Marine Institute, Ireland

PHYTOPLANKTON ENUMERATION AND IDENTIFICATION ANALYSIS

Ring Test Round 3 Exercise Report, March 2007

Siobhan Moran, Rafael Gallardo Salas and Joe Silke

Marine Institute
Rinville
Oranmore
Galway
Ireland

Phytoplankton Enumeration and Identification Proficiency Test

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

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1. Summary

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

The purpose of this exercise is to compare the performance of laboratories engaged in national official or non-official phytoplankton monitoring programmes throughout Ireland and the UK. The Marine Institute is accredited to ISO 17025 for Marine phytoplankton identification and enumeration, and recognises that regular Quality Control assessments are crucial to ensure a high standard of data.

In January 2007 an invitation to register for the scheme was issued to laboratories involved in phytoplankton analysis via the BEQUALM website. This included a timetable showing the dates samples would be sent to analysts and expected result dates.

At the beginning of Feb 2007, samples, identification sheets, instructions and results sheets were sent to all analysts who had registered.

Analysts were given until mid February to return results to the MI.

The inter-comparison has results from twenty-one analysts in ten labs throughout Ireland, Northern Ireland, Scotland and England.

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.

3. Participants

In total, twenty-one analysts from ten laboratories participated in the exercise PHY-ICN-07-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 and the Isle of Man. A complete list of the participating laboratories is given in Appendix I.

4. Materials and Methodology

4.1 Phytoplankton samples and micrographs

The inter-comparison exercise is comprised of two parts:

(a) **enumeration of cells** – micro-particles based on latex were used for enumeration, using the Utermöhl method. Initial trials were conducted to determine density of beads, and from this a stock solution was prepared. Further trials were conducted on homogenisation techniques.
A set of two sample bottles was prepared for each analyst.
Once prepared, each set of samples was couriered to the analyst.

(b) **identification of species** - a sheet containing 24 images of Marine phytoplankton species was given to each participating analyst for identification purposes. This was a multiple choice format. The analyst had to choose one of 3 possible answers. A correct identification was given a score of 5 and an incorrect answer was given 0.

4.2 Instructions for counting and identification

Detailed instructions had to be followed for PHY-ICN-07-MI1. These instructions are attached in Appendix II. Samples had to be settled and cells counted according to these instructions. Twenty-four micrographs had to be identified to an appropriate level using multiple choice questions.
All required results had to be returned in the official results sheets.

4.3 Statistical analysis

Independent statistical analysis was carried out by Dr John Newell from the Department of Mathematics at the National University of Ireland, Galway. The approach taken on this intercomparison was to compile the data from the enumeration and identification exercises of the different labs and calculate Z-scores and 95% Confidence limits against a reference or true

value. This reference value was calculated by the Galway lab from a set of 17 samples set up and analysed in the same way as the test samples sent to the participants.

Also a series of typical statistical analysis was carried out to test the data levels of agreement, difference and significance of the variance. Results are found in Appendix 3 and 4..

5. Results and Discussion

As all participants were given detailed instructions in the setting up and analysis of the samples and all the test samples were homogenized and set up in the same manner, the variance between the results should mainly be due to individual factors – such as sample set-up and counting bias in the analysis of the samples.

5.1 Phytoplankton Counts

All enumeration results were collated and different aspects of the data were examined statistically.

a) The Reference Galway data set (Table 1, Appendix 3) was plotted using the Anderson-Darling Normality test. See appendix 3 Graph 1. This test shows that the data suggests symmetry and therefore we can use either the mean or the median as the reference value for the Z-scores, as there is little difference between them. The mean in this case was used to calculate the Z-scores.

b) The 95% confidence intervals taken as twice the standard deviation were plotted using the mean and 2SD from the Galway reference data set. See Appendix 3, Graph 2. This plot shows that all analyst counts (Table 2, Appendix 3) were within the 95% confidence limits.

c) A test for equal variances was used to investigate whether there was a significant difference variance between Galway results and those from the other labs. Graph 3, Appendix 3 shows there is no significant difference between Galway results and the other labs.

d) The final results of all the analysts have been expressed as Z-scores. See Graph 4, Appendix 4. This shows that all results are within 2

standard deviations of the mean. The closest, the value is to zero the better, the agreement with the Galway data set.

5.2 Phytoplankton species identification

All the identification results have been tabulated and scores given for correct identifications,.

24 images were given for identification. 3 answers were given of which one was the correct one. The participants have to tick or circle the answer they thought was the right one

5 marks were given for a correct identification and 0 marks were given for an incorrect one.

The results have been tabulated as an overall percentage of correct answers. see Appendix 4, table 1

Other aspects of the identification exercise were examined. Graph 1 in Appendix 4 shows the number of incorrect answers to specific images. This reveals that 8 analysts named incorrectly image number 3 (38% of analysts), image number 5 was named incorrectly by 4 analysts (19% of analysts) and images 6 and 14 were incorrectly identified by 3 analysts (14% of analysts).

Also, we had a look at the correlation between the 2 skills tested in this exercise: identification and enumeration. See appendix 4, graph 2. This scatter plot shows that the two skills are independent of each other. There is no suggestion that if you are good at one skill you are good at the other, and there is no suggestion of the contrary. Therefore both skills can be studied separately.

5.3 Performance evaluation

In the Enumeration part of the exercise, all the analysts taking part performed to within 2 standard deviations of the mean as set through a reference value from the Galway Dataset.

In the identification part of the exercise, out of 21 analysts taking part in the exercise 17 of those got over 90% mark, 3 analysts got over 85% mark, and 1 analyst got over 70% mark.

This means that 95% of the analysts taking part in the exercise got a score of over 85% of correct identifications.

6. Conclusions and Recommendations

In summary, the enumeration exercise shows that there isn't systematic bias between the Galway Dataset and the counts from all the non-Galway labs. Also, that there is no significant difference in variation between the labs, and that all labs returned counts within 2 standard deviations of the Galway dataset.

The identification exercise shows that 14 out of the 24 images were correctly identified by all analysts, that images number 3,5,6 and 14 were the most difficult to classify and that 95% of the analysts got a mark over 85% of correct answers.

