Phytoplankton precision trials in the enumeration and identification of marine microalgae through the scheme "Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM)".

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"There are three kinds of lies: lies, damned lies and statistics"

Benjamin Disraeli, Mark Twain

"When it is not in our power to determine what is true, we ought to follow what is most probable"

Rene Descartes

Abstract

Scientists are coming under increased pressure in recent years to show that results they obtain arising from their scientific work are quality assured and stand up to scrutiny by independent expert auditors. This has meant that the methodologies used by laboratories involved in making these measurements have to be validated and fit for purpose and has led to the adoption of internationally recognised standard protocols.

These protocols must be underpinned by robust quality systems and must be accredited to an international standard. In order for laboratories to become accredited in particular methods, they have to fulfil a series of prerequisites but a compulsory one is the participation in a proficiency testing scheme.

Proficiency testing schemes are independent assessor organisations which coordinate regular inter-calibration and intercomparative studies between laboratories with a common purpose. What happens, though when proficiency testing schemes do not exist for a particular scientific measurement?

This study presents results from two inter-comparison exercises at European level between phytoplankton monitoring laboratories in the enumeration and identification of marine microalgae using the Utermöhl cell counting method.

Microalgae are a very important ecological component of the marine ecosystem and have also become important ecological indicators of hydro-climatic change, ocean acidification and eutrophication. Member states of the European Union are obliged to monitor for toxic and harmful algae which can cause problems and devastation in the natural environment, have detrimental effects on human health if contaminated fish and shellfish are eaten, can cause huge economic losses to the aquaculture industry and impact directly in coastal communities.

This study shows how an intercomparison of this kind is designed and organised, how samples are set up, materials homogenised and reference values obtained. It demonstrates the importance of using the right technique and best practice, based on experience, to analyse samples and how important it is to design the exercise to be statistically robust, both quantitatively and qualitatively.

The taxonomy quiz in 2009 showed that there was no evidence that video clips were better audit trail tools than images or vice versa. The quantitative measure suggested that there was evidence of good agreement between virtually all the analysts and the reference value for all species except one (*P.micans*). However, there was evidence of lack of reproducibility between and within laboratories. The qualitative measure calculated indicated that analysts are more likely to identify a toxic organism as a non toxic organism than the other way around.

The results from the enumeration data in the 2010 exercise showed that there was lack of reproducibility across laboratories using different counting strategies and volume sub-sampled and analysed. These results when compared to a set of hypothesised means used as reference values suggested that cell counts were potentially underestimated by as much as 30% and that this underestimation was most likely due to test method effects.

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1.1 Introduction to intercalibration exercises and proficiency testing schemes.

Intercalibration exercises across all disciplines of science are becoming important tests to support and assure the quality of results. These results need to be backed up by a good quality system (Quality manual, 2001) where the traceability and confidence in the results is important. Many difficult decisions in policy are made based on quantitative or qualitative measurements made by scientists. It is a good working practise that these results contain some indication of the quality of the results (EURACHEM/CITAC, 2000). Nowadays it is a requirement of laboratories to have quality assurance measures in place to provide data and measurements to a particular standard.

These measures include having a validated method in place, a series of internal quality controls, accreditation of the method, traceability of results and participation in proficiency testing schemes (ISO/IEC 17025, 1999).

The tendency in marine science is to move towards the use of standardised methods for the measurement of diverse environmental, chemical and biological variables (AOAC, 1995). These methods are sometimes prescribed in European legislation as official methods and are recognised internationally as the standard method for a particular measurement. ISO (International Standard Organisation) is the organisation dealing with providing the guidelines necessary to fulfil the quality requirements of analytical measurements.

Accreditation means that the test method used for a particular measurement has been validated and it is fit for purpose. A validation plan and report for the test method is fundamental towards providing a measure of the uncertainty of measurement for the test method (Ellison *et al.*, 1993) among other requirements.

There are six basic principles of best analytical practise in analytical measurements. Intercomparison exercises alone fulfil two of these principles "There should be a regular independent assessment of the technical performance of a laboratory" and "Analytical measurements made in one location should be consistent with those made elsewhere" (EURACHEM, 1998).

This means that laboratories must participate in proficiency testing schemes and also that they must have internal quality controls in the form of intercalibration and intercomparative studies within and between laboratories.

There are different types of interlaboratory comparison studies, from certification trials to method validation studies or collaborative trials to proficiency testing (PT) schemes. The latter are also known as External Quality Assessment (EQA) or Laboratory Performance (LP) studies.

The present study focuses on PT schemes as in this type of interlaboratory study laboratories can be assessed against other laboratories. It provides independent assessment of the quality of the routine analysis and provides comparative information about method and instrument performance.

1.2 Proficiency testing schemes

1.2.1 Quasimeme

In 1992, the QUASIMEME (Quality Assurance of Information in Marine Environmental monitoring) was founded. This was the first Proficiency Testing scheme for analytical procedures measuring chemical and environmental parameters in marine ecosystems. This project was initiated with EU funding (1992-1996) and continued by subscription of the participating institutes afterwards.

QUASIMEME was coordinated by the QUASIMEME Project Office at the Fisheries Research Scotland (FRS) Marine Laboratory in Aberdeen, United Kingdom until 2005. In 2005 the coordination was transferred to Wageningen University and Research Centre (Alterra/WUR) in The Netherlands.

The QUASIMEME Project Office operates under the guidelines provided in the ISO/IEC 17043 for the development and operation of proficiency testing schemes and in the Guidelines for the Requirements for the Competence of Providers of Proficiency Testing Schemes (ILAC G13:08/2007).

Some of the analysis that are carried out by QUASIMEME included nutrients in seawater, contaminants, metals, chlorinated organics, marine biotoxins, chlorophyll a among others in a broad spectrum of chemical measurements. These routine laboratory performance studies provided the basis of external quality assurance (EQA) for institutes that made regular chemical measurements in the marine environment.

The output from these studies is reviewed annually by the QUASIMEME Scientific Assessment Group, which is comprised of experts in each of the main areas of the QUASIMEME Laboratory Performance (LP) studies.

The QUASIMEME LP studies provide external quality assurance (QA) for national and/or international monitoring programmes, individual or collaborative research and for contract studies. These studies support quality management and quality measurement in the participating laboratories.

Participants may use the assessment of the studied data to validate internal laboratory QA, support accreditation, support QA of environmental monitoring data and provide data for national or international programmes.

1.2.2 Bequalm

Bequalm is a programme of biological measurements in the marine environment, this programme includes a diverse array of measurements, from biomarkers to whole organism assays (fish disease, bioassay, luminescent bacteria), to community analysis (benthic community, Phytoplankton assemblage). The work in this thesis is based on the work done only in the phytoplankton component of Bequalm that is the

identification and enumeration of marine microalgae, which is part of the community analysis component of Bequalm.

Marine biological measurements like the identification and quantification of marine microalgae through intercomparison exercises at a European level at least, had only started in the year 2000 (Reckermann and M., Colijn, F., 2000), to the best of my knowledge. The development of this type of exercises is generally not as advanced as other marine analytical measurements. Some of the reasons for this is that analytical methods based on the measurement of the concentration of particular analytes in chemistry is done by modern instrumentation and equipment with given tolerances, specifications and sensitivities. In some cases certified reference materials (ISO/IEC 33:1989) are available to provide reference values, standard curves and controls. In contrast, biological methodologies like the identification and enumeration of microalgae do not possess certified reference materials yet and the identification and enumeration of microalgae is done by the analysts not a piece of equipment, so there is a certain subjectivity to the identification of species.

The Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM) project, funded by the European Union through the Standards, Measurements and Testing programme of the European Commission, was initiated in 1998 (www.bequalm.org).

This was in direct response to the requirements of the Oslo-Paris Commission (OSPAR) to establish a European infrastructure for biological effects quality assurance and quality controls (QA/QC), in order that laboratories contributing to national and international marine monitoring programmes can attain defined quality standards.

Biological effects measurements are increasingly being incorporated into national and international environmental monitoring programmes to supplement chemical measurements.

The original project consisted of twelve work packages (WP) processed by nine partner experts in measurements on biological effects and monitoring techniques, the

phytoplankton component (WP 11) was entrusted to the Forschungs und Technologiezentrum Westkuste (FTZ) at Kiel University, Germany. They carried out two interlaboratory comparison exercises or ring trials in 2000 and 2001 (Reckermann and M., Colijn, F., 2000, 2001). These were the first intercomparison exercises in the enumeration and identification of microalgae at European level.

Some of the conclusions drawn from this first intercomparison were that the performance on cell counting of the laboratories was of a high standard but that a more standardized protocol for cell counting was recommended. Also, there were able to indicate that performances outside the confidence limits were exclusively associated with analysts using low volumes (1ml aliquots) on Sedgwick-Rafter cell counting chambers. They recommended not using these cell counting chambers or similar ones for counting phytoplankton field samples.

A standardized protocol was used in the second intercomparison (2001) and this appeared to improve the overall accuracy and precision of cell counting. The conclusion was that standardized methodologies are required to improve the quality and comparability of data at a European scale.

After the 2001 ring trial, there were no more phytoplankton intercomparisons at this level from FTZ. In 2003 there was an attempt by INTECMAR in Galicia, Spain to organise a phytoplankton ring trial worldwide. This intercomparison was set up in 2003 under the name Iberia 2003 and the results were published in the proceedings of the IX Iberian meeting on toxin phytoplankton and biotoxins in 2007, Spain (Pazos *et al.*, 2007).

Also, in 2003, BEQUALM adopted the UK NMBAQC (National Marine Biological Quality Control Scheme) as a model to progress the Community Analysis component which included Phytoplankton intercomparisons and launched the self-funded programme (participants pay a fee) in 2004. The BEQUALM phytoplankton work package initially comprised two parts: chlorophyll analysis and community analysis. The former was taken forward by QUASIMEME, whilst from 2005, the latter has been re-launched (initially for UK/Eire participants), through the BEQUALM/NMBAQC Scheme via the Marine Institute (MI) at Galway.

The MI phytoplankton unit under the BEQUALM/NMBAQC banner and with CTL (CEFAS Technology Limited) in the UK carrying out the administration and advertising, organised the first phytoplankton intercomparison exercise in 2005 with countries from Ireland and the UK initially. The MI has conducted a phytoplankton enumeration and identification ring trial, under the auspices of BEQUALM/NMBAQC annually since 2005.

The purpose of these exercises are to compare the performance of laboratories engaged in national official/non-official phytoplankton monitoring programmes and other laboratories working in the area of phytoplankton, to test the methodologies that are used for routine monitoring like the Utermöhl cell counting method (Utermöhl *et al.*, 1931, 1958). Each intercomparison exercise in a given year is designed to test one or various aspects of these methodologies.

The participation in this type of scheme is becoming an essential requirement for National phytoplankton monitoring laboratories in order to achieve accreditation for their methods. Since 2008, the participation of individual analysts on the scheme is certified by issuing statement performance certificates to each participant.

The Marine Institute phytoplankton laboratory is accredited to ISO 17025 for marine phytoplankton identification and enumeration since 2005, and it recognizes that regular quality control assessments are crucial to ensure a high quality output of phytoplankton data.

The exercise has served as a forum for phytoplankton taxonomists to meet and debate on matters regarding marine phytoplankton but also as a way to intercalibrate results using similar methodologies for the enumeration and identification of phytoplankton.

1.3 Regulations and monitoring in the EU

The concern of European directives and official methods for plankton analysis regarding the placing of bivalve molluscs in the market for human consumption is the identification and enumeration of toxin producing marine microalgae. As part of the national reference laboratory for Biotoxins, the MI carries out statutory work to meet

the EU requirements on bivalve molluscs through the hygiene directives 853/2004 with regards to the placing of bivalves molluscs in the market for human consumption and 854/2004 which lays the specific rules regarding the organisation of official controls for monitoring and the specific directives dealing with the different methodologies used for chemistry and mouse bioassay testing (91/492/EEC, 97/61/EC) of the different marine biotoxin groups. This entails the testing of shellfish chemically and through the mouse bioassay.

The hygiene directive 854/2004 states that plankton must be monitored in shellfish harvesting in relaying areas. This directive supersedes a previous directive (91/492/EEC) and it states that plankton should be monitored periodically to be able to detect changes in the composition of plankton containing marine biotoxins, also importantly it says that plankton samples need to be representative of the water column to be able to notice changes in population dynamics of toxin producing species. This latter point is a new addition to the 854/2004 EU directive.

1.4 Official methods for plankton analysis

While there are standardized official methods coming from the regulatory bodies in Europe regarding the testing of shellfish chemically and through the Mouse Bioassay, there are no official methods for the identification and quantification of plankton in samples.

It is up to the different phytoplankton monitoring programmes, to decide on a method/s that best equip them to identify the plankton species, found in their territorial coastal waters.

The MI phytoplankton monitoring programme, uses the Utermöhl cell counting method for phytoplankton analysis. This method is a light microscopy based method, where samples are preserved and settled in sedimentation chambers over time, before analysis is carried out.

This method, is commonly used among laboratories around Europe and it is useful in picking up all the known toxin producers in our waters. However, in Australia,

monitoring programmes use a filtration method (Wilkinson, 2006) of live material as they have problems associated with a group of organisms called raphydophyte flagellates (Throndsen, 1997; Hallegraeff *et al.*, 2004) these organisms are extremely fragile, lose their shape upon preservation and generally are best studied in unpreserved water samples to be identified correctly.

So, phytoplankton monitoring programmes around the world have to look at their potential targets before deciding on a methodology that suit their needs.
A variety of different methods have been developed to enumerate and identify phytoplankton over the years. Descriptions of these can be found in two UNESCO-produced volumes: The Phytoplankton manual, edited by Sournia in 1978 and The Manual on Harmful Marine Microalgae edited by Hallegraeff et al., first published in 1995, with a revised second edition published in 2003. The Utermöhl cell counting method is used as the standard methodology and it is chosen because it has been proven to be the most reliable method for cell counting and identifying phytoplankton.

After the publication of the Water Framework Directive (WFD) (2000/60/EC) a CEN document was prepared (CEN/TC 230) by a EU technical committee under the heading "water analysis" as a guidance paper on the need for a uniform procedure to assess the ecological quality of surface waters for phytoplankton abundance and composition. This document states that "A single standard procedure for the assessment of phytoplankton composition and abundance cannot be given as the questions underlying monitoring programmes are diverse in character and therefore require specific protocols." However later on asserts that "This European Standard is based on the analytical procedure of the standard settling technique as defined by Utermöhl (1958).

While contradictory in terms, this doesn't affect in practice what it is done in the ground by monitoring laboratories which already have been using this method for years. This is the first document to officially propose at a European level the Utermöhl cell counting method as the standard methodology for phytoplankton enumeration and identification.

An intercalibration workshop comparing a variety of different methods for the identification and enumeration of the dinoflagellate *Alexandrium fundyense* Balech was held at Kristineberg marine research station, Fiskebäckskil, Sweden in 2005. The results of this workshop were presented in Godhe *et al.* (2007) which conclude that the most reliable method for cell counting is the traditional count by the Utermöhl method.

Also, as recently as 2010 the Intergovernmental Oceanographic Commission (IOC) published a manual for microscopic and molecular methods for quantitative phytoplankton analysis (Karlson *et al.*, 2010). Here they discuss the merits of a wide array of methodologies and technologies, traditional and modern, for the advancement on the quantification of microalgae.

The implication of this publication is that monitoring programmes should move towards the use of multi-tools in monitoring programmes in conjunction with the more traditional microscopy techniques. Some of these tools are advanced in this manual like the use of molecular techniques (gene probes), epi-fluorescence techniques, flow cytometry and microarray detection.

1.5 Harmful algal blooms, their effect and the role of monitoring programmes

Marine phytoplankton blooms commonly known as 'red tides' or HABs (Harmful Algal Blooms) are naturally occurring phenomena. These blooms are a proliferation of algae to several millions of cells per liter which tend to discolour the water and cause negative effects in the environment. The frequency, intensity and distribution appear to have increased over the last few decades (Smayda, 1990, Hallegraeff 2004).

The term 'HABs' can be misleading and can be used loosely to refer to many different sets of events. For example, in some cases it is not necessary to have millions of cells per liter to have an event, or the bloom being visible to the human eye. Also, it should be noted that harmful effects are not always necessarily associated with toxin production by the microalgae. In some cases, the blooms may cause fish kills through

the production of ichthyotoxins, oxygen depletion, chemical release (acrylic acid, ammonia) or physical damage to fish gills.

The list of microalgal species that are involved in HABs comprises about 80 toxic species and 200 noxious species of an approximate total of 4000 marine phytoplankton algae described so far (Zingone and Enevoldsen, 2000).

Many of the marine microalgae that produce toxins in marine environments belong to the family Dinophyceae, but some other groups are also able to produce toxins (Diatomophyceae, Haptophyceae, Raphidophyceae and cyanophyceae). These toxins can cause direct damage to flora and fauna or accumulate in the food web causing harm to predators including humans (Backer *et al.* 2004, Landsberg 2002).

Most of these toxins are neurotoxic, but other toxins causing gastro intestinal effects, cytotoxins, hepatotoxins and dermatotoxins are also known. A detailed description of toxin chemistry and biological effects of these toxins can be found in Falconer (1993), Botana (2000), Landsberg (2002) and Hallegraeff *et al.* 2004.

Due to the potential health risks associated with the consumption of contaminated seafood, and the devastating effect that these toxins can have in the environment, which sometimes result in huge economic losses as well as human and environmental, and the perception that blooms have increased in frequency and distribution over the years have meant that governments which produce shellfish and finfish for human consumption have had to put in place restrictions on seafood products, these include the chemical analysis of algal toxins and the detection and monitoring of harmful algae (Anderson *et al.* 2001).

The appropriate design and establishment of HAB monitoring programmes is quintessential to the protection of public health, fisheries resources and coastal ecosystem function and structure (Andersen, 1996). This requires a good understanding of bloom dynamics, oceanographic knowledge of the area to be monitored, trained personnel for sampling, skilled taxonomists, knowledge of the organisms likely to be found in the water and the toxins they produce, and a good communication network for the dissemination of this information. The role of the

phytoplankton monitoring programmes is to prevent algal toxins from reaching human consumers of shellfish, to protect humans from aerosols, protect water resources (specially for freshwater species), minimise the damage cause to the environment and other flora and fauna, and ultimately, minimise the economic loss to the seafood industry. A successful monitoring programme needs to provide, therefore, an advance warning of the potential for biotoxins accumulation in shellfish.

1.6 The National Monitoring Programme (NMP) for Phytoplankton

The Marine Institute is the national agency for marine research and development in Ireland. It is a semi-state body, which was formally established by Statute (Marine Institute Act, 1991) in October 1992. Under this act the Marine Institute was given responsibility for and quote;

"*to undertake, to co-ordinate, to promote and to assist in marine research and development and to provide such services related to marine research and development, that in the opinion of the Institute will promote economic development and create employment and protect the marine environment".*

The Hygiene regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin and the regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption are the main directives underpinning the monitoring of toxic algae in shellfish production areas.

The Irish phytoplankton monitoring programme has been running since 1984 and in the year 2000 a big overhaul of the programme took place to allow the data generated by the programme to be stored in an easy an accessible way and it is at this point that the Harmful Algae Blooms database or commonly known as HABs database was created. This database became operational in 2002 and all phytoplankton records have been stored there since.

The phytoplankton monitoring programme covers shellfish and finfish production areas around the Irish coast, even though, originally the programme was intended specifically for the shellfish industry. The number of the samples collected in aquaculture finfish sites over the years has increased substantially, and at present these are approximately 50% of the sample total (Salas *et al.*, 2008).

This particular shift towards the inclusion of water samples from finfish growing sites, is a very important development because the species to be monitored for in finfish areas are very different to those involved in the intoxication of shellfish. Some species, may produce haemolytic substances that affect fish and can cause fish pathologies, like gill damage or bleeding for instance *Karlodinium micrum* (Leadbeater et Dodge) Larsen (Deeds *et al.*, 2002; Taylor *et al.*, 2004), or species that may cause clogging and/or physical damage (irritation and bleeding) to the fish gills, like *Chaetoceros* (Rensel, 1993; Fryxell & Hasle, 2004), also any species that bloom and proliferate in elevated numbers, and can cause the de-oxygenation of the water column (Silke *et al.,*2005) e.g. *Karenia mikimotoi* (Miyake & Kominami ex Oda) G.Hansen & Ø.Moestrup), or release other potentially harmful by-products of cell lysis, like ammonia e.g. *Noctiluca scintillans* (MacCartney) Kofoid & Swezy, (Landsberg, 2002) and others that harm fish indirectly by clogging the nets with mucus or oil like substances, that stop the flow of water through the finfish cages, like *Coscinodiscus wailesiii* Gran & Angst and *Coscinodiscus concinnus* Smith.

The sampling programme as it stands, depends on the Sea Fisheries Protection Officers (SFPOs) to collect samples periodically from shellfish production areas and it depends also, on the Aquaculture Catchment Monitoring Services (ACMS) to collect samples from finfish production areas. Samples from these sites are collected weekly with the potential to increase if a harmful algal event is occurring.

The phytoplankton monitoring programme receives on average over 1400 samples from all sampling sites annually (Salas *et al.,* 2009).

The Marine Institute phytoplankton unit developed a monitoring programme based on the quality principles used in other monitoring programmes around the world, but in particular, the system used in New Zealand by the Cawthorn Institute, where the Utermöhl cell counting phytoplankton method was already established and accredited.

The Marine Institute applied to INAB (Irish National Accreditation Board) for accreditation on the identification and enumeration of phytoplankton using the Utermöhl method in 2004. The test method was audited by INAB in December 2004 and was awarded accreditation under ISO/IEC 17025 in May 2005.

INAB raised three non conformances (NCFs) in order to award the accreditation. The first non conformance related to quality controls. INAB stated that a number of annual quality controls were needed, which could be in the form of internal intercomparison exercises to test the analysts' enumerating and identifying skills. The second issue raised was that an audit trail of the identification of toxic/harmful organisms was needed, and the third issue related to the participation of the unit in a proficiency testing scheme programme for phytoplankton.

The first and second issues were corrected by the incorporation of an annual internal intercomparison schedule, and an audit trail was integrated into the test method by capturing digital images of toxic species in the samples, as a traceable record of the identification. The third issue was different in that no proficiency testing scheme for phytoplankton existed at the time, as nearly all phytoplankton monitoring programmes in Europe at least weren't accredited then.

It is at this point that the MI phytoplankton monitoring programme took the lead under the BEQUALM/NMBAQC umbrella to organise the first interlaboratory comparison across Ireland and the UK in 2005, which over time have become a proficiency testing scheme for the quantification and qualification of phytoplankton.

1.7 Occurrence and detection of toxic algae: The Irish Perspective.

There are over 4000 known species of marine Phytoplankton and of these, around 80 species are able to synthesize biotoxins (Hallegreff *et al*, 2004). These are the primary species of interest in most monitoring programmes.

The Irish phytoplankton monitoring programme is also interested in other species, apart from those producing biotoxins, for example those able to cause problems to cultured finfish and those that can cause harmful algal blooms.

The main toxins can be classified depending on the nature of the symptoms they produce in humans. There are four main group of toxins found in Ireland at present: Diarrhetic Shellfish Poisoning (DSP) is produced mainly by the armoured dinoflagellates *Dinophysis* Ehrenberg and *Prorocentrum lima* (Ehrenberg) Stein. They produce OA (Okadaic Acid) and DTXs (Dinophysis Toxins). OA and DTXs are lipophilic polyether compounds that cause diarrhoea and vomiting in humans (Yasumoto *et al.*, 1985).

Paralytic Shellfish Poisoning (PSP) was named after the symptoms observed in animals of cramps, convulsions and respiratory paralysis (Shantz, 1986). This syndrome is produced by the armoured dinoflagellate *Alexandrium* Halim among other genera, but it is the only genus of marine species known to produce PSP toxins in Ireland, species belonging to this genus produce a number of compounds known as STXs (saxitoxins), GTXs (Gonyaulatoxins) and SPXs (Spirolides) (Taylor *et al.*, 1998; Cembella *et al.*, 2000; Richard *et al.*, 2001). These are powerful neurotoxins known to block the excitation current in nerve and muscle cells of animals resulting in paralysis (Shimizu *et al.*, 2000).

Azaspiracid Shellfish Poisoning (AZP) is produced by the small armoured dinoflagellate *Azadinium spinosum* Elbrächter & Tillmann. This recently erected genus *Azadinium* with the type species *Azadinium spinosum* (Tillmann et al., 2009) produces Azaspiracids. These compounds produce similar symptoms to those found in a typical DSP intoxication (James *et al.*, 2002), however recent pharmacological evidence (Draisci *et al.*, 1999; Volmer *et al.*, 2002; Hess *et al.*, 2003) suggests that these effects may contribute to causing chronic disorders in the intestinal tract in the human body like Crohn's disease or stomach or colon cancer.

Amnesic Shellfish Poisoning (ASP) is produced by the pennate diatom *Pseudonitzschia* H.Peragallo This genus produces Domoic Acid (DA). Domoic acid is a water soluble polyether compound that causes gastric upset, headache and dizziness, but the syndrome was named due to the persistent short-term memory impairment experienced by some patients (Perl *et al.*, 1990; Bates *et al.*, 1998). This is the only group of diatoms known to produce toxins in Irish waters.

The species referred to above are readily found in water samples from shellfish producing areas year on year in Irish coastal waters and therefore they need to be monitored for as there is an important Human health risk if people were to consume contaminated shellfish.

It is important to qualify that there are many other biotoxins found around the world that are not found in Irish coastal waters to date, e.g. ciguatera fish poisoning, palytoxins, tetrodotoxins, and other neurotoxins (Brevetoxins).

Other species that occur regularly in Irish waters with the potential to cause harmful algal events in marine ecosystems but do not produce toxins that effect humans include *Karenia mikimotoi*, *Phaeocystis* Lagerheim, *Nociluca scintillans, Emiliania huxleyi* (Lohmann) W.W.Hay & H.P.Mohler and Akashiwo sanguinea (Hirasaka) to name the more conspicuous from an Irish context.

1.8 Economic and Social impacts of marine biotoxins in Ireland

The social and economic impact of marine biotoxins and harmful algal events in mariculture operations in Ireland and the health risks attributed to these toxins can not be understated (Moestrup, 1994; Anderson *et al.*, 2001). It appears that the frequency, intensity and distribution have increased over the last few decades (Smayda, 1990; Hallegraeff, 2004). Several species of phytoplankton belonging in different taxonomic groups can produce toxins, which may result in extensive fish kills with major economic losses (Shumway 1990, Corrales & Maclean 1995, Zingone & Enevoldsen 2000). The shellfish industry in Ireland already suffers protracted periods of closure of their shellfish harvesting areas. This is an important economic loss to an indigenous industry that relies on export markets and also provides opportunities of employment in remote coastal areas along the Irish western seaboard.

The length of the closures for shellfish harvesting and the types of toxins found in the shellfish vary substantially from one year to another. For example 2005 was a particularly bad year with closures early in the year from ASP followed by AZP in the spring and early summer, DSP during the summer months and then AZP again in the

autumn-winter making some areas close for nearly the whole year (Clarke *et al.,* 2005).

On the other side of the social impact of marine biotoxins are the consumers which need to be protected from eating contaminated shellfish. There have been a number of high profile cases over the years of people becoming ill from marine biotoxins and in some cases dying from these. In 1987, there were 156 acute cases of intoxication for ASP after ingesting cultured mussels from Prince Edward Island off the coast of Eastern Canada. As a consequence of this, three people died. (US food and drug administration, 1992). In 1987, in Guatemala, 187 people became ill due to PSP intoxication from clams with 26 deaths recorded (US food and drug administration, 1992). In 1995, in the Netherlands several people became ill after ingesting blue mussels (M*ytilus edulis*) harvested in Killary Harbour, Galway Ireland (Mc Mahon *et al*., 1996). The symptoms were those of a gastrointestinal illness similar to DSP but the chemistry showed no DSP toxins present in the samples. Eventually this case would become the first case of AZP poisoning as later a new toxin called Azaspiracids was elucidated (Satake *et al.*, 1998; Ofuji *et al.*,1999) and found to be the causative toxin of this incident. In 1998, processed mussels from Bantry bay, Cork Ireland were implicated in an intoxication incident in France which were passed safe for DSP. The lack of confidence in the testing methods for AZAs led to a ban of all Irish shellfish in France in 1999 (EFSA Journal, 2009), in 2000 there were AZA poisoning incidents across the UK.

The amount of incidents recorded due to shellfish poisoning across Europe in the last decade have decreased dramatically thanks partly to the prevention through shellfish testing and phytoplankton identification in monitoring programmes across Europe and the protection of consumers from shellfish coming from outside Europe through the EU Hygiene regulations on imported shellfish and shellfish products.

The Directorate General for Health and Consumers in Europe (DGSANCO) is the food safety authority at European level and any potential contaminated product reaching the markets is raised as a European wide alert under the Rapid Alert System for Food and Feed (RASFF). This is an online searchable database (http://ec.europa.eu) that lists any notifications on shellfish products contaminated

with toxins, where they come from and what toxins are involved and can be accessed by the general public.

These examples show the need for more scientific research in the area of phytoplankton and also for better monitoring tools to be used to identify and quantify these organisms and predict using models the onset of harmful algal events in our coastal areas. The national monitoring programme for biotoxins is not only an obligation from the European directives but an important tool at national level to protect consumers from contaminated stock, to maintain the confidence in the consumption of safe shellfish in Ireland and abroad and help the industry to open up new export markets in Europe and further a field as their produce is underpinned by quality assured results.

2. Bequalm 2009 Intercomparison exercise in the enumeration and Identification of Phytoplankton

Abstract

The objective of this study was to test laboratories engaged in phytoplankton monitoring of toxic/harmful algae, in the enumeration and identification of marine microalgae through an intercomparative study or ring trial using light microscopy techniques. Samples were analysed by the Utermöhl cell counting method as the standard protocol for this exercise.

In the identification exercise, there was no evidence that video clips were better audit trail tools than images or vice versa. Analysts tended to have a higher success rate correctly identifying image sets than videos, but this was not consistent across analysts.

In the qualitative measure of the exercise, the degree of correctness of identifications had been measured for the method in terms of false positive and negative rates. These rates had been combined and expressed as a Bayesian likelihood ratio. The sensitivity and specificity of the method has been calculated as a Youden index.

The false positive rate of the identifications was calculated to be 1% and the false negative rate was 19%. This false negative rate indicates that analysts are more likely to identify a toxic organism as a non toxic organism than the other way around.

The specificity and sensitivity ratios (99% and 81%) respectively for the exercise were good. As the sensitivity of the test method was lower than the specificity, this indicates a higher number of false negative responses to the method than false positive responses, but overall the method has a 92% efficiency and high Youden index.

This exercise has shown the importance of intercomparison work between laboratories engaged in phytoplankton monitoring, to assure the quality of the results,

to validate the methodologies, to calculate the uncertainty of measurement for the method and ultimately to help the accreditation of the test method across laboratories.

This is the first time that a qualitative reliability measure for the identification of phytoplankton has been used in a phytoplankton intercomparison exercise. It is important for future exercises, that this reliability measure for the test method is used in conjunction with the quantitative measure.

2.1 Introduction

This exercise was designed to evaluate the performance of participating laboratories in the enumeration and identification of phytoplankton species from preserved culture material spiked in water samples, and through a taxonomic quiz to test participants' knowledge on phytoplankton taxonomy and evaluate the usefulness of graphic materials (images and video clips of phytoplankton species), as quality controls and audit trails in monitoring programmes.

The identification exercise was based on multimedia materials that are used regularly as monitoring tools in phytoplankton programmes for traceability purposes or audit trails of taxonomic identifications. Identification of phytoplankton species by analysts are subjective items in biological qualitative tests, as these identifications can not be corroborated by internal quality controls and/or auditors. Photographs and videos of phytoplankton species are an important record for traceability purposes of a phytoplankton taxonomic identification. Auditors have a tangible record that can be followed back to the sample and the result be queried if necessary.

During the design stage of the taxonomic quiz the use of video clips showing phytoplankton species in movement was thought to be a good idea, as it has never been used before in an exercise of this kind. A comparison could be made, whether videos could be better identifying tools than images, and whether these could also be used as monitoring tools. Videos give extra information to the analysts on the phytoplankton species by conveying the movement of the cell/s and show their tri-dimensional shape.

This identification exercise also gave analysts the option to identify different phytoplankton taxa other than diatoms (Werner, 1977; Round *et al.*, 1990; Mann, 1999) and dinoflagellates (Steidinger, 1997; Graham & Wilcox, 2000), like euglenophytes, chryptomonads, silicoflagellates and haptophytes (Green & Jordan, 1994; Edvarsen & Paasche, 1998), which regularly appear in samples and are ecologically important groups of marine algae. Since the publication of the Water Framework Directive (WFD) in Europe (which uses phytoplankton as one of the ecosystem components required to monitor the quality status of marine and freshwater bodies), monitoring of microalgae has expanded to other species which may not produce toxins, but can cause harmful algal events or are indicative of eutrophication processes. It is therefore important that analysts are able to identify a wider array of phytoplankton species.

Monitoring programmes, with a primary interest in the identification of toxin producing algae, have a greater emphasis on the identification of diatoms and dinoflagellates. This intercomparison exercise broadens the parameters to include other phytoplankton species, as well as toxin producing algae.

Environmental Protection Agencies interested in fulfilling the remit of the WFD, for example, are more interested in the water quality status of coastal/transitional waters and changes in the phytoplankton flora, due to pollution and eutrophication.

The enumeration exercise was designed taking into account the basic principles of experimental design for the life sciences (Ruxton and Colegrave, 2003) of replication (Hulbert, 1984), control, randomization (Harvey & Puvis, 1991) and blinding to be statistically robust, in order to obtain useful data for statistical analysis. This exercise was designed to be a controlled experimental study, where the treatments and factors were imposed on the individuals to observe their responses (Campbell, 1989; Dytham, 1998).

It could be argued that this exercise is in some ways, a method validation study, since all the participants were asked to use exactly the same techniques to analyse the samples.

Phytoplankton analysts identify and enumerate many phytoplankton species in water samples during routine analysis, so the exercise tried to replicate this by spiking several species into the samples. This was not only done to study the repeatability and reproducibility of the estimated cell counts, but also to evaluate a qualitative measure for the test method. The qualitative measure employed for this exercise was the use of false response rates (EURACHEM/CITAC "uncertainty in qualitative testing"), EURACHEM/CITAC are a European joint working group on the measurement of uncertainty and traceability in analytical chemistry. This was done, to establish and evaluate the risk of an incorrect classification of biological specimens from a monitoring perspective.

Based on experience, from previous phytoplankton intercomparison exercises; Bequalm 2000, 2001 (Reckermann and Colijn, 2000, 2001), IBERIA 2003 (Pazos *et al.*, 2007) Bequalm 2005, 2007 (Moran *et al.,*2005,2007) and Bequalm 2008 (Salas *et al.*, 2008) samples given to analysts are usually statistically analysed from a quantitative standpoint, but the qualitative measure of the exercise has never been developed, to provide a measure of the reliability of the test method and the correctness of the identification carried out by the analysts. In fact, there is very little in the literature that deals with this particular issue (de Ruig *et al.*, 1989; Ellison *et al.*, 1998; Milman *et al.*, 2000). This exercise tried to redress this imbalance by providing, through the analysis of the water samples, both a quantitative and qualitative measure for the test method. This is without detriment to the taxonomic quiz which is simply, a further qualitative measure of the taxonomic knowledge of the analysts.

A set of six samples was sent to each laboratory rather than to each analyst, this allowed, the investigation of the within laboratory reproducibility and inter observer variability. The treatments were, a negative control (one sample) containing no species, a positive control (one sample) containing one toxic phytoplankton species, two samples, containing four species and another two samples containing the four species plus the positive control species.

The negative control sample was used to investigate potential issues in the cleaning techniques employed at laboratories. Laboratories are known to re-use materials like sedimentation chamber glass plates due to the high cost of these materials. Glass

plates are cleaned thoroughly and dried before re-use, however remnants of the previous sample, can still be present on the glass surface. Also, an investigation of the 'placebo' effect, used regularly in medical drug trials, was thought to be useful; in this case are analysts seeing cells in samples when there are none?

The positive control sample was used to investigate, whether analysts, were able to identify a sample containing a toxin producing dinoflagellate. This same organism was spiked into two other samples, to investigate whether analysts would arrive at similar cell counts when the organism was confounded with other species in the same sample.

Samples containing four species were designed to give a wide array of phytoplankton cell types and sizes. Species included were: the diatom *Coscinodiscus granii* Gough which are large in size (>60microns), the toxic naked dinoflagellate *Gymnodinium catenatum* L.W.Graham a chain forming species medium in size (40-60microns), the armoured dinoflagellates *Scrippsiella trochoidea* (Stein) Balech ex Loeblich III and *Prorocentrum micans* Erhenberg non-chain forming species and small in size (<40microns).

Samples spiked with five species, contained the same four species described above and the armoured dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge which was spiked also into the 'positive control' sample.

Different cell densities were employed in this exercise for the various species, as there was an interest, on testing the range of the method from low to high cell concentrations and study the variation in results, due to cell density. It also, allowed an evaluation of the sample counting strategies that analysts would use as the density increased.

There are no certified reference materials in phytoplankton analysis that can be used for intercomparison purposes and this means that materials have to be homogenised and tested, in terms of the cell concentration and condition of the biomass, before they can be delivered to the participating laboratories, it also means that there are no validated true values or reference to work from. The true concentration can be either

be the mean +/- a specified multiple of the standard deviation of all the analysts' results or a 'gold standard' mean and standard deviation set up by the organising laboratory. It was decided that for this exercise the 'gold standard' approach would be used.

An advantage of using gold standards is that it creates a reference value for the data, where the experimenter had knowledge, *a priori,* of the relative cell densities and the materials used while the analysts are blinded to the experiment. Also, the amount of replicates analysed by the experimenter is larger, so theoretically, more faith can be had in the coefficient of variation of the sample population.

In theory, cell counts variability within laboratories should be small, that is to say the results would be reproducible.

BEQUALM/NMBAQC (Biological Effects Quality Assurance Programme/ National Marine Biological Analytical Quality Control Scheme), registers all participants through their website (www.bequalm.org) to the phytoplankton enumeration and identification ring trial for 2009.

2.2 Materials and Methods

This intercomparison exercise was coded in accordance with defined protocols in the Marine Institute, for the purposes of Quality traceability and auditing. The code assigned for the current study was PHY-ICN-09-MI1. PHY standing for phytoplankton, ICN for intercomparison, 09 refers to the year 2009, MI refers to the Marine Institute and 1 refers to the sequential number of intercomparisons for the year; so 1 indicates that is the first intercomparison carried out in 2009 by the Marine Institute.

Analysts were given until the 20th of March of 2009 (four weeks from sample receipt), to return enumeration and identification results to the Marine Institute (MI) Phytoplankton laboratory.

2.2.1 Phytoplankton samples

2.2.1.1 *Selecting culture materials*

Materials for the enumeration exercise were obtained from the Marine Institute algae culture collection. Decisions on species composition were influenced by the experimental design of the exercise and practical reasons, for example the cultures were initially assessed for viability of the cells, with special attention given to their morphology. Cultured material can contain typical vegetative cells, cells that are undergoing fusion or division, they can produce gametes which are smaller and sometimes even completely different in shape and size than the parent cell. Also, there are cultures that can produce resting cysts and others that can change shape completely under preservation.

Each species used in this intercomparison was screened for suitability in terms of shape, size and culture condition. Particular attention was paid to *Gimnodinium catenatum* so that chains of this organism were present in the samples rather than single cells as that would be typically how they would be recognized, despite this, single cells of this organism were also present in the sample as chains might break down through homogensation.

The final list of species was: *Prorocentrum lima* (Erhenberg) Dodge, *Scrippsiella trochoidea* (Stein) Balech ex Loeblich III, *Coscinodiscus granii* Gough, *Gymnodinium catenatum* L.W.Graham *and Prorocentrum micans* Erhenberg. The species *P.lima* and *G.catenatum* were chosen, because there are toxin producing microalgae. *P.lima* produces Diarrhetic shellfish toxins (Okadaic acid (OA) and Dinophysis toxin-1 (DTX-1)) (Yasumoto *et al.*, 1980; Tachibana *et al.*, 1981; Murata *et al.*, 1982) and *G.catenatum* produces neurotoxins, in particular Saxitoxins (STXs) and Gonyaulatoxins(GTXs) (Sommer *et al.*, 1937; Schantz *et al.*, 1966; Bates *et al.*, 1975; Sullivan *et al.*, 1984; Schantz *et al.*, 1986). The other three species were selected because there were non-toxin producing algae. It is important to identify toxic algae in a sample, but it is essential also to be able to discriminate between other species that are non-toxic. That is to avoid false positive and negative identifications.

2.2.1.2 Cell concentrations

Once the species were selected, experimental cell densities were decided for each species. The lowest final concentration was ~100 cells in a 25ml sample for *P. lima* and the highest was ~1000 cells in 25ml sample for *S. trochoidea.* The density of *G. catenatum* was around ~600 cells in 25ml, *P .micans* around ~400 cells in 25ml and *C. granii* around ~200 cells in 25ml. The cell concentration for each species was determined using a sedgewick rafter (Pyser-SGI, Kent, UK) cell counting chamber (Guillard, 1978; Guillard and Sieracki, 2005), before they were spiked into the samples. Ten sedgewick rafter measurements were carried out for each species, to ascertain the approximate cell concentration required.

2.2.1.3 Sample types, treatments and replicates

There were four different sample types: a negative control (Type d sample) containing sterile filtered seawater using GF/C filters (Whatmann^{TM}, Kent, UK), but no phytoplankton, a positive control containing the toxic phytoplankton *P .lima* (Type c sample), sample type a containing four species (*S. trochoidea, G. catenatum, C. granii* and *P. micans*) and sample type b containing five species (*S. trochoidea, G. catenatum, C .granii, P. micans* and *P. lima*). There were six samples in total with two replicates for sample types a and b and no replicates, for the positive and negative control samples. Cell concentrations of the spiked organisms were the same in all replicates.

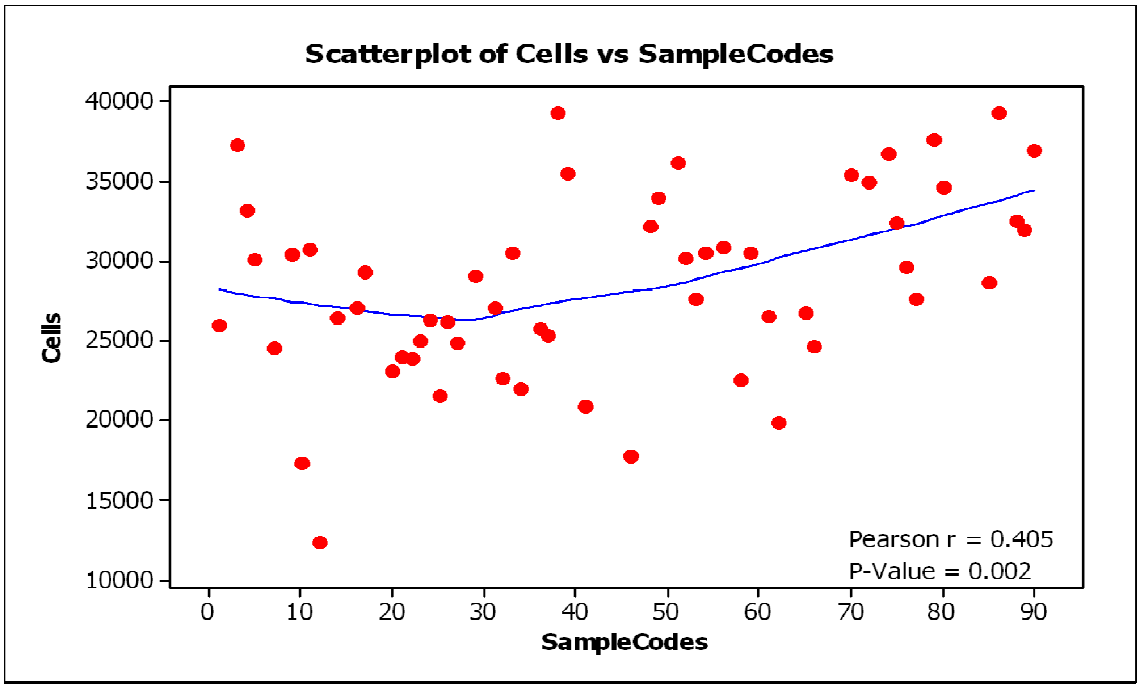
2.2.1.4 Sample preparation, homogenisation and spiking

All samples were prepared in the same way, except for the negative control (type d) sample as this sample did not contain cultured material. The seawater used in this experiment was natural field water collected from Carna, in county Galway, filtered through GF/C Whatmann filters, autoclaved, and preserved in a lugol's iodine solution (Clin-tech, Dublin, Ireland) as this is the most common preservative used in marine water samples (Utermöhl, 1958; Willen, 1962; Andersen & Throndsen, 2004).

Each of the species used in this experiment was spiked separately into a 250ml screw top Schott glass bottle (Duran®, Mainz, Germany), containing the filtered seawater. The 250ml bottles containing each of the species, were inverted 100 times to homogenate the sample and 1ml aliquots were taken after each 100 times inversion using a calibrated 1ml Gilson pipette (Gilson, Middleton, USA) with 1ml pipette tips (Eppendorf, Cambridge, UK). The 1ml aliquot was dispensed into a 30ml plastic sterilin tube (Sardstedt, Nümbrecht, Germany). This process was repeated for each sample and species. The rest of the sample was constituted with sterile filtered seawater.

The 250ml glass bottles were used in this experiment to avoid 'concentration effects' due to the master mix volume being too small or 'homogenisation effects' due to the volume of the master mix being too large. Experimental data from a previous intercomparison (2008), where the samples were aliquot sequentially, from sample 1 to sample 100 from a master mix with a 100ml volume indicated that the cell concentration of the samples tended to increase as the volume of the master mix decreased (Fig. 2.1).

Figure 2.1: 'Concentration effects' in samples from Bequalm intercomparison exercise 2008.



On the contrary, if the volume of the master mix is too large, there may be problems with the homogenisation of the mixture. In total, 60ml of the 250ml were used from the master mix of each species, less than 50% of the total volume.

The final volume of each sample was 25ml, which gives enough volume to fill a 25ml sedimentation chamber to the top. This was carried out using 25ml serological pipettes (Sardstedt, Nümbrecht, Germany) and a calibrated four place balance (ME414S Sartorius, AG Gottingen, Germany), to accurately measure each volume for each sample. For example for the type a samples, 21ml of preserved sterile filtered seawater was measured using this technique + 1ml aliquots of each of the four species. In the case of the type b samples, the 20ml volume of preserved sterile seawater was measured using a four place balance and pipette into the sterilin tubes, then the five species, each individually homogenised from the master mix, were added to the 20ml, 1ml of each species.

180 samples in total were produced for this exercise. There were 60 samples each for the types containing four species (Type a) and five species (Type b). Thirty samples prepared for the positive control (Type c) and 30 samples for the negative control (Type d) sample.

Seventeen laboratories were participating in this exercise and a set of six samples were sent to each laboratory which meant that 102 samples of the 180 sample population were couriered off to the participating laboratories. The other 78 samples were used for setting up the reference value or gold standard.

2.2.1.5 Sample randomization

All samples sent to the participating laboratories were randomly selected using Minitab statistical software version 15.0 (Minitab Inc., Pennsylvania, USA). Each sample was given a number and randomly assigned to a laboratory by the programme by using the randomization tool.

2.2.2 Taxonomic quiz

2.2.2.1 Images and video footage

The taxonomic quiz for this intercomparison was designed using footage from Cytographics Ltd. in Australia. This ensured that the images and video footage used were of the highest quality possible and also that the identification of the organisms used was done by independent phytoplankton taxonomy experts.

All the images and video clips came from two DVDs which are published work by cytographics Ltd. The titles are 'Diatoms: Life in glass houses' and 'The Kingdom protistan: The dazzling world of living cells'.

An agreement with cytographics Ltd. was signed for the use of this footage for a limited period of time. Images and video clips were taken from their DVDs to be used in the exercise.

2.2.2.2 Technical aspects

A website was set up for participants to view the videos. Under the license agreement the footage in the website had to be password protected and then dismantled soon after the exercise was over to avoid downloading of the material by third parties.

All registered participants for the exercise got a username and password that they had to use in order to access the content of this webpage.

2.2.2.3 Quiz content

Two sets of twenty images and videos were randomly separated using Minitab into four sets of five images/clips each. This was done to have a sufficient number of replicates. Most of the questions related to identifying the organism to a particular taxonomic level, while some questions related to morphological features of the organisms.

2.2.3 Forms and instructions

2.2.3.1 Couriers and materials

All the necessary forms and instructions were sent to all the participating laboratories. Laboratories with several analysts taking part in the exercise were asked to assign a sample manager for the exercise. The sample manager was in charge of receiving the materials and setting up the samples for all the analysts within that laboratory Each laboratory received a sample set (six samples), and a set of instructions (Appendix 1), a form to confirm receipt of materials (Appendix 2), an enumeration hard copy results form (Appendix 3), and a original master copy of the taxonomic quiz (Appendix 4) per analyst. These materials were sent via courier to all the laboratories on the same day. Upon receipt of materials laboratories were asked to check the samples and the documentation for missing forms or leaked samples.

2.2.3.2 Instructions

Laboratories were asked to read and follow the instructions before commencing the test and to give themselves plenty of time to limit the number of errors due to tiredness and stress. See instructions in Appendix 1.

2.2.4 Utermöhl cell counting test method

The Utermöhl cell counting method (Utermöhl 1931, 1958) was the standard technique used in this intercomparison. This method is a light microscopy based technique. The samples are collected in sterilin tubes or plastic bottles and preserved with lugol's iodine or formalin as the more typical preservation agents. Samples are then homogenised prior to the settlement step. These are settled into a sedimentation or Utermöhl chamber, where preserved organisms in the sample settle by gravity over time. Once the organisms are settled at the bottom of the chamber, these can be viewed, enumerated and identified using inverted light microscopy.

The inverted microscope should be equipped with bright field illumination and the optical quality of the microscope and its objectives is fundamental for the

identification of the species. The microscope should have objectives of different magnifications, the most typical arrangement is 4x, 10x, 20x, 40x and 60x. Other type of illumination like phase contrast or differential interference contrast (DIC) is also useful.

The sedimentation chambers consist of two parts, an upper cylinder made of plastic and a bottom plate made of stainless steel. A thin glass cover sits on the bottom plate and the cylinder is threaded into the plate. There are different volume chambers, but the most typical volumes are 2, 5, 10, 25 and 50ml. These are calibrated to make sure that they can hold the appropriate volume for analysis. Once samples are filled a top cover glass is slid across the top of the chamber to seal the sample. Air bubbles should be avoided.

Settling time depends on the height of the chamber and preservative (Lund *et al*., 1958, Nauwerk, 1963). The recommended settling times for lugol's preserved samples is of approximately 24 hours for a 50ml chamber of 10cm height. According to Hasle (1978a) formalin preserved samples need at least 40 hours settling time independent of the chamber used.

