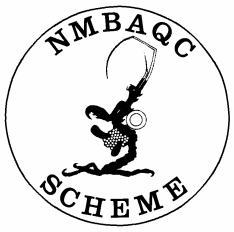
Marine Institute

Ireland



PHYTOPLANKTON ENUMERATION AND IDENTIFICATION ANALYSIS

Ring Test PHY-ICN-09-MI1 Exercise Report, June 2009

Rafael Gallardo Salas

Marine Institute
Rinville
Oranmore
Co. Galway
Ireland

PHY-ICN-09-MI1 VR1.0

Phytoplankton Enumeration and Identification Proficiency Test

Marine Institute (MI) and Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM):

Table of Contents:

1. Summary	Pages 3 to 4
2. Introduction	Pages 4 to 6
3. Participants	Page 6
4. Materials and Methodology	
4.1 Study design	Pages 6 to 10
4.2 Phytoplankton samples	Pages 10 to 13
4.3 Taxonomic quiz	Pages 13 to 16
4.4 Forms and instructions	Pages 16 to 17
4.5 Utermohl test method	Pages 17 to 18
4.6 Statistical analysis	Pages 18 to 19
5. Results and Discussion	
5.1 Phytoplankton enumeration results	Pages 19 to 25
5.2 Phytoplankton identification results	Pages 25 to 26
5.3 Performance evaluation	Pages 26 to 27
6. Conclusions and Recommendations	Pages 27 to 30
Annex I: Participating laboratories	Page 31
Annex II: Instructions	Pages 32 to 38
Annex III: Detailed results of the Enumeration test	Pages 39 to 56
Annex IV: Detailed results of the identification test	Pages 57 to 63
Annex V: Workshop Agenda	Pages 64 to 65
Annex VI: Taxonomic quiz	Pages 66 to 81
Annex VII: FORM 1_Checklist to Fax bequalm 08 MI1.pdf	Page 82

Annex VIII: FORM 3_Enumeration Hardcopy results Page 83

Annex IX: Statement of performance certificate Page 84 to 86

1. Summary

At the beginning of January 2009 contractual arrangements were put in place between the Marine Institute and CTL (CEFAS Technology Ltd) to administer and organize the Bequalm NMBAQC Phytoplankton programme for 2009.

The exercise was divided into an enumeration and identification exercise. The enumeration exercise was a number of seawater samples (6) spiked with live cultures of Phytoplankton that participants had to enumerate and identify and the identification exercise were a taxonomic quiz composed of set of images and video clips of phytoplankton species. These images and videos are copyright material belonging to Jeremy and Julianne Picket-Heaps of Cytographics Ltd in Australia. The Marine Institute bought the rights of this material to be used for this Intercomparison exercise only. As part of this deal the participating labs this year will receive a copy each of their 2 titles 'Diatoms: Life in Glass houses' and 'The kingdom Protistan: The dazzling world of living cells'.

This year the exercise has become slightly more complex than previous exercises. This year for the first time we have introduced samples with multiple species and we have introduced different types of samples, negative controls and positive controls.

Also, for the first time we have used video footage of Phytoplankton species for the identification part of the exercise to be used in conjunction with images and to compare both. We have extended the range of species to be identified to phytoplankton groups other than Diatoms and Dinoflagellates.

All the materials needed to complete this Intercomparison exercise PHY-ICN-09-MI1 were sent to all participants who had registered through the Bequalm website to this new round of the community analysis Phytoplankton component. The materials included spiked samples preserved in lugol's iodine, a Taxonomic quiz, a set of instructions, and forms to write results in and to confirm that materials had arrived in perfect conditions.

Analysts were given until the 20th of March (4 weeks from sample receipt) to return enumeration and identification results to the Marine Institute (MI) Phytoplankton laboratory.

The Bequalm workshop was set for the 16th of April 2009. The intercomparison enumeration and identification results were discussed at this workshop and statistical analysis was done on the results and presented at this fora.

The statistical analysis for the workshop was done by Dr. John Newell of the Biostatistics unit in the National University of Ireland Galway.
We also had invited Dr. Urban Tillmann of the Biosciences/Ecological Chemistry unit of the Alfred Wegener Institute (AWI) to give lectures on Azadinium spinosum and Protistan grazing.
Josephine Lyons of the Marine Institute Phytoplankton unit gave a talk on Accreditation in the phytoplankton lab.

Also, the Marine Institute is responsible for producing a report and certificates of the exercise which is later sent to all participating analysts.

This year 34 analysts in 17 labs from across Europe have taken part in this exercise. This is the first NMBAQC Bequalm exercise at a wider European level. This is the first time that labs from Holland and Germany have taken part on the exercise and 3 new labs from Spain had joined the scheme, One of them (IRTA) from the Mediterranean area in Spain. A total of 5 new labs took part on the exercise this year, these were AWI (Alfred Wegener Institute) in Germany, Koeman en Bijkerk in Holland, IRTA (Institut de Recerca i Tecnologia Agroalimentàries) in Catalunya, Spain. L.C.C.RR.PP. (Laboratorio de Control de Calidad de los Recursos Pesqueros) in Huelva, Spain and LVCC Egmasa (Laboratorios de Vigilancia y Control de la. Contaminación) en Cadiz, Spain.

2. Introduction

Biological effects measurements are increasingly being incorporated into national and international environmental monitoring programmes to supplement chemical measurements. 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. This was in direct response to the requirements of OSPAR to establish a European infrastructure for biological effects QA/QC, in order that laboratories contributing to national and international marine monitoring programmes can attain defined quality standards.

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 exercises are to compare the performance of laboratories engaged in national official/non-official phytoplankton monitoring programmes and other labs working in the area of phytoplankton (see bequalm website www.bequalm.org).

The objectives of the NMBAQC Bequalm intercomparison for phytoplankton are to mirror what we do in the lab and that is to analyse marine water samples for phytoplankton enumeration and identification to the highest taxonomic level possible.

Also, to test the method that we use for routine monitoring, most labs in Europe use the Utermohl method with slight variations, usually these variations have to do mainly with the volume of the Sedimentation chamber used and counting strategies. We are looking to test the method and its limitations in terms of their limit of detection, quantification, bias, robustness, accuracy, precision, specificity, reproducibility, repeatability, stability, etc. Each intercomparison exercise in a given year is designed to test one or various aspects of the test method.

So, what bequalm intends is to become a type of quality control for labs engaged in phytoplankton analysis, where labs and analysts can compare their results and see how they are all doing. Are we all enumerating in the same way and getting similar results? Are we all identifying correctly the same species?

Bequalm is slowly becoming a proficiency testing scheme, which is perceived by labs engaged in Phytoplankton monitoring programmes or for environmental reasons of being of good quality and where participating labs have an active input on the scheme through workshops and direct communications with the Marine Institute phytoplankton unit.

The participation in this type of schemes is becoming an essential requirement for National phytoplankton monitoring labs in order to achieve accreditation for their method.
Since last year, we also certify the participation of individual analysts on the scheme by issuing statement performance certificates.

This year for the first time we have participating labs from Holland and Germany, 3 new labs from Spain and other labs from other countries which have not participated this year have enquired about the possibility to take part in this type of exercise in the future.

The Marine Institute Phytoplankton lab is accredited to ISO 17025 for Toxic Marine phytoplankton identification and enumeration since 2004, and it recognizes that regular Quality Control assessments are crucial to ensure a high quality output of Phytoplankton data.

3. Participants

In total, thirty four analysts from seventeen laboratories participated in the exercise PHY-ICN-09-MI1. This code is in accordance to defined protocols in the Marine Institute for the purposes of Quality traceability and auditing. The laboratories taking part were located in Ireland, Northern Ireland, Scotland, England, the Isle of Man, Spain, Holland and Germany. A complete list of the participating laboratories is given in Annex I.

4. Materials and Methodology

4.1 Study design

The Bequalm 2009 intercomparison exercise was designed to be statistically robust in order to obtain useful data that could be used for statistical analysis. A number of hypotheses were asked a priori before choosing, manipulating and setting up the intercomparison materials.

The first question we asked ourselves was what type of study was more relevant to the type of exercise that we were carrying out and what type of questions or hypothesis we wanted answered.

The type of study used for this exercise was an experimental study. This is a study where one or more treatments are imposed on individuals to observe their responses.
The exercise should mirror what we do in the lab, in this case, this is to analyse marine water samples for the presence of toxic phytoplankton and enumerate and identify these organisms to the highest taxonomic level possible.

Analysts usually identify and enumerate at the same time, so in order to mirror that, a number of phytoplankton species were spiked into samples. There were 4 different sample types or treatments. Treatment 1 was a set of samples containing 4 different species; Treatment 2 was a set of samples containing the 4 species as in treatment 1 plus another. Treatment 3 was a set of samples containing just 1 species this is the extra species found in treatment 2 also (called positive control) and treatment 4 was a set of samples containing 0 species (called negative control).

The reason for using 4 different types of samples for the exercise goes back to the principles of experimental design in biological sciences. These are controls, randomization, replication and blinding.

The identification exercise was somewhat simpler in design with only 2 treatments: Treatment 1 was images and treatment 2 was video clips. There were 4 sets of 5 each. The factor of interest in this exercise was the use of 2D images compared to 3D moving images. Again the reason for the use of images and/or videos as part of the exercise hinges in the fact that images are used in practice as quality controls on monitoring programmes as an audit trial or record.

So, clearly the reasons why we are carrying out this research is first of all to figure out whether there are differences between labs and analysts enumerating and identifying toxic phytoplankton in marine water samples using the same methodology and to find the reasons why these differences exist.
Secondly, to test the robustness and limitations of the method used in order to standardise and fine tune these methodologies.

In order to address these questions we formulated several hypotheses:
Ho: There are no differences in the enumeration of cells between and within labs.
Ho: There are no differences in the identification trials between and within labs.

The fact that the null hypothesis states that there are no differences between and within labs this implies that the alternative hypothesis is that differences exist. If this is the case then we want to know a bit more about the reasons why this may be so. The most single important source of bias in this method is cell counting followed by false identification. Cell counting bias can be addressed by improving the techniques and standardising methodologies while reducing analyst error, false identifications need to be addressed through continue training on phytoplankton taxonomy.

This exercise did not set up to assess the technical expertise on the test method rather to study a factor that will influence the accuracy and precision of cell counting; this is the cell concentration or density.

The limit of detection for the method is one cell and the closest we get to this limit the harder it becomes to count for the analyst also the biggest the error as the difference between counting one cell or no cells is 100%. The same could be said about the limit of quantification, in theory this is limitless that is infinite. As the cell concentration increases in a sample, the hardest it becomes to count so you have a number of choices, one to dilute the sample or to choose a different counting method by counting a smaller area of the sample, both choices means accumulating errors.

In this exercise we are not really interested in the limit of detection as we already know that in the same way that a piece of equipment at their limit of detection may or may not miss an analyte, the analyst in this case suffers from the same problem.
We are not looking either at the limit of quantification this time but this may be interesting to explore further in the future.

In this exercise we are more interested in the range of the method that is different concentrations and to that end we are investigating low, medium and high concentrations hence the different cell concentrations of the spiked materials

So we can test our hypothesis further. What we are saying is that if there are differences in cell counts between and within labs, then these differences may have to do with concentration so we need to explore this relationship.

In this exercise, we are also using controls. A negative control (a sample with no cells in it) is used to spot methodology problems, that is whether analysts are seeing cells or/and whether analysts are having problems with the use of sedimentation chambers.

A positive control: that is a spiked sample with one toxic species (*Prorocentrum lima)* have been used to ascertain that we are all able to identify correctly a toxic organism on its own. In order to make the design more robust we have confounded this factor by including this same organism within 2 samples containing the four other organisms, this was done to observe whether when an organism is confounded is it more difficult to identify and are the cell counts less accurate.

This exercise is also designed so that labs with more than one analyst can compare results among themselves as they are all analysing exactly the same samples. So, each lab can look at their own lab intra and inter observer variability, and spot whether there are differences in their cell counts then this problem could be addressed.

In the identification part of the exercise we are more worried about making an assumption on a particular species from an image or video clip with a limited amount of information that is using an intuitive approach. This is what happens in reality, when someone wants your opinion on a particular organism, they may send you an image with a limited resolution, quality and very little information as you might not know where the sample was collected, when, the type of sample and of course the natural limitations of a two dimensional picture. At that point you may have to make a call as to what it is. This is a really important part of the process in identifying and it is a lot to do with using intuition as much as knowledge. Lest not forget that probably there isn't enough information to give the perfect answer.

In some ways, this is what we have tried to do in this exercise that is to force analysts to make a call with the information given. In some ways this may not appear to be fair but in the real day to day monitoring we have to make calls of

this kind all the time as monitoring programmes use light microscopy mostly. Fluorescence microscopy or SEM/TEM work is beyond the scope of most monitoring programmes and are seen more as research tools use when needed rather than on a daily basis. Again, this is to go back to mirroring what we do in the lab.

The other area of interest in this exercise was to compare the use of images to the use of videos that is two dimensional images compared to three dimensional moving images. In a way is like comparing more information against less information if we think that 3D videos contain extra information in terms of the movement of the cell and certain characteristics that accompany that movement.

So the null hypothesis here is that if there are differences in identifying between and within labs, then we want to know if these differences are in particular labs or are a more systemic problem across all the labs, that is are all the labs answering incorrectly particular questions or are particular labs better or worse at particular sets. Then the next hypothesis is: are labs better at identifying images or videos?

Finally, all through this process, we have attempted to avoid all the pitfalls that come with statistical designs, with special attention to pseudo-replication.

4.2 Phytoplankton samples: Enumeration exercise

4.2.1 Selecting culture material

The materials for the enumeration exercise were sought from the Marine Institute algal culture collection. The decision to use particular species were given by the experimental design of the exercise but also by practical reasons, for example the cultures used had to be in good working order in terms of their morphology so that their shape would be typical of a vegetative cell for that species.

Several phytoplankton species were short listed from a bigger pool of species. These were *Prorocentrum lima, Scrippsiella trochoidea, Coscinodiscus granii, Gymnodinium catenatum and Prorocentrum micans.*

In terms of cell density it was decided that *Scrippsiella and Gymnodinium catenatum* would be the high cell densities and *P.lima and C .granii* would be the low concentration species.

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

P.micans culture contained an amount of gametes, but analysts were warned on the instructions about the possibility of finding smaller cells in the samples. *P.lima and scrippsiella* did appear to be the easiest cells to identify a priori from the experimenter point of view, *Scrippsiella* mainly for its typical shape and *P.lima* because of being dorso-ventrally flattened and its characteristic morphology.

Overall great care was taken to choose the final candidate species for the exercise and a huge amount of effort was devoted to this end. However, it is true to say that cell cultures change morphologically with time and that many cultures kept in the marine institute would not be usable for that same reason, especially diatoms like *Pseudonitzschia spp.* and other armoured dinoflagellates. It would probably be better to use naturally occurring samples for some of these species.

4.2.2 Cell concentrations

Once the species were chosen, we had to decide about cell densities. The lowest concentration range would be around 100 cells in a 25ml sample (*P.lima)* and the highest range would be 1000 cells in 25ml sample (*Scrippsiella). G.catenatum* density would be around the 600 cells, *P.micans* around 400 cells and *C.granii* around 200 cells.

These densities were chosen to test lower densities 100 to 200 cells to higher densities 600 to 1000 cells. From the point of view of whole chamber cell counts 1000 cells would be about the range where analysts will start getting tired of counting a full sedimentation chamber and might decide to use a different counting strategy that is: Half chamber or even transect counts.

The cell concentrations for each species was pre-screened using a sedgewick Rafter cell counting chamber and carried out over 10 measurements for each species to ascertain the approximate cell concentration required before spiking the samples.

4.2.3 Sample types, treatments and replicates

In terms of the type of samples that was needed for the exercise, it was decided that a negative and positive control would be used. The negative control would be seawater which had been pre-filtered through 0.2µm GF/C whatmann filters and autoclaved. No species were added to this sample.

A positive control would be a sample containing the organism *P.lima* only. This was considered a positive control from the point of view of the organism being a well known toxin producer and also from the point of view that it was a single organism, so there were no other species and it was important to determine that analysts were only seeing the one organism and not multiples.

Replication is important in any mathematical design to be able to look at data variability. For this purpose we needed a minimum of two samples. As we used *P.lima* as confounding factor, we needed to at least double the amount of samples, that is 2 samples containing 4 species and 2 samples containing 5 species, the 4 plus *P.lima*. The 4 samples would contain the same concentration for each species that is the concentration of *P.lima* would have been the same in the positive control and also in the other 2 samples. Equally the concentration of the 4 species would be the same in the 4 samples.

This would total 2 controls (+ve, -ve) and 4 samples (2 samples 4sp, and 2 samples 5sp) 6 samples in total.

4.2.4 Sample preparation, homogenisation and spiking

All the sample types were prepared in the same way except the negative control as this sample type did not contain spiked cells.

In order to have enough samples for each lab, 180 samples were produced. There were 30 samples for the negative control, 30 samples for the positive control, 60 samples for the 4sp samples and 60 samples for the 5sp samples.

As there were 17 labs participating, a set of 6 samples each that meant that 102 samples of the 180 sample population were couriered off to the participating labs.

It also meant that the rest of the samples were used for setting up of the true value or gold standard. So, the totality of the sample population was used for this exercise.

The cell cultures were preserved with lugol's iodine as this is the most common preservative used by most monitoring programme. These were spiked into a 250ml screw top glass bottle and pre-screened for cell densities as aforesaid using sedgewick rafter cell counting chambers. The samples were inverted 100 times for sample homogenisation and 1ml aliquots were taking using a calibrated 1ml pipette. The process was repeated for each aliquot. The rest of the sample was constituted with sterile filtered seawater.

The final volume for each sample was 26ml so that there is enough volume to fill a 25ml sedimentation chamber to the top. This was done painstakingly using a pipette and a 4 place balance to accurately measure each volume for each sample type. For example for the 4sp sample type; 22ml of sterile filtered seawater was measured using this technique + 1ml aliquots of each of the 4 species. This was done for each sample type.

4.2.5 Sample randomization

All samples used in this exercise were chosen randomly using Minitab. Each sample was given a number and randomly assigned to a lab by the programme by using the randomization tool. What is important is that any sample from the sample population has the same chance as any other to end up in any participating lab and also the order of samples is randomized so that if there is any cell concentration quenching effect caused by the sample preparation procedure this would be minimised.

4.3 Taxonomic Quiz

4.3.1 Images and video Footage

The taxonomic quiz for this year's intercomparison was designed using footage from Cytographics Ltd in Australia. Cytographics Ltd (www.cytographics.com) is a company which dedicates themselves to the recording of live footage of cell biology. Their interest is geared more towards biological cell processes more than

taxonomy and their work is not only compounded to Phytoplankton rather they have a wider selection of subjects.

The reason for using footage from other sources, especially from sources that can provide really high quality images can't be underestimated. It is important to understand how difficult it is to come up with a test of enough quality of this kind from scratch using your own materials.

Also, images used in this type of tests should be certified to be what they are claimed to be and that can only be achieved by having a dedicated team to do this type of work or by engaging with taxonomists and biologists doing this type of work, and both are scarce on the ground.

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'.

The Marine Institute phytoplankton unit and cytographics Ltd. signed an agreement for the use of this footage for a limited period of time. This agreement allowed the Marine Institute to grab images and video clips from their DVDs to be used in the exercise. The only caveat to this agreement was the footage should be protected for downloading and copying by any parties.

Because we had to grab the images and clips from the DVDs rather than be able to use the originals that meant that a little bit of quality was lost in this process.

4.3.2 Technical aspects

The DVDs used for this taxonomy quiz had to be viewed several times to come up with a shortlist of images and video clips from phytoplankton organisms. While, the diatoms DVD is solely dedicated to diatoms the other DVD the Kingdom protistan only have a few chapters that are dedicated to phytoplankton groups.

Also, most of the footage is dedicated to biological processes like mitosis or cytoplasmic streaming, etc… which meant that only a small amount of footage was really related to taxonomic features of these organisms. This made it particularly difficult to come up with enough number of images to be used in the exercise.

Under the license agreement we could not send the taxonomic quiz by e-mail, so we had to do master copies that were to be returned by post, also the footage in the website had to be password protected and then dismantled soon after the exercise was over.

The Marine Institute phytoplankton lab contracted an Irish company Unique media that is dedicated to set up websites of this type to carry out the work. Once cytographics Ltd were happy with the amount of security to access the website and the images we could go ahead with the exercise. The video clips were secured from downloading.

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.

4.3.3 Quiz content

During the design stage we had thought of using video clips for this exercise, which is something that was recommended also from previous intercomparisons. We wanted to compare whether videos could be better identifying tools than images, as the amount of information given by video clips would be larger than images.

We had 2 sets of 20 images and videos randomly separated using Minitab into 4 sets of 5 images/clips each. This was done to have a sufficient number of replicates and to find out whether certain sets where more difficult than others.

It was decided, also that we should used images of other phytoplankton groups found in samples as it seems that in previous rounds the emphasis had been on diatoms and dinoflagellates only. There is definitely a bias towards those 2 groups of organisms probably because most national phytoplankton monitoring programmes are concerned with species that produce biotoxins, and most toxin producers would fall into these two groups.

So for the first time, we had used images of euglenophytes, chryptomonads, haptophytes plus others.

Most of the questions related to identifying the organism to a particular taxonomic level, while some questions related to taxonomic features of the organisms.

As all the sets being images or video clips were randomly selected, there wasn't a predetermined idea of making some sets more difficult than others. Neither there was a predetermination to make images more difficult than videos or vice versa.

There was however an inherent bias in that the range an amount of images that could be used for the exercise was quite limited, to the point that most images that could be used were used. So, there wasn't a big pool from which to randomly select.

4.4 Forms and Instructions

4.4.1 Couriers and materials

All the necessary forms and instructions were sent to all the participating labs. This year it was indicated for labs with more than one analysts taking part to assign a sample manager for the exercise. This sample manager would be in charge of receiving the materials and of setting up the materials for all the analysts within that lab.

Each lab received apart from the sample set (6 samples), a taxonomic quiz (Form 2, Annex VI) original (Master copy) per analyst, a set of instructions (Annex II) and several forms, a form for writing the enumeration results in (Form 3 : Enumeration hardcopy results. Annex VIII) and a form to confirm receipt of materials (Form 1: Fax checklist. Annex VII).

These materials were sent via courier to all the labs on the same day. Upon receipt of materials labs were asked to check the samples and the documentation for missing forms or leaked samples. Usually, a small number of samples had to be replaced each year. This is done on an individual basis with the labs.

4.4.2 Instructions

A set of concise instructions was sent with the rest of the materials, labs 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 annex II.

