Harmful Algae 42 (2015) 52–59

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Examination of six commonly used laboratory fixatives in HAB monitoring programs for their use in quantitative PCR based on Taqman probe technology

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ARTICLE INFO

Article history:
Received 6 August 2014
Received in revised form 9 December 2014
Accepted 17 December 2014
Available online 30 January 2015

Keywords:
Algal fixatives
Harmful algal blooms
Monitoring programs
qPCR
Taqman probe
Time series

ABSTRACT

Due to the need for more rapid and reliable detection, quantification and enumeration of harmful algal species the use of molecular methods are increasingly being used in monitoring and field studies. However, many studies often require sample fixation to allow for transportation before analyses are conducted. Here, we describe the effects of six fixatives (acidified Lugol's iodine with or without sodium thiosulphate, glutaraldehyde, paraformaldehyde (PFA), formalin and ethanol) on quantitative real-time polymerase chain reaction (qPCR) amplification with Taqman probes. We applied extracted total genomic DNA from four harmful algal species from Danish waters, representing three dinoflagellates (*Alexandrium tamarense*, *Karenia mikimotoi*, *Karlodinium veneficum* and a haptophyte (*Prymnesium parvum*). The C_q values generated on the qPCR amplification plot were compared to those of an unfixed sample that acted as a control. For all species positive amplifications were achieved from DNA templates from all preserved samples. However, amplification efficiencies between fixatives and species varied. Yet it was found that Lugol's iodine was the most ideal short-term fixative for enumeration of cells by qPCR as well as being the safest to handle. The effect of age on Lugol's iodine fixed samples was also addressed. Samples were fixed and stored at 5 °C in the dark and total genomic DNA extracted after 24 h, 72 h, 1 week, 2 weeks, 1 month and 2 months. Samples remained stable for 1 month for *A. tamarense* and *K. veneficum* and 2 months for *K. mikimotoi* and *P. parvum*.

1. Introduction

Phytoplankton is the focus of interest for both scientists and policy makers as the size and make up of the phytoplankton community can be affected by a variety of environmental and anthopogenic factors such as climate change or eutrophication. The make up of phytoplankton communities can provide a benchmark for future environmental changes, thus increasing our understanding of any future changes that may be occurring (Suthers and Rissik, 2009). Marine environmental policy means that EU member states are required under a number of different directives to monitor the aquatic environment. Phytoplankton monitoring is a key component of a number of these directives, and include: the water framework directive (2000/60/EC), the EU marine strategy framework directive (2008/56/EC) and the EU Shellfish Hygiene Directive (91/492/EEC). Under these directives EU member states must survey, monitor and record the presence and geographical distribution of phytoplankton. Many counties worldwide are now faced with a large number of toxic or harmful microalgal species and their associated effects, which may have an impact on resources and cause large economic losses (Anderson et al., 2000). Many coastal waters experience a range of HAB-based events of relevance to both shellfish and finfish production. Data collected from phytoplankton monitoring programs provide early warning of harmful or toxic events, thus alerting industry and minimising financial losses whilst at the same time safeguarding human health. The health and economic problems associated with harmful phytoplankton demonstrate the need for fast and reliable methods for detecting and enumerating harmful phytoplankton species (e.g. Eckford-Soper et al., 2013).

Traditionally, the standard method for identification and enumeration of phytoplankton is by light microscopy, most commonly using the Utermöhl technique (Utermöhl, 1958). However, this technique requires high levels of skills by the operator and is highly time consuming (Karlson et al., 2010; Medlin, 2013). Furthermore, it can be nearly impossible to

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http://dx.doi.org/10.1016/j.hal.2014.12.007
1568-9883/

differentiate between species that are morphologically similar or toxic from non-toxic microalgal populations.

Developments in molecular biology are increasingly allowing the genetic characterisation of phytoplankton populations (Anderson et al., 2012; Bertazzoni et al., 2005). Not only can RNA and DNA be used to examine phytoplankton communities but also population genetics, trophic interactions, stress responses, genome structure and gene function. The majority of these fields require preserved nucleic acids. Molecular techniques allow us to rapidly and more reliably identify phytoplankton species, significantly increasing sample throughput compared to traditional light microscopy methods (Medlin, 2013). Some of these innovative molecular methodologies include: fluorescent *in situ* hybridisation (FISH) (Touzet et al., 2010), FISH-FC (Eckford-Soper et al., 2013), ELIZA, MIDTAL (Medlin et al., 2013) and qPCR (Penna and Galluzzi, 2013).

Quantitative real-time PCR (qPCR) is a powerful molecular tool for providing an accurate and sensitive enumeration of phytoplankton species (e.g. Kamikawa et al., 2006; Galluzzi et al., 2010). During a qPCR reaction the sequence of interest is amplified using a specific primer pair with product formation being monitored in real time by measuring the increase in fluorescence. The higher the starting concentration of the target the earlier the fluorescence signal begins to significantly increase. The amount of target can then be calculated from the number of PCR cycles (C_q) required to cross a fixed threshold. The amount of the target sequence can then be deduced by using a standard curve as a reference. The standard curve can be established either from a target sequence inserted into a plasmid or from genomic DNA extracted from a known amount of cultured material (Galluzzi et al., 2010). The two most commonly used approaches adopt either an intercalating fluorescent dye such as SYBR green or a probe based platform labelled with a fluorochrome and a quencher (Taqman technology) (Goyer and Dandie, 2012). Taqman chemistry adds an additional level of specificity to the assay, as each probe can be labelled with different distinguishable reporter dyes, allowing amplification of two or more distinct sequences (often equivalent to species) in one reaction thus providing high throughput.