The sample after the settling period is placed on the inverted microscope and analysed. Counting of the organisms starts by a series of sinuous parallel movements up and down or across the chamber, avoiding overlapping or missing parts of the chamber. The counting strategy and magnification used will depend on the sample, the type of organisms and the cell density found. A preliminary scan before counting start is recommended.

Organisms should be identified to the lowest taxonomic level possible (Hasle 1978b) that time and skill permits. The counting strategy will depend on the biomass of the sample. Usually, analysts aim to do a whole chamber count, otherwise a half chamber, transect or even field of view count may be chosen. If a half chamber count is chosen, every second transect of the whole chamber is not counted. If a transect count is used, the diameter of the chamber is counted three times and the count averaged.

When cells are too numerous to count, field of view counts can be carried out. Randomly chose at least 10 fields of view and count each individual field of view, then average the results.

In order to obtain statistically robust data for the quantification a certain number of counting units are necessary. The precision is usually expressed as the 95% confidence limit as a proportion of the mean. The precision increases with the amount of units counted (Venrick, 1978, Edler, 1979).

After analysis, chambers should be cleaned appropriately. The thin glass cover should be disposed of, as even with a good cleaning, organisms tend to stick to the glass and sides of the chamber. Given the results of the negative sample from the 2009 exercise, I would recommend the disassembly, disposal of glass and cleaning of the chamber with a neutral detergent as the chambers will deteriorate with corrosive agents.

The Utermöhl method is probably the most widely used for the quantitative analysis of phytoplankton. It has an advantage over other methods of phytoplankton analysis in that algal cells can be both identified and enumerated. Using this method, it is also possible to determine individual cell size, form, biovolume and resting stage (Edler & Elbrachter, 2010).

The Utermöhl method is based on the assumption that cells are poisson distributed in the counting chamber. To quantify the result as cells per litre, a conversion factor must be determined.

2.2.5 Statistical analysis

The first step was to check the enumeration data. Particular attention was paid to outliers, errors in the data and missing data (Gotelli & Ellison, 2004). Once this has been done the summary statistics for the data concerning each species has been explored. This was done using Anderson-Darling Normality plots, showing the graphical summary and the descriptive statistics (mean, median, interquartile range

and shape of the curve) for each species. Our null hypothesis was: is the cell densities found normally distributed?

Then a reference value was calculated by the organizing laboratory. This reference value from a random set of samples was set up and analysed in the same manner as the participating laboratories.

The analysts' data were then compared using box plots (Tukey, 1977; McGill, 1978; Benjamini, 1988) for each species against the 'gold standard' to look at significant differences between the mean and median of the analysts' results against the reference values. A general linear model (2 way ANOVA) (Fisher, 1925; Mardia, 1979) was fitted to calculate significant differences between the analysts and the reference value and between treatments, and the interaction treatment and analyst type. Main effect plots were used to illustrate graphically the differences between the main factors under consideration, and pareto charts to observe the influence of these factors.

Data were also analysed using the t-test to study differences between replicate samples (paired t-test) and sample types (Two sample t-test) for the analysts' results and the reference values.

Also, I charts of the individual mean values for each organism were plotted against the 'gold standard' mean and 3 standard deviations or sigma limits. Analysts results should fall within the limits set up for the exercise. Any out of specification results are shown in the graphs.

Z-scores (+/- 3 sigma limits) of each analyst and laboratories were plotted against the reference value for each organism, to show how each analyst has performed against the 'gold standard'.

Finally, scatterplots were used to study reproducibility within laboratories with two or more analysts. All analysts within the same laboratory analysed the same sample set, so theoretically, all the results should be the same for all analysts within that laboratory. Analysts' results have been plotted against each other to investigate how reproducible results are within a laboratory.

The results of the taxonomic quiz were analysed using percentages of correct answers for each analyst. The main analysis of this data was to compare the results from the image sets against the video clip sets, and also within sets of images and videos to investigate, whether some sets were more difficult to identify than others.

The descriptive statistics for the data for each treatment were compared using box plots. Also each laboratory and analyst results were compared for each treatment, and a percentage of correct answers were calculated for each analyst, laboratory and their cumulative percentage. The individual results in terms of percentage of correct answers were plotted in a league table.

As a qualitative test, the degree of correctness of the identifications in the samples has been measured for the method in terms of false positive and negative rates.

These positive and negative rates, based on false positive and negative responses, were combined and expressed as a Bayesian likelihood ratio (Albert, 1996, 1997; Gelman, 1995). The sensitivity and specificity of the method has been calculated as a Youden index (Youden, 1975): a single statistic measure that uses the specificity and sensitivity of a diagnostic test, in this case the Utermöhl counting method to give a reliability measure of how good the test is, for what it is intended, in this case the identification of marine microalgae. The test result always falls between -1 and +1 with +1 as the optimum result.

In order to calculate the positive and negative rates of this intercomparison, a definition was needed to describe what makes a false positive rate and what makes a false negative rate. A false positive rate is the number of false positive results divided by the number of true negative results + false positive results. Equally the false negative rate is the number of false negative results divided by the number of true positive results + false negatives results.

A **true positive** (TP) result in this case is the number of toxic/harmful species correctly identified. A **false positive** (FP) result is the number of non-toxic/non-harmful species identified incorrectly or identified as toxic/harmful. A **true negative** (TN) is the number of non-toxic/non-harmful species correctly identified and a **False**

negative (FN) is the number of toxic/harmful species identified incorrectly or identified as non-toxic/non-harmful.

This provided a very powerful reliability measure for the intercomparison (Table 2.1). These rates were then used to construct a measure of how sensitive, specific and efficient was the method.

Table 2.1: Expression of reliability measure for identification

Realiability measure	Expression
False Positive Rate	FP/(TN+FP)
False Negative Rate	FN/(TP+FN)
Sensitivity	TP/(TP+FN)
Specificity	TN/(TN+FP)
Efficiency	TP+TN/(TP+TN+FP+FN)
Youden Index	Sensitivity+Specificity-1
Likelihood ratio	1-False Negative rate/False
Bayes Posterior probability	Bayes Rule

2.3 Results

2.3.1 Participants

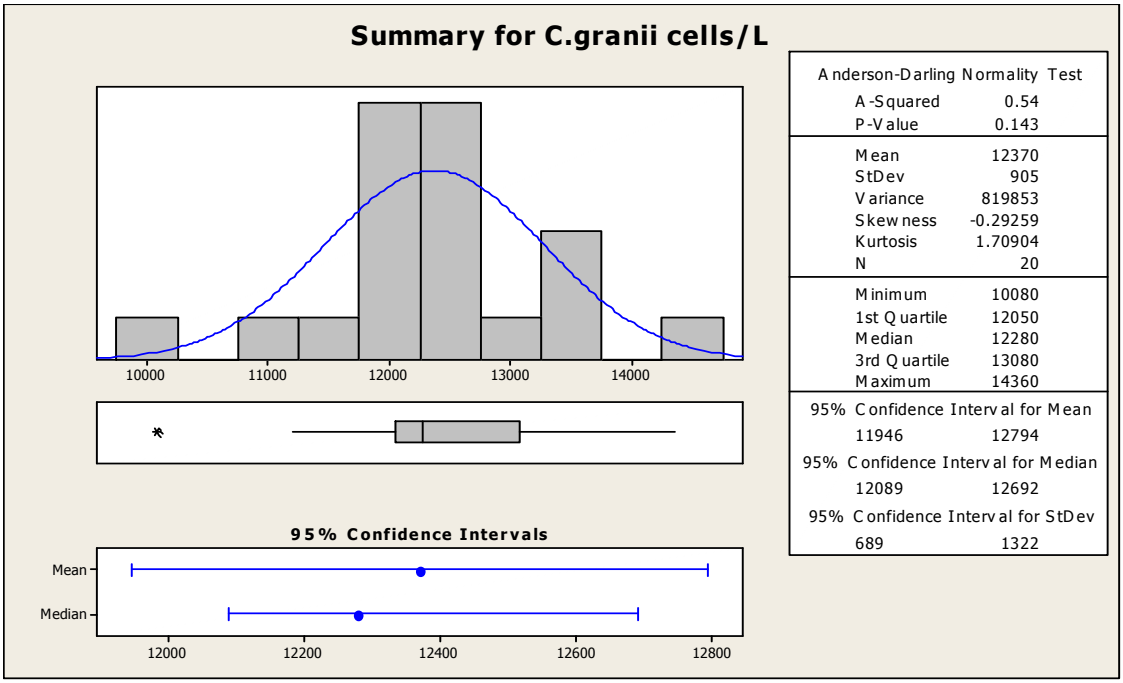
Thirty analysts from seventeen laboratories across Europe took part in this exercise. These included laboratories from Ireland, Northern Ireland, UK, Scotland, Spain, Germany, and the Netherlands. The list of participants is included in Table 1 (Appendix 5). Most of the laboratories participating in this exercise are phytoplankton monitoring laboratories in their respective countries and marine areas. Some laboratories are environmental protection agencies and a few laboratories are environmental companies.

2.3.2 Phytoplankton quantification results

2.3.2.1 Gold standard or reference data

The 'True value' or 'gold standard' results are shown in (Appendix 6); these were determined by the organising laboratory, to establish a reference value or gold standard for each organism.

Figure 2.2: Anderson-Darling normality test for *Coscinodiscus granii* cell counts

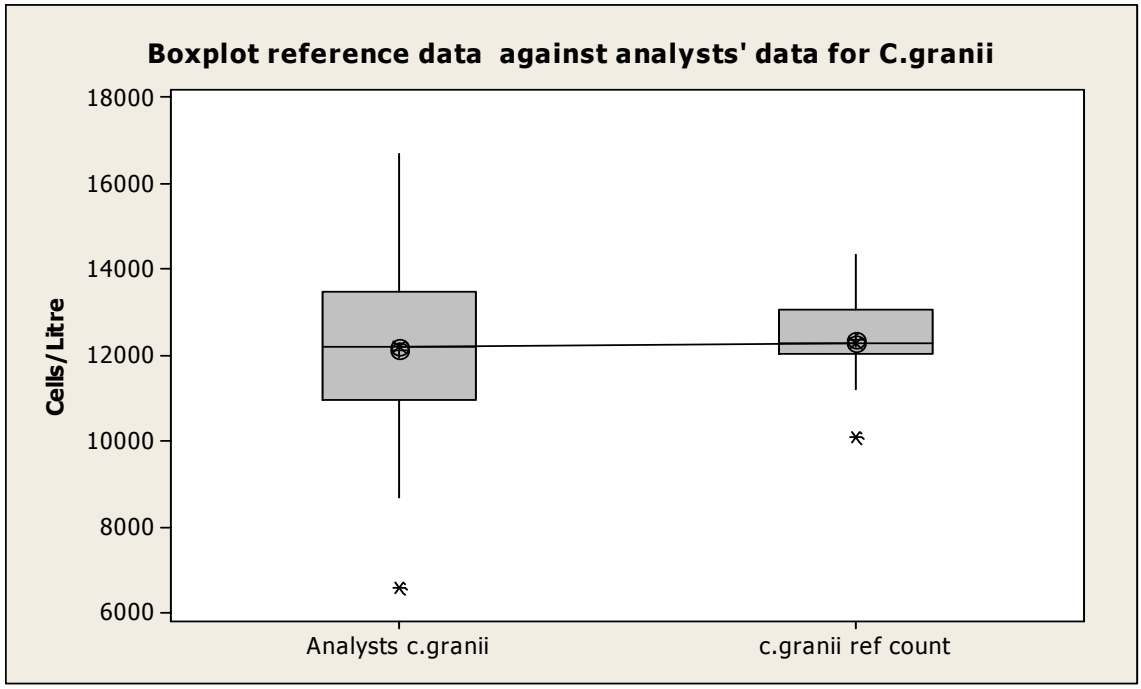


The reference data obtained was analysed for normality using the Anderson-Darling normality test for each organism. The summary of the cell counts descriptive statistics for *Coscinodiscus granii* are shown in figure 2.2. The figure shows, the typical bell shape of a poisson distribution, slightly flattened indicating a larger spread of the values but the mean and the median are comparable. The P-value 0.143 is greater than the level of significance 0.05. So, we reject the null hypothesis, therefore the data is normally distributed. This analysis was carried out for all the other species and it was shown that the reference data was normal.

2.3.2.2 Reference data versus Analysts' data

Figures 2.3 to 2.7 show the box plot of the analysts' results for each organism against the reference values. The box plots of *C. granii* (Fig.2.3), *S. trochoidea* (Fig.2.5), and *G. catenatum* (Fig.2.6) suggested that there were no significant differences between the analysts' mean and the reference mean, in fact the spread of results and the median and mean were very close for all the cell counts.

Figure 2.3: Box plot of analysts' versus reference cell counts for *C. granii*



The *P. micans* (Fig.2.4) and *P .lima* (Fig. 2.7) box plots indicate that there are significant differences between the analysts and the reference cell counts.

Figure 2.4: Box plot of analysts' versus reference cell counts for *P.micans*

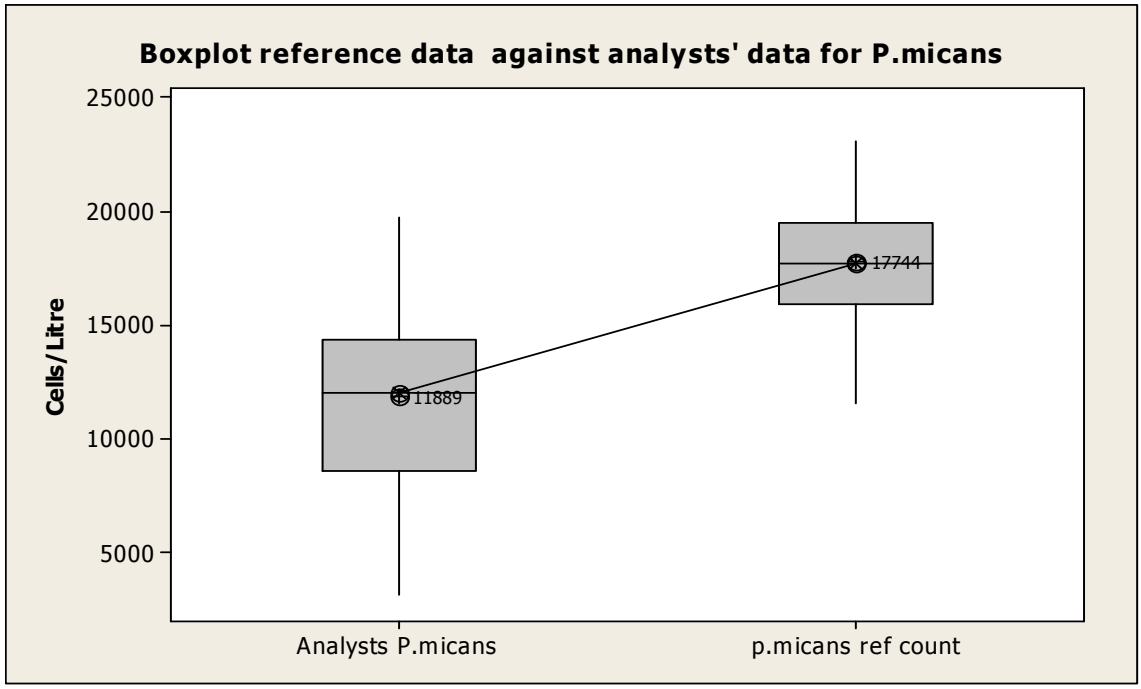


Figure 2.5: Box plot of analysts' versus reference cell counts for *Scrippsiella sp.*

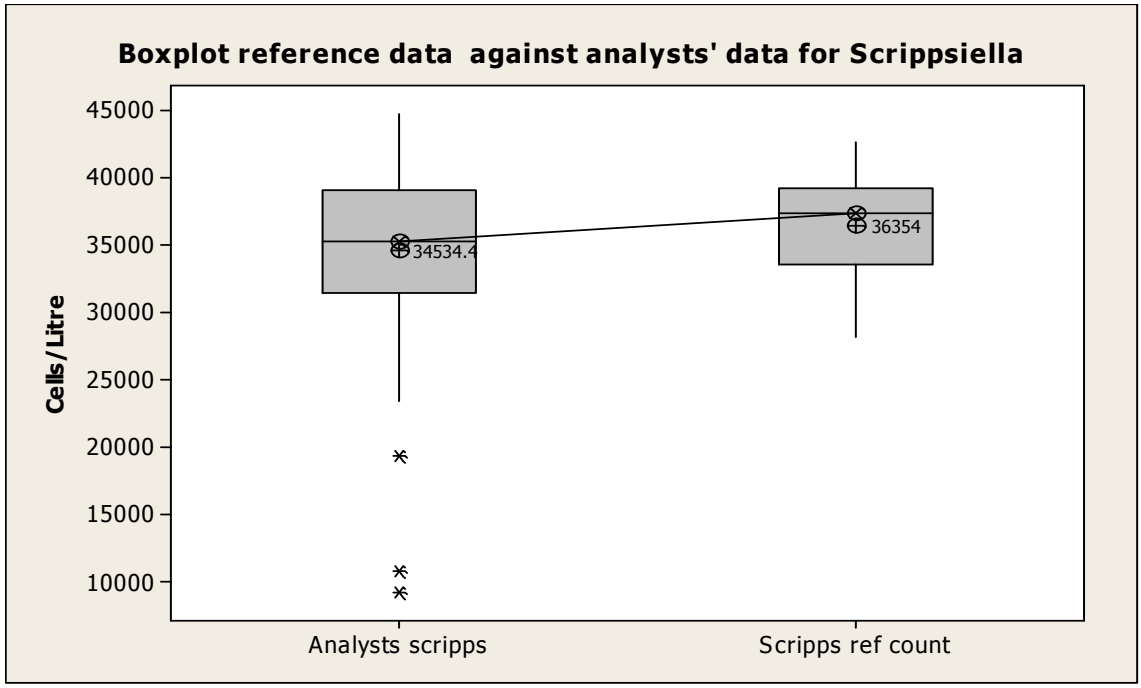


Figure 2.6: Box plot of analysts' versus reference cell counts for *G.catenatum*

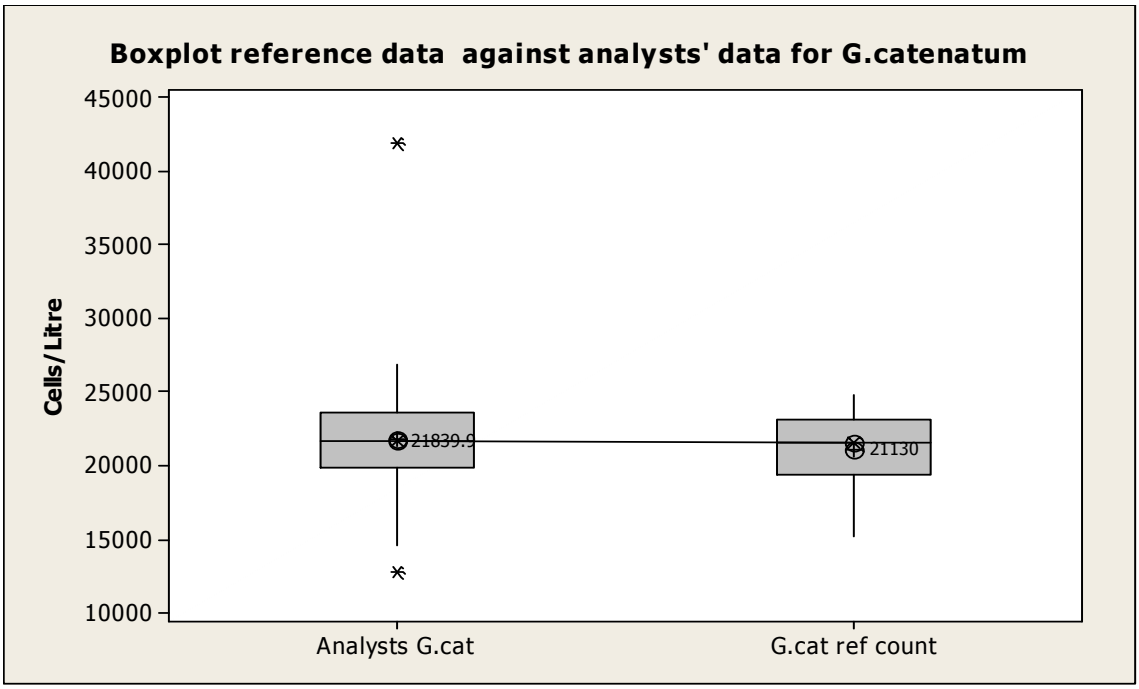
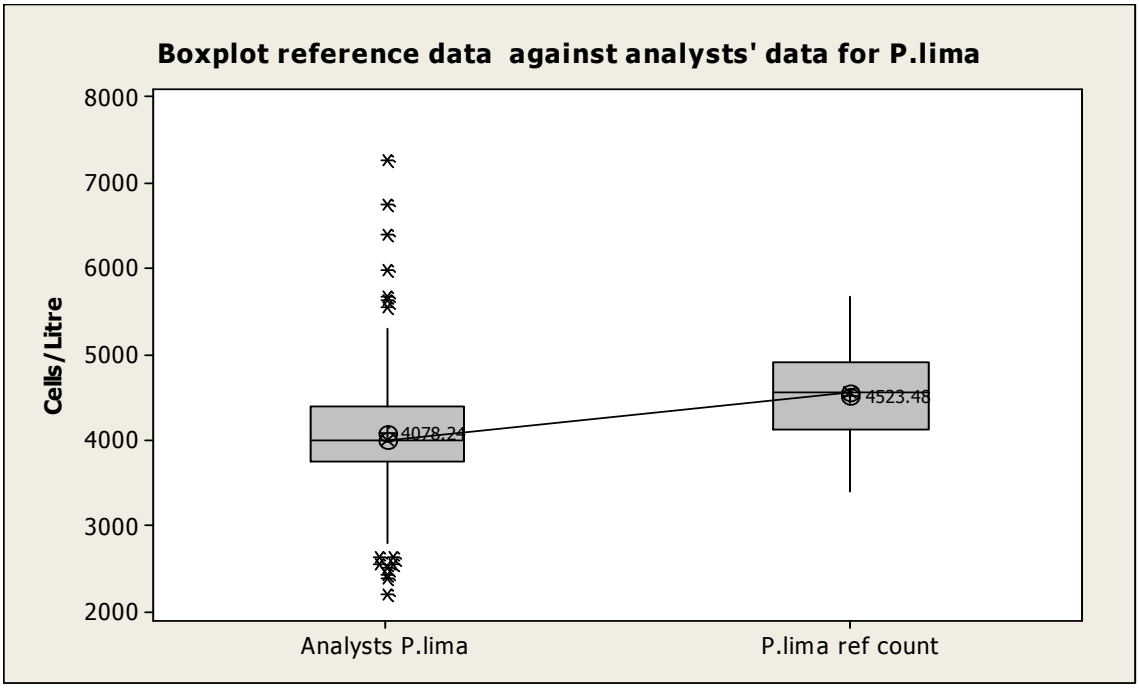


Figure 2.7: Box plot of analysts' versus reference cell counts for *P.lima*



2.3.2.3 P.lima reference value versus P.lima analysts' results

The species *P.lima* was spiked into two different sample types, the Type b sample contained *P.lima* and the other four species. The type c sample contained only *P.lima*, the idea was to test, if cell counts would be different in the type b and type c samples and to test, if the cell counts would be different to the reference cell counts. The analysts examined one type c sample and two type b samples, so all analysts should have three counts in total for *P.lima*.

A comparison was made between the type b analysts' replicate counts using a paired t-test (Table 2.2). The results indicate that there are no significant differences between the analysts' replicate counts.

Table 2.2: Paired T-Test and CI for *P.lima*: Analysts (type b) 1st vs. 2nd sample

Paired T for 1st sample - 2nd sample				
	N	Mean	StDev	SE Mean
1st sample	34	4067	1125	193
2nd sample	34	4256	860	148
Difference	34	-189	731	125
95% CI for mean difference: (-444, 66)				
P-Value = 0.140				

In order to establish whether there were differences between the type b and type c analysts' *P. lima* cell counts a two-sample T-test was used (Table 2.3). As there were no significant differences between the analysts' counts for the type b samples as it has been shown in Table 2.2, the mean of the replicate counts for type b samples were used to compare with the result from the type c sample.

The mean of the type b replicates is 4162 and the mean of the type c sample also called 'positive control' is 3911, the P-value = 0.167 suggests that the null hypothesis can be accepted and there are no significant differences between analysts' type b and type c *P.lima* samples.

Table 2.3: Two-Sample T for *P.lima*: Analysts type b mean vs. type c sample (+ve)

Two-sample T for 1st + 2nd mean vs. +ve				
	N	Mean	StDev	SE Mean
1st + 2nd mean	34	4162	932	160
+ve	34	3911	461	79
Difference = mu (1st + 2nd mean) - mu (+ve)				
Estimate for difference: 251				
95% CI for difference: (-108, 609)				
P-Value = 0.167				

The analysts' results were also compared against the reference values for both treatments (type b and type c samples) of *P.lima*. A paired T-test between the reference and the analysts values for type c samples (Table 2.4), suggests that differences exist between the reference and the analysts count of the positive control sample (type c). However there are no differences between the reference and the analysts count for *P.lima* type b samples (Table 2.5).

Table 2.4: Paired T-Test and CI for *P.lima*: Reference (ref) versus Analysts (+ve) results.				
Paired T for +ve – ref				
	N	Mean	StDev	SE Mean
+ve	23	3900	454	95
ref	23	4523	540	113
Difference	23	-623	716	149
95% CI for mean difference: (-933, -314)				
P-Value = 0.000				

Table 2.5: Paired T-Test and CI for *P.lima*: Analysts type b mean vs. reference type b mean				
Paired T for 1st + 2nd mean - ref tp b				
	N	Mean	StDev	SE Mean
1st + 2nd mean	10	3994	696	220
ref tp b	10	4220	419	133
Difference	10	-226	796	252
95% CI for mean difference: (-795, 343)				
P-Value = 0.393				

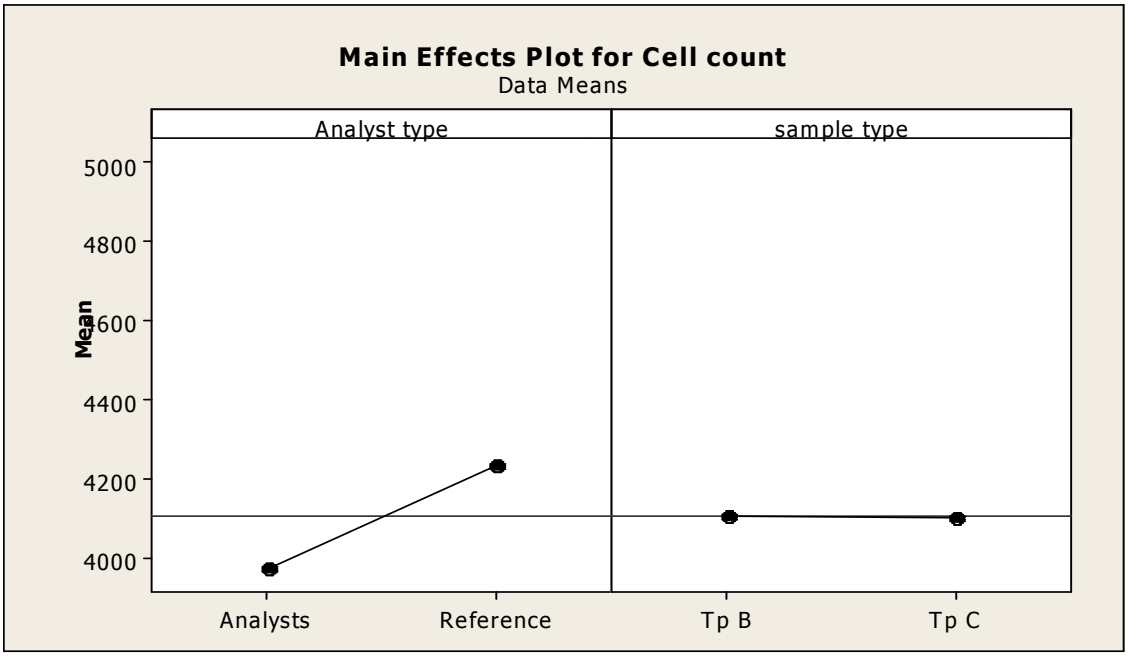
As some significant differences exist between the treatment (type c) across the factors (analysts and reference values), a two way ANOVA was carried out using a general linear model (GLM) to study if this effect between sample types (type b and type c samples for *P.lima*), across the factor Analyst type (reference and analysts), was significant (Table 2.6). The analysis of variance suggests that there were no significant differences between cell counts on sample types (P-value= 0.436), between analysts' types (P-value= 0.260), or across the 2 way interaction analyst type*sample type (P-value=0.442).

Table 2.6: General Linear Model for *P.lima* results: Cell count versus Analyst Type, Sample Type						
Factor	Type	Levels	Values			
Analyst Type	fixed	2	Analyst, Reference			
Sample Type	fixed	2	Tp B, Tp C			
Analysis of Variance for Cell count, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Analyst Type*Sample Type	1	873755	263104	263104	0.60	0.442
Analyst Type	1	497551	566732	566732	1.29	0.260
Sample Type	1	269645	269645	269645	0.61	0.436
Error	87	38361616	38361616	440938		
Total	90	40002567				
S = 664.032 R-Sq = 4.10% R-Sq(adj) = 0.80%						

The main effect plot in figure 2.8 compares the cell counts for *P.lima* by sample and analyst type. This plot illustrates that there were no differences across sample types suggesting that analysts were able to count *P.lima* cells equally well in samples containing *P.lima* alone and samples containing *P.lima* confounded with other species. However some differences exist between the reference count and the analysts' count, although as demonstrated in the general linear model (Table 2.6), this effect was not significant.

Figure 2.9 is a graphical representation of the ANOVA interaction showing that there were no significant effects across the treatments Sample type and analyst type and the interaction sample type*analyst type. The pareto chart (figure 2.10) highlights the source of major effects of the set of factors for *P.lima*. The bars in the graph correspond to the size of the effects for the count of *P.lima.* It shows that the main factor of variation by far was due to the quantification of the analysts (A) followed by the interaction of the analysts and the sample type (AB). The smallest effect was due to the sample types (B).

Figure 2.8: Main effects plot by sample type and analyst type for *P.lima*



It was therefore demonstrated that there were no significant differences between sample replicates, sample types, and analysts type values for *P.lima*, although some

variation existed in cell counts across the factor analyst type, this variation was not sufficiently big to reject the null hypothesis.

Figure 2.9: Standardized effects for factors and levels for *P.lima*

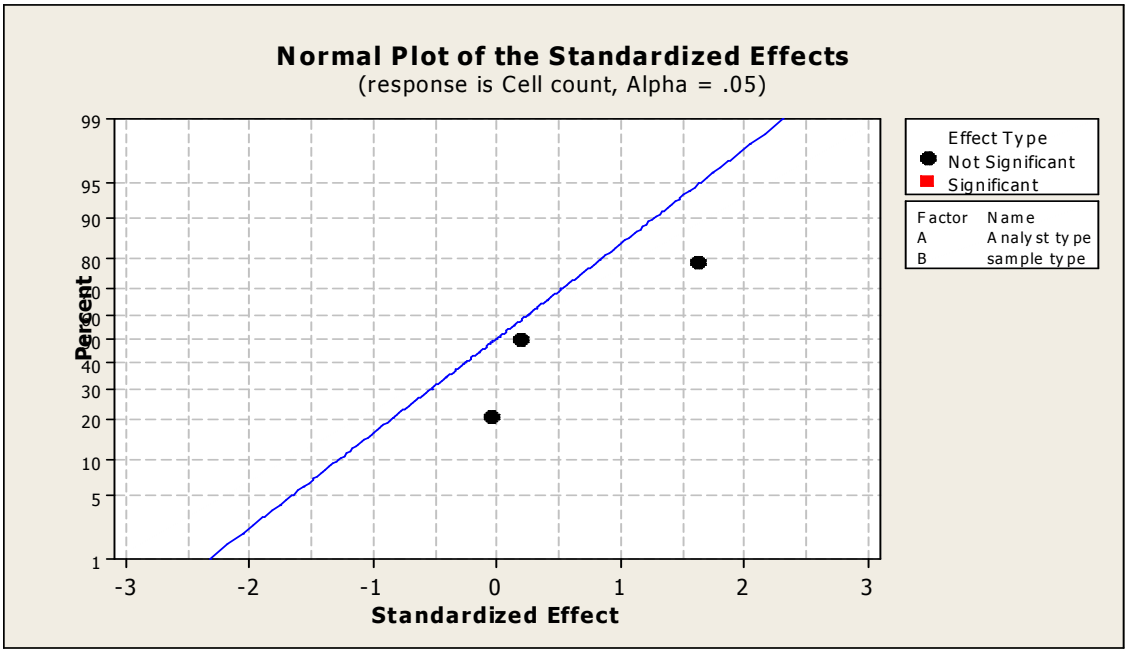
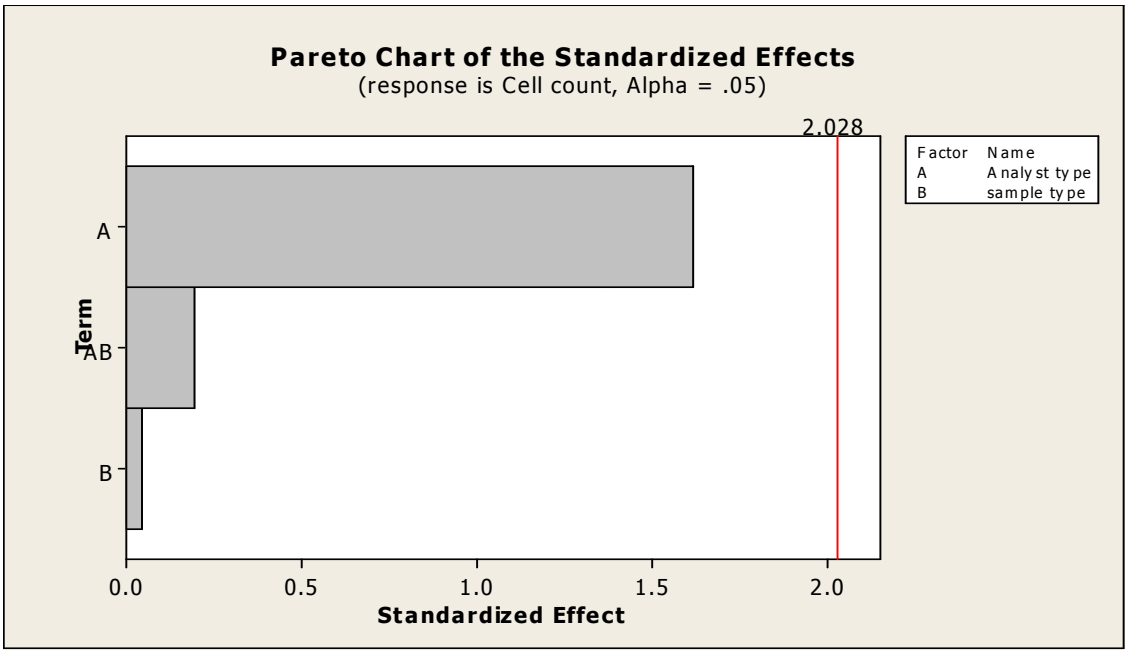


Figure 2.10: Pareto chart of effects *P.lima*



The results above permitted the use of the full set of data to calculate Z-scores and use the reference mean and coefficient of variation to set the upper and lower confidence limits for *P.lima* cell counts.

2.3.2.4 Prorocentrum micans cell counts variability

The *Prorocentrum micans* box plot of analysts' cell counts against the reference value for *P.micans* (Fig. 2.4) showed that there were differences between the analysts and the reference counts and that these differences were significant.

In order to determine if differences exist between the analysts' and the reference data, the analysts' data was studied for differences between replicates and sample types. In this case the sample types were type a samples containing four species and type b samples containing exactly the same four species and concentrations plus one extra species (*P.lima*) which is the only difference between the samples.

Tables 2.7 and 2.8 are the paired t-tests for the sample types a and b first and second *P.micans* cell count, the P-value in both cases is larger than the level of significance (P>0.05) and we cannot reject the null hypothesis in this case, that there were no significant differences in cell counts between sample replicates.

Table 2.7: Paired T-test of analysts' replicate *P.micans* cell counts type a samples				
Paired T for P.mic_1_TpA - P.mic_2_TpA				
	N	Mean	StDev	SE Mean
P.mic_1_TpA	34	11953	3285	563
P.mic_2_TpA	34	11825	3538	607
Difference	34	128	2286	392
95% CI for mean difference: (-669, 926)				
P-Value = 0.745				

Table 2.8: Paired T-test of analysts' replicate *P.micans* cell counts type b samples				
Paired T for P.mic_1_TpB - P.mic_2_TpB				
	N	Mean	StDev	SE Mean
P.mic_1_TpB	34	10422	3208	550
P.mic_2_TpB	34	11040	3523	604
Difference	34	-617	2613	448
95% CI for mean difference: (-1529, 295)				
P-Value = 0.178				

As there were no significant differences between analysts' replicate a or b types, the mean of these results were used in a two sample t-test, to compare whether differences existed between sample types. The differences were not significant between sample types a and b (Table 2.9) for analysts counting *P.micans* as the P-value (P=0.136) and confidence interval indicates.

Table 2.9: Two sample T-test of analysts *P.micans* cell counts for type a vs. type b samples.				
Two-sample T for P.mic TpA mean vs. P.mic TpB mean				
	N	Mean	StDev	SE Mean
P.mic TpA mean	34	11889	3217	552
P.mic TpB mean	34	10731	3105	533
Difference = mu (P.mic TpA mean) - mu (P.mic TpB mean)				
Estimate for difference: 1158				
95% CI for difference: (-373, 2689)				
P-Value = 0.136				

Because there were no significant differences between sample types for analysts cell counts, the mean of all the data was used to compare the analysts' *P.micans* mean results against the mean reference results (Fig. 2.4), this illustrates that there were significant differences between the reference mean and the analysts mean. The reference mean is higher than the analysts mean. As the number of observations was

different across the reference and analysts count, the design was not balanced, so a general linear model (Table 2.10) was used to test, whether there were mean differences across the factors: analyst type, sample types and the interaction. The full model indicates that we can not reject the null hypothesis at the alpha=0.1 level that the operator to sample interaction is equal to zero; however there are significant differences between the individual components of the model: analyst types and sample types.

Table 2.10: General Linear Model for *P.micans* cell counts across the factors analyst and sample type with interaction

Factor	Type	Levels	Values			
Analyst Type	fixed	2	Analyst, Reference			
Sample Type	fixed	2	Tp A, Tp B			
Analysis of Variance for Cell count, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Analyst Type	1	639754554	639754554	639754554	72.97	0.000
Sample Type	1	58141697	73916127	73916127	8.43	0.005
Analyst Type*Sample Type	1	16365076	16365076	16365076	1.87	0.176
Error	84	736451667	736451667	8767282		
Total	87	1450712994				
S = 2960.96 R-Sq = 49.24% R-Sq(adj) = 47.42%						

The main effects plot (fig.2.11) suggests that the differences were larger across the factor analyst type, but both were significant as the normal plot of standardised effects show (fig.2.12). This indicates that the mean population of the reference cell count was significantly different to the analysts' cell counts for *P. micans*.

Figure 2.11: Main effects for *P. micans* cell counts across factors analyst & sample type.

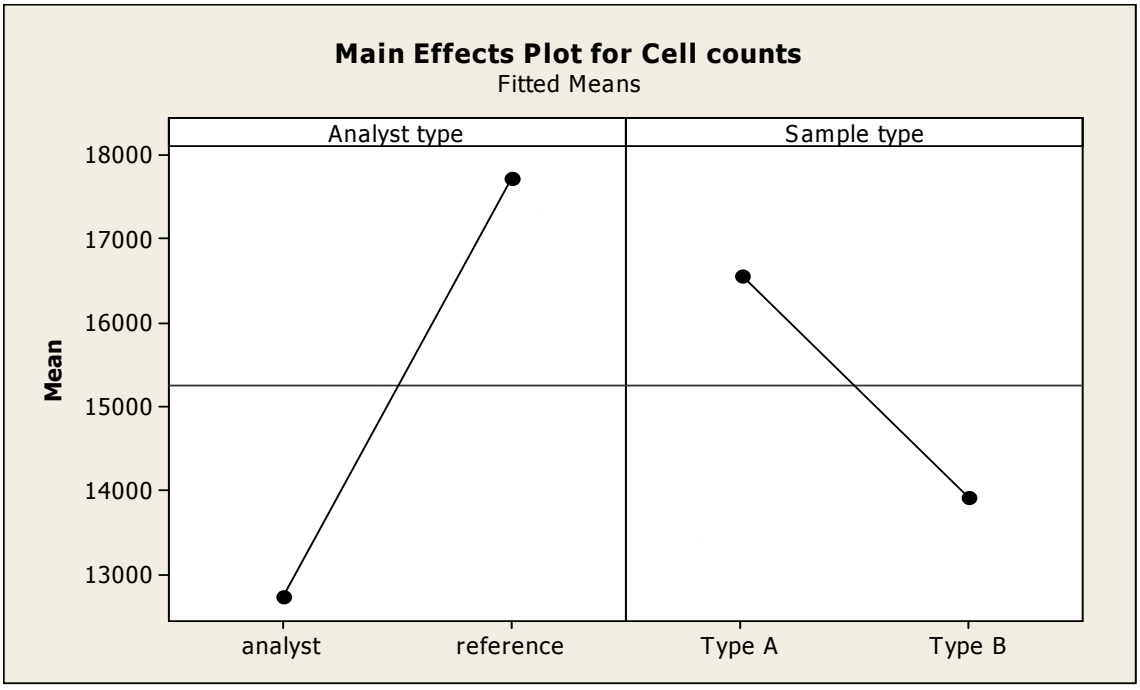
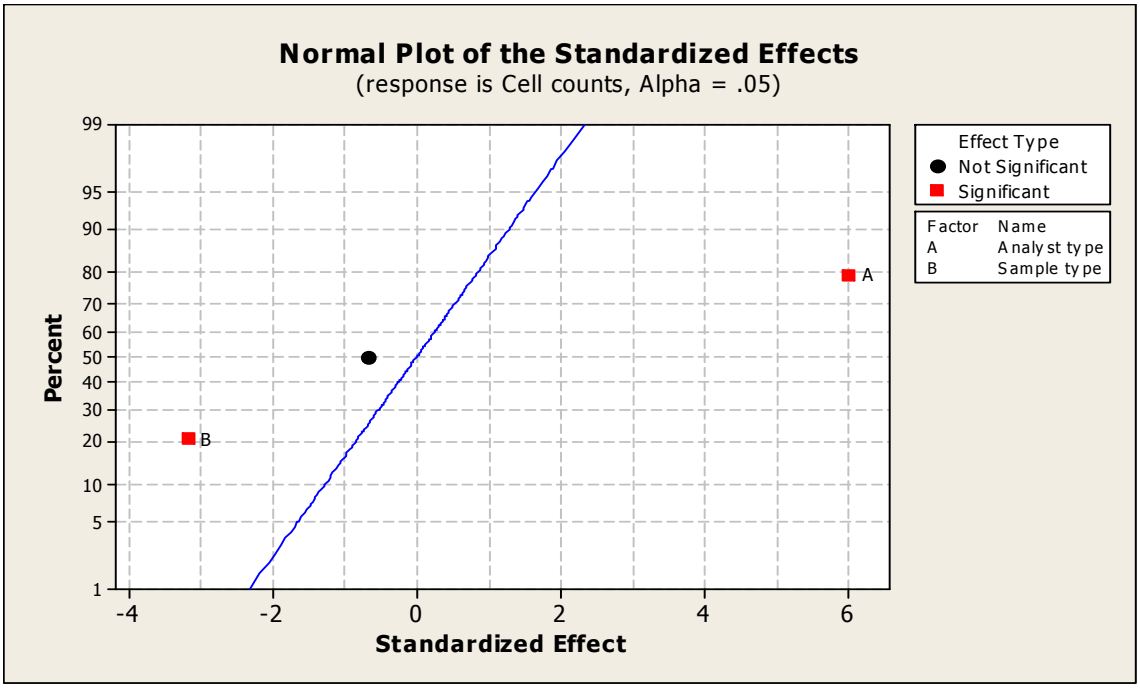


Figure 2.12: Normal plot of standardised effects for *P. micans* cell counts across factors



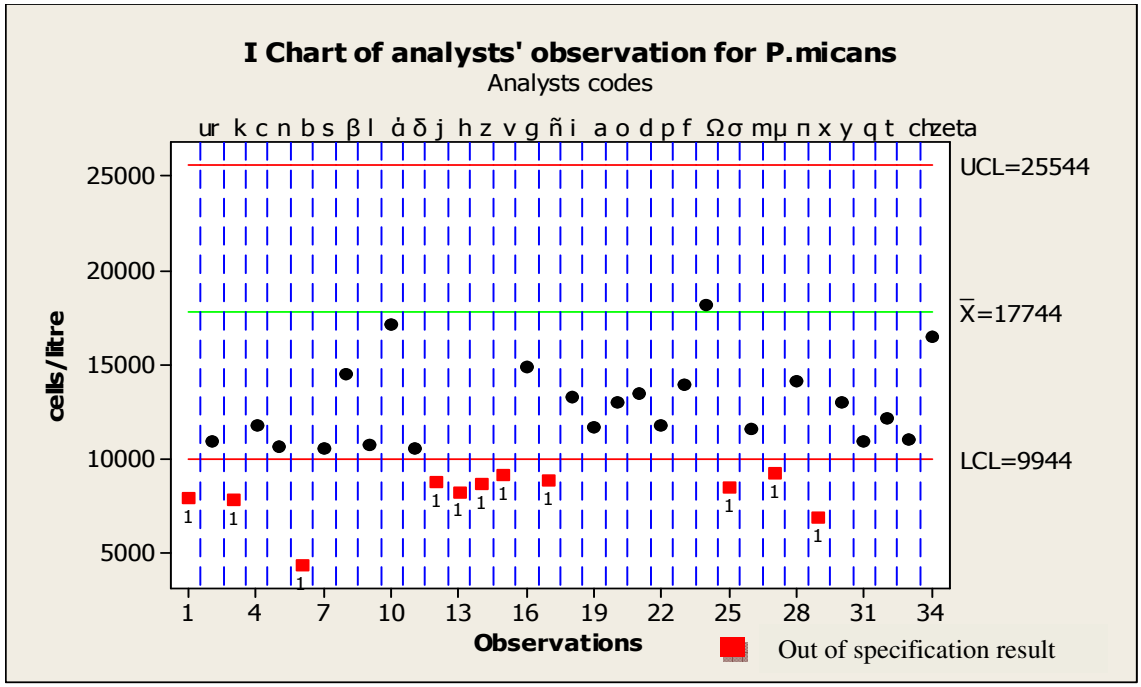
2.3.2.5. I-charts

In order to compare the individual measurements provided by the thirty four analysts, I-charts were produced using the analysts' mean of the replicates for each analyst by species, where the mean was justified given the small variance component due to replicates, against the reference mean and upper and lower control limits (UCL and LCL respectively). Note that the UCL and LCL were generated using 3 times the reference sample standard deviation.

The individual I-charts for each species show that in four species counts the analysts results were within 3 standard deviations of the reference mean value.

The *P. micans* I-chart (fig.2.13) for the individual mean values of the species against the reference value indicates there were significant differences between the reference value and most laboratories; there were systematic differences between the mean counts. Most laboratories' values were below the expected reference value indicating that the reference laboratory and the analysts had quantified *P.micans* differently.

Figure 2.13: I chart of analysts' observations for *Prorocentrum micans*



UCL= Upper confidence limit, LCL= Lower confidence limit, X= mean

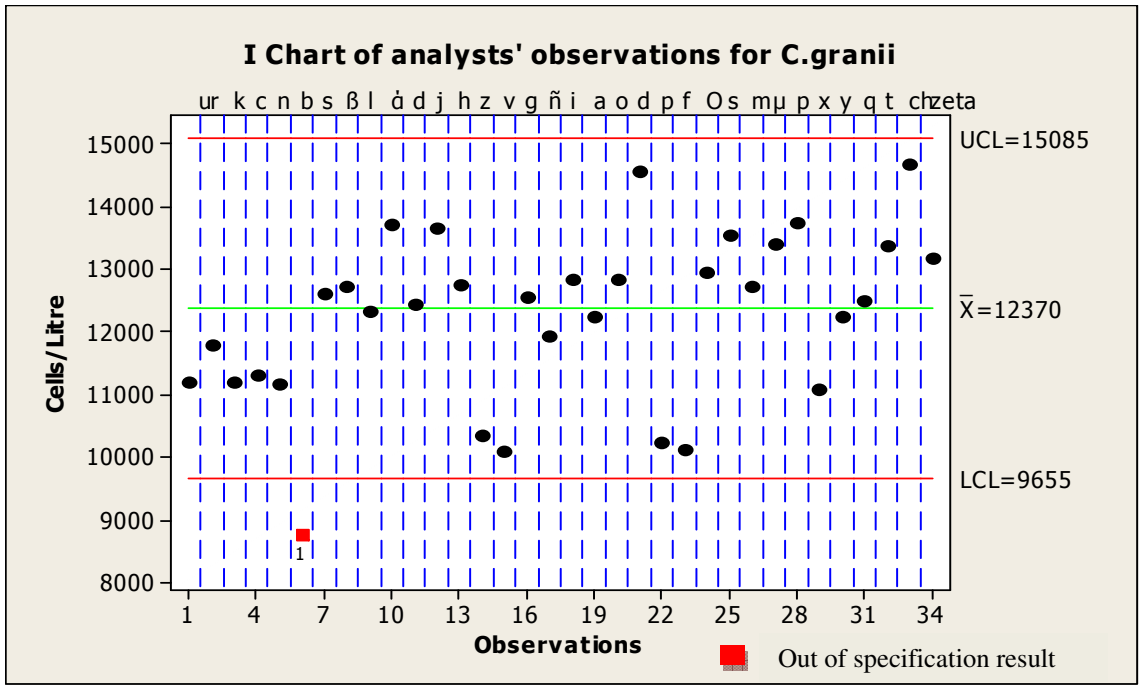
Eleven analysts performed outside (red dots) the 3 standard deviations of the reference data, while the rest have performed within the limits but tended to underestimate the mean reference value. The analysts or the experimenter have done something consistently different in these counts. This is a systematic error.

Figures 2.14 to 2.17 illustrate the remaining I charts of analysts' mean measurements across laboratories for each species compared to the reference value, mean and 3 standard deviations.

There was good agreement between the reference data and the analysts' data for each species except for *P.micans* where virtually all laboratories underestimated the reference measurement.