4.5 Ütermohl cell counting method

The ütermohl cell counting method is the standard methodology used in this intercomparison. This methodology is based on preserved marine water samples that are settled into a sedimentation chamber or ütermohl chamber where Phytoplankton organisms in the sample settle by gravity overtime. Once the organisms are settled at the bottom of the chamber, these can be viewed using inverted light microscopy.

There are different type of sedimentation chambers and chamber volumes. We are not prescribing the use of one over the other at this stage. The most usual chamber volumes used are 10ml, 25ml and 50ml and within these you have sliding chambers and fixed chambers.

In the Marine Institute phytoplankton lab we are accredited to use 25ml fixed sedimentation chambers and this is what we asked participants to use as well.

It is up to participants to use this volume but is not compulsory. Over the years it has been proven that 10ml and 50ml sedimentation chambers can be used equally well and give reliable results.

The ütermohl cell counting method is used as the standard methodology as it is chosen because it has been proven to be the most reliable method for cell counting and identifying phytoplankton (picoplankton is not included in this definition).

A variety of different methods have been developed to enumerate 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.* was first published in 1995, with a revised second edition published in 2003.

An intercalibration workshop comparing a variety of different methods for the identification and enumeration of the dinoflagellate *Alexandrium fundyense* was held at Kristineberg Marine Research Station, Fiskebäckskil, Sweden in 2005. The results of this workshop are presented in Godhe *et al.* (2007). This concludes that the most reliable method for cell counting is the traditional count by the Utermöhl method.

4.6 Statistical analysis

Independent statistical analysis of the data was carried out by Dr John Newell from the Biostatistics unit, School of Medicine at the National University of Ireland, Galway.

The approach taken on this intercomparison was to compile the data from the enumeration results of the different labs and calculate Z-scores (+/- 3 sigma limits) against a reference or true value for each of the species.

This reference value was calculated by the Galway lab from a set number of samples randomly chosen, set up and analysed in the same manner as included in the instructions for all the participating labs.

The reference data was analysed for normality and bias before it was used to compare this value with the participants' results. The results were then plotted against the mean reference value +/- 3 sigma limits. Results within the 3 sigma limits would show repeatability between participating labs and the Galway lab.

The results of the identification part of the exercise were analysed using percentages of correct answers for each analyst. 70% was considered an acceptable pass mark. The main analysis of this data was to compare the results from the image sets against the video clip sets. Also, we wanted to know whether some sets were more difficult than others and ultimately we wanted to test the taxonomic skill level of the participants.

The descriptive statistics of the data for each treatment were compared using box plots. We also compared each lab and analyst results for each treatment and then we calculated the percentage of correct answers for each analyst and their lab

and their cumulative percentage. The individual results in terms of percentage of correct answers were plotted in a league table.

5. Results and discussion

5.1 Phytoplankton enumeration results

Annex III Figure 1 True value cell counts, shows the values obtained by the experimenter that is the Galway lab and that were used to obtain a reference value or gold standard for the exercise.

There are certain advantages associated with using this gold standard or reference value against using all the data available from the participating labs.

One clear advantage is that the experimenter had knowledge a priori of the likely numbers used in the experiment that is the number of species used in the samples and their cell concentrations. This is a clear advantage against the participants as they are blinded to the experiment. The other advantage is that the experimenter is more likely to follow the instructions for the exercise very closely in terms of sample set up, homogenisation and counting strategies. This is very clear in the fact that some analysts choose to use different cell counting strategies for their samples even though they were asked to used whole chamber cell counts (see Annex II: instructions for the exercise, section 5).

There are also some disadvantages to using all the participants' data as part of the reference value in this case since not everyone had followed the instructions. This doesn't mean that this data can't be used, it means that by using all the data in that manner we would muddle any interesting information that could come out of the exercise. For example, it could tell us that there are cell counts differences between analysts using different counting strategies or on the contrary it may tell us that it is possible to use these different counting strategies and not have significant differences.

If it can be proven that there are no differences in the outcome of this exercise according to the different cell counting strategies, then there could be a call for using all the data to generate the mean and 3 sigma limits for the exercise, if not then we'll have to investigate what these differences are and why are the occurring.

So, a priori the Galway data will be used as the reference point and further on we will discuss whether we should consider pooling all the data as the reference value.

First of all, we looked at the Galway lab reference data and discuss whether the data is normal, what the variability and the spread of the data is. Figure 3 in annex III shows a summary of the descriptive statistics for the cell counts of *Coscinodiscus granii*. As the data appears symmetrical the mean can be used to set up our limits. The P-value 0.143 is greater than the level of significance 0.01, so we accept the null hypothesis that the data is normal. Another way to look at this data is to carry out a Normality test, see Figure 4 in annex III using the same data shows that the data fits well with the normality line.

This type of analysis was done for each species and it was found that the reference or gold standard data was normal. Figure 5 in annex III shows the cell counts for each species as a box plot. The box plot shows 5 numerical summaries of the data distribution, the median, the first and third quartile, and the minimum and maximum. As you can see in the box plot, the data appears normal for all the cell counts.

Figures 6 to 10 shows the box plots of the cell counts by analysts against the reference cell counts. Figures 6, 8 and 9 shows that there are no differences between the analysts' cell counts and the reference value, in fact the spread of results and the median and mean are very close for all the counts. Figure 10 *P.*lima box plot illustrates that there may be differences between the analysts cell counts and the reference cell counts. The box plots are not to far off from each other and the mean and median may be ok, but what is obvious from this box plot is that there are quite a few outliers, so we need to look further into these results and the individual cell counts to find out what is happening here and whether there are any significant differences between cell counts of *P.lima*.

The box plot in figure 7: *P.micans* box plot shows that the reference cell counts and the analysts' cell counts are different; it is very obvious that we are looking at two different populations.

So, it is important to look at the *P.lima* and *P.micans* cell counts further and also we need to look at the individual values as there are several outliers in all the cell counts.

So far what we are seeing is that all analysts have performed very well in 3 out of the 5 cell counts, but we have to look at the other 2. The *P.lima* cell count appears to have too many outliers and we need to find out whether this is due to a particular lab result, a particular set of samples or down to a much tighter spread of results. If you look at figure 5: Galway reference results, you can see that the box plot of *P.lima* is very tight, so we need to investigate this further. Regarding the *P.micans* cell counts it is clear that there are differences in the cell counts so we need to figure out what had occurred here.

These box plots give us a general feel for the overall results but we still have to look at the individual results, at the lab results, the inter- and intra-variability of the cell counts within labs. Then we may be able to compare these with other labs and particularly with the reference lab.

So, lets look at the within lab variability of cell counts, as an example I took the *G.*catenatum cell counts of 3 analysts in Lab K. We want to know how close analysts is to each other on same sample counts that is reproducibility within lab and how close their repeat analysis is.

Figure 11 in annex III shows the scatter plot of the results of 3 analysts from lab K on the cell counts of *G.catenatum*. We are comparing same sample results and the scatter plot indicates that results are not comparable this is because the results should be closer to the median line. Figure 12: Bias box plot of analysts r,c & k shows that there are differences between analysts cell counts, this is because there isn't symmetry above the zero, so there is a bias between these counts.

So, let's compare these values against the reference value for *G.catenatum*. In figure 13, the box plot of the reference value versus the mean of the analyst's value indicates that there are no differences in cell counts between lab K and the reference value.

It is up to all the participating labs to look at their own data and the reproducibility within their lab, as we have demonstrated that there is variability

between same sample counts by different analysts, it is up to the different participating labs to look critically at their own data and decide what it is to be done to improve the reproducibility.

At the same time the cell counts are within the 3 sigma limits, so we have to decide whether we can live with the fact that even though we are all analysing samples using the same methodology and techniques there is always going to be variability within and between samples, that the variability between samples may be a naturally occurring variability due to the handling and homogenising of the samples and that there is also variability due to the actual counting by analysts.

Figure 14 which is the case profile of all the replicate cell counts per species per analyst demonstrates this fact very clearly that there is no reproducibility within or between labs.

Figure 15 is the scatter plot of a particular lab, in this case lab C to illustrate the same thing, that replicate cell counts are not reproducible within labs. This can be seeing particularly well in the panel sp4IIa (*scrippsiella* cell counts).

Figure 16 shows that there is no reproducibility of cell counts between labs. This graph shows all the mean results per species per lab.

However, the variability of the cell counts are within the variability of the Galway lab or reference value and the test for equal variances for each lab confirms this (See figure 17).

Lest look at the particular I charts for each lab and species and see how this generalisation works at a more defined level. Figures 18 to 22 illustrate the I chart of individual measurements across labs compared to the Galway reference value, the mean and 3 sigma limits.

The cell counts for *coscinodiscus granii, Scrippsiella trochoidea, gymnodinium catenatum and Prorocentrum lima* look good that is within the 3 sigma limits for all the labs apart from lab E which have underestimated most of the cell counts. The I chart for *Prorocentrum micans* is slightly different and appears that most labs have underestimated the cell counts or that the reference lab had overestimated the cell counts.

It is important to spend a bit of time in this issue as it appears that the reference mean for *P.micans* is different to the analysts mean. Let look at the two sample means using the 2 sample t-test in figure 23. This test null hypothesis is that there are no differences in the sample populations mean, as the figure illustrates the sample means are different, the 95% confidence interval is negative(-7765, -5103) the t-value =-9.89 and the P-value is 0.000 so the alternative hypothesis is true in this case which indicates that the sample population means are different.

The I chart of the analysts observations for *P.micans* against the reference value shows that 11 analysts have performed outside the 3 sigma limits, while the rest have performed within the limits but have tended to underestimate. It is clear from this data that either the analysts or the experimenter have done something consistently different in these counts, this is a systematic error.

If we consider that the experimenter have done something different to the analysts, this could not be related to the sample set up, spiking or homogenisation as randomization would have taken care of that, it would have to do most probably with cell counting. Since, the experimenter is not blinded to the experimental design then the error must be with cell counting rather than identification in any case. The only drawback of this assumption is that the experimenter had already done concentration trials for these species and also analysts had performed within the mean and 3 sigma limits for the other species.

On the other hand if we assume analysts error, then how could this be if most analysts have already performed well in the other 4 species counts and replicates. As the error is so systematic across all the labs the question is, is it possible that most analysts have underestimated the cell counts for the same reasons? Is it possible that analysts may have confused *P.micans* with one of the other species spiked in the samples? It is difficult to see how as *P.micans* is not a particularly difficult species to identify. The reasons for these results are not very clear, but it was decided at the workshop held on the 16th of April 2009 in the Marine Institute that the mean of all the results should be used to calculate the sigma limits instead for this particular cell count. See Z-score for *P.micans* figure 29.

The following z scores in figures 26 to 30 shows that most labs performed within the 3 sigma limits or reference cell counts apart from analyst b which tended to

underestimate in most cell counts. Analyst x was outside the limits on the *P.lima* cell count and analyst σ on the *scripsiella* cell count.

Regarding the negative control sample, that is the sample with no species, 2 analysts observed cells in this sample, these were analysts x and analyst J. These observations were low, in the case of analyst J was one cell of *scripsiella* and in the case of analyst x was 8 cells in the negative sample and 3 cells in the positive sample of *Scripsiella*. This would suggest perhaps a problem with contamination at the particular lab as great care was taken in making up these samples.

Most analysts performed well in the identification of one species in the positive control (+Ve). The *P.lima* cell counts on the +Ve sample were compared to the *P.lima* cell counts on the other 2 samples where *P.lima* cells were confounded with the other 4 species called 1st and 2nd sample in figure 24. We want to look at whether analysts counted differently *P.lima* cells. Figure 24 shows the box plot of *P.lima* counts in the 1st and 2nd sample, the positive control, the mean of these 3 counts, all the individual counts and the reference counts. The box plots indicate differences in the mean of the counts against the reference counts. Are these differences significant? Figure 25 are paired T-test for the different counts. This gives us a measure of the confidence intervals of the populations. The comparison of confidence intervals shows that the 1st and 2nd sample are not significantly different to the reference count but there is a significant difference between the +Ve sample and the reference count and also between all the individual counts and the reference values which indicates that there is a difference in the mean analyst count compare to the reference count. Also there seem to be a significant difference between the 2nd sample count and the +Ve sample, which indicates a systematic counting effect on counting *P.lima* in samples with other organisms present compare to counting a sample with only *P.lima* present.

All analysts identified correctly to species level the organisms *Prorocentrum lima and Prorocentrum micans*. The organism *Scrippsiella sp.* 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 ensicullifera as answer which was given as correct. The differences between these genera would need at least calcofluor staining to see the cingular plate

pattern that would help their identification. Therefore, here we are giving *Scrippsiella sp.* as the typical answer and *Ensicullifera* as synonym.

The organism *Coscinodiscus granii* 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. These are some of the species name given: *C.asteromphalus, C.radiatus, C.centralis and C.wailesii* by a number of analysts. This suggests different approaches to the identification of large organisms like *Coscinodiscus* by the different labs.

The organism *Gymnodinium catenatum* was identified correctly to species level by 21 out of 34 analysts, a further 5 analysts identified to genus level and 8 analysts identified incorrectly this organism. Some names given incorrectly were *Cochlodinium, A.catanella* and the non-name 'Chain forming organism'. These were all incorrect answers.

As *G.catenatum* is a highly toxic organism, the correct identification should have been to species level in this case as the identification to genus only would be not enough.

5.2 Phytoplankton identification results

Annex IV contains the table of results of the identification exercise (Figures 32 & 33). The identification exercise was divided into 2 sub sets, one of still images and one of video clips, each subset contained 4 sets of 5 images/videos.

The reason for this was to compare how video clips would perform against images as quality controls for phytoplankton identifications and also to see whether some sets were more difficult than others. The question of interest was whether still images or videos are better quality control tools. A priori the null hypothesis would be that there is no difference between sets and within sets and no differences between still images and videos.

Figure 34: box plot of image results versus video results suggest that the null hypothesis is true. There are no differences between identifying images or videos; we seem to be able to do both well. The descriptive statistics of the box plot shows that we are 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 Video (see figure 35).

Figure 36: The box plot of correct answers by sets 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 image sets and set C got the highest score for both images and videos, which suggests that this sets were found to be 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.

A General Linear model, fitted to compare the mean percentage correct across the four factors namely species, lab, analysts and replicate, indicated (see figure 39) a significant difference in the mean percent correct between type and analysts only.

There was a borderline significant difference between Type ($p=0.09$) and no significant difference between labs ($p=0.38$). 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.

5.3 Performance evaluation

Overall in the enumeration part of the exercise most analysts performed well and within the 3 sigma limits set by the reference lab.

Only lab E seems to underestimate 4 out of the 5 counts, also Lab F and lab O underestimated one of their counts.

Most labs underestimated the count for *Prorocentrum micans*. It was decided at the workshop that all analyst counts would be used to set the reference value for this count as there was a systemic error and it wasn't appropriate to use only the reference lab count as we could not point out at where and how this error could have occurred.

Although most analysts performed well and within the prescribed 3 sigma limits, there was overall evidence of lack of reproducibility within and between labs.

In general there appears to be variability between analysts when measuring the same species with some variability between replicates within analyst and between analysts.

On the identification exercise, most analysts exceeded the 70% overall pass mark. 7 analysts performed above 90%, 15 analysts between 80 and 90%, 11 analysts between 70 and 80% and 1 analyst below the pass mark.

Overall, the standard on the enumeration and identification exercise was very high by all of the participating labs.

6. Conclusions and recommendations

In summary, there was evidence of a good agreement in the Enumeration Study between virtually all the analysts to the Galway Gold Standard for all species bar one.

The Galway Laboratory provided data for 33 replicates on each of 5 species while 34 analysts across 17 labs each provided either 4 or 3 replicate measures for each of the 5 species. The first aim was to investigate whether the measurements provided by the analysts were comparable to the Gold Standard (i.e. as estimated by the Galway data).

The data suggest that the variability between replicates within species is plausibly normal given the symmetry present in each box plot (Figure 5). There appears to be a difference in variability however between species where 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

concentration and *Scripsiella sp.* being the highest concentration. Given the symmetry present it is reasonable to assume that the mean and standard deviation of the measurements provided are valid estimates of their population counterparts.

A General Linear Model was fitted to compare the mean measurement across four factors namely species, lab, analysts and replicate (see figure 31). The results of fitting the model are that there was evidence of a systematic difference in the mean measurement for all of the factors under consideration. The overall variability explained by these four factors, as given by the R^2 (adjusted) statistic, is estimated to be 91% i.e. 9% of the variability in measurement evident in the data is not explained by these factors.

The components of variation explained by the model can be attributed to the factors as follows: 68% due to difference between species, 19% due is due to Labs, 11% due to the Analysts and the remainder (1%) to the replicates.

In order to compare the individual measurements provided by the 34 analysts in question plots of the mean of the replicates for each analyst by species, where the mean is justified given the small variance component due to replicates, against the Galway mean and upper and lower control limits (UCL and LCL respectively) are given in Figures 18 to 22. Note that the UCL and LCL are generated using 3 times the Galway sample standard deviation.

There is evidence of good agreement for each species except *P.micans* where virtually all labs underestimate the Galway measurement. It was decided that all the data from the participating labs should be used as reference data to calculate the z-score on this count as the reasons for the systematic error could not be pinned down to one particular reason. There is evidence that the Lab (labelled as E) tends to underestimate the Galway measurement for all species bar *G.catenatum* count.

The results returned for the Identification study were excellent with no evidence of systematic difference in the percentage correct between the Image and Video formats of presentation.

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, Set D the hardest.

The identification exercise shows that overall all participants did quite well, with 68% of the participants scoring over 80%.
Also, it doesn't appear to be any differences between image sets and video sets as identification tools, with a slightly higher percentage mark for images over videos.

Overall, this proficiency test has proven very successful both in terms of interest from labs involved in phytoplankton analysis and overall results.

On the 16th of April, 2009 the Marine Institute hosted the 4th workshop for the BEQUALM Phytoplankton Intercomparison PHY-ICN-09-MI1. At this meeting, the results of the intercomparison and future directions of the exercise were discussed. See Annex V: Agenda for the workshop.

Some recommendations were put forward by the participants to improve and further enhance this proficiency testing scheme.

Overall, most participants found the exercise excessively long and it was difficult to get the time to finish it. So, fewer samples would be better and more time for the exercise.

It was suggested that the exercise should be double blinded so that the reference value results would go through the same process as the samples sent to all the participants that is to be couriered off for an analyst to count, which doesn't know what the samples contain.

It was suggested that all the cell counts from all the analysts should be used for the reference count or true value. This is valid as long as everyone is using the same methodologies and counting strategies for the samples as the results of this intercomparison indicate that analysts using different sub sampling and counting techniques were outside the 3 sigma limits in some cell counts.

When using spike materials, not to use organisms that don't preserve too well.

Use wild samples for the exercise so that the effect of cultured species would be completely avoided. Use different spiking aliquots to see how the reference counts variability changes.

There is a new CEN document published recently called 'water quality. Guidance standard on the enumeration of phytoplankton using inverted microscope'. Most labs seem to be unaware of this guide. It was recommended to read this guide and come back to discuss.

Each lab could submit images for the identification part of the exercise that the other labs would have to identify.

The Marine Institute will be discussing these ideas in preparation for the next exercise which will take place in the 1st quarter of 2010.

Annex I: Participating labs in NMBAQC Bequalm Phytoplankton Intercomparison 2009

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	

Annex II: 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
2. Preliminary Check and deadlines
3. Equipment
4. Sample Preparation
5. Counting Strategy
6. Samples
7. Conversion Calculations of Cell Counts
8. Identification
9. Points to Remember

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 labs 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 lab for this exercise should make sure that the lab 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 lab. 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 lab.

Once you have received the samples, your lab 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.

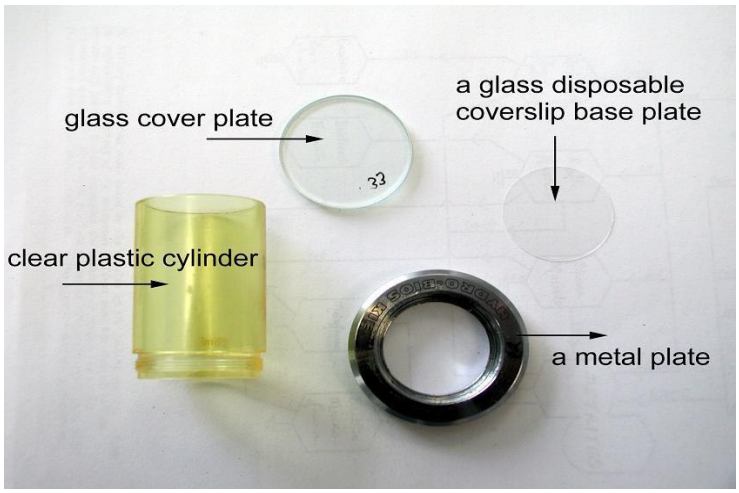
3. Equipment

- 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 Utermohl 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.

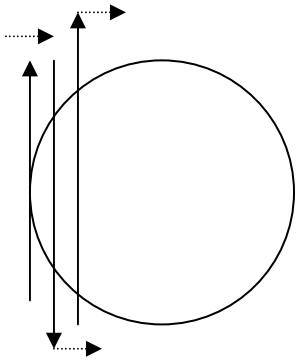


Fig 2

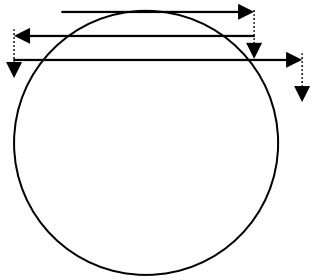


Fig 3

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 sub samples has been used to set the true value of the sample population within 3 SD. The results obtained by all the labs 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 labs.

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 lab will receive the parcel with samples and forms for all the participants from their lab. The sample manager should make sure that their lab 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 lab. As this year we have to analyse 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 labs 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 litre, use 25ml as the chamber volume.

