Comparison by light microscopy and qPCR of potentially ichthyotoxic microalgae in Danish on-shore lagoons producing European flounder (*Platichthys flesus*): Pros and cons of microscopical and molecular methods

Fig. 1. Lagoon used for production of European flounder at Fishlab, Denmark.

Evaluation of phytoplankton communities is frequently used to determine the ecological status of water bodies. Hence, species diversity of phytoplankton has become an integral component of national assessment programs. The most commonly used technique for counting phytoplankton is the Utermöhl method [1] and the precision of the enumeration is evaluated with standard statistical analyses techniques [2]. Despite long-term reliance on the Utermöhl method, new techniques for algal identification and enumeration are continuously being explored. Particularly, the recent explosion in molecular tools has resulted in an influx of alternative methods (e.g. real-time qPCR, microarrays and FISH-FC). While many of these methods are promising, their results often differ from those of the conventional method that they were intended to supplement (or replace). Given the importance of algal community assessments, significant efforts have been put into the quality assurance of phytoplankton counts [3]. This has led to the development of standardised procedures.

This study aimed to compare cell counts of seawater samples by light microscopy (LM) and qPCR from on-shore production lagoons of European Flounder (Fig. 1). A total of six lagoons had been filled with untreated seawater from the nearby Limfjorden. Samples for phytoplankton analyses were taken twice weekly from 7 March to 18 May, 2017. A total of 55 samples were examined by LM and qPCR. Potentially ichthyotoxic species (Table 1) were identified either quantitatively or qualitatively by qPCR using species-specific primer sets and hydrolysis (Taqman) probes. Results from qPCR were compared to microscopic cell counts performed by Fishlab. Examples of two representative lagoons are provided in Fig. 2A-D.

Data gathered from all six lagoons revealed ca. 20 groups or species by LM (not shown) and the qPCR assays available detected 8 out of the 11 potentially fish killing species (Fig. 2A, C). There were very few cross overs in terms of species identification between the two methods. LM failed to detect seven of the potentially ichthyotoxic species and *Dictyocha* was the only microalga detected by both methods. Despite this, the cell densities differed markedly. The qPCR assay only detected *Dictyocha speculum* cells in lagoon six at a density of 2,600 cell L^{-1}, whereas LM detected much higher numbers of 56,560 and 36,210 *Dictyocha* 'sp.' in lagoons 1 and 6, respectively. *Pseudochattonella farcimen* and *P. verruculosa* were detected in all lagoons by qPCR and a succession pattern for *P. farcimen* to *P. verruculosa* was evident. When temperatures were low (8-9 °C) *P. farcimen* was dominant but as temperatures increased above 9 °C a switch occurred and a decline in *P. farcimen* concentration coincided with an increase in *P. verruculosa* cell numbers (Fig. 2A, C). During the production period, lagoons were replenished with an addition of ca. 10% of newly collected seawater. The additional water appeared to re-inoculate each lagoon with additional *Pseudochattonella* cells. Fish survival rates in the lagoons were between 0.5 and 13 %.

In terms of monitoring, the discrep-

Species	Lagoon number 1	2	3	4	5	6
Alexandrium tamarense						
Alexandrium ostenfeldii	+			+	+	+
Karenia mikimotoi						
Prymnesium parvum				+	+	
Pseudochattonella farcimen	+	+	+	+	+	+
Pseudochattonella verrculosa	+	+	+	+	+	+
Karlodinium veneficum	+	+	+	+	+	+
Pfiesteria shumwayae	+		+	+		+
Pfiesteria piscicida	+	+	+		+	+
Luciella masanensis						
Dictyocha speculum						+

Table 1. List of species for which qPCR assays are available for quantitative (cell abundance) and qualitative (presence/absence) of potentially ichthyotoxic microalgae in this study. '+' = presence of a species in the lagoons used for production of European flounder.