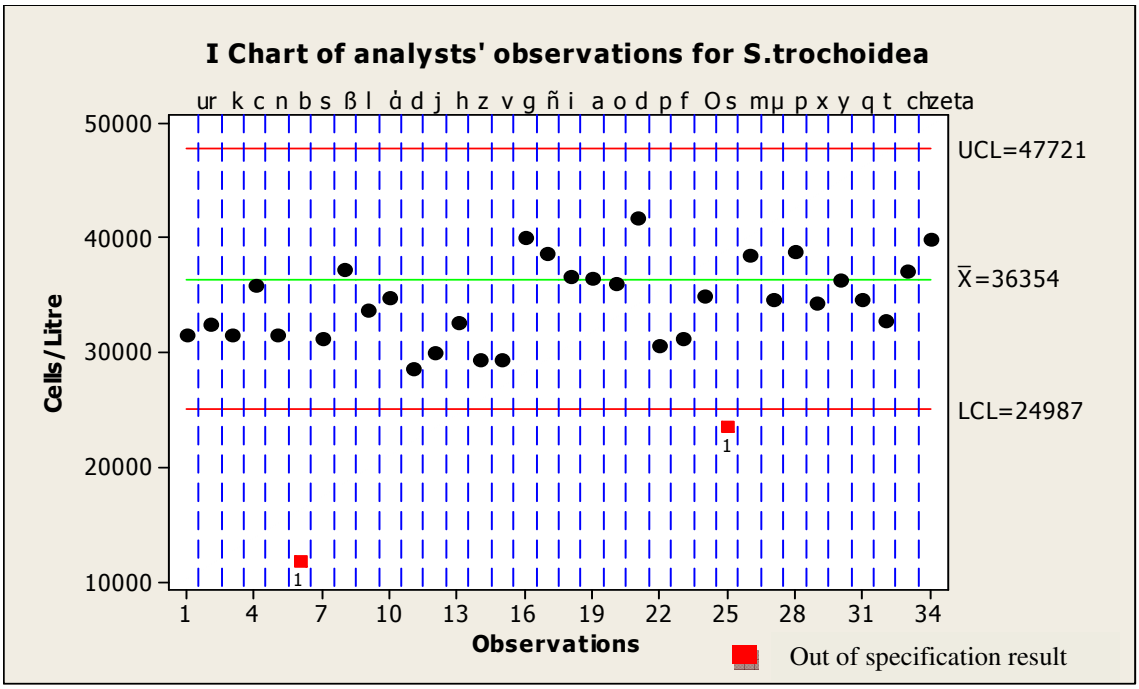
Analyst b underestimated the reference measurement for all species except the *G .catenatum* cell count. Also, analyst s in the *Scrippsiella* count and analyst x in the *P.lima* count were outside the lower confidence limits. These are shown as red dots in the figures indicating out of specification results, that is results outside the 3 standard deviations.

Figure 2.14: I chart of analysts' observations for *Coscinodiscus granii*



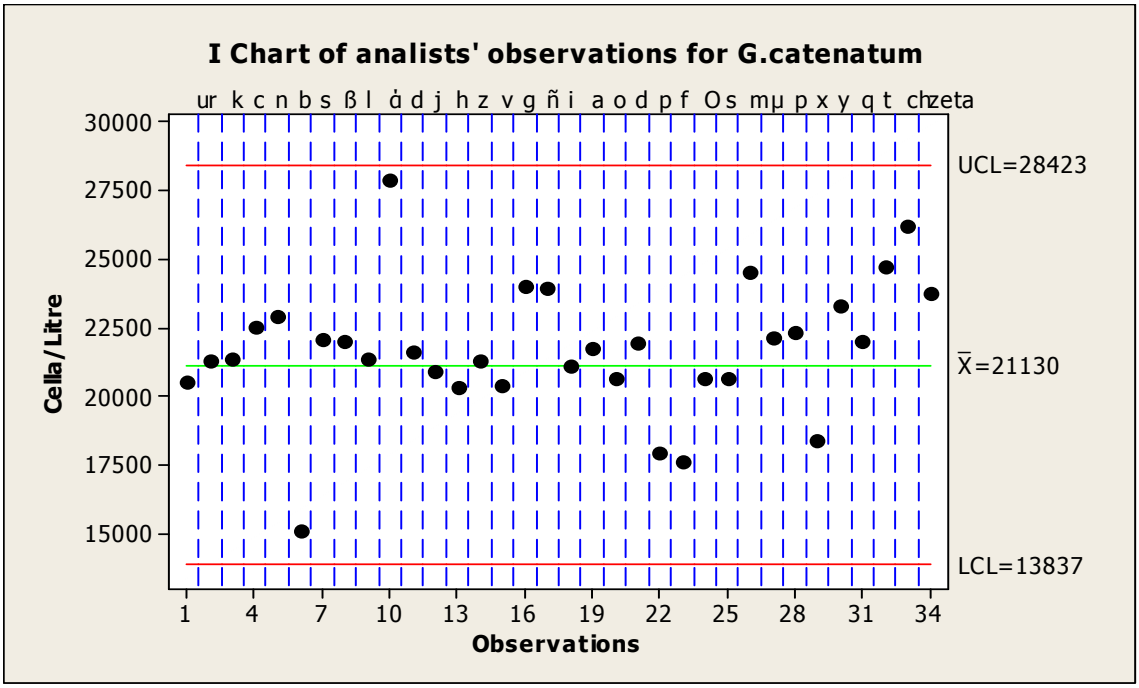
UCL= Upper confidence limit, LCL= Lower confidence limit, X= mean

Figure 2.15: I chart of analysts' observations for *Scrippsiella trochoidea*



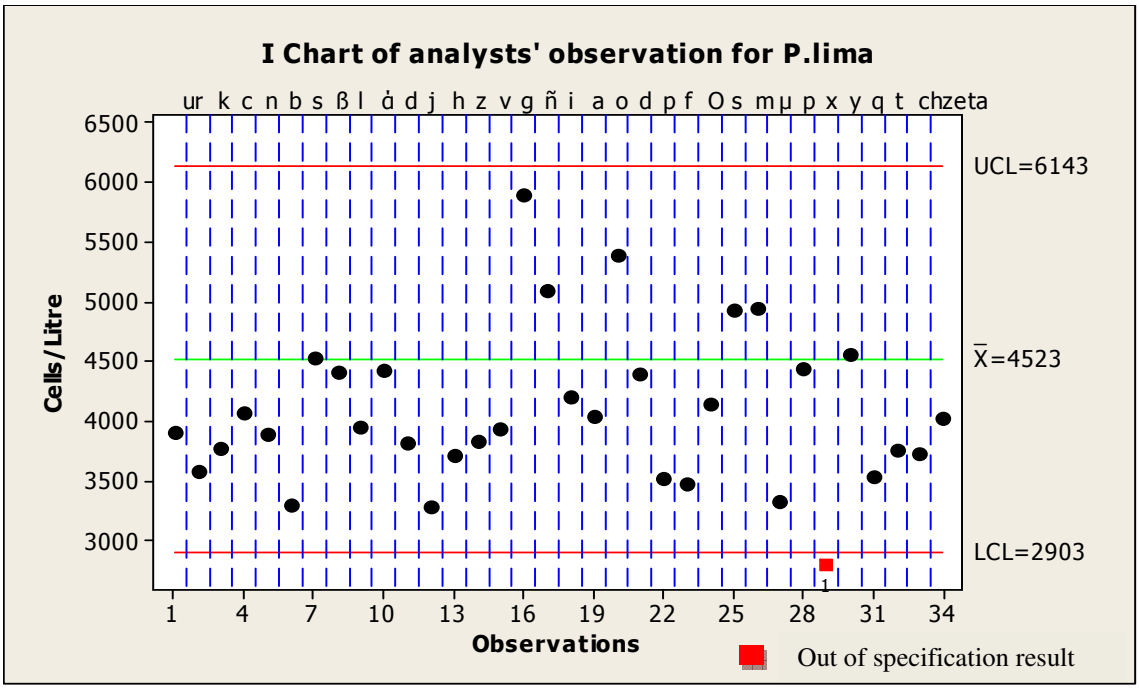
UCL= Upper confidence limit, LCL= Lower confidence limit, X= mean

Figure 2.16: I chart of analysts' observations for *Gymnodinium catenatum*



UCL= Upper confidence limit, LCL= Lower confidence limit, X= mean

Figure 2.17: I chart of analysts' observations for *Prorocentrum lima*



UCL= Upper confidence limit, LCL= Lower confidence limit, X= mean

2.3.2.6 Z-Scores

The following z scores in figures 2.18 to 2.22 show that most analysts performed within the 3 sigma limits of the reference cell counts.

Figure 2.18: Z-score for *Coscinodiscus granii*

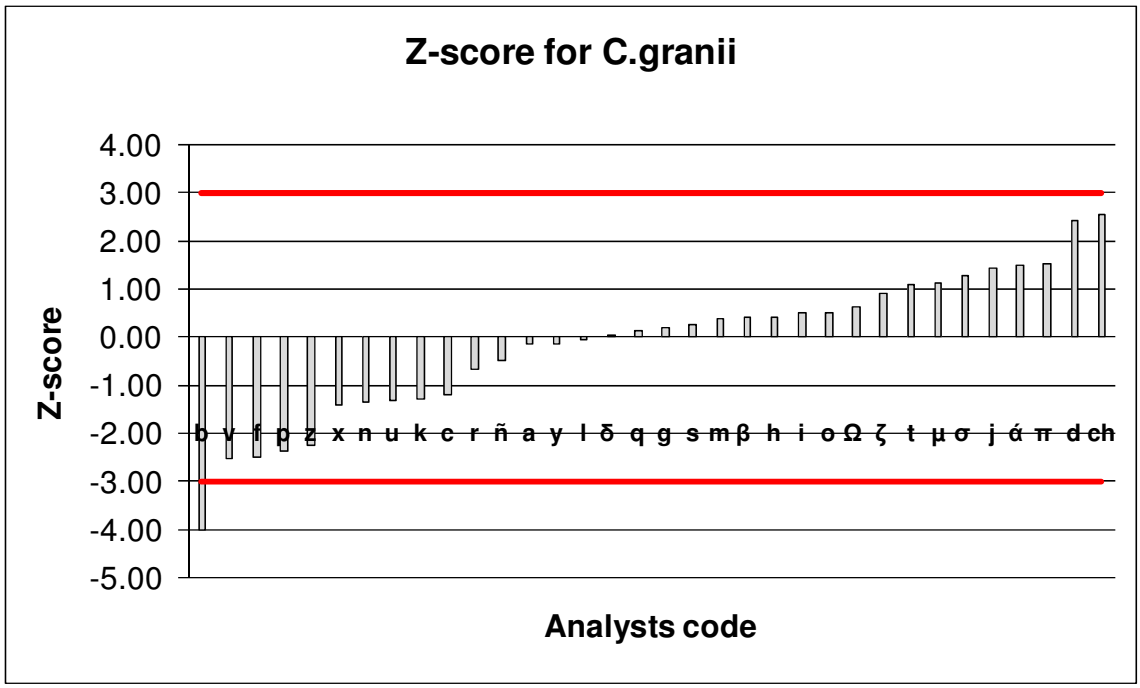


Figure 2.19: Z-score for *Gimnodinium catenatum*

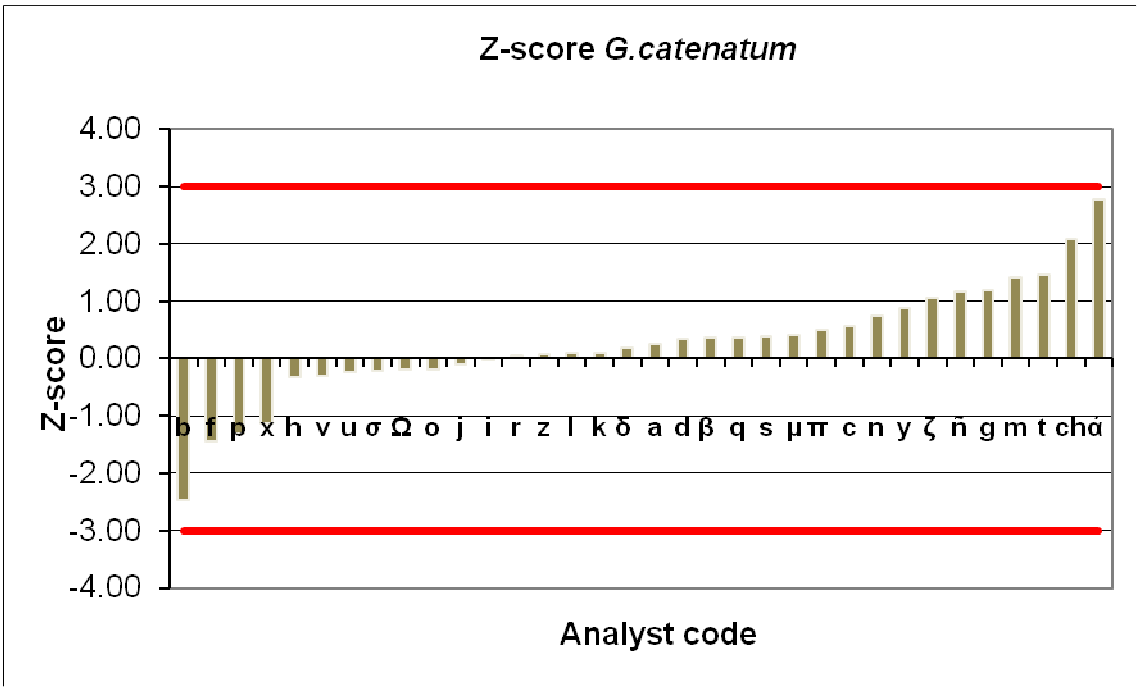


Figure 2.20: Z-score for *Scrippsiella sp.*

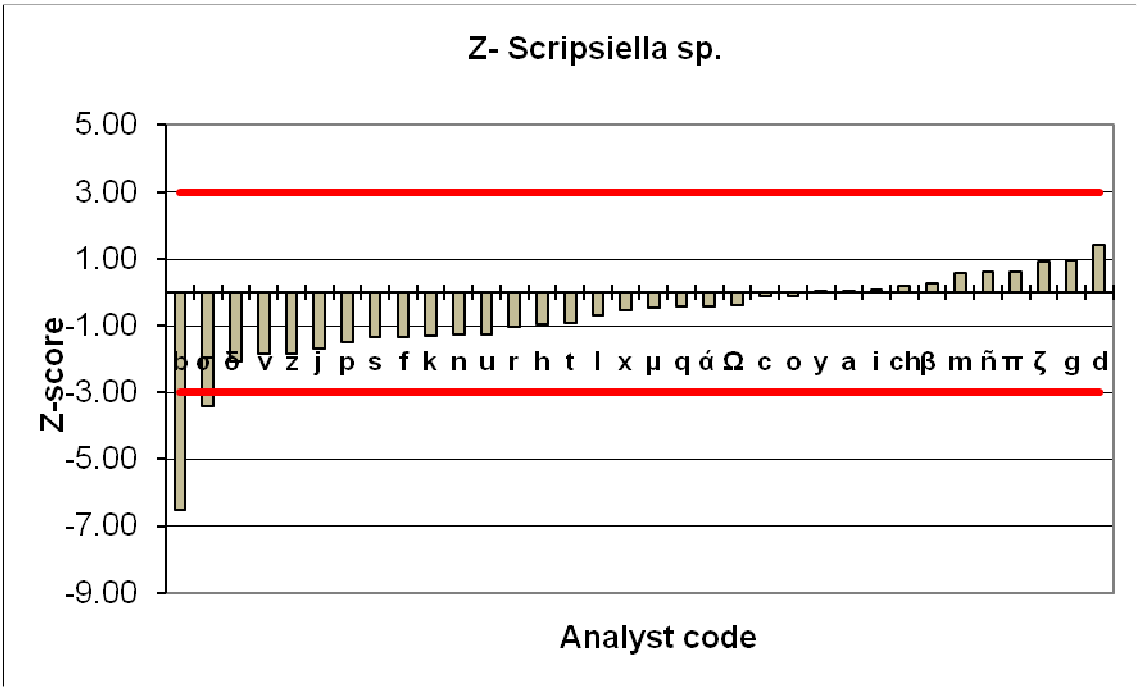


Figure 2.21: Z-score for *P micans*

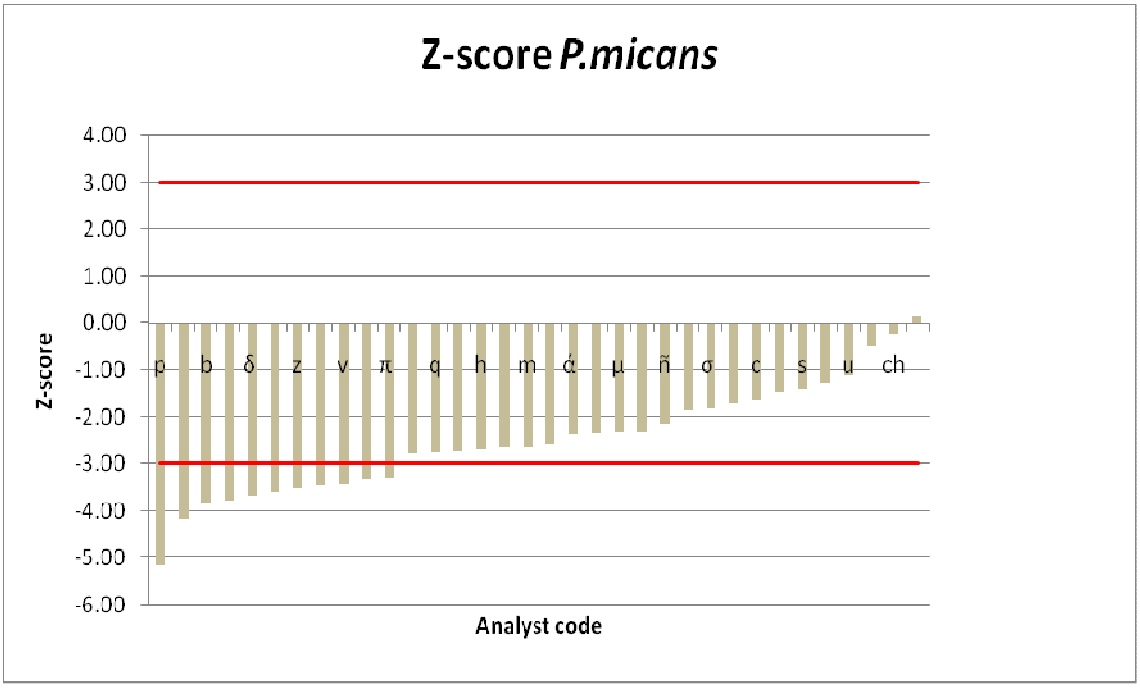
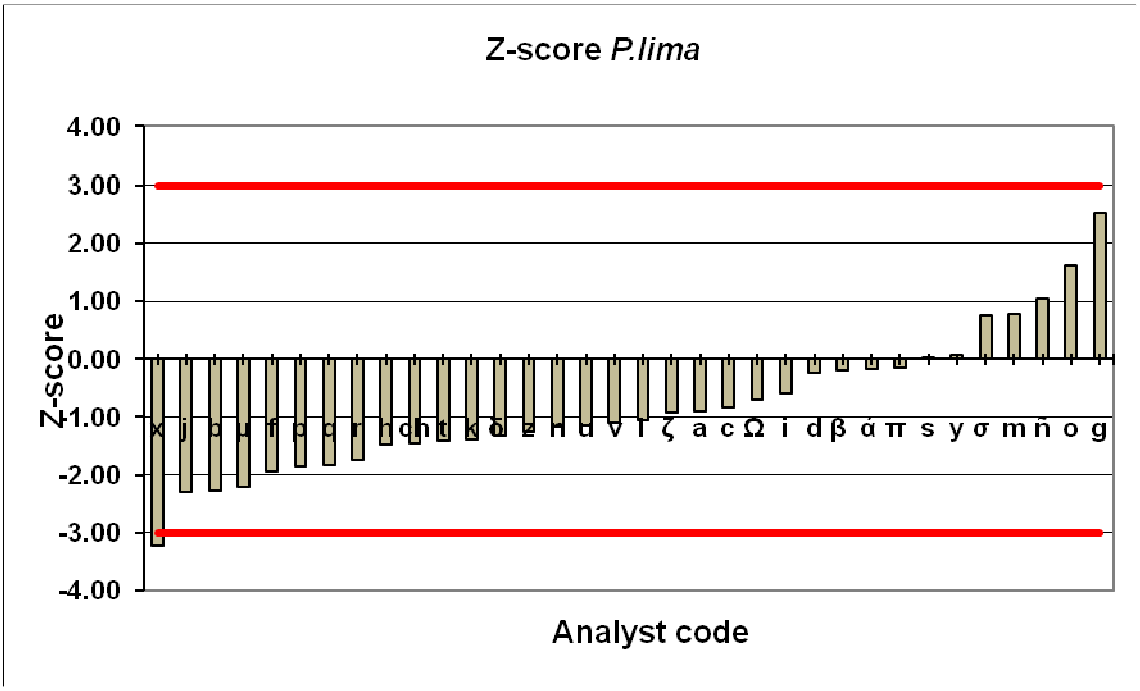


Figure 2.22: Z-score for *P.lima*



2.3.2.7 Reproducibility within laboratories

This exercise was designed to study inter-observer variability within laboratories. Laboratories were divided between single analyst laboratories and multiple analyst laboratories (minimum two and maximum five analysts).

The interest was in testing within laboratory inter-observer variability in laboratories with more than one analyst and between laboratory repeatability. Each laboratory was given a set of samples, which were homogenised and settled by the laboratories' sample manager and analysed successively by the analysts under the same conditions.

Repeatability between analysts from the same laboratory was studied using scatterplots. Three analysts (r, c, k) from Laboratory K (fig. 2.23) were used here as an example. The scatterplot (r-c, r-k, c-k) compares all pair-wise comparisons of four replicate cell counts by subtracting each analyst value. The perfect value from all the pairs would be close to zero and would be a similar cell concentration. Data points in the graph should appear close to the reference line (x=y) and they should be bunched together. However, the scatterplot indicates that results from these analysts are not reproducible.

Figure 2.23: Scatterplot of analysts' r, c and k from lab K against each other

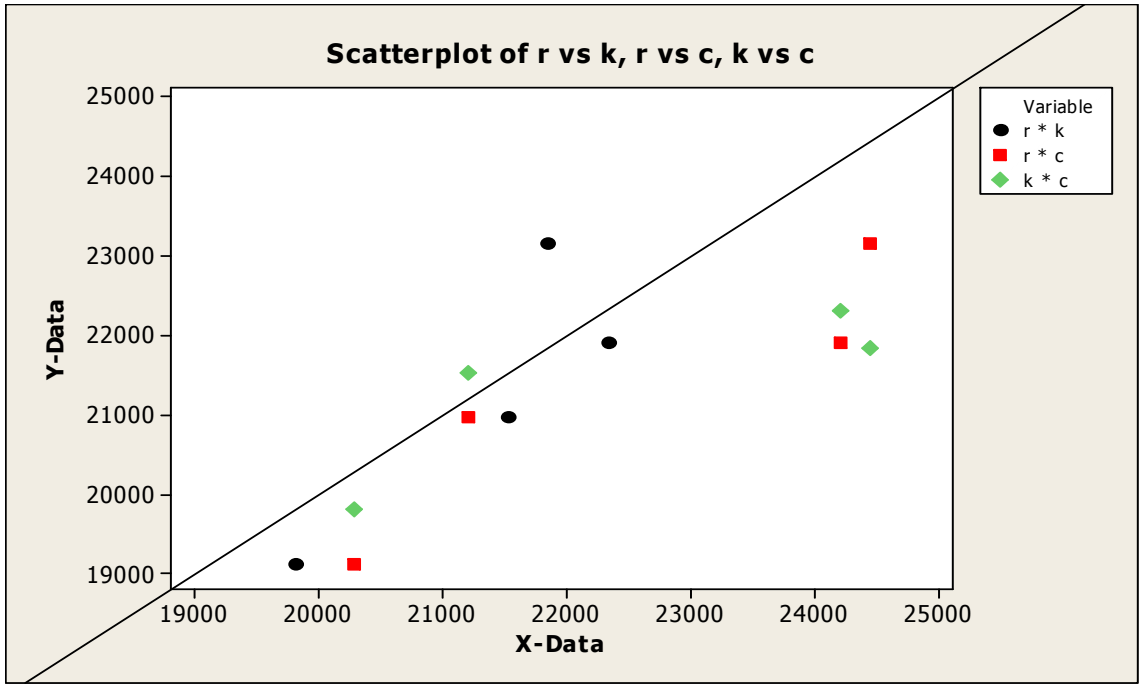
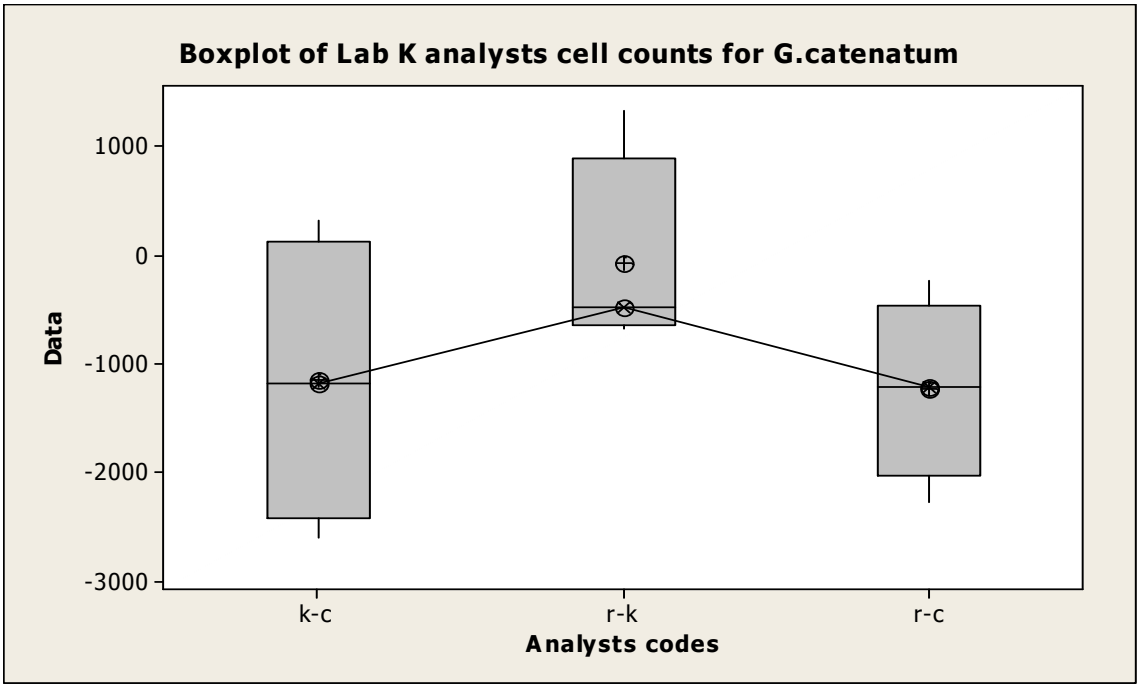


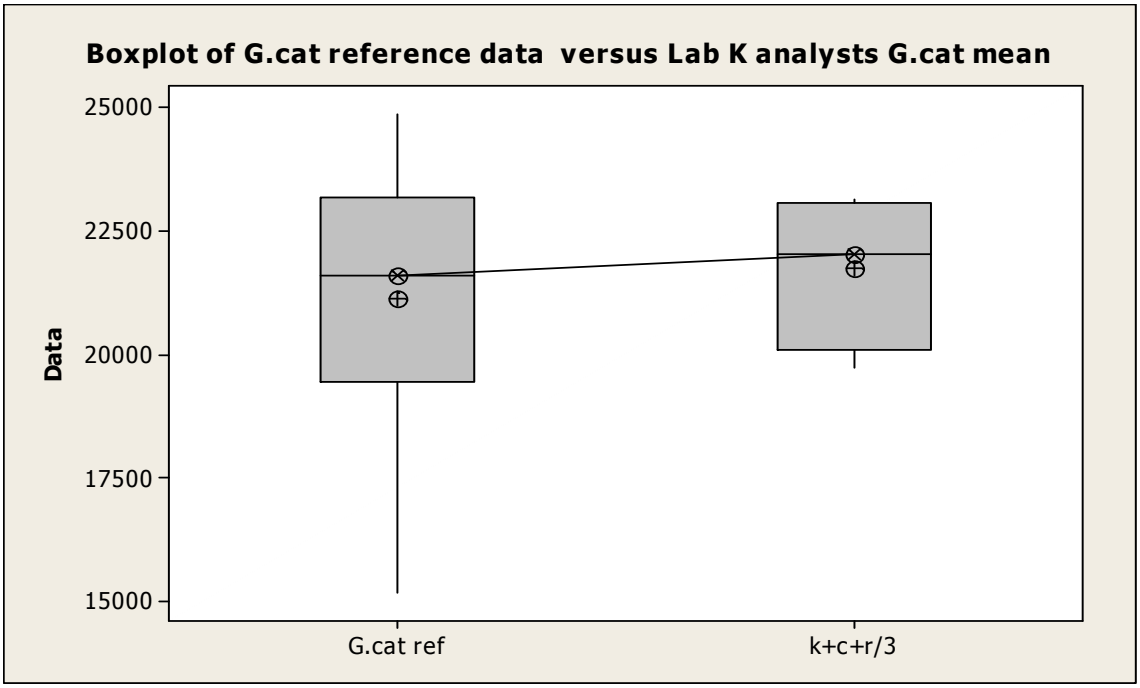
Figure 2.24: Bias box plot of analysts' r, c and k from lab K



Another way of looking at this variability is through the use of bias box plots of the mean cell counts for each analyst; the bias box plot (Fig. 2.24) of analysts' r, c & k shows that there were significant differences between analysts as there isn't symmetry about the zero. This demonstrates that there was significant inter-observer cell count variability in same sample analysis, which suggests that analysts working in the same laboratory are analysing samples differently.

However, when the mean results from analysts (r, c and k) from laboratory K were compared against the reference mean value for *G.catenatum* in figure 2.25, the box plot of the reference mean versus the mean of the analysts indicates that there were no significant differences in cell counts between laboratory K and the reference value. This suggests that values found in the samples by laboratory K analysts were within the variability found in the reference value samples. So, the variation found is within the sample population variation.

Figure 2.25: Box plot of analysts' k,c and r from lab K versus reference value for *G. catenatum*.



This is generally the case across laboratories and between analysts, the case profile plot (Fig. 2.26) shows that the replicate measurements across species were not reproducible between observers. Each label in the plot is an analyst result, the graph is broken down in panels, each panel correspond with one organism and there are four replicate counts for each analyst except for Sp.5.I.c (*P.*lima) where there are only three replicates. The x axis is the cell concentration expressed in cells per litre. The plot is a quick graphical representation of all the counting results, the interpretation of the profile would be that all replicates for each analyst should be on a straight line across the replicates for each panel and all labels should be tightly together, this would represent a good correlation in the variation between parallel samples by the same analysts and between analysts. The interaction plot for each mean measurement (Fig. 2.27) illustrates that not only the results are not reproducible but also a pattern emerges which suggests that laboratories that typically tended to underestimate or overestimate results for one of the species tended to do the same on all the species counts independently of the cell concentration indicating that there are quantification methodology differences between laboratories.

Figure 2.26: Analysts replicate cell counts by species. Sp1.I.b= *C.granii*; Sp2.I.b= *G.catenatum*; Sp3.II.b= *P.micans*; Sp4.IIa= *S.trochoidea*; Sp5.I.c= *P.lima*.

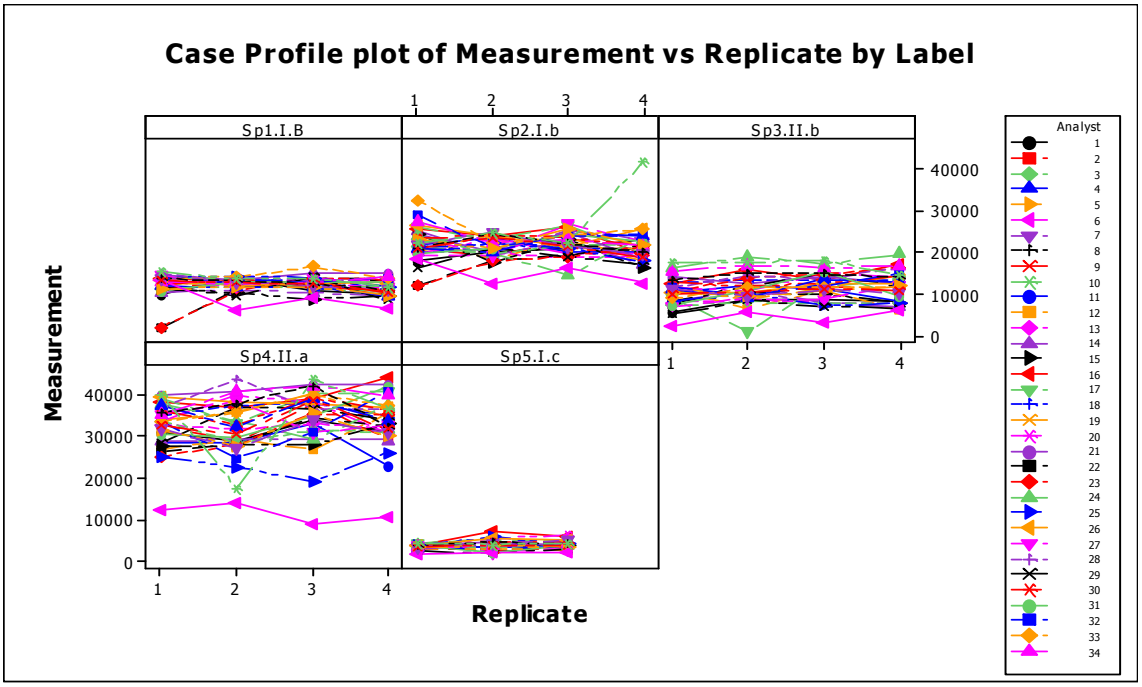
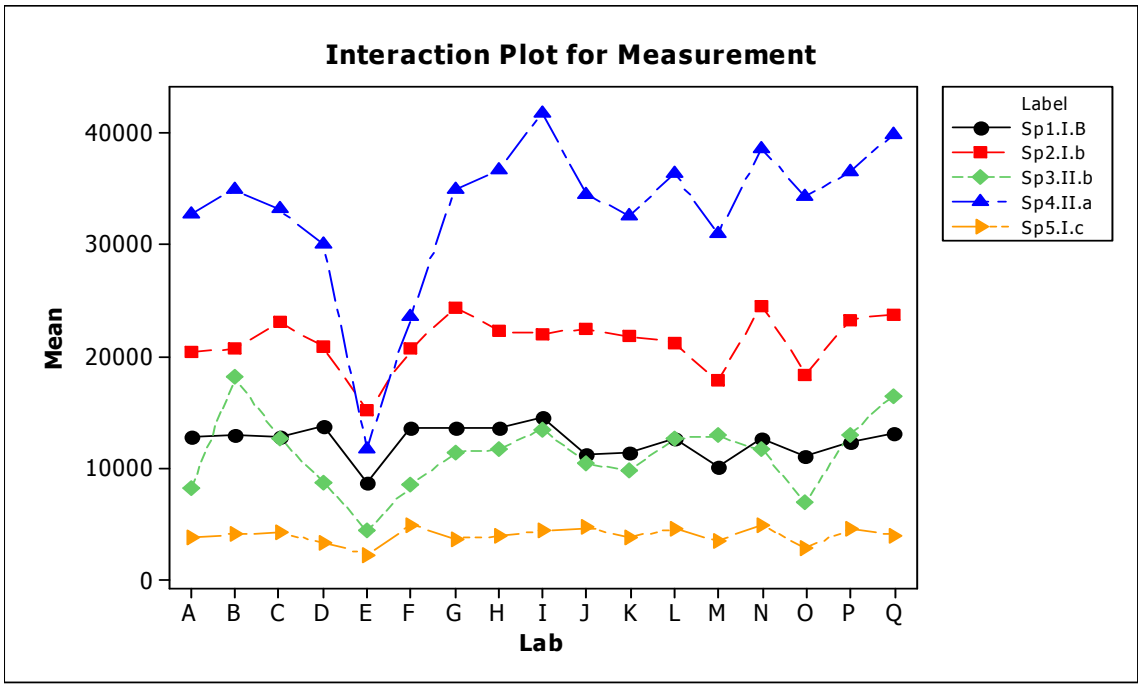


Figure 2.27: Mean measurements of species per lab Sp1.I.b= *C.granii*; Sp2.I.b= *G.catenatum*; Sp3.II.b= *P.micans*; Sp4.IIa= *S.trochoidea*; Sp5.I.c= *P.lima*.



2.3.2.8 Type d sample (negative control sample)

The results obtained from the negative control sample (Appendix 8) indicate that two analysts out of thirty four observed cells within the negative control sample which they identified and counted. Analyst x produced a count of 8 cells or 320 cells L^{-1} of *Scrippsiella sp.* in the sample and analyst j observed one cell of the same species in this sample and three cells in the positive control.

2.3.3 Phytoplankton identification results

Appendix 9 and 10 contain the results of the identification exercise. The identification exercise was divided into two sub sets, one comprising still images and the other video clips, each subset contained four sets of five images/videos.

The reason for this was to compare how video clips would perform against images as quality controls for phytoplankton identifications and whether some sets were more difficult than others. Our null hypothesis was that there were no differences between set types (Images –videos).

The box plot of image results versus video results (Fig. 2.28) suggests that the null hypothesis is true. There are no differences between identifying images or videos, both are done equally well. The descriptive statistics of the box plot (Table 2.11) shows that analysts were slightly better at identifying images (84% mean) compare to videos (81% videos), around 3 to 4% higher success rate. This pattern however was not consistent across analysts where some analysts scored higher for images and others scored higher for videos (Fig. 2.29).

Table 2.11: Descriptive statistics of identifications

Descriptive Statistics: Percentage

Variable	Type	Mean	StDev	Minimum	Maximum
Percentage	Image	84.78	7.79	65.00	100.00
	Video	81.10	9.83	65.00	95.00

Figure 2.28: Box plot of percent (%) correct images versus video clips

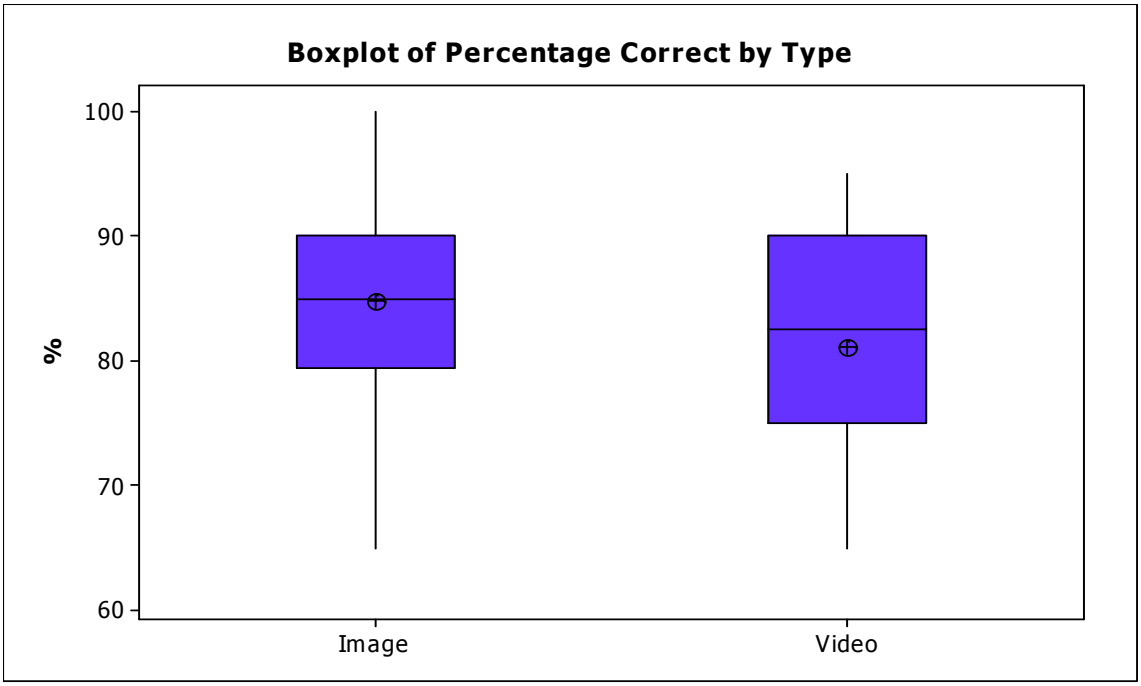
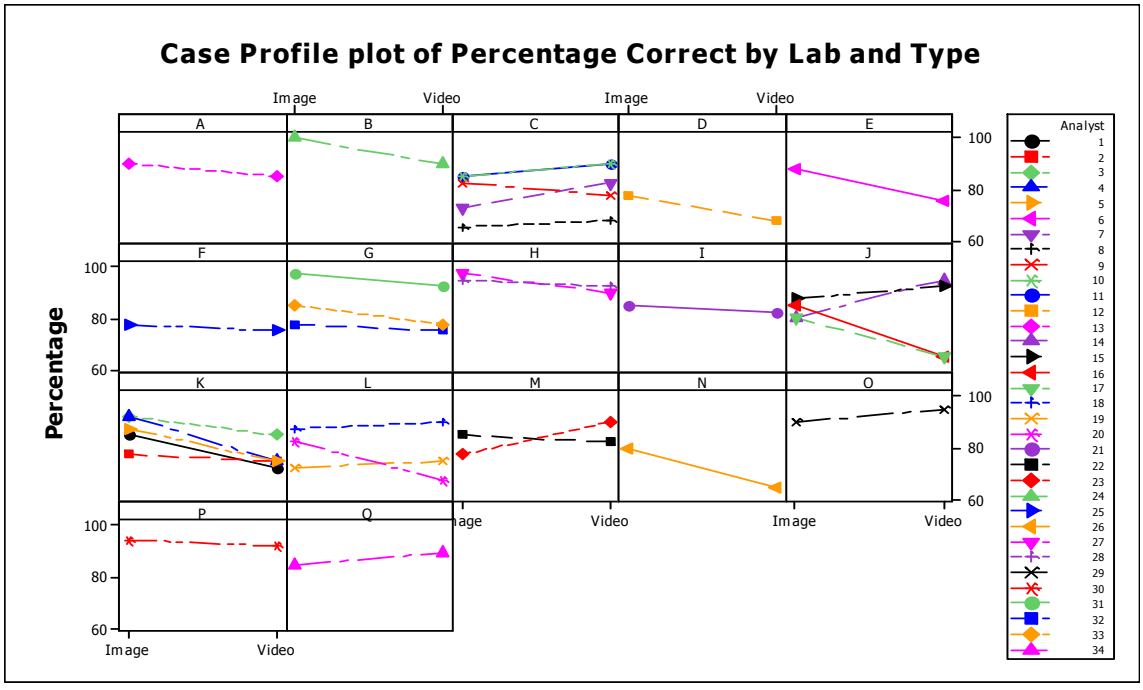


Figure 2.29: case profile by lab of images versus videos

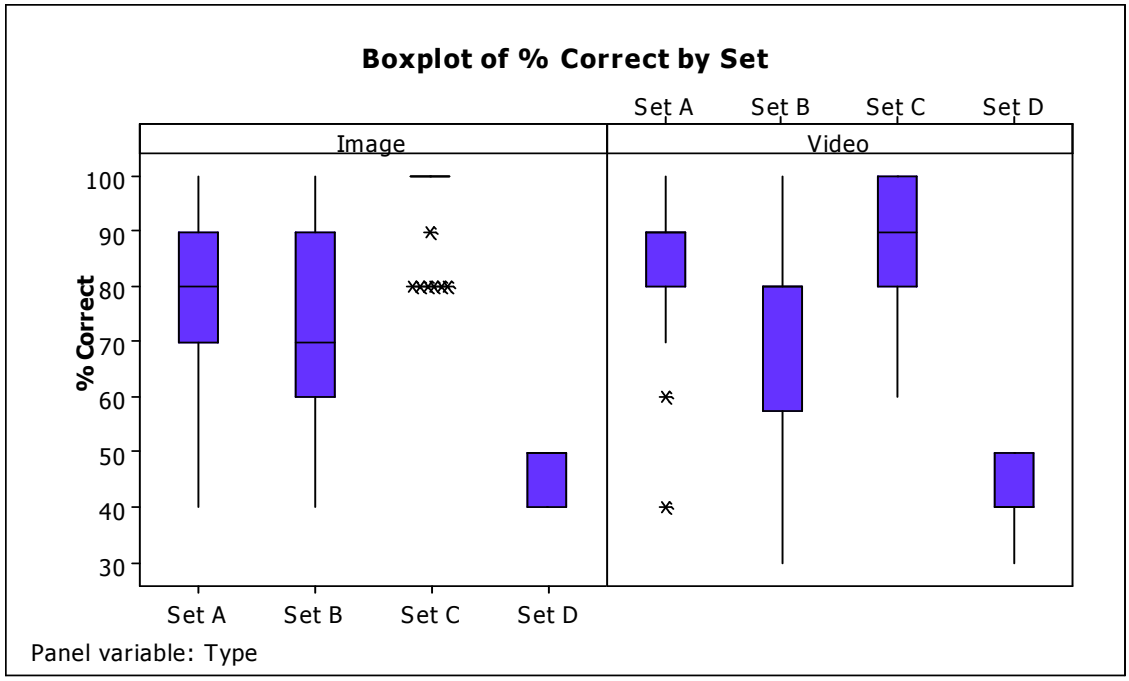


The box plot of correct answers by sets (Fig. 2.30) illustrates that some sets were found to be easier to identify than others. For example set D on both images and

videos got the lowest percentage of all the sets and set C got the highest score for both images and videos, which suggests that this set was the easiest to identify.

The hardest image to identify was A1 where 71% of analysts scored a zero. Video B2 and D1 were the joint hardest video where 65% of analysts scored a zero. There were perfect scores recorded by all analysts for images C2, C3 and C5 and for videos C1 and D4.

Figure 2.30: Box plot of correct answers by sets



A general linear model, fitted to compare the mean percentage correct answers across the four factors namely species, laboratories, analysts and replicates, indicated (Table 2.12) a significant difference in the mean percent correct between type and analysts only.

There was no significant difference between type (image or video) ($p=0.09$) and no significant difference between laboratories ($p=0.38$). The model did identify a significant difference in the mean percentage correct between the sets ($p<0.001$) where the percentage correct differed between the sets on average. Set C has the highest mean percentage correct (92%) followed by set A (81%), set B (70%) and finally set D (44%). There was also a significant difference between analysts

(p=0.002) where analysts tended to have different percentage correct scores on average.

Table 2.12: General linear model for the identification exercise

General Linear Model: Correct versus Types, Set, Laboratories, Analysts

Factor	Type	Levels	Values
Types	fixed	2	Image, Video
Set	fixed	4	Set A (%), Set B (%), Set C (%), Set D (%)
Laboratories	fixed	17	A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P,Q
Analysts(Laboratories)	random	34	13, 24, 7, 8, 9, 10, 11, 12, 6, 25, 31, 32, 33,27, 28, 21, 14, 15, 16, 17, 1, 2, 3, 4, 5, 18,19, 20, 22, 23, 26, 29, 30, 34

Analysis of Variance for Correct, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Types	1	412.6	412.6	412.6	2.86	0.092
Set	3	85903.2	85903.2	28634.4	198.35	0.000
Labs	16	6512.2	6512.2	407.0	1.16	0.378
Analysts(Labs)	17	5940.8	5940.8	349.5	2.42	0.002
Error	234	33781.1	33781.1	144.4		
Total	271	132549.9				

S = 12.0151 R-Sq = 74.51% R-Sq(adj) = 70.48%

2.3.4 Qualitative reliability measure results

All analysts correctly identified to species level the organisms *P. lima and P. micans* in the samples. The organism *Scrippsiella* was also identified correctly by all analysts either to genus or species level. In this case genus level would have been sufficient as there was not enough information to go to species level. Analysts going to species level over-identified this organism. One analyst gave *Ensiculifera* Balech as the

answer which was given as correct. In order to observe the differences between the genus *Ensiculifera* and *Scrippsiella* analysts would need to use epi-fluorescence microscopy and brightener (Fritz & Triemer, 1985; Elbrächter, 1994) to see the cingular plate pattern that would help their identification (Elbrachter *pers comm.*). Therefore, *Scrippsiella sp.* was given as the typical answer and *Ensiculifera* as synonym but both correct.

The organism *C. granii* Gough was again identified correctly to genus level by all analysts. Most analysts tried to identify this organism to species level but many answers were given for this e.g.: *C.asteromphalus* Erhenberg*, C.radiatus* H.L.Smith*, C.centralis* Erhenberg *and C.wailesii* Gran & Angst by a number of analysts. This suggests different approaches to the identification of large organisms like *Coscinodiscus* by the different analysts.

The organism *G. catenatum* was identified correctly to species level by twenty one out of thirty four analysts, a further five analysts identified it correctly to genus level and eight analysts incorrectly identified this organism. Some names given incorrectly were *Cochlodinium* Schütt*, A.catenella* (Whedon & Kofoid) Balech and the non-name 'Chain forming organism'.

As *G. catenatum* is a highly toxic organism, the correct identification of this organism should have been to species level in this case as the identification to genus only would not be enough to discriminate between toxic and non-toxic species of this genus incurring on a false negative answer.

P. lima and *G. catenatum* were considered true positive results as both these organisms are toxin producers. *C. granii, Scrippsiella sp. and P. micans* were true negative results as there are non-toxin producer species.

Any incorrect answers for *P. lima* and *G. catenatum* were considered false negative results and any incorrect answers for *C .granii, Scrippsiella sp. and P. micans* were considered false positive results.

The identification of *Coscinodiscus and Scrippsiella* to genus level is given as correct, for the other three species the identification to species level is given as correct. All other answers are given as incorrect including not going to the adequate taxon level as could be the case with *G.catenatum*.

Table 2.13 show the results of the five species by the analysts. 1 is given for correct identification (id) and 0 for incorrect identification. The table gives the false positive and negative rates for each species and for the exercise, the false positive rate was only 1% and the false negative rate was 19%.

The sensitivity of the method in this case was 81%; the specificity of the method was 99 % and the efficiency 92%. Efficiency was measured by dividing the true positive and negative results (all the correct ids) by all the answers (correct and incorrect ids).

Youden index was calculated using the sensitivity and specificity and subtracting 1, if the sensitivity and specificity were to be perfect then the Youden index would be +1. The closest to this number the more specific and sensitive the method is. In this case is +0.8. This index does not indicate if the method is more sensitive than specific or *vice versa* as it pools both measures in the same equation.

This work suggests that the Utermöhl method expressed as a reliability measure has a very good specificity and sensitivity ratios (99% and 81%) respectively. As the sensitivity is lower this indicates a higher number of false negative responses to the method than false positive responses but overall the method has a 92% efficiency and high Youden index.