7. Conversion Calculations of Cell Counts

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

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.**

Annex III: Detailed results of the Enumeration test PHY-ICN-09-MI1

Figure 1: True value counts

Sample number	Sp1	Sp2	Sp3	Sp4	Sp5	Species		Sp5	Species
	I	I	II	II	I	Size		I	Size
	b	b	b	a	c	Concentration		c	Concentration
16	11400	19920	17080	37480		Type A	68	4120	Type C
114	12720	22040	20200	39280			86	4640	
75	12040	21480	19560	37920			23	5200	
126	12280	17760	15760	35320			107	5200	
51	13200	23240	19400	37120			135	4560	
32	14360	23760	17640	39240			129	4240	
19	13280	24840	21720	39160			59	4360	
170	10080	21720	19880	39000			175	5680	
37	11200	22400	23080	40360			100	5040	
22	11920	23200	19200	42520			76	4800	
150	12120	23320	16480	35720	4040	Type B	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				
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				

Figure 2: Analysts Enumeration results

	D	B	B	A	A	B	B	A	A	B	B	A	A	B	B	A	A	C	B	B
ANALYST CODE	Sp0	Sp1	Sp1	Sp1	Sp1	Sp2	Sp2	Sp2	Sp2	Sp3	Sp3	Sp3	Sp3	Sp4	Sp4	Sp4	Sp4	Sp5	Sp5	Sp5
		I	I	I	I	I	I	I	I	II	II	II	II	II	II	II	II	I	I	I
		b	b	b	b	b	b	b	b	b	b	b	b	a	a	a	a	c	c	c
u	0	10000	13720	11040	9960	17960	20080	20920	23280	5880	8800	8560	8520	31320	29240	34280	31440	3720	3640	4360
r	0	11600	12560	13080	9840	20960	19120	23160	21920	10680	10360	12000	10640	33440	28600	38120	29640	3280	3280	4200
k	0	11360	11760	12040	9600	21520	19800	21840	22320	7360	8200	7760	7760	34080	28760	31680	31440	3680	3600	4040
c	0	12200	11640	11640	9720	21200	20280	24440	24200	11120	9160	13800	12840	37760	32520	39320	33960	3800	4040	4360
n	0	11320	11760	11760	9760	23040	21000	25840	21920	10400	9480	10720	12080	31920	27960	35840	30320	3680	4160	3840
b	0	13300	6100	9000	6600	18600	12500	16600	12800	2400	5900	3200	6000	12700	14100	9300	10800	5300	2400	2200
s	0	14000	13200	12000	11200	22000	24800	21600	20000	11120	9600	11200	10400	32000	27600	34200	31200	3840	4560	5200
β	0	13800	13240	12640	11240	21040	24600	22160	20240	13440	15280	14960	14200	36000	38120	42560	32600	3920	4920	4400
l	0	13240	12840	12680	10520	21200	23840	21600	18760	10200	10640	11480	10720	32880	30880	39160	31960	3840	3960	4040
ά	0	15400	13760	13920	11800	22440	24720	22360	42000	17840	17720	18280	14640	40080	17800	44120	37040	4600	4280	4400
δ	0	14080	12400	12600	10600	22040	23480	20440	20440	10400	12160	11120	8360	28800	28800	33280	23440	3840	3800	3800
j	40	14400	13000	13080	14160	23560	18400	23560	18040	11200	6600	11040	6200	27200	29720	27200	35800	3240	3800	2800
h	0	13280	11800	13080	12800	23400	19880	18800	19320	8040	8720	8120	8040	33200	32000	33400	32120	3720	3880	3560
z	0	10360	11040	10360	9560	25520	19400	23160	17160	8480	10680	8120	7200	29000	29720	29680	29400	3120	3960	4440
v	0	11320	10360	8880	9800	24320	17800	23080	16360	8120	10360	10120	7920	27960	28160	28200	33400	3960	3920	3920
g	0	13720	13640	11920	10920	25960	24040	26040	20000	12760	15960	13600	17240	38760	37320	39360	44640	4000	7280	6400
ñ	0	12800	12720	11480	10720	26360	23240	26440	19840	9080	1160	12040	13080	39560	33480	40480	41120	4480	5560	5240
i	0	12880	13480	13680	11240	19920	22360	21400	20800	13120	13360	13360	13360	35160	37760	38320	35200	4080	4360	4160
a	0	12440	13080	13240	10200	20080	24720	21240	20960	12040	8720	11640	14360	34280	36080	40800	34560	3800	4480	3840
o	0	12440	14320	13280	11320	19640	23360	19880	19840	11640	10880	15120	14200	34040	40080	38680	31160	3760	5680	6760
d	0	14520	13440	15280	14960	24320	20040	24840	18720	14320	12520	14400	12720	40320	41080	42760	42600	4120	4880	4200
p	0	2160	11240	13960	13520	12120	18280	19320	22080	8280	11920	15000	11920	26560	28520	34840	32840	3960	2640	3960
f	0	2120	10760	14000	13600	12000	18000	19080	21440	10600	13960	14920	16400	25480	28320	35720	35520	3960	2560	3920
Ω	0	14440	13440	11800	12040	24000	20000	14560	24000	16320	19160	17320	19720	38120	34000	29600	37880	4000	4360	4080
σ	0	14000	14200	14040	11880	21440	22160	21000	17920	8400	10120	7320	8000	25160	22960	19360	26400	4160	6000	4640
m	0	13440	12200	12400	12800	26800	23920	21560	25920	10640	10600	12640	12560	39960	38680	38040	37320	4040	5640	5160
μ	0	14800	11960	13760	13080	19440	19440	26920	22720	7000	9000	8880	12040	35400	38840	31280	33040	4240	2440	3320
π	0	15280	12440	13440	13800	20400	20520	25360	23200	11680	14520	13600	16520	37000	44200	37000	36840	4560	3920	4840
x	320	11561	9793	14284	8706	16457	21081	19044	17005	5164	8569	7074	6802	28804	37322	36917	34077	2640	2520	3200
y	0	12280	13840	11760	11080	23520	24280	23680	21640	12920	11960	12720	14280	36400	33080	39600	36520	3960	4920	4800
q	0	11000	12800	13040	13160	19920	21160	24360	22736	7400	10800	15776	9800	31088	30240	35264	42224	4040	2560	4000
t	0	12992	13560	12992	13920	29000	21320	24128	24360	11600	9800	12160	15080	33872	24920	31320	41296	4080	3120	4080
ch	0	13920	14200	16704	13920	32712	22280	24128	25752	9040	11680	11880	11600	33872	36200	40368	37816	4080	3040	4080
ζ	0	13160	12520	12920	14120	27600	22440	23160	21720	15680	17000	16480	16840	35720	41040	42520	40160	3480	4120	4480

Figure 3: Anderson-Darling Normality Test for *Coscinodiscus granii* cell counts

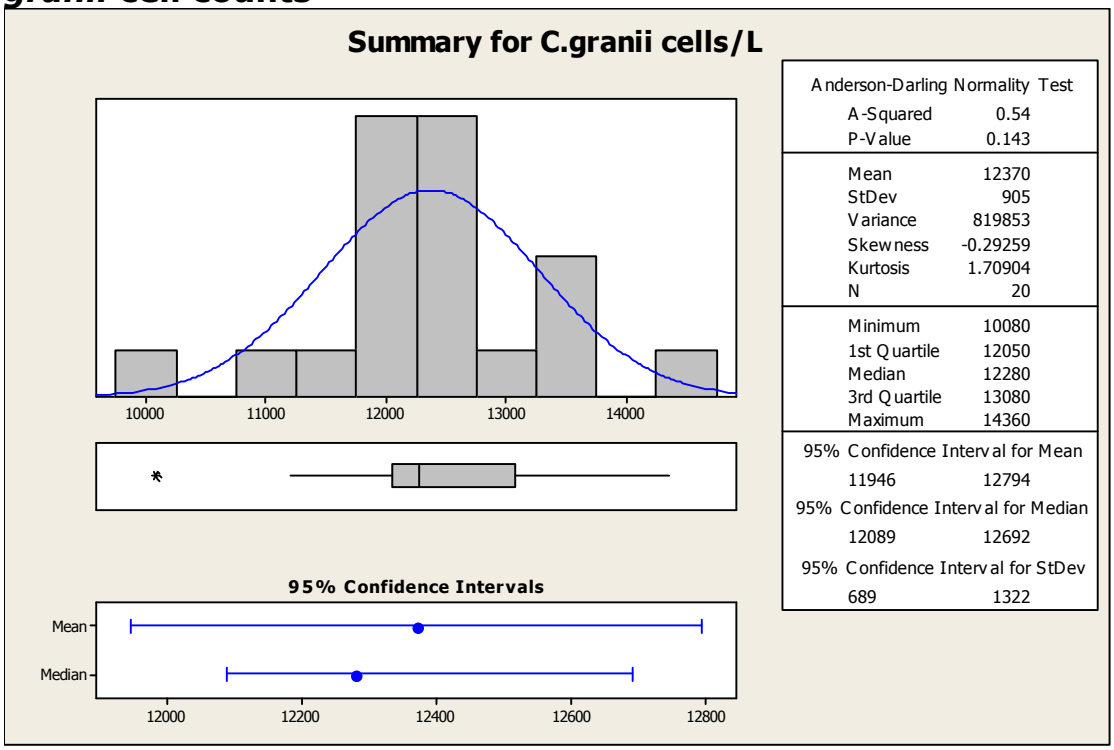


Figure 4: Normality test for *C.granii* cell counts

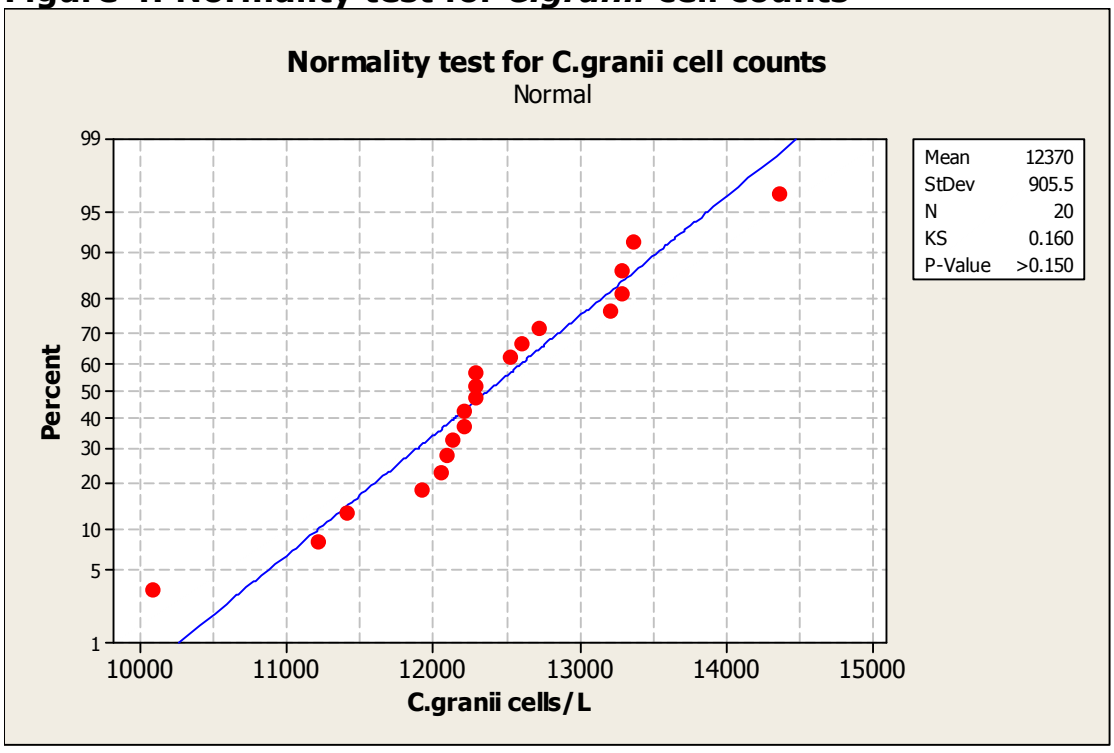


Figure 5: Box plot of Galway reference data

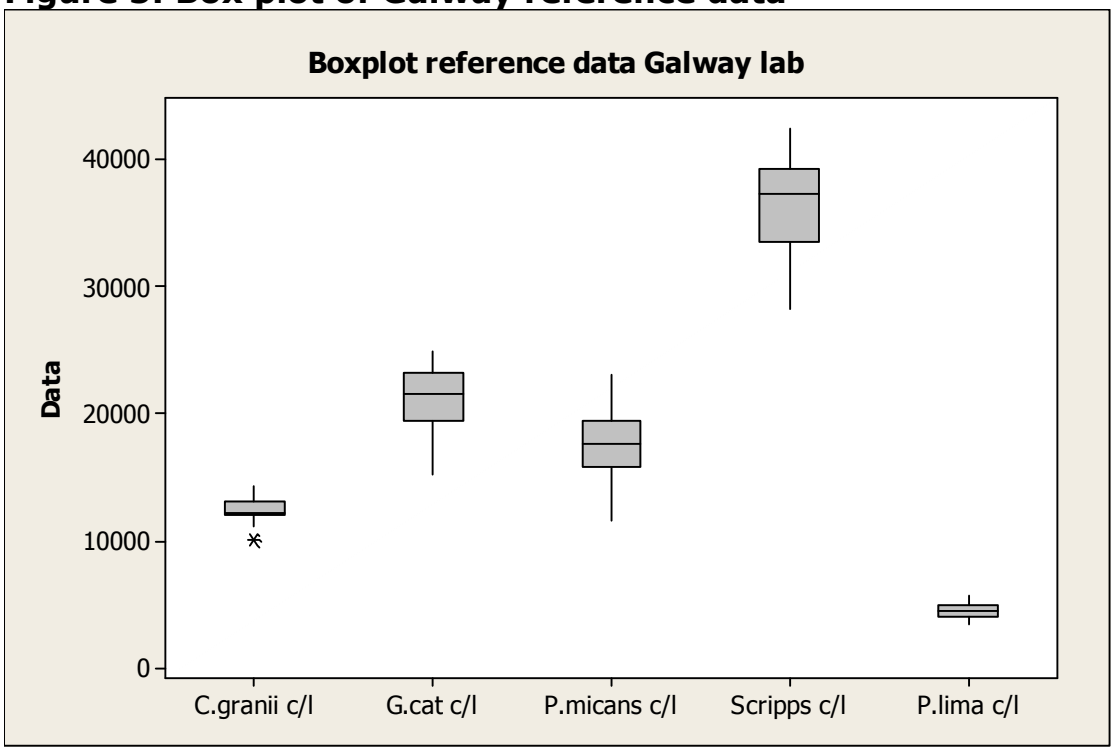


Figure 6: Box plot of analysts versus reference cell counts for *C.granii*

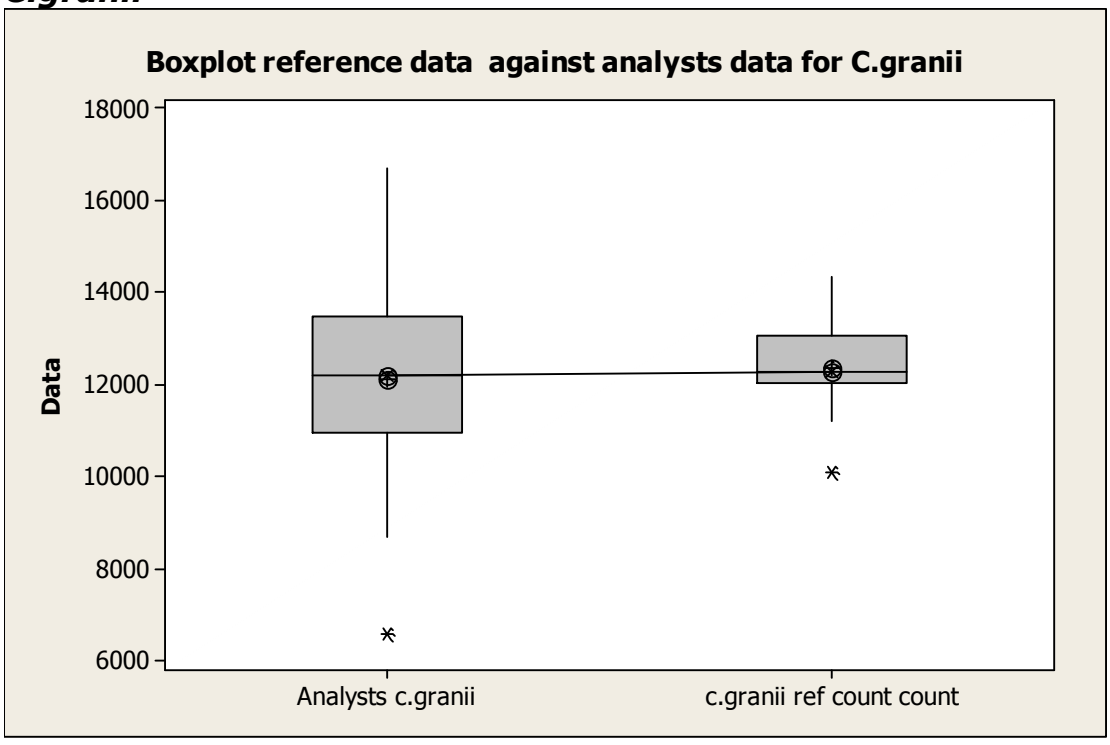


Figure 7: Box plot of analysts versus reference cell counts for *P.micans*

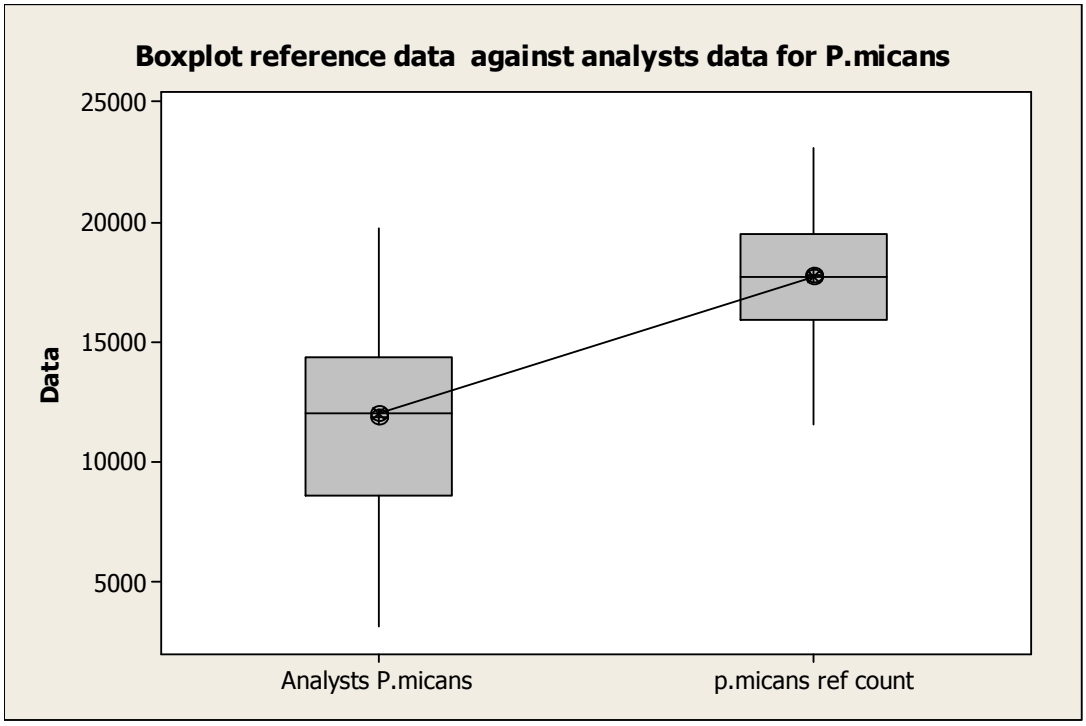


Figure 8: Box plot of analysts versus reference cell counts for *Scrippsiella sp.*

