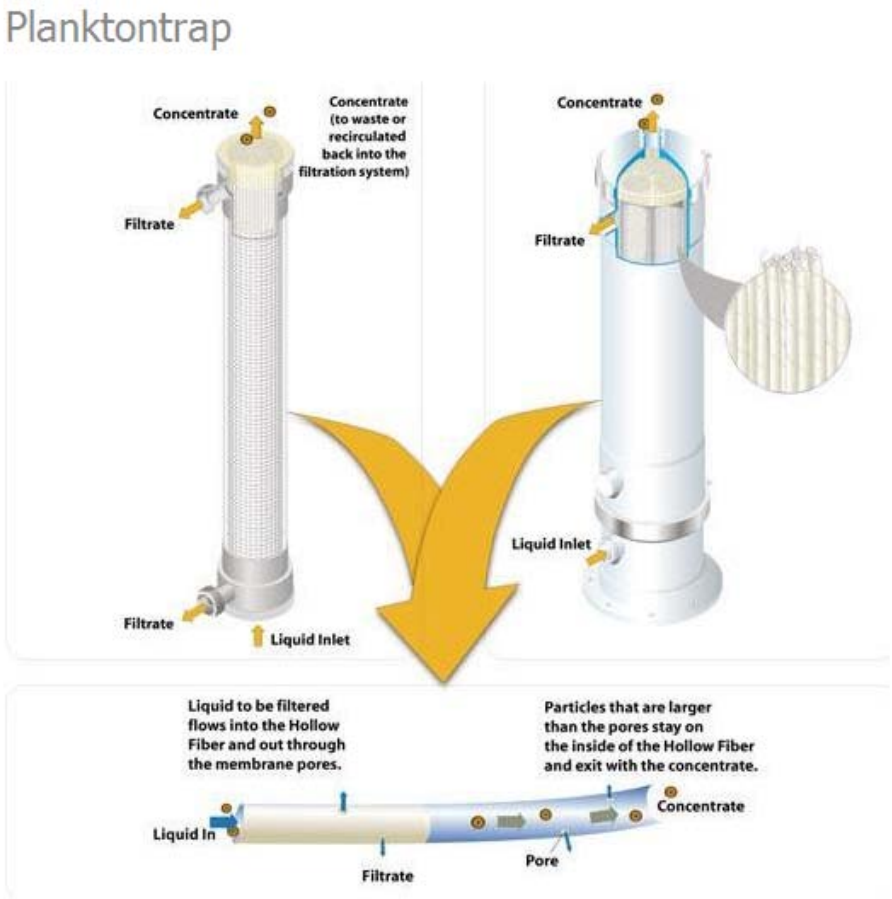


Figure 55 The Planktontrap (Photo modified from www.ballast-water.co.uk).

5.11.1 IDEXX METHOD 1

The IDEXX Colilert reagent is used around the world for the detection of coliform bacteria including an *E. coli* test and *Enterococci* in water and the method was developed to meet the relevant ISO and other standards. This patented method requires an incubation time of 24 hours and special equipment, such as an especially designed sealer (Figure 56) to seal the Quanti-Tray test plates (Figure 57). The sealer has a weight of more than 10 kg. This method delivers quantitative numbers of bacteria by a growth experiment and based upon this a table is used to calculate colony forming units.

For analysis a water sample is transferred to a pre-prepared IDEXX sample bottle and selective media are added (Figure 56). As a second step this sample is transferred into IDEXX Quanti-Tray test plates which is thereafter sealed and placed in an incubator (Figure 58).

The number of bacteria is confirmed by a colour change of the sample water. Additional information may be found at www.idexx.com.

Figure 56 IDEXX sealer, test plate tray and sample bottle.

Figure 57 Sample transfer into IDEXX incubation plates (Quanti-Tray test plates).

Figure 58 Sealing of IDEXX incubation plates.

5.11.2 IDEXX METHOD 2

A second IDEXX method was considered to check quantitatively for total heterotrophic bacteria (including indicator microbes *E. coli* and Enterococci as referred to in Regulation D-2) presence (Figure 59) and the method was developed to meet the relevant ISO and other standards. A volume of 4 ml of the water to be tested is pipetted on the Quanti-disc plates which are incubated for 2 days. The analysis is done by exposure of the plates to UV light. Each coloured cell shows bacteriological activity and with an IDEXX concentration table the density per ml can be calculated. Figure 59 shows the results from one experiment where three dilutions were applied to the tested ballast water.