Before any analysis can take place many studies and monitoring programs often require sample fixation to allow transportation. For instance, samples need to be shipped from a sampling site to the place of analysis. Artefacts due to fixation have previously been a significant factor of bias in phytoplankton sampling (Naik et al., 2010). This has culminated in the need for better sample preservation methods. The ideal fixative should be cost effective and safe for the user. The reason for this is that many samples are fixed in the field away from appropriate equipment such as fume hoods. They should be easy to administer and not destroy cell morphology or the integrity of DNA (Godhe et al., 2002).

However many fixative formulations, especially those that contain acids or alcohol can influence the ability to retrieve DNA (Koleśarová et al., 2012). The most common fixatives in phytoplankton research have traditionally been formaldehyde and Lugol's iodine solution. When carrying out molecular analysis, formaldehyde has been shown to be problematical as it acts by forming crosslinks, thus altering the DNA structure and reducing PCR performance (Auinger et al., 2008).

Iodine based Lugol's solution (1–2% final concentration) is the most common fixative used in monitoring programs that require light microscopy. However, some studies have shown Lugol's to inhibit PCR reactions at high Lugol's concentrations (Auinger et al., 2008; Godhe et al., 2002). Despite this, a number of studies using both PCR and qPCR have shown successful amplification of samples fixed with Lugol's (Collins et al., 2009; Connell, 2002; Tengs et al., 2001).

Whilst a number of studies have reviewed the effects of various fixatives on PCR amplification and qPCR (Godhe et al., 2002; Yuan et al., 2012), no studies have assessed the effects of fixatives on qPCR with Taqman probe technology. Here, we describe the effects of a number of fixatives: acidified Lugol's iodine (with and without the addition of sodium thiosulphate), glutaraldehyde, paraformaldehyde (PFA), formalin and ethanol on qPCR amplification using the Taqman probe technology. We used DNA extracted from four different harmful algal species from coastal waters. We also addressed if the age of Lugol's fixed samples over periods of days, weeks, months alter the efficiency and thus end results of the qPCR reaction.

2. Materials and methods

2.1. Cultures

The following microalgal cultures were used: the thecate, dinoflagellate *Alexandrium tamarense* (CCAP 1119/28) obtained from the Culture Collection of Algae and Protozoa, Oban, Scotland, the naked dinoflagellates *Karenia mikimotoi* (K-0260), *Karlodinium veneficum* (K-1661) and the haptophyte *Prymnesium parvum* (K-0081) obtained from the Scandinavian Culture Collection of Algae and Protozoa, Copenhagen, Denmark. *A. tamarense*, *K. mikimotoi* and *K. veneficum* were grown in L1 media and *P. parvum* in TL10 media at 15 °C under a light intensity of 110 μmol E m^{-2} s^{-1} (16:8 h light:dark cycle).

2.2. Comparison of fixatives

The following fixative treatments were used: (i) acidified Lugol's (660 μg I_2), (ii) acidified Lugol's (660 μg I_2) plus sodium thiosulphate (390 μg $Na_2SO_2O_3$ ml^{-1}), (iii) glutaraldehyde (0.1%), (iv) paraformaldehyde (PFA) in BPS, (1%), (v) formalin (1%) plus ice cold methanol (100%) and (vi) ice cold ethanol (70%). An unfixed sample was used as a control.

Aliquots (1 ml) containing approximately 10,000 cells for *Alexandrium tamarense* and *Karenia mikimotoi* and 100,000 cells for *Karlodinium veneficum* and *Prymnesium parvum* were removed aseptically from exponentially growing cultures. Samples were then fixed in triplicate using the fixatives described above. Samples fixed in acidified Lugol's (660 μg I_2) were fixed in sextuplicate; three for the acidified Lugol's treatment and three for Lugol's plus sodium thiosulphate treatment. For all treatments except the 70% ethanol treatment, the fixatives were added directly to the sample. For the ethanol treatment the samples were first pelleted by centrifugation (4000 × *g*, 10 min), the supernatant removed and then re-suspended in 1 ml of 70% ice-cold ethanol. All samples were then stored for 24 h before being pelleted by centrifugation (4000 × *g*, 10 min) and the supernatant removed. The formalin samples were then re-suspended in 1 ml of ice-cold methanol and stored over night at −20 °C. Three of the Lugol's fixed samples were re-suspended in 20 μl $Na_2SO_2O_3$ ml^{-1} and left for a few minutes until the solution was clear. All samples were then centrifuged (4000 × *g*, 10 min) and the supernatant removed. The cell pellets were washed twice in PBS buffer, centrifuged (4000 × *g*, 10 min) and the supernatant removed. Cell pellets were then stored at −20 °C until DNA extraction (Fig. 1).