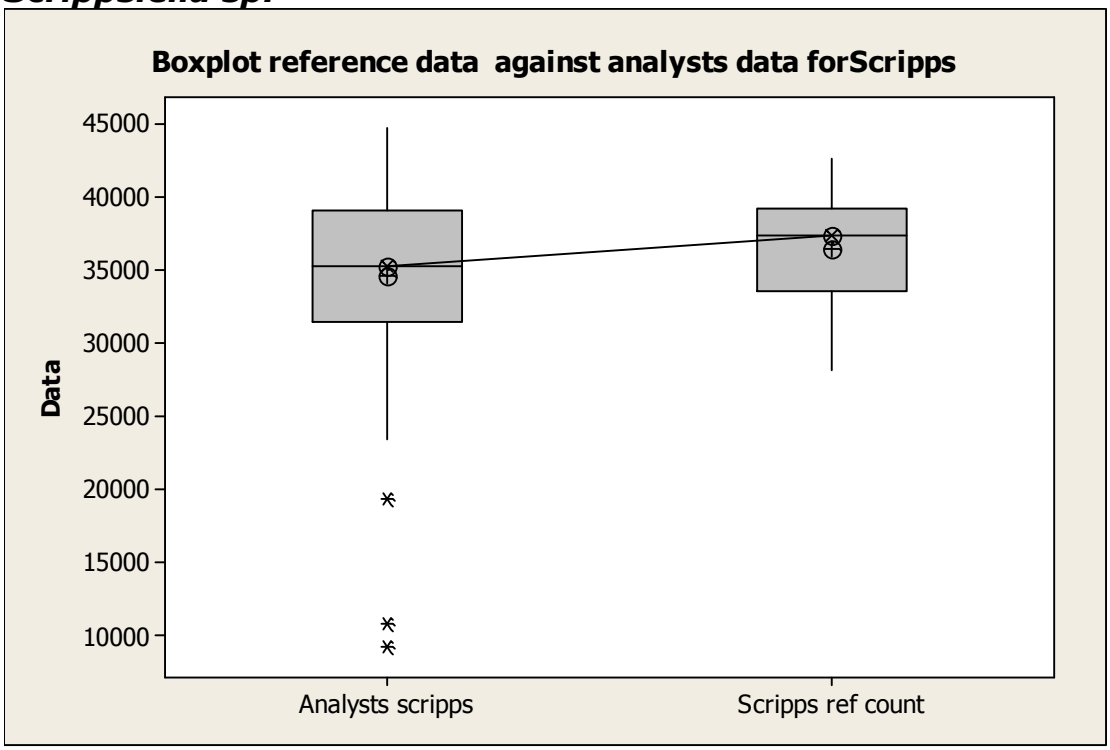


Figure 9: Box plot of analysts versus reference cell counts for *G.catenatum*

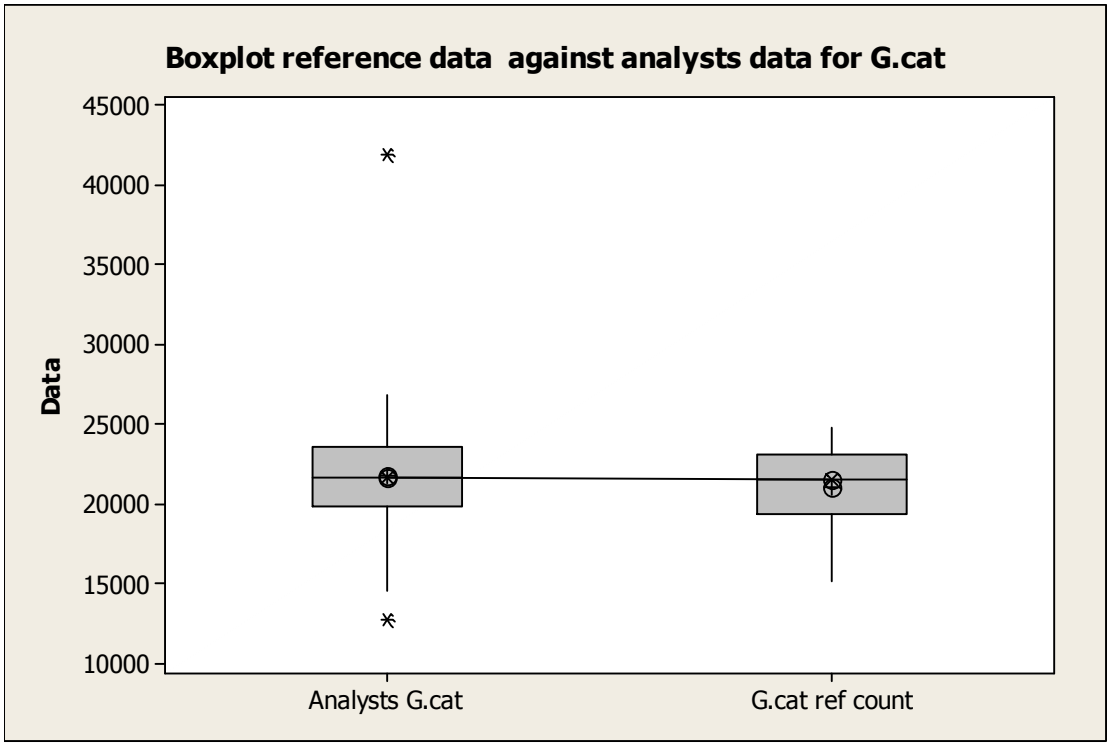


Figure 10: Box plot of analysts versus reference cell counts for *P.lima*

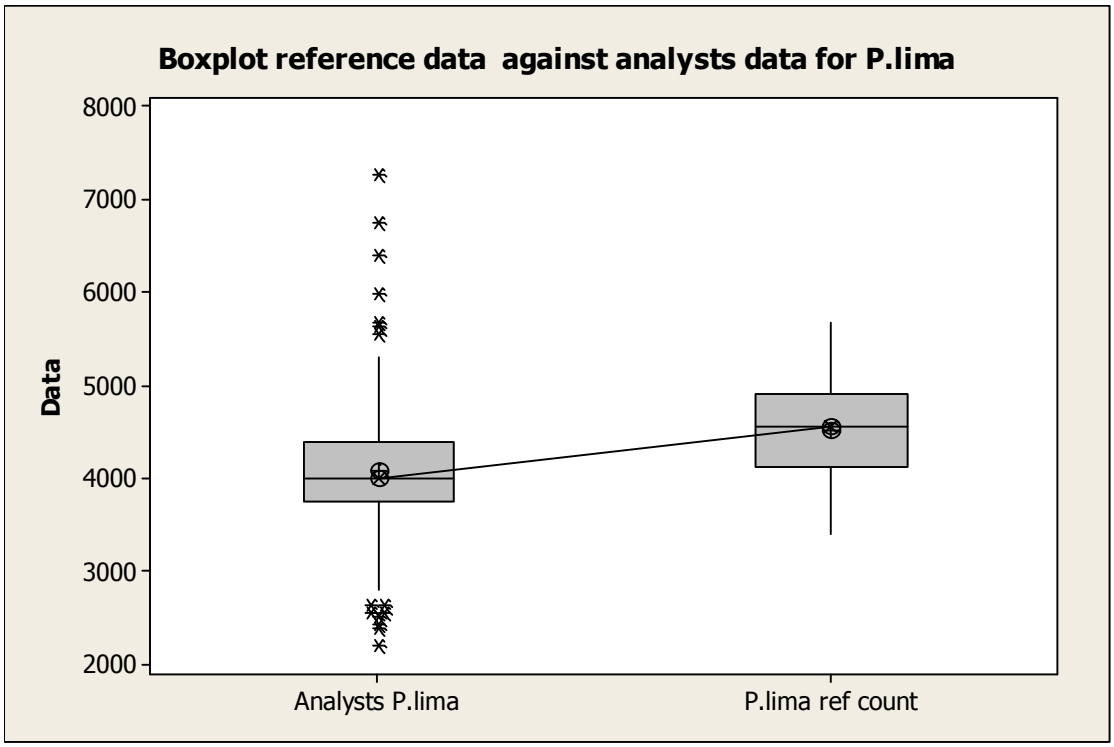


Figure 11: Scatter plot of analyst's r, c and k from lab K against each other

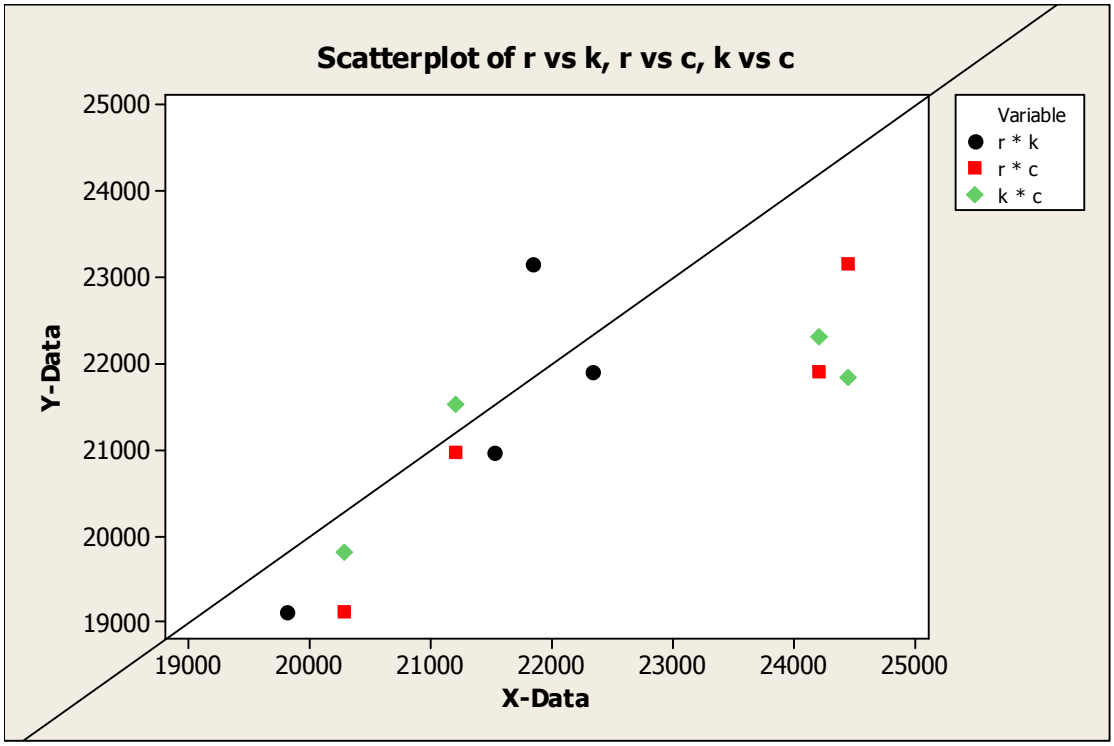


Figure 12: Bias box plot of analyst's r, c and k from lab K

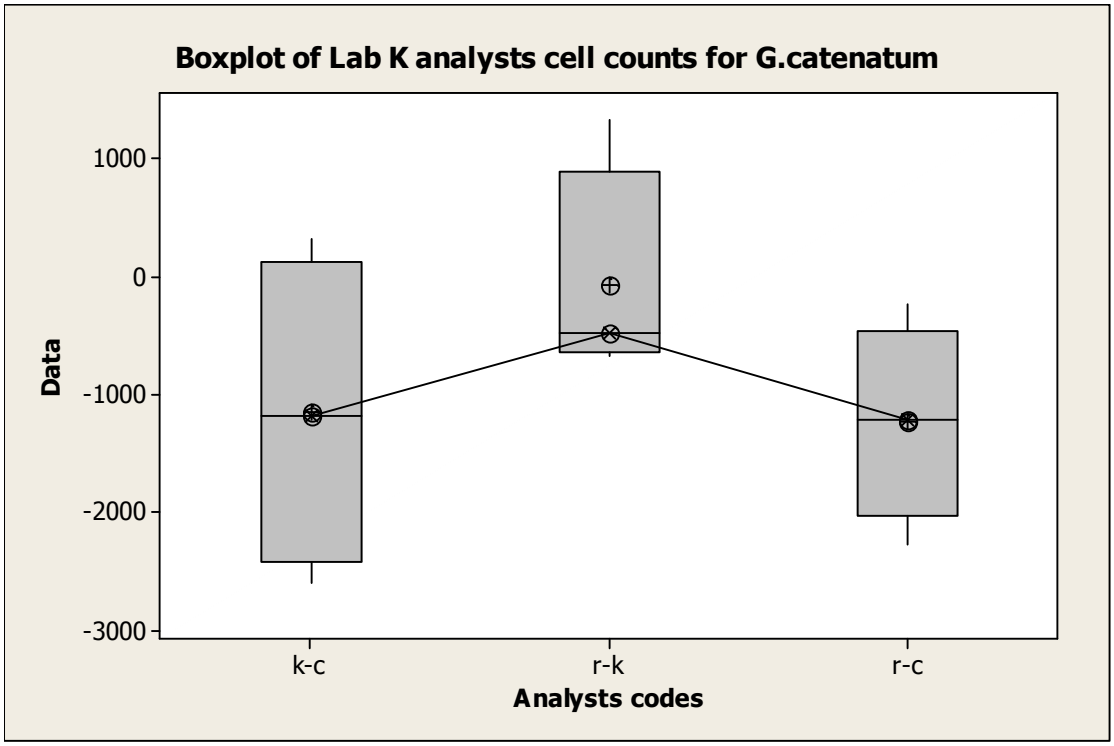


Figure 13: Box plot of analysts k,c and r from lab K versus reference value

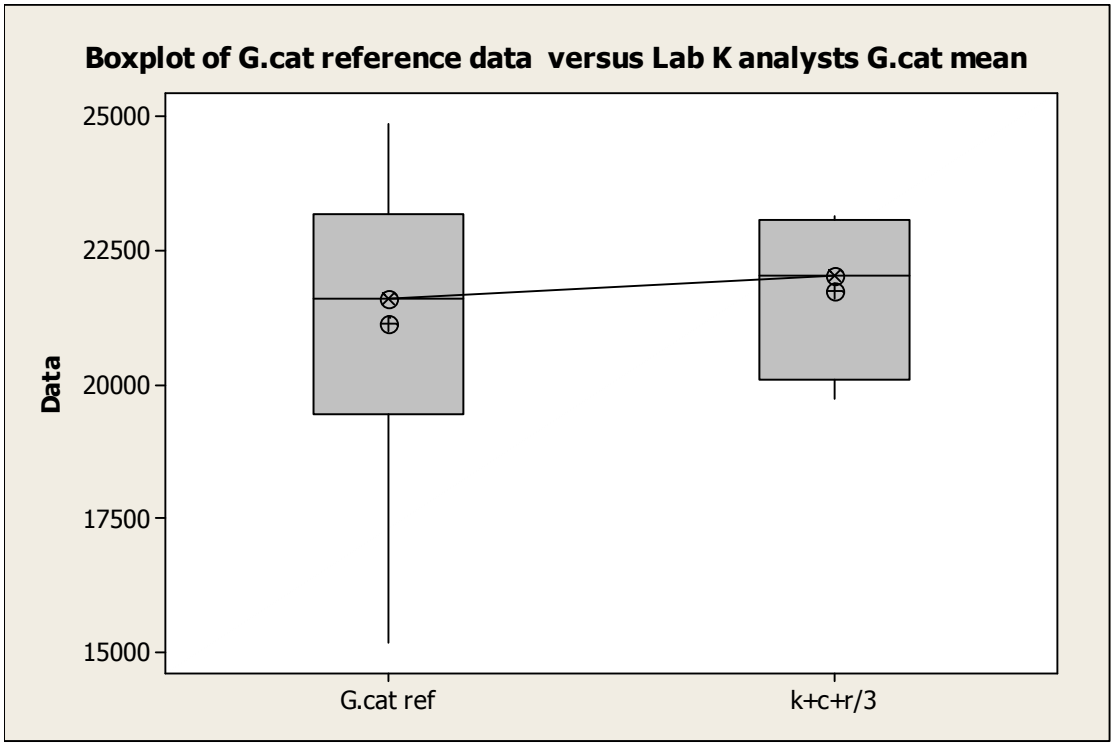


Figure 14: Analysts replicate cell counts by species

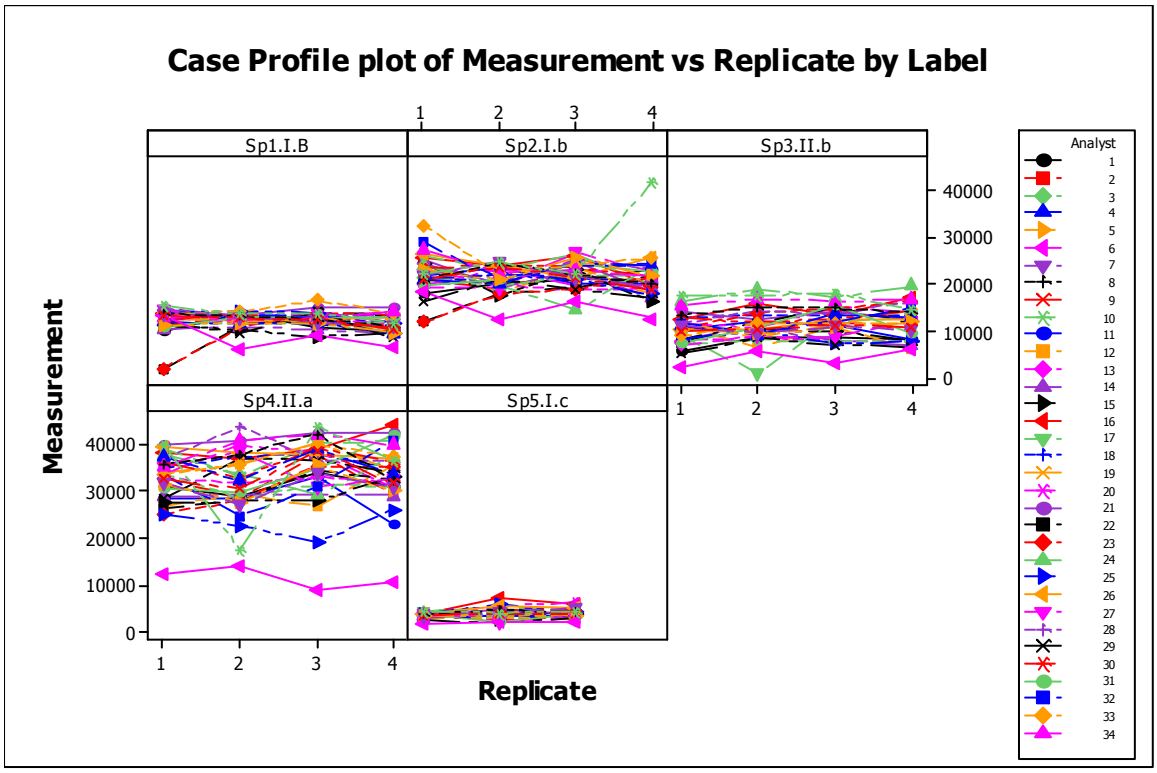


Figure 15: scatter plot of analysts in Lab C measurements versus replicate

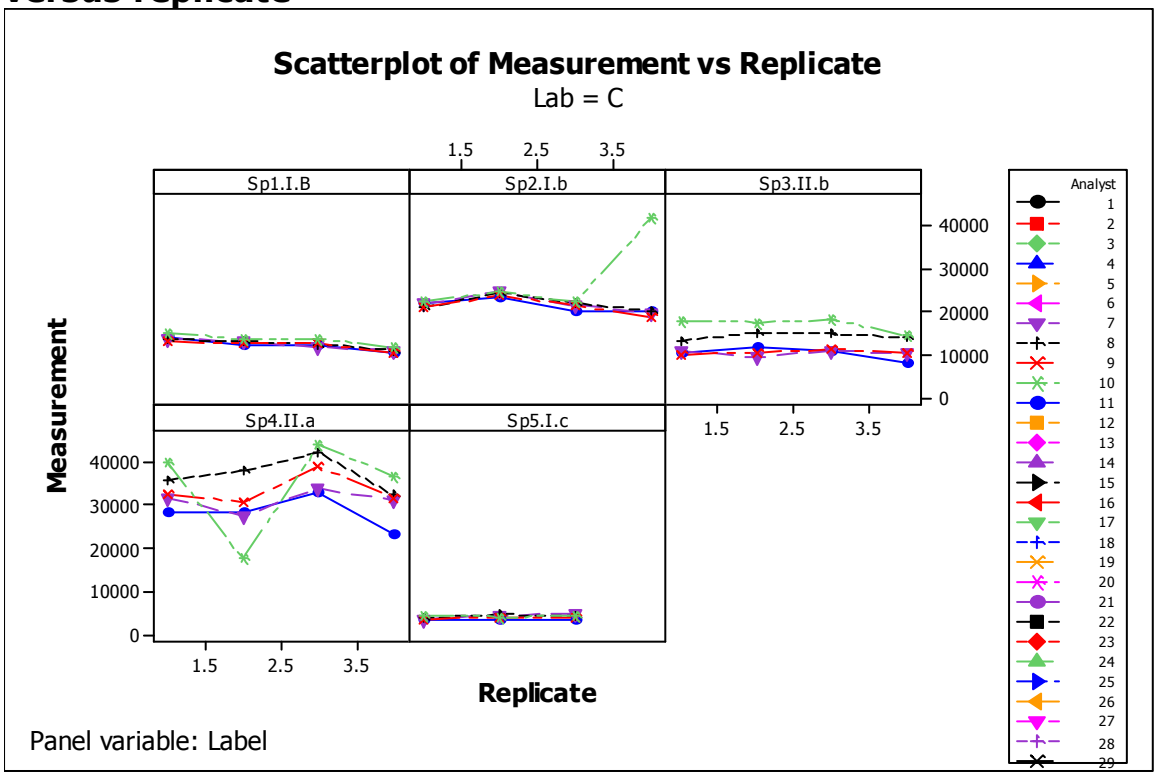


Figure 16: Mean measurements of species per lab

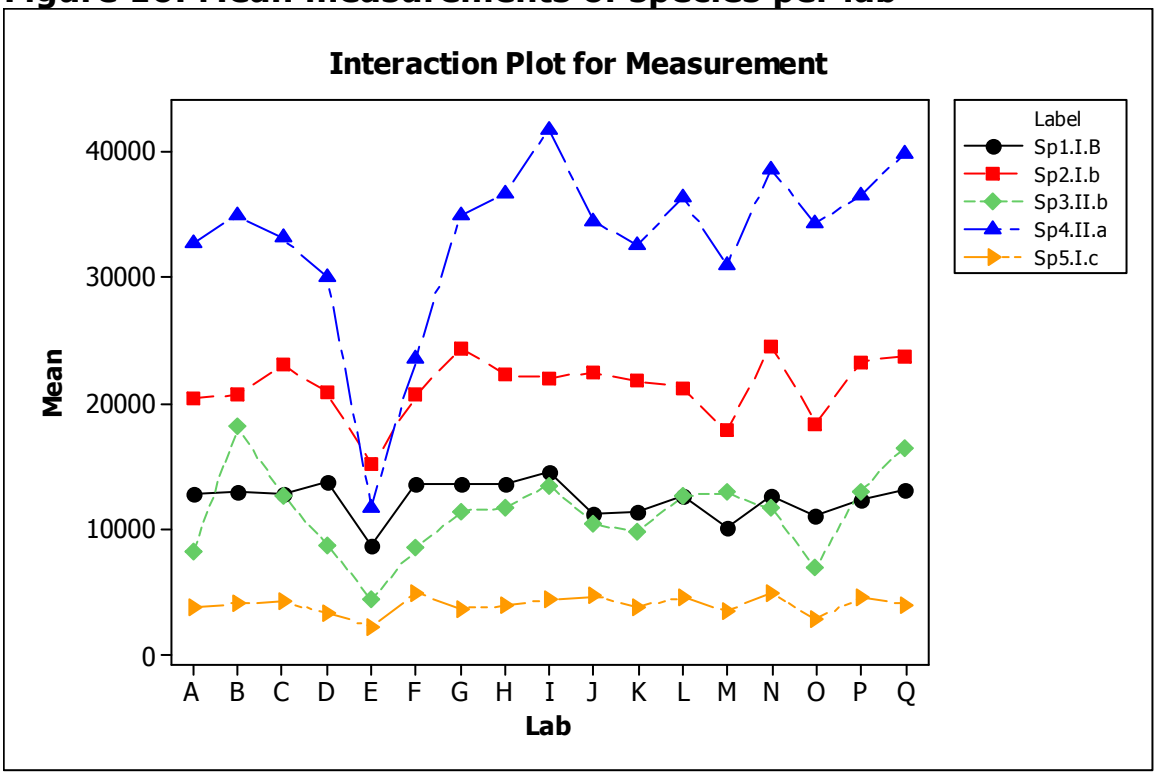


Figure 17: Test for equal variances

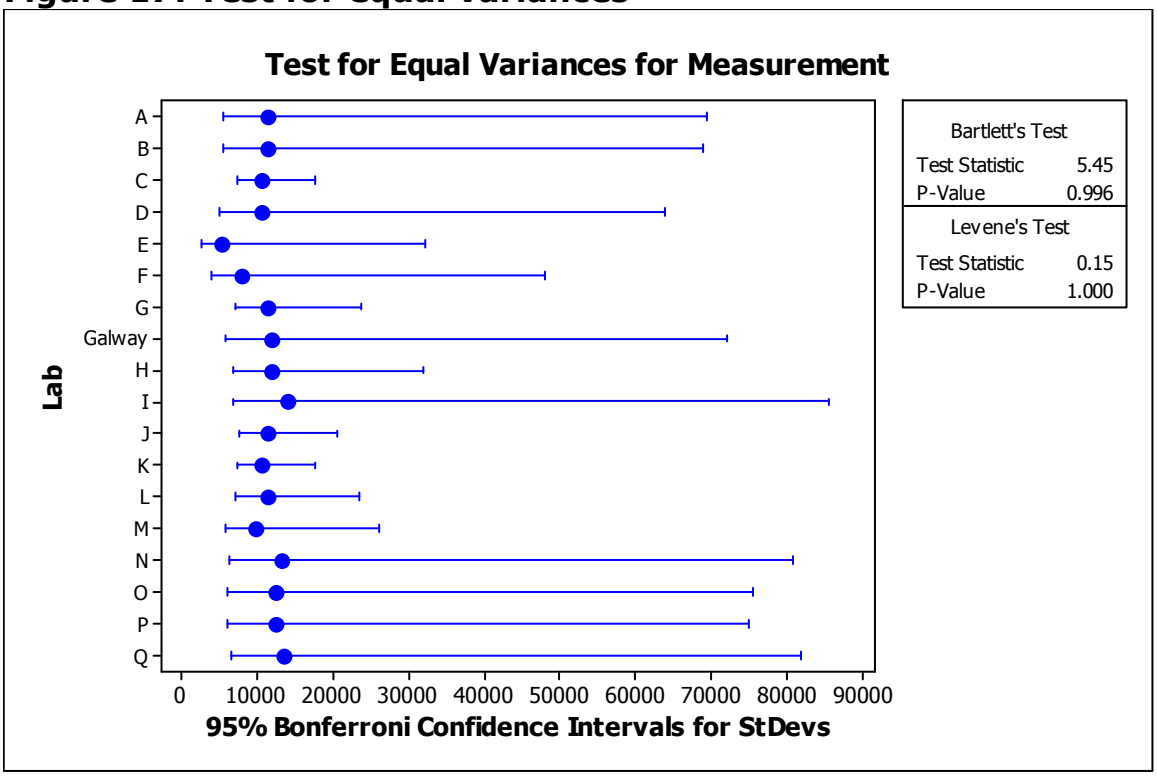


Figure 18: I chart of lab observations for *Coscinodiscus granii*

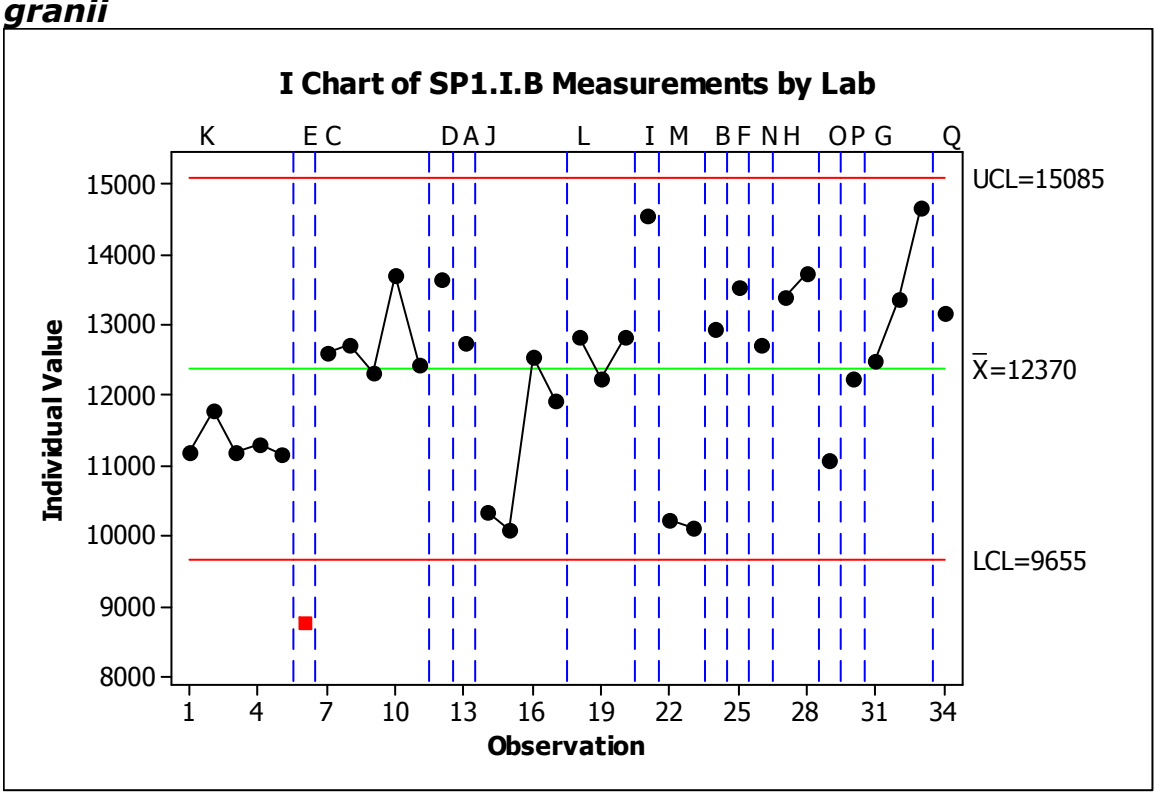


Figure 19: I chart of lab observations for *Scrippsiella trochoidea*

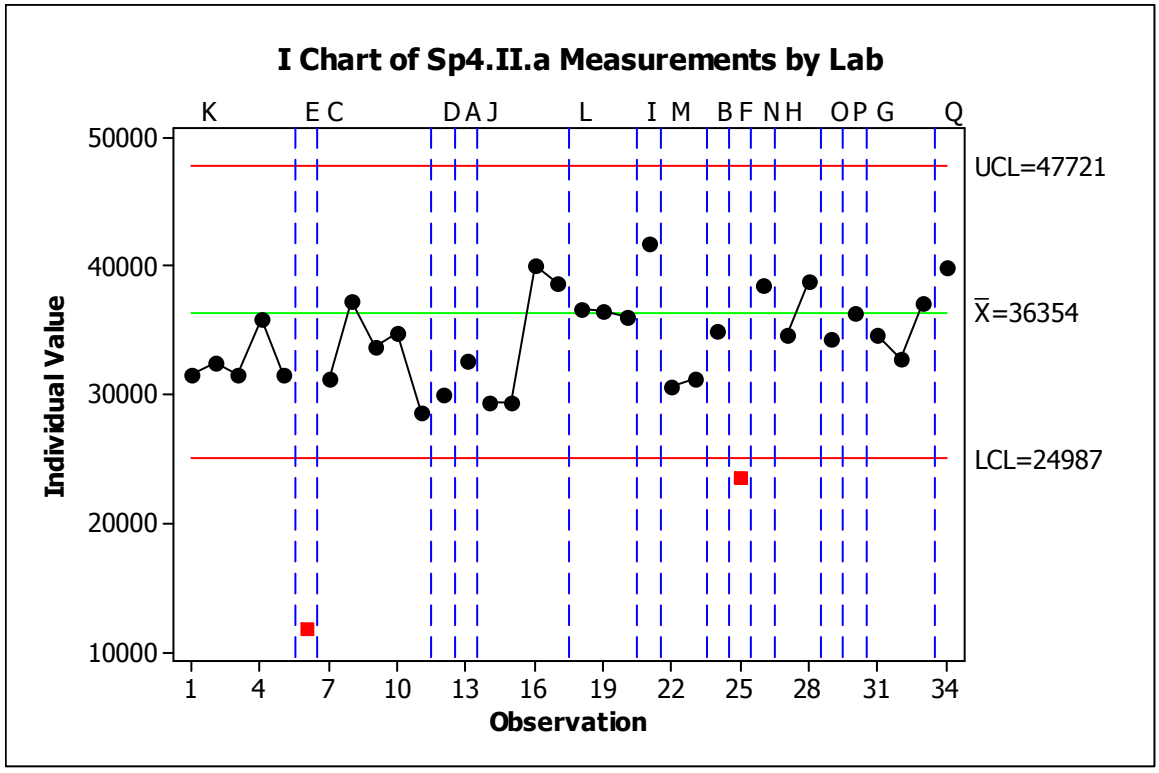


Figure 20: I chart of lab observations for *Gymnodinium catenatum*

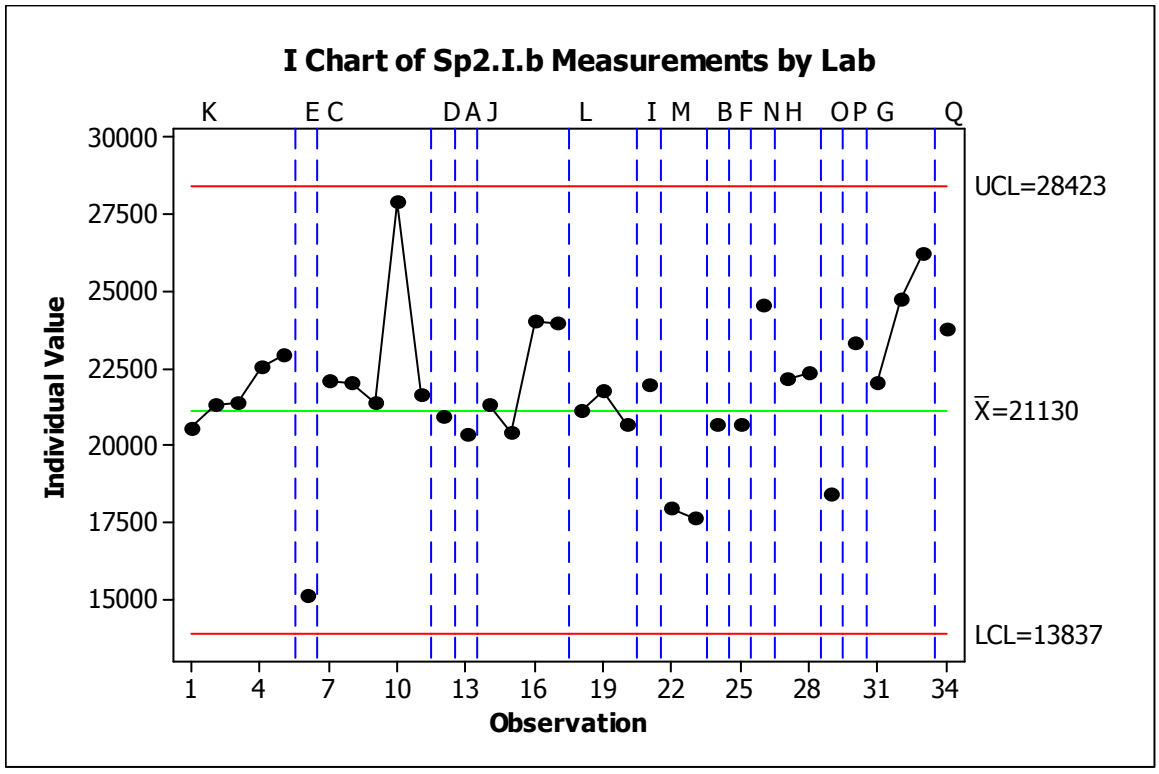


Figure 21: I chart of lab observations for *Prorocentrum micans*

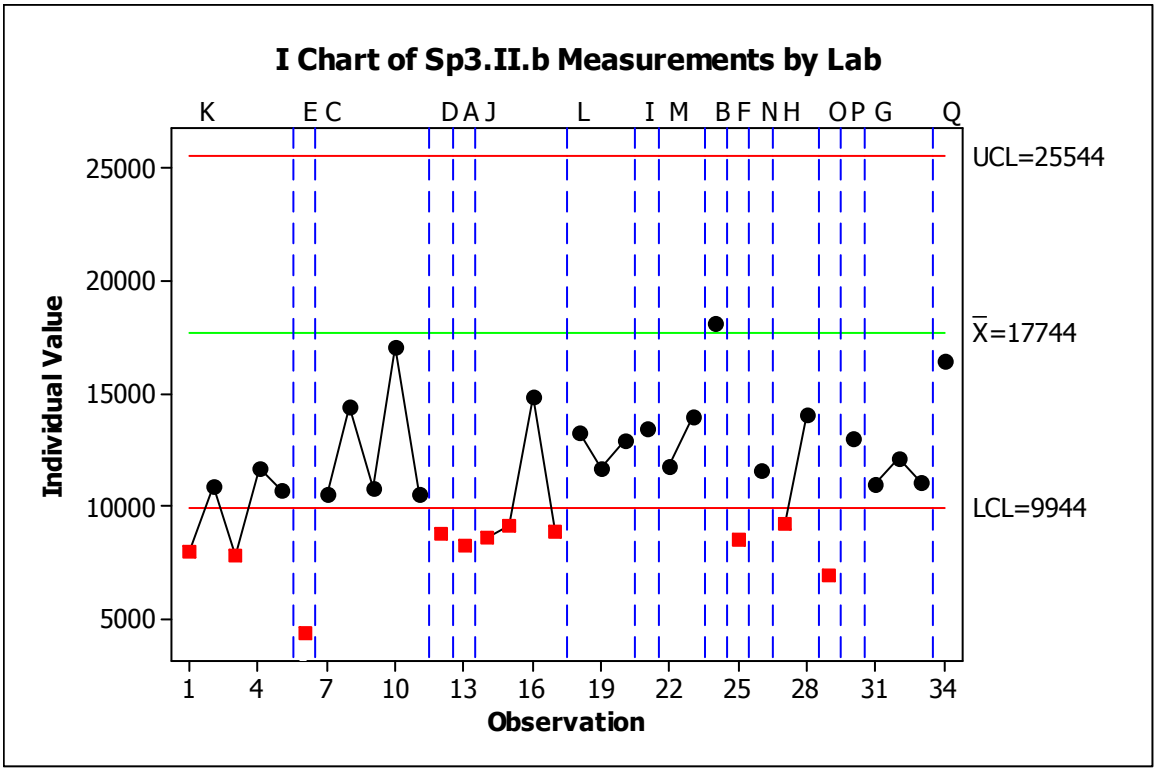


Figure 22: I chart of lab observations for *Prorocentrum lima*

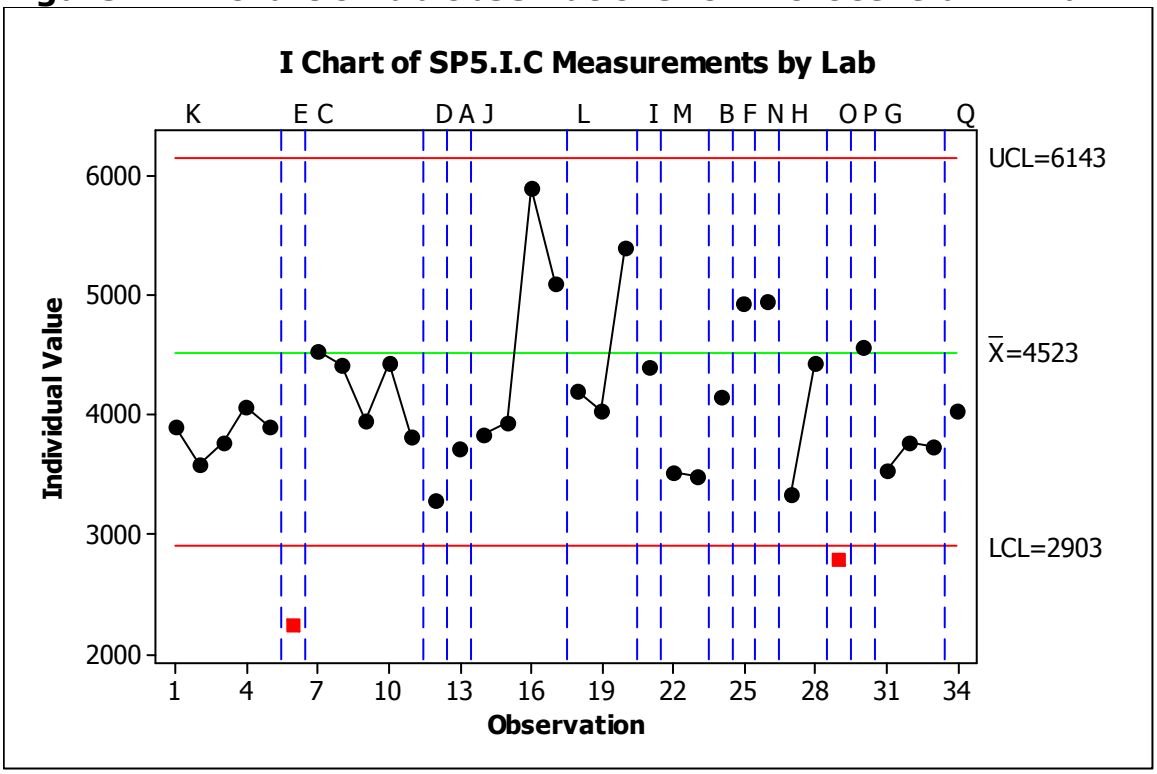


Figure 23: T-test for P.micans results of analysts versus true value

One-Sample T: Analysts data
Variable N Mean StDev SE Mean 95% CI
Analysts data 136 11310 3411 292 (10732, 11888)
One-Sample T: True value
Variable N Mean StDev SE Mean 95% CI
True value 20 17744 2600 581 (16527, 18961)
Two-Sample T-Test and CI: Analysts data, True value
Two-sample T for Analysts data vs True value
N Mean StDev SE Mean
Analysts data 136 11310 3411 292
True value 20 17744 2600 581
Difference = mu (Analysts data) - mu (True value)