However, this method results in bacteria counts, but the number of cfu cannot be shown.

Figure 59 IDEXX Quanti-Disc trial for total bacteria. Test with three dilutions (bottom left, left disc undiluted, middle 10 ml and right 1 ml bacteria solution filled to 100 ml with distilled water). Bottom right is a close up of the left image.

5.11.3 MÖLLER AND SCHMELZ MEDIA

This selective media method to proof cfu of *E. coli* and Enterococci was developed to meet the relevant ISO and other standards. Nutrient Pad Sets are provided which are sterile with dehydrated nutrient culture media, ready for immediate use after addition of 3,5ml of distilled water. The water to be tested needs to be filtered which may be done by using a Millipore stand which should be flamed between tests to avoid contamination (Figure 60). We used filter plates with 0,45 micrometre pore size (Figure 61). After filtration the filter plate is placed on the Nutrient Pad Sets and incubated.

For *E. coli* tests the incubation time is 24 hours followed by a second incubation step of another 24 hours. The Enterococci incubation time is 48 hours (see below).

Figure 60 Cleaning of Millipore filter stand.

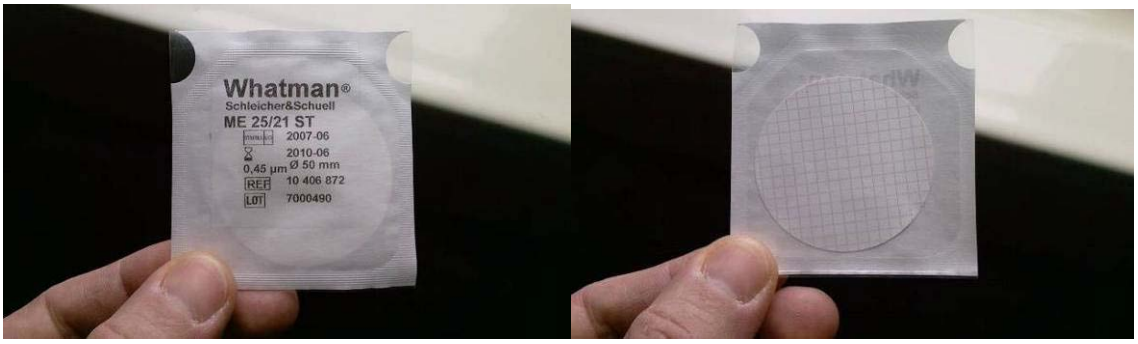


Figure 61 Whatman filters (0.45 micrometre pore size).

5.11.3.1 E. coli

E. coli "suspects" are found in case transparent colonies develop which shown a yellow halo visible from top and bottom of the Nutrient Pad Sets (Figure 62).

As a second step to positively identify viable *E. coli* colonies on the selective medium, the suspect colonies are transferred to Tryptophane broth medium and incubated for 1 day at 44 °C. Positive *E. coli* are indicated by a colour change of the Tryptophane broth from yellow to red/purple on the surface when adding two drops of Kovacs solution (Figure 63).