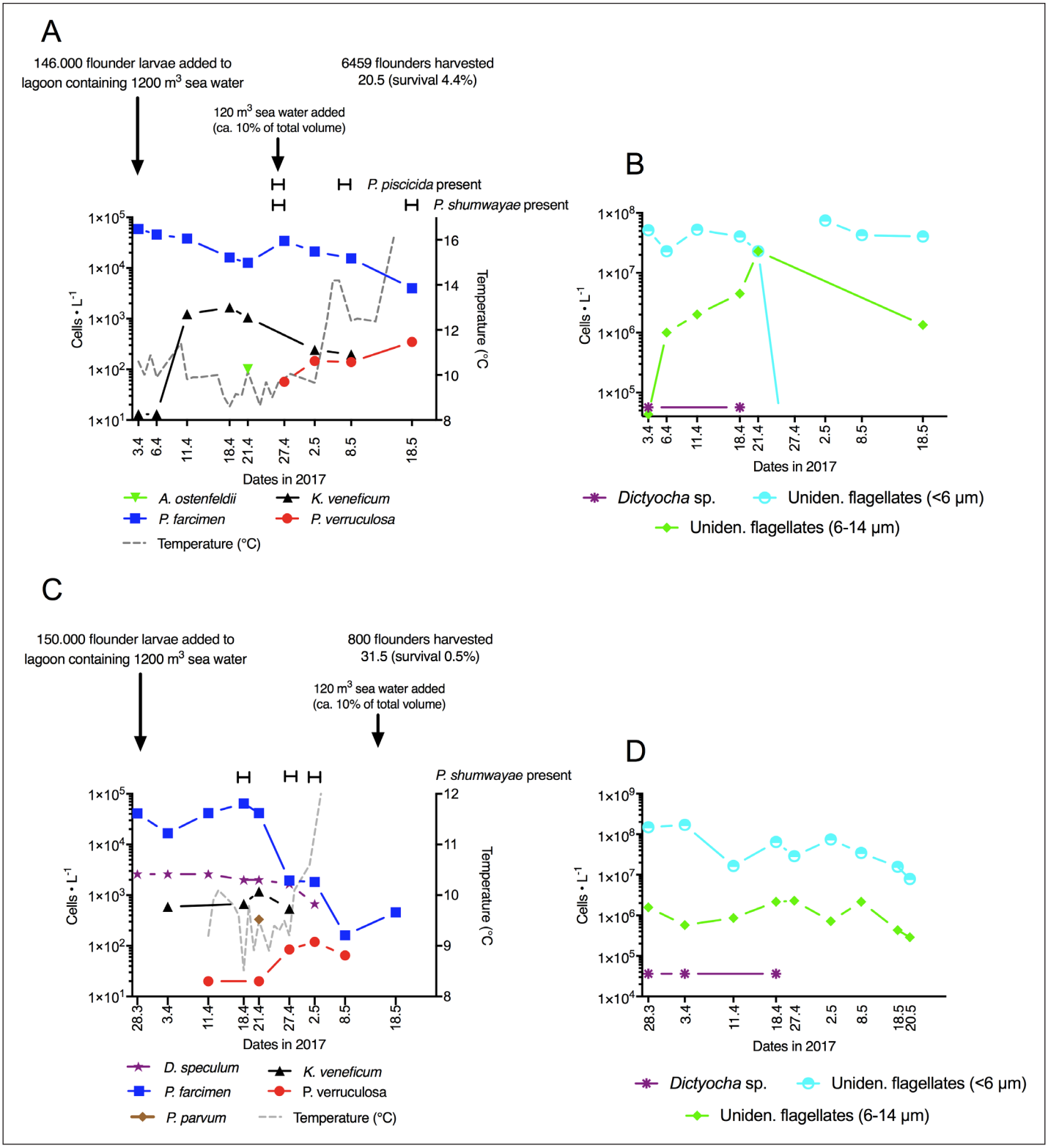

Fig. 2. Comparison of phytoplankton species identification and cell densities (cells L^{-1}) by qPCR (A and C) and light microscopy (target species and groups which potentially could comprise ichthyotoxic organisms) (B and D) in lagoon 1 (A and B) and lagoon 6 (C and D), respectively. The right Y-axis shows the water temperature during the growth period. Presence of Pfiesteria piscicida and P. shumwayae at specific dates are indicated by H. Information on the number of flounder larvae added and the survival rates in percentage at the harvest dates are provided at the top of A and C.

ancy between the qPCR and LM results is obviously quite worrying. There are clearly problems with one or even both methods and it raises many questions over their accuracy. When evaluating phytoplankton data, each inaccuracy associated with sampling, sub-sampling and sample preparation should be taken into account. As each individual step from sampling to counting comes with its own forms of variation, we will critically analyse each method and discuss potential problems and sources of error.