Estimate for difference: -6434
95% CI for difference: (-7765, -5103)
T-Test of difference = 0 (vs not =): T-Value = -9.89 P-Value = 0.000 DF = 29

Figure 24: *P.lima* counts, mean of samples versus reference value

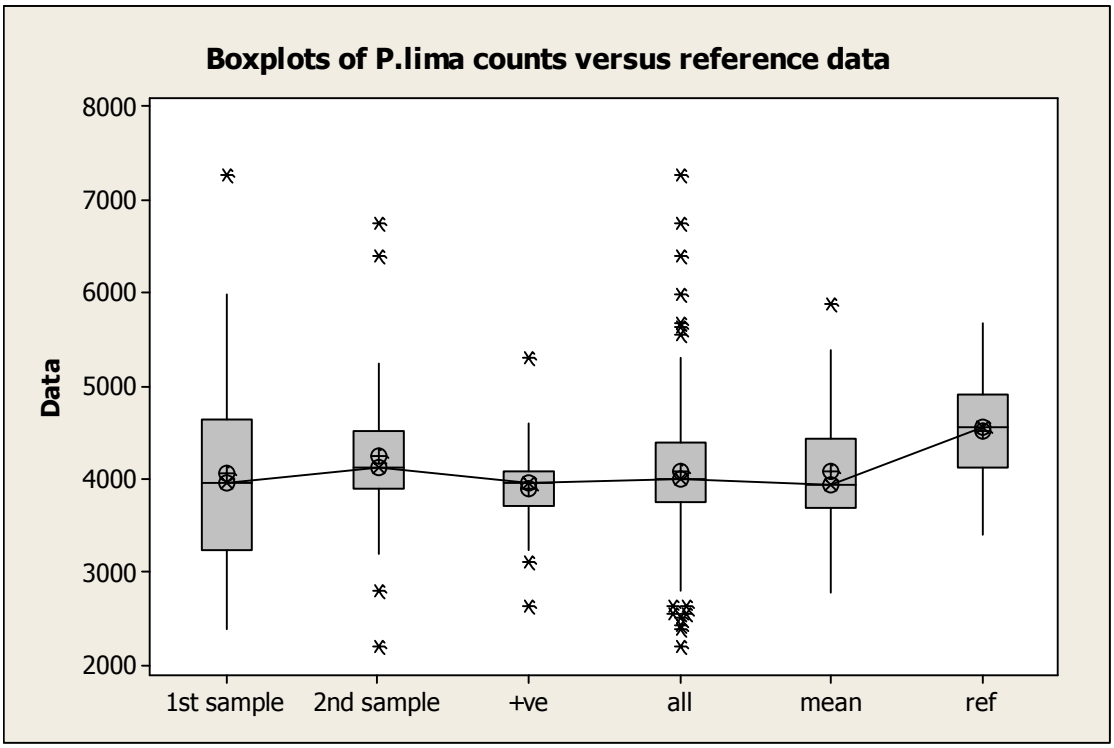


Figure 25:

Paired T-Test and CI: 1st sample, ref

Paired T for 1st sample - ref

	N	Mean	StDev	SE Mean
1st sample	23	4158	1071	223
ref	23	4523	540	113
Difference	23	-365	1171	244

95% CI for mean difference: (-871, 141)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.50 P-Value = 0.149

Paired T-Test and CI: 2nd sample, ref

Paired T for 2nd sample - ref

	N	Mean	StDev	SE Mean
2nd sample	23	4263	968	202
ref	23	4523	540	113
Difference	23	-261	1117	233

95% CI for mean difference: (-744, 222)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.12 P-Value = 0.275

Paired T-Test and CI: +ve, ref

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)
T-Test of mean difference = 0 (vs not = 0): T-Value = -4.17 P-Value = 0.000

Paired T-Test and CI: all, ref

Paired T for all - ref

	N	Mean	StDev	SE Mean
all	23	4158	1071	223
ref	23	4523	540	113
Difference	23	-365	1171	244

95% CI for mean difference: (-871, 141)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.50 P-Value = 0.149

Paired T-Test and CI: mean, ref

Paired T for mean - ref

	N	Mean	StDev	SE Mean
mean	23	4107	647	135
ref	23	4523	540	113
Difference	23	-417	836	174

95% CI for mean difference: (-778, -55)
T-Test of mean difference = 0 (vs not = 0): T-Value = -2.39 P-Value = 0.026

Paired T-Test and CI: 1st sample, +ve

Paired T for 1st sample - +ve

	N	Mean	StDev	SE Mean
1st sample	34	4067	1125	193
+ve	34	3911	461	79
Difference	34	156	1184	203

95% CI for mean difference: (-257, 569)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.77 P-Value = 0.448

Paired T-Test and CI: 2nd sample, +ve

Paired T for 2nd sample - +ve

	N	Mean	StDev	SE Mean
2nd sample	34	4256	860	148
+ve	34	3911	461	79
Difference	34	345	972	167

95% CI for mean difference: (6, 685)
T-Test of mean difference = 0 (vs not = 0): T-Value = 2.07 P-Value = 0.046