Finally, that there is no correlation between the skills of identifying and enumerating, these are independent skills.

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

On the 5th of March, 2007 the Marine Institute hosted the 2nd workshop of the BEQUALM Phytoplankton ring test round 3. Here, the results of the intercomparison and future directions were discussed.

Some recommendations were put forward by the participants to improve and further enhance this proficiency testing scheme. It was suggested that a study should be designed to find sources of variability, this was in relation to homogenization procedures, counting strategies and other probable sources of bias.

It was also suggested that other materials other than beads could be used for enumeration purposes, as beads do not resemble the way real phytoplankton species look in samples.

One idea that was put forward was the use of standard reference materials. As these standards don't exist at present, it was proposed that some standards could be fabricated using new technologies like micro laser drilling or photo microlithography.

These standards would take the shape of a disc with engraved or drilled figures resembling phytoplankton species. This disc would sit into the sedimentation chamber and be analysed by all the analysts. This way, homogeneity issues wouldn't be a problem and a modal value could be used statistically.

The Marine Institute is progressing these suggestions in preparation for the next exercise which will take place in quarter 3 of 2007.

Appendix I: Participating laboratories

Table showing participating laboratories in the proficiency test PHY-ICN-07-MI1.

Please note that some labs submitted multiple data sets.

Laboratory	Country	No. Of Participants
Marine Institute, Galway	Ireland	3
Environmental Protection Agency, Dublin	Ireland	1
Environment & Heritage Service, Lisburn	N. Ireland	1
AFBI, Belfast	N. Ireland	2
FRS Marine Laboratory, Aberdeen	Scotland	4
SEPA, Riccarton	Scotland	3
SAMS, Oban	Scotland	1
CEFAS Laboratory, Lowestoft	England	4
CEFAS Laboratory, Weymouth	England	1
Department of Local Government and the Environment	Isle of Man	1

Appendix II: Instructions

Marine Institute BEQUALM Phytoplankton Proficiency Test 2007

Instructions for Sample Preparation, Counting, Calculations and Identification

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

1. Introduction

This 3rd Phytoplankton Ring Test is being conducted to determine any inter-laboratory variations for enumeration and identification between labs in Ireland and the United Kingdom. Please adhere to the following instructions strictly. Please note that these instructions are specific for this ring test.

2. Preliminary Check and Time Restrictions

Upon receipt of the samples please make sure that you have received everything listed in the Return Slip form (Form 1). Complete the form 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.

Hard copy results **must be received** by the Marine Institute by **February 20th 2007**. Results will be accepted by e-mail, on the electronic form supplied by the Marine Institute, up to Feb 16th. This is only to facilitate compiling the results. Results have to be validated by hard copy. Hard copy results received after Feb 20th will be not be included in the final report.

3. Equipment

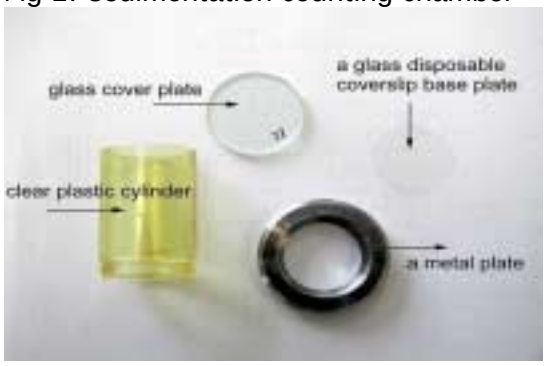
- Two Utermöhl counting chambers. Both 10ml and 25ml chambers can be used.
- Base plates and glass covers.

- Inverted Microscope.

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). Two sets will be required. For this test 10ml or 25ml chambers can be used.

Fig 1: Sedimentation counting chamber



4.1 Place a disposable glass base plate on a cleaned metal plate.

4.2 Screw the plastic cylinder into the metal plate. Extra care should be taken when setting up chambers. Glass 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 **must be tested** for the possibility of leaks by filling the completed chamber with water and allowing it to rest for a few minutes. If no leakage occurs, pour out the water and proceed with the next step. There is no need to dry the chamber.

4.3 To set up a sample for analyses invert the sample tube gently at least five times to ensure that the beads are evenly distributed throughout the sample. Do not shake the tube to avoid air bubbles.

4.3.1 Pour the sample into the counting chamber.

4.3.2 Wash the sample tube with the water provided and pour into the chamber.

4.3.3 Wash the lid of the sample tube also, and pour into the chamber.

4.3.4 Top up the sedimentation chamber with the water provided and cover with a glass cover plate to complete the vacuum and avoid air pockets.

4.3.5 Give the chamber a label corresponding to the label of the sample in question.

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

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

4.6 Use the 20X objective to count the beads.

4.7 Enumeration results for each sample are to be entered on the Results Sheet - **Form 2** E-mail Results Sheet and **Form 3** Enumeration Hardcopy Results Sheet.

5. Counting Strategy

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

5.1 The whole base plate of the chamber is counted by enumerating all beads 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.

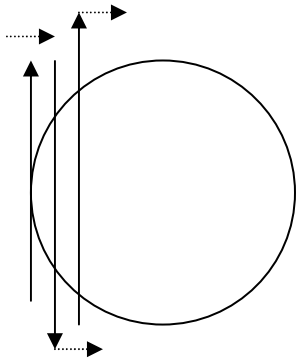


Fig 2

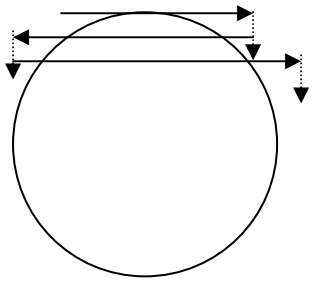


Fig 3

6. Polystyrene Micro-particles

The beads used in this test are micro-particles based on polystyrene. They have a diameter of 30µm.

Before counting for the test, it is very important to become familiar with their structure and size.

6.1 Being spherical, the beads initially may seem difficult to focus. Figures 4 to 6 show how the beads look under three different focal planes.