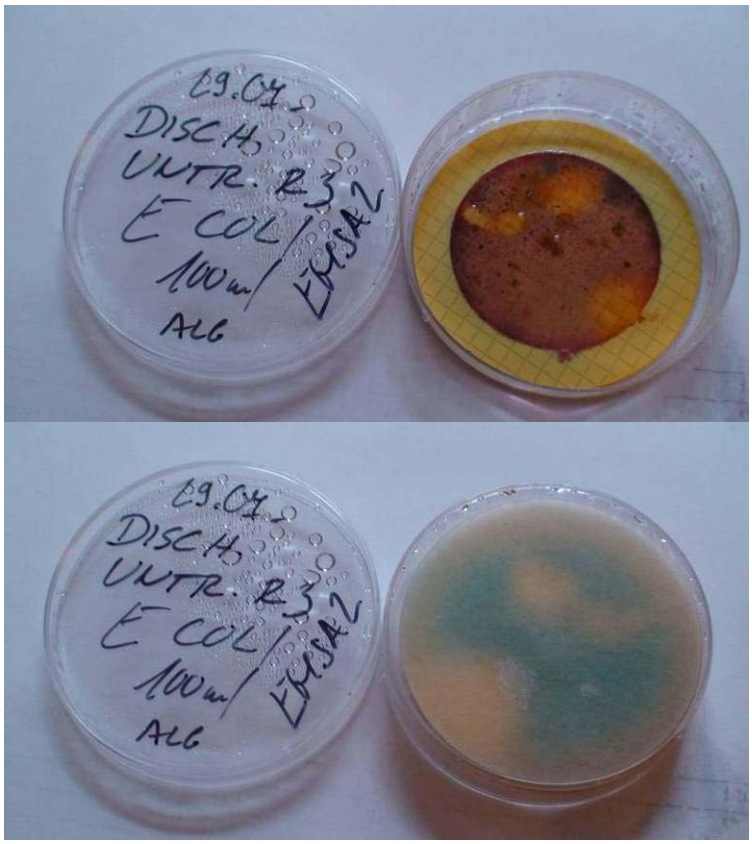


Figure 62 Positive *E. coli* colonies on selective medium. Upper photo = view from top, lower photo = bottom view of Petri disc.

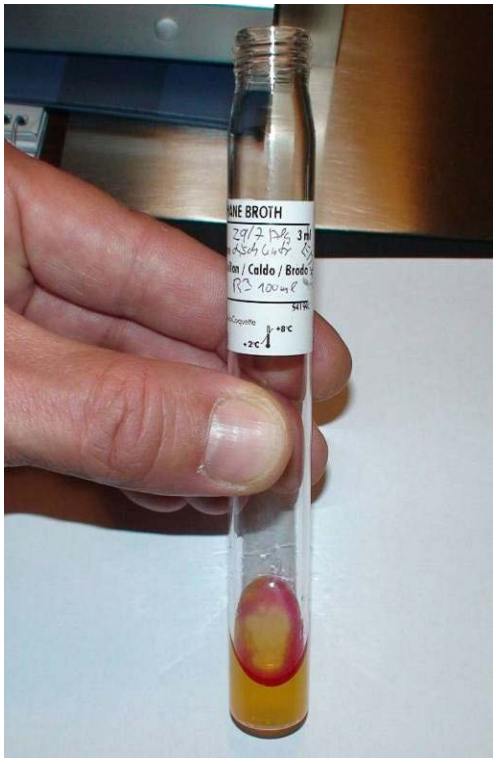


Figure 63 Tryptophane medium incubated to proof *E. coli*.

5.11.3.2 Enterococci

Positive Enterococci are identified as dark red colonies which are ca. 2 mm in diameter after 48 hours of incubation (Figure 64).

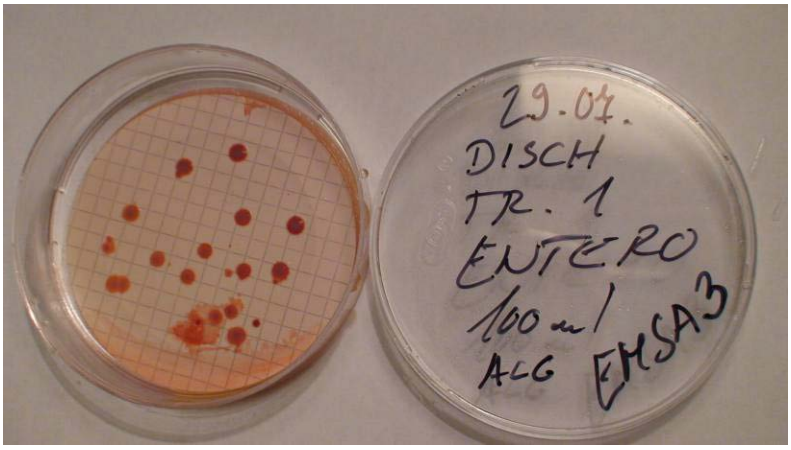


Figure 64 Positive Enterococci on selective medium.

5.11.4 PETRIFILM – 3M TESTS

Two Petrifilm products were tested on previous sea voyages, i.e. one method to show the number of colony forming coliform bacteria and another for total bacteria counts. Both methods were developed to meet the relevant ISO and other standards. For both methods 1 ml of test water is pipetted onto the plates and the plates are sealed with a transparent tape lid (Figure 65). The total bacteria count test takes 2-3 days and the coliform test needs a 1 day incubation time. However, the first results may already be visible after 6 hours incubation time.