2.3. Efficiency of Lugol's over time

To test the effectiveness of acidified Lugol's iodine as a fixative of qPCR over time aliquots (1 ml) containing approximately 10,000 cells for *Alexandrium tamarense* and *Karenia mikimotoi* and 100,000 cells for *Karlodinium veneficum* and *Prymnesium parvum* were removed aseptically from exponentially growing cultures. Samples were then fixed in triplicate and left for 24 h, 72 h, 1 week, 2 weeks, 1 month and 2 months in Lugol's iodine (660 μg I_2). Samples were then stored in the dark at 5 °C before being pelleted by

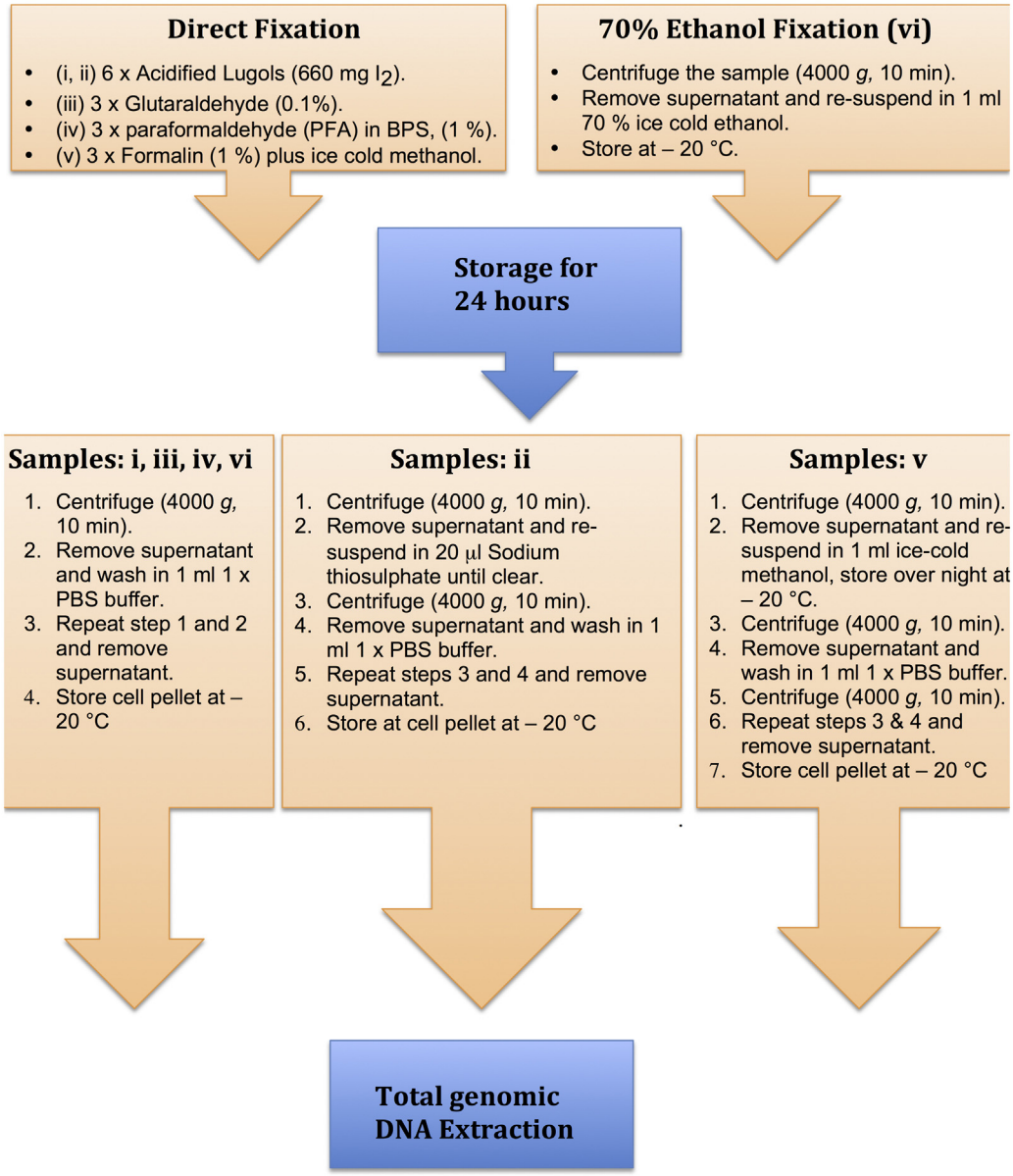

Fig. 1. The workflow for sample fixation, for example 24 h.

centrifugation (4000 × *g*, 10 min) and the supernatant removed. The samples were then washed twice in PBF buffer and pelleted once more. Cell pellets were finally stored at −20 °C overnight until DNA extraction.

2.4. Extraction of total genomic DNA

Cell pellets were re-suspended in 10 µl of ddH_2O and then transferred to a reaction tube. This was followed by two further washes using 10 µl of ddH_2O to ensure all cells had been transferred. For all experiments, extraction was carried out using the Powerplant Pro DNA isolation Kit (MO BIO) according to manufacturers recommendations (total elution volume of DNA = 50 µl) and stored at −20 °C.