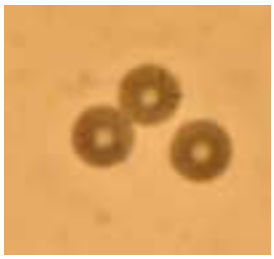


Fig 4

Fig 5

Fig 6

6.2 It is very important to **spend some time** becoming familiar with how the beads appear on the base plate before any count is done as part of the test.

6.3 Please note that although the beads are 30µm diameter, there are some which are slightly smaller (Fig 7). **These should also be counted**.

Fig 7

7. Conversion Calculations of Cell Counts

The number of cells found is converted to cells.L^{-1} .

Please show calculation step in Form 3, section A.

8. Identification

Photographs of cells are on the Identification Sheet (Form 4).

Please identify and include your results on the Identification Sheet (**Form 4**). Results should also be included on **Form 2**: E-mail Results Sheet.

9. Points to Remember:

1. All results must be the analysts **own work**. Conferring with other analysts is **not** allowed.
2. Form 2: E-mail Results Sheet, must be e-mailed to siobhan.moran@marine.ie by Friday Feb 16th 2007.
3. Form 3: Enumeration Hardcopy Results Sheet, and Form 4: Species Identification Sheet must be received by the Marine Institute by Tuesday Feb 20th 2007

Appendix III: Detailed results of the enumeration test

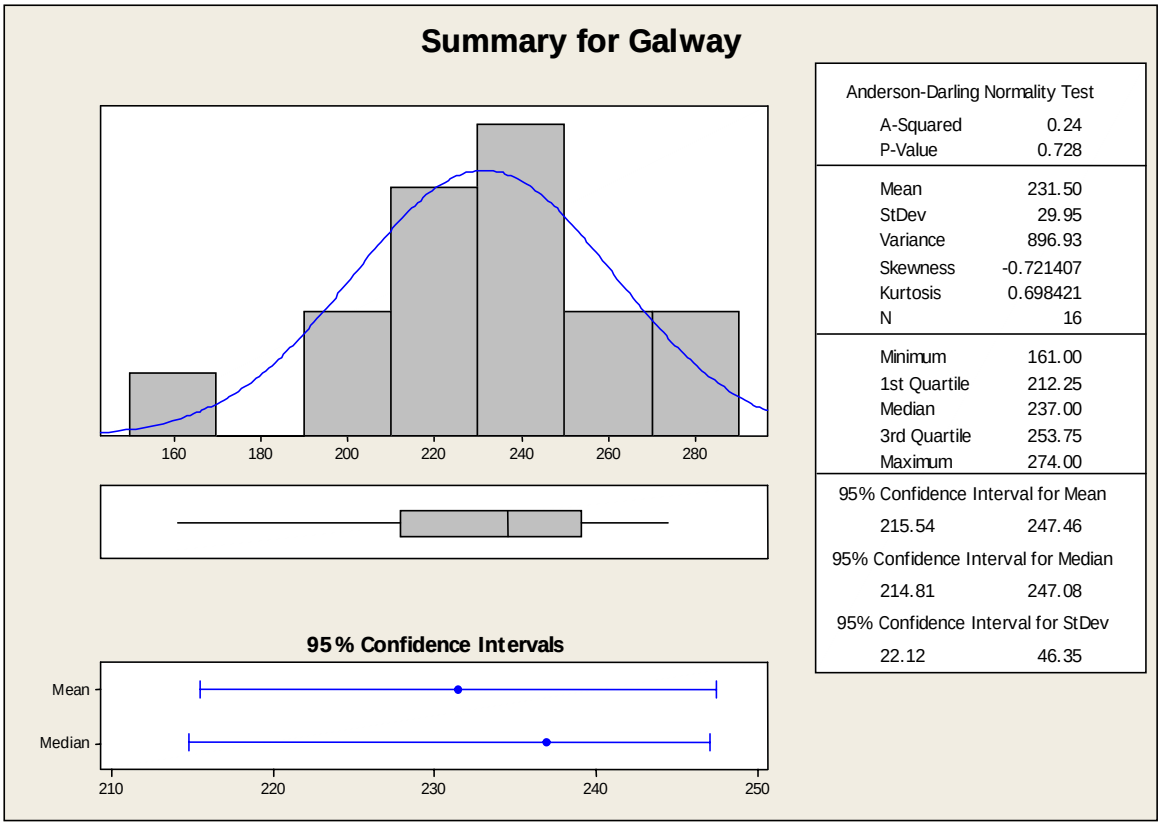
Table 1: Galway Lab Sample dataset count used as reference value

Sample No	Method	Count
40	1	257
57	1	240
17	1	244
5	1	
60	1	274
45	1	260
16	1	273
15	1	244
9	1	227
54	2	224
30	2	241
56	2	161
55	2	216
19	2	208
10	2	234
7	2	190
33	2	211