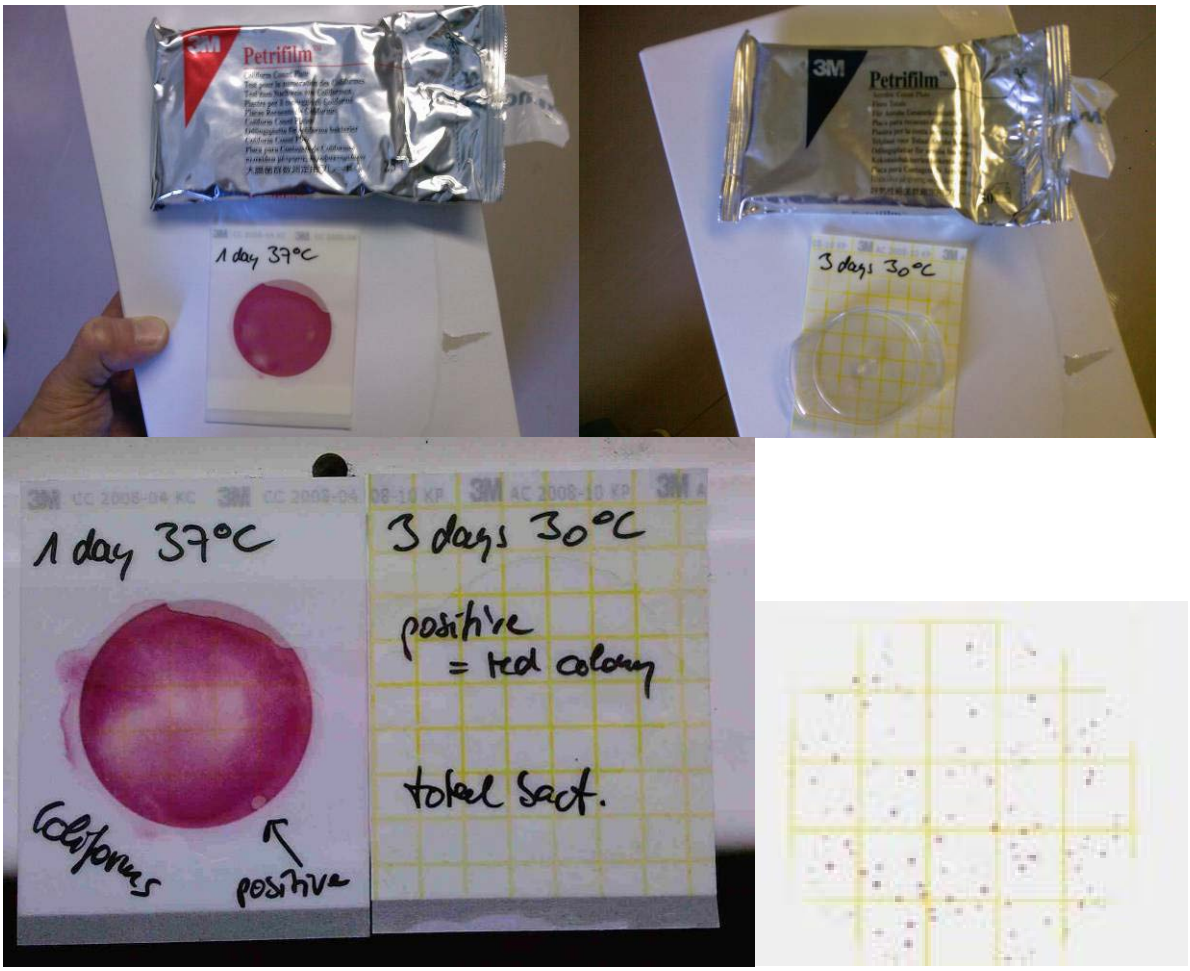


Figure 65 Petrifilms to test for coliform (top left) and total bacteria (top right). The bottom photo shows positives (bottom right photo modified from http://solutions.3m.com).

5.11.5 QUANTITUBE WITH EASYGEL

A simple test for *E. coli* was developed by Micrology Laboratories, USA (Figure 66). 5 ml of the test water are mixed with the Easygel, the test tube inverted three times to allow proper mixing of sample water and gel and transferred into the test tube. Incubation time is 18-48 hours. Positive *E. coli* are indicated by green coloured colonies in the gel (Figure 67).

The recognition of this test in comparison with other methods and the acceptance of the results are unknown.

Figure 66 *E. coli* test kit developed by Micrology Laboratories, USA.