2.5. qPCR

The species specific qPCR primers and probe sets used in this study for *Alexandrium tamarense* and *Karenia mikimotoi* were developed by Toebe et al. (2013) and Yuan et al. (2012) respectively. Primers and probes for *Karlodinium veneficum* and *Prymnesium parvum* were designed for this study using the online service provided by Integrated DNA technologies (https://eu.idtdna.com) and following their recommendations. For *K. veneficum* we used previously published LSU rDNA sequences (Daugbjerg et al., 2000) and for *P. parvum* we determined the internal transcribed spacer region (ITS) from three Scandinavian strains. PCR conditions and sequence determination of ITS was as outlined in Craveiro et al. (2013).

Each species was amplified using the primer and probe sequences. Modifications and concentrations are described in Table 1. Primer and probe concentrations were optimised for the CFX96 Touch Real-time PCR detection system (Biorad). Optimised conditions for qPCR were with 20 µl reactions with 4 µl of HOT FIREPol® Probe qPCR Mix Plus (no ROX) (1× final concentration) (Solis BioDyne), 2 µl of template, 0.5 µl of each appropriate primer, 1 µl of probe and 12 µl ddH_2O. The cycling parameters for all reactions were: 15 min at 95 °C for activation of the polymerase, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Table 1
Species-specific primers and Taqman probes use in this study.

Species	Target	Primer/probe sequence 5′–3′	Final concentration (nm)	Amplicon size (bp)	Reference
A. tamarense	28s rDNA	Forward primer TamF: ccacagcccaaagctcttgt Reverse primer TamR: ccatgagggaaatatgaaaagga Probe ATNA279: CY5-aacactcccaccaagca-BHQ2	375 375 250	131	Toebe et al. (2013)
K. mikimotoi	ITS	Forward primer KMF: ctttgtgtgtaacctgttgctttgt Reverse primer KMR: tcagcgggtttgcttacct Probe KMP: FAM-tcagcgggtttgcttacct-BHQ1	225 225 250	111	Yuan et al. (2012)
P. parvum	ITS	Forward primer PrymF: ggatcattaccggtctttccac Reverse primer PrymR: ctcaagcaagagcacagatga Probe PrymP: Texas Red-tgcgtaccactcgtccctttgg-BHQ2	100 100 250	100	This paper
K. veneficum	28s rDNA	Forward primer KarveneF: gcctggtagaactcatgtctaaa Reverse primer KarveneR: cgacgagtaacagaagctacaa Probe HEX-tcattacctgcgtctgggttcgtg-BHQ1	200 200 250	101	This paper

2.6. Statistical analysis

Statistical procedures were carried out in Minitab statistical software. A one-way ANOVA was used on log-transformed data to test the differences between the C_q values generated for each fixative. $P < 0.05$ was considered significant and variability was measured by standard error of the mean (S.E.M.).

3. Results

To examine the state of preservation of phytoplankton genomic DNA, a qPCR amplification plot was used to measure the various phytoplankton fixed samples. We analysed the C_q value on the qPCR amplification plot because the C_q value reflects the total amount of target DNA, with a smaller C_q value indicating a larger amount of target DNA in the sample. All fixative C_q values were compared to those of the unfixed sample and this acted as the control. We were able to achieve positive amplification of DNA fragments from all preserved samples from all species. However, amplification between fixatives and species was variable, see below.

3.1. Comparison of fixatives

Over a short fixation period (24 h), the DNA yield varied with species and fixative. For *Alexandrium tamarense* C_q values all of the fixatives with the exception of formalin (1%) and ethanol (70%) were similar compared to the control ($P > 0.05$) (Fig. 2A). Formalin and 70% ethanol had a detrimental effect on the total DNA concentrations with the C_q values being significantly (25% and 7%, respectively) greater than the control ($P < 0.05$).

Whilst for *K. mikimotoi* cell samples fixed in ethanol (70%), glutaraldehyde (0.1%) and paraformaldehyde (1%) generated C_q values, which were comparable to the unfixed control ($P > 0.05$) (Fig. 2B). Both Lugol's treatments (with and without sodium thiosulphate) and formalin (1%) had a slight (between 6 and 8%) but significantly higher C_q value ($P < 0.05$).

The most effective fixative for *K. veneficum* proved to be the Lugol's based treatments, which were similar to that of the control ($P > 0.05$). All other fixatives had significantly larger C_q values ($P < 0.05$), with glutaraldehyde and formalin fixed samples being the highest and 31% and 32% greater than the control (Fig. 2C).

None of the fixatives acted to preserve *Prymnesium parvum* without significantly reducing qPCR results. For all the fixatives the C_q values were significantly higher compared to the control ($P < 0.05$) (Fig. 2D). Out of all the fixatives described the ethanol (70%) fixed sample had the closest C_q value which was 14% greater than the control followed by the Lugol's fixed samples which increased the C_q value by 17–19%.

3.2. Medium-term storage effects of Lugol's

DNA concentrations remained stable for up to 1 month after fixation in Lugol's iodine for *Alexandrium tamarense* and *Karlodinium veneficum* and after an initial decline in qPCR value, samples were stable for 2 months for *Karenia mikimotoi* and *Prymnesium parvum*. The C_q values of *A. tamamrense* fixed samples remained similar to the control for the first month post-fixation ($P > 0.05$), but after 2 months qPCR results had declined further with a 11% increase in C_q value compared to the control (Fig. 3A).

Karenia mikimotoi samples preserved for 24 h had a slight increase in C_q value of 6%, which increased to 8% after two days. C_q values then remained stable ($P > 0.05$) with a further 4% reduction in concentrations being observed after 2 months (Fig. 3B).