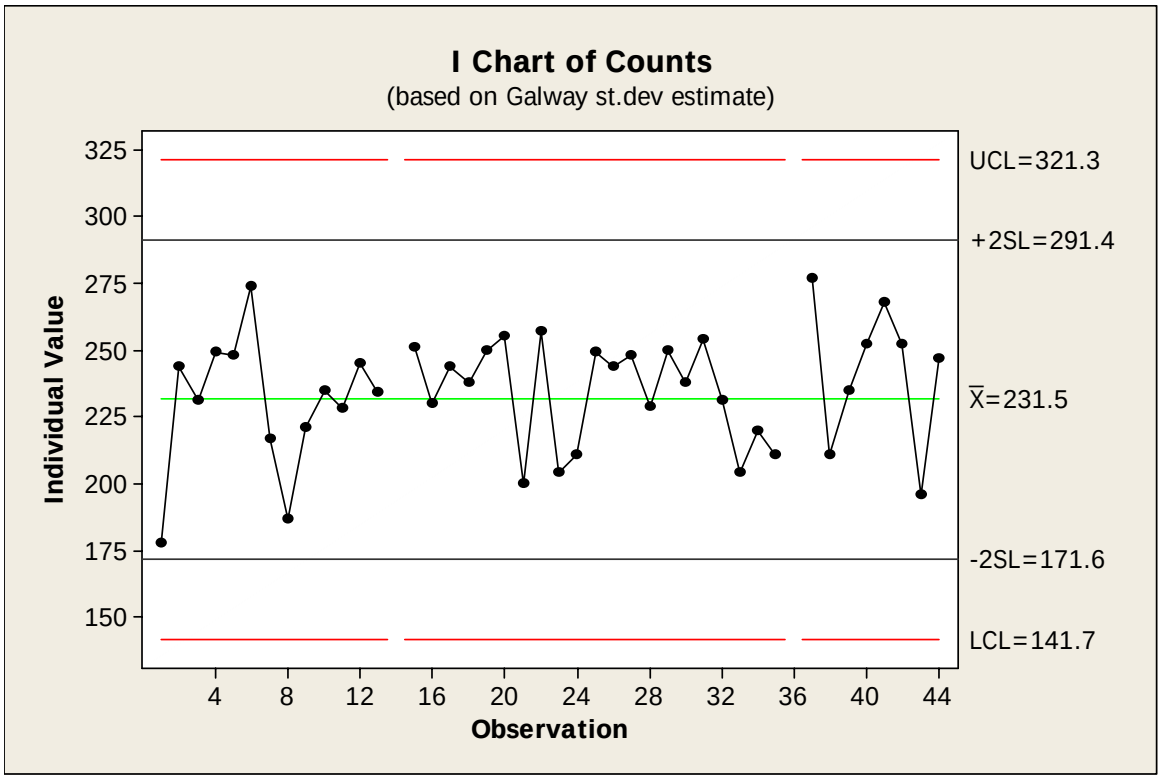
Table 2: Analysts sample counts

ANALYST	Sample A	Sample B
a	244	235
b	245	220
c	200	196
d	187	238
e	228	204
f	251	277
g	274	229
h	235	231
i	248	248
j	221	254
k	238	252
m	255	252
n	249	244
p	250	268
q	230	211
r	234	211
s	217	250
t	244	211
u		
v	178	204
w	231	249
y	257	247