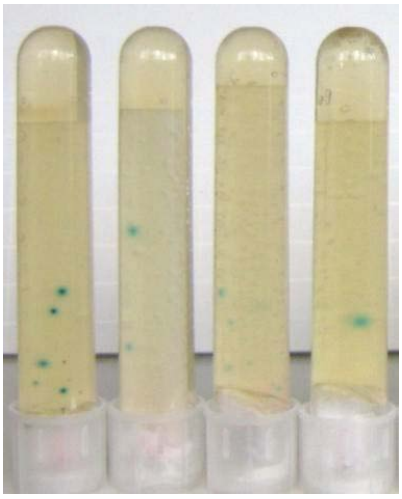


Figure 67 Positive *E. coli* colonies in the test kit developed by Micrology Laboratories, USA. (Photo modified from http://yeasttesting.com).

5.11.6 ENVIROCHECK CONTACT SLIDES

Another very simple method to identify *E. coli* and Enterococci was developed by VWR/Merck (www.vwr.com) to meet the relevant ISO and other standards. The method was originally developed for hygiene monitoring of surfaces and liquids. A test slide, which is installed in a plastic test tube simply needs to be dipped into water for 5 – 10 seconds, than be sealed and incubated. The incubation time is 24 – 48 hours. Unfortunately the detection limit is 1000 cfu per ml and this is much higher than the acceptable bacteria concentrations in D-2 (see Figure 68).

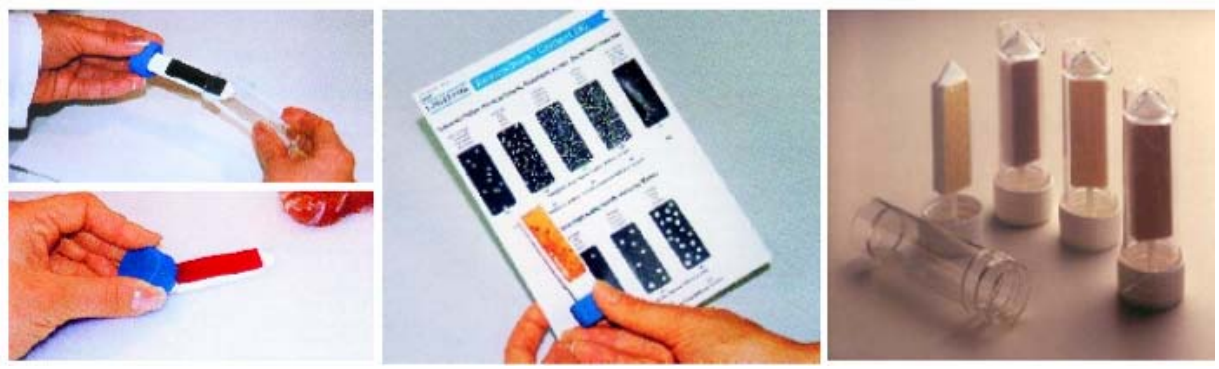


Figure 68 Envirocheck contact slides (Photo modified from www.vwr.com).

5.11.7 BIOMERIEUX API METHOD

The API method developed by Biomerieux identifies the presence and absence of *E. coli*, Enterococci and also *Vibrio cholerae* in water by colour changes of selective media (www.biomerieux.com). the methods were developed to meet the relevant ISO and other standards, however colony forming units cannot be identified and an incubation time of 14 to 48 hours is required (see Figure 69).

Figure 69 Biomerieux bacteria identification strips (Photo modified from www.biomerieux.com).

5.11.8 HAND-HELD FLUOROMETER

Fluorometric diagnostic systems can deliver results of low levels of bacteria in water samples in less than 10 minutes provided the samples do not require incubation time. Should an incubation be needed (e.g. in cases of very low levels of bacteria) a growth time of ca. 4 hours is applied which is the shortest incubation time required of all methods considered here to show colony forming units. The actual test is very simple and limited to adding a reagent to the water sample and a reading is recorded on the fluorometer.

The systems are based upon selective detection of enzymes produced by the target bacteria and test kits for *E. coli* and Enterococci are available. The detection limit is 1 cfu in 100 ml, but it is a presence/absence test, i.e. no quantification of cfu. However, the reading is semiquantitative, i.e. low level readings equate to lower level of bacteria enzymes present. It works with small volumes of an unconcentrated freshwater or marine sample (www.fluorovei.com). However, although the presence of bacteria can be revealed, whether or not these form cfu cannot be proven with this method, i.e. this means that the presence of enzyme activity is not identical bacteria viability.

