

Difficulties in obtaining representative samples for compliance with the Ballast Water Management Convention

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Abstract

As implementation of the Ballast Water Convention draws nearer a major challenge is the development of protocols which accurately assess compliance with the D-2 Standard. Many factors affect the accuracy of assessment: e.g. large volume of ballast water, the shape, size and number of ballast tanks and the heterogeneous distribution of organisms within tanks. These factors hinder efforts to obtain samples that truly represent the total ballast water onboard a vessel.

A known cell density of *Tetraselmis suecica* was added to a storage tank and sampled at discharge. The factors holding period, initial cell density and sampling interval affected representativeness. Most samples underestimated cell density, and some tanks with an initial cell density of 100 cells ml⁻¹ showed <10 cells ml⁻¹ at discharge, i.e. met the D-2 standard. This highlights difficulties in achieving sample representativeness and when applied to a real ballast tank this will be much harder to achieve.

Key words

Ballast water, D-2 Discharge Standard, Ballast water management convention, ballast water sampling, *Tetraselmis suecica*

1. Introduction

In 2004 the International Convention for the Control and Management of Ships' Ballast Water and Sediments ('the Convention') was adopted by the IMO. This Convention aims to reduce the transportation of species across the globe in ballast water by eliminating viable organisms prior to discharge. As part of the Convention, threshold levels, also known as standards, were set to state the allowable number of viable organisms and indicator microbes within the ballast water discharged at port (IMO 2004). These levels are stated in Regulation D-2 (the 'Ballast Water Discharge Standard') and when the Convention enters into force will become the standard vessels have to meet to legally discharge their ballast water. Crew on any ship found to violate this level could face prosecution, costing port authorities, port state controls, ship operators, ship owners and cargo owners millions of pounds. The convention will enter into force 12 months after ratification by 30 states which represent 35% of the world's merchant shipping tonnage (IMO 2004), and at the time of writing (October

2012) 36 states representing 29.07% of the world's tonnage had ratified the Convention. As the ratification date comes closer, researchers and regulators need to start deciphering the meaning and handling of the representativeness of the samples.

Compliance with standards is essential for effective implementation of any environmental regulation. Sampling, as well as adequate inspection and monitoring, are equally essential in any environmental pollution control or prevention policy (RCEP 1998). To ensure ballast discharges meet Regulation D-2, sampling is required to determine the number of viable organisms present (Pazouki, Basurko et al. 2009). In addition, according to the G2 guideline, those samples used to determine a ship's compliance must be 'representative' of the 'whole' ballast water to be discharged (IMO 2008). Representativeness of ballast water samples has not, however, yet been discussed clearly and while G2 guideline states that representative samples are required it does not provide clear guidelines on how to obtain these samples.

To define representativeness of samples the following definition from the Royal Commission on Environmental Protection (1998) can be considered:

“any numerical environmental standard needs to be robust, recognise scientific assessment and should be specified in a way that takes full account of the nature of the substance to which it relates, the extent of statistical variation in the parameter to which it relates and the requirements for verification”.

From this definition two types of sample 'representativeness': biological and statistical, can be identified. To obtain biological representativeness samples should 'take full account of the nature of the substance to which it relates', and so in ballast water sampling this should show a true account of the diversity and living status of organisms contained within ballast tanks. For statistical representativeness samples should 'take full account of the extent of statistical variation in the parameter to which it relates', hence, in ballast water studies it refers to the number of organisms. The idea of samples being both statistically and biologically representative is recent in ballast water research (i.e. Pazouki et al 2009). Therefore to try and comply properly with G2 samples must satisfy both aspects of representativeness.

Statistical representation enables findings to be generalised to a larger population, and so if a sample is being used to generalise a population it must truly represent it (Stuart 1984). To obtain data which is statistically representative of an entire ship the volume of ballast water to be sampled must first be determined. This has been addressed by Basurko and Mesbahi (2011) and Miller et al (2011) using

different statistical approaches to determine the volume of water required for statistical representation. The results obtained in each study varied widely, highlighting the difficulty in determining a standard approach. Even once statistical representation is determined there is a further hurdle: how do we know that the samples collected are biologically representative of the entire contents of the ballast water? The distribution of organisms within ballast tanks is known to be heterogeneous (Murphy, Ritz et al. 2002; Gollasch and David 2010) and this will hinder the collection of samples which are biologically representative of an entire ship. Further problems encountered while trying to obtain biologically representative samples from ballast tanks include: the large volume of ballast water present in vessels, differential locations of ballast water uptake, the presence of sediments and irregular shapes of the tanks (Murphy, Ritz et al. 2002).

The frequency of collecting samples from the tank discharge will affect the accuracy of the data obtained. Ideally, samples would be taken at frequent intervals, e.g. 2 minutes, throughout the whole discharge of the tank to obtain an accurate idea of organism distribution. In doing this the number of samples which require analysing would be very high and the time required to do this would cause 'undue delay to the ships operation, departure or movement' (IMO 2008). By reducing the sampling frequency the accuracy of data decreases, but sampling a vessel for compliance would be feasible, this is the compromise which must be made. However, the required sampling frequency is not conclusively determined in the G2 requirements. G8 details the sampling required in shipboard approval testing as: 3 replicate samples at the 'beginning', 'middle' and 'end' of discharge for each influent water, control discharge and treated discharge water. A total of 27 samples (9 sampling points x 3 replicates) would be collected and require processing, with 9 samples collected per water type (3 sampling points x 3 replicates). This number of samples is much more feasible, and the results could be determined within 1 day with sufficient biological expertise, equipment and biologists available to perform the analysis. The effect that this reduction in sample collection has on the accuracy of the data obtained will be addressed in this study.