Graph 1: Anderson-Darling Normality Test of Galway Sample set count

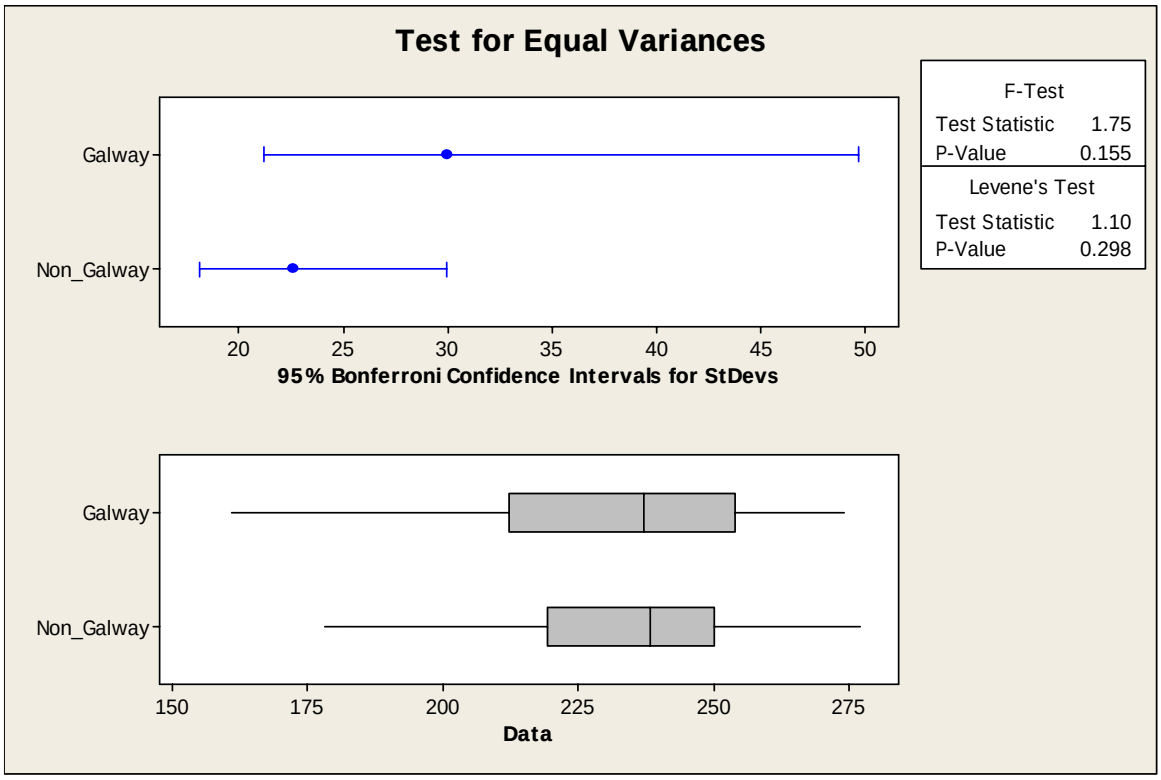


Graph 2: Plot of the individual counts against the reference mean +2SD



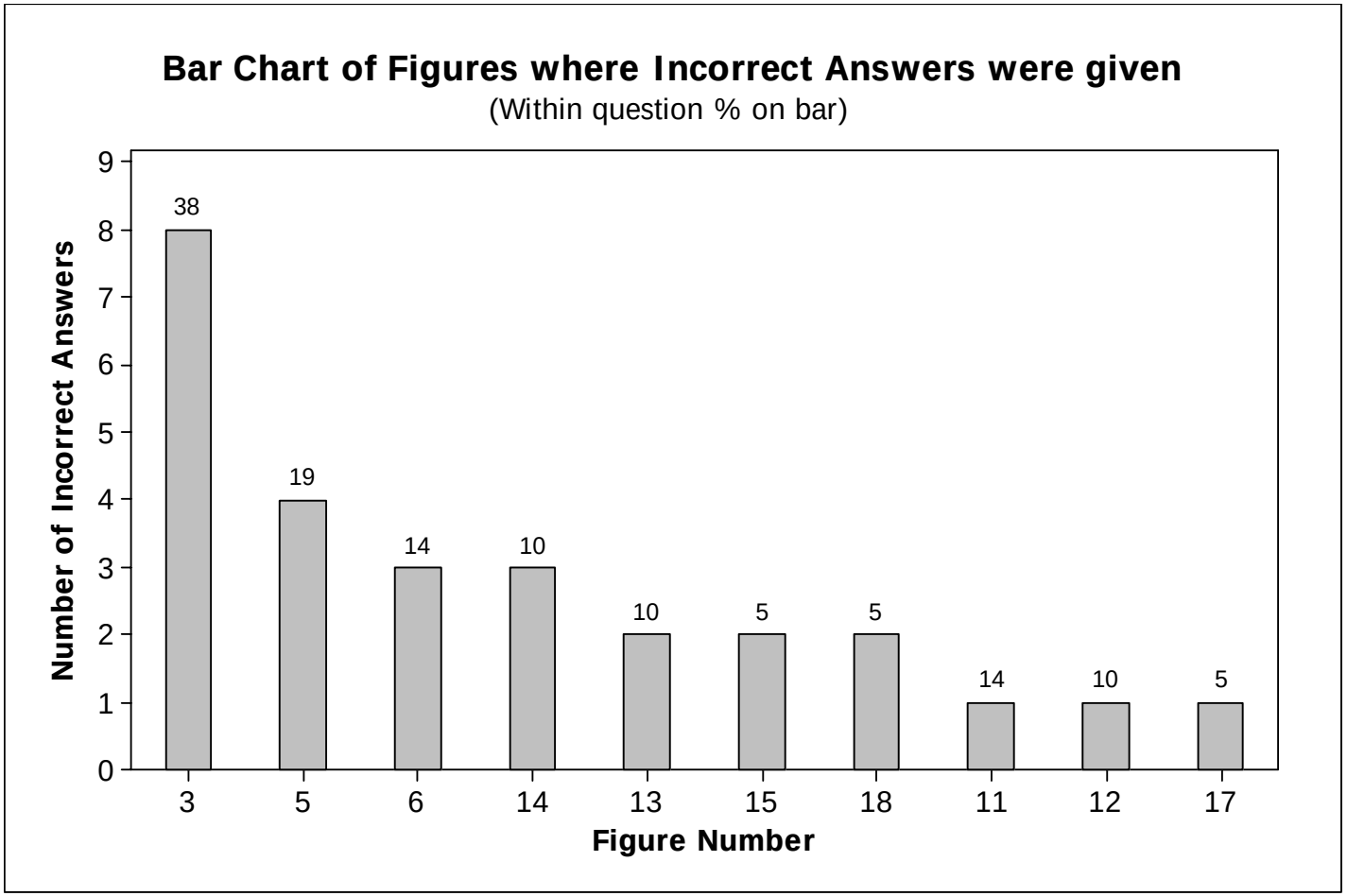
UCL= Upper Confidence Limit LCL= Lower Confidence Limit