Table 2.13: Qualitative measurements

Reliability measure for Intercomparison exercise PHY-ICN-09-MI1						
Analysts	*C.granii*	*Scrippsiella*	*P.micans*	*P.lima*	*G.catenatum*	
u	1	1	1	1	0	
r	1	1	1	1	0	
k	1	1	1	1	0	
c	1	1	1	1	0	
n	1	1	1	1	0	
b	1	1	1	1	0	
s	1	1	1	1	1	
β (Beta)	0	1	1	1	1	
l	1	1	1	1	1	
ά (Alpha)	1	1	1	1	1	
δ (delta)	1	1	1	1	1	
j	1	1	1	1	1	
h	1	1	1	1	0	
z	1	1	1	1	1	
v	1	1	1	1	1	
g	1	1	1	1	0	
ñ	1	1	1	1	1	
i	1	1	1	1	1	
a	1	1	1	1	0	
o	1	1	1	1	1	
d	1	1	1	1	0	
p	1	1	1	1	0	
f	1	1	1	1	0	
Ω (omega)	1	1	1	1	1	
σ (sigma)	1	1	1	1	0	
m	1	1	1	1	1	
µ	1	1	1	1	1	
π (pi)	1	1	1	1	1	
x	1	1	1	1	1	
y	1	1	1	1	1	
q	1	1	1	1	1	
t	1	1	1	1	1	
ch	1	1	1	1	1	
ζ (zeta)	1	1	1	1	1	**Total**
TP= True positives	0	0	0	34	21	55
TN= True Negatives	33	34	34	0	0	101
FP= False Positives	1	0	0	0	0	1
FN= False Negatives	0	0	0	0	13	13
FP rate	0	0	0	0	0.38	0.01
FN rate	0.03	0.00	0.00	0	0	0.19
				Sensitivity		**0.81**
				specificity		**0.99**
				Efficiency		**0.92**
				Youden Index		**0.80**

1%
19%

2.4 Performance evaluation

Overall in the enumeration part of the exercise most analysts performed well and within the 3 standard deviations set by the reference data or 'gold standard'.

Only analyst b seems to underestimate four out of the five counts, also analysts s and x underestimated one of their counts. Most laboratories underestimated the count for *Prorocentrum micans*.

Although most analysts performed well and within the prescribed 3 standard deviations, there was overall evidence of lack of reproducibility within and between laboratories. There appears to be variability between analysts when measuring the same sample and species with some variability also between laboratories.

On the identification exercise, most analysts exceeded the 70% overall pass mark. Seven analysts performed above 90%, fifteen analysts between 80 and 90%, eleven analysts between 70 and 80% and one analyst below the pass mark.

The reliability measure for the test method qualitative component showed that thirteen analysts out of thirty four committed false negative responses, this is 38% of the analysts; one analyst gave a false positive response which is 3% of the analysts. The false negative rate is quite high compared to the false positive rate which raises concerns about the reliability of the identification of toxic species. When all the results are taken into account, the reliability measure in terms of the sensitivity, specificity and efficiency of the test method to detect phytoplankton species is quite robust.

Overall, the standard in the enumeration and identification exercise was very high for all of the participating laboratories. A statement of performance (Appendix 5) was issued to each individual participant and given a mark for each component of the exercise.

2.5 Discussion

In summary, there was evidence of a good agreement in the enumeration study between virtually all the analysts when compared with the gold standard set up by the organising laboratory for all species except one. The experimental design for this exercise allowed the results of the analysts to be compared against a reference value.

The organising laboratory provided data from twenty replicate counts on four species (*C. granii, Scrippsiella sp., G. catenatum, P. micans*) and twenty three replicates on one species (*P. lima*) while thirty four analysts across seventeen laboratories each provided four replicate counts on the four species and three replicate counts on *P. lima*. The main goal was to investigate whether the measurements provided by the analysts were comparable to the gold standard (i.e. as estimated by the reference laboratory).

Results from data analyses suggested that the variability between replicates across species is normal given the symmetry present in each box plot (Figures 2.3 to 2.7). There appears to be a difference in variability between species, the largest variability is evident in *Scripsiella sp.* while the smallest is evident in *P. lima.* This may be an effect of the cell concentration, with *P. lima* being the lowest density sample and *Scripsiella sp.* being the highest.

Given the symmetry present, it is reasonable to assume that the mean and standard deviation of the measurements are valid estimates of their population counterparts.

As there are no reference materials available for phytoplankton intercomparison exercises, I set up a reference value or gold standard for each species (using the best available methods to homogenise and spike the samples). Thus, the experimenter was not blinded to the species and densities used in the experiment. The gold standard provided a true value of the sample population which all the analysts then tried to replicate.

While the likelihood of an analyst obtaining a sample, which contained a smaller or larger number of cells, than the reference mean, due to the variability on homogenisation techniques, was a consideration that needed to be taken into account, the use of sample replicates and randomization should have minimised this issue. However, it is possible that an analyst could be very accurate yet fall far from the expected mean result due to out of specification samples. This is one of the reasons why reference materials and validated methodologies are necessary for the future advancement of these studies.

The results of *P. micans* counts indicate that the reference count deviates systematically from the analysts. This data is difficult to interpret as this, did not happen with any of the other organisms counted. The mean cell density of the counts by the analysts was found to be lower than the mean cell density found by the reference value. Most analysts had already performed well in the other four organisms counted so it was difficult to understand how many analysts would have returned lower cell counts on an organism not particularly difficult to identify. The expectation would have been that particular laboratories or analysts within a laboratory might have a number of out of specification results, which would point to problems in the techniques used. This was the case with analyst b, which tended to underestimate most species counts and was outside the reference value and 3 standard deviations of the mean. Also analysts x and s had one out of specification result each. This may indicate methodology issues or problems counting particular species or cell concentrations. There is also a possibility that cell losses may have occurred during transport and time before settlement and analysis of samples for this particular organism.

Overall, the enumeration exercise showed that laboratories performing the same test method achieved comparable results (mean +/- 3 standard deviations) in exercises using spiked preserved and homogeneous biological materials in samples. However, when the within and between laboratory reproducibility was studied, it was found that there was a significant inter-observer cell count variability in same sample analysis, indicating differences in counting and microscopic techniques in analysts from the same laboratory. This variability was also found between laboratories, which may indicate different approaches to cell counting, homogenising, settling samples and microscope set up.

The use of 3 standard deviations was designated for this exercise due to the lack of reference certified homogeneous materials for the exercise. The materials for this exercise were prepared, homogenised and divided into sub-samples using best practice, however these techniques have not yet been validated. Therefore, erring in the side of caution as to the homogeneity and quality of the materials provided using the technique described in the study it was decided to use 3 standard deviations for the mean as the upper and lower confidence limits.

Correct identification of the species spiked in the samples was used in this exercise to provide some sort of reliability measure on the quality of the identifications by the analysts. This is generally an overlooked area on phytoplankton intercomparison exercises where cell counting is usually seen as the principal data.

The qualitative data was studied using the Bayes posterior probability theorem through the use of false positive and negative rates based on correct or incorrect classification of the species spiked in the samples.

Results of these analyses showed that the test method was quite sensitive and specific and therefore efficient at the identification of phytoplankton species. A large percentage (38%) of false negative responses compared to false positive responses (3%) indicated that analysts were more likely to identify a toxic organism as a non toxic organism than the other way around. This seems to indicate a tendency by analysts to be more cautious identifying toxic species.

This is an interesting finding as the false negative rate could have profound implications on human health given that phytoplankton results are used by monitoring programmes to provide advance warning on potential harmful algal events and the possibility of accumulation of toxins in shellfish.

False positive and negative responses for the purpose of this study were given the same weighing, that is given the same importance. However, at least theoretically the false negative responses should have a greater weighting than false positive responses as they are more critical to humans and should be minimised. This could mean that the 38% of false negative responses is actually a larger number.

Also, two analysts identified and counted *Scrippsiella sp.* in the negative sample (type d) which contained only filtered sterile seawater and lugol's iodine. This seems to indicate in both cases a contamination or carry over problems perhaps due to the cleaning protocols used by these laboratories as this species were one of the target species in the exercise. This demonstrates how intercomparison trials can be used to

indicate where laboratories may be having problems in implementing techniques or protocols.

The results returned for the identification study were excellent with no evidence of systematic difference in the percentage of correct answers between the image and video formats of presentation. There was a higher mean success rate when identifying images although this was not consistent across analysts.

There was evidence of a significant difference between sets where set C appeared the easiest and set D the hardest for both formats. This may be a normal outcome as analysts would generally be used to looking at images more so than videos. In terms of correct identifications, all participants did quite well, with 68% of the participants scoring over 80%.

All these results were feedback to the participants at the Bequalm phytoplankton annual workshop which took place in the Marine Institute, auditorium space in May 2009, where the results were presented and discussed. Although there was variability between analysts and laboratories, the values obtained on the samples using the techniques described were mostly comparable between the gold standard and analysts; this has acted as a kind of validation on the preparation of the materials for future exercises. This meant that all analysts using the same aliquots and counting techniques could come up with reasonable results and that these results were within what was expected by the gold standard.

This exercise showed that the use of a gold standard only becomes truly valuable when the preparation of the materials are assessed and validated and can be used as a true reference value, that is a certified reference material. Otherwise, we won't ultimately really know how much of the variation encountered can be apportioned to the analysts or the technique employed to prepare the materials. This is a challenge that needs to be addressed in future exercises. All the information, gathered from this exercise has been used to inform some of the decisions made for the preparation of the following exercise. Overall, this intercomparison exercise was very successful both in terms of interest from laboratories involved in phytoplankton analysis and the results obtained.

3. Bequalm 2010 Intercomparison exercise in the enumeration and Identification of Phytoplankton

Abstract

The objective of the present study was to test laboratories involved in phytoplankton monitoring in the enumeration and identification of marine microalgae through an intercomparative study using light microscopy techniques.

The primary objective in the identification exercise was to test analyst's knowledge of phytoplankton taxonomy using a quiz or written exam where analysts were given several questions containing images and illustrations of marine microalgae that needed to be identified. These exams are tools used to assess the taxonomic knowledge of analysts across laboratories and identify where their strengths and weaknesses lie.

The objectives in the enumeration exercise were: to compare results between laboratories using their own test methods, to investigate learning effects by analysts on replicate samples, to examine method effects between laboratories using different sample volumes and cell counting strategies, to compare laboratory results against a set of hypothesised means or reference values and to calculate Z-scores for each laboratory.

Theoretically, it would be better to standardise the techniques used by the different laboratories into a common protocol to reduce the variability found between laboratories in the enumeration and identification of microalgae. In practice each laboratory uses slightly different protocols sample volumes and counting strategies. In this study, the interest is on calculating the variability caused by the volume and the counting strategy used by the laboratories. This measure would give us information about how laboratories are actually monitoring microalgae and help us in the future to move towards best practice for the test method.

The enumeration exercise consisted of seawater samples preserved in lugol's iodine and spiked with one cultured species. There were triplicate samples at two different cell concentrations.

The results from the identification exercise indicated that analysts have a good level of theoretical expertise in the identification of common marine microalgae across the laboratories.

The enumeration data demonstrates that there was no evidence of learning effects by analysts, neither between the three replicates nor at the two cell concentrations.

The results from the enumeration exercise illustrate that laboratories settle different sub-sample volumes for analysis and use different counting strategies depending on the cell concentration of the analyte. There is evidence of lack of reproducibility across laboratories using different volumes and counting techniques.

The results obtained were compared with hypothesized means or reference values for each concentration. The comparison suggested that cell counts were potentially underestimated by as much as 30% and that this underestimation was due to test method effects. Should correction factors be introduced in the test method? Or how can these effects are minimized?

Future work should investigate further these effects to understand where these losses occur, how to minimize them and perhaps to introduce correction factors if necessary.

3.1 Introduction

The 2010 Bequalm exercise was divided in two sections: an enumeration exercise comprising six samples spiked with cultured material at two cell concentrations and an identification exercise consisting of a taxonomic quiz (Appendix 14).

The identification exercise was a basic test purposely biased towards marine diatoms and dinoflagellates species, towards toxic/harmful species and designed to test the participants' basic theoretical knowledge of phytoplankton taxonomy. The exercise

included three general questions on armoured dinoflagellates and three questions on diatoms in order to compare whether analysts were better at identifying one group more so than the other. One question on the genus *Dinophysis* and one question on 'naked' dinoflagellates.

All the species chosen for the exercise are species commonly found in marine water samples. The illustrations also depicted typical marine phytoplankton species and questions were asked on morphological features typical of the type species to be able to distinguish between different genera or even species.

Do significant differences in cell counts between laboratories exist dependent on sample volume analysed and cell counting strategies? The main objective of the enumeration study was to observe method effects in the analysis of the samples and compare these results. In order to do this we needed at least two cell densities and a minimum number of sample replicates per analyst to obtain statistically robust data. Two different cell densities were used to test the behaviour of the test method and analysts at particular concentration ranges. We were interested in the method choices that participants would make in the analysis depending on the cell concentration found in the samples.

Secondary objectives included the study of learning effects by analysts; can analysts perform better on successive replicates? Learning effects can be caused by the ability of analysts to improve their performance when analysing successive replicate samples. For this reason, analysts were asked to number sequentially their samples as these were analysed.

Also, hypothetical mean values were proposed for both cell densities. The hypothesised means study was based on raw cell counts of 1ml aliquots dispensed from the two cell concentration's master mix into sedgewick rafter counting chambers to calculate values that, theoretically speaking, could be the true values of the cell concentration in the samples. It is important to clarify at this point that these hypothetical values are not to be used as reference or true value for the purpose of calculating Z-scores but rather as a way of highlighting potential method effects by the system which may occur once aliquots are dispensed into sterilin tubes.

The enumeration exercise was designed to be strictly a counting exercise. No identification of the spiked cell culture material was needed. No gold standard or reference values were assigned in this exercise by the organising laboratory instead all the data generated from the participating laboratories was used to generate the mean and standard deviation for the sample population.

This intercomparison exercise did not stipulate that analysts must use one particular counting strategy, sample volume or cell counting method. The exercise allowed the participants to use the standard test method that each laboratory routinely uses. The only limiting factor with regards to this point was the volume of sample sent to the participants (30ml), in some cases laboratories would use larger sample volumes (50ml).

Participants were asked to read carefully the instructions and were also asked on receipt of the samples to send back the return slip and checklist to the organising laboratory to ascertain that the samples have been received in good condition. Analysts had to return results within a four week deadline.

3.2 Materials and Methods

3.2.1 Taxonomic Quiz

The identification exercise or taxonomic quiz (Appendix 14) was custom made from 'scratch' and comprised eight questions and 300 marks. The pass mark for the exercise was set at 70%.

This exercise used photographs and line drawings of marine phytoplankton species that participants needed to identify to a particular taxon. Some questions related to morphological features typical of the genus/species which are commonly used to key out microalgal species (e.g. dinoflagellates thecal plate structure).

The digital images were taken using an Olympus camera (Camedia 3030, Olympus, UK) attached to a side port of an IX-50 inverted Olympus microscope (Olympus,

Mason Technology, Ireland)). The images were taken using Köeller illumination settings with some images captured using phase contrast. The drawings and illustrations were replicated from publications using Adobe photoshop 6.0 (Adobe systems, San Jose, USA) and Microsoft office (Microsoft, USA) editing software.

Question one (Q1) dealt specifically with the armoured dinoflagellates of the genus *Dinophysis*. The analysts had to identify the images provided to species level and name a morphological feature marked with a red arrow in the images. Species within this genus are known to produce diarrhetic shellfish toxins and they are geographically cosmopolitan; therefore they are a very important group of algae. Although these species have been known for decades, their life cycle was unknown partly due to the failure to grow and maintain cultures alive for any length of time. This was until recently when the organism *Dinophysis acuminata* Claparède & Lachmann was successfully cultured by Park *et al.,* 2006. This development helped to unravel some of the mysteries surrounding the life cycle of species in this genus.

The *Dinophysis* species concept needs reviewing at present and from a monitoring point of view, we need to be aware of these changes, so it is important to know how laboratories confront the dilemma on the identification of these species.

Questions two (Q2), three (Q3) and four (Q4) depicted armoured dinoflagellates. Q2 asked analysts, using illustrations, to differentiate between the Kofoidean tabulation (Kofoid, 1909, 1911; Fensome, 1993; Steidinger, 1997) of two similar armoured dinoflagellates (one toxic and one non toxic) and to name the genus they represented. Q3 showed an illustration of the thecal structure of the genus *Alexandrium* (Halim, 1960; Balech, 1989) in ventral and apical view. Analysts were asked to name certain plates and structures typical of this genus. Q4 asked analysts to identify to species level a number of images representing armoured dinoflagellates (Lebour, 1925; Schiller 1933; Dodge, 1982; Taylor *et al.,* 2004), these are typical species found regularly in marine water samples around the world. There were two images of the dinoflagellate *Protoperidinium crassipes* (Kofoid) Balech, the first image shows the organism in ventral view and the second image in apical view to be able to show the 1’ apical plate of *P. crassipes* episome.

Questions five (Q5), six (Q6) and seven (Q7) were questions on diatoms. Q5 had images of mostly centric planktonic diatoms (Simonsen, 1979; Mann & Droop, 1996) regularly found in samples and participants were asked to identify these to species level. Q6 asked participants to name the image that was the 'odd one out'. The question contained 8 images, one of which was not a diatom. Q7 was specific to the genus *Pseudo-nitzschia* (Cleve) H.Peragallo participants were asked using the illustrations depicting a silica frustule of this pennate diatom in valve and girdle view (Hasle & Syvertsen, 1997) to draw where a cell width measurement should be taken and in which view. The follow up question asked participants to choose from four images which one showed the correct view.

The importance of this question relies on the fact that *Pseudo-nitzschia* are discriminated by size into two main sub-groups; the *P. seriata* complex (Cleve) H.Peragallo and the *P. delicatissima* complex (Cleve) Heiden (Priisholm *et al.*, 2002; Lundholm *et al.*, 2003) based on the size of the cell valve. This need to be measured in valve view to be a correct measurement as in girdle view the size of diatoms changes depending on how many girdle bands are present (Hasle & Syvertsen, 1997).

The last question (Q8) dealt with the identification of 'naked' dinoflagellates (Kofoid & Swezy, 1921; Schiller, 1933). This group of organisms is differentiated from the group of armoured dinoflagellates in that the cells appear 'naked' that is they don't have cellulose plates (Larsen & Sournia, 1991) enveloping the episome and hyposome; and when viewed under the microscope the cells appear to be rounded. The species concept of this group of microalgae has changed recently with the introduction of new morphological features that need to be seen in order to place the organism in one genus or another (Larsen, 1994, 1996; Hansen *et al.*, 2000; Haywood *et al.*, 2004). The previous species concept was based on different characters of the cingulum (girdle) of the cells, i.e. the position, displacement and number of turns around the cell body (Dodge, 1982) which caused confusion regarding the use of these relative characters. The new concept added the shape of the apical pore complex and the presence/absence of a ventral pore (Takayama, 1985; De salas *et al.*, 2003). It is essential that taxonomists are aware of these changes.

3.2.2 Phytoplankton samples: Enumeration exercise

The enumeration exercise consisted of six marine water samples spiked with the armoured dinoflagellate *Scrippsiella* from a culture kept in the Marine Institute culture collection at two different cell densities. The samples were preserved using Lugol's iodine.

A master mix was made for each density using a 500ml borosilicate glass screw top Schott bottle and the aliquots were taken with a 1ml Gilson Pipette after homogenising the sample at least 100 times for each aliquot by inversion of the Master mix. The six samples were triplicate samples of two densities. Low density samples contained ~200 cells/30ml and the high density samples contained ~1000 cells/30ml.

Preliminary cell counts were carried out using a sedgewick-rafter cell counting chamber to ascertain the approximate densities in the samples (Appendix 18). The Hypothetical values for the low density samples were based on ten replicate cell counts of the total area of the chamber. The estimate for the high density samples was based again on ten replicates but only 1/10 of the area of the chamber was counted (Venrick, 1978; Edler, 1979).

Aliquots were dispensed into sterilin tubes containing a volume of 29ml sterile filtered sterile seawater. The final concentration was hypothesised to be approximately 6 cells/ml and 333 cells/ml for each density.

Overall, 300 samples were prepared for each cell density, that is 300ml were taken from a master mix of 500ml or 60% of the total.

All samples used in this exercise were chosen randomly using Minitab software Vr15.0. Each sample was given a number and randomly assigned to a laboratory by the programme by using the randomization tool.

3.2.3 Forms and Instructions

3.2.3.1 Couriers and materials

All the necessary forms and instructions (Appendix 11) to complete the exercise were sent to all the participating analysts.

Each analyst received apart from a set of six samples, a set of instructions (Appendix 11), a form to confirm receipt of materials (Form 1, Appendix 12), a form for writing in the enumeration results (Form 2, Appendix 13) and a taxonomic quiz (Form 3, Appendix 14).

Samples were sent via courier to all the laboratories on the same day and the forms were sent to all the participants via e-mail. Upon receipt of these materials all participants were asked to check the samples and the documentation for missing forms or leaked samples.

3.2.3.2 Instructions

A set of concise instructions was sent with the rest of the materials, laboratories and analysts were asked to read and follow the instructions before commencing the test and to give themselves plenty of time to limit the number of errors due to tiredness and stress. See instructions in Appendix 11.

3.2.4 Statistical analysis

The qualitative exercise was analysed statistically as a percentage of correct identifications. Some set of questions in the exercise were compared against other sets, for example questions two, three and four on dinoflagellates were compared against questions five, six and seven on diatoms (Appendix 15). The main statistics used for this exercise were descriptive statistics to ascertain whether analysts answered particular questions better than others. Box plots to show graphically whether there were any differences between answers to certain questions, the cumulative percentage of correct answers to provide a yardstick for the exercise on

where the pass mark for the exercise should be set, the individual values of each analyst to compare how well participants did and finally the ranking of analysts in the exercise.

The objective of the enumeration exercise was to compare the variability of cell counts at different cell concentrations. There were triplicate samples at two cell concentrations (~200 cells/30ml and ~1000 cells/30ml) these were sent on triplicates to obtain robust statistical data and enable us to carry out ANOVA statistics.

Learning effects between replicates were investigated through box plots, individual value plots and descriptive statistics, also significant differences in cell concentrations depending on the volume sub-sampled and the cell counting strategy used were studied through the use of two sample T-tests, paired T-tests, 2-way ANOVA, interaction plots for factors and general linear models.

Hypothesised values were also used to make assumptions about the samples' true value and how these values compared with the analysts'. This allowed us to discuss method effects.

Finally, mean values for each concentration were plotted and results were compared between analysts and laboratories. The final score was given as a Z-score using the mean of all the results and 3 sigma limits as a measure of dispersion.

3.3 Results

3.3.1 Participants

There were thirty nine analysts from twenty one laboratories in total participating in this exercise (Appendix 17) mostly from Europe with two laboratories for the first time from South America (Peru and Argentina).

3.3.2 Phytoplankton identification results

The total number of marks for this identification exercise was 300. Incorrect answers were given a zero, but no negative marks were given. Only nineteen analysts of the total of thirty six had to return results for the taxonomic quiz as the other twenty analysts had already done this same exercise back in 2008.

A correct answer for each species (sp.) and feature (ft) was given 5 marks in question 1 for a perfect score of 60 marks. Most analysts returned near perfect scores, with six analysts making one error each (Table 3.1).

There were 20 marks for a perfect score in each of questions 2 and 3 (Table 3.2). Q2 was subdivided into Q2 1a, Q2 1b (5 marks each) and Q2 2a (10 marks). There were no errors in Q2 1a and Q2 1b with four errors made in Q2 2a. A correct answer in this section was given 10 marks with one analyst (33) given half the marks for a not fully correct answer. Q3 was sub divided into four questions (a, b, c, d), each was worth 5 marks. Most analysts performed very well in this question only two analysts made a mistake.

Table 3.1: Question 1 results of identification exercise Bequalm 2010

ANALYST CODE	Question 1 (60 marks)											
	A		B		C		D		E		F	
	sp.	ft	sp.	ft	sp.	ft	sp.	ft	sp.	ft	sp.	ft
33	5	5	5	5	5	5	5	5	5	5	5	5
4	5	5	5	5	5	5	5	5	5	5	5	5
38	5	5	5	5	5	0	5	5	5	5	5	5
27	5	5	5	5	5	5	5	5	5	5	5	5
16	5	5	5	5	5	5	5	5	5	5	5	5
8	5	5	5	5	5	5	5	5	5	5	5	5
2	5	5	5	5	5	5	5	5	5	0	5	5
35	5	5	5	5	5	5	5	5	5	5	5	5
9	5	5	5	5	0	5	5	5	5	5	5	5
11	5	5	5	5	5	5	5	5	5	5	5	5
10	5	5	5	5	5	5	5	5	5	5	5	5
19	5	5	5	5	5	5	5	5	5	5	5	5
3	5	5	0	5	5	5	5	5	5	5	5	5
28	5	5	5	5	5	5	5	5	5	0	5	5
15	5	5	5	5	5	5	0	5	5	5	5	5
12	5	5	5	5	5	5	5	5	5	5	5	5
22	5	5	5	5	5	5	5	5	5	5	5	5
29	5	5	5	5	5	5	5	5	5	5	5	5
40	5	5	5	5	5	5	5	5	5	5	5	5

Table 3.2: Questions 2 and 3 results identification exercise Bequalm2010

ANALYST CODE	Question 2 (20 marks)			Question 3 (20 marks)			
	1a	1b	2a	a	b	c	d
33	5	5	5	5	5	5	5
4	5	5	10	5	5	5	5
38	5	5	10	5	5	5	5
27	5	5	10	5	5	5	5
16	5	5	10	5	5	5	5
8	5	5	10	5	5	5	5
2	5	5	10	5	5	5	5
35	5	5	10	5	5	5	5
9	5	5	0	5	5	5	0
11	5	5	10	5	5	5	5
10	5	5	10	5	5	5	5
19	5	5	10	5	5	5	5
3	5	5	0	5	5	5	5
28	5	5	0	5	5	5	0
15	5	5	10	5	5	5	5
12	5	5	10	5	5	5	5
22	5	5	10	5	5	5	5
29	5	5	10	5	5	5	5
40	5	5	10	5	5	5	5

Table 3.3: Question 4 results identification exercise Bequalm 2010

ANALYST CODE	Question 4 (50 marks)									
	A		B		C		D		E	
	gen	sp.	gen	sp.	gen	sp.	gen	sp.	gen	sp.
33	5	5	5	5	5	5	5	5	5	5
4	5	5	5	5	5	5	5	5	5	5
38	5	5	5	5	5	5	5	5	5	5
27	5	0	5	5	5	5	5	5	5	5
16	5	5	5	5	5	5	5	5	5	5
8	5	5	5	5	5	5	5	5	5	5
2	5	5	5	5	5	0	5	5	5	5
35	5	5	5	5	5	5	5	5	5	5
9	5	5	5	5	5	5	5	5	5	5
11	5	5	5	5	5	5	5	5	5	5
10	0	0	5	5	5	5	5	5	5	5
19	5	5	5	5	5	5	5	5	5	5
3	5	5	5	5	5	5	5	5	5	5
28	5	5	5	5	5	5	5	5	5	5
15	5	5	5	5	5	5	5	5	5	5
12	5	5	5	5	5	5	5	5	5	5
22	5	5	5	5	5	5	5	5	5	5
29	0	0	5	5	5	5	5	5	5	5
40	0	0	5	5	5	5	5	5	5	5

Q4 depicted five armoured dinoflagellates species (A, B, C, D, E) and 5 marks were given for a correct genus and 5 marks for a correct species name, 10 marks for each image and 50 in total for a perfect score (Table 3.3). All analysts performed very well in this question. Only five analysts made an error in Q4 four analysts in Q4A and one

analyst in Q4C, Q4A corresponds to the small armoured dinoflagellate *Heterocapsa triquetra* (Ehrenberg) Stein.

Q5 depicts the images of seven species of diatoms, each had to be identified to species level with 5 marks given for correct genus and 5 marks for correct species name (Table 3.4). A perfect score would give 70 marks.

Eight analysts had difficulty going to species level with image B which correspond to the diatom *Chaetoceros densus* (Cleve) Cleve, two images were used for this organism the first one, a general view to show a chain of this organism in girdle view and the second one to show a defining characteristic which is typical of these species and that is the angle at which the setae (spines) diverge from the cells valve (Rines & Hargraves, 1988), in this organism the setae diverge at equal angles from the apical plane of the cells.

Table 3.4: Question 5 results identification exercise Bequalm 2010

ANALYST CODE	Question 5 (70 marks)													
	A		B		C		D		E		F		G	
	gen	sp.	gen	sp.	gen	sp.	gen	sp.	gen	sp.	gen	sp.	gen	sp.
33	5	5	5	5	5	5	0	0	5	5	5	5	5	5
4	5	0	5	0	5	5	5	5	5	5	5	5	5	5
38	5	5	5	5	5	5	5	5	5	5	5	5	5	5
27	5	0	5	0	5	5	5	5	5	5	5	5	5	5
16	5	5	5	0	5	5	5	5	5	5	5	5	5	5
8	5	5	5	0	5	5	5	5	5	5	5	5	5	5
2	5	5	5	0	5	5	5	5	5	5	5	5	5	5
35	5	5	5	5	5	5	5	5	5	5	5	5	5	5
9	5	5	5	5	5	5	5	5	5	5	5	5	5	5
11	5	0	5	5	5	5	5	5	5	5	5	5	5	5
10	5	5	5	0	5	5	5	5	5	5	5	5	5	5
19	5	5	5	5	5	5	5	5	5	5	5	5	5	5
3	5	5	5	5	5	5	5	5	5	5	5	5	5	5
28	5	0	5	5	5	5	5	5	5	5	5	5	5	5
15	5	5	5	5	5	5	5	5	5	5	5	5	5	5
12	5	5	5	0	5	5	5	5	5	5	5	5	5	5
22	5	5	5	0	5	5	5	5	5	5	5	5	5	5
29	5	5	5	5	5	5	5	5	5	5	5	5	5	5
40	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Also, four analysts had problems identifying to species level image A, which corresponds to the diatom *Chaetoceros peruvianus* Brightwell. Analysts were warned that this organism was not a chain forming organism (Appendix 15), this was done because the images show this organism dividing and 2 cells can be seen together

which could create the impression that the organism was a chain forming species. One analyst answered incorrectly image D (*Neocaliptrella robusta* Norman).

The correct answer to question six (Q6) was image E depicting a silicoflagellate; *Dictyocha speculum* Ehrenberg which is not a diatom. All analysts answered correctly this question (Table 3.5) which was worth 15 marks.

Q7 was sub divided in three sections (A, B, C) (Table 3.5), each correct answer was worth 10 marks and 30 in total. Q7A and B asked where and which view should analysts use to measure the width of a *Pseudo-nitzschia* cell. The diagram shows two views a girdle view and a valve view. The correct answer is in valve view and in the center of the valve. Critically a correct answer to Q7A and Q7B should give the right answer to Q7C which shows four digital images of chains of this organism one of which is in valve view. Two analysts gave incorrect answers to Q7C even though they were correct to Q7A and Q7B.

Table 3.5: Q6, 7 & 8: Participants results intercomparison Bequalm 2010

ANALYST CODE	Question 6 (20 marks)	Question 7 (30 marks)			Question 8 (30 marks)						
	Circle answer	A	B	C	A	B	C	D	E	F	G
33	15	10	10	10	5	5	5	5	5	5	5
4	15	10	10	10	5	5	5	5	5	5	5
38	15	10	10	10	5	5	5	5	5	5	5
27	15	10	10	10	5	5	5	5	5	5	5
16	15	10	10	10	5	5	5	5	5	5	5
8	15	10	10	10	5	5	5	5	5	5	5
2	15	10	10	0	5	5	5	5	5	5	5
35	15	10	10	10	5	5	5	5	5	5	5
9	15	10	10	10	5	5	5	5	5	5	5
11	15	10	10	10	5	5	5	5	5	5	5
10	15	10	10	10	5	5	5	5	5	5	5
19	15	10	10	10	5	5	5	5	5	5	5
3	15	10	10	10	5	5	5	5	5	5	5
28	15	10	10	0	5	5	5	5	5	5	5
15	15	10	10	10	5	5	5	5	5	5	5
12	15	10	10	10	5	5	5	5	5	5	5
22	15	10	10	10	5	5	5	5	5	5	5
29	15	10	10	10	5	5	5	5	5	5	5
40	15	10	10	10	5	5	5	5	5	5	5

Q8 depicts illustrations of seven different genera of 'naked' dinoflagellates. Each correct answer was worth 5 marks for a perfect score of 35 marks. All analysts received full marks on this question (Table 3.5). The overall results had been ranked

per analyst and tabulated as a percentage of correct answers from the total (Table 3.6 & 3.7).

Table 3.6: Analysts overall score identification exercise

Analyst Code	Q1(%)	Q2,3,4(%)	Q5,6,7(%)	Q8(%)	Total(%)
33	100	94	93	100	**97**
4	100	100	93	100	**98**
38	92	100	100	100	**98**
27	100	94	93	100	**97**
16	100	100	96	100	**99**
8	100	100	96	100	**99**
2	92	94	85	100	**93**
35	100	100	100	100	**100**
9	92	83	100	100	**94**
11	100	100	96	100	**99**
10	100	89	96	100	**96**
19	100	100	100	100	**100**
3	92	89	100	100	**95**
28	92	83	85	100	**90**
15	92	100	100	100	**98**
12	100	100	96	100	**99**
22	100	100	96	100	**99**
29	100	89	100	100	**97**
40	100	89	100	100	**97**

Table 3.7: Analysts overall rank

%	ANALYST CODE	Position
100	35	1
100	19	1
99	16	2
99	8	2
99	11	2
99	12	2
99	22	2
98	4	3
98	38	3
98	15	3
97	29	4
97	40	4
97	33	4
97	27	4
96	10	5
95	3	6
94	9	7
93	2	8
90	28	9

Table 3.8 shows the descriptive statistics of all the participants' results in the intercomparison. The overall mean of correct answers for all the questions was very high indicating nearly perfect results. The highest result is for Q8 followed by Q1, the groupings Q2, 3, 4 (armoured dinoflagellates) and Q5, 6, 7 (diatoms) are slightly lower than the other two with a higher percentage of correct answers on diatoms but not significantly higher. This is shown graphically with box plots (Fig.3.1).

Table 3.8: Descriptive statistics of identification results Bequalm 2010

Descriptive Statistics: Q1 (%), Q2,3,4 (%), Q5,6,7 (%), Q8 (%)							
Variable	N	Mean	SE Mean	StDev	Q1	Median	Q3
Q1 (%)	19	97.368	0.913	3.980	91.667	100.000	100.000
Q2,3,4 (%)	19	95.03	1.40	6.11	88.89	100.00	100.00
Q5,6,7 (%)	19	96.20	1.06	4.63	92.86	96.43	100.00
Q8 (%)	19	100.00	0.000000	0.000000	100.00	100.00	100.00

Figure 3.1: Box plot of identification scores.

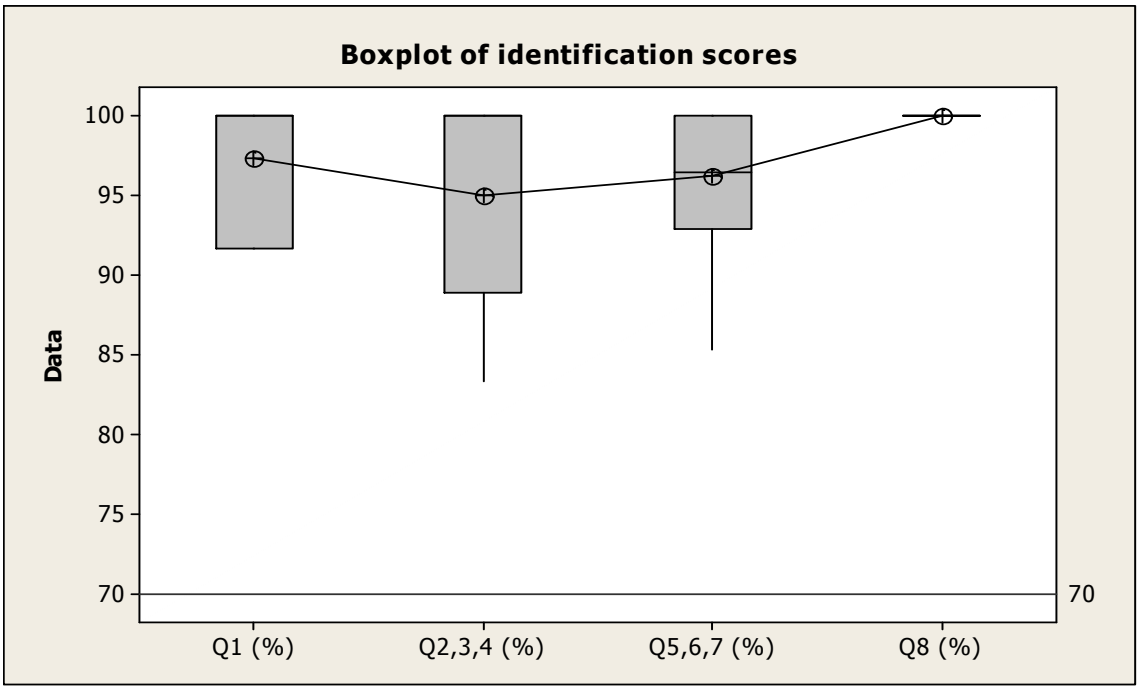


Table 3.9 shows the cumulative percentage of correct answers. The tally for discrete variables shows that most analysts would be above the 90% mark in most questions with a small number of analysts just below the 90% mark in Q2, 3, 4 and Q5, 6, 7.

Table 3.9: Cumulative percentage of correct answers

Tally for Discrete Variables: Q1 (%), Q2,3,4 (%), Q5,6,7 (%), Q8 (%)			
n=19	%	Analysts	Cumulative %
Q1 (%)	92	6	31.58
	100	13	100
Q2,3,4 (%)	83	2	10.53
	89	4	31.58
	94	3	47.37
	100	10	100
Q5,6,7 (%)	85	2	10.53
	93	3	26.32
	96	6	57.89
	100	8	100
Q8 (%)	100	19	100

If the overall pass mark was 90% (Fig.3.2) all analysts would still have passed the test with most analysts in the high 95-96% mark and one analyst around the 90% mark.

Figure 3.2: Overall % correct answers by individual analysts

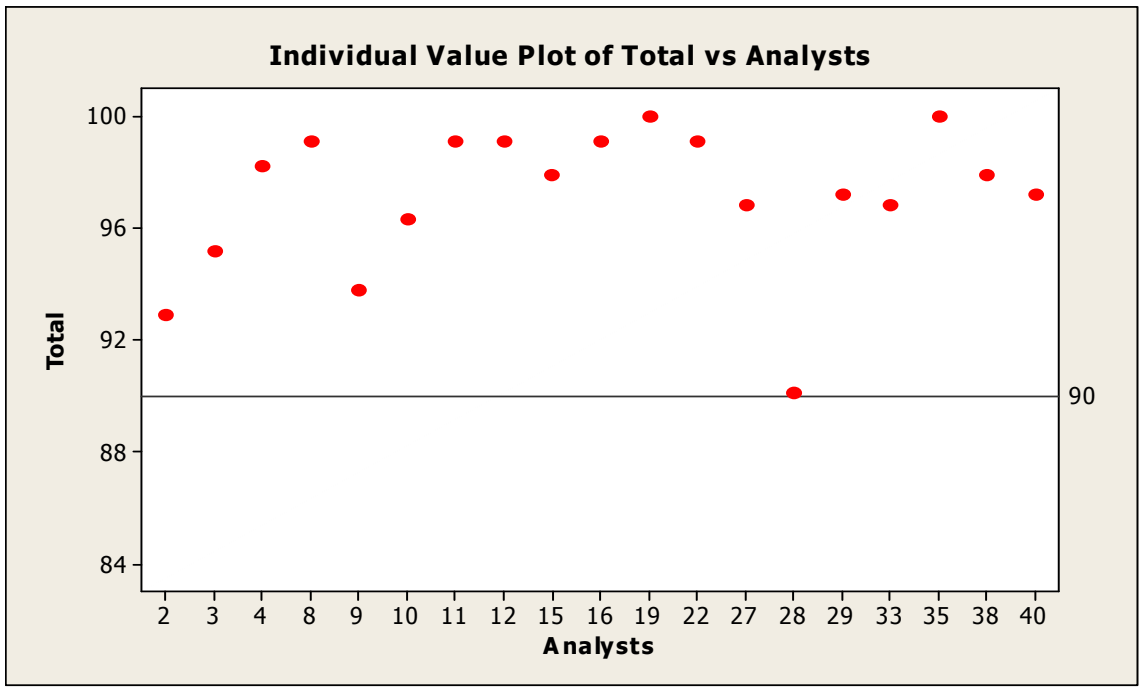
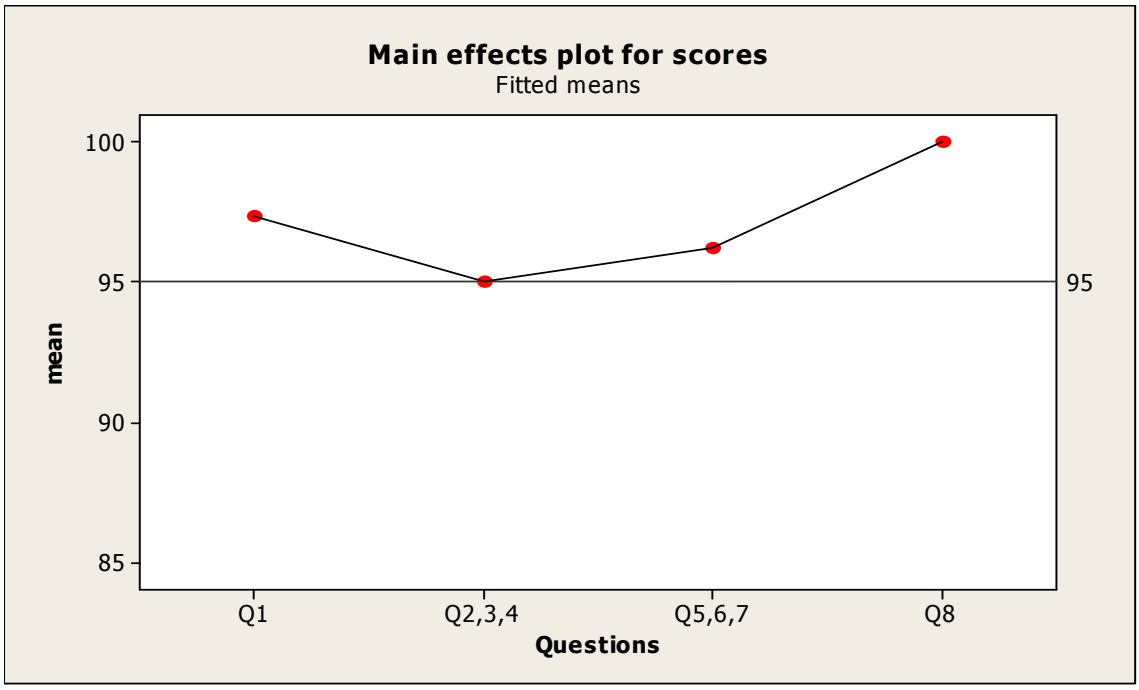


Figure 3.3: Main effect plot for identification scores by questions



The main effects plot for scores (Fig.3.3) shows that the mean of correct answers per question or group of questions is above 95%.

3.3.3 Phytoplankton enumeration results

All analysts used the Utermöhl test method as the preferred method for this exercise and all analysts used sedimentation chambers to carry out the cell counts however analysts used different sub-sample volumes and counting strategies for their samples.

Appendix 19 shows the analyst codes, sample codes and the cell concentrations in the triplicate low and high density samples. There are a few data points missing and this indicates that analysts did not return all the cell counts, in the case of analyst 37 and 24 their samples leaked from the sedimentation chamber and in the case of analyst 3 this analyst received four samples instead of three at the high cell concentration and only two at the low concentration. In all cases it was too late to send a replacement sample before submission of results.

Appendix 20 shows the sub-sampled volume used by the different analysts and the cell counting strategies used for each cell concentration. Most analysts used either 10ml or 25ml aliquots to be analysed except one analyst that used 2ml aliquots. One analyst used a 27ml aliquot instead of 25ml but for statistical purposes the results have been pooled together with the 25ml analysts.

The cell counting strategy mainly used for the low cell concentration samples was a Whole chamber (WC) count by the majority of the analysts, although one analyst used a half chamber (HC) and another a Transect (TR) count. At the high concentration analysts were divided mainly between using WC counts or TR counts, while one analyst used a HC count and two analysts used a Field of View (FoV) count.

3.3.3.1 Learning effects

The analysts mean cell count per replicate on low cell concentration samples (Fig.3.4) indicates that there are no significant learning effects between replicates, that there is no improvement in the performance either towards higher or lower cell numbers. This was done for the high cell concentration samples as well with similar results (Fig.3.5). However, there were more outliers in the high cell counts.

Figure 3.4: Analysts learning effects box plots for Low cell density samples

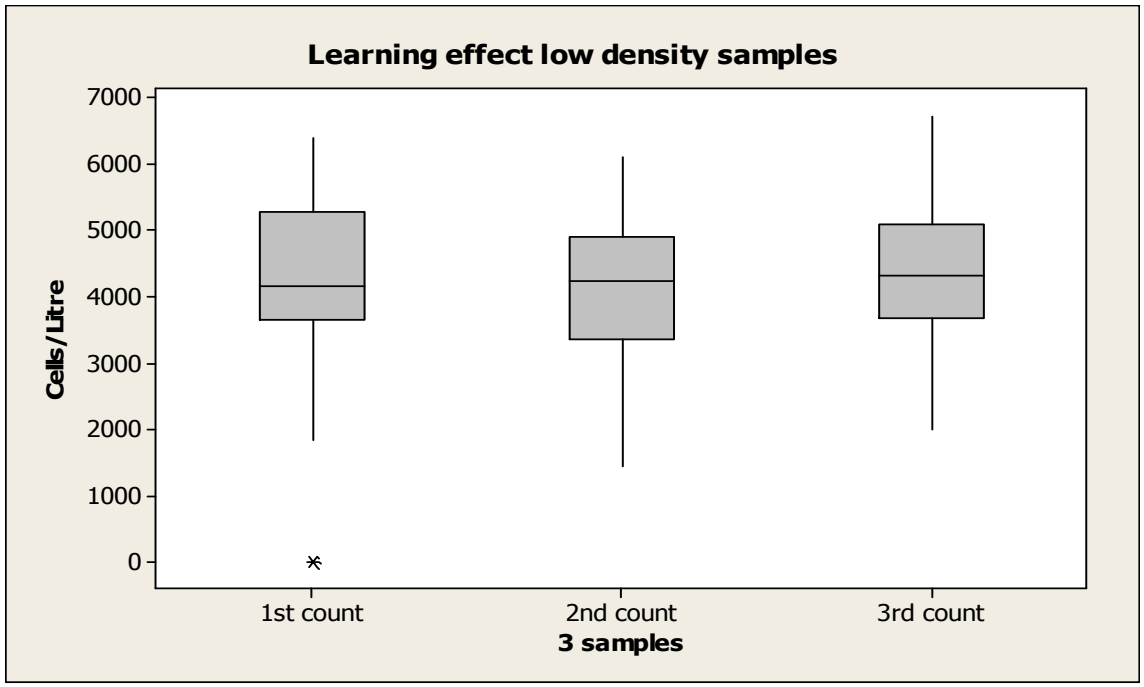
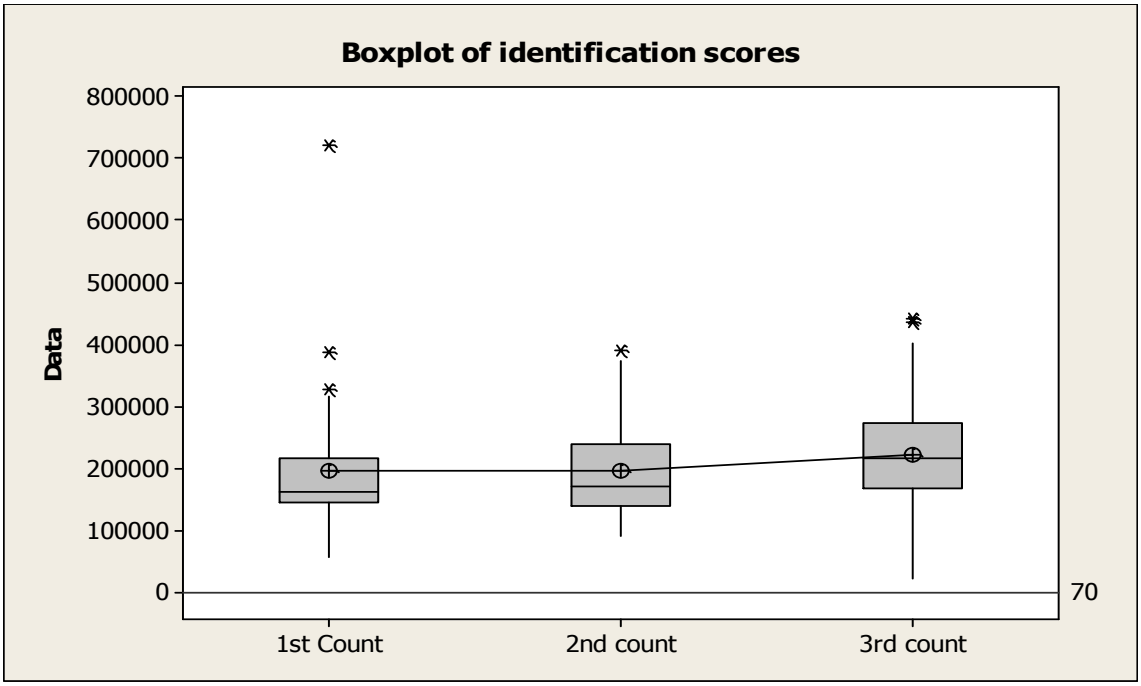


Figure 3.5: Analysts learning effects box plots for High cell density samples



A paired T-test was performed to compare the analysts replicate counts. Table 3.10 shows the Paired T-test between the 1st and 2nd count at low concentration. The 95% Confidence Interval for the mean difference of these two counts is (-175,653) and a P-value of 0.248 which demonstrates that the null hypothesis is true and that there is no significant differences between replicate counts.

Table 3.10: Paired T-test of 1st and 2nd count of low cell density samples

Paired T for 1st count - 2nd count				
	N	Mean	StDev	SE Mean
1st count	34	4385	1260	216
2nd count	34	4146	1025	176
Difference	34	239	1186	203
95% CI for mean difference: (-175, 653)				
P-Value = 0.248				

This Paired T-test was performed for all cell concentrations Low and High (Table 3.11) and all possible permutations of sample replicates (1st Vs 2nd, 1st vs. 3rd, 2nd vs.