In order to demonstrate the difficulty in obtaining samples which are statistically representative of a whole tank this study performed tests to assess the variability in the abundance of the alga *Tetraselmis suecica* during complete discharge of a 1 m³ storage tank. The distribution of organisms throughout the tank could be affected by the length of holding time e.g. increased holding duration could allow organisms to settle to the bottom of the tank or attach to the walls. This was investigated by assessing organism distribution in tanks which had been stored for 1, 3 and 5 days. The effect of sampling frequency was investigated by considering 4 'scenarios': sample collection every 2, 6, 12

and 18 minutes throughout the duration of the tank discharge, i.e. from ‘continuous’ to reduced sampling intervals. The tanks used were regularly shaped and contained a known inoculation density of the test organism.

2. Methods

2.2.1 Test organism and inoculation concentration

The test organism *Tetraselmis suecica*, a single celled green alga representative of the $\geq 10 < 50 \mu\text{m}$ size class (defined in Regulation D-2 of the Convention), was used in tests. This organism was used due to its size, ease of culture and due to its common use as a test species for ballast water treatment system testing in G8 approval tests. The experiments were conducted in two sets and a known concentration of *T.suecica* (Set 1: 10 cells ml^{-1} ; Set 2: 100 cells ml^{-1}) was inoculated into a 1 m^3 storage tank. The seawater was filtered by 50 μm prior to inoculating with *T.suecica*.

The concentrations 10 cells ml^{-1} and 100 cells ml^{-1} were chosen due to their applicability in compliance testing. 10 viable cells ml^{-1} is the borderline level for a vessel to fail to comply with the D-2 discharge standard. 100 cells ml^{-1} was subsequently identified as it was 10 times above the level at which a vessel would fail to meet the standard. The aim was to determine whether it would be possible for a vessel known to be substantially over the discharge standard to ‘pass’ compliance testing.

2.2.2 Test setup

Three identical, replicate tanks were used (Tanks 1, 2 and 3). The tanks were square, 1 m^3 , plastic storage tanks with a discharge valve located at the base of the tank. The interior design of the tanks was sloped in order to direct all water to the valve, facilitating full discharge.

The tanks were covered for different holding periods (1, 3 and 5 days) in the dark mimicking ballast tank conditions. A total period of 5 days was used as that is the time period specified in the G8 guidelines for ballast water testing. Samples were also taken on days 1 and 3 to look at the trends observed within this period.

After the required holding time the seawater was discharged at a constant flow rate (1 m^3hr^{-1}) from the bottom of the tank for the three replicate tanks. This flow rate was the fastest flow rate that could be sustained for the entire discharge period to ensure constant conditions throughout.

Samples (70 ml) were collected from each tank simultaneously, every 2 minutes, to perform as near to continuous (or ‘on-line’) sampling as was feasible and to monitor the discharge as closely as possible, taking into account the time required between samples to collect, fix and seal each sample. The sampling size was determined based on a down-sizing calculation for the scaled-down version of our experiment (1 tonne) relative to the volume specified in the G8 guidelines (200 tonnes, sample size 10 L). After collection Lugols iodine was added to preserve samples for analysis.

2.2.3 Data analysis

Analysis was completed using FlowCAM, an automated particle analyser used to count and identify plankton in seawater samples. The samples were run in AutoImage mode to capture images of all particles in the cameras field of view seven times per second. After identifying all *T.suecica* images captured the cell density was determined and expressed as cells ml⁻¹. Results are given in four scenarios; each scenario representing the four sampling frequencies i.e. every 2, 6, 12, and 18 minutes.

2.2.4 Statistical analysis

For each set (10 cells ml⁻¹ and 100 cells ml⁻¹) within each scenario (2, 6, 12 and 18 minutes), the cell density of *T.suecica* was compared both between holding duration and between sampling days using Scheirer-Ray-Hare tests. Cell density data were ranked and the ranked values were used in two-way ANOVAs with holding duration and sample day as factors. The total mean square values were then calculated to determine the χ^2 value for each factor, which were compared to the critical values in a χ^2 table. *Post-hoc* Tukey pairwise comparisons ($P = 0.05$) were used to determine differences observed within holding durations and between sampling days.

Scheirer-Ray-Hare tests using the method outlined above were also used to determine if there were any differences in cell density between the initial and final sampling points between sampling days.

3. Results

3.1 Scenario 1: samples collected every 2 minutes

3.1.1 Set 1: 10 cells ml⁻¹:

In Scenario 1 a total of 32 samples were collected from each of the three tanks. Throughout the tank discharge period (64 minutes) the density of cells found in samples reduced (Figure 1). Regardless of holding period, there was a significant difference in cell density between the initial sampling point

compared to the final sampling point (Table 1), with a higher density recorded at 2 minutes. As the holding period increased, the density of cells present reduced significantly (Figure 1; Table 2). Samples taken after both 2 minutes and 4 minutes had significantly higher cell densities than samples taken after 22, 40, 54, 56 and 62 minutes. In addition, samples taken after 2 minutes had significantly higher cell densities than samples taken at 24, 26, 32, 34, 36, 42, 46, 48, 50 and 58 minutes (*Post-hoc* Tukey pair-wise comparison, $P = 0.05$). On day 1 no sample contained the inoculum level of 10 cells ml^{-1} , and on days 3 and 5 only 3% of samples contained the inoculum level. Although this was observed on all three sampling days, the interaction between holding duration and sampling day had no significant effect on cell density. There was however, a significant difference in cell density between sampling days (Table 2), with significantly more cells recorded on day 3 compared to day 5. Cell density was underestimated in 35%, 56% and 72% of samples on days 1, 3 and 5 respectively. In addition, many samples did not contain any *T.suecica*, and yet a known density of organisms was present in the tanks. These observances of 0 cells ml^{-1} were in 29%, 21% and 41% of samples on days 1, 3 and 5 respectively.