Graph 3: Test for equal variances between Galway results and the other labs



Appendix IV: Detailed results of the identification test

GRAPH 1: Number of Incorrect answers in respect to Image numbers



GRAPH 2: Correlation between Identification and enumeration skills

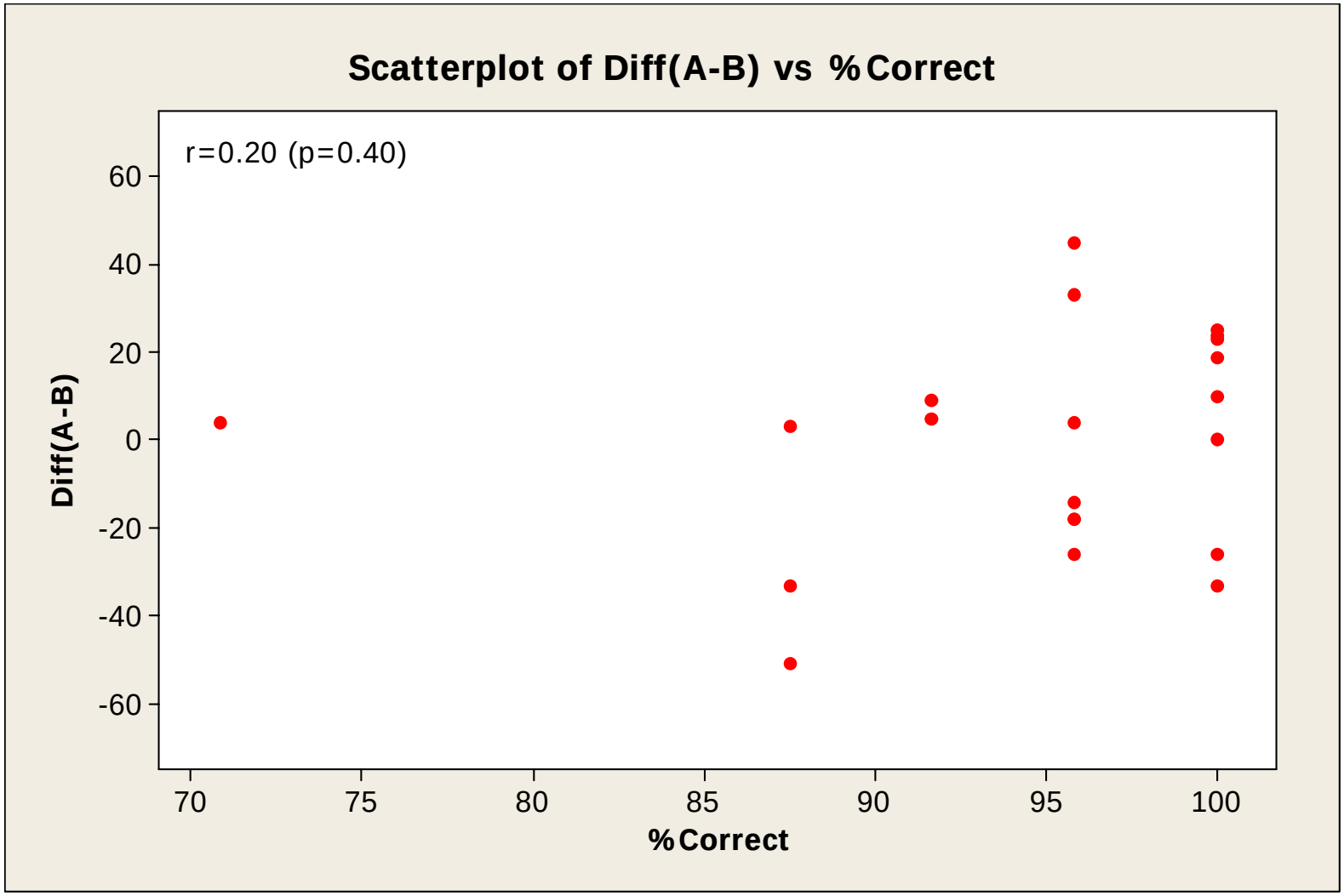
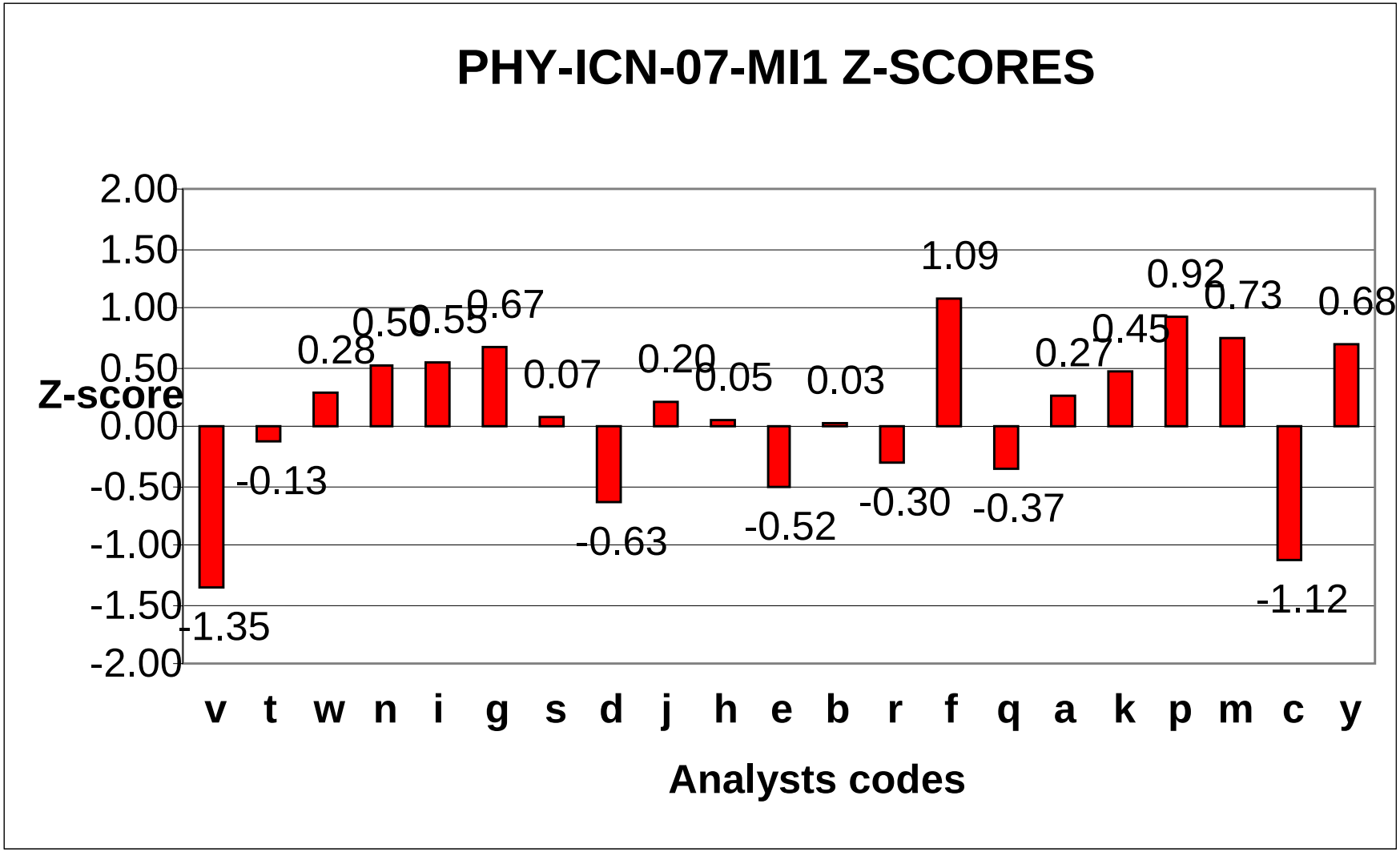


Table 1: Identification results

ANALYST CODE	Fig No:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	Number Correct	Marks	% Score
	Correct Answer	B	C	B	A	A	C	B	C	A	C	A	C	B	A	C	B	A	A	C	C	B	C	A	A	B		
v		B	C	C	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	23	115	96
t		B	C	C	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	23	115	96
w		B	C	C	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	23	115	96
n		B	C	C	A	B	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	22	110	92
i		B	C	B	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	24	120	100
g		B	C	B	A	A	C	B	C	A	C	A	C	B	A	C	B	A	A	C	B	C	A	A	B	23	115	96
s		B	C	B	A	A	C	B	C	A	C	A	C	C	B	C	B	A	A	C	B	C	A	A	B	21	105	88
d		B	C	C	A	C	A	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	21	105	88
j		B	C	B	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	24	120	100
h		B	C	B	A	C	A	B	C	A	C	C	B	B	B	B	B	C	C	C	B	C	A	A	B	17	85	71
e		B	C	B	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	24	120	100
b		B	C	B	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	24	120	100
r		B	C	B	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	24	120	100
u																											0	0
f		B	C	B	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	24	120	100
q		B	C	B	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	24	120	100
a		B	C	C	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	23	115	96
k		B	C	C	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	23	115	96
p		B	C	B	A	A	C	B	C	A	C	A	C	B	B	C	B	A	C	C	B	C	A	A	B	23	115	96
m		B	C	C	A	A	C	B	C	A	C	A	C	A	A	B	B	A	C	C	B	C	A	A	B	21	105	88
c		B	C	B	A	A	A	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	23	115	96
y		B	C	B	A	A	C	B	C	A	C	A	C	B	A	C	B	A	C	C	B	C	A	A	B	24	120	100