Karlodinium veneficum samples were stable for up to 1 month post-fixation ($P > 0.05$) with only a 8% increase in C_q value compared to the control after this period. However, after 2 months a 32% decline in qPCR values was observed (Fig. 3C).

After an initial decline of 17% after 24 h ($P < 0.05$) C_q values for *Prymnesium parvum* remained stable for 2 months post-fixation, with all samples between 24 h and 2 months being similar ($P > 0.05$). DNA concentrations had declined by 18% after 2 months in the Lugol's iodine (Fig. 3D).

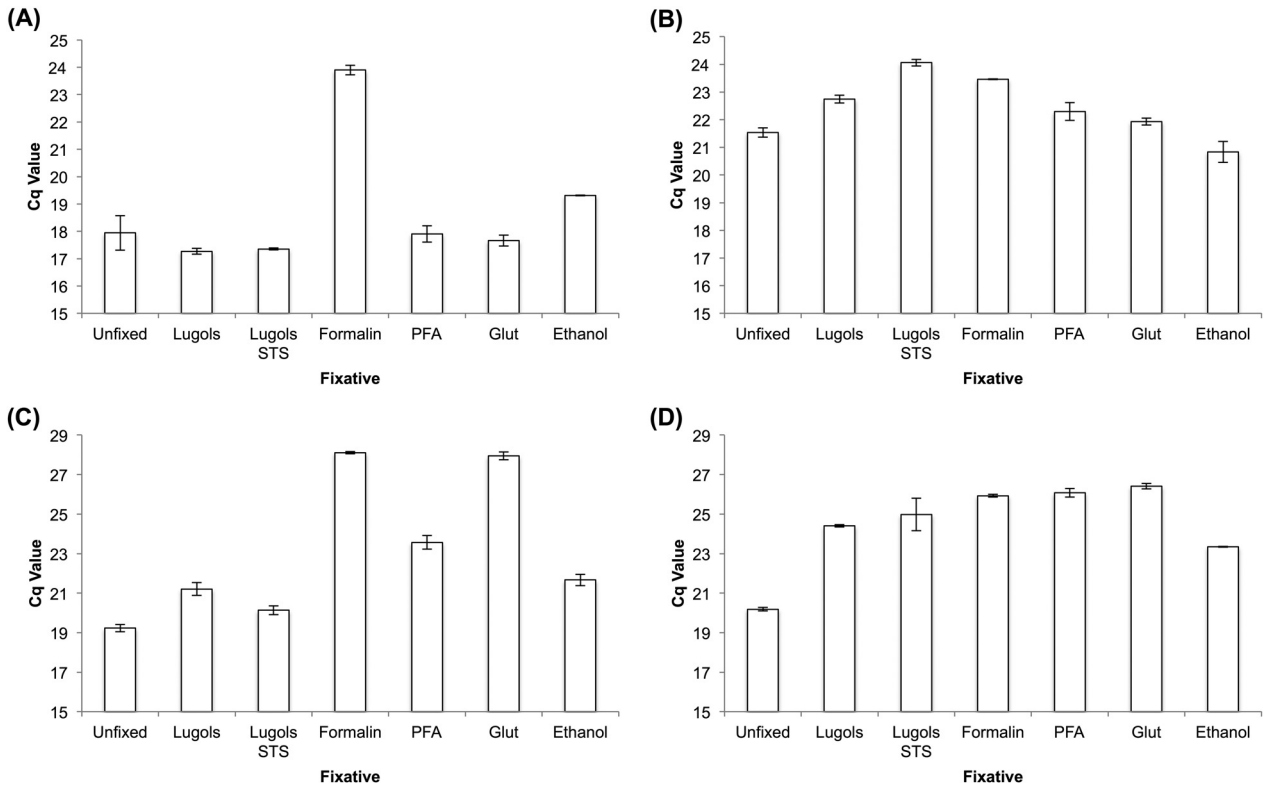

Fig. 2. Comparison of the C_q values generated from cultured cells of (A) *A. tamarense*, (B) *K. mikimotoi*, (C) *K. veneficum* and (d) *P. parvum* preserved for 24 h. (i) Acidified Lugol's (660 μg I_2), (ii) acidified Lugol's (660 μg I_2) plus sodium thiosulphate (390 μg $Na_2SO_2O_3$ ml^{-1}), (iii) gluteraldehyde (0.1%), (iv) paraformaldehyde (PFA) in BPS, (1%), (v) formalin (1%) plus ice cold methanol (100%) and (vi) ice cold ethanol (70%). An unfixed sample was used as a control. All results are means of triplicate samples. Error bars represent SE.