Microscopic analysis

Traditionally light microscopy has been the gold standard for phytoplankton identification due to its relatively low costs and equipment requirements (basically an inverted microscope and a settling chamber). One of the main advantages of LM over qPCR is its ability to identify at least theoretically all organisms present in the sample in contrast to qPCR, which will only target species of interest, e.g. toxic or nuisance organisms. If there were any new species

present in a sample then qPCR would miss those probably due to lack of a developed assay. However, LM does require high levels of taxonomic skills and the precision in identification is only as accurate as the taxonomist allows. Different taxonomists trained in different ways using different identification literature can cause large person-person differences. The ease of identification is also species dependent. For example highly plastic species, or those with a variable life cycle are harder to identify and can often be easily misidentified. The naked form of *Dictyocha speculum* can easily be confused with the rounded cells of *Pseudochattonella*. Some species of the genus *Alexandrium* cannot be identified to species level due their very subtle morphological differences in their thecal plates. When dealing with toxic species, false positives are less problematic but can cause substantial financial losses if they result in the closure of a fishery, but when toxic or problematic organisms are missed completely this could have dire consequences. To reduce confusion, each taxonomist should be provided with a checklist of common species with up to date taxonomic names.

Undertaking frequent inter-comparison exercises, e.g. the ring test or the International Phytoplankton Intercomparison (IPI) exercise, provide feedback on how individual laboratories and taxonomists perform. This forum also provides an opportunity to convene a discussion on nomenclatural changes and new technological advances in monitoring techniques.

As error can be introduced in various different forms, in order to get the most accurate and reproducible results each individual step from: collection, storage, subsampling, homogenisation, filling the chamber, settling and counting strategies all require their own standardised protocols.

All aspects of the protocol need to be considered from the storage containers to the type of fixative used. Many cells e.g. *Pseudochatonella* spp. are sticky and can adhere to plastic walls and as plastic bottles is often preferred over glass especially when transporting samples this can become problematic for accurate cell enumeration. The choice of preservative is important and often the optimal preservation methods are taxa specific. It is not always easy to obtain reliable estimates from fixed material; preservatives can alter the sample in various ways creating a biased measurement. Lugol's iodine [4] has long been the fixative of choice due to its relatively low toxicity and high stability. However, it is known to introduce artefacts such as changes to cell size, a reduction in cell number and in some instances it may fail to preserve certain taxa all together [5-6]. Each alternative fixative comes with its own issues [7].

Settling chambers themselves can be another source of variation. For reliable cell counts, specimens must be completely randomly distributed within the chamber. If cells do not follow a poisson distribution then it will bias the enumeration and any statistical analysis will be affected.

To prevent uneven settling, the samples must be at a constant temperature during the settling period. For a higher chance of getting a well-mixed distribution then samples must first be homogenised. The best way to homogenise a sample is the 'Paul-Schatz' figure of eight rotation method where samples are mixed 100 times in a rhythmic pulsating motion. Even when all precautions are taken, it is still almost impossible for cells to be randomly distributed due to issues such as cell clumping caused by polysaccharide fibrils or inconsistent settling conditions. Due to radial abundance gradients cell abundances at the periphery can be up to 50% lower than at the center, causing a settling bias. Uneven settling will affect the counting strategy. For any counting strategy a predetermined number of units must be observed. The number of units differs depending on the organism and the research objectives. Typically to reach an accuracy of 10% at least 400 cells must be counted [8]. Whole chamber cell counts should be carried out where possible but other counting strategies are often used such as transects and random fields.

qPCR

When carefully designed, with optimisation and validation, qPCR assays are highly accurate and sensitive, but without due care and optimisation, qPCR can be plagued by reproducibility and reliability problems.