This fluorometer is available in hand-held format, not bigger than a salinity meter (see Figure 70 and Figure 71).

Figure 70 Fluoroquik hand-held fluorometer of Vista Enterprises Inc. (modified from www.fluorovei.com).

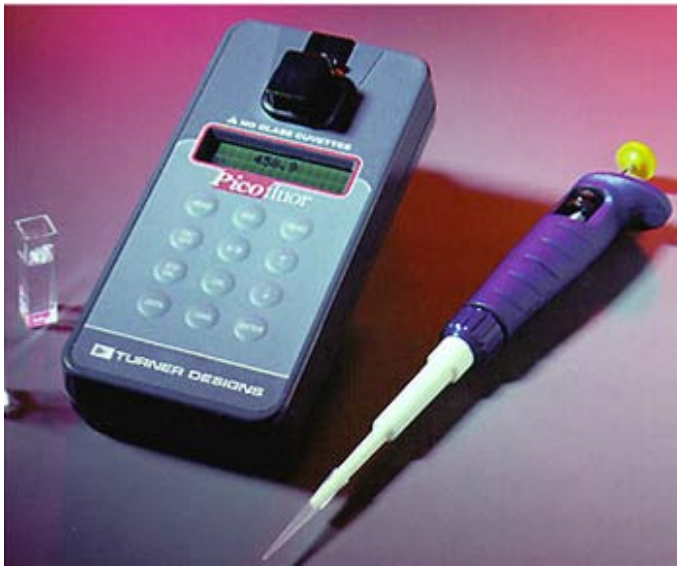


Figure 71 Picofluor hand-held fluorometer of Turner Designs (www.topac.com/picofluor.html).

5.11.9 TECTA-ENDETEC

The TECTA™ approach adapts traditional enzyme-chemistry methods with an entirely automated measurement system which eliminates visual interpretation. It provides for easy on-site microbiological tests with rapid results, but the full time of analysis still takes 2 to 18 hours. The system uses a modified spectrophotometry method enabled by a patented partitioning technology. Although the presence of specific bacteria can be shown it remains unproven if cfu are formed (see Figure 72).

Figure 72 The TECTA-Endetec *E. coli* analyser (modified from www.endetec.com).

5.11.10 NEW HORIZONS DIAGNOSTICS

The qualitative New Horizons Diagnostics detection technology kits were prepared to deliver rapid results of bacteria absence/presence (www.nhdiag.com), i.e. cfu cannot be documented. The kits were developed as an adjunct to classical culture methods. Kits are available for *E. coli* and Cholera. The Cholera and Bengal SMART™ (Sensitive Membrane Antigen Rapid Test) tests are rapid colorimetric immunoassays designed for the direct detection of *Vibrio cholerae* 01 and O139. The tests work with strain specific monoclonal antibodies to identify V. cholerae from clinical and environmental samples. The test is simple and can be performed in less than 15 minutes.

Simply place a few drops of the sample in the tube of lyophilized reagent to reconstitute, transfer the sample from the reagent tube with a swab and place the swab into the plastic SMART™ device. A few drops of a Chase buffer need to be added and after 15 – 30 minutes a distinct colour reaction becomes visible if a positive result occurs. Alternatively water may be filtered through a filter plate which, after filtration, should be placed in an APW medium for 6 – 20 hours incubation (according to the test medium) at 36 °C (see Figure 73).

Figure 73 Cholera detection method as developed by New Horizons Diagnostics. Right image shows a positive (right test plate, two lines) and negative (left test plate, one line) test result (Photo modified from www.nhdiag.com)

It needs to be proven that this method also works with waters of different salinities and what the bacteria detection limit is.

5.12 SUMMARY OF INDICATIVE ANALYSIS METHODS

Various methods for indicative analysis were considered in this overview. The following sections summarise the findings of the system evaluation structured by the three organism groups of the Ballast Water Performance Standard of Regulation D-2 of the BWM Convention.

We like to repeat that this summary is not meant to deliver a fully comprehensive inventory of all methods available on the market, but we tried to deliver a first overview showing the different approaches.

This summary is structured by the organism groups as stated in Regulation D-2 of the BWM Convention, i.e. smaller and bigger organisms and indicator microbes (bacteria).