FIGURE 1

TABLE 1

3.1.2 Set 2: 100 cells ml^{-1} :

When the inoculum density was increased to 100 cells ml^{-1} there was again an overall reduction in cell density throughout the discharge period on all sampling days (Figure 2); however, the interaction between holding duration and sampling day had no significant effect on cell density (Table 2). There was a significant difference in cell density between sampling days (Table 2) with day 1 having significantly higher densities than days 3 and 5; and day 5 having significantly higher densities than day 3. On day 1, 97% of the samples collected underestimated the density of cells, and on days 3 and 5 all samples underestimated cell density. As the holding duration increased, the density of cells in the discharge samples decreased significantly (Table 2). *Post-hoc* Tukey pair-wise comparison showed that samples taken at 50 and 56 minutes had significantly lower cell densities than samples taken at 2, 6, 8, 10, 20 and 26 minutes. In addition, samples taken at 56 minutes had significantly lower densities than samples taken at 4, 14, 22 and 24 minutes. Samples taken at 60 and 62 minutes had significantly lower densities than samples taken at 8, 10 and 20 minutes. Samples taken at 20 minutes also had significantly higher densities than samples taken at 38 minutes. However, while there was no significant difference in cell density between the initial sampling point and the final sampling point, there were differences between sampling days (Table 1).

A further important observation was the number of samples on Days 3 and 5 which underestimated the cell density to the extent that they actually met the IMO D-2 Discharge Standard. Their occurrence was observed as 37% and 19% of samples on days 3 and 5 respectively. If these samples were collected for checking compliance the ship would meet the D-2 standard and discharge would be allowed. However, the tank was known to be in violation of D-2 due to the density of organisms that were added to the tank.

FIGURE 2

TABLE 2

3.2 Scenario 2: samples collected every 6 minutes

3.2.1 Set 1: 10 cells ml⁻¹:

In Scenario 2 a total of 10 samples were collected from each of the tanks. As was observed in Scenario 1 a general decline in cell abundance was observed throughout the discharge period in all tests, however this was not significantly different (Table 2), with no significant difference between the initial cell density and the final cell density on any of the sampling days (Table 1). Increased holding duration showed an overall decrease in cell numbers (Figure 3), though again this was not significantly different (Table 2).

FIGURE 3

3.2.2 Set 2: 100 cells ml⁻¹:

All samples underestimated the inoculation density of *T.suecica*. As holding duration increased the cell density observed in samples reduced (Figure 4), though this was not significant over the discharge period (Table 2). There were differences in cell density between sampling days with day 1 have significantly higher cell densities than days 3 and 5 (Table 2). On Days 1 and 3 the trendlines showed a relatively consistent cell density (Figure 4), however these levels were > 60% lower than the initial inoculation density, with a significant difference between the initial sampling point and the final (Table 1).

FIGURE 4

3.3 Scenario 3: samples collected every 12 minutes

3.3.1 Set 1: 10 cells ml⁻¹:

In Scenario 3 a total of five samples were collected from each of the three tanks. All tests showed low cell density, although in Scenario 3 this was not clearly related to holding duration (Figure 5) as the cell density at the initial sampling point was not significantly different from the final sampling point on any of the sampling days (Table 1). On Day 1 80% of samples underestimated the cell density, and of these 60% did not record any *T.suecica* cells. There was no significant difference in cell density over holding duration or between sampling days (Table 2).

FIGURE 5

3.3.2 Set 2: 100 cells ml⁻¹:

All samples were again below the inoculation level and underestimated cell density by <72%. After 5 days holding duration 40% of samples showed <10 *T.suecica* per ml, (i.e. met the D-2 Discharge Standard), although there was no significant difference in cell density between sampling days (Table 2). Increased holding duration showed a slight decrease in cell density (Figure 6), however this was not significant (Table 2) with no significant difference in cell density between the initial sampling point and the final sampling point on any of the sampling days (Table 1).

FIGURE 6

3.4 Scenario 4: samples collected every 18 minutes

3.4.1 Set 1: 10 cells ml⁻¹:

In Scenario 4 a total of 3 samples were collected from each tank. This could be considered the equivalent of sampling the ‘beginning’, ‘middle’ and ‘end’ of the ballast water discharge as described in the IMO G8 guidelines. On all days ≥66% of samples underestimated the cell density, although there was no significant difference in cell density between days (Table 2). Only one sample was equal to the inoculation density and on each sampling day one sample did not contain any *T.suecica* cells (Figure 7). There was no significant difference in cell density between the initial sampling point and the final sampling point for any of the sampling days (Table 1). Holding duration had no significant effect on cell density (Table 2).

FIGURE 7

3.4.2 Set 2: 100 cells ml⁻¹:

In all samples the inoculation density was underestimated by >58%. Increased holding duration resulted in lower numbers of *T.suecica* in samples; however there were no significant differences in cell density with holding duration (Table 2), with the cell density at the initial sampling point not being significantly different to the final sampling point (Table 1). On Days 3 and 5 33% of samples were lower than the D-2 discharge standard, and there was a significant difference in cell density between sampling days (Table 2), with samples collected on day 1 having higher cell densities than samples collected on days 3 and 5. While lower numbers were observed, all samples contained *T.suecica* cells (Figure 8).