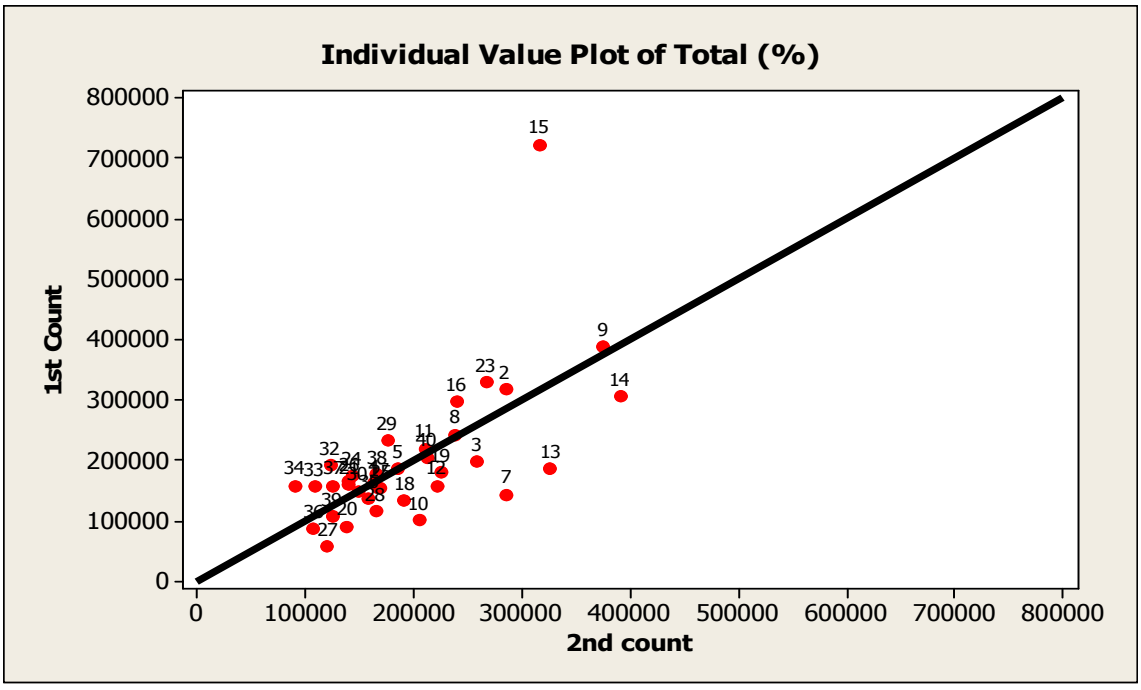
3rd) and the results indicated that there are not learning effects between replicate counts overall.

Table 3.11: Paired T-test of 1st and 2nd count of high cell density samples

Paired T for 1st Count - 2nd count				
	N	Mean	StDev	SE Mean
1st Count	36	196906	115574	19262
2nd count	36	197207	75624	12604
Difference	36	-301	88649	14775
95% CI for mean difference: (-30295, 29693)				
P-Value = 0.984				

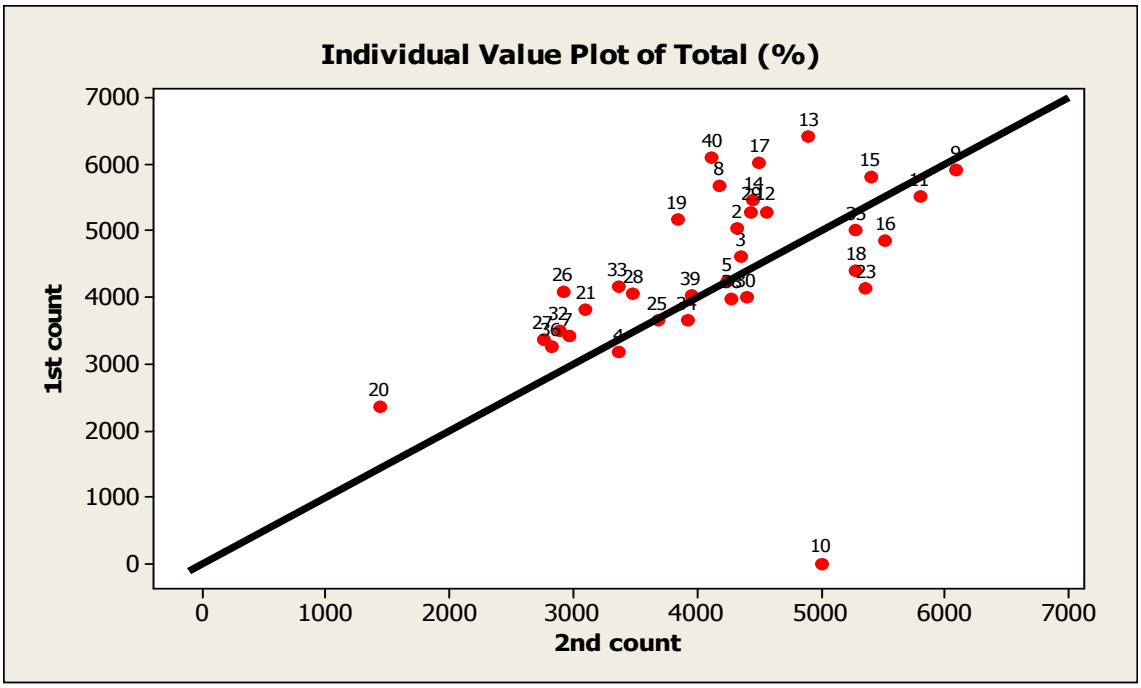
The comparison of the first cell count against the second cell count at both densities (Figs. 3.6 & 3.7) shows good agreement between replicates (red dots are close to the reference line).However, there are some outliers; analyst 15 in figure 3.6 where the 1st count was close to 700000 cells L^{-1} and the second was just over 300000 cells L^{-1} and analyst 10 in figure 3.7 are outliers.

Figure 3.6: individual values by analysts 1st count-2nd count at high concentration



Overall, it can be summarised that learning effects have not been observed in the analysis of both cell density sample replicates and that while there is variability between replicates of analysts the paired T-test indicates that this difference is not significant.

Figure 3.7: individual values by analysts 1st count-2nd count at low concentration



3.3.3.2 Method effects

The Whole Chamber (WC) cell counting strategy was used by most analysts on the samples containing low cell concentrations (Appendix 20). 75% of analysts chose the 25ml sub-sample volume and 24% used the 10ml sub-sample volume. One analyst used a different sample volume (2ml). Two analysts from those using the 25ml volume, two used different counting strategies, one did a transect count and one did a half chamber count (Fig.3.8).

There is insufficient data to compare all the different counting strategies and volumes settled with the low cell concentration samples but we can compare the results of those using the whole chamber counting strategy at 10 and 25ml volumes (Fig.3.9).

Figure 3.8: Box plot of 10ml, 25ml, 2ml sub-samples at low concentrations against counting strategies

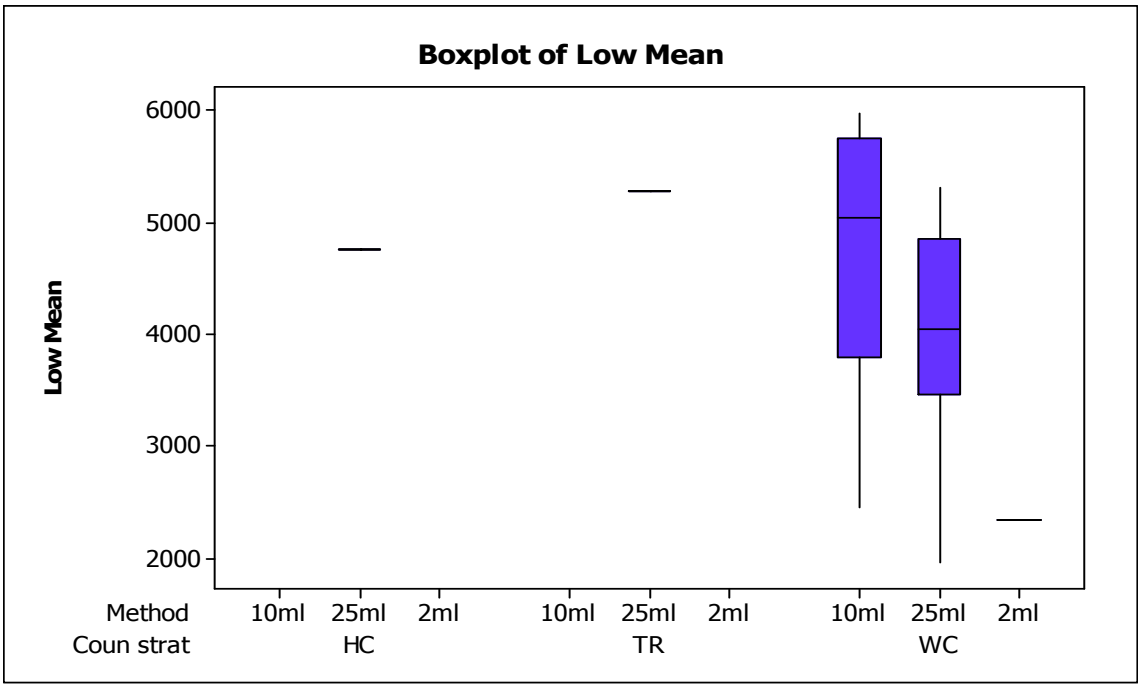
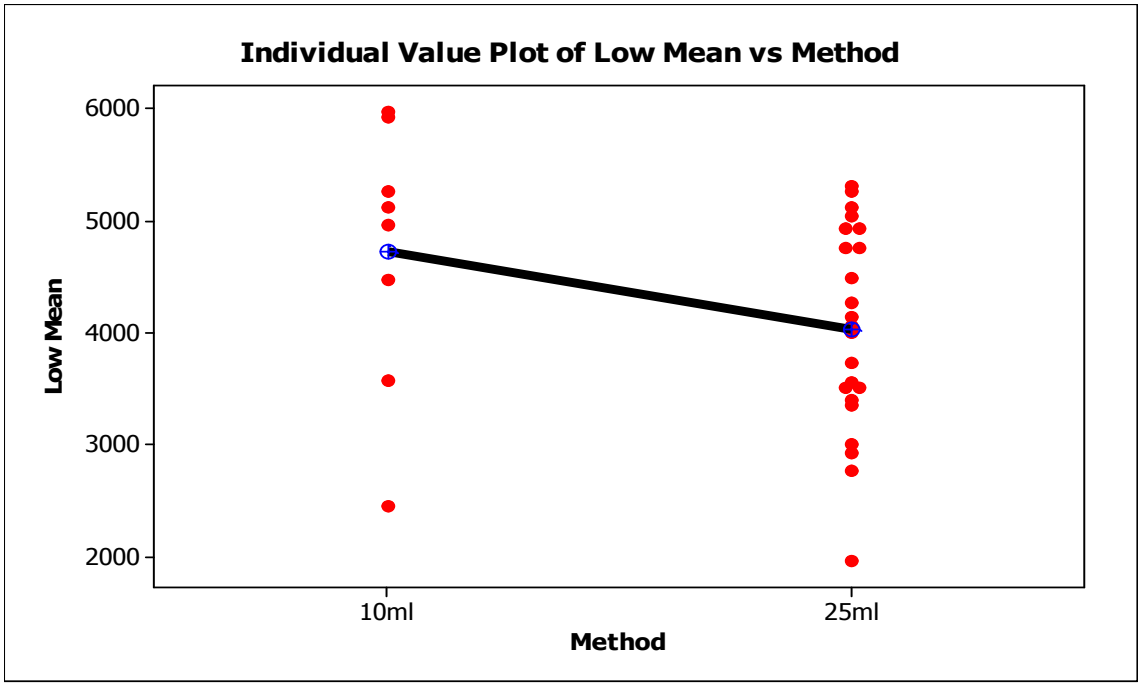


Figure 3.9: Individual value plot for low density samples



The mean values of all analysts using 10ml or 25ml volumes (Fig.3.9) suggests that there may be some differences between the mean of analysts using 10ml and 25ml

samples volume. A two sample T-test was carried out (Table 3.12) to test whether the differences in the mean values of 10ml versus 25ml analysts were significant. The result (P-value=0.170) and confidence interval (-353, 1720) suggests that the null hypothesis is true; there is no significant differences between 10 and 25ml volume methods at low cell concentrations. The standard deviation was smaller in the 25ml (872) compared to the 10ml analysts (1198) suggesting that the variation tends to decrease if a larger volume is analysed at this cell concentration.

Table 3.12: Two sample T-test 10ml versus 25ml volume

Two-sample T for Low Mean				
Method	N	Mean	StDev	SE Mean
10ml	8	4715	1198	424
25ml	25	4032	872	174
Difference = mu (10ml) - mu (25ml)				
Estimate for difference: 684				
95% CI for difference: (-353, 1720)				
P-Value = 0.170 DF = 9				

The samples containing high cell concentration analysts chose to use different sample volumes (10ml and 25ml) and counting strategies. Table 3.13 show the number of analysts and the settlement volume and counting strategy used. There were three different volumes used for analysis (10ml, 25ml, 2 ml) and four counting strategies (WC= Whole Chamber; HC= Half Chamber; Tr= Transect; FoV= Field of view). The counting strategies refer to the surface area of the chamber that was counted.

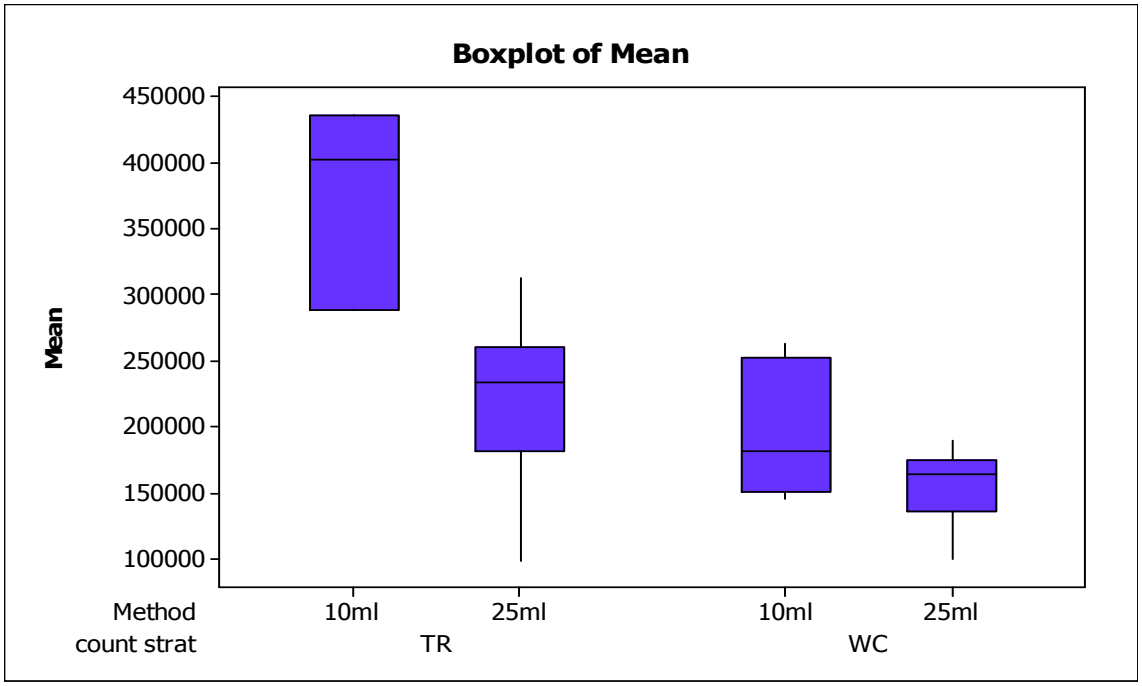
Eight analysts used a 10ml volume settled of which three used a transect count and five a whole chamber count. Twenty seven analysts chose the 25ml volume settled of which thirteen used a transect count and eleven used a whole chamber count. Two analysts used a field of view count and one a half chamber count. Therefore, the 25ml and 10ml volumes and the transect count and whole chamber count were compared.

Table 3.13: ANOVA statistics for high cell concentration samples

Rows: count strat Columns: Method				
	10ml	25ml	2ml	All
FV	0	2	0	2
HC	0	1	1	2
TR	3	13	0	16
WC	5	11	0	16
All	8	27	1	36
Cell Contents:		Count		

The Box plot of the mean results of all analysts using these volumes and counting strategies (Fig.3.10) shows that there are differences between volumes and also between counting strategies. Is this mean difference significant?

Figure 3.10: Box plot of Methods and counting strategies for high density



A general linear model (two way ANOVA with interaction) (Table 3.14) was fitted to compare the results of the analysts across the factors volume and counting strategy and the interaction volume*counting strategy. The P-value in all cases was <0.05 which is significant and we reject the null hypothesis that there are no significant differences.

Table 3.14: General Linear Model of method and counting strategy for high density samples

Factor	Type	Levels	Values
Method	fixed	2	10ml, 25ml
count strat	fixed	2	TR, WC

Analysis of Variance for Mean, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Method	1	33260934694	56425214036	56425214036	22.87	0.000
count strat	1	68233708189	85258666854	85258666854	34.55	0.000
Method*count strat	1	17557939388	17557939388	17557939388	7.12	0.013
Error	28	69092177089	69092177089	2467577753		
Total	31	1.88145E+11				

S = 49674.7 R-Sq = 63.28% R-Sq(adj) = 59.34%

The residual plots of the mean results (Fig.3.11) suggests that the results are normal and randomly distributed and the values in the versus fits and order value graphs are randomly scattered across the zero which indicate that there is no observation order effects or no constant variance effects. The four in one residual plots indicate that the data does not violate the statistical assumptions and that the model fits the data reasonably well.

Figure 3.11: Residual plots of high density samples mean analysts results

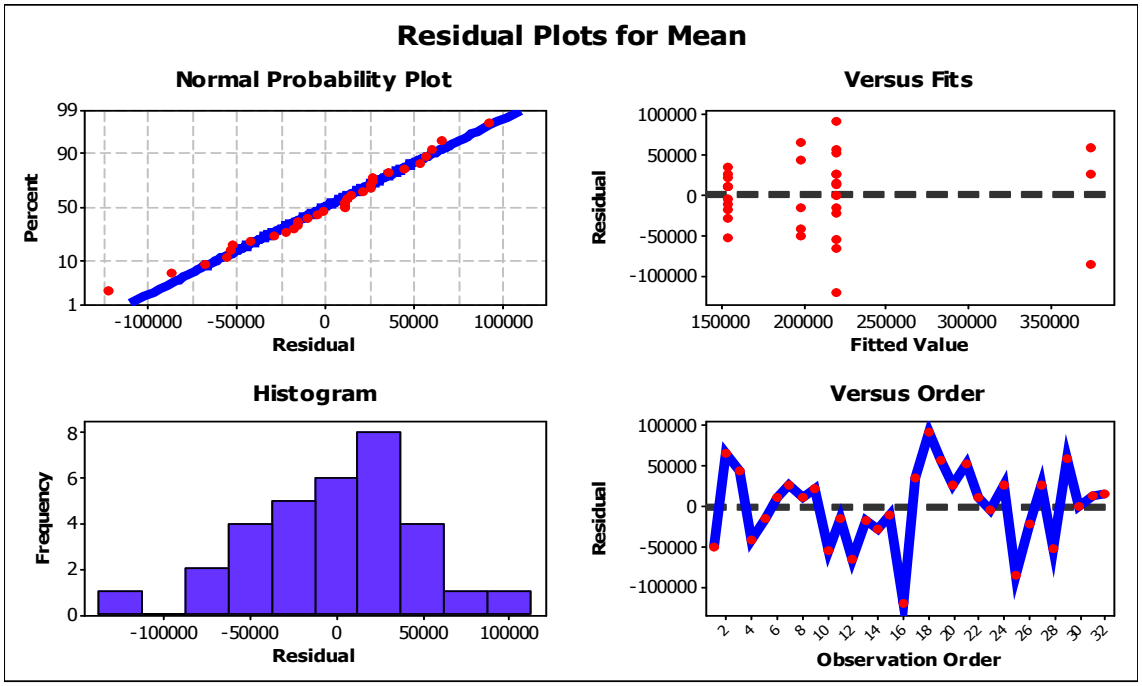
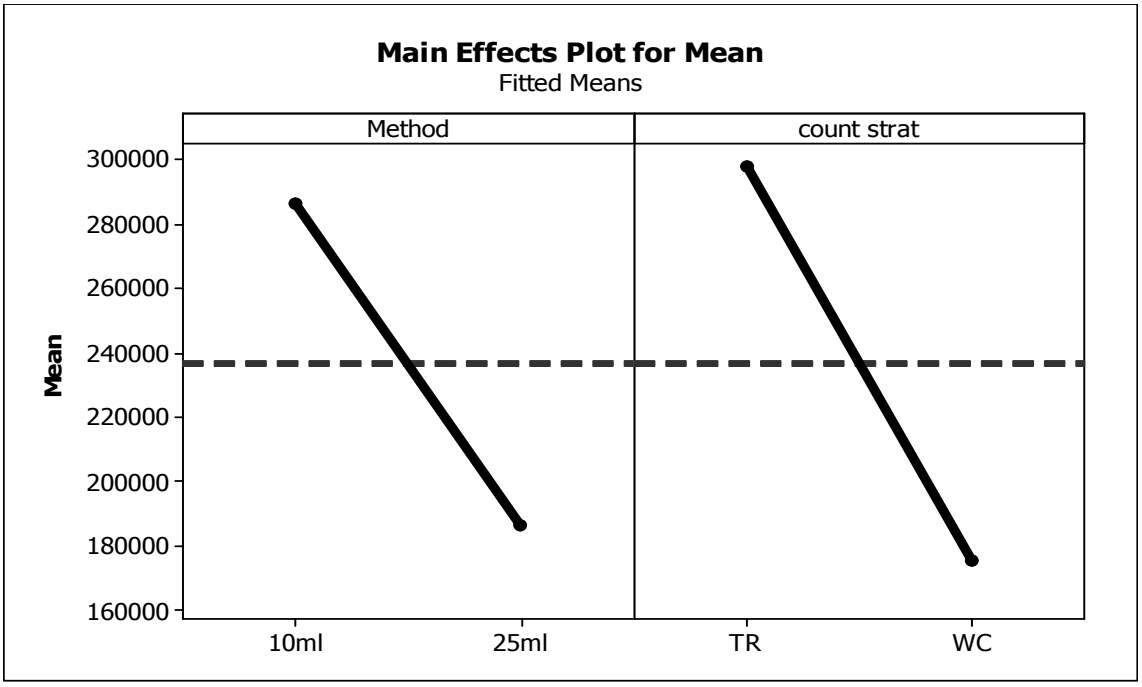


Figure 3.12: Main effects plot of high density samples by method and by counting strategy



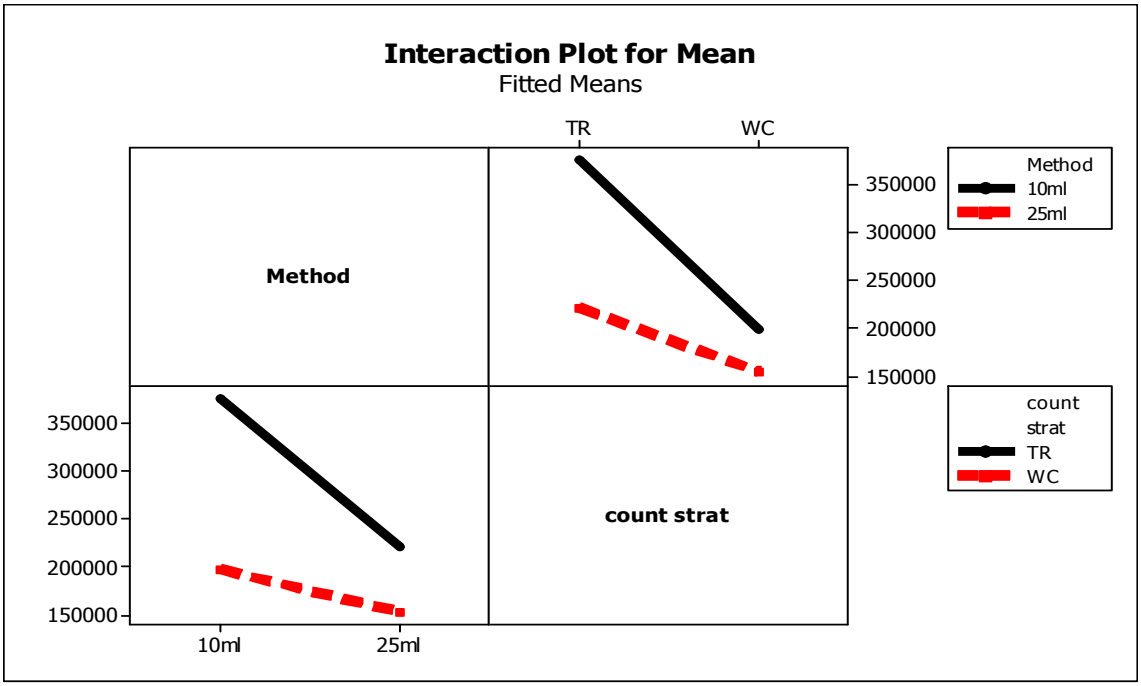
The main effects plot (Volume method and counting strategy) (Fig.3.12) illustrates that there are differences between volume methods (10ml, 25ml) and counting

strategies (WC, TR). Higher cell concentrations on average are found by analysts using 10ml volumes than 25ml volumes and by analysts using the transect count rather than the whole chamber count. The difference between counting strategies is however larger than the differences between volume methods.

The interaction plot (Fig.3.13) which shows the interaction between factors and suggests that the differences in mean cell concentrations between volume methods is smaller when the whole chamber counting strategy is used and that the differences in mean cell concentrations between counting strategies is also smaller when the 25ml volume method is used.

Summarising, the mean cell concentration of high density samples are more comparable when the 25ml volume and whole chamber counting strategy are used in conjunction.

Figure 3.13: Interaction plot of counting strategy, Method for high density samples

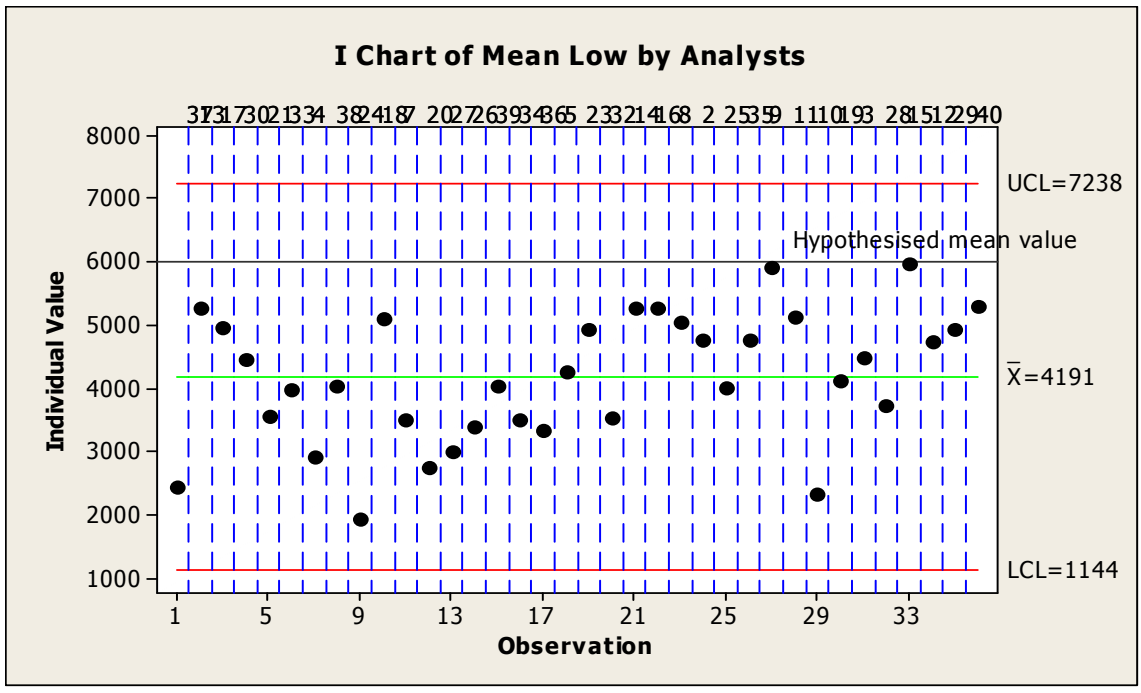


3.3.3.3 Hypothesised means

The hypothetical values obtained by the organising laboratory (Appendix 18) for the low density samples was rounded to 6000 cells L^{-1} final concentration and for the high density samples was 333333 cells L^{-1}. This is the samples theoretical real values calculated before they are dispensed into sterilin tubes.

The I-chart of the mean results of all analysts for the low density samples (Fig.3.14) compared to the hypothesized true value (blue line) suggests that most analysts tend to underestimate this value on average by 2000 cells L^{-1}.

Figure 3.14: I chart of mean results (Low) by analysts and hypothesised mean



Only two analysts, codes 9 and 15 were close to the potential real value of the sample. This underestimation is independent of the volume used (Fig.3.15). The I-chart of the mean results of all analysts for the high cell density samples (Fig.3.16) suggests that the underestimation of the hypothesised values (yellow line) occurs independently of the cell density of the sample.

Figure 3.15: Individual plot (low) of 10ml and 25ml minus hypothesised mean

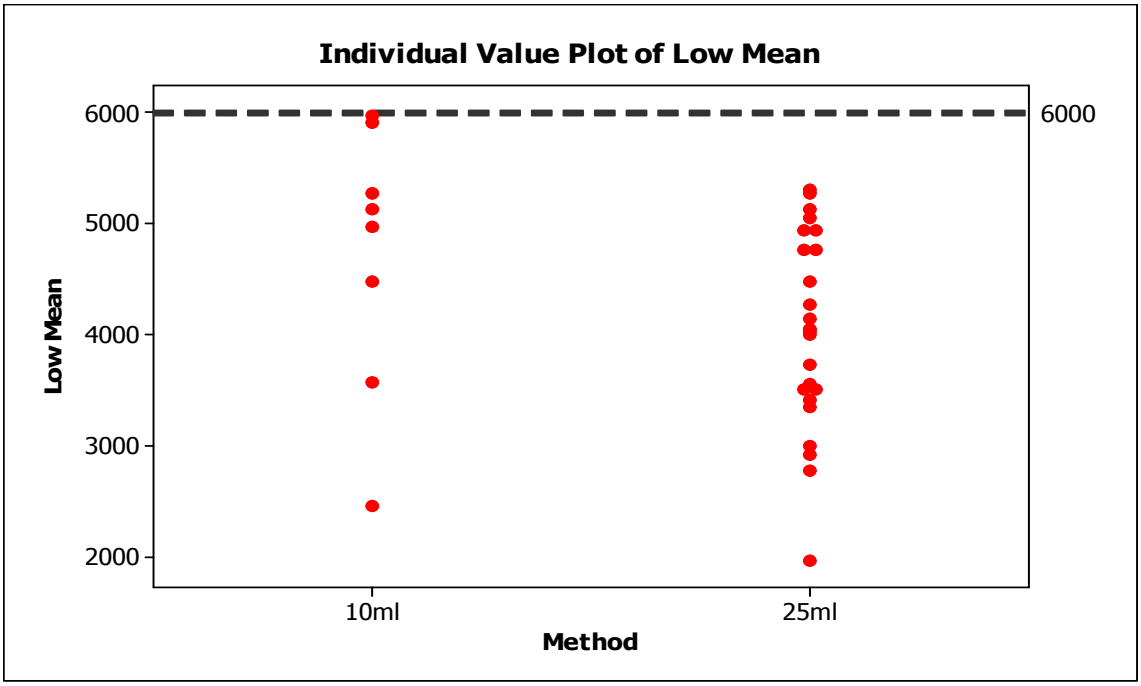
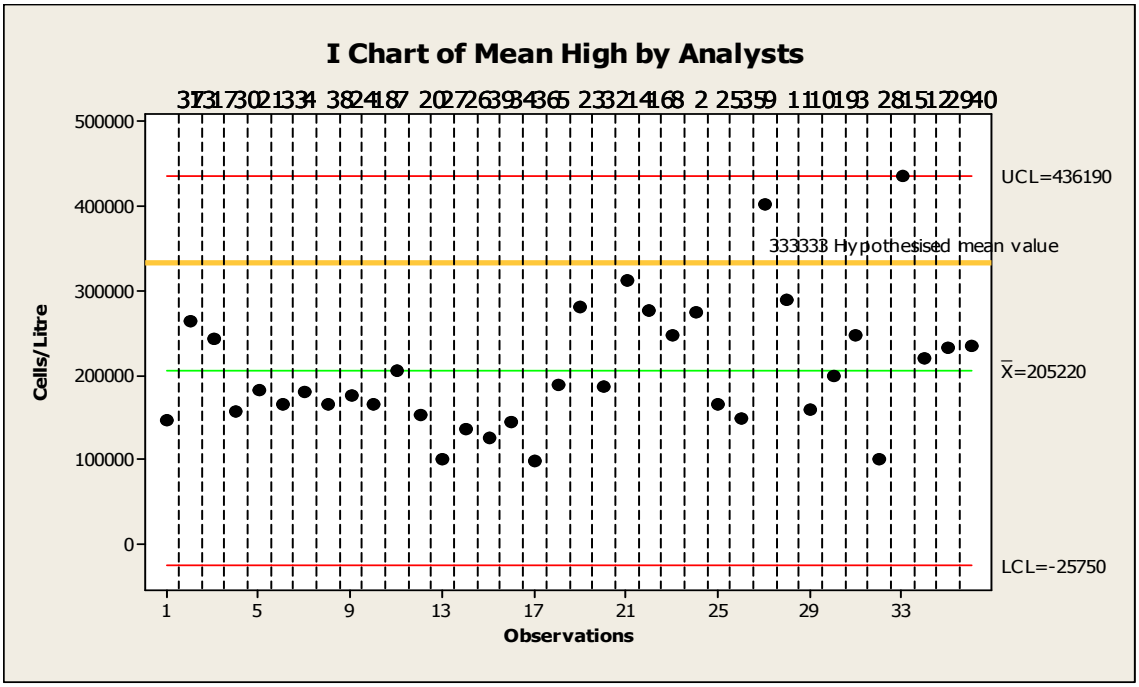
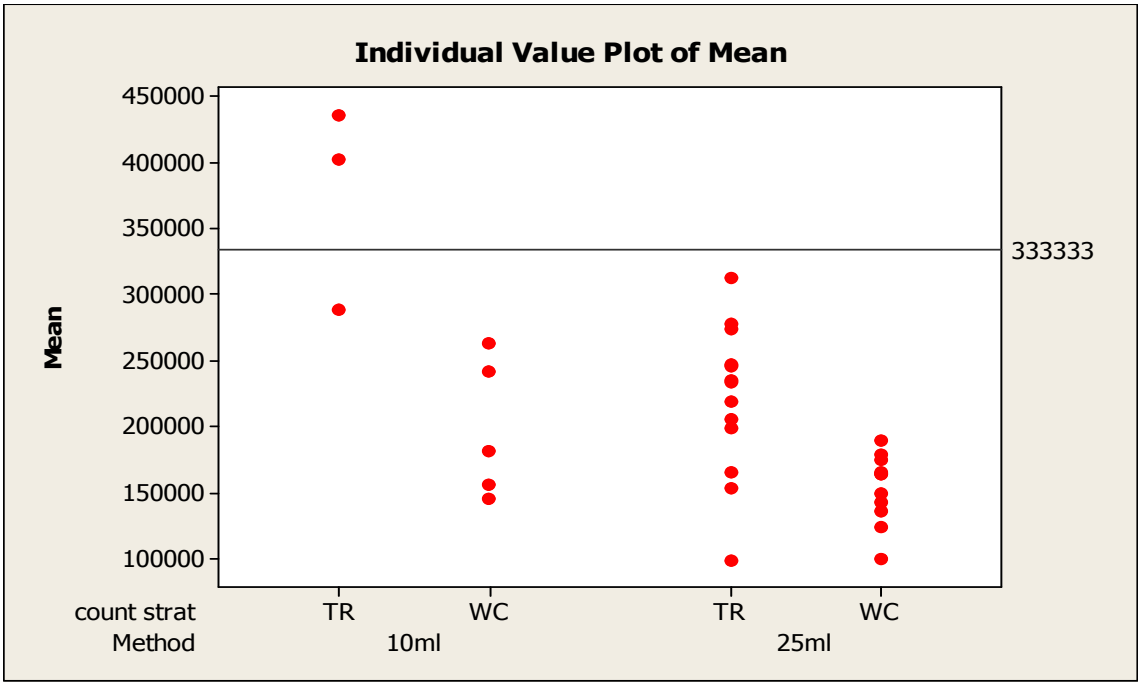


Figure 3.16: I chart of mean results (High) by analysts and hypothesised mean



The hypothesised value for the high cell density samples is on average around 130000 cells L^{-1} higher than the average value found by the analysts. This underestimation if found independent of the volume method and counting strategy used in the samples (Fig.3.17) apart from the analysts using the 10ml volume and transect cell counts.

Figure 3.17: Individual plot (high) by method and counting strategy minus hypothesised mean.



The Bias plots for both cell density samples (Figs. 3.18 & 3.19) against the Hypothesised means clearly illustrate that the bias is always negative.

Figure 3.18: Individual value plot low bias

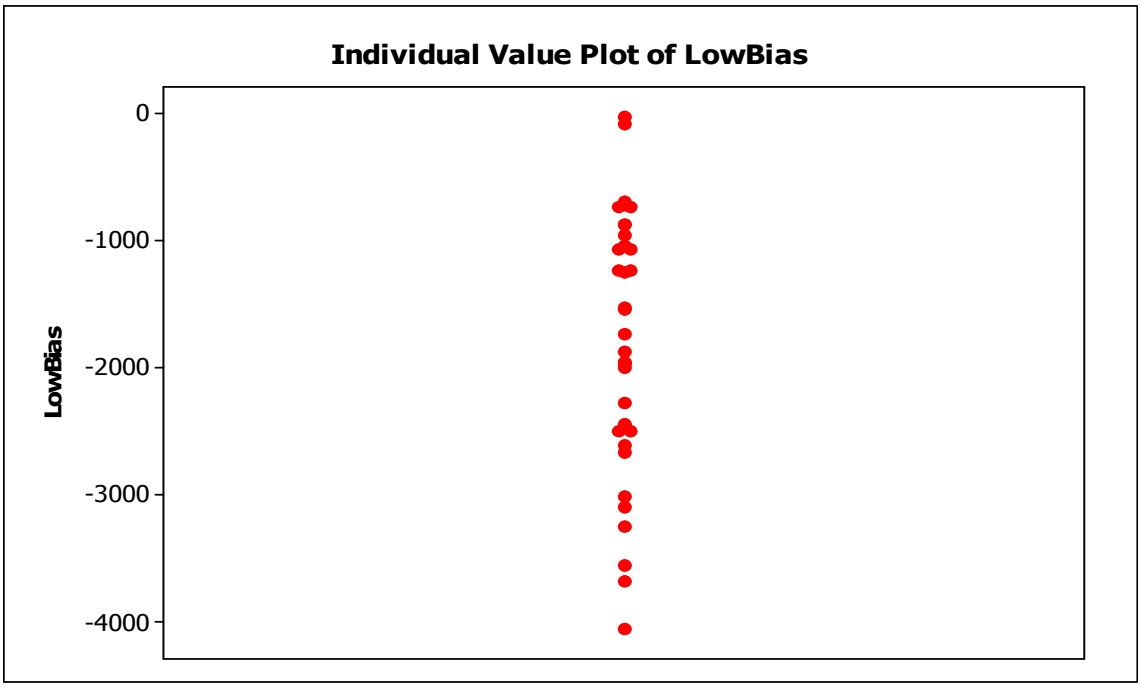
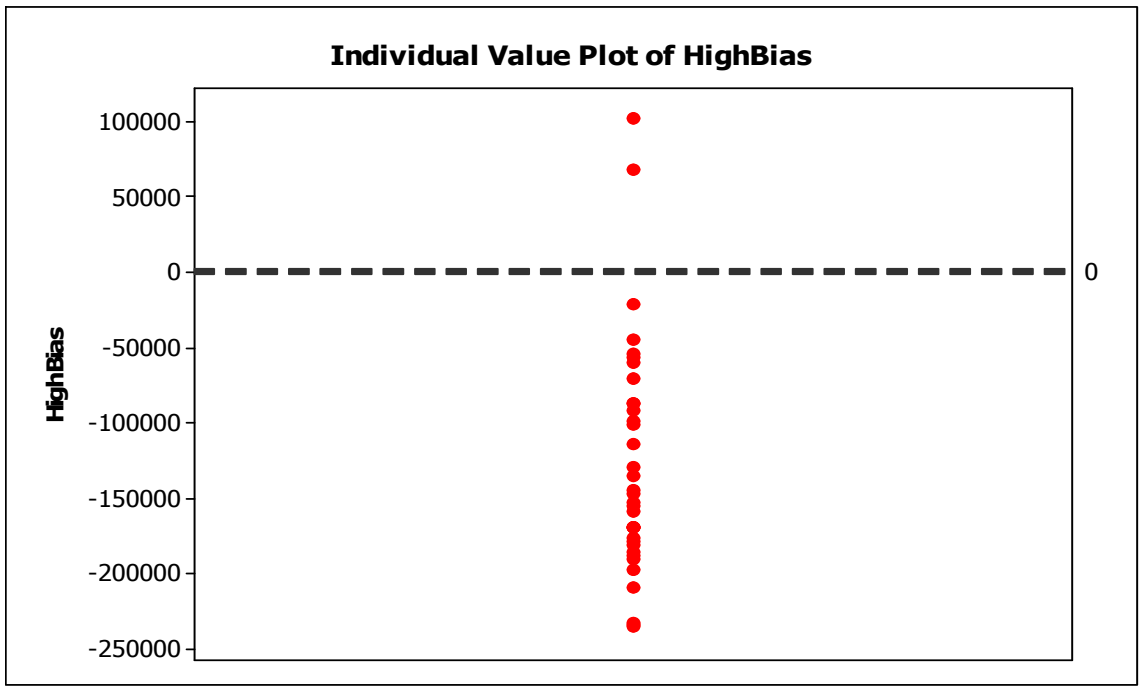
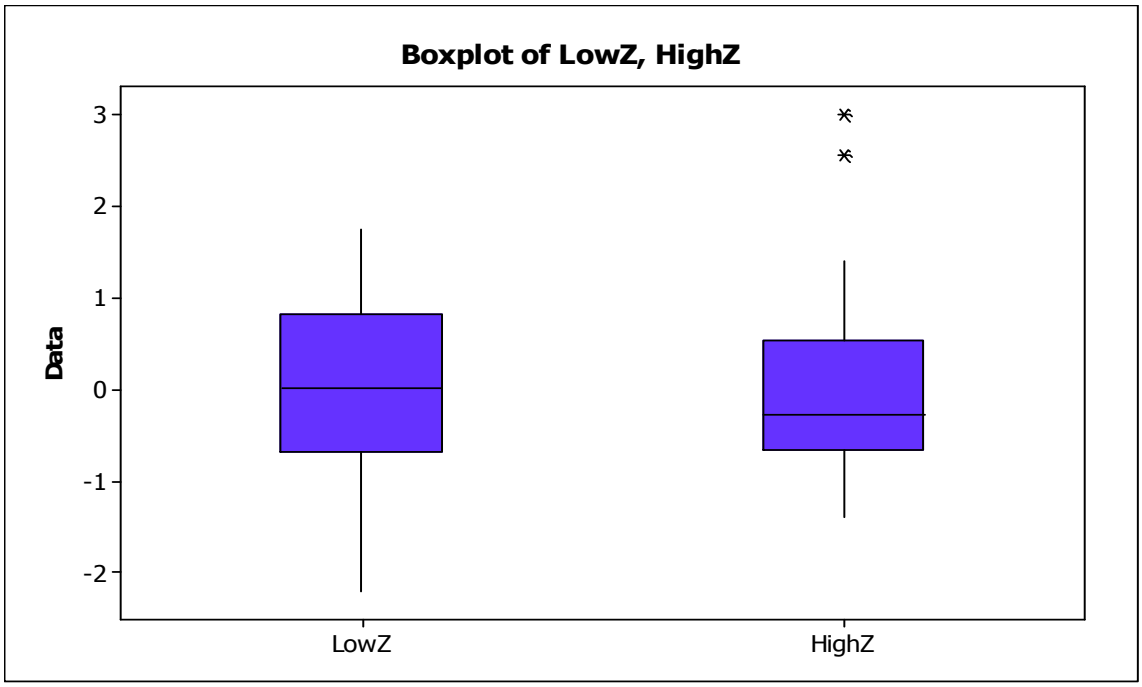


Figure 3.19: Individual value plot high bias



These results can be converted into Z-scores using the hypothesised means, when this is done the bias box plot of the Z-scores (Fig.3.20) for both densities these are comparable. This underestimation is approximately 30% of the hypothesised value.

Figure 3.20: Box plot of Low versus High Z



3.3.3.4 Z-scores

All the results have been used in this exercise to calculate the mean and 3 Standard deviations of the mean. Z-scores have been calculated for each analyst and laboratory for both cell density samples (Figs. 3.21 & 3.22).

These results show that there is variability within and between laboratories at both cell densities but that this variability is within the estimated parameters (3 standard deviations) set for the exercise.

The pattern observed in the I-charts suggests that laboratories tend to perform similarly regardless of the cell density of the samples. This means that laboratories that obtained a positive Z-score for one density usually obtained a positive Z-score for the other. This pattern was observed in most laboratories except in laboratory M and perhaps not so clearly in laboratories V and K.

Figure 3.21: Z-score (Low) by lab code

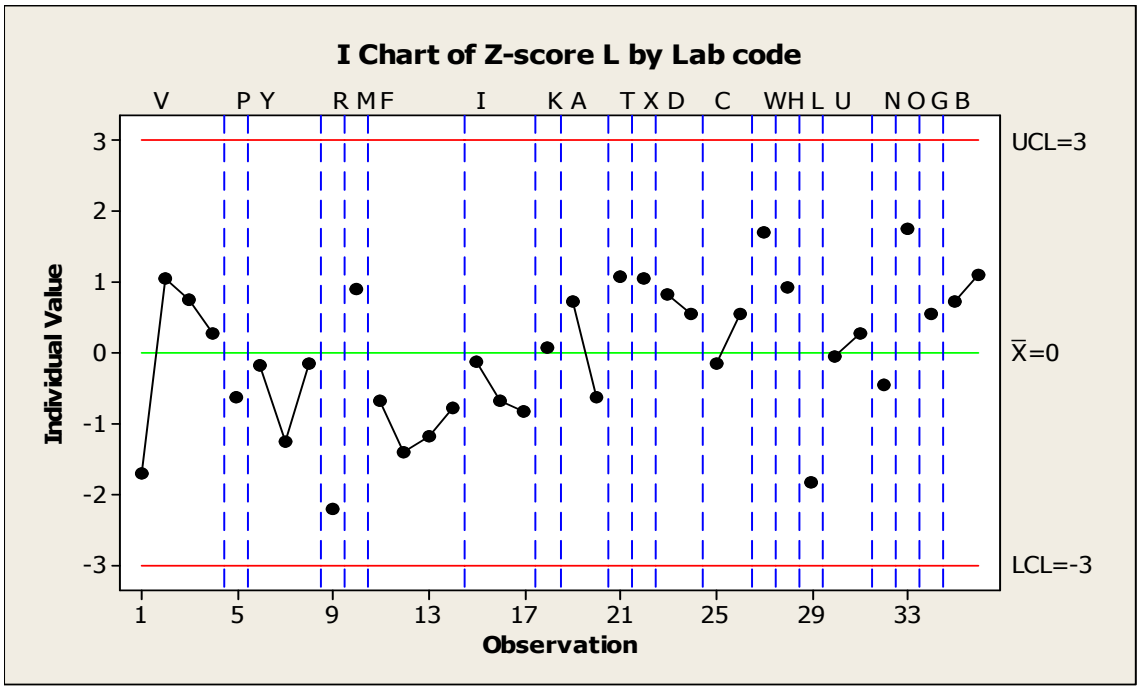
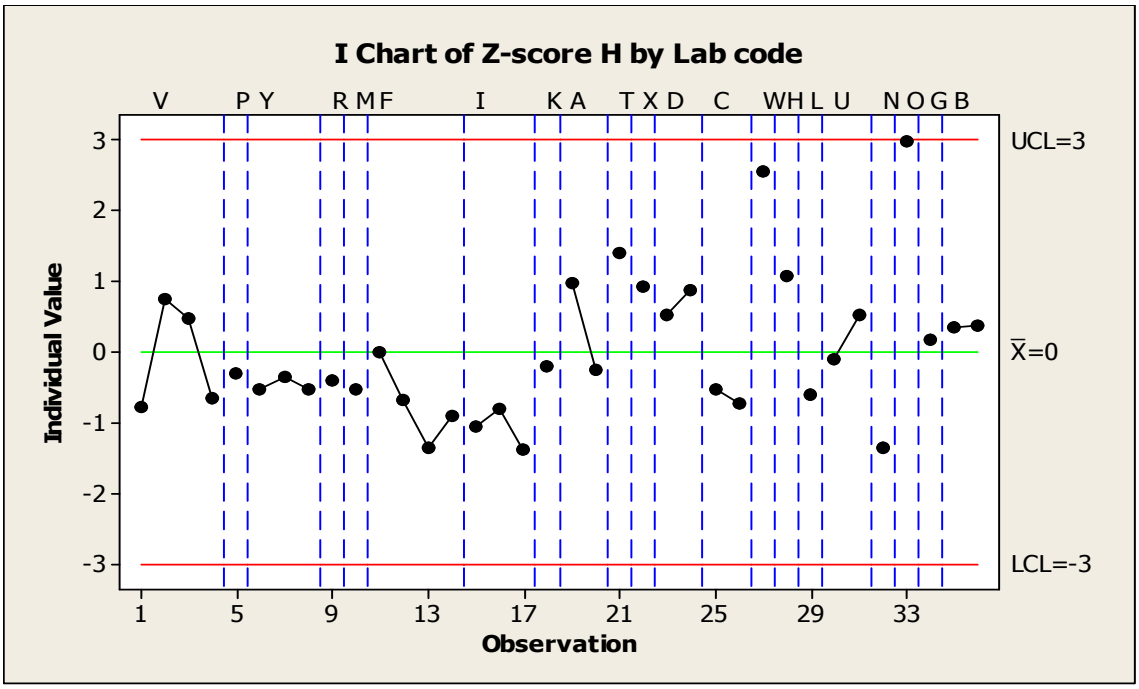


Figure 3.22: Z-score (high) by lab code



3.4 Performance evaluation

On the identification exercise, all analysts exceeded the 70% overall pass mark. All analysts performed above 90%, two analysts achieved full marks (100%), five analysts 99%, three analysts 98% and the remaining analysts between 90 and 97%. Overall, the standard on the identification exercise was very high by all of the participating laboratories and analysts.

On the enumeration exercise all analysts performed within the mean and +/-3 standard deviations prescribed for this intercomparison exercise.

While the analysts and laboratories performed within the parameters for the exercise there is evidence of lack of reproducibility of results between and within laboratories.

All laboratories participating in this exercise used the Utermöhl cell counting method. There is evidence that laboratories will use different methodologies to analyse the samples in terms of the volume aliquot and the counting strategy used. The volume depends on the laboratories and their internal operating procedures and the counting strategy appear to depend on the cell concentration of the analyte.

3.5 Discussion

3.5.1 Identification exercise

The quiz results were analysed to study how participants did overall and also to investigate whether some participants performed better at identifying particular phytoplankton species over others. All analysts completing the exercise did exceedingly well with an overall score of over 90% of correct answers for all analysts.

There were no significant differences in the way analysts answered certain questions compared to others. Analysts did slightly better in the questions on diatoms than questions on dinoflagellates but not significantly.

The question on 'naked dinoflagellates' (Q8) was the best scored question of the exercise with full marks for all analysts. This perhaps was due to the fact that the illustration used was probably known to the analysts.

