

**ASSESSMENT OF THE FEASIBILITY  
OF USING IMAGE ANALYSIS IN  
THE OYSTER EMBRYO-LARVAL  
DEVELOPMENT AND *DAPHNIA*  
*MAGNA* GROWTH TESTS**

**WRc plc.**

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## SUMMARY

At present the majority of ecotoxicity tests involve some form of visual assessment to be made during or at the end of the test, for the determination of the endpoint. These assessments may require the correct recognition and enumeration of different stages of development of the test organism, a measurement of size (such as area or length) or numbers, or whether or not the organism is motile after given exposure periods.

These visual assessments are, inevitably, judgmental and may be time consuming. Adoption of the latest electronic imaging technology is expected to benefit many of these tests technically, by improvements in accuracy, reproducibility and lower error rates; and in terms of reduced costs because of improved processing rates. Further benefits may be accrued in areas in which electronic imaging becomes an enabling technology: permitting procedures or tests which require a non-invasive approach or which cannot be performed manually. Image analysis may also facilitate measurements during the time course of a response which could not easily and/or cost-effectively be obtained under other circumstances.

However, there remain a number of practical issues which must be addressed before electronic imaging techniques can be applied to ecotoxicity testing. The aims of the study were to address the former issues by applying the latest image capture, processing and analysis techniques to two ecotoxicity tests: the oyster embryo-larval development test and the *Daphnia magna* growth test integrated with measurements of immobilisation and juvenile production which, between them, encompass most of the required assessments.

The principal objectives of the study were to:

- test the application of image analysis to the oyster embryo-larval development test and quantify the technical and cost benefits;
- establish the feasibility of incorporating a growth test endpoint, based on non-invasive measurements, into an integrated *Daphnia magna* immobilisation and juvenile production test;
- ascertain the constraints imposed on electronic imaging techniques by current methodologies.

On the basis of the zinc reference toxicant test data derived in this study for each type of method the following conclusions can be drawn:

- the OEL test can be effectively carried out in multiwell plates which provides the opportunity to count objects using image analysis software;
- the use of the image analysis parameters area and size (length) in combination results in mean control abnormalities and  $EC_{50}$  values which are not significantly different statistically from corresponding values derived using conventional visual observations. Discrimination using the area and length parameters may be improved by the inclusion of other parameters into a measurement 'macro' which would eliminate extraneous material or lighting artefacts;

- the use of multiwell plates and image analysis can eliminate the variability associated with sub-sampling and inter-operator differences in the counts of D and non-D larvae. Image analysis also opens up the possibility of assessing egg quality at the start of the test by comparing measured values for parameters such as length and area against defined acceptability criteria. It also allows initial inoculum number to be determined as a quality check of the numbers of objects measured at the end of the test;
- in terms of cost-benefit using image analysis the current system only resulted in a small time saving. However, there could be major saving in staff time if a partially or fully automated X-Y stage was added to the system and test replicates could be counted without the requirement for the presence of a human operator.
- growth of *D.magna*, as measured by image analysis, can be a sensitive sub-lethal indicator of toxicity and can be integrated with existing 48 h immobilisation and a short-term or 21 d juvenile production endpoints in a single test procedure. This would result in a more cost-effective test method which would yield greater amounts of information on the toxicity of test substances than the existing approaches and would remove the need to conduct a suite of tests, thereby removing inter-test variability as a problem in data interpretation.

## 1. INTRODUCTION

At present the majority of ecotoxicity tests involve some form of visual assessment to be made throughout the course of the test or at the end of the test, for the determination of the endpoint. These assessments may require a) the correct recognition and enumeration of different stages in the development of the test organism (for example the oyster embryo-larval development test, or the *Daphnia* reproduction test), b) the measurement of size, for example area (the *Lemna* growth inhibition test) or length (the larval fish growth test) or numbers (in the *Daphnia magna* or *Tisbe battagliai* reproduction test) or c) the motility of test organisms (for example the *Daphnia magna* immobilisation test).