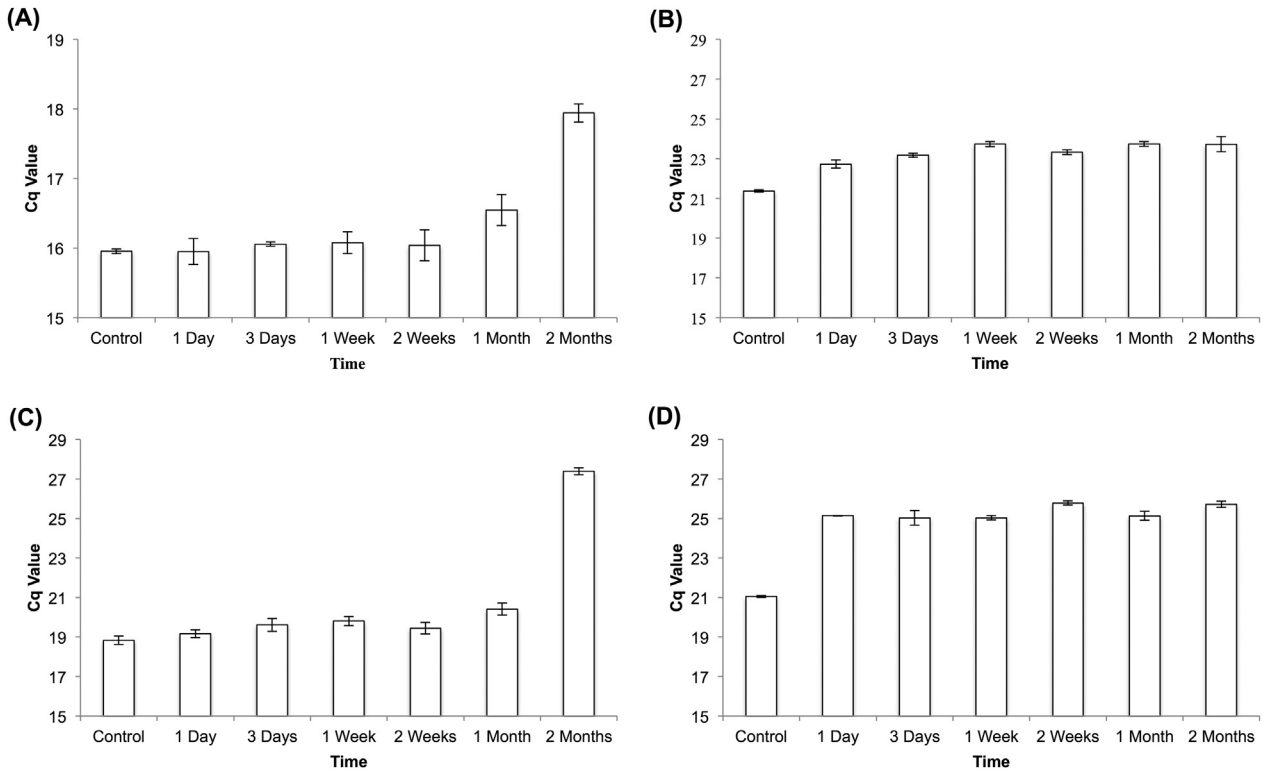

Fig. 3. Change in the C_q values over time from cultured cells of (a) *A. tamarense*, (b) *K. mikimotoi*, (c) *K. veneficum* and (d) *P. parvum* preserved in acidified Lugol's (660 μg I_2) for (i) 24 h, (ii) 72 h, (iii) 1 week, (iv) 2 weeks, (v) 1 month and (vi) 2 months. An unfixed sample was used as a control. All results are means of triplicate samples. Error bars represent SE.

4. Discussion

There are a number of distinct challenges when sampling for molecular, morphological or ecological studies. The first is the rapid deterioration of the samples to minimise any quantitative or qualitative changes, so samples should be processed or fixed immediately after collection. Processing freshly collected samples can often be impractical due to either the number of samples or the distance from the point of collection to the point of processing. The fixative must be suitable to overcome a number of challenges. Often the samples need to be preserved in large volumes, meaning that the fixative of choice must be affordable for bulk use. It must also maintain the important physical characteristics required for identification as well as preserve genomic DNA integrity. Lastly, the fixative must be safe for the user. A common method for the fixation of biological materials is storage at ultra cool temperatures or in liquid nitrogen, but these are not always available especially when collecting samples during field surveys (Fonseca and Fehlauer-Ale, 2012). Therefore, a wide range of chemical treatments have also been used for sample fixation with the effectiveness varying across taxonomic groups.

To simulate the conditions of a HAB monitoring program, we fixed four different but HAB species (representing three dinoflagellates and a haptophyte) in a number of different commonly used laboratory-preserving agents. These fixatives were either Lugol's iodine, alcohol or aldehyde based fixatives. The performance of each fixative in preserving DNA for molecular analysis was species dependent. For all the fixatives *Prymnesium parvum* was the most sensitive in terms of increased C_q value compared to the three dinoflagellates. An increased C_q indicates that the DNA has been affected, either by degradation, cross-linking or denaturation making it impossible for the Taq-polymerase to bind during the qPCR reaction. The species-specific differences are most likely due to the different chemical and physical properties of each fixative combined with the individual biochemical makeup of each species. Factors such as: target functional groups, lipid content, presence or absence of a cell plate, membrane surface charges and numbers of hydrophobic domains will affect fixation (Wood, 1973; Migneault et al., 2004; Rhodes, 2013). For example, paraformaldehyde and gluteraldehyde are able to react with phospholipids, whilst formalin is not, but it is able to cross-link DNA (Migneault et al., 2004). In contrast the coagulant fixatives ethanol and methanol work by denaturing proteins by displacing the water and disrupting hydrophobic and hydrogen bonding (Rhodes, 2013). So each fixation method will affect the molecular fixation of each individual species differently.