There were a number of images which analysts found more difficult to identify than others in this exercise. These were image A (Q4) (*Heterocapsa triquetra)* and images A and B (Q5) (*Chaetoceros peruvianus* and *Chaetoceros densus*). The erroneous identification of images A and B in Q5 may be related to laboratories which put more emphasis on the identification of toxin producing phytoplankton species. These images depict diatoms that may be able to cause damage to finfish through physical and mechanical irritation of the fish gills, therefore laboratories and monitoring programmes dealing exclusively with toxin producing algae would have found difficulties in identifying this group of organisms. The difficulty with identifying *H.triguetra* in Q4 may be related to a geographical issue. The reason for this is that all participating laboratories and analysts from America got this identification wrong, while the rest of laboratories from Europe got it right, but it could be only a coincidence.

The specific question on *Dinophysis* (Q1) returned good results from all analysts with correct percentage answers above 90% This demonstrates a good knowledge at a global level of this group of organisms.

The question on *Pseudo-nitzschia* (Q7) asked analysts which view (and where) of the silica frustule of this pennate diatom should be used to carry out a width measurement of this organism. There were given two views (girdle and valve view). All participants answered correctly the theoretical part of this question however the follow up question asked analysts which of the following images of *Pseudo- nitzschia* should be used to make that measurement? Two analysts responded incorrectly to this question suggesting that the theoretical knowledge of phytoplankton taxonomy not always matches the experiential knowledge.

These results raised several questions which should be studied in further experiments and intercomparisons. One of the questions was whether the pass mark for the exercise should be raised? All analysts seem to be well above the 70% pass mark, perhaps for this type of exercise the mark should be raised slightly from here, maybe to 80 or even 90%. Or is the exercise too easy? Should it be harder questions?

Should graphics and illustrations be used in these exercises? The answer to this question should be yes as we have already seen in question 7 that sometimes, the theoretical knowledge, does not correspond with the practical knowledge, or in question 8 it is easy to find illustrations in publications that are known to most participants, so the use of Scanning Electron Microscopy (SEM) or Transmission Electron Microscopy (TEM) images of organisms to show particular taxonomical details may be more advantageous.

Also, it would be a good idea to have a group of taxonomy experts in the different algal groups validating the identification exercise to have more confidence in the exercise and also that the questions asked from a particular image are fair and that the image itself has enough quality to be in the exercise.

Which algal groups should be included in an intercomparison of this kind? When most laboratories participating in this scheme are only interested in toxin producing algae. Is it fair to include other species? My opinion would be that technicians identifying algae should be made aware of as many toxic, harmful, nuisance algae around the world as possible as it is difficult to predict which future challenges lie ahead for their monitoring programmes. The use of diatoms, for example which can

be harmful to fish and are cosmopolitan like *Chaetoceros spp.* and other species that become nuisance and produce algal blooms like *Phaeocystis spp.* for example are important groups of algae that should be recognized by all.

3.5.2 Enumeration exercise

The analysed data demonstrates that there is no evidence of learning effects by analysts on replicate samples at both cell densities.

Most analysts chose different sub-sample volumes to analyse their low density samples, these were either 10ml or 25ml aliquots. Independently of the volume used, most analysts decided to use a Whole Chamber (WC) counting strategy for the low density samples. This was the most likely counting strategy choice because for this concentration it would be typical based on experience to count the total cell number in the whole area of the sedimentation chamber.

It is possible that if a bigger volume had been given instead of 30ml samples, some laboratories would have used other volumes.

The data suggests that there are differences in the mean concentration between 10ml and 25ml sub-sample volumes but that this difference is not significant. The Standard deviation of results for the 25ml sub-sample is smaller suggesting that the variation is smaller in 25ml sample volumes at this cell concentration.

Analysts chose different counting strategies to analyse the high cell concentration samples but they continue using the same volume independently of the cell concentration of the samples. The main counting strategies used were Whole Chamber (WC) and Transect (TR) counts. Analysts were divided as to whether they should use one counting strategy or another.

The results obtained suggest that there are significant differences in the mean concentration between 10ml and 25ml sub-sample volumes and between TR and WC counting strategies. This difference is significantly larger in 10ml than in 25ml sub-samples and larger again in TR than in WC counting strategies.

This indicates lack of reproducibility between laboratories using different volumes and counting strategies for their samples.

Results of the high density samples by volume sub-sampled or counting strategy are significantly different. Should protocols based on experience be set in terms of the volume sub-sampled and the cell counting strategy depending on the cell concentration of the samples?

The bias test using the hypothesised values against the Low and High cell concentration results show that all the values are below zero. This would indicate that there are other methodology effects that potentially have an influence in this underestimation independently of the volume and the counting strategy used.

While the bias is larger at the high cell concentration compare to the bias at the low concentration both bias are still comparable as a Z-score. The underestimation is similar at both cell densities (approximately 30%) which suggests that these effects are related to the sample setup rather than the analysts as the hypothesised values are based on raw counts taken directly from the master mix compare to the analysts results based on 1ml aliquots spiked into 29ml sterilin tubes This means that cells are being lost potentially due to the materials used (sterilin tubes) homogenisation and settling technique of the sample after these have been prepared.

Preliminary studies not published here suggest that how the sample is homogenised accounts for some of this variability, also some cells seem to get stuck onto the walls of plastic surfaces, and it has been found that some cells are found left behind in sterilin tubes and sedimentation chambers which would account for some of the differences between the hypothesised values and the analysts values.

Since the test method tends to undererestimate by as much as 30% the hypothesised values, should correction factors be introduced for this method? or How these effects can be minimised? Should the values be validated using another method like flow cytometry?

To avoid methodology effects caused by factors like counting strategies, guidelines should be given as to which counting strategy should be used depending on cell concentrations. Perhaps for high cell concentrations fields of view or transect counts should be used instead of whole chamber cell counts.

Some recommendations for future studies include the use of larger sample volumes (100ml). The cell concentration ranges should depend on the species used and take into account the ranges found in nature. *Dinophysis* are more likely to be found in small cell densities in samples and they are known to cause toxic events even at these small quantities where *Pseudo-nitzschia* cell densities are only relevant when they reach higher cell concentrations (50000 cells L^{-1}).

The use of reference materials or validated counting material needs to be studied to study more critically the variability in cell counts found in the different laboratories.

4. General Conclusions and future work

This study was designed to investigate how laboratories across Europe involved in monitoring microalgae would perform using inter laboratory comparison tests in the enumeration and identification of microalgae.

The main conclusions from this work are that most laboratories engaged in phytoplankton monitoring use the Utermöhl cell counting method as the standard technique for analysis. This technique allows both the identification and enumeration of microalgae in the same sample at the same time which is an advantage over other methods, however in the literature this method is mostly described as a quantitative method.

This study used a qualitative expression of reliability for this test method. It is in fact the first time that such measure had been used in a phytoplankton intercomparison exercise.

This measure has provided us with interesting information on the reliability of microalgal identification by analysts through the use of false positive and negative

responses and the results indicate that the false negative rate is much higher than anticipated a priori. This means that analysts are more likely to identify a toxic organism as a non-toxic organism than vice versa creating a potentially critical problem on the management and release of information of monitoring programmes.

Also, this study has shown that analysts and laboratories using the same methodologies and techniques to analyse a set of samples from a sample population are comparable to the mean and standard deviation of the sample population. This is not the case when analysts and laboratories use different techniques to analyse the samples, which suggests that a uniform standard methodology is necessary to validate the results between laboratories.

The inter analyst reproducibility is poor indicating that even within the same laboratories analysts are performing differently, which suggests that regular within laboratory quality controls are necessary to improve the test method analysis technique. Positive results in the negative samples suggests that some laboratories are using poor cleaning techniques and have carry over problems which need to be addressed, therefore the importance of a good quality programme underpinning the test method.

The use of a reference value or gold standard based on best practice and repeatability studies as a kind of laboratory reference material for the exercise can be useful, but this is limited, as these values are not validated. There is, therefore, an imperative that certified reference materials are produced for future works containing a true value with a corresponding coefficient of variation, for these standards to be truly useful on the improvement of the laboratory techniques on the identification and enumeration of microalgae.

Method effects caused by the materials and techniques used on the samples indicate that analysts could be underestimating their cell counts by as much as 30%. This underestimation seems to relate to a number of issues that have not been addressed in these exercises but they should be part of any future work. These effects include the analyst technique, the homogenisation of the samples and losses due to the container

used. This issue is not critical for the exercise itself as these effects happen to all samples equally, but it does affect the results of real monitoring samples, where the, accurate quantification of toxic algae in a sample is fundamental for monitoring programmes. The question is whether correction factors should be included in the method, should the materials be changed or the technique improved to take account of these effects.

The results obtained in the 2009 exercise informed how the 2010 exercise was developed. The gold standard was not used in the latter exercise, but the technique used for preparing the materials seemed to work, so this was replicated in 2010. There was no need, then for a gold standard as we had already shown in 2009 that the results would be comparable if prepared in the same manner and used the same techniques. What it was done in 2010 was to allow the participating laboratories to decide what counting technique and volume aliquot to use based on their best practice. These effects were then studied through ANOVA statistics like main effect plots and interaction plots that differences exist in cell counts between laboratories using different aliquots and counting techniques. The critical factor remains, that while we proved that significant differences exist, using general linear models and simple t-test statistics, ultimately we cannot tell which counting strategies and aliquots are best as we have no validated reference materials to compare against. So, once more the improvement of future exercises passes through the production of these certified materials.

The taxonomic quizzes developed for this study have proved very useful for analysts. It provides a measurement of the theoretical knowledge of analysts engaged in the identification of microalgae and have proved very popular among the participants to discuss issues related to phytoplankton taxonomy.

These taxonomic exercises should be validated by a panel of experts, so that the questions asked are relevant and the images shown are of the necessary quality for the participants to answer the question.

Phytoplankton analysts appeared to have a good theoretical knowledge of phytoplankton taxonomy. Most analysts have performed exceedingly well in both taxonomic exercises. Images of phytoplankton species have proved useful tools for monitoring programmes as audit trails of correct identifications of the species which can be used for quality traceability purposes.

Future phytoplankton intercomparison exercises should take into consideration some of these findings as they provide the basis for the improvement of the scheme. In summary enumeration exercises success hinges on the preparation and production of certified reference materials in the future, for use in these interlaboratory exercises. The techniques used by analysts can then be compared and best practice be used. Method effects due to the containers used and homogenisation techniques of the subsamples need to be studied further through validation exercises to ascertain the losses (bias) due to these effects and make recommendations to monitoring laboratories on the use of correction factors.

In the identification exercise, the use of images and videos have proved very successful and a nice way of training and testing analysts taxonomical knowledge. These exercises need to be also validated and high standards set on the quality of the images used and that these should be assessed independently by an expert panel to provide a fair and robust exercise.

Overall the exercises have been very successful not only in terms of the quantity but also in terms of the quality of the laboratories and analysts taken part. In 2010, a survey was undertaking to obtain some feedback on the completed intercomparison exercises, some of the main conclusions from this survey were that the exercises were useful tools to develop the analysts taxonomic and enumeration skills, that helped quality and technical competency of the laboratories, that it was helpful to obtain accreditation for their method, and that the uncertainty of measurement could be used to update their own validation.

All surveyed also stated that it was very important that this type of exercise would continue into the future on a regular basis.

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Appendix 1

Instructions for phytoplankton intercomparison exercise PHY-ICN-09-MI1

Marine Institute BEQUALM Phytoplankton Proficiency Test PHY-ICN-09-MI1

Instructions for Sample Preparation, Cell counting, calculations & Identification

Please note that these instructions are designed strictly for use in this intercomparison.

1. Introduction

This Phytoplankton Ring Test is being conducted to determine any inter-laboratory variability in the enumeration and identification of Marine Phytoplankton species within and between laboratories from a number of samples spiked with cultured material. Please adhere to the following instructions strictly. Please note that these instructions are specific to this ring test.

2. Preliminary Checks and Deadlines

Upon receipt of the samples, the sample manager assigned to your laboratory for this exercise should make sure that the laboratory has received everything listed in the Return Slip and checklist form (Form 1). Make sure that all the samples are intact and sealed and check that you have received enough Taxonomic quiz forms (Form 2) for all the analysts registered in this exercise for your laboratory once you are happy that you have received everything you need to complete this exercise and samples and forms are in working order. Complete this form (Form 1) and send it by Fax to the Marine Institute, Galway. Fax No. 00353 91 387237. A receipt of Fax is necessary for the Marine Institute to validate the test process for your laboratory

Once you have received the samples, your laboratory has 4 weeks to complete the exercise and return the results of all the Bequalm registered analysts. The hardcopy of enumeration results (Form 3) and the Taxonomic quiz (Form 2) **must be received** by the Marine Institute by **March 20th, 2009**.

Please note: Hardcopy results and Taxonomic quiz results received after the March 20th 2009 date will not be included in the final report.

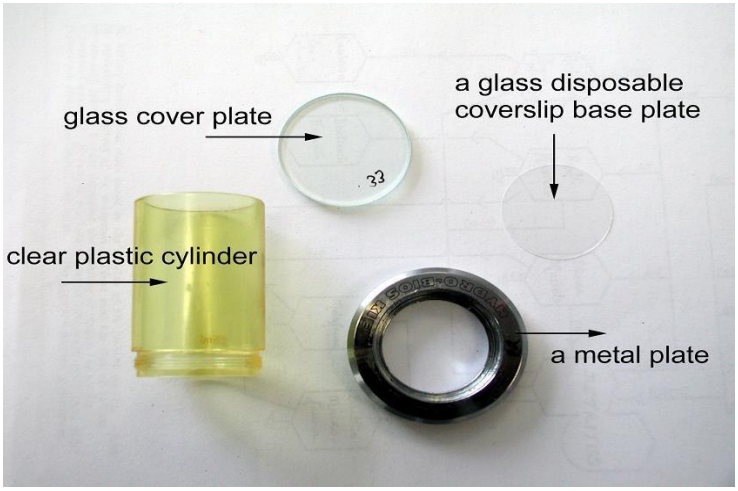
3. Equipment

- 6 Utermöhl cell counting chambers. **25ml sedimentation chambers should be used preferably.**
- Base plates and glass covers.
- Inverted Microscope equipped with long distance working lenses and condenser of Numerical Aperture (NA) of 0.3 or similar.

4. Sample Preparation

Sedimentation counting chambers consist of a clear plastic cylinder, a metal plate, a glass disposable cover-slip base plate and a glass cover plate (Fig 1). 6 sedimentation chambers will be required.

Fig 1: Sedimentation counting chamber



4.1 Place a clean disposable cover slip base plate inside a cleaned metal plate.

4.2 Screw the plastic cylinder into the metal plate. Extra care should be taken when setting up chambers. Disposable cover slip base plates are fragile

and break easily causing cuts and grazes. Careless handling can easily damage metal plates, and render them unusable.

4.3 **Important:** Once the chamber is set up, it should be tested for the possibility of leaks by filling the completed chamber with sterile seawater and allowing it to rest for a few minutes. If no leakage occurs, pour out the water and proceed with the next step.

4.3 To set up a sample for analysis **firmly invert the sample at least 20 times** to ensure that the contents are homogenised properly. Do not shake the sample.

4.3.1 Pour the sample into the counting chamber. (samples must be adapted to room temperature to reduce the risk of air bubbles in the chambers)

4.3.2 There should be enough sample volume in each sample to fill a 25ml Utermöhl sedimentation chamber. Top up the sedimentation chamber and cover with a glass cover plate to complete the vacuum and avoid air pockets.

4.3.3 If the sample volume just about fills the sedimentation chamber, top up with sterile seawater as this won't affect the concentration of the sample for this particular exercise.

4.3.4 Label the sedimentation chamber with the sample number from the sterilin tube.

4.4 Use a horizontal surface to place chambers protected from vibration and strong sunlight.

4.4 Allow the sample to settle for a minimum of twelve hours.

4.5 Set the chamber on the inverted microscope and analyse.

4.6 Enumeration results for each sample are to be entered on **Form 3 Enumeration Hardcopy Results Sheet.**

5. Counting Strategy

For this test a whole base plate count should be conducted.

5.1 The whole base plate of the chamber is counted by enumerating all cells within a continuous motion of field of view for the entire area of the base plate. This can be done by going from left to right or top to bottom, in a continuous series of sinuous movements in such a manner that the whole base plate is observed (Fig 2 and 3). Make sure the field of view does not exclude any uncounted area or overlap any area already counted.

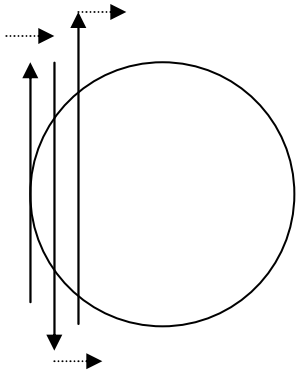


Fig 2

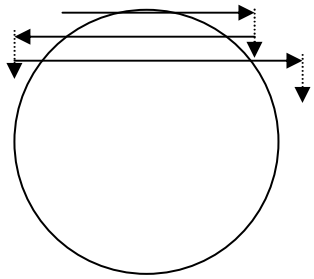


Fig 3

6. Samples

The samples for this intercomparison have been spiked with live cell culture material. This material have been preserved using acidic lugol's iodine and then homogenized following the IOC Manual on Harmful Marine Algae technique of 100 times sample inversion to extract sub-samples.

A set of subsamples has been used to set the true value of the sample population within 3 SD. The results obtained by all the laboratories will be compared against this true value. The purpose of this exercise is to study reproducibility of results in enumeration and identification of marine phytoplankton species between and within laboratories.

It is very important to spend some time becoming familiar with the sample and how the cells appear on the base plate before any count is done as part of the test. The reason for this is that cultured cells could be undergoing division or fusion and look slightly different to the known standard vegetative cell type. Also note that cells from

one species may vary in size. Some cells will appear smaller than others, this is normal in culture conditions, please make sure to count these.

Aberration of cell morphology can occur naturally in culture conditions and also upon preservation of samples with lugol's iodine. A big effort has been made to minimize this effect but take this into account when analyzing the samples. Cells have to be identified to species level, where this is possible.

As soon as samples are received, the sample manager is asked to check the samples for leaks or breakages. If a sample appears half full or completely broken, please inform Rafael.salas@marine.ie so we can send you another set of samples straightaway.

The sample manager assigned by each laboratory will receive the parcel with samples and forms for all the participants from their laboratory the sample manager should make sure that their laboratory has everything they need to complete the exercise. The sample manager should fill form 1: Return slip and Fax and send back to the Marine institute.

The most important task for the sample manager is to organize the settlement and analysis of the samples for everyone else in the laboratory As this year we have to analyze 6 samples it is important that once samples are settled analysts complete the analysis within one or two days maximum from sample settlement.

In order to do this the sample manager may decide to settle only a small number of samples at one time to avoid samples from leaking in the chambers before continuing with the rest. Remember everyone will have to analyse the same set of samples. This is particularly important for laboratories with 3 or more analysts.

Each sample should contain approximately a volume of 26ml; this means that a very small amount of sample may be left behind in the sample tube when the sample is poured into the 25ml sedimentation chamber. This is normal and should be the same for all the samples. A 26ml sample should be sufficient to fill a 25ml sedimentation

chamber to the top. Although some evaporation may occur during transport and settlement this should be minimal.

Please note: when converting cells per sample to cells per liter, use 25ml as the chamber volume.

7. Conversion Calculations of Cell Counts

The number of cells found should be converted to cells per Liter.

Please show calculation step in Form 3: Hardcopy enumeration results sheet

8. Identification

A taxonomic quiz has been designed for the identification part of the exercise. A number of photomicrographs and video clips of high quality will be provided for the exercise. All the images and video clips are copyright material.

The purpose of this exercise is to identify the marine phytoplankton shown in these images and video clips to genus or/and species level but also to identify correctly morphological and taxonomic characteristics unique to these marine phytoplankton species.

This year for the first time we have introduced the use of video clips. All participants will have to go onto the web to the following address: www.unique-media.tv/mie001 and log on using the **username: marine** and **password: bus7xehe**. Remember username and passwords are case sensitive.

The still images have been printed onto an authorized copy of Form 2: Taxonomic quiz. There should be one for each analyst. All the questions on the images and videos are printed on this form and all the answers should be written on this form too. Once you have finished the test you will have to post the original authorized copy back to us. We enclosed self addressed envelopes for this purpose. Make sure you keep a copy of your results before you send the form in the post.

Please identify and include your results on the Taxonomic quiz (**Form 2**).

The identification exercise carries a total of 400 marks.

Participants should name phytoplankton species according to the current literature and scientific name for that species. Where species have been named using a synonym to the current name and if this synonym is still valid or recognized the answer will be accepted as correct.

Examples of this are: *Prorocentrum cordatum* better known as *P.minimum* or *Akashiwo sanguinea* also known as *Gymnodinium splendens*

9. <u>Points to Remember</u>:

1. **All results must be the analysts own work. Conferring with other analysts is not allowed.**
2. **Before sending the original results in the post, make a copy of your own results just in case they get lost in the post.**
3. Form 3: Enumeration Hardcopy Results Sheet and Form 2: taxonomic quiz must be received by the Marine Institute, Phytoplankton unit by **Friday March 20th 2009.**

Appendix 2

Form 1: RETURN SLIP AND CHECKLIST

ATTENTION: Rafael Salas

Please ensure to complete the table below upon receipt of samples, and fax immediately to the Marine Institute. 00353 91 387237		
Sample Manager:		
Laboratory Name:		
Contact Tel. No. / e-mail		
CHECKLIST OF ITEMS RECEIVED	**(Please circle the relevant answer)**	
Sample number: _____	YES	NO
Sample number: _____	YES	NO
Sample number: _____	YES	NO
Sample number: _____	YES	NO
Sample number: _____	YES	NO
Sample number: _____	YES	NO
Set of Instructions	YES	NO
Enumeration Result Sheet **(Form 3)**	YES	NO
Taxonomic quiz **(Form 2)**	YES	NO
One MI Addressed Envelope	YES	NO

I confirm that I have received all items, as detailed above. Samples arrived intact and sealed.

(If any of the above items are missing, please contact Rafael.salas@marine.ie)

SIGNED (Sample manager): _____

DATE: _____

Appendix 3

Form 3_Enumeration Hardcopy results

Bequalm Intercomparison PHYI-CN-09-MI1
FORM 3: ENUMERATION HARD COPY RESULTS SHEET

Analyst Name:	
Laboratory Code:	
Analyst Code :	

	Organism	Cell count	Multiplication factor	Number cells/L
Sample No:				
Settlement date:				
Analysis date:				
Volume Chamber (ml):				

	Organism	Cell count	Multiplication factor	Number cells/L
Sample No:				
Settlement date:				
Analysis date:				
Volume Chamber (ml):				

Appendix 4

Form 2: Taxonomic quiz

ANALYST CODE:_____

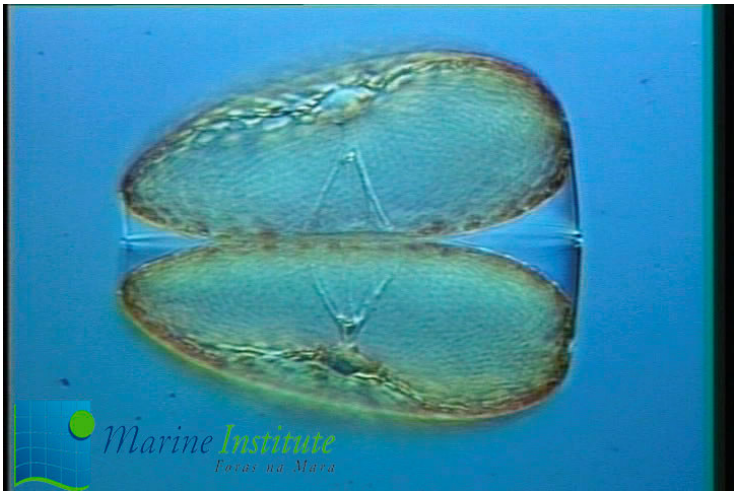
FORM 2: TAXONOMIC QUIZ BEQUALM PHY-ICN-09-MI1

SET A (image 1)

1A Question: Where is the raphe slit in this pennate diatom? Point using arrows (10 marks)

Answer:_____

SET A (image 2)



2A Question: Name this organism to species level, Typical size: 30 to 210µm (Diameter) (10 marks)

Answer:_____

SET A (image 3)

3A Question: Name this organism to species level. Typical size: 35 to 65µm in Length (10 marks)

Answer:_____

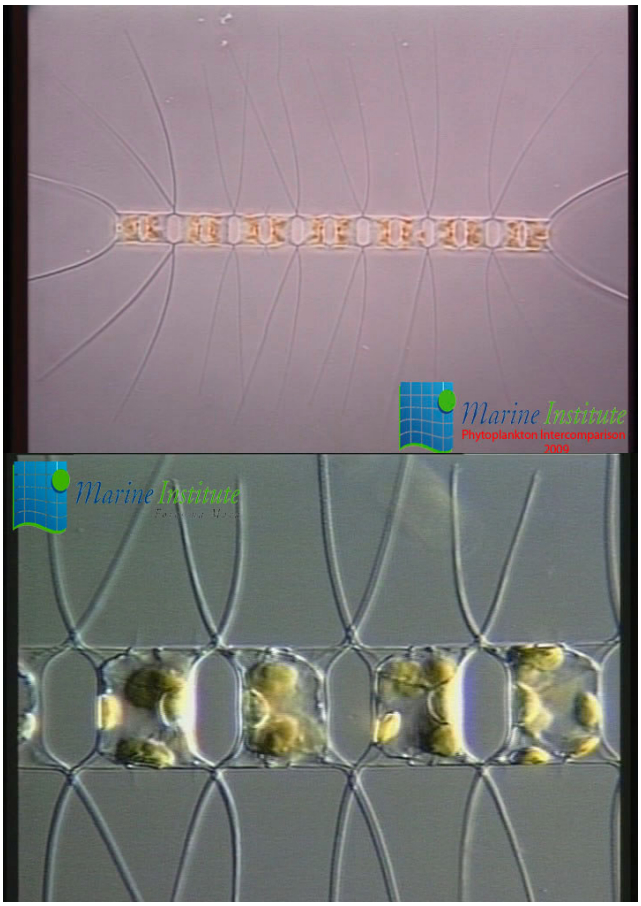
SET A (image 4)

4A Question: Name the parts of this pennate diatom coloured in blue and pink (10 marks)

Answer:

_____

SET A (image 5)



5A Question: a) Name this organism to species level. b) Point using arrows to the 'foramen' in this chain. (10 marks) Answer:_____

To answer the following questions, you need to go to the website www.unique-media.tv/mie001 username: marine and password: bus7xehe and watch the **SET A** videos. You have the choice of low or high resolution viewing.

6A) Video A1: **Question: Name these organisms to species level. Typical size: 25 to 35µm Long (10 marks)**

Answer_____

7A) Video A2: **Question: Name these organisms to species level. Typical size: 34 to 65µm long (10 marks)**

Answer_____

8A) Video A3: **Question: Which Class and genus do this organism belongs to? Typical size: 8 to 15µm long (10 marks)**

Answer_____

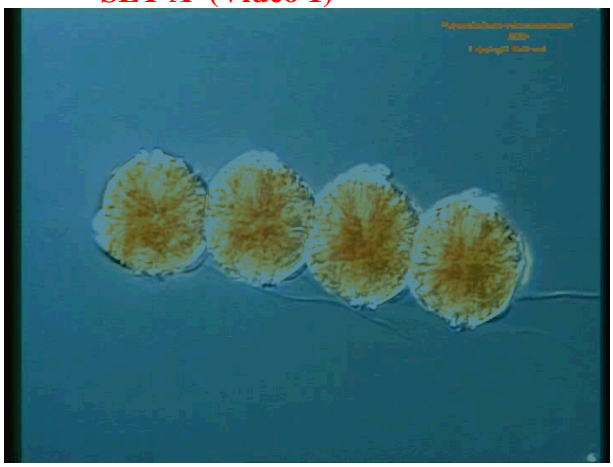
9A) Video A4: **Question: Name this organism to species level. Typical size: 12 to 18µm (10 marks)**

Answer_____

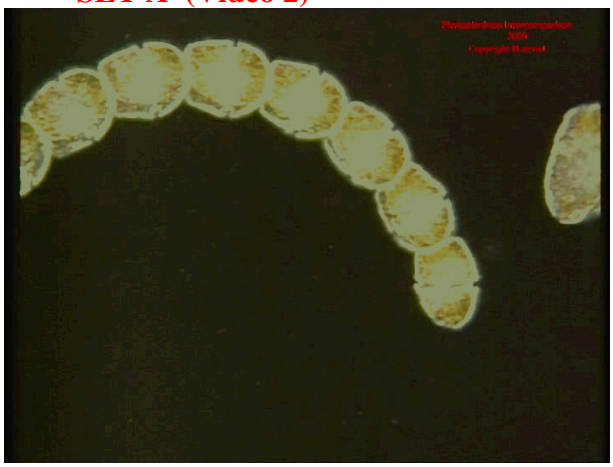
10A) Video A5: **Q: Name this organism to species level. Typical size: 140-180µm long (10 marks)**

Answer_____

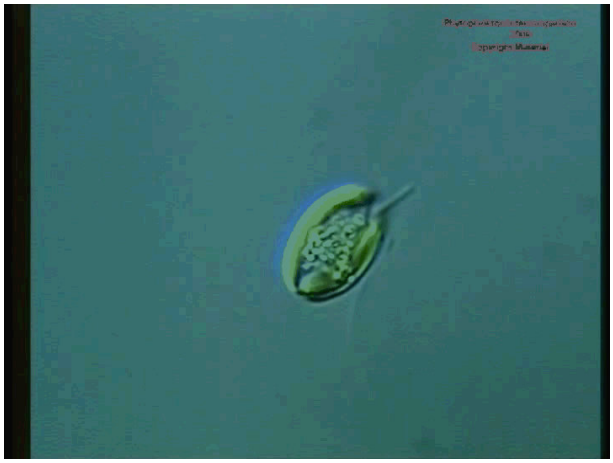
SET A (Video 1)



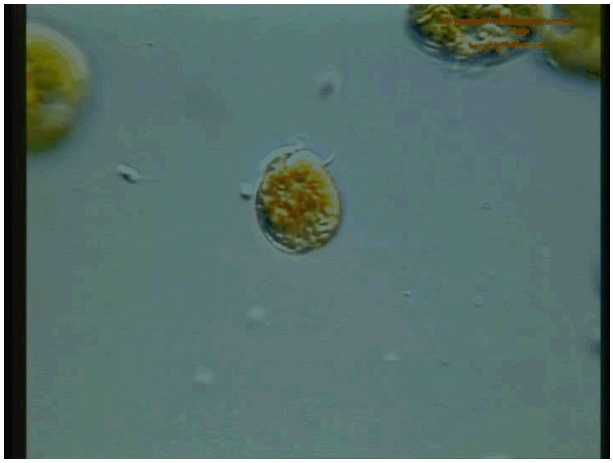
SET A (Video 2)



SET A (Video 3)

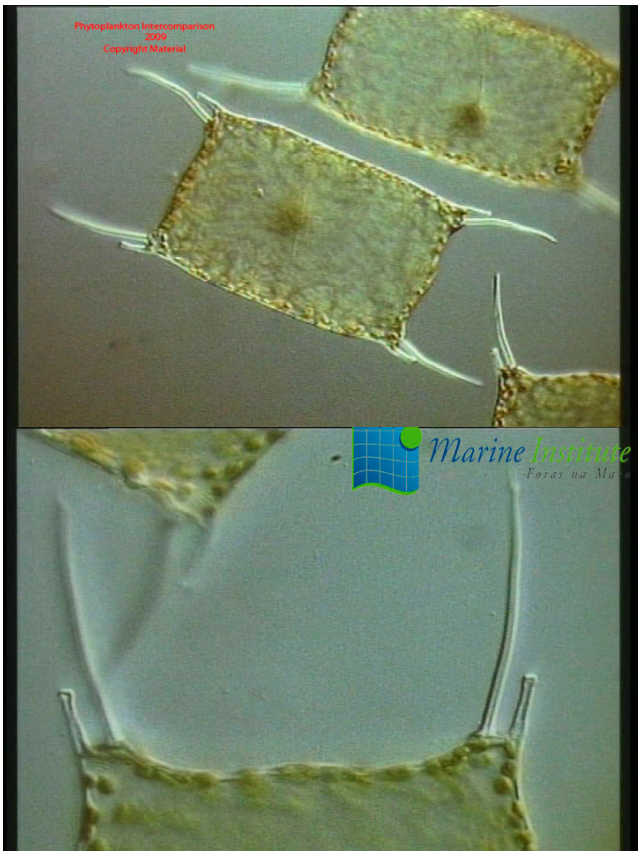


SET A (Video 4)



SET A (Video 5)

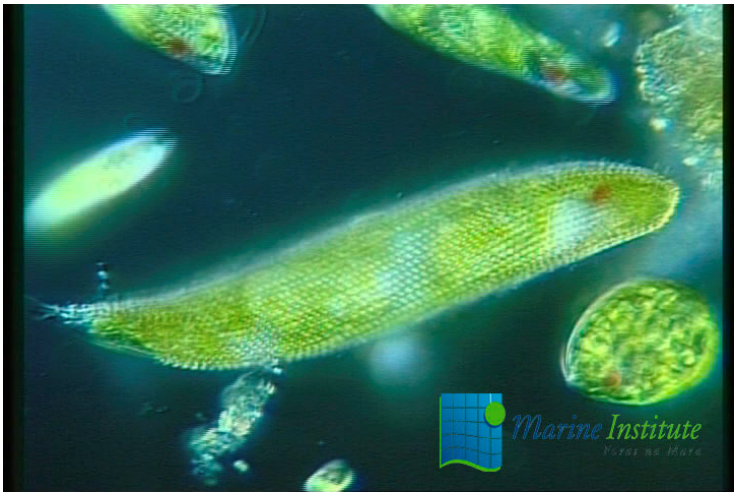
SET B (image 1)



1B Question: Name this organism to species level and point an arrow to the labiate process. Typical size: 90 to 260µm long (10 marks)

Answer:_____

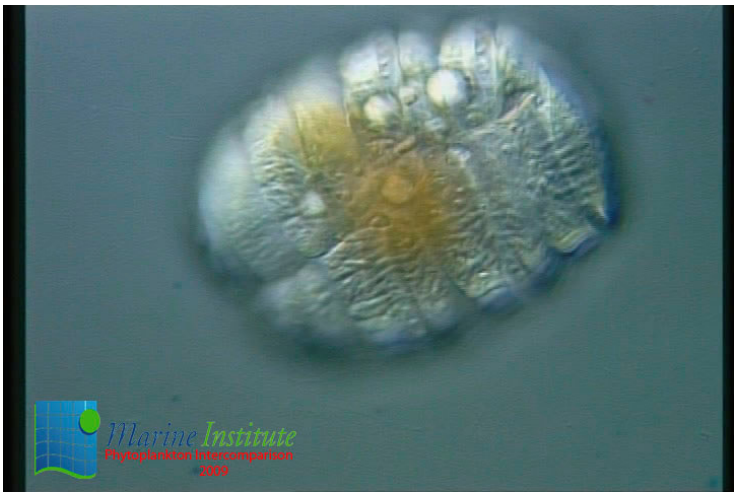
SET B (image 2)



2B Question: Name the order these group of organisms shown in the photo belong too. Marks (10 marks)

Answer:_____

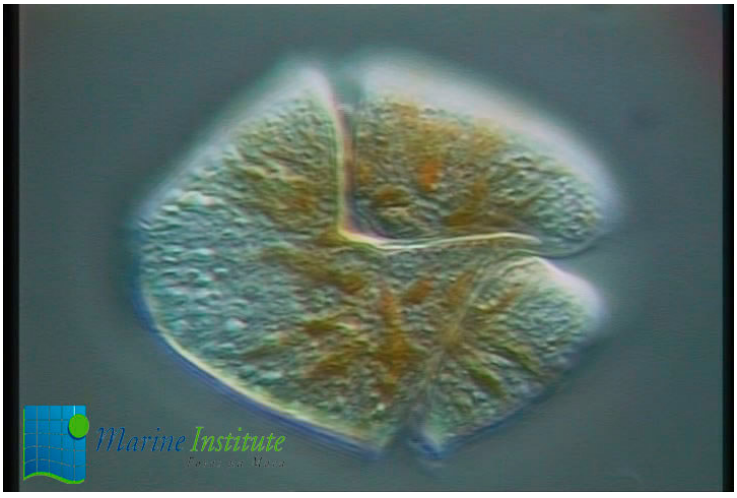
SET B (image 3)



3B Question: a) Name this organism to species level. b) What is unusual about this dinoflagellate compared to other dinoflagellates with relation to motility. Typical size: 100 to 150µm long (10 marks)

Answer:_____

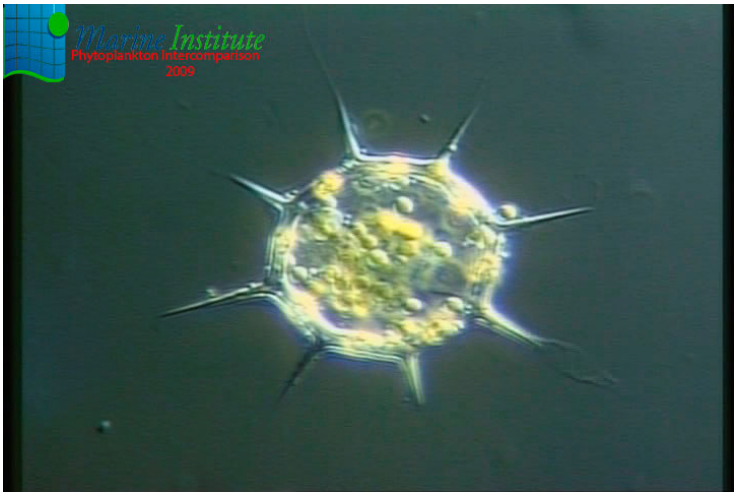
SET B (image 4)



4B Question: Name this organism to genus level. Typical size: 50 to 80µm long(10 marks)

Answer:_____

SET B (image 5)



5B Question: Name this organism to species level. Typical size: 20 to 30µm diameter (10 marks)

Answer:_____

To answer the following questions, you need to go to the website www.unique-media.tv/mie001 username: marine and password: bus7xehe and watch the **SET B** videos. You have the choice of low or high resolution viewing.

6B) Video B1: **Q: Name this organism to species level. Typical size: up to 1mm long**

(10 marks)

Answer_____

7B) Video B2: **Q: Name this organism to genus level. Typical size: 12 to 18µm long (10 marks)**

Answer_____

8B) Video B3: **Q: Name this organism to species level. Typical size: 25 to 35µm long (10 marks)**

Answer_____

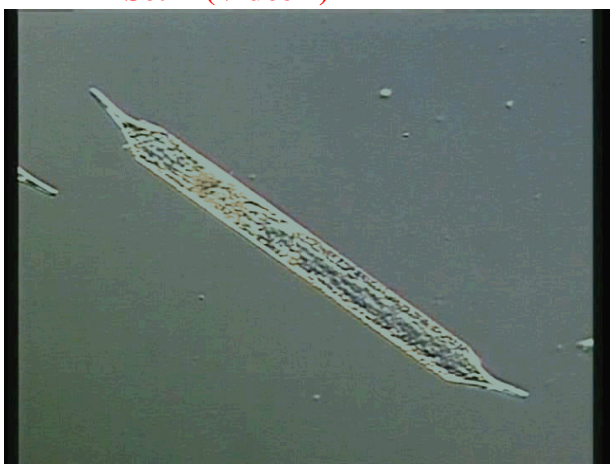
9B) Video B4: **Q: Name this organism to genus level. Typical size: up to 1.5mm long (10 marks)**

Answer_____

10B) Video B5: **Question: Name this organism to genus level. Typical size: 250µm long (10 marks)**

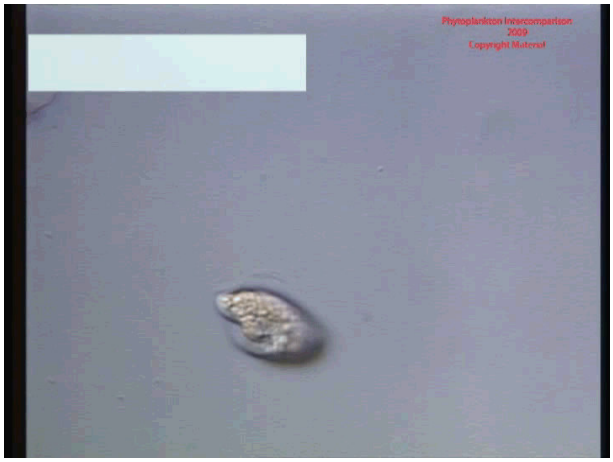
Answer_____

Set B (Video 1)

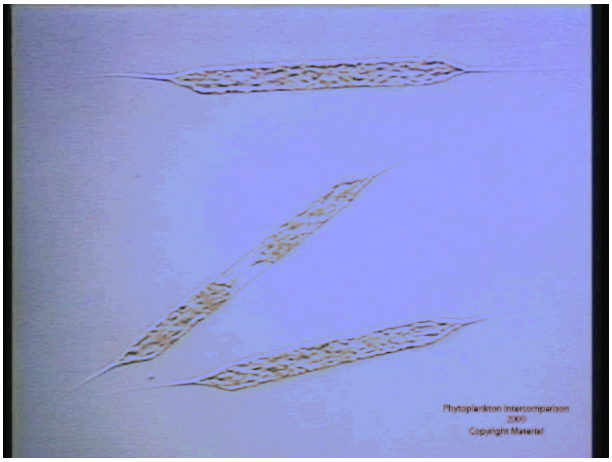


Set B (Video 2)

Set B (Video 3)

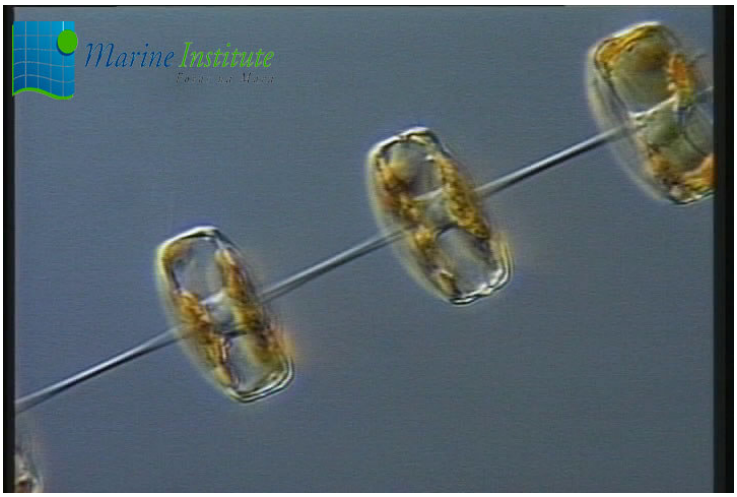


Set B (Video 4)



Set B (Video 5)

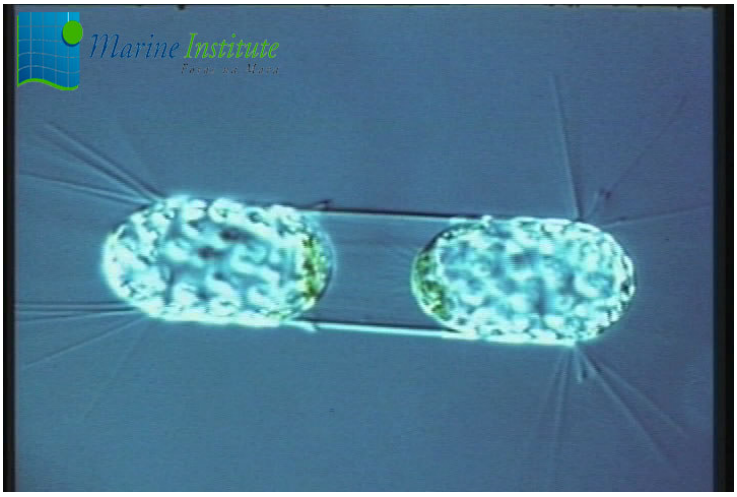
SET C (image 1)



1C Question: a) Name this organism to genus level. b) how do you call the thread joining the cells? Typical size: 10 to 60µm in diameter (10 marks).

Answer:_____

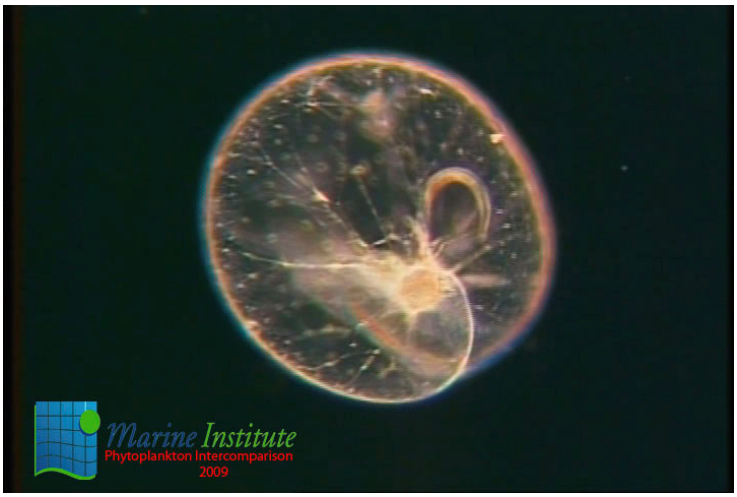
SET C (image 2)



2C Question: Name this organism to genus level. Typical size: 20 to 40µm in diameter (10 marks)

Answer:_____

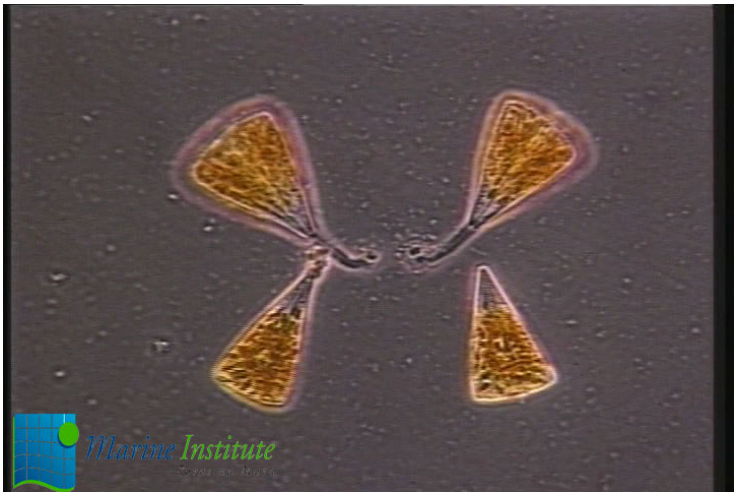
SET C (image 3)



3C Question: Name this organism to species level. Typical size: up to 2mm in diameter (10 marks)

Answer:_____

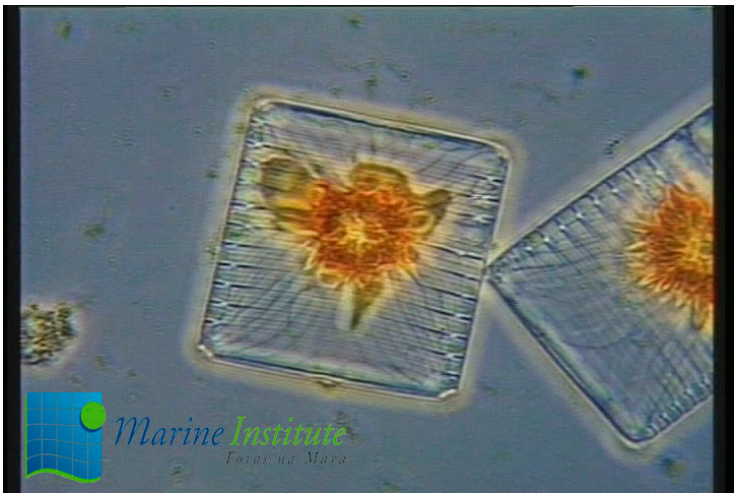
SET C (image 4)



4C Question: Name this organism to genus level. (10 marks)

Answer:_____

SET C (image 5)



5C Question: Name this organism to species level, Typical size: 30 to 120µm (Width) (10 marks)

Answer:_____

To answer the following questions, you need to go to the website www.unique-media.tv/mie001 username: marine and password: bus7xehe and watch the **SET C** videos. You have the choice of low or high resolution viewing.