A detailed overview of the capital and running costs to operate the systems could not be completed within the tight time frame of this project.

5.12.1 METHODS FOR ORGANISMS LESS THAN 50 AND GREATER THAN OR EQUAL TO 10 MICROMETRES IN MINIMUM DIMENSION

The majority of organisms in this size class are plankton algae. Eight phytoplankton analysis methods were considered (Table 26).

Table 26 Methods for analysis of organisms less than 50 and greater than or equal to 10 micrometres in minimum dimension indicating the ease of handling, time needed to get a result, and also indicating whether or not the method is portable, i.e. can the analysis be undertaken at the sampling point on-board. The ease of handling is shown as "+" = not easy to use to "+++" = very easy to apply. The very right columns indicate if the method was tested on-board and the level of biological expertise needed to undertake a test.

Method	Ease of Handling	Time to result (for sample processing)	Portable	Tested Onboard	Level of biological expertise needed
DNA	+	< 60 min	no	no	high
RNA	+	< 60 min	no	no	high
ATP	++	< 30 min	no	no	high
Chl a	+	< 30 min	yes	no	low
Oxygen	++	< 90 min	yes	no	low
PAM	+++	< 10 min	yes	yes, EMSA voyages	low
Flow camera	++	< 60 min	no	yes	high
Holographic microscopy	++	< 20 min	no	no	high

5.12.2 METHODS FOR ORGANISMS GREATER THAN OR EQUAL TO 50 MICROMETRES IN MINIMUM DIMENSION

Zooplankton dominates this size class and 6 methods were located (Table 27).

Table 27 Methods for analysis of greater than or equal to 50 micrometres in minimum dimension indicating the ease of handling, time needed get to a result, and also indicating whether or not the method is portable, i.e. can the analysis be undertaken at the sampling point on-board. The ease of handling is shown as "+" = not easy to use to "+++" = very easy to apply. The very right columns indicate if the method was tested on-board and the level of biological expertise needed to undertake a test.

Method	Ease of handling	Time to result (for sample processing)	Portable	Tested Onboard	Level of biological expertise needed
DNA	+	< 60 min	no	no	high
RNA	+	< 60 min	no	no	high
ATP	++	< 30 min	no	no	high
Visual inspection	+++	< 5 min	yes	yes, EMSA voyages	medium
Stereomicroscope	++	< 40 min	yes	yes, EMSA voyages	high
Flow camera (hand-held)	+	< 30 min	yes	yes	high

5.12.3 METHODS FOR BACTERIA ANALYSIS

Eleven methods were identified as potential approaches to analyse for indicator microbes (Table 28).

Table 28 Methods for bacteria analysis indicating the ease of handling, time needed get to a result, and also indicating whether or not the method is portable, i.e. can the analysis be undertaken at the sampling point on-board. The ease of handling is shown as "+" = not easy to use to "+++" = very easy to apply. The very right columns indicate if the method was tested on-board and the level of biological expertise needed to undertake a test.

Method	Ease of Handling	Time to Result (for sample processing)	Detection of cfu	Portable	Tested on-board	Level of biological expertise needed
DNA	+	< 60 min	no	no	no	high
RNA	+	< 60 min	no	no	no	high
ATP	++	< 30 min	no	no	no	high
IDEXX 1	+ (sealer needed)	incubation time ca. 24 hrs.	yes (by calculation)	yes, sealer and incubator needed	yes	medium
IDEXX 2	+	incubation time ca. 48 hrs	no	yes, incubator needed	yes	medium
Möller & Schmelz	+	incubation time 24-48 hrs	yes (counts)	yes, incubator needed	yes, EMSA voyages	medium
Petrifilm 3M	++	incubation time 24 - 72 hrs	yes (counts)	yes, incubator needed	yes	low
Quantitube Easygel	+	incubation time 18 – 48 hrs	yes (counts)	yes, incubator needed	yes	low
Hand-held fluorometer	+++	with incubation time ca. 4 hrs, without incubation time < 10 min	no	yes	no	low
TECTA Endetec	++	2 – 18 hrs	no	yes	no	low
New Horizons Diagnostics	+++	30 min to 20 hrs depending on medium	no	yes	no	low

5.13 INDICATIVE ANALYSIS AND INDICATIVE SAMPLING

Indicative sample analysis is addressed in the Ballast Water Sampling Guideline G2.