Figure 26: Z-score for *Coscinodiscus granii*

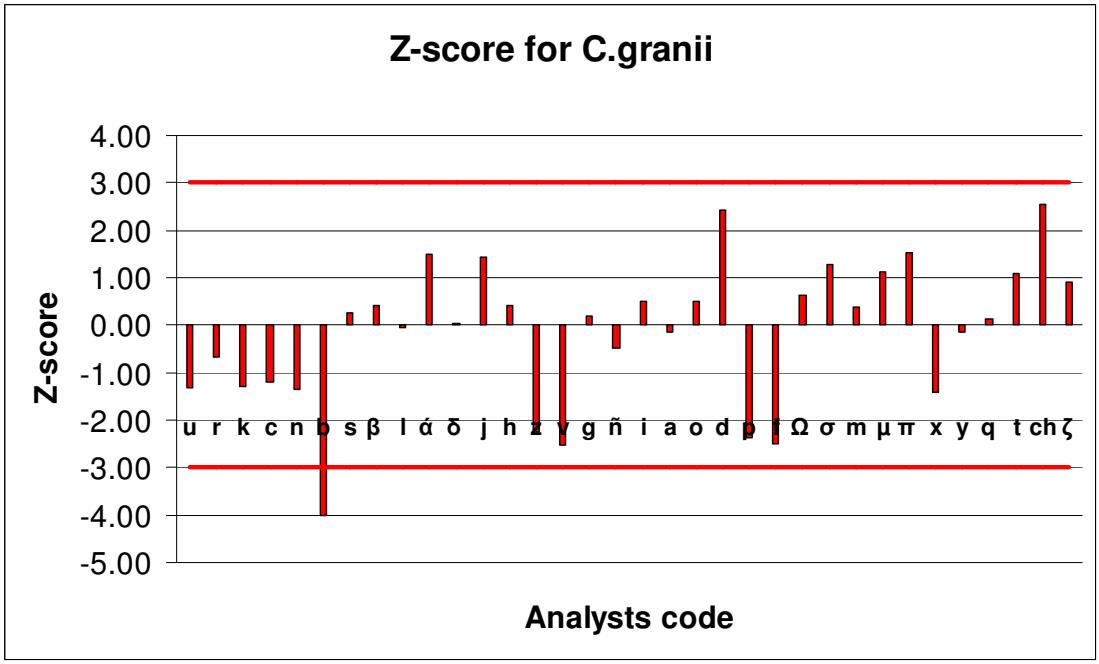


Figure 27: Z-score for *Gimnodinium catenatum*

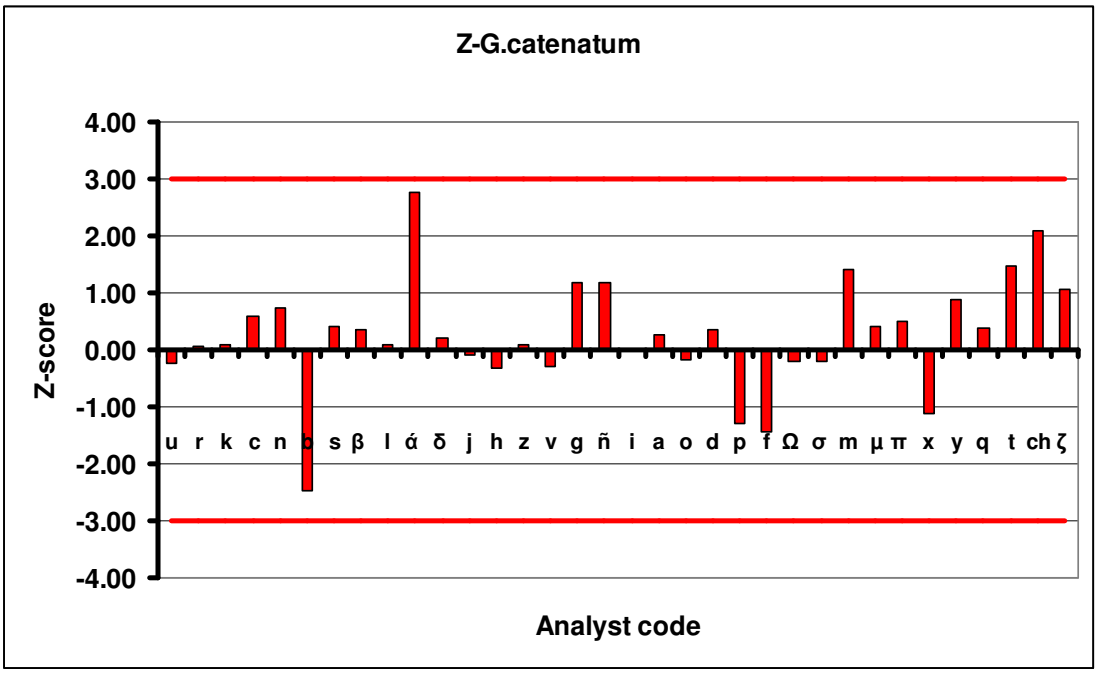


Figure 28: Z-score for *Scripsiella sp.*

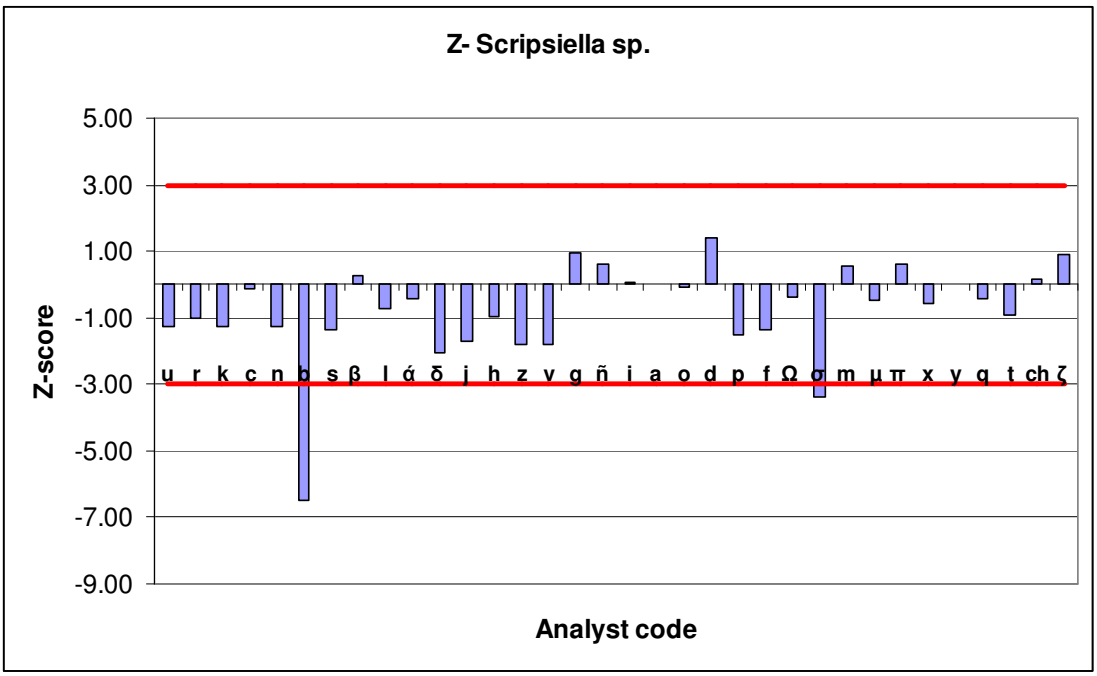


Figure 29: Z-score for *P micans*

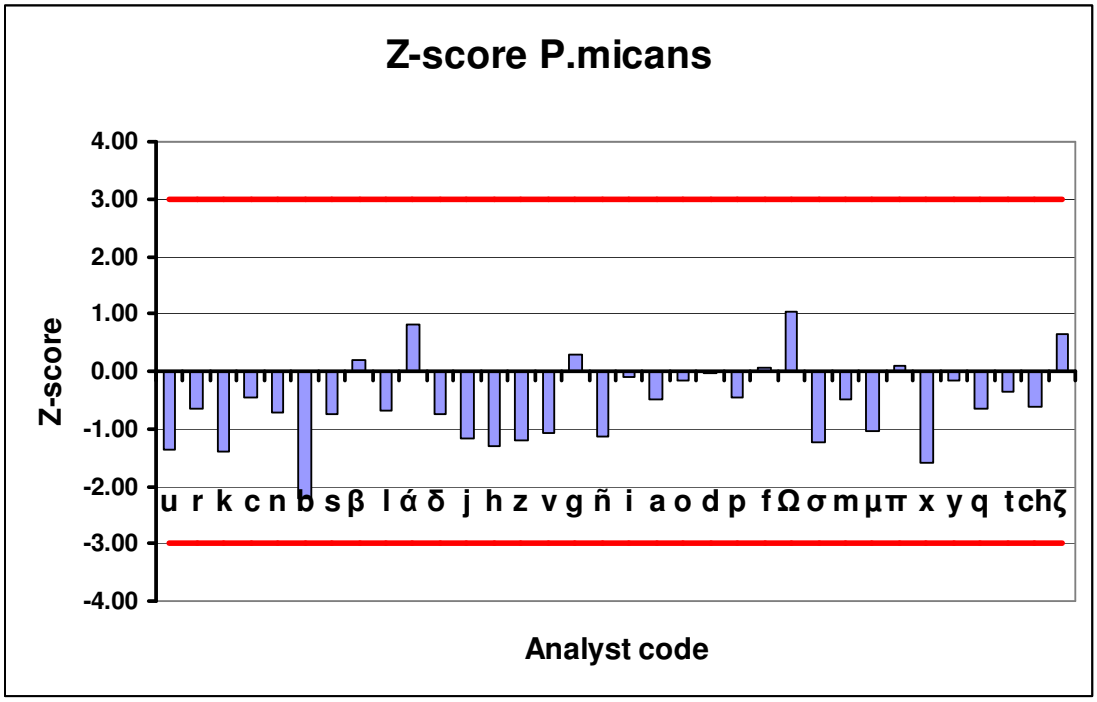


Figure 30: Z-score for *P.lima*

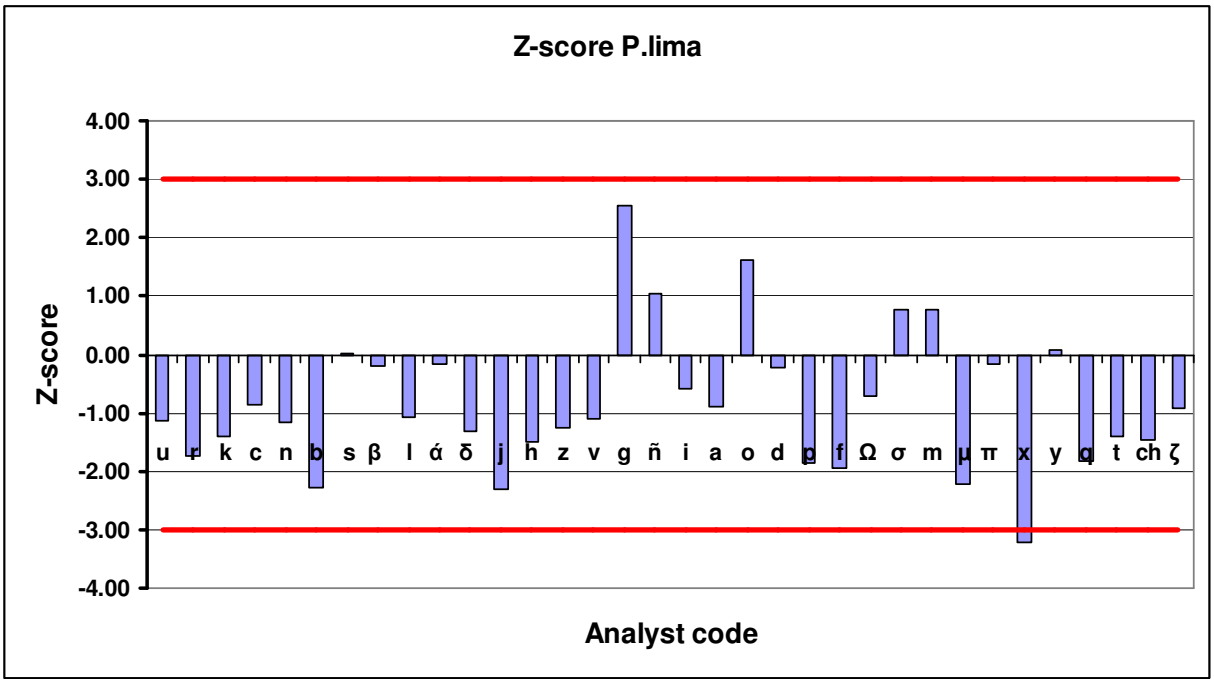


Figure 31: General linear model for the enumeration exercise

Analysis of Variance for Measurement, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Species	4	64587150193	63533787673	15883446918	1540.20	0.000
Lab	16	2423347878	2423347878	151459242	3.54	0.007
Analyst (Lab)	17	727453541	727453541	42791385	4.15	0.000
Replicate	3	117430908	117430908	39143636	3.80	0.010
Error	605	6239101794	6239101794	10312565		
Total	645	74094484313				

S = 3211.32 R-Sq = 91.58% R-Sq(adj) = 91.02%

Annex IV: Detailed results of the identification test

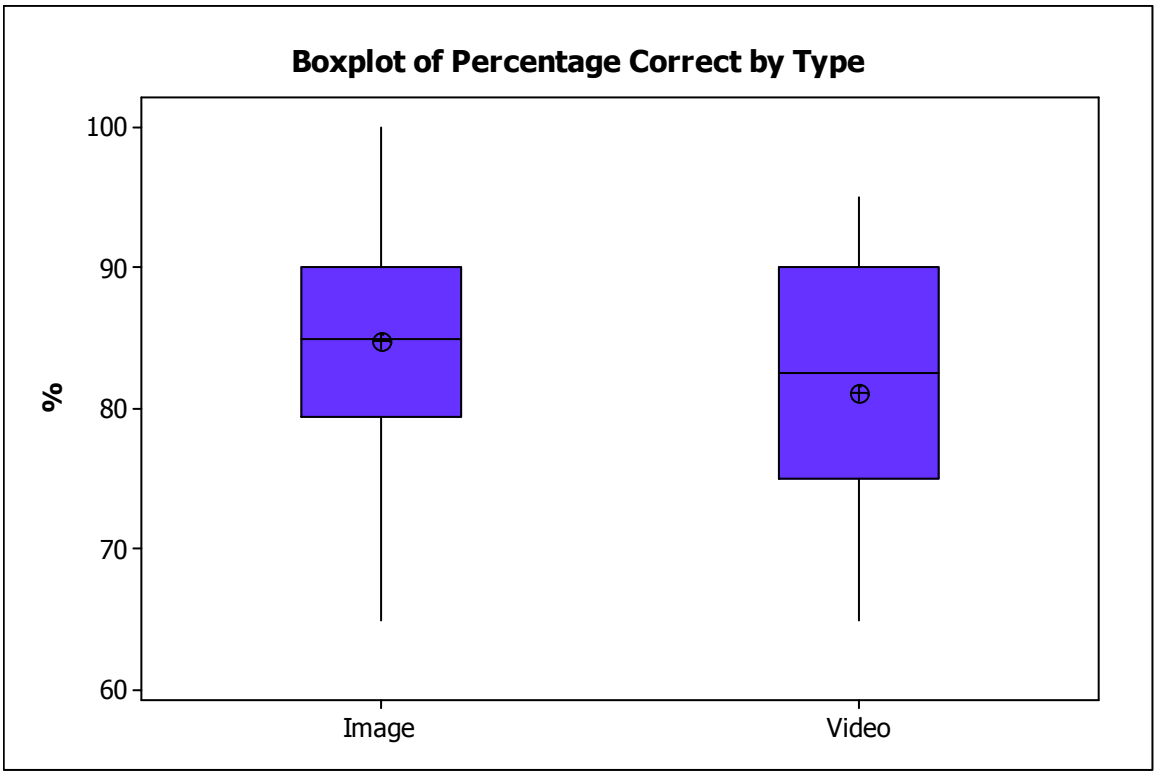
Figure 32: Identification results Taxonomic quiz images

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

Figure 33: 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

Figure 34: Box plot of percentage correct images versus video clips



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 35: case profile by lab of images versus videos

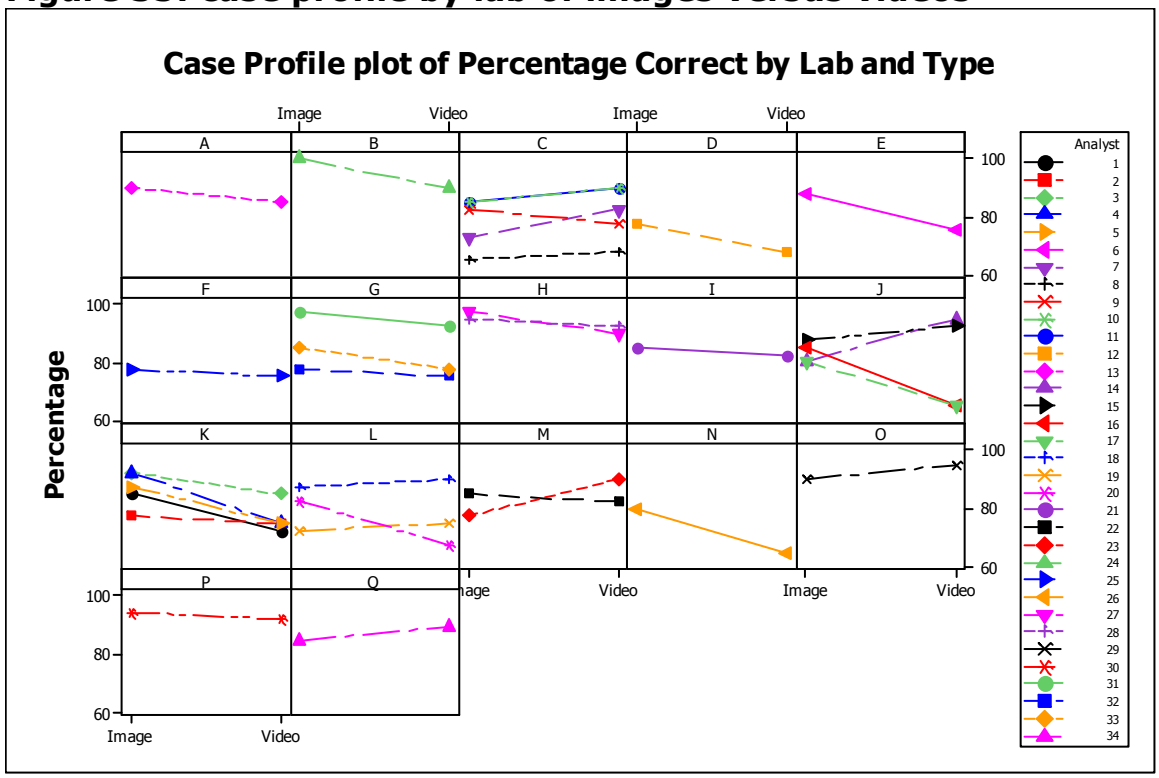


Figure 36: Box plot of correct answers by sets

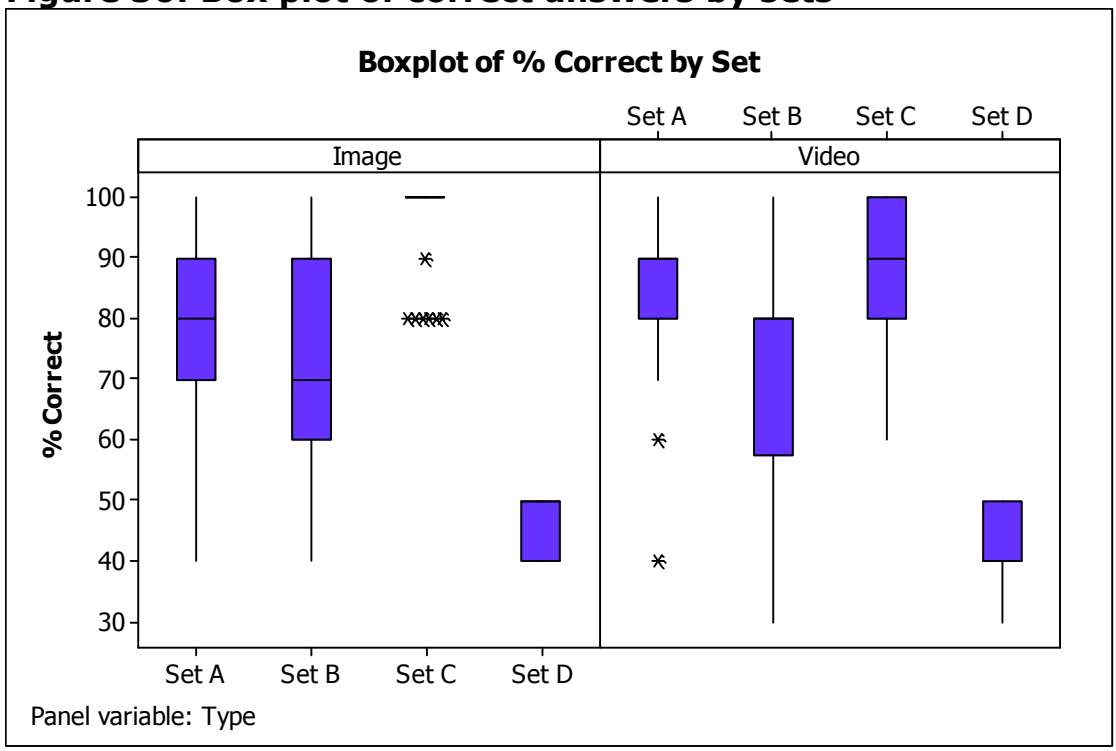
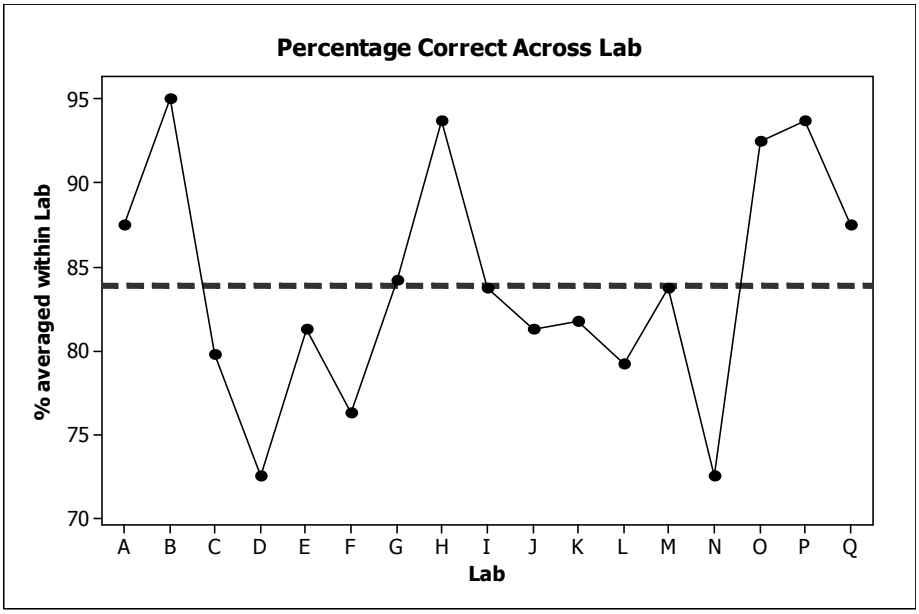