Graph 4: Analysts Z-scores



BEQUALM / NATIONAL MARINE BIOLOGICAL ANALYTICAL QUALITY CONTROL SCHEME

BEQUALM / National Marine Biological Analytical Quality Control Scheme
Phytoplankton Ring Trial Exercise 2007

Workshop

Monday 5th March 2007,
Marine Institute Rockall Meeting Room

Agenda

10:00 Introductions / Welcome

10:15 Development of Phytoplankton Intercomparison

Methods and Materials supplied
A: Identification exercise
B: Enumeration exercise
Lessons Learned / Discussion

11:00 Coffee Break

11:30 Results Submitted

12:00 Discussion

12:30 Lab Tour

13::00 Lunch in Marine Institute Restaurant

14:00 The Genus Alexandrium (Nicolas Touzet)

14:45 The Statistical analysis of Intercomparison Exercise (John Newell)

15:30 Coffee

16:00 Discussion / Plans for 2007 and future of Phytoplankton intercomparisons

Form 4: Species Identification Sheet and Results Sheet

Please circle the relevant answer.
Identification aids can be used. Analysts can not confer with other analysts.

There is only **one correct** answer per photo.

Fig 1

Fig 1:	
A	*Gymnodinium spp.*
B	*Akashiwo sanguinea*
C	*Gyrodinium spirale*

Fig 2

Fig 2:	
A	*Kofoidinium velloides*
B	*Pronoctiluca spp.*
C	*Noctiluca scintillans*

Fig 3

Fig 3:	
A	*Ceratium azoricum*
B	*Ceratium tripos*
C	*Ceratium symmetricum*

Fig 4

Fig 4:	
A	*Ceratium platycorne*
B	*Ceratium compressum*
C	*Ceratium tripos*

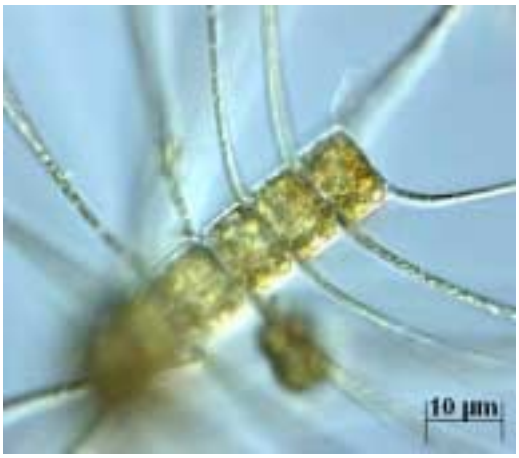


Fig 5

Fig 5: This is a Chaetocerous spp. Does it belong to the	
A	Phaeocerous group
B	Hyalochaete group
C	Bacteriastrum group

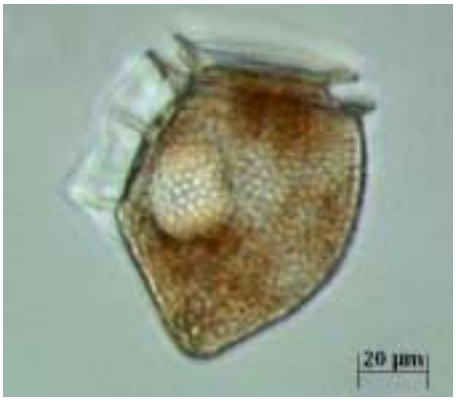


Fig 6

Fig 6:	
A	*Dinophysis acuta*
B	*Dinophysis acuminata*
C	*Dinophysis norvegica*

Fig 7

Fig 7: What genus does this armoured dinoflagellate belong to?	
A	*Gonyaulux spp.*
B	*Alexandrium spp.*
C	*Gymnodinium spp.*

Fig 8

Fig 8:	
A	*Amphidinium pellucidum*
B	*Amphidinium micrum*
C	*Amphidinium carterae*

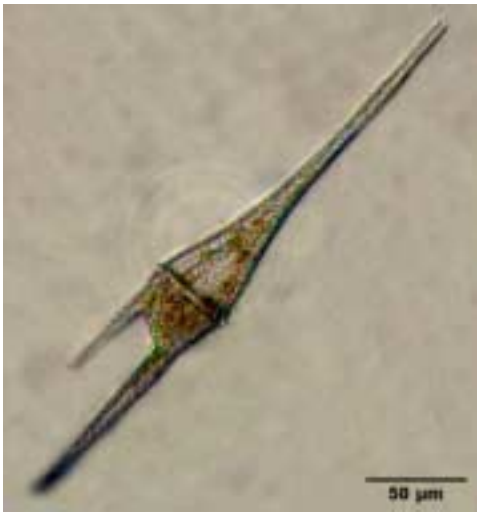


Fig 9

Fig 9:	
A	*Ceratium furca*
B	*Ceratium lineatum*
C	*Ceratium pentagonum*

Fig 10

Fig 10:	
A	*Ceratium inflatum*
B	*Ceratium extensum*
C	*Ceratium fusus*

Fig 11

Fig 11:	
A	*Protoperidinium bipes (Minuscula bipes)*
B	*Protoperidinium brevipes*
C	*Protoperidinium granii*

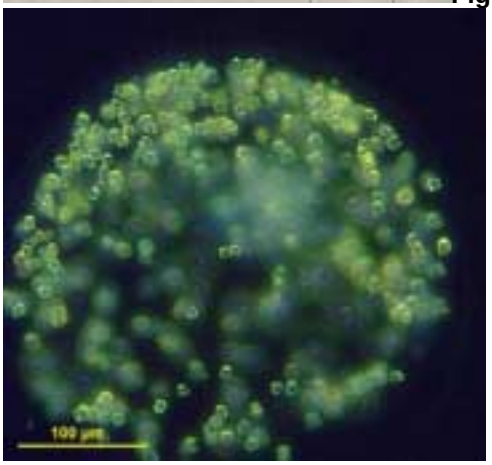


Fig 12A

Fig 12B

Fig 12A & 12B: What is this colony forming species?	
A	*Corymbellus aureus*
B	*Prymesium parvum*
C	Phaeocystis spp.