6C) Video C1: **Question: Name this colonial organism to species level (10 marks)**

Answer_____

7C) Video C2: **Question: Which class and order this organism belongs to? (10 marks)**

Answer_____

8C) Video C3: **Question: Name this organism to species level (10 marks)**

Answer_____

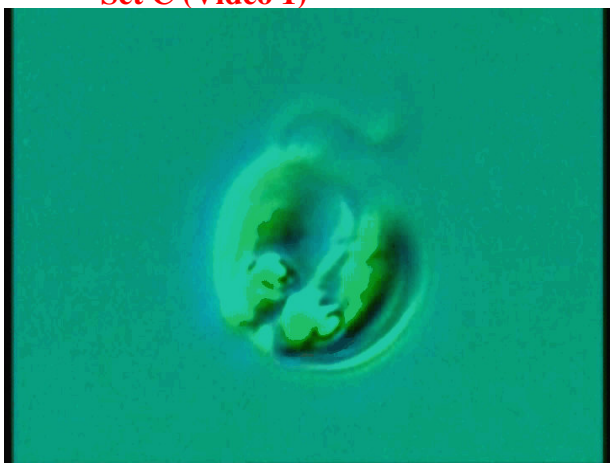
9C) Video C4: **Question: a) Which family this organism belongs to? b) Name the structural feature circled in red in the video. Typical size: 10 to 40µm long (10 marks)**

Answer_____

10C) Video C5: **Question: This video shows details of an euglenophyte. Could you name the feature circled in red in the video(10 marks)**

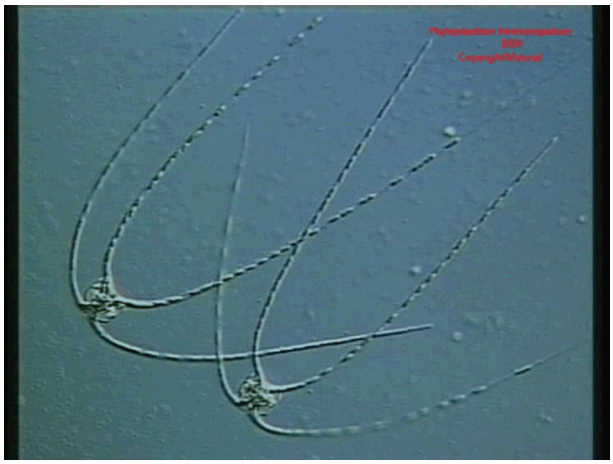
Answer_____

Set C (Video 1)

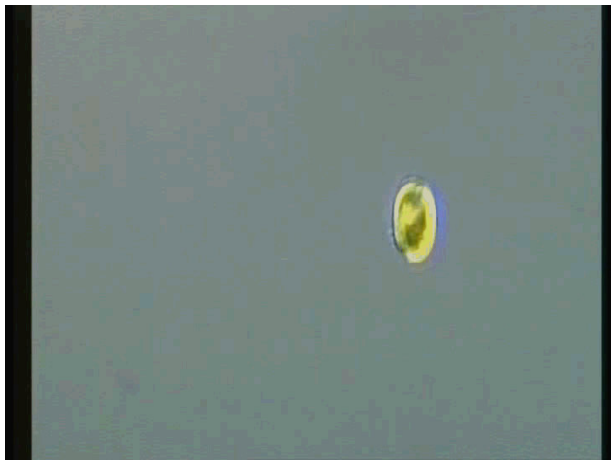


Set C (Video 2)

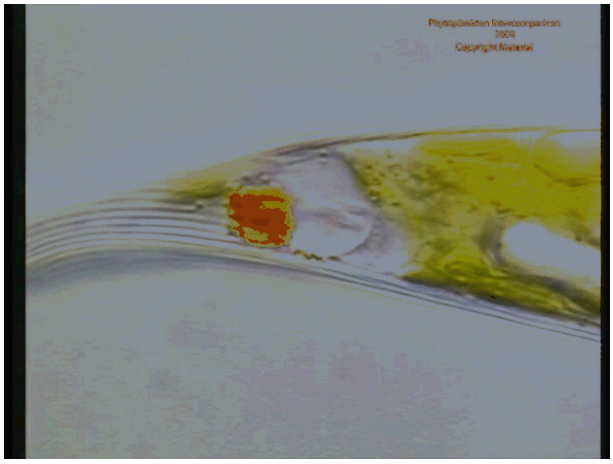
Set C (Video 3)



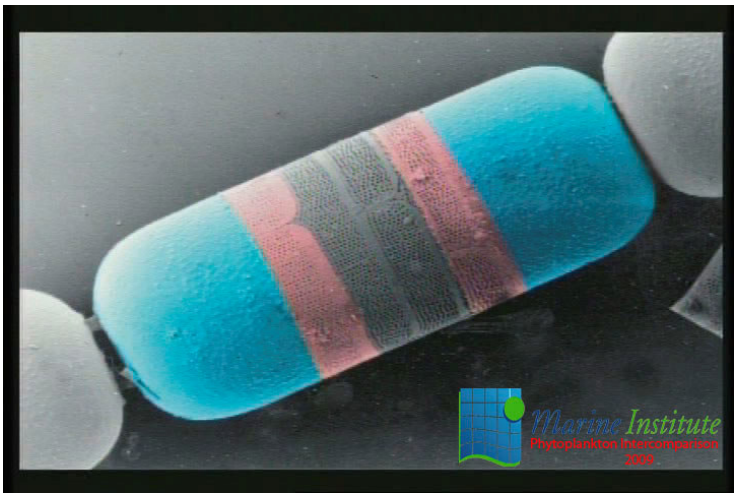
Set C (Video 4)



Set C (Video 5)



SET D (image 1)



1D Question: Name the parts of this Centric diatom coloured in blue and pink (10 marks)

Answer:

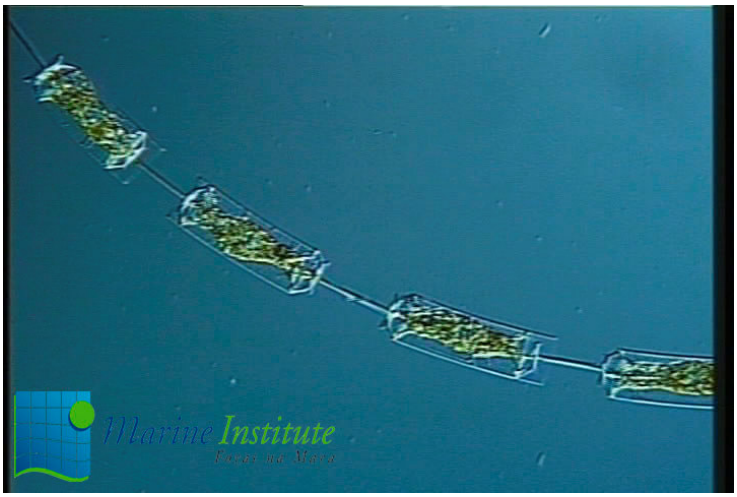
_____

SET D (image 2)

2D Question: Name this organism to genus level. (10 marks)

Answer:_____

SET D (image 3)



3D Question: Name this organism to species level, Typical size: 70 to 140µm (Length) (10 marks)

Answer:_____

SET D (image 4)

4D Question: Name this organism to species level, Typical size: 30 to 60µm long (10 marks)

Answer:_____

SET D (image 5)

5D Question: Name this organism to species level. Typical size: 200 to 400µm long (10 marks)

Answer:_____

To answer the following questions, you need to go to the website www.unique-media.tv/mie001 username: marine and password: bus7xehe and watch the **SET D** videos. You have the choice of low or high resolution viewing.

6D) Video D1: **Question: This organism is dorsoventrally flattened. Name this organism to species level. Typical size: 15 to 50µm long (10 marks)**

Answer_____

7D) Video D2: **Question: a) Name this organism to genus level. b) What is the arrow in the video pointing at? Typical size: 4 to 26µm (10 marks)**

Answer_____

8D) Video D3: **Question: Name this organism to genus level (10 marks)**

Answer_____

9D) Video D4: **Question: Name this organism to species level (10 marks)**

Answer_____

10D) Video D5: **Question: what is happening in this sequence. Choose one of the following:**

(10 marks)

a) gametes fuse becoming an Hypnozygote and then a planozygote

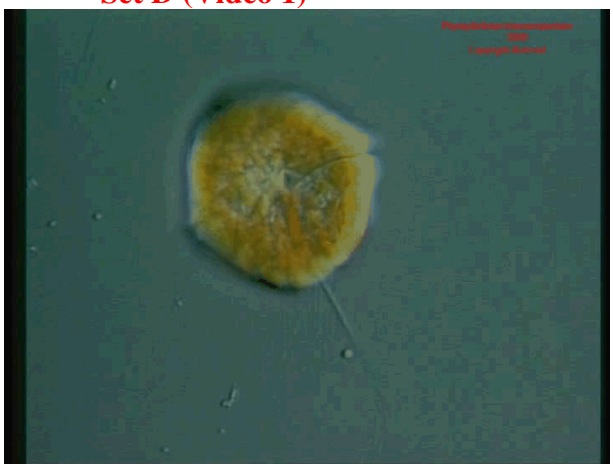
b) vegetative cells fuse becoming an hypnozygote and then a planozygote

c) gametes fuse becoming a planozygote and then a hypnozygote

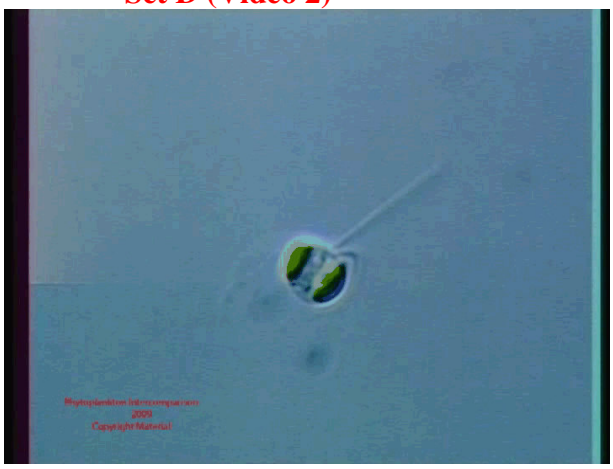
d) vegetative cells fuse becoming a planozygote and then a hypnozygote

Answer_____

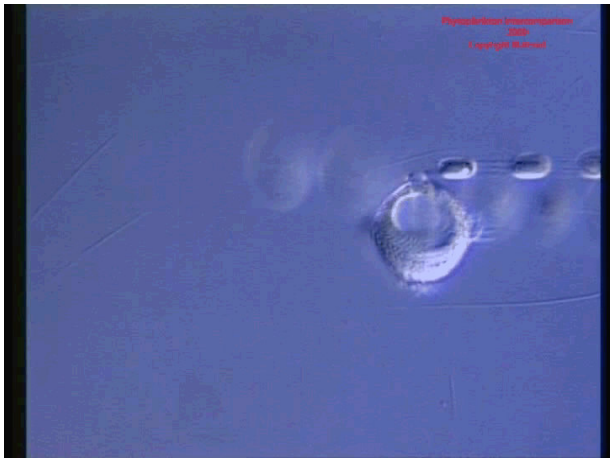
Set D (Video 1)



Set D (Video 2)



Set D (Video 3)



Set D (Video 4)

Set D (Video 5)

ANALYST SIGNATURE:_____

DATE: _____

Appendix 5: Statement of performance certificate

Biological Effects Quality Assurance in Monitoring Programmes /
National Marine Biological Analytical Quality Control Scheme /
Marine Institute
STATEMENT OF PERFORMANCE
Phytoplankton Component of Community Analysis
Year 2009

Participant details:
Name of organisation:
Participant:
Year of joining:
Years of participation:

Statement Issued: 26/06/2009
Statement Number: MI-BQM-09

Summary of results:

Component Name	Exercise	Subcontracted	Results		identification
			Z-score (+/- 3 Sigma limits)		
Phytoplankton Enumeration PHY-ICN-09-MI1		Marine Institute	*Gymnodinium catenatum*		
			Prorocentrum micans		
			Prorocentrum lima		
			Scripsiella sp.		
			Coscinodiscus granii		
Results (Pass Mark 70%, over 90% proficient)					
Phytoplankton Identification PHY-ICN-09-MI1		Marine Institute			

n/a: component not applicable to the participant; n/p: Participant not participating in this component;
n/r: no data received from participant
The list shows the results for all components in which the laboratory participated. See over for details.
Notes:

Details certified by:

Section Manager
Joe Silke (MI)

Scientific Technical Officer
Rafael Salas (MI)

Description of Scheme components and associated performance standards

In the table overleaf, for those components on which a standard has been set, 'Proficient', 'Good', and ' "Pass" flags indicate that the participants results met or exceeded the standards set by the Bequalm Phytoplankton scheme; 'Participated' flag indicates that the candidate participated in the exercise but did not reach these standards. The Scheme standards are under continuous review.

Component	Annual exercises	Purpose	Description	Standard
Phytoplankton Enumeration Exercise	1	To assess the performance of participants when undertaking analysis of a natural or prepared sample/s of Seawater preserved in Lugol's iodine and spiked using biological or synthetic subjects using the Utermöhl cell counting method.	Natural or prepared marine water sample/s distributed to participants for Phytoplankton enumeration analysis and calculation of counts in cells per litre	Participants are required to identify and enumerate the spiked material and give a result to within ±3SD or sigma limits of the true value. The true value and 3 sigma limits are usually calculated from a randomly selected sample population of the total and calculated by the organising laboratory. This data has to demonstrate normality to become the reference data for the exercise.
Phytoplankton identification exercise	1	To assess the accuracy of identification of a wide range of Marine phytoplankton organisms.	This is a proficiency test in the identification of marine phytoplankton. The exercise tests the participant's ability to identify organisms from photographs, videos and/or diagrams supplied. In addition to the identification to the particular taxon required, certain taxonomic features of these organisms may be required to be identified.	The pass mark for the identification exercise is 70%. Results above 90% are deemed proficient, results above 80% are deemed good, results above 70% are deemed acceptable, and results below 70% are reported as "Participated". There are no standards for phytoplankton identification. These exercises are unique and made from scratch.

Appendix 6: Reference values or 'Gold Standard'

True value counts or gold standard in cells per liter for species Sp1 to Sp5. Sp1= *C.granii*; Sp2= *G.catenatum*; Sp3= *P.micans*; Sp4= *S.trochoidea*; Sp5= *P.lima*. I= Size < 50µm; II= Size >50µm. a= High cell concentration; b= medium cell concentration; c= low cell concentration. Type A= Samples containing four species; Type B= samples containing five species; Type C= +ve (positive control).

Sample number	Sp1	Sp2	Sp3	Sp4	Sp5	Species	Sample number	Sp5	Species
	I	I	II	II	I	Size		I	Size
	b	b	b	a	c	Cells/L		C	Cells/L
16	11400	19920	17080	37480	N/A		68	4120	
114	12720	22040	20200	39280	N/A		86	4640	
75	12040	21480	19560	37920	N/A		23	5200	
126	12280	17760	15760	35320	N/A		107	5200	
51	13200	23240	19400	37120	N/A	Type A	135	4560	
32	14360	23760	17640	39240	N/A		129	4240	
19	13280	24840	21720	39160	N/A		59	4360	Type C
170	10080	21720	19880	39000	N/A		175	5680	
37	11200	22400	23080	40360	N/A		100	5040	
22	11920	23200	19200	42520	N/A		76	4800	
150	12120	23320	16480	35720	4040		132	4920	
20	12280	22240	18880	37920	4280		88	5200	
83	12200	17560	17800	40400	3400		141	3880	
117	12600	21440	17840	34120	3920				
46	12080	23120	16160	29200	4040	Type B			
30	12200	19280	16000	35760	4560				
98	12520	21040	15440	33320	4920				
127	12280	19000	15840	33120	4240				
154	13360	20040	11600	28200	4600				
79	13280	15200	15320	31920	4200				

Appendix 7

Participant laboratories of Bequalm exercise 2009

PHY-ICN-09MI1

Appendix 1: BEQUALM 09 LABS	
AWI/BAH Kurpromenade Helgoland 27498 Germany	**IRTA Carretera del Poblenou km 5,5** Sant Carles de la Ràpita 43540 Spain
AFBI HQ, Newforge Lane Belfast Northern Ireland BT9 5PX United Kingdom	**Isle of Man Government Laboratory** Ballakermeen Road Douglas, Isle of Man IM1 4BR United Kingdom
Fisheries Research Services, Marine Laboratory Victoria Road Aberdeen Scotland AB11 9DB United Kingdom	**INTECMAR NIF- Q3600376B** Peirao de Vilaxoán s/n. Vilagarcía de Arousa Pontevedra, Galicia 36611 Spain
SAMS Research Services Ltd Dunstaffnage Marine Laboratory Oban Argyll PA37 1QA United Kingdom	**LVCC Palmones. Egmasa** c/Trasmallo s/n. Palmones Los Barrios Cádiz 11379 Spain
L.C.C.RR.PP. Ctra. PUNTA UMBRÍA - CARTAYA km 12 CARTAYA HUELVA 21459 Spain	**Marine Institute** Phytoplankton lab Gortalassa Bantry Co.Cork Ireland
CEFAS Barrack Road, The Nothe Weymouth Dorset DT4 8UB United Kingdom	**Jacobs Engineering UK Ltd** Kenneth Dibben House, Southampton Science Park Southampton,Hampshire, SO167NS United Kingdom
The Water Management Unit Northern Ireland Environment Agency 17 Antrim road, Lisburn Down BT283AL United Kingdom	**Marine Institute** Phytoplankton lab Rinville, Oranmore Co. Galway Ireland
CEFAS Laboratory Pakefield Rd Lowestoft NR33 0HT United Kingdom	**Marine Phytoplankton Ecologist** Scottish Environment Protection Agency Clearwater House, Heriot Watt Research Park Avenue North, Riccarton EDINBURGH EH14 4AP
Koeman en Bijkerk bv Kerklaan30 Haren 9751NN Netherlands	

Appendix 8

Analysts Negative control sample (type d) results intercomparison Bequalm 2009

ANALYST CODE	Number of cells	Cells/L
	Cell count	Cell count
u	0	0
r	0	0
k	0	0
c	0	0
n	0	0
b	0	0
s	0	0
β (Beta)	0	0
l	0	0
ά (Alpha)	0	0
δ (delta)	0	0
j	1	40
h	0	0
z	0	0
v	0	0
g	0	0
ñ	0	0
i	0	0
a	0	0
o	0	0
d	0	0
p	0	0
f	0	0
Ω (omega)	0	0
σ (sigma)	0	0
m	0	0
μ	0	0
π (pi)	0	0
x	8	320
y	0	0
q	0	0
t	0	0
ch	0	0
ζ (zeta)	0	0

Appendix 9: Identification results Taxonomic quiz images

ANALYST CODE	SET A					SET B					SET C					SET D					Total	%
	img 1	img 2	img 3	img 4	img 5	img 1	img 2	img 3	img 4	img 5	img 1	img 2	img 3	img 4	img 5	img 1	img 2	img 3	img 4	img 5		
u	0	10	10	10	5	5	10	5	10	5	10	10	10	10	10	10	10	10	10	10	170	85
r	0	10	10	10	5	5	10	5	0	10	10	10	10	10	10	10	0	10	10	10	155	77.5
k	0	10	10	10	10	5	10	10	10	10	10	10	10	10	10	10	10	10	10	10	185	92.5
c	0	10	10	10	10	5	10	10	10	10	10	10	10	10	10	10	10	10	10	10	185	92.5
n	0	10	10	10	10	10	10	10	10	5	10	10	10	10	10	10	10	10	5	5	175	87.5
b	0	10	10	10	10	5	10	10	10	10	10	10	10	10	10	5	10	10	10	5	175	87.5
s	0	10	10	5	10	5	0	10	0	10	10	10	10	0	10	5	10	10	10	10	145	72.5
β	0	10	5	0	5	10	0	5	0	5	10	10	10	10	10	0	10	10	10	10	130	65
l	0	10	10	10	5	10	0	10	0	10	10	10	10	10	10	10	10	10	10	10	165	82.5
ά	0	10	10	5	5	10	0	10	10	10	10	10	10	10	10	10	10	10	10	10	170	85
δ	0	10	10	10	5	10	10	5	10	10	10	10	10	10	10	10	0	10	10	10	170	85
j	0	10	10	10	5	5	10	5	0	5	10	10	10	10	10	5	10	10	10	10	155	77.5
h	10	10	10	10	5	10	10	10	0	10	10	10	10	10	10	5	10	10	10	10	180	90
z	0	10	10	5	10	5	10	5	0	5	10	10	10	10	10	10	10	10	10	10	160	80
v	10	10	10	10	5	5	10	10	0	5	10	10	10	10	10	10	10	10	10	10	175	87.5
g	0	10	10	10	10	10	10	10	10	10	10	10	10	0	10	10	10	0	10	10	170	85
ñ	0	10	10	10	10	5	0	10	10	5	10	10	10	0	10	10	10	10	10	10	160	80
i	0	10	10	10	10	5	0	10	10	10	10	10	10	10	10	10	10	10	10	10	175	87.5
a	0	10	10	10	5	5	0	5	0	10	10	10	10	10	10	0	10	10	10	10	145	72.5
o	0	10	5	10	10	5	0	10	10	10	10	10	10	10	10	10	10	10	5	10	165	82.5
d	0	10	10	10	10	5	10	10	10	5	10	10	10	0	10	10	10	10	10	10	170	85
p	0	10	10	10	5	10	10	10	0	5	10	10	10	10	10	10	10	10	10	10	170	85
f	10	5	10	10	10	10	0	5	0	5	10	10	10	10	10	10	0	10	10	10	155	77.5
Ω	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	200	100
σ	0	10	10	10	5	5	10	5	0	10	10	10	10	0	10	10	10	10	10	10	155	77.5
m	10	10	10	10	10	5	0	10	0	5	10	10	10	0	10	10	10	10	10	10	160	80
μ	10	10	10	10	10	5	10	10	10	10	10	10	10	10	10	10	10	10	10	10	195	97.5
π	10	10	10	10	10	5	10	5	10	10	10	10	10	10	10	10	10	10	10	10	190	95
x	10	10	5	10	10	5	10	5	10	5	10	10	10	10	10	10	10	10	10	10	180	90
y	10	10	10	10	5	5	10	10	10	10	10	10	10	10	10	10	10	10	10	10	190	95
q	10	10	10	10	10	5	10	10	10	10	10	10	10	10	10	10	10	10	10	10	195	97.5
t	0	10	10	10	5	10	10	5	0	10	5	10	10	10	10	10	10	10	5	5	155	77.5
ch	0	10	10	10	10	10	10	10	0	5	10	10	10	10	10	10	10	10	5	10	170	85
ζ	0	10	10	0	10	5	10	10	10	5	10	10	10	10	10	10	10	10	10	10	170	85

Appendix 10: Identification results Taxonomic quiz video clips

ANALYST CODE	SET A					SET B					SET C					SET D					Total	%
	vid 1	vid 2	vid 3	vid 4	vid 5	vid 1	vid 2	vid 3	vid 4	vid 5	vid 1	vid 2	vid 3	vid 4	vid 5	vid 1	vid 2	vid 3	vid 4	vid 5		
u	10	10	10	10	5	10	0	10	10	0	10	10	10	10	0	0	10	10	10	0	145	72.5
r	5	10	10	10	5	10	10	0	10	0	10	10	10	0	10	0	10	10	10	10	150	75
k	5	10	10	10	10	10	0	0	10	10	10	10	5	10	10	10	10	10	10	10	170	85
c	5	10	10	10	10	10	0	0	10	0	10	10	5	10	10	0	10	10	10	10	150	75
n	10	10	10	10	5	10	0	0	10	0	10	10	10	5	10	0	10	10	10	10	150	75
b	10	10	10	10	5	10	0	0	10	0	10	10	5	10	10	0	10	10	10	10	150	75
s	10	10	10	10	5	10	10	10	10	0	10	10	5	5	10	0	10	10	10	10	165	82.5
β	10	0	0	5	5	10	10	0	10	0	10	10	5	10	10	0	10	10	10	10	135	67.5
l	10	10	0	10	5	10	10	0	10	10	10	10	10	10	0	0	10	10	10	10	155	77.5
ά	10	10	10	10	5	10	10	10	10	10	10	10	5	10	10	0	10	10	10	10	180	90
δ	10	10	10	10	5	10	10	10	10	10	10	10	5	10	10	0	10	10	10	10	180	90
j	10	10	5	10	5	5	0	0	10	0	10	10	5	5	10	0	10	10	10	10	135	67.5
h	10	10	10	10	10	10	0	10	10	10	10	10	10	0	10	0	10	10	10	10	170	85
z	10	10	10	10	5	10	10	10	10	10	10	5	10	10	10	10	10	10	10	10	190	95
v	10	10	10	10	5	10	0	10	10	10	10	10	10	10	10	10	10	10	10	10	185	92.5
g	10	10	10	10	5	5	0	0	10	0	10	0	5	5	10	0	10	10	10	10	130	65
ñ	10	10	5	10	5	10	0	10	10	0	10	10	10	0	0	0	0	10	10	10	130	65
i	5	10	10	10	5	10	10	10	10	0	10	10	10	10	10	10	10	10	10	10	180	90
a	5	10	10	10	5	10	10	10	10	0	10	10	10	0	10	0	10	10	10	0	150	75
o	10	0	10	5	5	10	0	0	10	0	10	10	5	10	10	0	10	10	10	10	135	67.5
d	10	10	0	10	5	10	0	10	10	10	10	10	10	10	10	0	10	10	10	10	165	82.5
p	10	10	10	10	10	10	0	10	10	10	10	10	5	0	10	0	10	10	10	10	165	82.5
f	10	10	10	5	5	10	10	10	10	0	10	10	10	10	10	10	10	10	10	10	180	90
Ω	5	10	10	10	5	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	190	95
σ	10	10	10	5	10	10	0	10	10	0	10	10	5	10	10	0	10	0	10	10	150	75
m	5	10	10	5	5	10	0	10	10	0	10	0	5	10	10	0	10	10	10	0	130	65
μ	10	10	10	10	5	10	10	10	10	10	10	10	5	10	10	10	10	10	10	10	190	95
π	10	10	10	10	5	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	195	97.5
x	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	0	10	10	10	10	190	95
y	10	0	10	10	5	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	185	92.5
q	10	10	10	10	5	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	195	97.5
t	10	10	10	10	5	0	0	0	10	10	10	10	5	10	10	5	5	10	10	10	150	75
ch	10	10	10	10	5	5	0	0	10	10	10	10	5	10	10	0	10	10	10	10	155	77.5
ζ	5	10	10	10	5	10	0	10	10	10	10	10	10	10	10	10	10	10	10	10	180	90

Appendix 11

Instructions for the phytoplankton Intercomparison Bequalm PHY-ICN-10-MI1

Marine Institute BEQUALM Phytoplankton Proficiency Test PHY-ICN-10-MI1

Instructions for Sample Preparation, Cell counting, calculations & Identification

Please note that these instructions are designed strictly for use in this Intercomparison only.

1. Introduction

The Marine Institute, Galway, Ireland, has conducted a Phytoplankton Enumeration and Identification ring trial, under the auspices of BEQUALM annually since 2005.

The purpose of this exercise is to compare the performance of laboratories engaged in national official/non-official phytoplankton monitoring programmes and other laboratories working in the area of phytoplankton analysis.

The Marine Institute is accredited to ISO 17025 for Marine phytoplankton identification and enumeration since 2005 and recognizes that regular Quality Control assessments are crucial to ensure a high quality output of Phytoplankton data.

This Phytoplankton Ring Test is being conducted to determine any inter-laboratory and inter-analyst variability in the enumeration of Marine Phytoplankton species within and between laboratories from a number of samples spiked with cultured material.

A taxonomic quiz has been designed to test analysts' knowledge on phytoplankton species and in important morphological and structural characteristics that could help in their identification. This quiz is an updated version of the quiz sent in the 2008 round. Analysts which have previously completed this exercise are exempted from doing it again. Only analysts that have not completed this quiz before should do the taxonomy quiz.

Please adhere to the following instructions strictly. Please note that these instructions are specific to this ring test only.

2. Preliminary Checks and Deadlines

Upon receipt of the samples, every analyst should make sure that they have received everything listed in the Return Slip and checklist form (Form 1). Make sure that all the samples are intact and sealed properly and check that you have received the Taxonomic quiz (Form 3) and the Enumeration Hard copy results sheet (Form 2). Once you are happy that you have received everything you need to complete this exercise and samples are in working order. Complete form 1: Return slip and checklist form and send it by Fax or e-mail to the Marine Institute, Galway. Fax No +353 91 387237 or Rafael.salas@marine.ie A receipt of Fax/e-mail is necessary for the Marine Institute to validate the test process for each analyst.

Once you have received the samples, each analyst has four weeks to complete the exercise and return the results to Rafael Salas, Marine Institute, Phytoplankton laboratory, Rinville, Oranmore, Co. Galway, Ireland. The hardcopy of enumeration

results (Form 2) and the Taxonomic quiz (Form 3) **must be received** by the Marine Institute by **April 16th, 2010**.

Please note: Hardcopy results and Taxonomic quiz results received after the April 16th, 2010 date will not be included in the final report.

3. Test Method

The Utermöhl cell counting method is the standard method used in the Marine Institute Phytoplankton programme in Ireland. Our method uses 25ml sedimentation chambers and our laboratory is accredited to ISO 17025 standard for this method since 2005.

In previous years, we have advised others laboratories taking part in the exercise to use the Utermöhl cell counting method to analyse the samples. This year, we are asking laboratories to use their own in house cell counting methodologies to carry out the analysis of the samples.

Those laboratories using the Utermöhl cell counting method can if they wish sub-sample to analyse different sample volumes.

Those laboratories using methodologies other than the Utermöhl method should describe briefly, which method they use, how it works and how they carry out their calculations to obtain the final density in cells per liter. These laboratories should send their Standard Operating Procedures along with their results to us in order to understand better how they analysed their samples and compare fairly the results at the workshop.

4. Equipment

Those laboratories using the Utermöhl method will need to complete the exercise:
Six Utermöhl cell counting chambers
Base plates and glass covers.
Inverted Microscope equipped with long distance working lenses and condenser of Numerical Aperture (NA) of 0.3 or similar.

5. Sample Preparation

Sedimentation counting chambers consist of a clear plastic cylinder, a metal plate, a glass disposable cover-slip base plate and a glass cover plate (Fig 1). Six sedimentation chambers will be required.

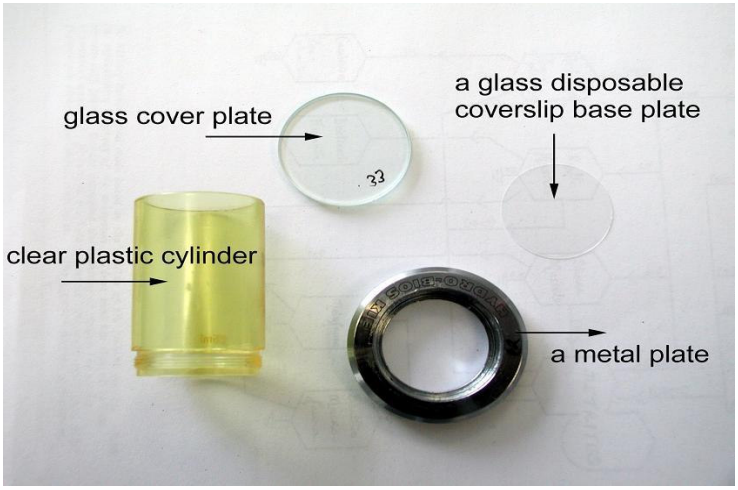


Fig 1: Sedimentation counting chamber

If using the Utermöhl method follow the following instructions:

5.1 Place a clean disposable cover slip base plate inside a cleaned metal plate.

5.2 Screw the plastic cylinder into the metal plate. Extra care should be taken when setting up chambers. Disposable cover slip base plates are fragile and break easily causing cuts and grazes.

5.3 Important: Once the chamber is set up, it should be tested for the possibility of leaks by filling the completed chamber with sterile seawater and allowing it to rest for a few minutes. If no leakage occurs, pour out the water and proceed with the next step.

5.4 To set up a sample for analysis or sub-sample. Firmly invert the sample at least 20 times to ensure that the contents are homogenised properly.

5.4.1 Pour the sample into the counting chamber. (Samples must be adapted to room temperature to reduce the risk of air bubbles in the chambers)

5.4.2 There should be enough sample volume in each sample to fill an Utermöhl sedimentation chamber. Top up the sedimentation chamber and cover with a glass cover plate to complete the vacuum and avoid air pockets.

5.4.3 Label the sedimentation chamber with the sample number from the sterilin tube.

5.5 Use a horizontal surface to place chambers protected from vibration and strong sunlight.

5.6 Allow the sample to settle for a minimum of twelve hours.

5.7 Set the chamber on the inverted microscope and analyse.

5.8 Enumeration results for each sample are to be entered on **Form 2 Enumeration Hardcopy Results Sheet.**

5.9 If using a different method to the Utermöhl method, please send the Standard Operating Procedure for your method with your results. Explain briefly how it works and how samples are homogenized, set up, analysed, counted and how you calculate the final concentration.

6. Counting strategy

Each analyst should carry out the cell counting according to their own laboratory procedures. This could be in the case of the Utermöhl method a whole chamber cell count (WC), a half chamber count (HC), a transect count (TR) or a field of view count (FV). If counting transects or field of view on a sedimentation chamber the analyst should average at least three counts.

If the analysts are using a different methodology to the Utermöhl method, these should provide information on their counting strategy and calculations to obtain cells/Liter counts.

7. Sample types

This Intercomparison exercise comprises six samples spiked with cell culture material kept in the Marine Institute Phytoplankton culture collection. All the materials have been preserved using lugol's iodine and then homogenized following the IOC Manual on Harmful Marine Algae technique of 100 times sample inversion to extract sub-samples.

It is very important to spend some time becoming familiar with the samples and how the cells appear on the base plate before any count is done as part of the test. The reason for this is that cultured cells could be undergoing division or fusion and look different to the known standard vegetative cell type. See figure 1.

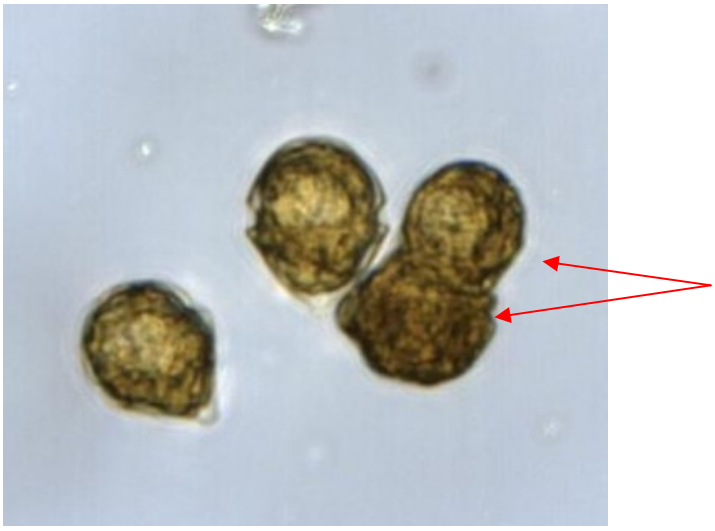


Figure 1: Two Cells fusing

Also note that cells empty theca may appear in the sample (see figure 2),

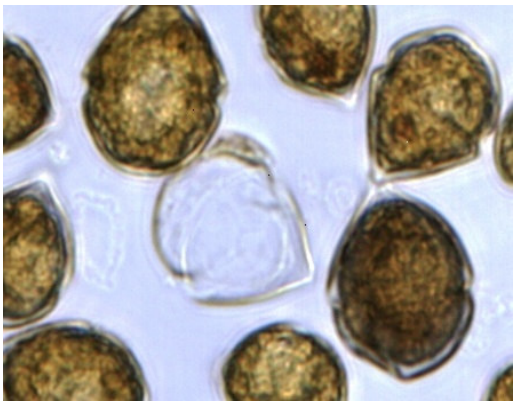


Figure 2: Empty theca

Cells may also vary in size, some cells will appear smaller than others, this is normal in culture conditions (see figure 3). Sometimes Plasmolysis may occur and the cells appear naked and rounded (see figure 4). Aberration of cell morphology can occur also in culture conditions and upon preservation of samples with lugol's iodine.

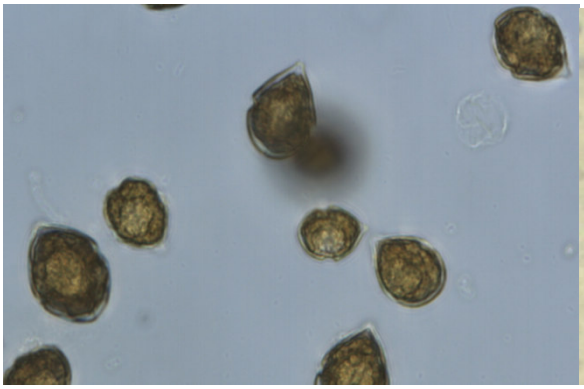


Figure 3: Big versus small cells

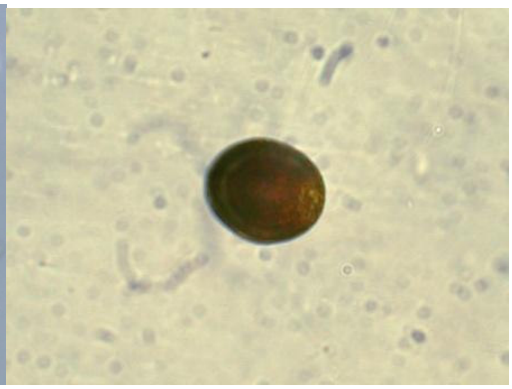


Figure 4: Plasmolised cell

The following rules should be applied for cell counting in this exercise:

a) Any cells that are dividing or fusing, no matter how advance the stage of division or fusion is should be counted as one cell.
b) Empty theca should not be counted
c) Cells should be counted regardless of size
d) Plasmolised cells should be counted
e) Aberrant forms should be counted
f) There is no need to identify the cultured organism in the set of six samples as this is purely an enumeration exercise.

These rules are only applicable to this Intercomparison exercise to avoid bias due to cell counting cultured material.

8. Cell counts Conversion calculations

The number of cells found should be converted to cells per Liter.
Please show the calculation step in Form 2: Hardcopy enumeration results sheet

9. Identification

The Taxonomic quiz for the exercise in 2010 is an updated version of the exercise completed in the Bequalm exercise 2008. Analysts which had already completed this exercise back in 2008 won't have to take part on the identification part on this years exercise.

Analysts that have not participated before or analysts from laboratories which have participated before but did not take part on this particular exercise should complete the taxonomy quiz.

The quiz has been designed to test the general taxonomic skills of the participants. The quiz comprises the use of images, figures and diagrams in various ways to test participants' knowledge of species morphological characteristics and identification.

Please identify and include your results on the Taxonomic quiz (**Form 3**).
The identification exercise carries a total of 300 marks. Make sure you keep a copy of your results before you send the original form in the post.

Participants should name phytoplankton species according to the current literature and scientific name for that species. Where species have been named using a synonym to the current name and if this synonym is still valid or recognized the answer will be accepted as correct.

10. Points to Remember

1. All results must be the analysts own work. Conferring with other analysts is not allowed.

2. Before sending the original results in the post, make a copy of your own results just in case they get lost in the post.

3. Form 2: Enumeration Hardcopy Results Sheet and Form 3: taxonomic quiz must be received by the Marine Institute, Phytoplankton unit by Friday April 16th 2010.

Appendix 12: Form 1_Checklist to Fax Bequalm PHY-ICN-10 MI1

BEQUALM / NATIONAL MARINE BIOLOGICAL ANALYTICAL QUALITY CONTROL SCHEME

Bequalm Intercomparison PHY-ICN-10-MI1

FORM 1: RETURN SLIP AND CHECKLIST

Please ensure to complete the table below upon receipt of samples, and fax or e-mail immediately to the Marine Institute. + 353 91 387237 or rafael.salas@marine.ie		
Analyst Name:		
Laboratory Name:		
Analyst Code Assigned :		
Contact Tel. No. / e-mail		
CHECKLIST OF ITEMS RECEIVED (Please circle the relevant answer)		
Sample numbers _____	YES	NO
Wild Sample number _____	YES	NO
Set of Instructions	YES	NO
Enumeration Result Sheet **(Form 2)**	YES	NO
Taxonomic Quiz **(Form 3)**	YES	NO

I confirm that I have received the items, as detailed above.

(If any of the above items are missing, please contact Rafael.salas@marine.ie)

SIGNED: _____
DATE: _____

Form 1: Return Slip and Checklist

Appendix 13: Form 2_Enumeration Hardcopy results

BEQUALM / NATIONAL MARINE BIOLOGICAL ANALYTICAL QUALITY CONTROL SCHEME

Analyst Name:	
Laboratory Name:	
Analyst Code :	

Enumeration exercise

Sample No	Date of Settlement	Date of Analysis	No. of cells	Volume Chamber (ml)	Calculations	Number cells/L

Describe briefly methodology used:_____

_____

_____

_____

_____

Signed: _____

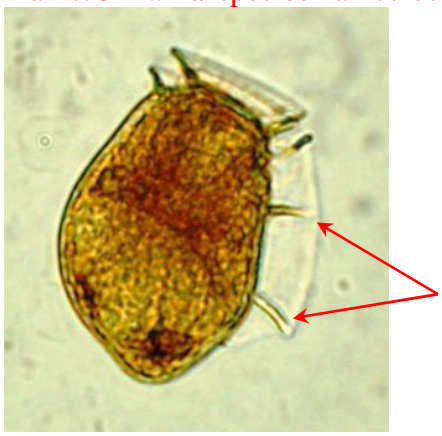
Date: _____

Form 2: Enumeration Hardcopy results sheet

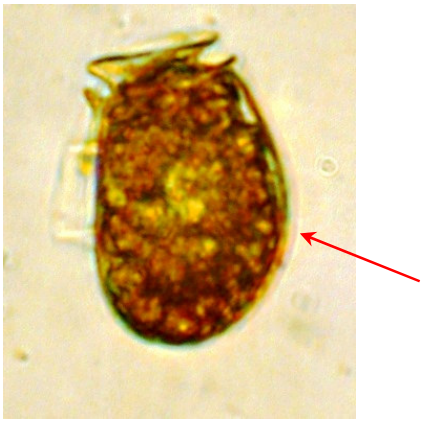
Appendix 14: Form 3: Taxonomic quiz

Form 3: TAXONOMIC QUIZ BEQUALM PHY-ICN-10-MI1

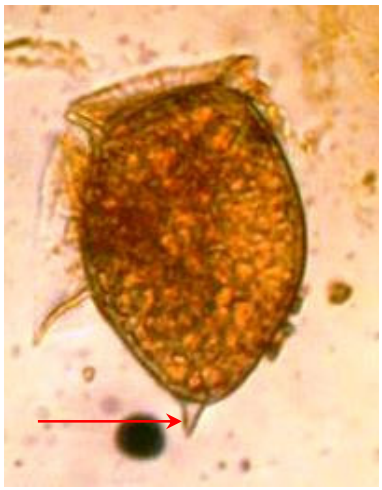
QUESTION 1: The following photographs belong to the genus *Dinophysis*. Participants are asked to name the species and the morphological features that the arrows are pointing at. This question is worth 60 marks. 5 marks/ species named correctly and 5 marks/ features named properly.



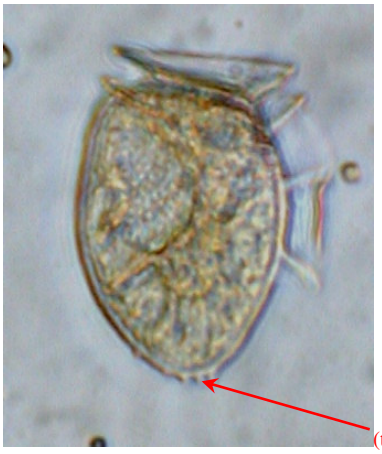
A. ***Dinophysis***_____
Size: L: 85.0, W: 55.0 µm



B. ***Dinophysis***_____
Size: L: 65.0, W: 43.0 µm

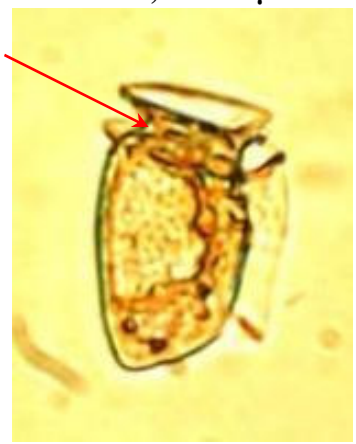


C. ***Dinophysis***_____
Size: L: 74, W: 58 µm

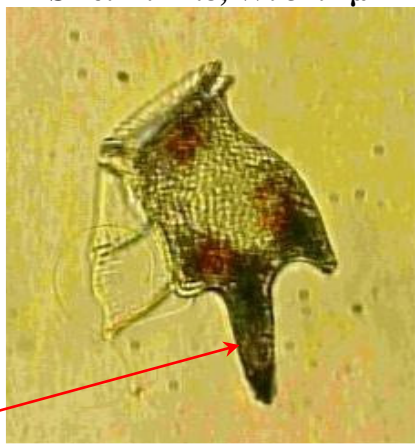


(the small bulgy things)

D. ***Dinophysis***_____
Size: L: 44.8, W: 31.2 µm



E. ***Dinophysis***_____
Size: L: 52.5, W: 32.5µm



F. ***Dinophysis***_____
Size: L: 95.0, W: 55.0 µm

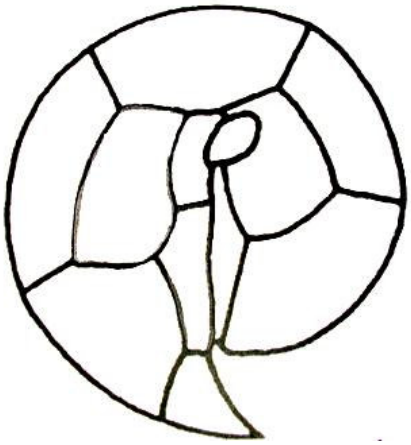
QUESTION 2: The following diagrams show the **Kofoidean tabulation** of two different armoured dinoflagellates in apical view.

This question is worth 20 marks. 10 marks/question

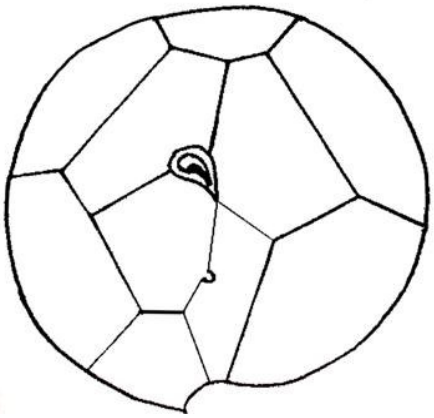
You are asked:

1) Which armoured dinoflagellates genera do these diagrams represent? ***Write answer under each diagram***

2) Which are the main epithecal plate differences between these two genera? ***Name the plates that are different and point at them with arrows***



Answer:_____



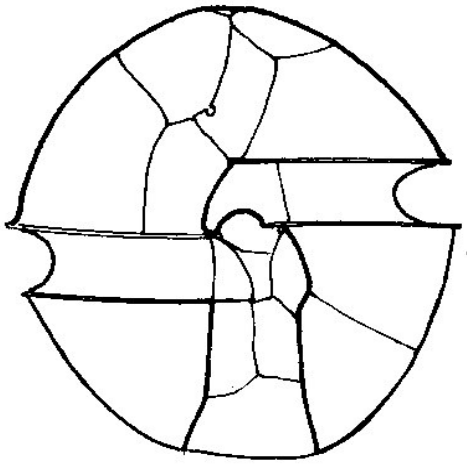
Answer:_____

QUESTION 3: The following diagrams represent an armoured dinoflagellate plate structure in ventral and apical view. Could you with the help of arrows point to the following features:

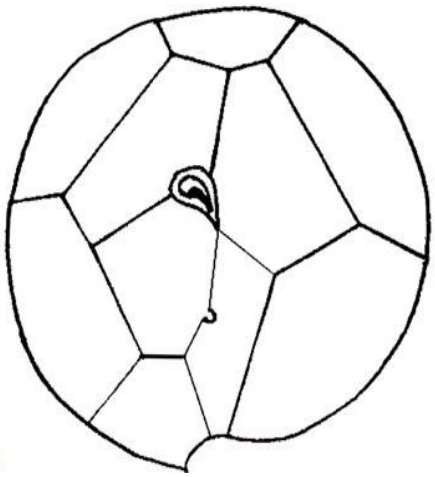
a) the 1' (apical) plate
b) the 6'' (pre-cingular) plate
c) the ventral pore (vp)
d) the sulcal plate (sp)

Use either diagram to point to the features

(**This question is worth 20 marks, 5 marks/correct feature**)



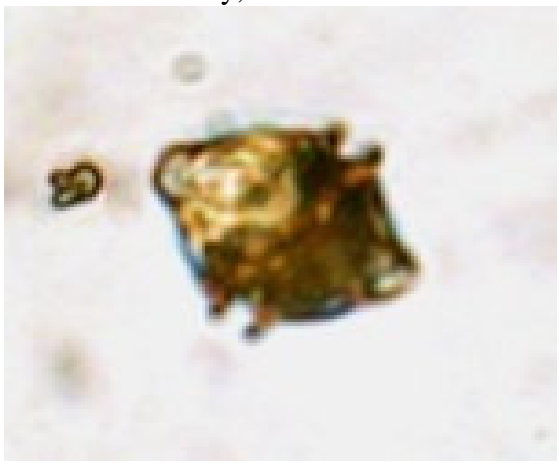
Ventral view



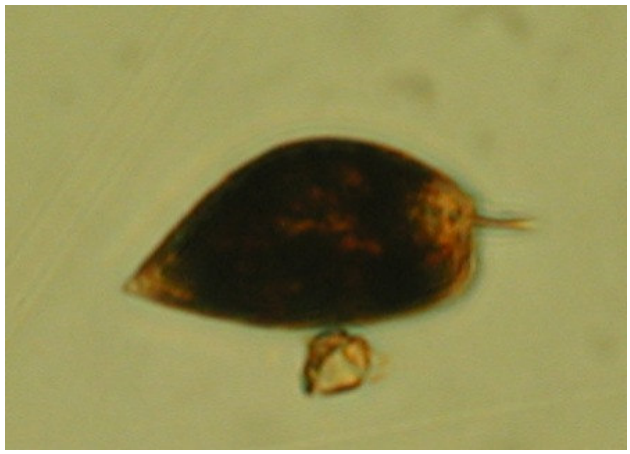
Apical view

QUESTION 4: **Identify to species level** the following pictures of armoured dinoflagellates.

Cell size is given in microns, first number indicates length and second number is width of the cell. Each correct genus answer carries 5 marks. Each correct species answer carries 5 marks. If the genus is named incorrectly, no marks will be awarded for the species name. This question is worth 50 marks.

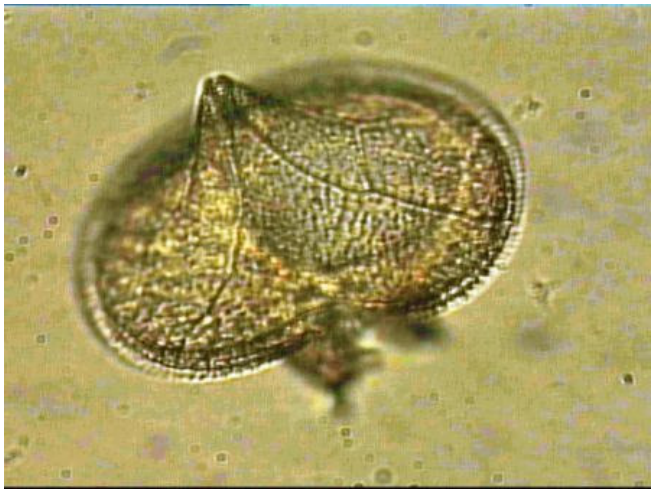


A. Size: L:25, W:20 µm
Name:



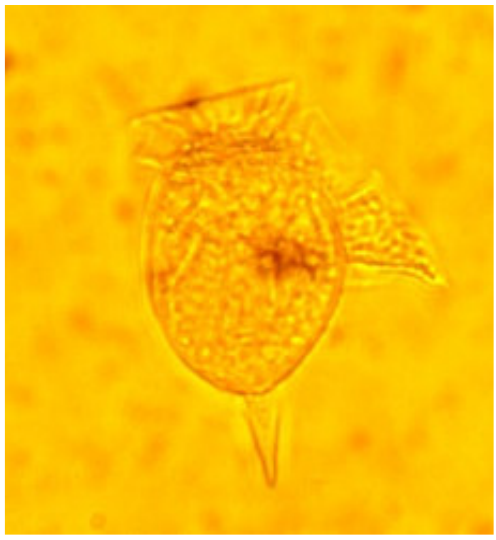
B. Size: L: 65, W: 30 µm
Name:

C. Size: L: 100, W: 105 µm
Name:



second image showing plate structure

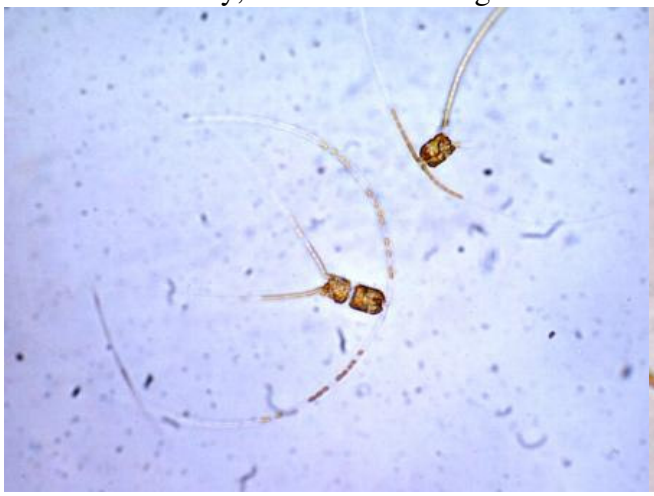
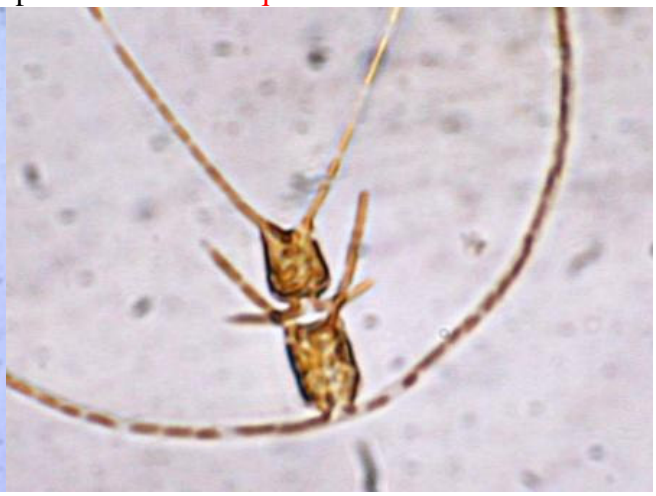
D. Size: L: 47.5, W: 32.5 µm
Name:



E. Size: L: 64, W: 38 µm
Name:

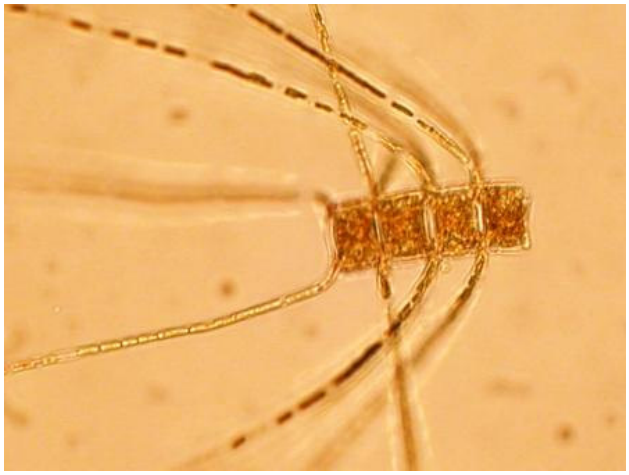
QUESTION 5: Name the following diatoms to **species level**

Each correct genus answer carries 5 marks. Each correct species answer carries 5 marks. If the genus is named incorrectly, no marks will be given for the species name. This question is worth 70 marks.