The quality of the starting material is one of the key determinants for obtaining reliable and reproducible data. As with microscopy fixatives and storage techniques play a large part in quality of the samples. Fortunately for short term storage Lugol's iodine is the most ideal fixative and the same sample can be used for both microscopic and qPCR analysis [7]. Before amplification DNA must be extracted from the cellular material and commonly commercial extraction kits are applied. To get purified genomic DNA the sample must undergo a number of steps to lyse the cells, remove contaminants and purify the resulting DNA. In cases where the purification step is inefficient the resulting DNA may not be representative of the sample and/or contain compound(s) that will cause assay interference. In these cases the performance of the reaction will be sub-optimal, causing a reduction in the sensitivity and/or amplification efficiency. Inhibition of amplification can occur in different ways. Firstly, when high molecular weight compounds, e.g. humic acids or complex carbohydrates, combine with metal ions to sequester the nucleic acids away from the polymerases and prevent amplification. While some molecules block or inhibit the polymerase or alter the specificity of the primers, inhibitors which block or delay polymerase activity are highly problematic and they can lead to an underestimation of material in the sample or false negatives [9]. Typical approaches to combat inhibition include alternative DNA extraction kits, dilution, specialised polymerases, addition of adjuvants and internal controls.

When designing a qPCR assay it is important to select an appropriate target and to design specific primers with no cross reactivity with other organisms. This study used previously validated species-specific hydrolysis probes in combination with primers to add an extra level of specificity. To ensure accuracy, time is required to optimise the efficiency of the assay and validate it multiple times. This sometimes means altering the constituents of the master-mix used, e.g. nucleotides, magnesium chloride or polymerase concentrations. This is extremely relevant when tackling issues arising from multiplexing assays [9-10].

Once optimised users can still face a number of precision related challenges.

As qPCR measures genetic material rather than viable cells an over estimation of cell numbers can occur due to the inclusion of dead or dying cells. Problems may also occur when targeting multiple copy genes where the organism carries different numbers of the target depending on nutritional status, stress or replication stage. This can lead to an over or under estimation of total cell numbers. Common problems associated with cell number enumeration and copy number do not occur until late exponential-stationary phase meaning that cell numbers can be accurately quantified until this point [11]. One way of potentially overcoming this issue it to use standards created using cells from all parts of the growth curve to produce an 'average' copy number. However, this will decease the overall accuracy of the assay.

As with microscopic analysis qPCR requires standardisation/normalisation for both the laboratory protocols and statistical analysis strategies. To aid this, in 2009 the MIQE guidelines, *Minimum information for publication of Quantitative Real-time PCR Experiments*, were published. These guidelines are designed to 'encourage better experimental practice'. The guidelines establishes a framework for conducting qPCR experiments in the laboratory and are designed to improve experimental workflow and should be followed when designing any qPCR assay [12].

Conclusions

Clearly despite efforts to standardise procedures for both techniques there are still many problems affecting the accuracy and the quality of the results. The comparison of enumeration techniques that was carried out in this study has highlighted the difficulties in obtaining comparative data especially of small-sized, ichthyotoxic microalgae. Enumeration by LM missed many important species, which emphasizes how difficult it is to identify phytoplankton from Lugol's fixed material. We are now moving into the era of 'bio-monitoring 2.0' and with the reduction in costs for meta-barcoding based techniques it is still to be seen if these molecular techniques will eventually replace LM. Yet improvements need to be made across the board for all techniques. The low survival rate of European flounder observed in the 2017 production may be explained by the diverse assemblage of potentially ichthyotoxic microalgae in the lagoons. In previous years the survival rate has been 40-50%.

References

1. *Utermöhl H 1958. Limnol 9:1–38*
2. *Lund J W G et al 1958. Hydrobiologia 11: 143–170*
3. *Rott E et al 2007. Hydrobiologia 578: 141–146*
4. *Throndsen J & A Sournia 1978. Phytoplankton Manual (Monographs on Oceanographic Methodology, UNESCO, Paris), 337 pp.*
5. *Leakey R J G et al 1994. J Plankton Res 16:375–389*
6. *Stoecker DK et al 1994. Mar Ecol Prog Ser 110: 293–299*
7. *Eckford-Soper L K & N Daugbjerg 2015. Harmful Algae 42: 52–59*
8. *Karlson et al 2010. IOC Manuals and Guides 55, UNESCO, Paris, 110 pp*
9. *Webb S 2013. Biotechniques 55: 165–168*
10. *Eckford-Soper L K & N Daugbjerg 2015. Harmful Algae 48: 37-43*
11. *Eckford-Soper L K & N Daugbjerg 2016. J Phycol 52: 174-183*
2. *Bustin S A et al 2009. Clin Chem 55: 611–622*

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