____50µm Fig 13

Fig 13:	
A	*Ceratium longipes*
B	*Ceratium horridum*
C	*Ceratium macroceros*

____8µm **Fig 14**

Fig 14:	
A	*Gonyaulax verior*
B	*Gonyaulax digitale*
C	*Gonyaulax spinifera*

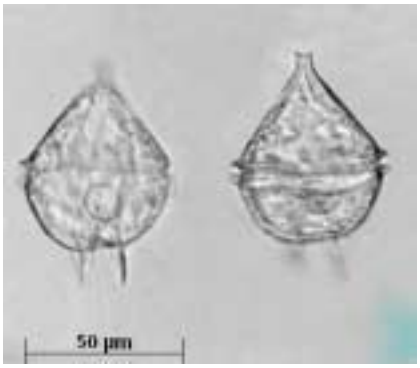


Fig 15

Fig 15:	
A	*Protoperidinium breve*
B	*Protoperidinium steinii*
C	*Protoperidinium pyriforme*

Fig 16

Fig 16:	
A	*Ceratium azoricum*
B	*Ceratium tripos*
C	*Ceratium symmetricum*

Fig 17

Fig 17:	
A	*Chaetocerous danicus*
B	*Chaetocerous convolutus*
C	*Chaetocerous densus*

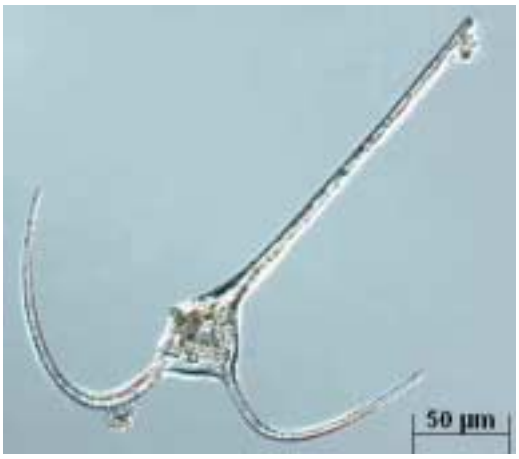
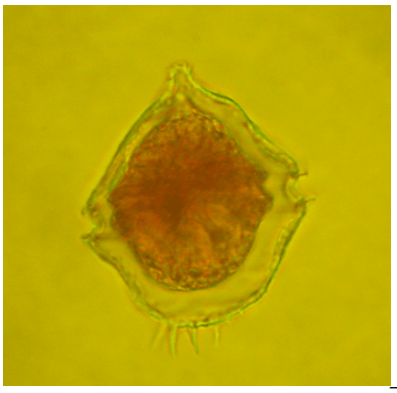


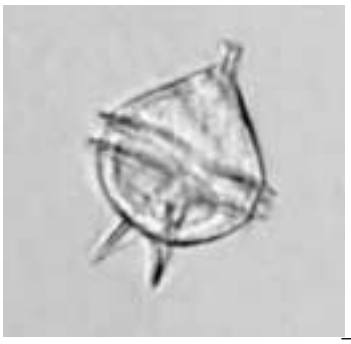
Fig 18

Fig 18:	
A	*Ceratium longipes*
B	*Ceratium horridum*
C	*Ceratium macroceros*



10µm **Fig 19**

Fig 19:	
A	*Gonyaulax verior*
B	*Gonyaulax digitale*
C	*Gonyaulax spinifera*

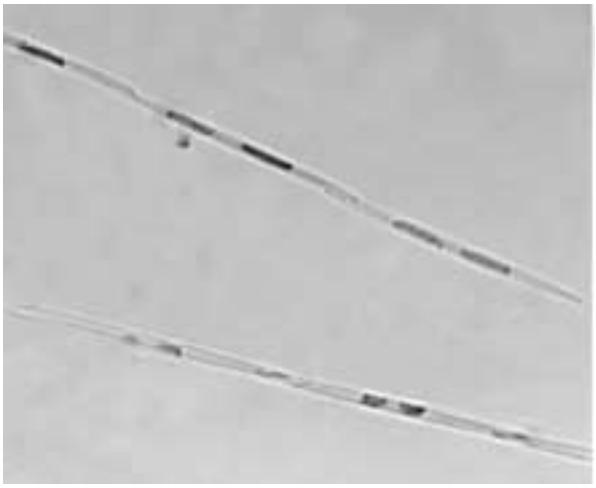


10µm **Fig 20**

Fig 20:	
A	*Protoperidinium mite*
B	*Protoperidinium steinii*
C	*Protoperidinium pyriforme*

Fig 21

Fig 21:	
A	*Ceratium minutum*
B	*Ceratium lineatum*
C	*Ceratium pentagonum*



_____10µm

Fig 22

Fig 22:	
A	*Pseudo-nitzschia delicitissima group*
B	*Pseudo-nitzschia seriata group*
C	*Nitzschia spp.*

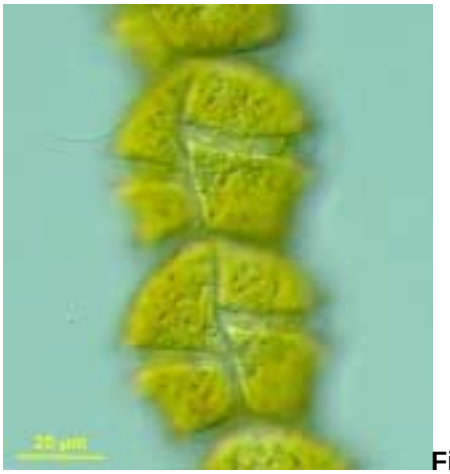


Fig 23

Fig 23:	
A	*Gymnodinium catenatum*
B	*Alexandrium tamerense*
C	*Alexandrium ostenfeldii*

Fig 24

Fig 24:	
A	*Ceratium furca*
B	*Ceratium lineatum*
C	*Ceratium pentagonum*

PLEASE NOTE:
Please circle the relevant answer. Identification aids can be used.
There is only **one correct** answer per photo.

I declare that the above identifications are my own work, and I have not conferred with any other person.

Signed: _____

Analyst Code No:_____

Date: _____