FIGURE 8

4. Discussion and conclusions

When determining compliance with Regulation D-2 the G2 guidelines require sampling regimes to provide samples which are representative of the whole discharge of ballast water. This study was performed to show the difficulties in obtaining representative samples of a model tank, without incorporating the factors which are involved with ballast water tank sampling, such as large size, volume of water, wide diversity of organisms and irregular shape of tanks. The sample size (70ml) used in this study demonstrated these difficulties on a small scale, and in real scenarios a larger sample size would be required. The variability in organism distribution within a standard tank was demonstrated through the following observations:

- High levels of overestimation (>35%) and underestimation (>28%) of cell density (in Scenario 1),
- The frequency of obtaining (approximately) the input number of cells in the samples was very rare (Figures 1-8),
- Many samples contained no cells,
- Overall decrease in cell density over the duration of tank discharge – this may be due to the settling of organisms in the tank,
- Decrease in cell density with longer holding periods (3 and 5 days),
- The ‘compliance’ of samples known to contain a cell density >10 cells ml⁻¹.

These outcomes were supported through statistical tests and highlight the problems involved in obtaining representative samples, also showing that no one single sample could be used to represent a tank. When applied to a ballast tank which is much larger, irregularly shaped and holds an

unknown density of organisms it is clear that obtaining data which represents the entire tank will be difficult to achieve.

The effect of sampling frequency on the accuracy of the data obtained was clearly observed in the four Scenarios. Scenario 1, in which the sampling frequency was every 2 minutes, showed a clear picture of the heterogeneous distribution of organisms within the tank. The effect of holding time was observed as a decrease in cell density as the holding period increased. But, as the sampling frequency was decreased to 6, 12 and 18 minutes the distribution of organisms within the tank was not accurately shown (Figures 3-8). In Scenario 4 the organism distribution for the whole tank could not be accurately depicted due to only three samples being collected i.e. 'beginning', 'middle' and 'end' of tank discharge (Figures 7 and 8). This study has shown the effect that low sampling frequency has on the accuracy of data obtained from a 1 tonne storage tank. In reality ships can carry between 100 and 100,000 tonnes of ballast water and discharge at flow rates of 100 to over 3000 m³hr⁻¹. The discharge flow rate of a single vessel could vary on every voyage due to the type of cargo carried and the rate of loading in order to prevent excessive shearing force and bending moment on the ship's hull. The variability in organism distribution and uncertainty in the accuracy of data obtained from collecting only three replicate samples at three sampling points on discharge of ballast water will, in reality, be much greater than that seen in this study.

Previous studies have used different statistical analysis methods to determine the volume of water which must be sampled to obtain statistically representative data for organism's $\geq 50\mu\text{m}$. One example is presented in Basurko and Mesbahi (2011). In the mentioned work, the authors determined the volume of water which must be sampled to obtain data representative of the entire population (with 95% confidence). This study concluded that a large volume of ballast water must be sampled, for example, for a ballast water discharge of 216, 5,000, 10,000 and 50,000 m³, the required sample volumes are 211, 3,288, 4,899 and 8,000 m³ respectively (Basurko and Mesbahi 2011). These sample volumes are not practical due to the time and space required for sampling, which would cause delays to the vessel, and the associated costs involved in both the sampling itself and interruptions to normal operations.

In addition to determining the water volume required to obtain representative data there are further uncertainties regarding data accuracy due to the range of methods employed to count organisms and assess their viability. The counting methods used by previous studies include common microscopic methods e.g. Sedgwick-rafter cells and Utermohl counting chambers (e.g. Tamburri, Smith et al. 2006; Baek, Jung et al. 2012), and automated methods such as flowcytometry (Veldhuis, Fuhr et al.

2006). Viability has been determined using chlorophyll α levels (e.g. McCollin, Quilez-Badia et al. 2007; Wright, Dawson et al. 2007; Wright, Dawson et al. 2007; Quilez-Badia, McCollin et al. 2008; Klein, MacIntosh et al. 2010) and viability stains such as Evans blue, FDA and SYTOX green (e.g. Jochem 1999; Veldhuis, Fuhr et al. 2006; Reavie, Cangelosi et al. 2010). When dealing with low concentrations of organisms, as will likely be present in samples tested for compliance, the error itself involved in the processing of samples by using these different assessment methods could be the difference between a vessel passing or failing compliance (For example, see Godhe, Cusack et al. 2007; Steinberg, Lemieux et al. 2011). Thus it is vital that standard methods are developed for use in laboratories performing compliance testing.

Therefore, in light of the closeness of ratification of the Ballast Water Convention it is vital that not only are effective treatment systems developed, but also that accurate sampling methods for ensuring compliance are determined. This study has discussed the difficulties in obtaining statistically and biologically representative samples from ballast tank. Although a challenging task, it is necessary that such methods are developed and implemented for the accurate detection of ships violating the D-2 Standard.