Figure 37: Cumulative percentage correct images and videos

Percentage_**Image**	Count	CumPct
65.0	1	2.94
72.5	2	8.82
77.5	5	23.53
80.0	3	32.35
82.5	2	38.24
85.0	8	61.76
87.5	4	73.53
90.0	2	79.41
92.5	2	85.29
95.0	2	91.18
97.5	2	97.06
100.0	1	100.00
N=	34	

Percentage_**Video**	Count	CumPct
65.0	3	8.82
67.5	3	17.65
72.5	1	20.59
75.0	7	41.18
77.5	2	47.06
82.5	3	55.88
85.0	2	61.76
90.0	7	82.35
92.5	4	94.12
95.0	2	100.00
N=	34	

Figure 38: Percentage correct answers by lab



Analysis of Variance for Percentage, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Type	1	229.78	229.78	229.78	3.63	**0.063**
Lab	16	2026.35	2026.35	126.65	2.00	**0.032**
Error	50	3168.14	3168.14	63.36		
Total	67	5424.26				

S = 7.96007 R-Sq = 41.59% R-Sq(adj) = 21.73%

Figure 39: General linear model for identification exercise

General Linear Model: PerCorrect versus Types, Set, Labs, Analysts

Factor	Type	Levels	Values
Types	fixed	2	Image, Video
Set	fixed	4	Set A (%), Set B (%), Set C (%), Set D (%)
Labs	fixed	17	A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P,Q
Analysts(Labs)	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 PerCorrect, 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%

Figure 40: Image identifications results by analyst

Analysts	Score	Analysts	Score
Ω	100	g	85
q	97.5	ζ	85
μ	97.5	d	85
π	95	p	85
y	95	o	82.5
k	92.5	l	82.5
c	92.5	m	80
h	90	ñ	80
x	90	z	80
b	87.5	r	77.5
n	87.5	j	77.5
i	87.5	f	77.5
v	87.5	σ	77.5
u	85	t	77.5
ά	85	a	72.5
δ	85	s	72.5
c h	85	β	65

Figure 41: Video identifications results by analyst

Analysts	Score	Analysts	Score
π	97.5	p	82.5
q	97.5	l	77.5
z	95	c h	77.5
Ω	95	r	75
μ	95	c	75
x	95	n	75
v	92.5	b	75
y	92.5	a	75
ά	90	σ	75
δ	90	t	75
i	90	u	72.5
f	90	β	67.5
ζ	90	j	67.5
k	85	o	67.5
h	85	g	65
s	82.5	ñ	65
d	82.5	m	65

Annex V: Workshop Agenda

BEQUALM / National Marine Biological Analytical Quality Control Scheme
Phytoplankton ring test PHY-ICN-09-MI1 2009

Workshop

Thursday, 16th April 2009,
Marine Institute Brendan the Navigator Meeting room

Agenda

09:45 **Introductions / Welcome**

10:00 **Intercomparison exercise PHY-ICN-09-MI1**

Materials and Methodology

A: Enumeration exercise results

B: Identification exercise results

11:00 **Statistical analysis of ICN exercise: results of enumeration and identification exercise**
Dr. John Newell
National University of Ireland Galway
Biostatistics unit, School of medicine

11:30 Coffee Break

12:00 **Discussion: Questions and answers session**

12:30 **Accreditation in the phytoplankton lab**
Josephine Lyons
Phytoplankton analyst
Marine Institute

13:00 Lunch in Marine Institute Restaurant

14:00 **"Characterization of a novel azaspiracid-producing dinoflagellate from the North Sea "**
Doctor Urban Tillmann
Biosciences | Ecological Chemistry
Alfred Wegener Institute

14:45 **"Phagotrophy of planktonic protists - video observations"**
Doctor Urban Tillmann
Biosciences | Ecological Chemistry
Alfred Wegener Institute

15:30 Coffee Break

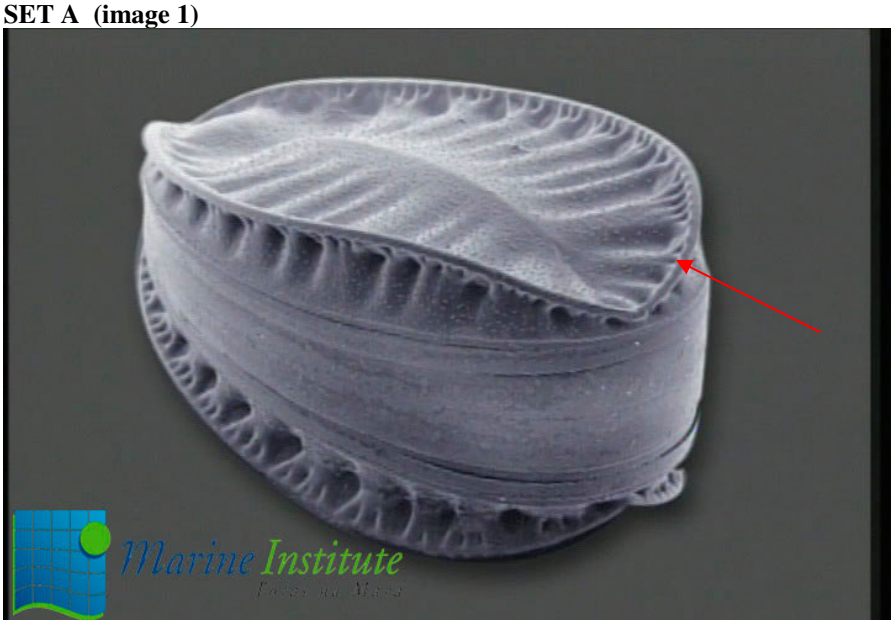
16:00 **Results Discussion: Future developments of ICN 2010**

Annex VI: Form 2: Taxonomic quiz

ANALYST CODE:_____

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

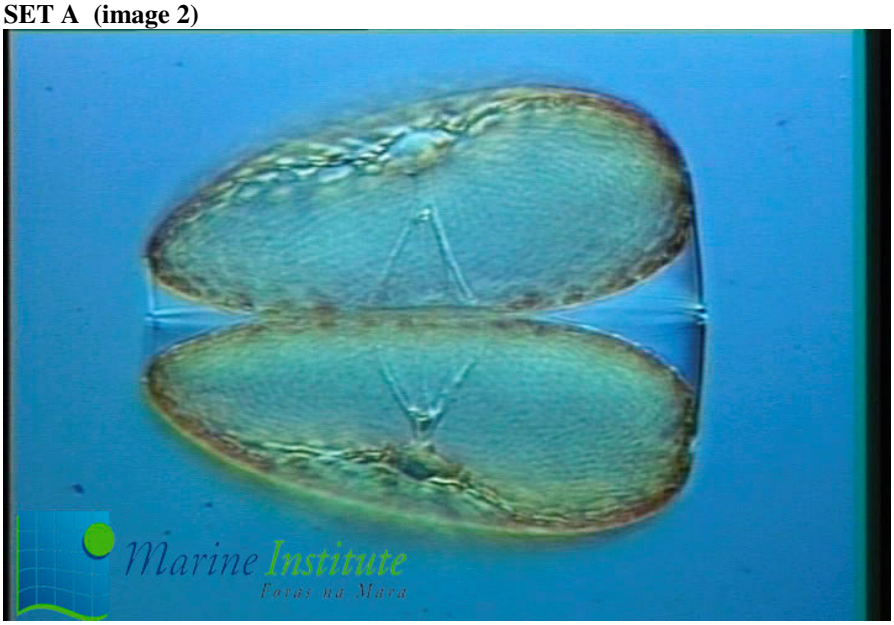
SET A (image 1)



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:_____*Coscinodiscus granii*_____

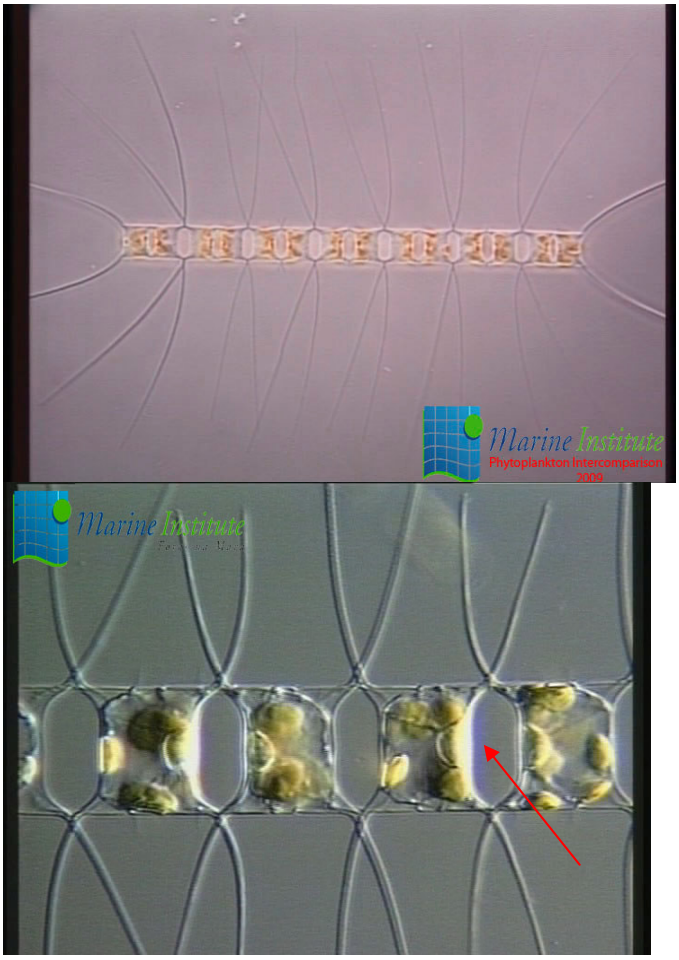
SET A (image 3)

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

SET A (image 4)

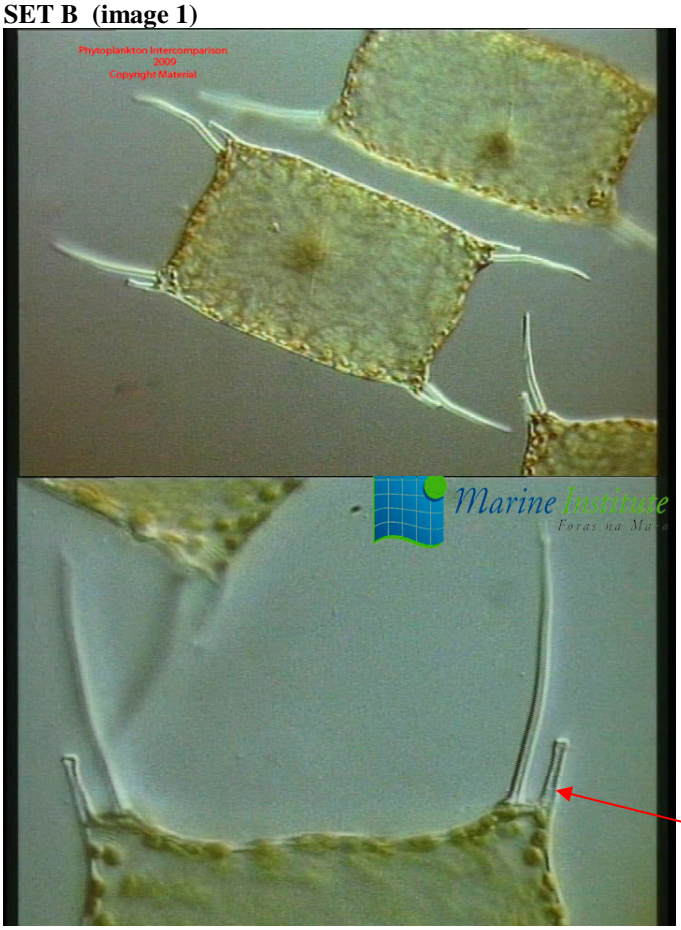
4A Question: Name the parts of this pennate diatom coloured in blue and pink (10 marks)
Answer: _____girdle bands (pink) and valves (blue)_____

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:___*C. decipiens/lorenzianus*_____
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_____*A.catanella*_____
7A) Video A2: **Question: Name these organisms to species level. Typical size: 34 to 65µm long (10 marks)**
Answer_____*G.catenatum*_____
8A) Video A3: **Question: Which Class and genus do this organism belongs to? Typical size: 8 to 15µm long (10 marks)**
Answer_____Haptophyte, primnesium_____
9A) Video A4: **Question: Name this organism to species level. Typical size: 12 to 18µm (10 marks)**
Answer_____*Amphidinium carterae*_____
10A) Video A5: **Question: Name this organism to species level. Typical size: 140 to 180µm long (10 marks)**
Answer_____*Gyrodinium britanicum*_____

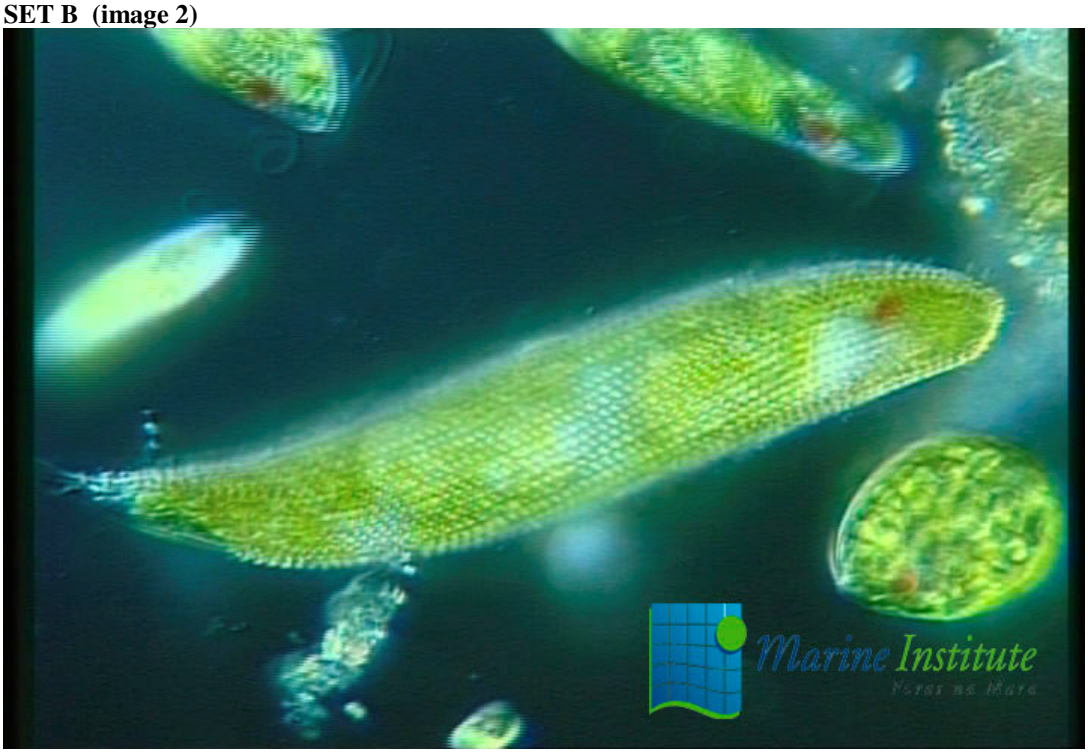
SET B (image 1)



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:_____*Odontella sinensis*_____

SET B (image 2)



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

Answer:_____Euglenales_____

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)

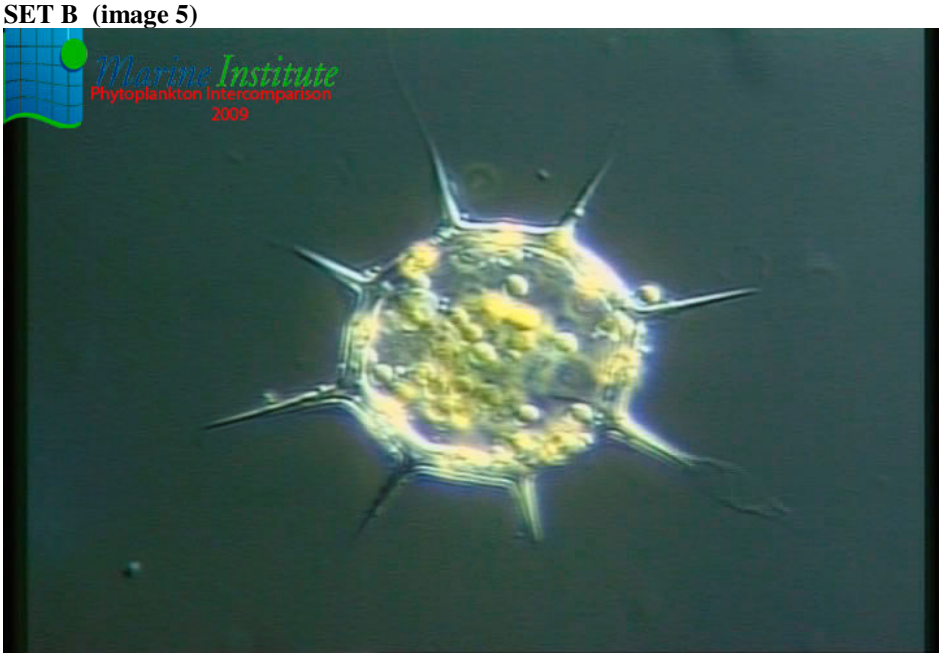
Answer:_____*Polykrikos schwarzii*_____

SET B (image 4)

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

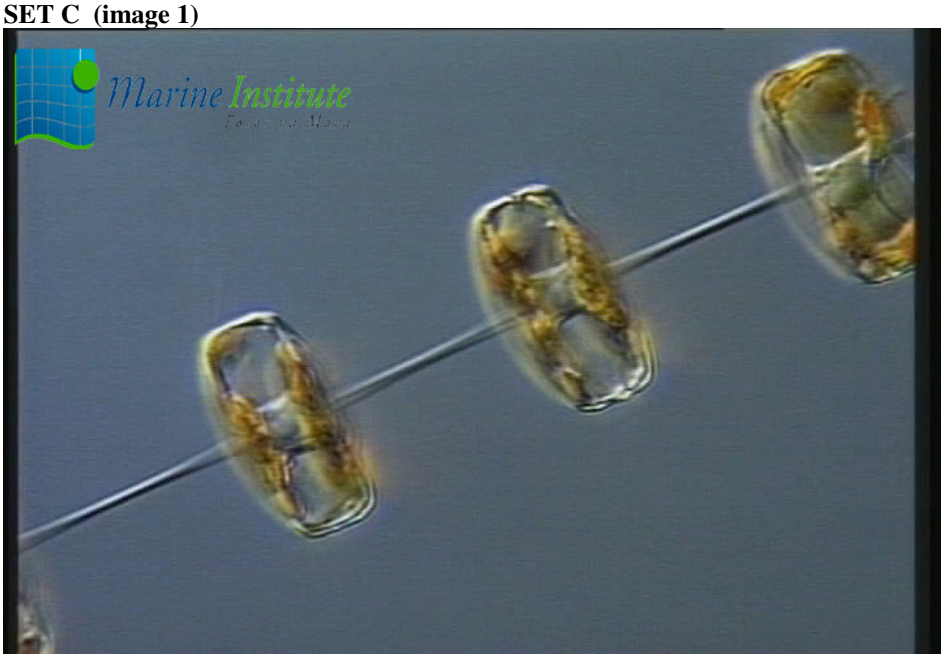
Answer:_____*Gyrodinium*_____

SET B (image 5)



5B Question: Name this organism to species level. Typical size: 20 to 30µm diameter (10 marks)
Answer:_____***Dyctiocha octonaria***_____
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_____*P. alata/indica*_____
7B) Video B2: **Q: Name this organism to genus level. Typical size: 12 to 18µm long (10 marks)**
Answer_____*Amphidinium*_____
8B) Video B3: **Q: Name this organism to species level. Typical size: 25 to 35µm long (10 marks)**
Answer_____*Osirrhys marina*_____
9B) Video B4: **Q: Name this organism to genus level. Typical size: up to 1.5mm long (10 marks)**
Answer_____*Rhizosolenia*_____
10B) Video B5: **Question: Name this organism to genus level. Typical size: 250µm long (10 marks)**
Answer_____*Pirocystis*_____

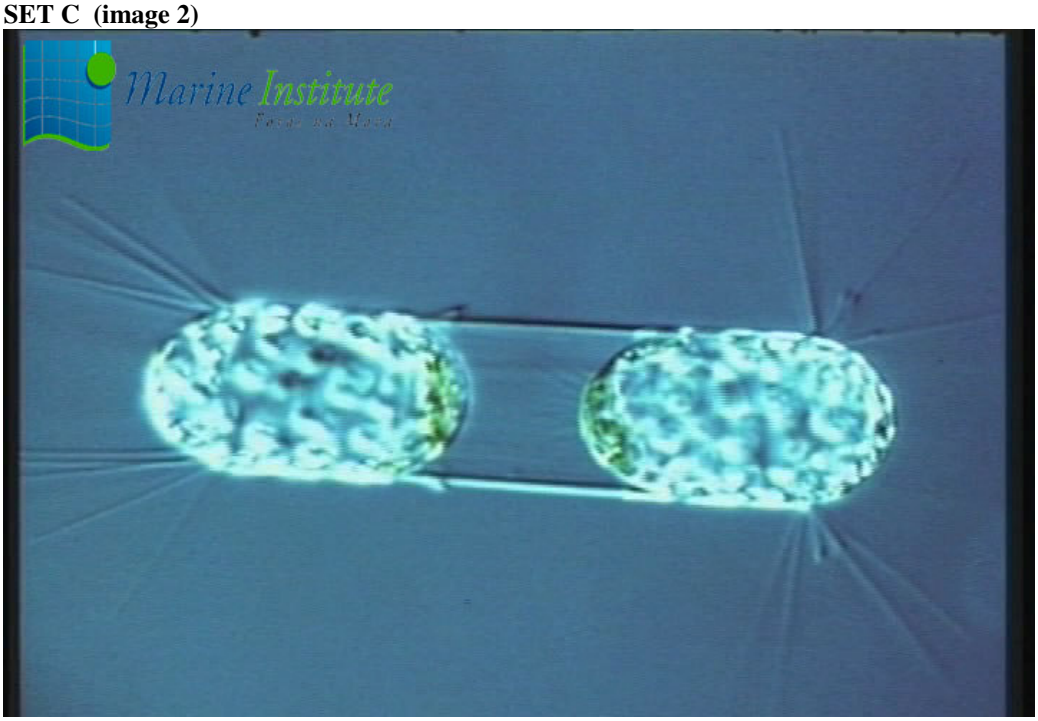
SET C (image 1)