The paragraph 6.3 reads: *Prior to testing for compliance with the D-2 standard, it is recommended that, as a first step, an* ***indicative analysis of ballast water discharge*** *may be undertaken to establish whether a ship is potentially compliant or non-compliant. Such a test could help the Party identify immediate mitigation measures, within their existing powers, to avoid any additional impact from a possible non-compliant ballast water discharge from the ship.*

For a ballast water sample to be analysed, certainly, as a very first step, sampling needs to be conducted. The ballast water sampling guideline does not address explicitly how indicative sampling would need to be undertaken. Implicitly, an indicative analyses could be conducted on a sample, or on a part of a sample, taken during the complete D-2 compliance control sampling process, or just on a stand-alone sample.

It is important to understand that an indicative sampling may be focussed only on one group of organisms (i.e., smaller and bigger organisms or bacteria). While results from each of these organism groups may give an indication that a BWTS is not performing properly, from our experience of on-board sampling, it easily can happen that, e.g., bacteria and smaller organisms would be in acceptable limits, however bigger organisms may be in too high concentrations to meet the D-2 standard or vice versa.

Different groups of organisms in general require different sampling approaches (e.g., in general bigger organisms require bigger water quantities to be sampled than when focussing on smaller organisms), as there are relatively lower concentrations of bigger organisms in the water than the smaller ones. Therefore, indicative sampling methods may be very different for each organism group, differing. in e.g. sample duration, timing, volume, and at which sampling point it was taken.

It would be very difficult to predict in advance which group of organisms to focus on to identify possible non-compliance with the D-2 standard, as this would require a risk assessment conducted in advance. Hence, from this perspective it would be most helpful to use a sampling method which would allow conducting analyses on all organism groups. This would also offer a step-by-step process, where one analyses method may be applied first. If this shows some indication or even does not give an indication of non-compliance, another sample analysis method can be applied (e.g., start with the fastest available analysis method, and proceed with the next available method).

The next issue to consider is: What consequences may arise from an indicative analysis? May an indication require further tests, i.e. complete D-2 compliance test? or May a vessel be banned from discharging ballast water based on this indication? Basically, based upon paragraph 6.3 of the G2 guideline, it is understood that an indicative analysis is meant to give a Party an opportunity to identify a potential non-compliant ballast water in an early stage (i.e., as the full compliance test is expected to show results only after all ballast water was already discharged) to avoid any additional impact from a possible non-compliant ballast

water discharge from the ship. The answer to this is related to the level of non compliance of the BWTS identified by using reliable indicative sampling and analysis methods (see below).

Nevertheless, noting all the above and after the tests and analyses conducted during this study, supported by experience and results from previous voyages, we **recommended** that for **indicative** ballast water **sampling, one sequential sample** is taken using the **same sampling methodology as for a full D-2 compliance test** (as described in subchapters 4.5.1.1, 4.5.1.2, 4.5.1.4 and 4.5.1.5).

When taking one sequential sample, the sampling time is short and the sample analysis could be conducted with a range of different methods. The results obtained from this approach can also represent very solid grounds for different actions PSC may have available in case of indicated non-compliance with the D-2 standard, e.g. (a) from an indication that more tests are needed and to proceed to complete full compliance D-2 tests, (b) to send a vessel to a designated ballast water discharge area, (c) require to discharge the ballast water in a port reception facility, or even (d) to ban a ship from further ballast water discharge, all depending on the result obtained. For instance, if the concentration of organisms identified is just above the D-2 standard, this would be an indication possibly requiring further tests. However if much higher concentrations of organisms than the D-2 standard are identified, a ship may be banned from continuing the ballast water discharge.

We also believe that in certain occasions it may be required not to take a sample from the ballast water discharge line as G2 recommends. This can only be done while the ballast water is pumped overboard. Should a vessel carry ballast water from areas known to contain outbreaks, infestations, or populations of Harmful Aquatic Organisms and Pathogens (e.g., toxic algal blooms) it should be avoided to sample this ballast water from the discharge line while being discharged. Should non-compliance be proven in such a case the water was already pumped overboard posing a risk to the environment, human health, property or resources. Instead we **recommend** that in such cases an **indicative** ballast water **sample** is taken **directly from the ballast water tank prior discharge**. Although such sampling methods may not be representative of the whole discharge an indicative compliance control analysis is enabled without discharging the ballast water.

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