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Table 1. Scheirer-Ray-Hare test to investigate the effects of holding duration and sampling day between the initial and final sampling point on the cell density (ml^{-1}) of *Tetraselmis suecica* for each set within each scenario, where inoculation densities in set 1 were 10 cells ml^{-1} and set 2 100 cells ml^{-1}

Scenario	Set	Source of Variation	Cell density			
			χ^2	df	<i>P</i>	Tukey (<i>P</i> = 0.05)
Sc. 1	1	Holding Duration	5.39	1	< 0.05 ^s	$t_{\text{initial}} > t_{\text{final}}$
		Sampling Day	1.09	2	> 0.05 ^{ns}	(d1 = d3 = d5)
		Holding Duration*Sampling Day	2.16	2	> 0.05 ^{ns}	
	2	Holding Duration	0.002	1	> 0.05 ^{ns}	$t_{\text{initial}} = t_{\text{final}}$
		Sampling Day	11.31	2	< 0.01 ^s	(d3 > (d1 = d5))
		Holding Duration*Sampling Day	0.52	2	> 0.05 ^{ns}	
Sc. 2	1	Holding Duration	0.72	1	> 0.05 ^{ns}	$t_{\text{initial}} = t_{\text{final}}$
		Sampling Day	0.64	2	> 0.05 ^{ns}	(d1 = d3 = d5)
		Holding Duration*Sampling Day	0.90	2	> 0.05 ^{ns}	
	2	Holding Duration	4.17	1	< 0.05 ^s	$t_{\text{initial}} > t_{\text{final}}$
		Sampling Day	3.50	2	> 0.05 ^{ns}	(d1 = d3 = d5)
		Holding Duration*Sampling Day	3.68	2	> 0.05 ^{ns}	
Sc. 3	1	Holding Duration	0.28	1	> 0.05 ^{ns}	$t_{\text{initial}} = t_{\text{final}}$
		Sampling Day	0.96	2	> 0.05 ^{ns}	(d1 = d3 = d5)
		Holding Duration*Sampling Day	1.44	2	> 0.05 ^{ns}	
	2	Holding Duration	2.16	1	> 0.05 ^{ns}	$t_{\text{initial}} = t_{\text{final}}$
		Sampling Day	1.21	2	> 0.05 ^{ns}	(d1 = d3 = d5)
		Holding Duration*Sampling Day	1.56	2	> 0.05 ^{ns}	
Sc. 4	1	Holding Duration	2.63	1	> 0.05 ^{ns}	$t_{\text{initial}} = t_{\text{final}}$
		Sampling Day	0.39	2	> 0.05 ^{ns}	(d1 = d3 = d5)
		Holding Duration*Sampling Day	0.79	2	> 0.05 ^{ns}	
	2	Holding Duration	0.50	1	> 0.05 ^{ns}	$t_{\text{initial}} = t_{\text{final}}$
		Sampling Day	9.72	2	< 0.01 ^s	(d1 > (d3 = d5))
		Holding Duration*Sampling Day	0.19	2	> 0.05 ^{ns}	

s: significant effect on cell density

ns: no significant effect on cell density

d1: sampling day 1; d3 = sampling day 3; d5 = sampling day 5

ml^{-1}

Table 2. Scheirer-Ray-Hare test to investigate the effects of holding duration and sampling day on the cell density (ml^{-1}) of *Tetraselmis suecica* throughout the discharge period. Comparisons were made for each set within each scenario, where inoculation densities in set 1 were 10 cells ml^{-1} and set 2 100 cells ml^{-1}

Scenario	Set	Source of Variation	Cell density			
			χ^2	df	<i>P</i>	
Sc. 1	1	Holding Duration	71.39	31	< 0.01 ^s	
		2 mins	Sampling Day	6.62	2	< 0.05 ^s
		Holding Duration*Sampling Day	45.75	62	> 0.05 ^{ns}	
	2	Holding Duration	65.21	31	< 0.01 ^s	
		Sampling Day	63.29	2	< 0.01 ^s	
		Holding Duration*Sampling Day	51.42	60	> 0.05 ^{ns}	
Sc. 2	1	Holding Duration	8.29	9	> 0.05 ^{ns}	
		6 mins	Sampling Day	1.48	2	> 0.05 ^{ns}
		Holding Duration*Sampling Day	15.26	18	> 0.05 ^{ns}	
	2	Holding Duration	11.62	9	> 0.05 ^{ns}	
		Sampling Day	24.51	2	< 0.01 ^s	
		Holding Duration*Sampling Day	18.54	18	> 0.05 ^{ns}	
Sc. 3	1	Holding Duration	2.49	4	> 0.05 ^{ns}	
		12 mins	Sampling Day	3.13	2	> 0.05 ^{ns}
		Holding Duration*Sampling Day	8.92	8	> 0.05 ^{ns}	
	2	Holding Duration	7.03	4	> 0.05 ^{ns}	
		Sampling Day	4.89	2	> 0.05 ^{ns}	
		Holding Duration*Sampling Day	8.09	8	> 0.05 ^{ns}	
Sc. 4	1	Holding Duration	2.77	2	> 0.05 ^{ns}	
		18 mins	Sampling Day	0.08	2	> 0.05 ^{ns}
		Holding Duration*Sampling Day	2.68	4	> 0.05 ^{ns}	
	2	Holding Duration	0.73	2	> 0.05 ^{ns}	
		Sampling Day	14.34	2	< 0.01 ^s	
		Holding Duration*Sampling Day	1.93	4	> 0.05 ^{ns}	

Captions for figures

Figure 1. Number of *Tetraselmis suecica* at an inoculation cell density of 10 cells ml⁻¹ for 1 day, 3 days and 5 days holding period in three replicate tanks (1, 2 and 3). Values are the mean of 3 replicates ± SE, for scenario 1 (sampling frequency 2 minutes). The shaded section indicates >10 *T.suecica* per ml i.e. samples which do not meet the D-2 Discharge Standard.

Figure 2. Number of *Tetraselmis suecica* at an inoculation cell density of 100 cells ml⁻¹ for 1 day, 3 days and 5 days holding period in three replicate tanks (1, 2 and 3). Values are the mean of 3 replicates ± SE, for scenario 1 (sampling frequency 2 minutes). The shaded section indicates >10 *T.suecica* per ml i.e. samples which do not meet the D-2 Discharge Standard.