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:_____*Thalassiosira*_____strutted process_____

SET C (image 2)



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

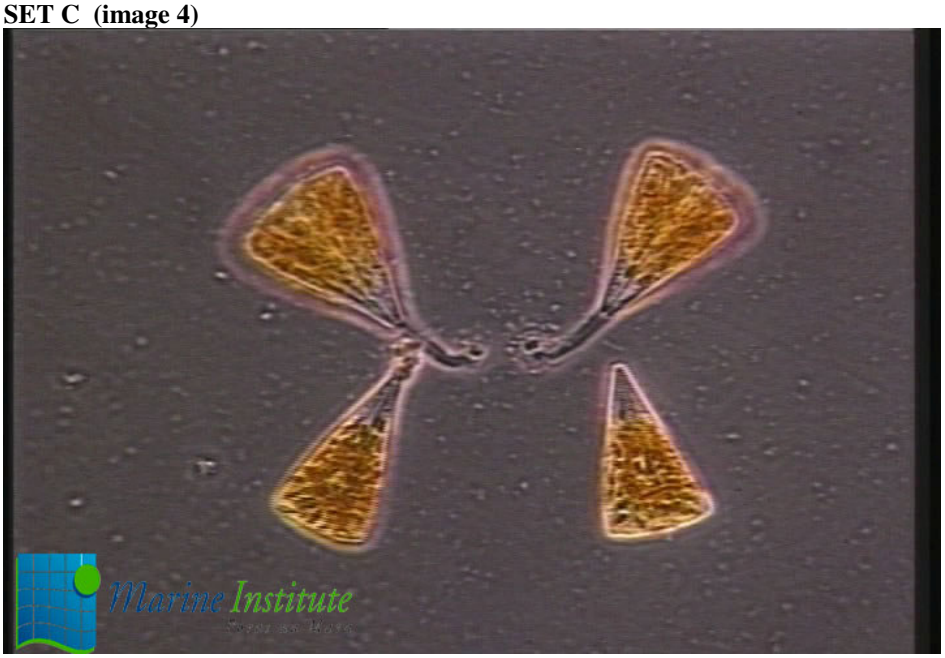
Answer:_____*Corethron*_____

SET C (image 3)

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

Answer:_____*Noctiluca scintillans*_____

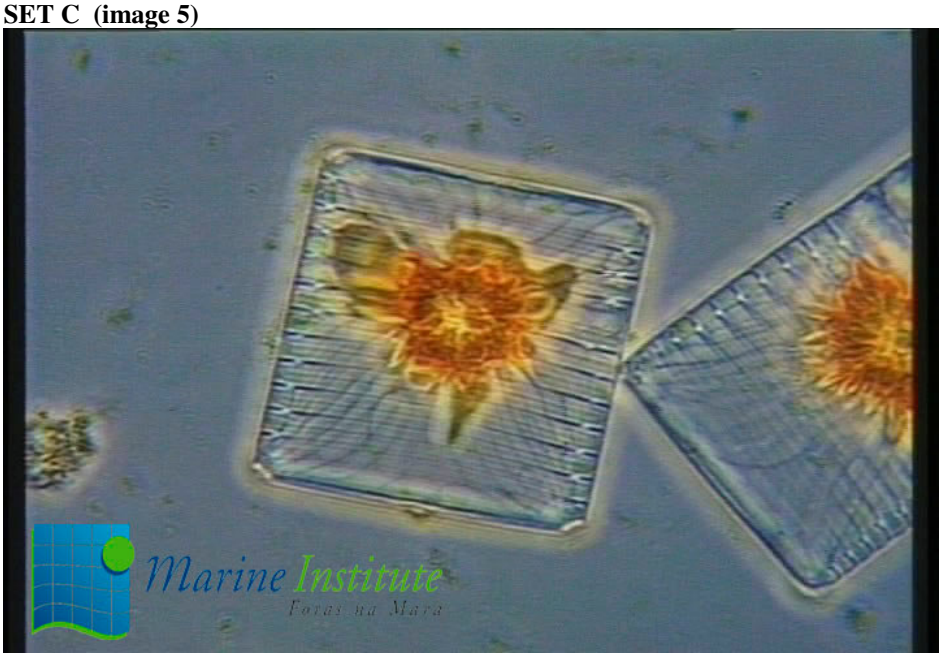
SET C (image 4)



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

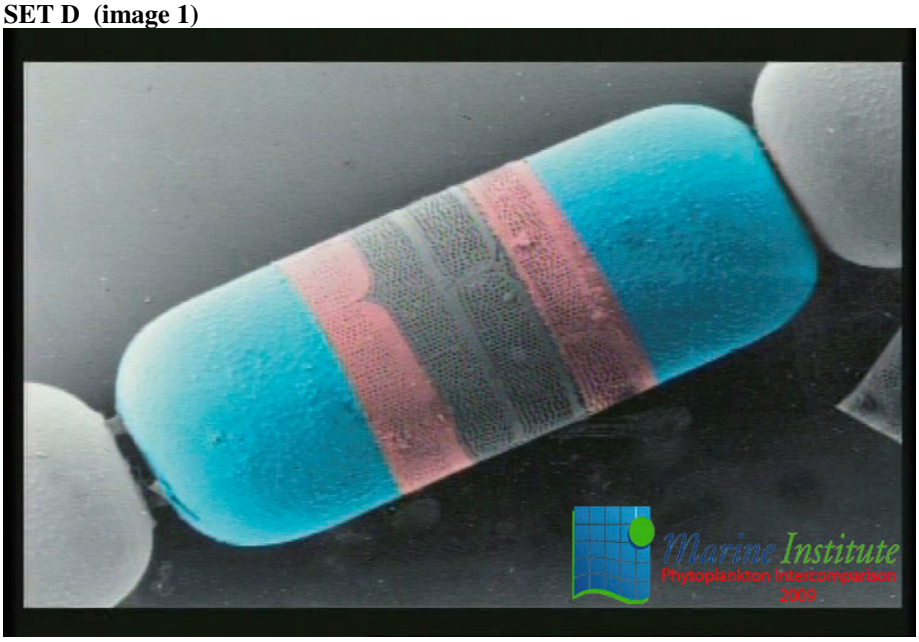
Answer:_____*Licmophora*_____

SET C (image 5)



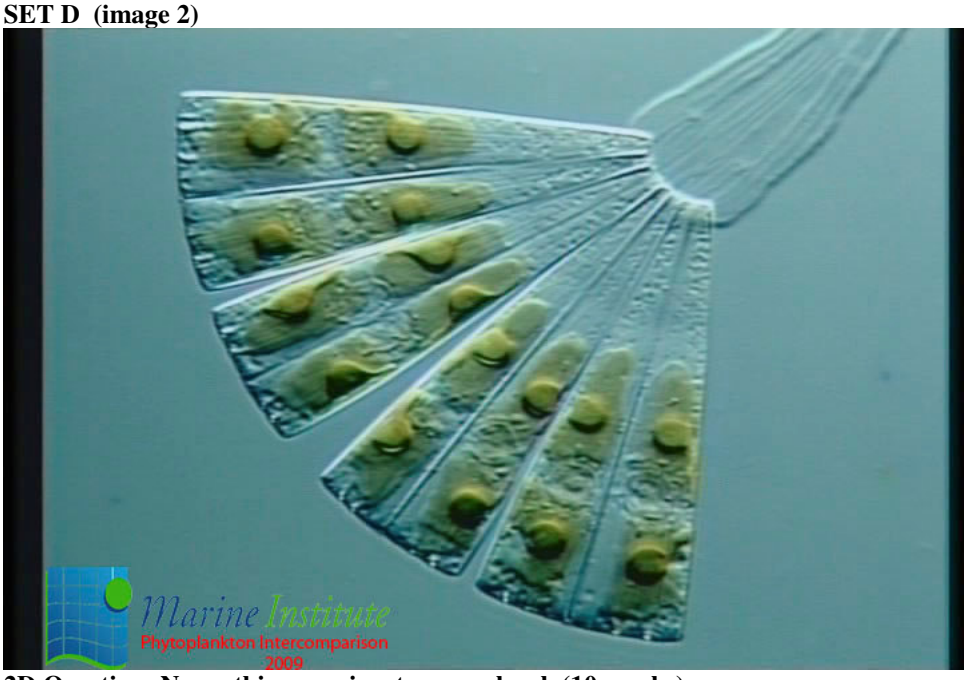
5C Question: Name this organism to species level, Typical size: 30 to 120µm (Width) (10 marks)
Answer:_____***striatella unipunctata***_____
To answer the following questions, you need to go to the website www.unique-media.tv/mie001 username: marine and password: bus7xehe and watch the **SET C** videos. You have the choice of low or high resolution viewing.
6C) Video C1: **Question: Name this colonial organism to species level (10 marks)**
Answer_____***Phaeocystis pouchetti/globosa***_____
7C) Video C2: **Question: Which class and order this organism belongs to? (10 marks)**
Answer_____Euglenida, euglenales_____
8C) Video C3: **Question: Name this organism to species level (10 marks)**
Answer_____*chaetoceros peruvianus*_____
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_____Cryptomonads**,** ejectosomes _____
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_____eyespot_____

SET D (image 1)



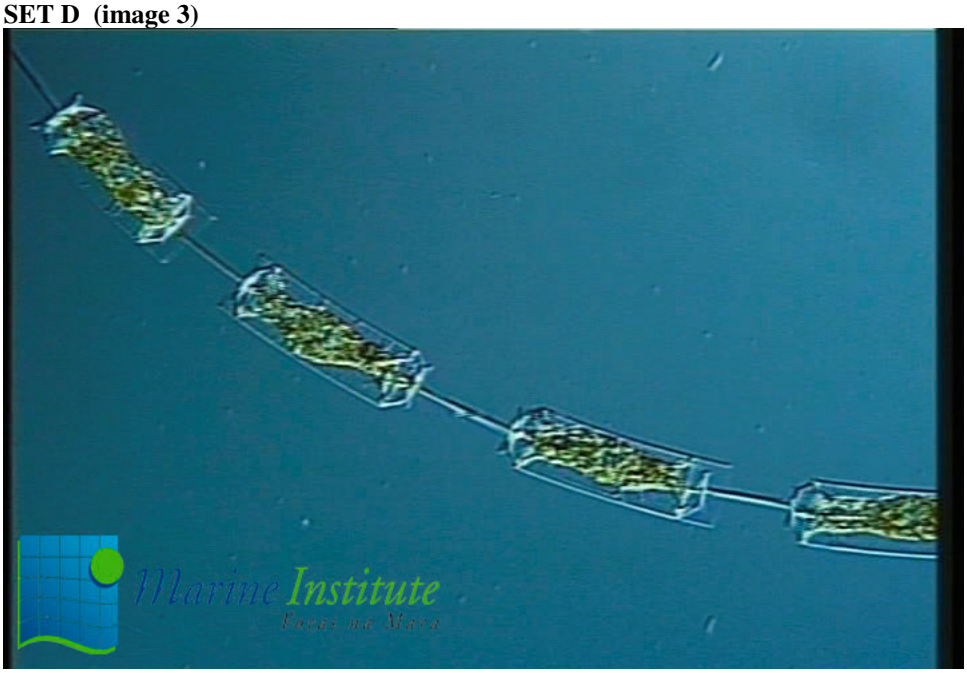
1D Question: Name the parts of this Centric diatom coloured in blue and pink (10 marks)
Answer: _____Valves (blue) and girdle bands (pink)_____

SET D (image 2)



2D Question: Name this organism to genus level. (10 marks)
Answer:_____***Licmophora***_____

SET D (image 3)



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

SET D (image 4)

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

SET D (image 5)

5D Question: Name this organism to species level. Typical size: 200 to 400µm long (10 marks)
Answer:_____*Ceratium horridum/ macroceros*_____
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_____***Glenodinium foliaceum***_____
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_____*Chrysochromulina, haptonema*_____
8D) Video D3: **Question: Name this organism to genus level (10 marks)**
Answer_____*Protoperidinium*_____
9D) Video D4: **Question: Name this organism to species level (10 marks)**
Answer_____*Bacillaria paradoxa*_____
10D) Video D5: **Question: what is happening in this sequence. Choose one of the following: (10 marks)**

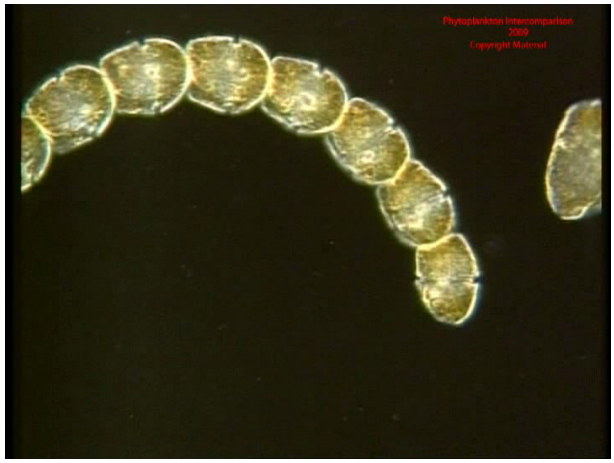
a) gametes fuse becoming an Hypnozygote and then a planozygote
b) vegetative cells fuse becoming an hypnozygote and then a planozygote
c) gametes fuse becoming a planozygote and then a hypnozygote
d) vegetative cells fuse becoming a planozygote and then a hypnozygote
Answer_____

ANALYST CODE:_____
ANALYST SIGNATURE:_____
DATE: _____

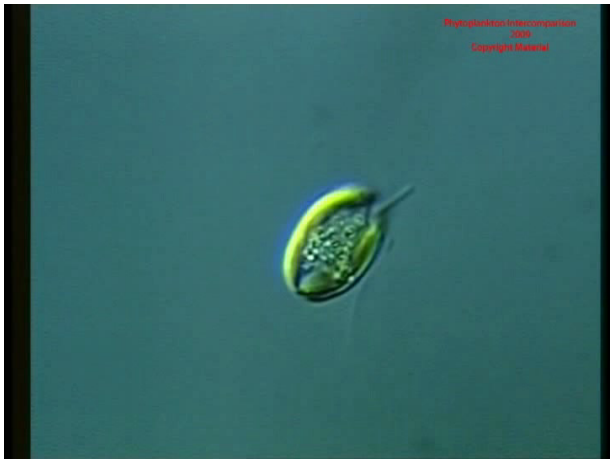
Video sets

SET A (Video 1)

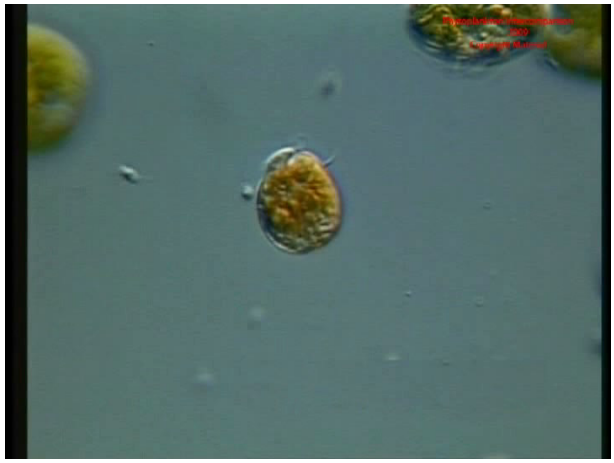
SET A (Video 2)



SET A (Video 3)



SET A (Video 4)

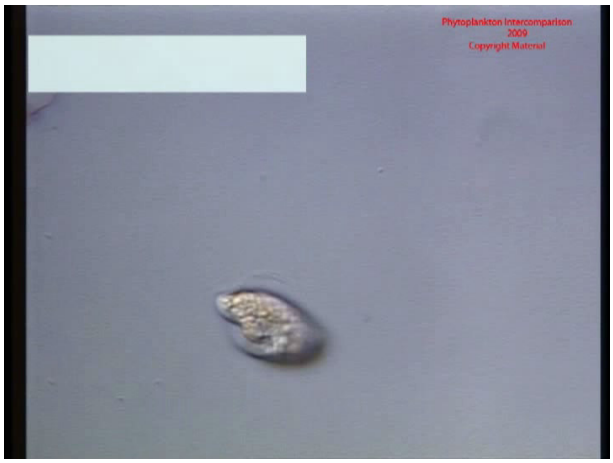


SET A (Video 5)

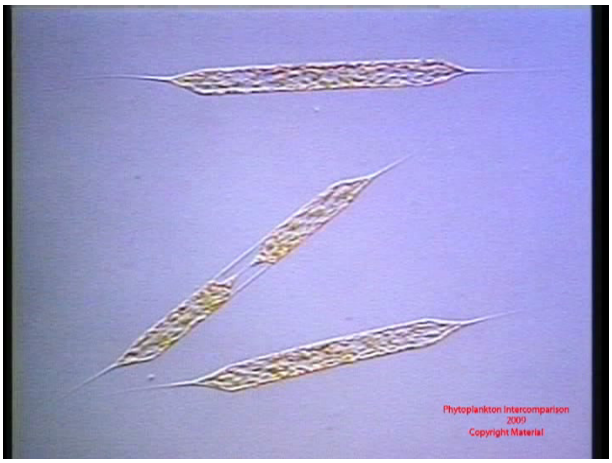
Set B (Video 1)

Set B (Video 2)

Set B (Video 3)

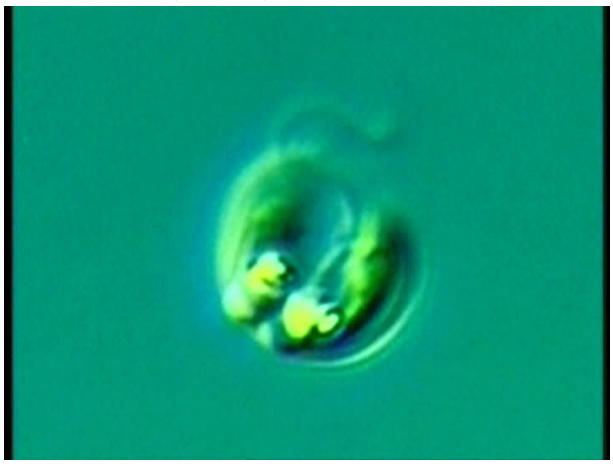


Set B (Video 4)



Set B (Video 5)

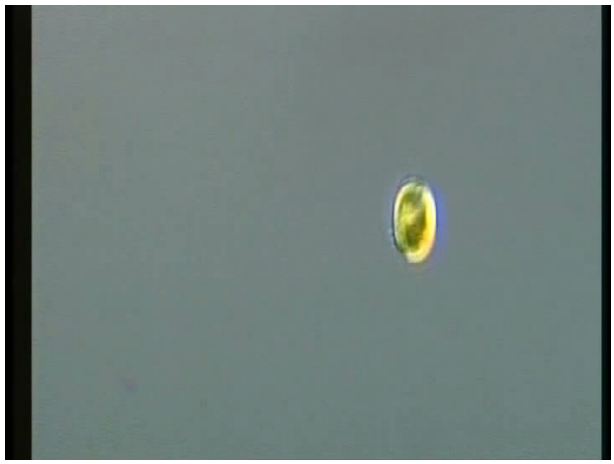
Set C (Video 1)



Set C (Video 2)

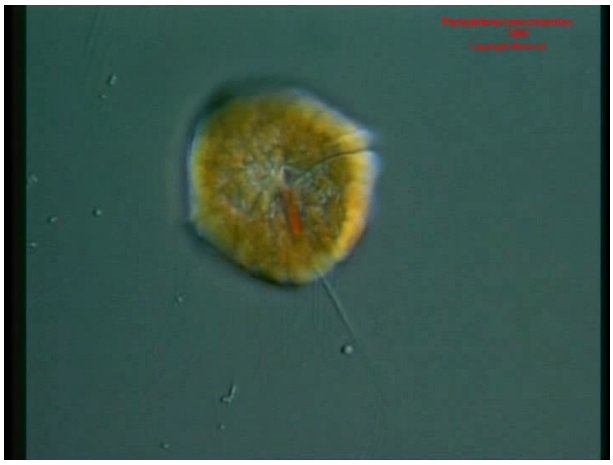
Set C (Video 3)

Set C (Video 4)

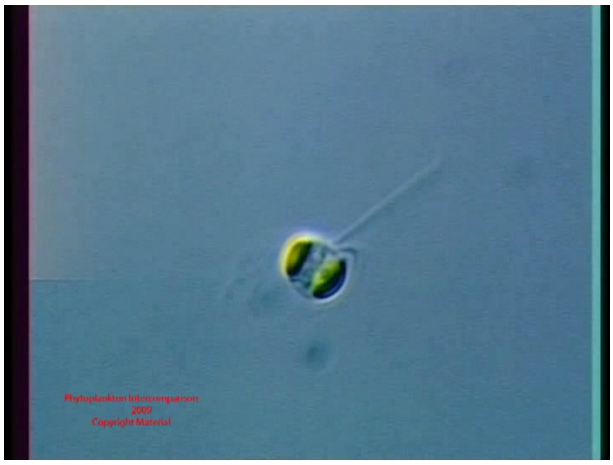


Set C (Video 5)

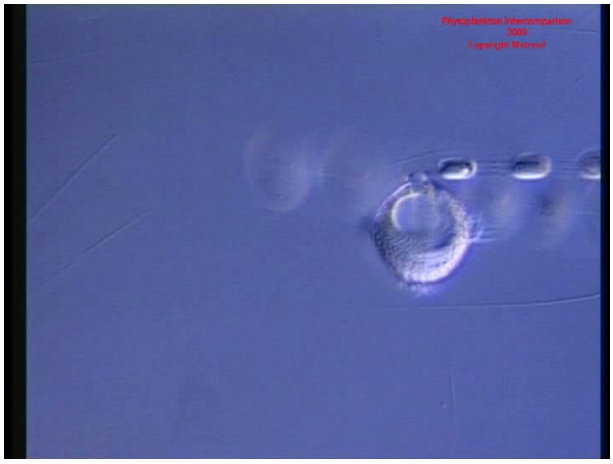
Set D (Video 1)



Set D (Video 2)



Set D (Video 3)



Set D (Video 4)

Set D (Video 5)

Annex VII: FORM 1_Checklist to Fax bequalm 09 MI1.pdf

Bequalm Intercomparison PHY-ICN-09-MI1
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: _____

Annex VIII: 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):				

Annex IX: 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 Utermohl 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.