Lugol's iodine is the most commonly used fixative in HAB monitoring programs as it is relatively safe and thought to be better for accurately quantifying cells than many of the aldehyde based fixatives (Moncheva and Parr, 2010; Ohman and Snyder, 1991). Ingredients for the preparation of Lugol's iodine are relatively safe and easy to obtain, with the stock solution keeping for many years (Naik et al., 2010). It has been previously recommended for the fixation of ciliates and flagellates (Throndsen, 1978). Naik et al. (2010) observed that Lugol's iodine introduced fewer artefacts to samples when used for light microscopy, thus there were fewer identification errors compared to formalin or glutaraldehyde fixed samples. However, it has two disadvantages for morphological studies: the dark brown stain can mask key identifying features and the flagella are frequently lost from the cells (Xia et al., 2013) although much of the stain can be removed by an additional sodium thiosulphate wash step (Auinger et al., 2008; Naik et al., 2010). Auinger et al. (2008) successfully performed PCR on phytoplankton samples preserved in Lugol's, using an additional sodium thiosulphate wash. Here we tested samples fixed in Lugol's with and without a sodium thiosulphate wash and we discovered that this additional wash step was not required for *Alexandrium tamarense*, *Karlodinium veneficum* and *Prymnesium parvum* as the C_q values were similar between the two Lugol's iodine treatments. The additional wash step even decreased the amount of target DNA and thus increased the C_q value for *Karenia mikimotoi* compared to the control. Cembella and Rafuse (2010) found that a sodium thiosulphate wash could result in an additional loss in morphological details and even cell lysis, making this extra step redundant for samples used in qPCR analysis.

Lugol's can also dissolve hard structures over time making it impractical for long-term storage (Naik et al., 2010). Samples fixed in Lugol's iodine should be monitored during storage as iodine oxidises over time (Naik et al., 2010). Storing samples below 5 °C will slow down the rate of physical and chemical processes that can cause reduction in sample quality. The maximum storage time for Lugol's fixed samples is thought to be between 4 (Doll et al., 2014) and 6 months (Moncheva and Parr, 2010).

In an accompanying study we tested the validity of the procedure on artificially spiked environmental field samples collected from Danish coastal waters (Øresund) in August 2014 to assess whether the environmental matrix would affect quantification. The results showed that the assay was not negatively affected by any unidentified contaminants and the cell estimates by qPCR did not statistically differ from microscopic cell counts over a range of cell concentrations (Eckford-Soper and Daugbjerg, unpublished data).

So, as shown in this study, and studies by others (Doll et al., 2014), Lugol's appears to be the safest and most effective short term fixative for analysis by qPCR using Taqman probes, provided that the samples are processed and analysed quickly (within 1 month).

Formalin is an aqueous solution containing formaldehyde plus a stabiliser, in this case methanol. It is an inexpensive, effective and low maintenance fixative, which maintains morphological integrity in most species for long periods (several decades) (Fonseca and Fehlauer-Ale, 2012), making it a practical fixative for microscopy. However, attempts to extract usable DNA from formalin fixed samples for molecular analysis has had variable success (Srinivasan et al., 2002). Molecular studies using formalin to preserve cells have shown a number of direct and indirect impacts on the structure of DNA such as covalent cross-linking, denaturation, modification and fragmentation (Bucklin and Allen, 2003). When compared to DNA from frozen samples, formalin fixed samples exhibit a higher frequency of sequence alteration. This is thought to be due to the cross-links formed between cytosine nucleotides, causing Taq polymerase to not recognise cytosine and add an adenine instead of a guanine creating a C-T or G-A base change (Srinivasan et al., 2002). Formalin is reported by some (Bik et al., 2009; Schlander and Halanych, 2003) to be not suitable for PCR amplification, whilst others have shown successful amplification of DNA extracted from samples preserved in formalin (Bucklin and Allen, 2003; Douglas and Rogers, 1998; Richlen and Barber, 2005), but with a high rate of DNA damage (De Giorgi et al., 1994; Godhe et al., 2002; Richlen and Barber, 2005). Bucklin and Allen (2003) believed that short-term exposure along with only amplifying short fragments (100–200 bp) will allow successful amplification of DNA, as it was thought that up to 1 base change per 500 bases can occur (Srinivasan et al., 2002). The rate of DNA modification is also thought to be dependent on concentration and temperature. Fixing a high molecular weight sample at room temperature can cause a 30% loss in nucleic acid integrity, whilst samples fixed at 4 °C have the least amount of degradation (Srinivasan et al., 2002). As we used a protocol only requiring short fragments (100–131 bp) that were only exposed to formalin for a period of 24 h at 5 °C, we were able to achieve product amplification in all formalin fixed

samples, but with an increase in C_q value for all species studied compared to the control. This increase in C_q value is most likely due to DNA becoming cross-linked during fixation. Therefore we would not recommend using formalin as a fixative for DNA samples used in molecular studies. Also, Xia et al. (2013) have reported severe cell loss in formalin fixed samples, making it a poor fixative for molecular and quantitative studies. In addition to the problems described above, formalin is also a highly toxic irritant and carcinogenic even at low concentrations, so appropriate protective wear and handling techniques must be used. This may be problematic when samples are treated in the field away from relevant safety equipment.