Figure 3. Number of *Tetraselmis suecica* at an inoculation cell density of 10 cells ml⁻¹ for 1 day, 3 days and 5 days holding period in three replicate tanks (1, 2 and 3). Values are the mean of 3 replicates ± SE, for scenario 2 (sampling frequency 6 minutes). The shaded section indicates >10 *T.suecica* per ml i.e. samples which do not meet the D-2 Discharge Standard.

Figure 4. Number of *Tetraselmis suecica* at an inoculation cell density of 100 cells ml⁻¹ for 1 day, 3 days and 5 days holding period in three replicate tanks (1, 2 and 3). Values are the mean of 3 replicates ± SE, for scenario 2 (sampling frequency 6 minutes). The shaded section indicates >10 *T.suecica* per ml i.e. samples which do not meet the D-2 Discharge Standard.

Figure 5. Number of *Tetraselmis suecica* at an inoculation cell density of 10 cells ml⁻¹ for 1 day, 3 days and 5 days holding period in three replicate tanks (1, 2 and 3). Values are the mean of 3 replicates ± SE, for scenario 3 (sampling frequency 12 minutes). The shaded section indicates >10 *T.suecica* per ml i.e. samples which do not meet the D-2 Discharge Standard.

Figure 6. Number of *Tetraselmis suecica* at an inoculation cell density of 100 cells ml⁻¹ for 1 day, 3 days and 5 days holding period in three replicate tanks (1, 2 and 3). Values are the mean of 3 replicates ± SE, for scenario 3 (sampling frequency 12 minutes). The shaded section indicates >10 *T.suecica* per ml i.e. samples which do not meet the D-2 Discharge Standard.

Figure 7. Number of *Tetraselmis suecica* at an inoculation cell density of 10 cells ml⁻¹ for 1 day, 3 days and 5 days holding period in three replicate tanks (1, 2 and 3). Values are the mean of 3 replicates ± SE, for scenario 4 (sampling frequency 18 minutes). The shaded section indicates >10 *T.suecica* per ml i.e. samples which do not meet the D-2 Discharge Standard.

Figure 8. Number of *Tetraselmis suecica* at an inoculation cell density of 100 cells ml⁻¹ for 1 day, 3 days and 5 days holding period in three replicate tanks (1, 2 and 3). Values are the mean of 3 replicates ± SE, for scenario 4 (sampling frequency 18 minutes). The shaded section indicates >10 *T.suecica* per ml i.e. samples which do not meet the D-2 Discharge Standard.