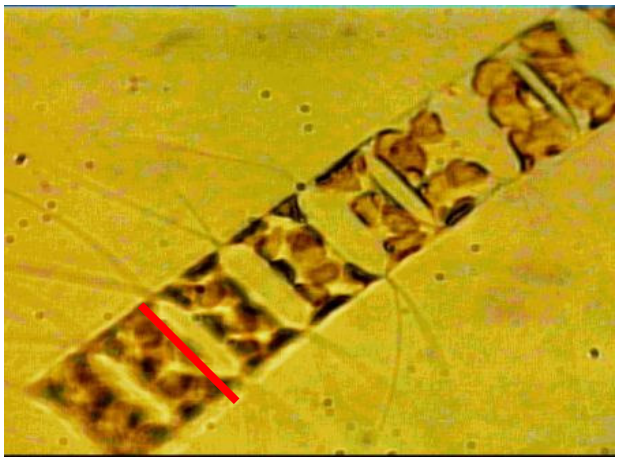
2 images of the same organism (This organism doesn't form chains, Images show organism undergoing division): Size: 35µm length of valve in girdle view Transapical plane

A. Name:



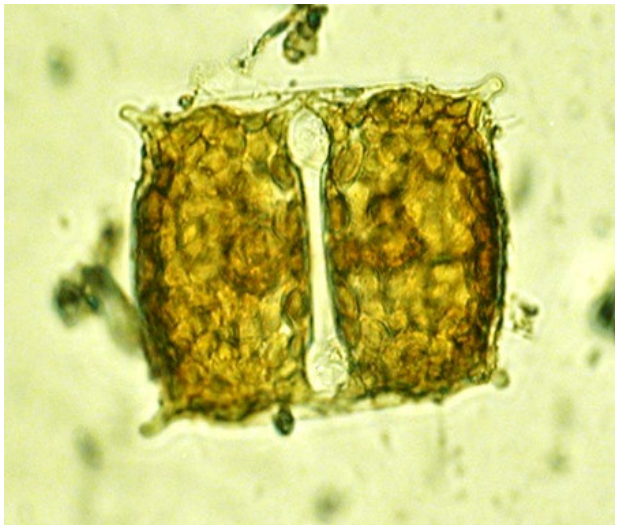

2 images of the same organism. Setae diverge equally from the apical plane. Size: 45µm Length of valve in girdle view transapical plane

B. Name:

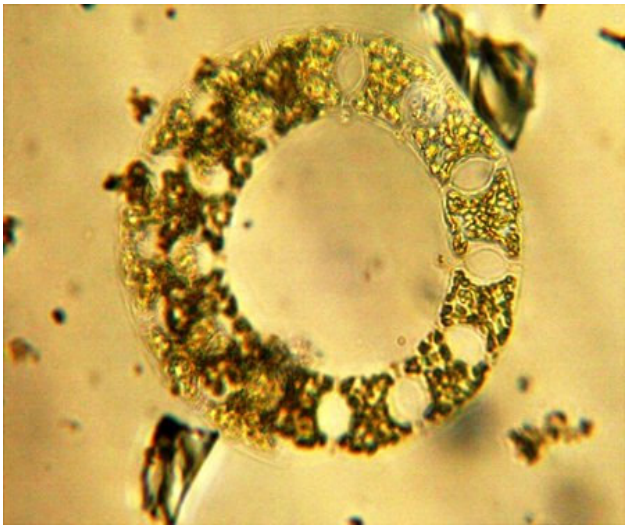


C. Name:
Size: 56µm

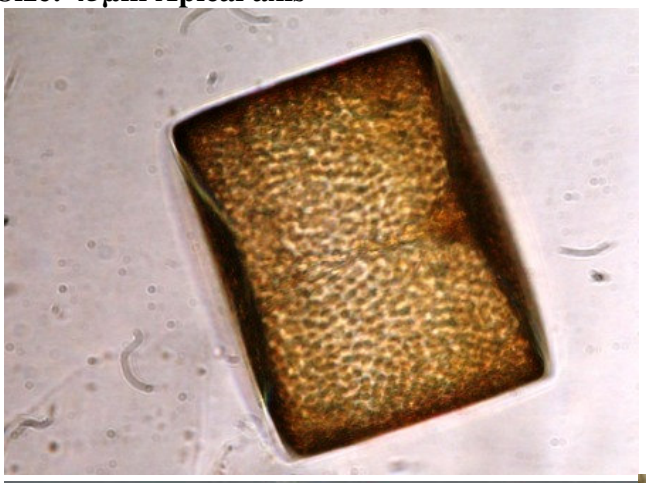
D. Name:
Size: L: 650, W: 100 µm

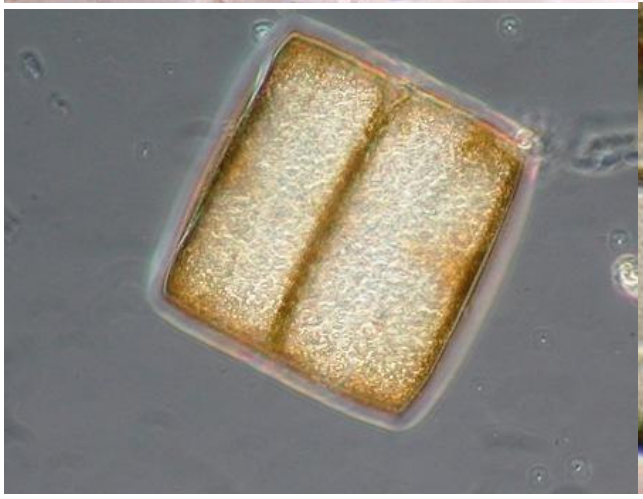


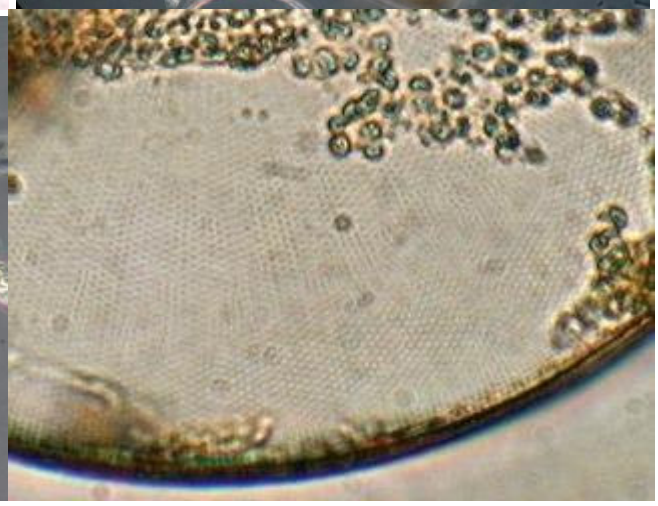
E. Name:
Size: 45µm Apical axis



F. Name:
Size: 65µm wide





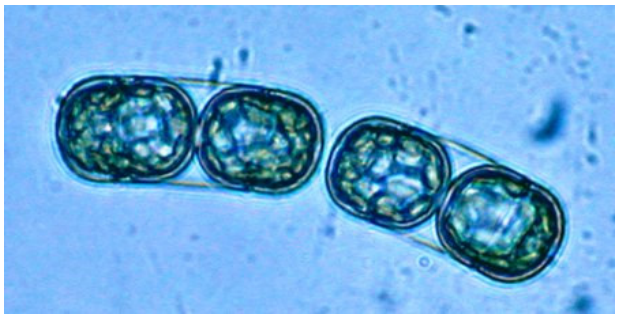


4 images of the same organism. (300 µm diameter) **Areolae details**

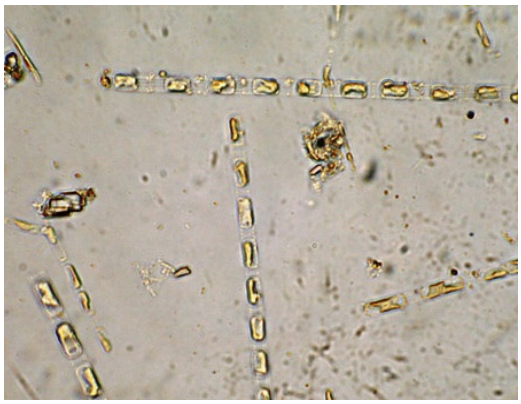
G. Name:

QUESTION 6: Could you circle the odd one out?

This question is worth 15 marks

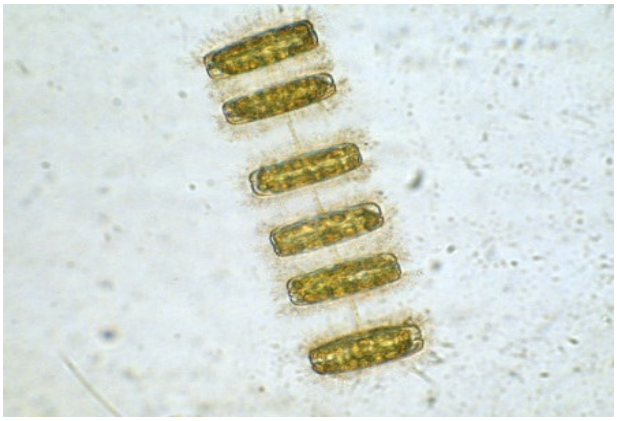


A

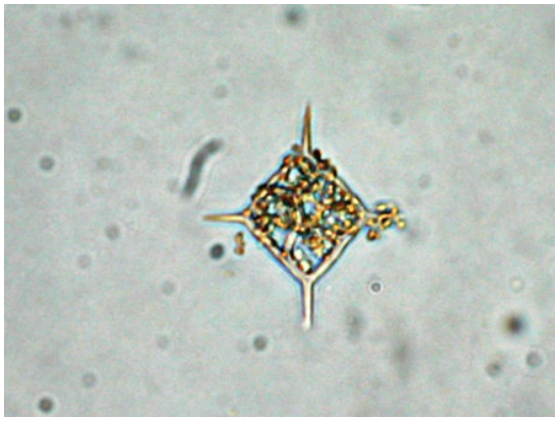


B

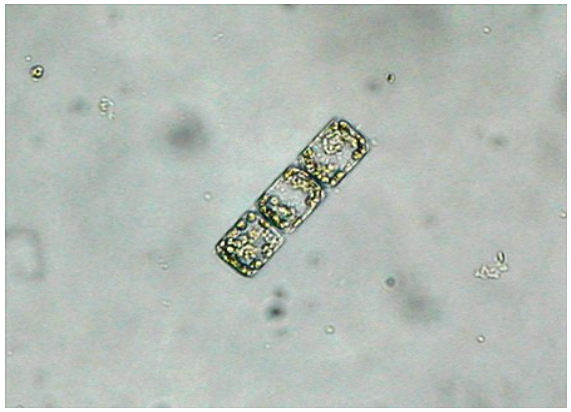
C



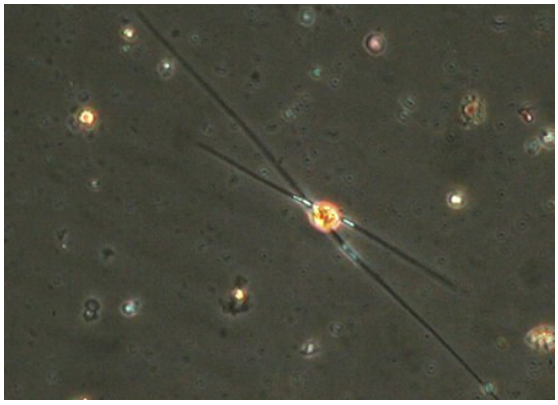
D



E



F



G

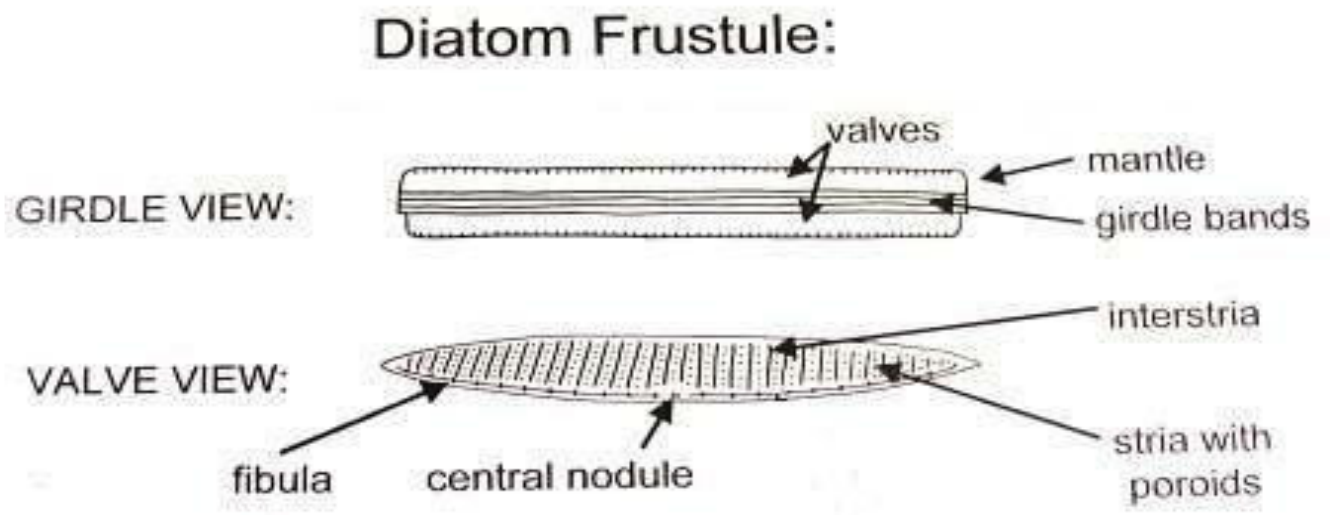
H

QUESTION 7: The following diagrams show a schematic picture of a *Pseudo-nitzschia* cell in valve and girdle view.

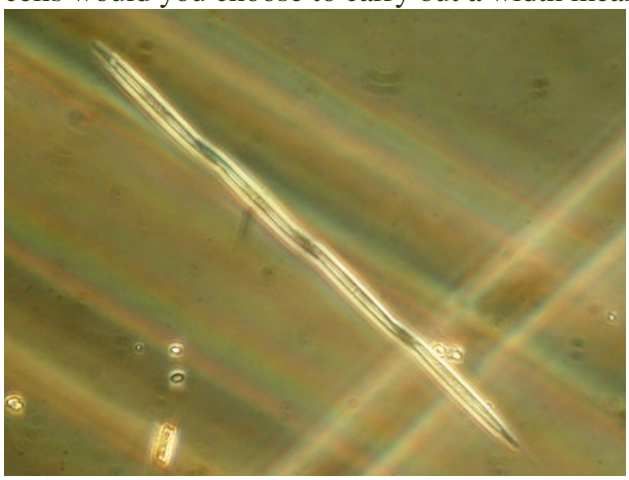
A) If you were to measure the 'width' of a *pseudo-nitzschia* cell, which **view** would you choose to do this? (Draw a line showing where you would measure the cell's 'width')

B) And give a reason why you would choose that particular view to measure the width of the cell?

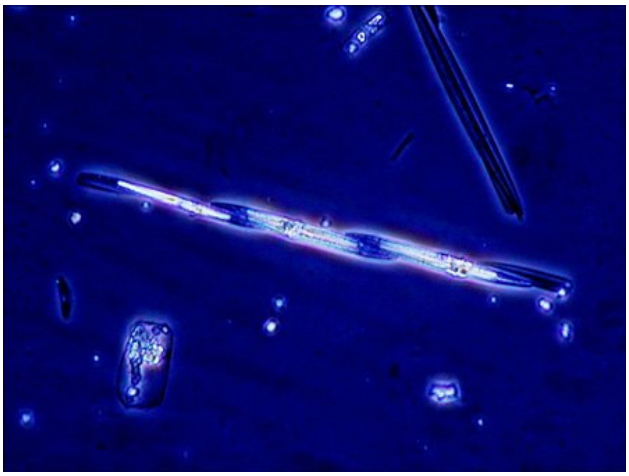
This question is worth 30 marks. 10 marks/correct answer.



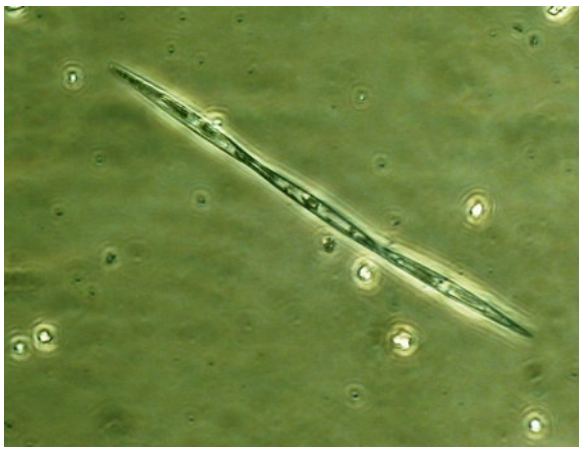
C) Taking into account the answers to **A** and **B**. which of the following photographs of *pseudonitzschia* cells would you choose to carry out a width measurement?



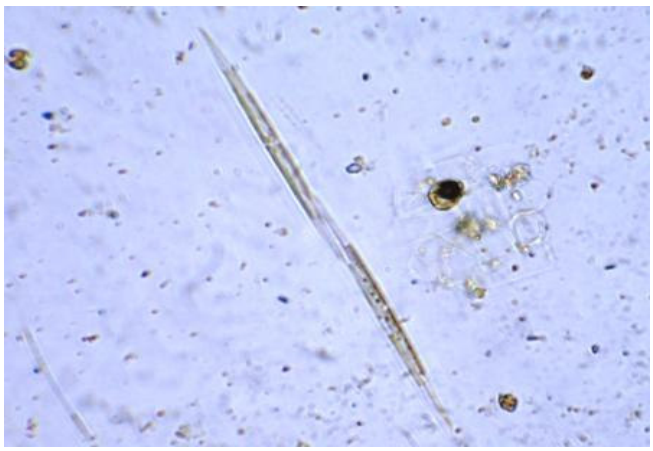
A



B



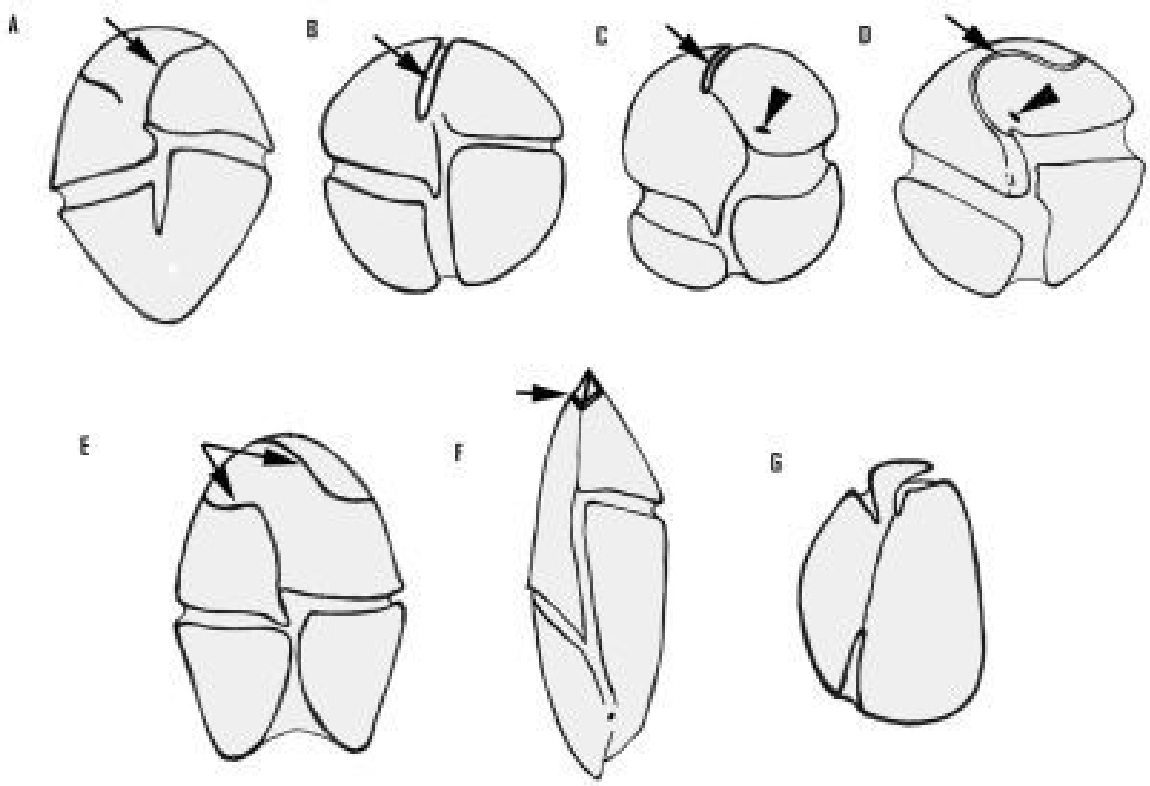
C



D

QUESTION 8: Which **Genera** do these diagrams of naked dinoflagellates represent?
This question is worth 35 marks. 5 marks/correct answer

A:
B:
C:
D:
E:
F:
G:

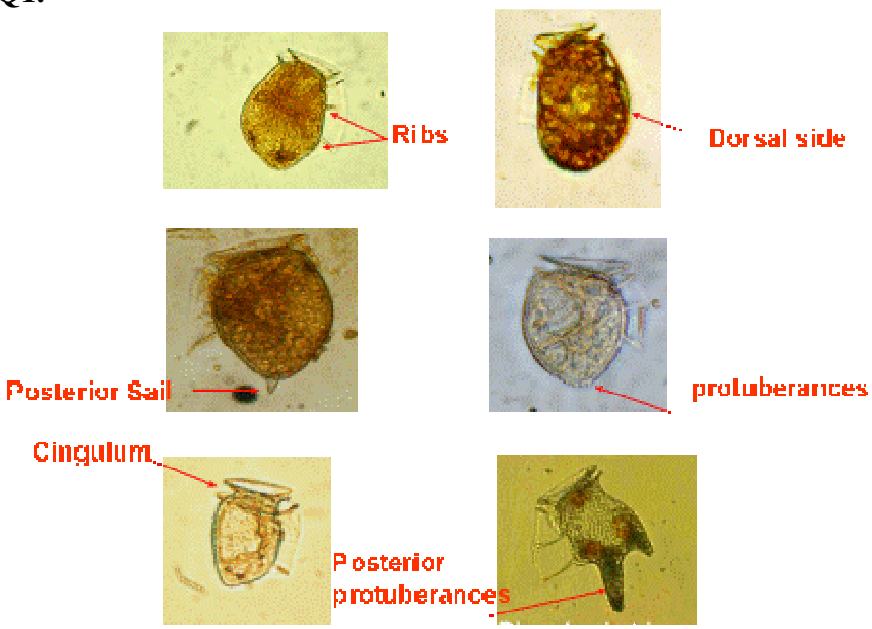


Analyst code:_____

Date:_____

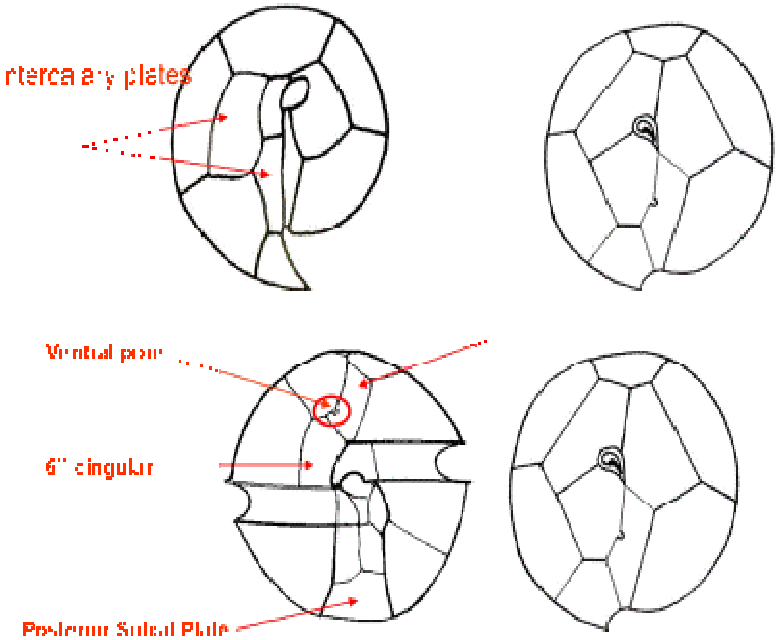
Appendix 15
Correct answers to the identification test PHY-ICN-10-MI1

Q1.

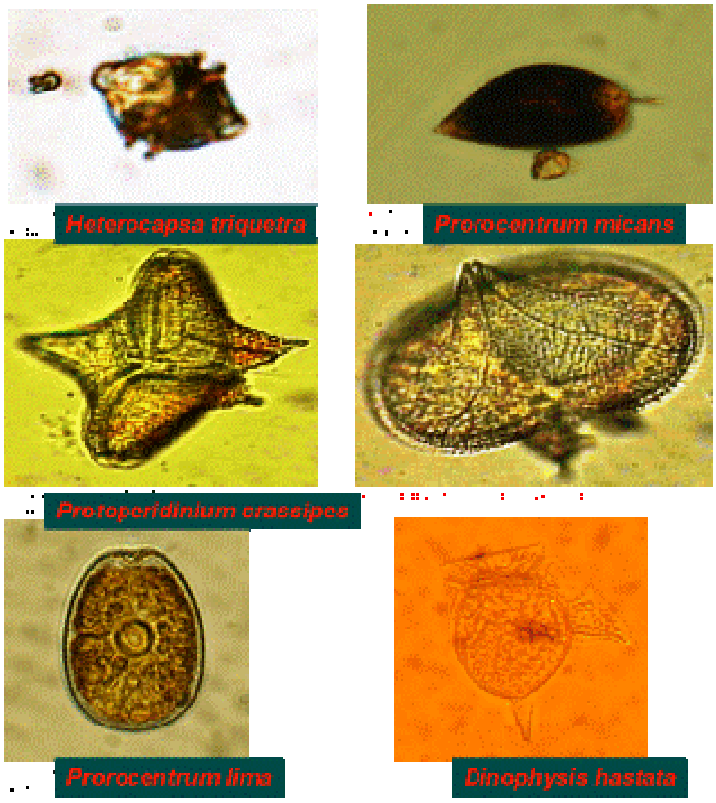


Q2 & Q3

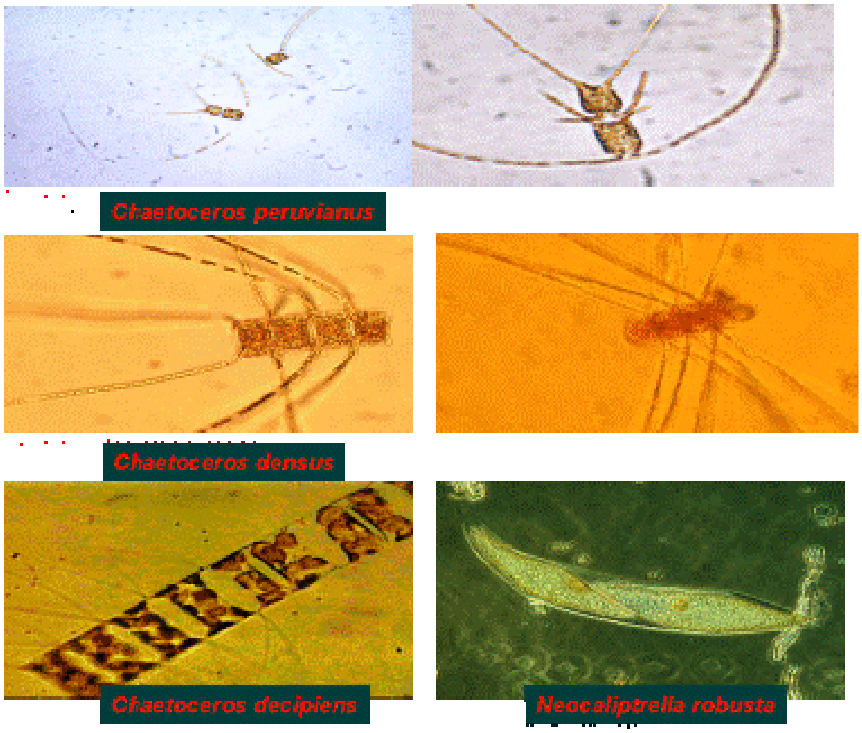
Gonyaulax ***Alexandrium***



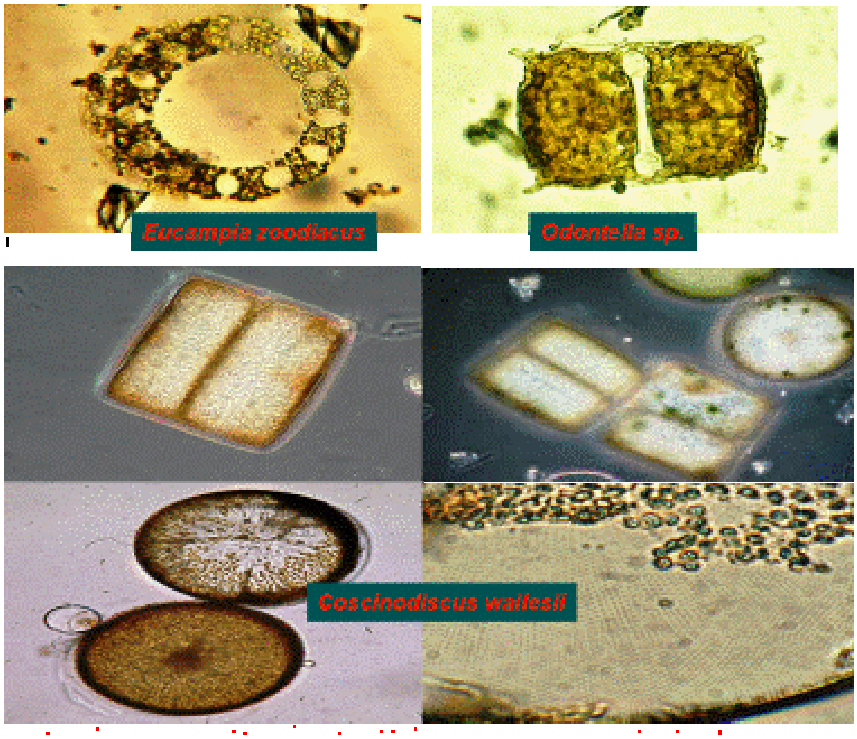
Q4.


Heterocapsa triquetra
Prorocentrum micans
Protoperidinium crassipes
Prorocentrum lima
Dinophysis hastata

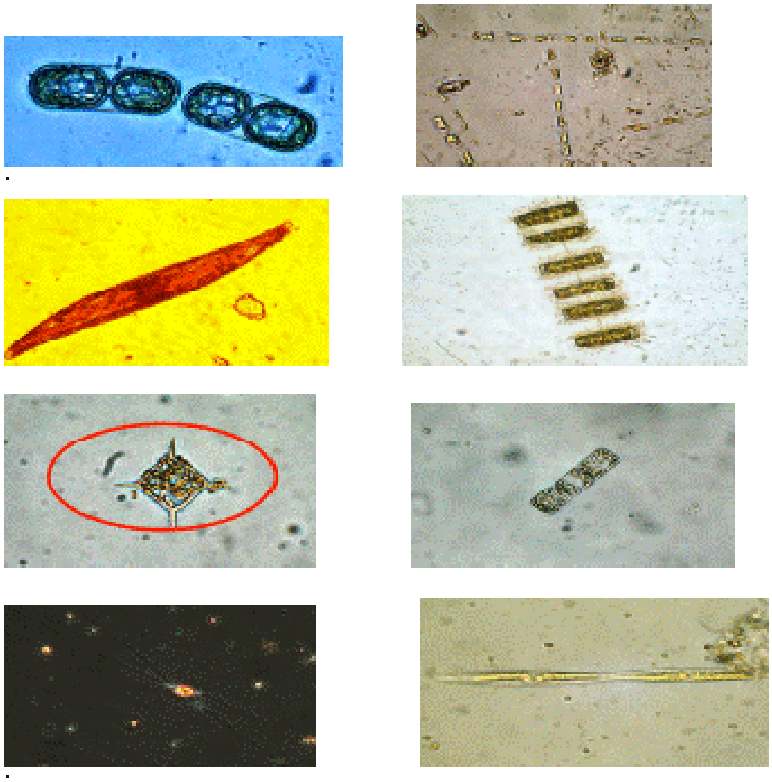
Q5.


Chaetoceros peruvianus
Chaetoceros densus
Chaetoceros decipiens
Neocaliptrella robusta

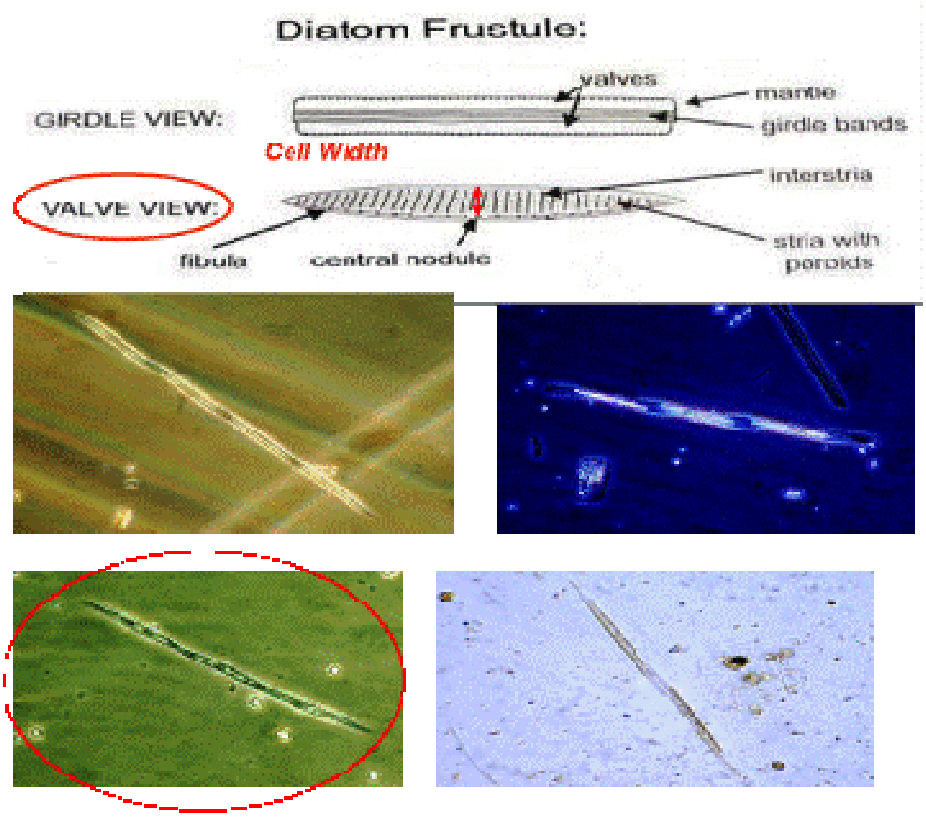
Q5.



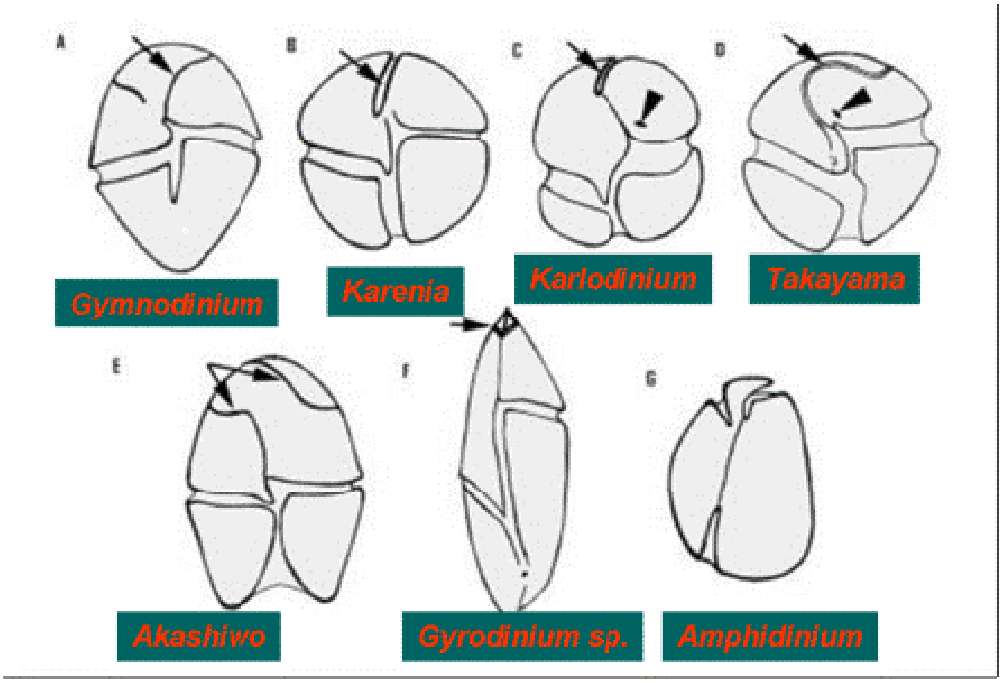
Q6.



Q7.


Diatom Frustule:
GIRDLE VIEW:
valves
mantle
girdle bands
Cell Width
VALVE VIEW:
interstria
fibula
central nodule
stria with
poroids

Q8.


A
B
C
D
Gymnodinium
Karenia
Karlodinium
Takayama
E
F
G
Akashiwo
Gyrodinium sp.
Amphidinium

Appendix 16: Statement of performance certificate

Biological Effects Quality Assurance in Monitoring Programmes / National Marine Biological Analytical Quality Control Scheme / Marine Institute
STATEMENT OF PERFORMANCE
Phytoplankton Component of Community Analysis
Year 2010

Participant details:
Name of organisation:
Participant:
Year of joining:
Years of participation:

Statement Issued:
Statement Number: MI-BQM-10-

Summary of results:

Component Name	Exercise	Subcontracted	Results Z-score (+/- 3 Sigma limits)	
Phytoplankton Enumeration	PHY-ICN-10-MI1	Marine Institute	**Low density samples**	**High density samples**
			Results Pass Mark 70% (over 90% proficient)	
Phytoplankton Identification	PHY-ICN-10-MI1	Marine Institute		

n/a: component not applicable to the participant; n/p: Participant not participating in this component; n/r: no data received from participant

The list shows the results for all components in which the laboratory participated. See over for details.

Notes:

Details certified by:

Section Manager
Joe Silke (MI)

Senior Lab Analyst
Rafael Salas (MI)

Description of Scheme components and associated performance standards

In the table overleaf, for those components on which a standard has been set, 'Proficient', 'Good', and ' "Pass" flags indicate that the participants results met or exceeded the standards set by the Bequalm Phytoplankton scheme; 'Participated' flag indicates that the candidate participated in the exercise but did not reach these standards. The Scheme standards are under continuous review.

Component	Annual exercises	Purpose	Description	Standard
Phytoplankton Enumeration Exercise	1	To assess the performance of participants when undertaking analysis of a prepared sample/s of Seawater preserved in Lugol's iodine and spiked using biological or synthetic subjects using the Utermöhl cell counting method.	Prepared marine water sample/s distributed to participants for Phytoplankton enumeration analysis and calculation of counts in cells per litre	Participants are required to enumerate the spiked material and give a result to within ±3SD or sigma limits of the true value. The true value is the mean calculated from a sample population of the total by the participating laboratories
Phytoplankton identification exercise	1	To assess the accuracy of identification of a wide range of Marine phytoplankton organisms.	This is a proficiency test in the identification of marine phytoplankton The exercise tests the participant's ability to identify organisms from photographs and/or diagrams supplied. In addition, certain taxonomic details need to be identified as well as in some cases genus and species name of the organism. This exercise may also include a combined identification plus enumeration exercise.	The pass mark for the identification exercise is 70%. Results above 90% are deemed proficient, results above 80% are deemed good, results above 70% are deemed acceptable, and results below 70% are reported as "Participated". There are no standards for phytoplankton identification. These exercises are unique and made from scratch.

Appendix 17

Participating laboratories in the Phytoplankton Intercomparison exercise

Bequalm 2010

Appendix 1: BEQUALM 10 LABS	
Marine Institute Phytoplankton lab Rinville, Oranmore Co. Galway Ireland	**IRTA** Carretera del Poblenou km 5,5 Sant Carles de la Ràpita 43540 Spain
AFBI HQ, Newforge Lane Belfast Northern Ireland BT9 5PX United Kingdom	**DLGE** Ballakermeen Road Douglas, Isle of Man IM1 4BR United Kingdom
FRS Marina Laboratory Victoria Road Aberdeen Scotland AB11 9DB United Kingdom	**INTECMAR NIF- Q3600376B** Peirao de Vilaxoán s/n. Vilagarcía de Arousa Pontevedra, Galicia 36611 Spain
SAMS Research Services Ltd Dunstaffnage Marine Laboratory Oban Argyll PA37 1QA United Kingdom	**WEAQ AB** Doktorsgatan 9 d Angelholm SE-26252 Sweden
L.C.C.RR.PP. Ctra. PUNTA UMBRÍA - CARTAYA km 12 CARTAYA HUELVA 21459 Spain	**Marine Institute** Phytoplankton lab Gortalassa Bantry Co.Cork Ireland
CEFAS Barrack Road, The Nothe Weymouth Dorset DT4 8UB United Kingdom	**IZOR** Setaliste I. Mestrovica 63 P.O. Box 500 Split 21000 Croatia
The Water Management Unit Northern Ireland Environment Agency 17 Antrim road, Lisburn Down BT283AL United Kingdom	**SEPA** Clearwater House, Heriot Watt Research Park Avenue North, Riccarton EDINBURGH EH14 4AP
CEFAS Laboratory Pakefield Rd Lowestoft NR33 0HT United Kingdom	**SAHFOS, The Laboratory** Citadel Hill Plymouth Devon PL1 2PB United Kingdom
IRB G. Paliaga 5 Rovinj 52210 Croatia	**Departamento Científico Ficología** Facultad de Ciencias Naturales y Museo Paseo del Bosque s/n 1900 La Plata Argentina
Certificaciones del Perú S.A. Av. Santa Rosa No. 601 La Perla Callao Callao 4 Peru	**IMARES** Haringkade 1 Ijmuiden NH 1976 CP Netherlands
Apem Ltd Riverview A17 Embankment Business Park Heaton Mersey, Stockport Cheshire SK4 3GN United Kingdom	**AquaEcology GmbH & Co. KG** Marie-Curie-Str. 1 Oldenburg 26129 Germany

Appendix 18

Hypothesised values based on Sedgewick-Rafter cell counts

Sedgewick-Rafter cell counts		
Sample number	Cell number in 1ml	Final Cell conc. (Cells/L)
1	210	7000
2	195	6500
3	198	6600
4	189	6300
5	213	7100
6	188	6267
7	205	6833
8	202	6733
9	206	6867
10	208	6933
Mean	201	6713

Hypothesised mean= 6000
200 cells in 30ml

Sedgewick-Rafter cell counts			
Sample number	Cell number	Cell number* 10	Final Cell conc. (Cells/L)
1	982	9820	327333
2	1035	10350	345000
3	957	9570	319000
4	982	9820	327333
5	1001	10010	333667
6	1025	10250	341667
7	995	9950	331667
8	1015	10150	338333
9	975	9750	325000
10	1036	10360	345333
Mean	1000	10003	333433

Hypothesised mean= 333333
10000 cells approx. in 30ml

Appendix 19

Analysts cell concentration counts Bequalm 2010

ANALYST CODE	SAMPLE CODES						Cells/L					
							Cell count (Low			Cell count (High density)		
10	35	39	151	82	90	112	0	5000	2000	101000	205000	168000
37	88	132	133	15	17	25		1600	3300	158000	125200	153000
13	86	201	293	190	255	276	6400	4900	4500	186100	326000	277000
17	55	161	265	11	99	245	6000	4500	4400	155200	167000	402486
30	13	249	275	43	80	93	4000	4400	5000	149300	148000	169500
21	21	77	124	69	147	289	3800	3100	3800	160000	139700	244700
9	129	250	287	254	257	297	5915	6097	5733	387512	374376	443340
11	3	18	66	44	120	185	5500	5800	4067	219567	209833	436333
15	2	54	159	283	16	67	5800	5400	6700	723900	315400	266000
33	64	179	246	97	177	272	4160	3360	4480	157480	108600	227400
4	72	87	209	14	169	238	3160	3360	2225	161480	163480	211920
38	176	230	291	103	237	292	3960	4280	3880	175880	166000	151920
24	49	101	294	126	212	296	1840		2080	173160	142880	207500
26	119	140	223	52	23	228	4080	2920	3200	166120	139640	102240
39	102	150	225	183	187	229	4010	3952	4192	105752	124930	141915
34	38	236	279	5	123	298	3640	3920	2960	157560	90280	181320
5	24	211	273	28	85	197	4240	4240	4320	184560	184400	197520
25	47	36	263	40	51	58	3640	3680	4760	153520	169320	170400
35	232	60	12	207	252	194	5000	5280	4000	136200	158200	151440
28	29	94	121	4	10	239	4038	3482	3667	114334	164815	21260
27	41	37	171	248	160	186	3360	2760	2880	55720	120080	126240
18	95	226	166	56	19	135	4400	5280	5680	132183	190931	171606
7	193	235	26	1	266	227	3400	2960	4160	142975	284760	187093
20	100	231	281	62	167	240	2360	1440	4494	90629	138565	229447
36	68	259	284	163	195	234	3240	2824	3960	86216	107448	101795
16	111	46	61	261	27	92	4840	5520	5440	296678	239659	294413
8	115	162	241	7	84	131	5667	4173	5280	243041	238374	256734
2	152	157	203	148	224	274	5040	4320	4920	317130	284456	219108
19	110	130	286	114	153	256	5160	3840	3400	180112	224726	190026
3	8	210	191	105	50	178	4600	4360		197000	258280	284760
29	75	196	214	6	53	220	5280	4440	5080	231679	175473	291997
40	280	113	138	206	70	117	6080	4120	5720	204330	211870	288776
12	34	149	180	59	202	300	5280	4560	4400	158064	221432	278392
14	108	144	264	122	158	174	5445	4455	5940	305415	390555	242055
23	104	258	290	73	127	295	4120	5360	5320	329618	266875	242149
32	143	156	285	165	189	253	3480	2880	4280	191212	122922	245844

Appendix 20

Analysts methodologies Bequalm 2010

ANALYST CODE	Methodology	Counting strategy	
		Low	High
10	2ml sub-sample in Utermohl chamber	WC	HC
37	10 ml sub-sample in utermohl chamber	WC	WC
13	10 ml sub-sample in utermohl chamber	WC	WC
17	10 ml sub-sample in utermohl chamber	WC	WC
30	10 ml sub-sample in utermohl chamber	WC	WC
21	10 ml sub-sample in utermohl chamber	WC	WC
9	10 ml sub-sample in utermohl chamber	WC	TR
11	10 ml sub-sample in utermohl chamber	WC	TR
15	10 ml sub-sample in utermohl chamber	WC	TR
33	25ml sub-sample in Utermohl chamber	WC	WC
4	25ml sub-sample in Utermohl chamber	WC	WC
38	25ml sub-sample in Utermohl chamber	WC	WC
24	25ml sub-sample in Utermohl chamber	WC	WC
26	25ml sub-sample in Utermohl chamber	WC	WC
39	25ml sub-sample in Utermohl chamber	WC	WC
34	25ml sub-sample in Utermohl chamber	WC	WC
5	25ml sub-sample in Utermohl chamber	WC	WC
25	25ml sub-sample in Utermohl chamber	WC	WC
35	25ml sub-sample in Utermohl chamber	WC	WC
28	25ml sub-sample in Utermohl chamber	WC	WC
27	25ml sub-sample in Utermohl chamber	WC	HC
18	25ml sub-sample in Utermohl chamber	WC	TR
7	25ml sub-sample in Utermohl chamber	WC	TR
20	25ml sub-sample in Utermohl chamber	WC	TR
36	25ml sub-sample in Utermohl chamber	WC	TR
16	25ml sub-sample in Utermohl chamber	WC	TR
8	25ml sub-sample in Utermohl chamber	WC	TR
2	25ml sub-sample in Utermohl chamber	WC	TR
19	25ml sub-sample in Utermohl chamber	WC	TR
3	25ml sub-sample in Utermohl chamber	WC	TR
29	25ml sub-sample in Utermohl chamber	WC	TR
40	25ml sub-sample in Utermohl chamber	WC	TR
12	25ml sub-sample in Utermohl chamber	HC	TR
14	25ml sub-sample in Utermohl chamber	TR	TR
23	25ml sub-sample in Utermohl chamber	WC	FoV
32	25ml sub-sample in Utermohl chamber	WC	FoV

WC= Whole chamber; HC= Half chamber; TR= Transect; FoV= Field of View