Paraformaldehyde (PFA) is similar to formalin but does not contain methanol and is produced directly from a polymerised formaldehyde powder. Here we used PFA in phosphate buffered saline (PBS). PBS buffers the tissues from enzymatic damage during fixation. Unlike formalin, PFA did not negatively affect the C_q value for *Alexandrium tamarense* and *Karenia mikimotoi* but it did cause an increase in C_q value for *Prymnesium parvum* and *Karlodinium veneficum*. A few studies have been able to successfully amplify DNA from a PFA fixed samples, but with more laborious extraction protocols (Shi et al., 2014; Khodosevich et al., 2007). Khodosevich et al. (2007) successfully reversed cross linking in the PFA preserved samples using an optimised proteinase K/SDS lysis solution with subsequent treatment with phenol pH 4.2. Despite this, PFA has proved to be a poor fixative for medium and long-term storage as over-fixation can cause molecules to become over cross-linked, thus preventing hybridisation and increasing auto-fluorescence, which in turn increases background-fluorescence when using fluorescent probes. Also, like formalin, PFA is toxic and as the preparation process involves heating which causes considerable vaporisation, thus increasing the hazard.

Glutaraldehyde is another cross-linking fixative and is widely used in scanning (SEM) and transmission electron microscopy (TEM), as it preserves delicate cell structures better (Cembella and Rafuse, 2010). Nonetheless its practical use as a fixative for microscopy is diminished as it is often thought of as a poor fixative for successful qPCR amplification (Morel and Raccurt, 2003). Here we were able to successfully amplify a DNA product from a glutaraldehyde preserved sample. However, fixation using glutaraldehyde had a species-specific effect on qPCR results. It had little effect on *Alexandrium tamarense* and *Karenia mikimotoi*, but negatively affected *Karlodinium veneficum* and *Prymnesium parvum*. The difference in effect is most likely due to the *K. veneficum* and *P. parvum*'s high lipid content (Fuentes-Grünewald et al., 2012), as gluteraldehyde is known to cross-link proteins, phospholipids and lipid containing amino acid groups (Wood, 1973). Xia et al. (2013) were also able to amplify a DNA sequence from a glutaraldehyde fixed sample but only by first grinding the sample in liquid nitrogen. Not only is glutaraldehyde a poor short-term fixative for samples used in molecular studies but it is also a poor long-term fixative for cell enumeration studies as over time it gradually becomes volatilised, causing cell lysis and a reduction in cell number (Xia et al., 2013). Also, glutaraldehyde like the other aldehyde-based fixatives is toxic, causing throat, skin and eye irritation as well as breathing difficulties and like formalin and PFA it is problematic when using it in the field.

Ethanol is a potent enough fixative for most applications and it is much safer to deal with compared to some of the more toxic fixatives. Many studies have demonstrated that non-cross linking alcohol based fixatives are better for nucleic acid fixation compared to aldehydes. Alcohol fixation is thought to cause little chemical change as the DNA collapses and after rehydration it returns to its original form (Srinivasan et al., 2002). However, here ethanol (70%) had no effect the C_q value of *Karenia mikimotoi*, but increased the C_q values of *Alexandrium tamarense*, *Karlodinium veneficum* and *Prymnesium parvum*, indicating ethanol might not be the best fixative for a number of HAB species. Goetze and Jungbluth (2013) also reported DNA degradation in ethanol fixed samples, with DNA losses increasing with storage time, and also in samples with a high water content. Godhe et al. (2002) were able to successfully amplify DNA from samples fixed in 75–95% ethanol but with a significant loss of cell numbers making ethanol an unsuitable fixative for phytoplankton monitoring programs.

5. Conclusions

Each fixative has its own advantages and disadvantages and it is generally accepted that no single fixative provides the perfect solution. Many fixatives are now being mixed to try and counteract the shortcomings of single components e.g. Carnoy's, Methacarn, etc. (Srinivasan et al., 2002). There are also a number of commercially available fixatives that are designed to better preserve molecular material (FineFIX), which has been used with varying degrees of success (Dotti et al., 2010). However, these fixatives are often expensive, making them impractical for large numbers of samples.

Many sampling and long-term monitoring programs require preserving agents for conservation of samples before morphological or molecular studies. In most weekly HAB monitoring programs water samples are fixed with Lugol's iodine and then sent, usually overnight, to a place of analysis where they are processed and analysed by light microscopy. Yet with the development of the polymerase chain reaction (PCR) and other techniques analysing nucleic acids, we may be able to more rapidly analyse phytoplankton communities, thus allowing us to better describe temporal and spatial trends of phytoplankton species composition, abundance and biomass, as well as identifying dominant, harmful and indicator species. Therefore, knowledge about the effects of different fixatives on the integrity of preserved DNA and RNA is of the greatest importance.

In view of everything described above, the method of preservation should depend of the objectives of the work and the target taxa (Naik et al., 2010). Here we have shown that Lugol's iodine is the most ideal short term fixative for enumeration of cells by qPCR as it is the safest to handle and is often the fixative of choice for ciliates and flagellates including dinoflagellates (Naik et al., 2010). In this study Lugol's iodine had no or very little effect on DNA compared to the other fixatives examined.

Acknowledgements

This study was supported by the Danish Strategic Research Council (project HABFISH). We thank SCCAP and CCAP for providing the microalgal cultures used here.[TS]

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