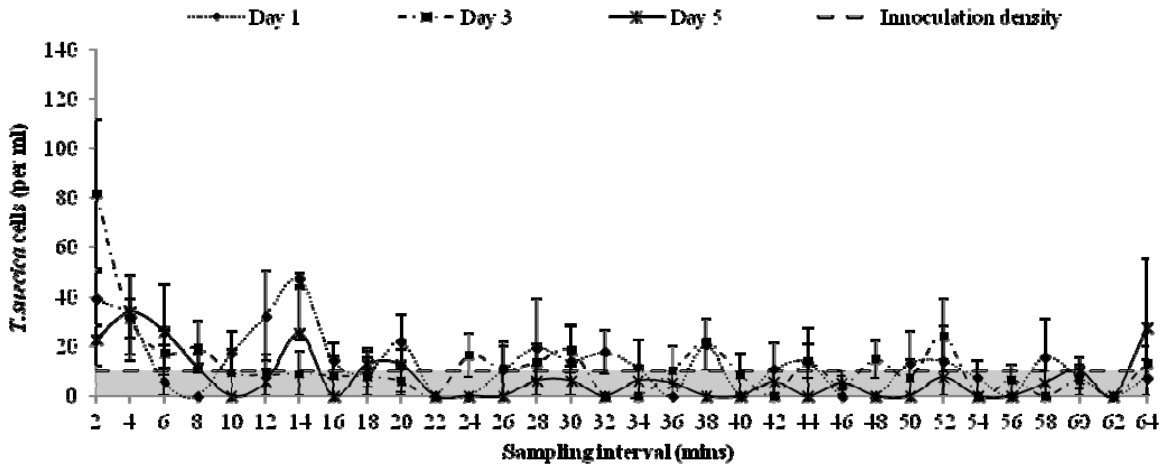


Figure 1

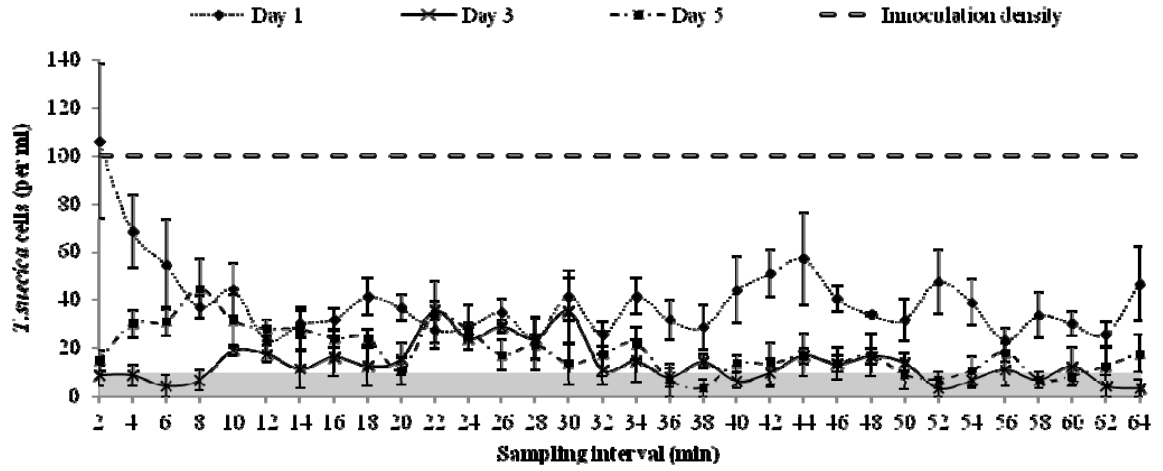


Figure 2

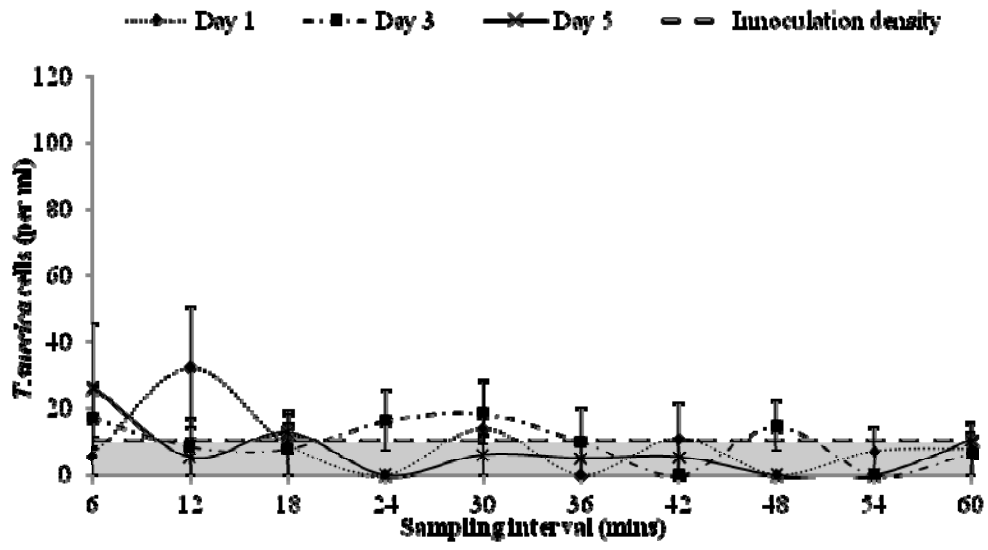


Figure 3

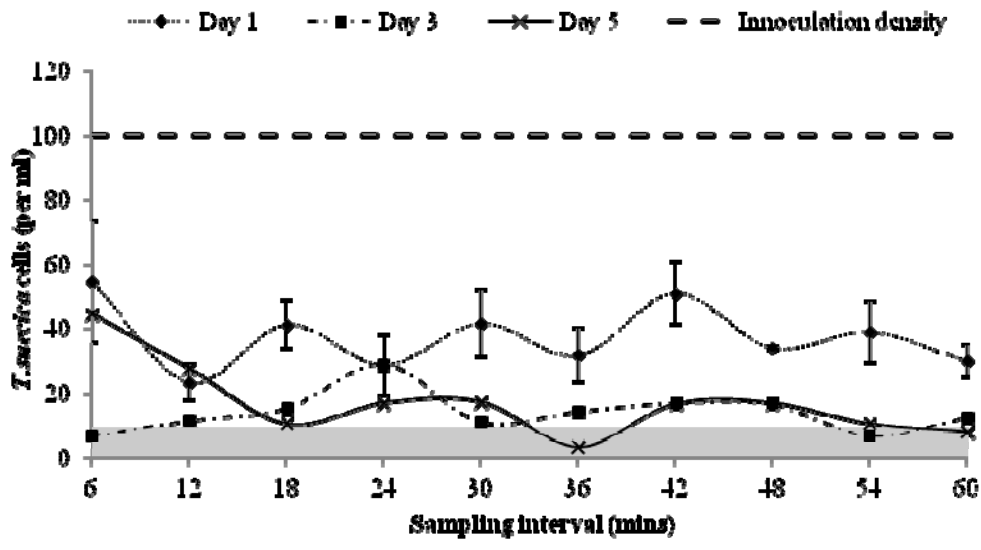


Figure 4

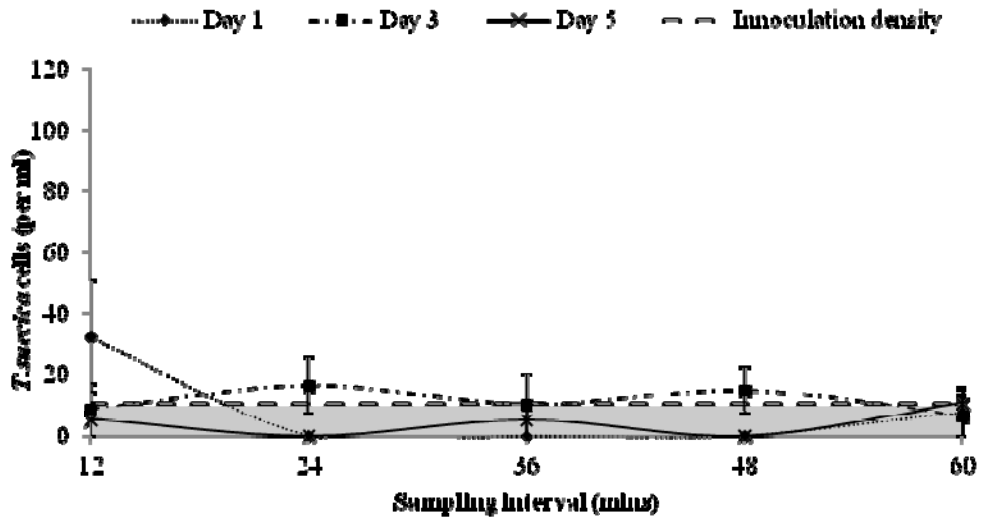


Figure 5

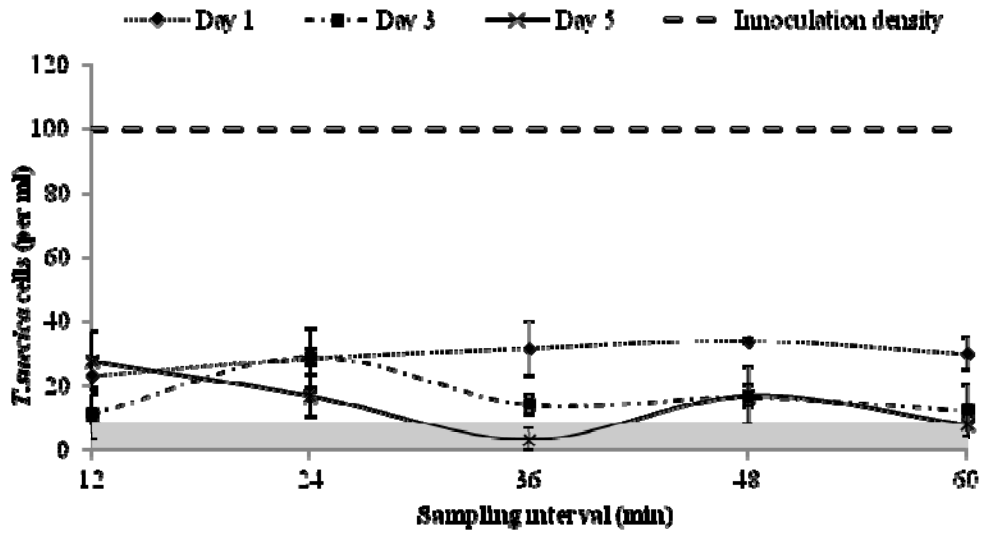


Figure 6

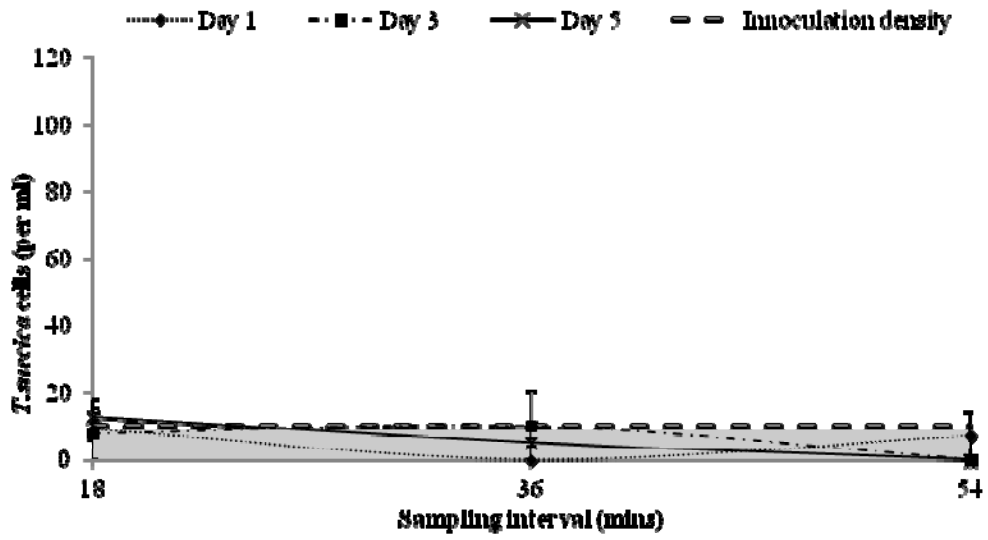


Figure 7

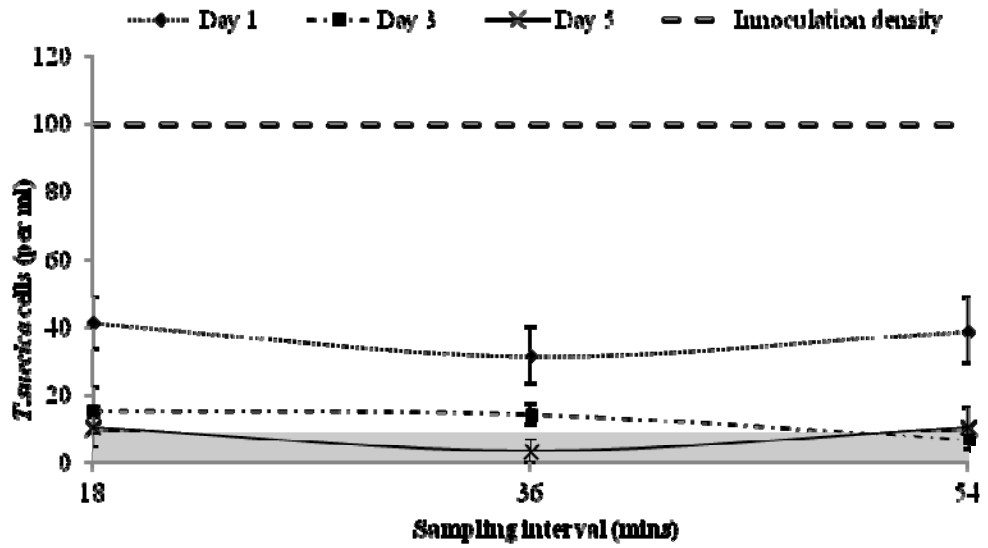


Figure 8