These visual assessments are, inevitably, judgmental and may be time consuming. Adoption of the latest electronic imaging technology is expected to benefit many of these tests technically, by improvements in accuracy (because it is more objective), reproducibility and lower error rates; and in terms of reduced costs because of improved processing rates. Further benefits may be accrued in areas in which electronic imaging becomes an enabling technology: permitting procedures or tests which require a non-invasive approach or which cannot be done manually (for example measurements of size in the *Daphnia magna* growth test). Image analysis may also facilitate or make cost-effective the measurements of endpoints during the time course of experiments (for example time-to-event analysis), thereby enhancing the information derived from a test.

However, there remain a number of practical issues which must be addressed before electronic imaging techniques can be applied to ecotoxicity testing. Some issues are concerned with the technical capability of the apparatus and software itself to discriminate between different forms of test organisms, or to make accurate measurements from the images obtained. Other issues concern the amenability of the existing ecotoxicity test systems towards the application of such electronic imaging techniques.

This study aims to address the former issues by applying the latest image capture, processing and analysis techniques to two ecotoxicity tests: the oyster embryo-larval development test and the *Daphnia magna* growth test integrated with measurements of immobilisation and juvenile production which, between them, encompass most of the required assessments. In the oyster embryo-larval development test the imaging system will be required to discriminate between different forms of microscopic organisms with the objective of classifying and enumerating them. This test serves, therefore, as an example of a widely used and time consuming method for which electronic imaging techniques might offer significant cost savings in addition to improved determination of the endpoint. Furthermore, the introduction of the objective approach used for the oyster embryo-larval test could be transferred to other methods such as the *Daphnia magna* and *Tisbe battagliai* reproduction test, where microscopic larval stages have to be discriminated from adults and other material. In the *Daphnia magna* growth test the imaging system will be required to define and measure macroscopic animals. This serves as an example of a test for which electronic imaging becomes the enabling technology, permitting non-invasive determination of this endpoint along with the subsequent measurement of juvenile production. However, it is important to recognise that the application of image analysis to the tests described in this study does not represent the extent of the potential utility of the approach within the field of ecotoxicology.



A recent demonstration given by Data Cell Limited at WRc Medmenham has shown the broad potential of the Image-Pro Plus image processing package to this application. The electronic imaging apparatus used in the study was generously provided by Data Cell Limited (Blackbushe Business Park, Saxony Way, Yateley, Hampshire, GU46 6GB).

Initially the extent to which imaging techniques are constrained by the existing test systems (for example optical aberrations in images obtained from glass beakers or large depth of field) will be investigated.

For the oyster embryo-larval development test the performance of the imaging system will be compared with the method defined in the DTA Ecotoxicology Methods Guidelines (Environment Agency 1997). Measurements made in the *Daphnia* growth test will be compared with those obtained in Johnson and Delaney (1998).

### **1.1 Objectives**

The principal objectives of the proposed study are to:

- test the application of image analysis to the oyster embryo-larval development test and quantify the technical and cost-benefits;
- establish the feasibility of incorporating a growth test endpoint based on non-invasive measurements into the *Daphnia magna* juvenile production test;
- ascertain the constraints imposed on electronic imaging techniques by current methodologies.

### **1.2 Benefits**

The principal benefits of the proposed study will be to:

- establish the feasibility of performing the oyster embryo-larval development test more quickly, with reduced error and greater accuracy and precision;
- assess the potential of electronic imaging techniques as an 'enabling technology' which promotes the development of new test procedures such as the *Daphnia* growth test, but which may also be applied to established tests in the determination of intermediate endpoints.

### **1.3 Limitations of the Study**

The inherent limitations of this study stem from the fact that only two ecotoxicity tests will be considered. Given that some other tests may pose their own unique and particular problems it will not be possible to deduce with certainty whether or not electronic imaging technology would be beneficial to all such tests.

## 2. PRELIMINARY DEVELOPMENT WORK FOR THE OYSTER EMBRYO LARVAL DEVELOPMENT TEST

### 2.1 Introduction

The oyster embryo-larval development (OEL) test assesses the effects of environmental samples on the development of oyster larvae over a 24 h period during which naked embryos would normally be transformed into larvae having a protective 'D' shaped shell where the paired hinged shells are visible. Although the exposure time is short, it encompasses a period of intense cellular activity, in which the impairment of a number of critical physiological and biochemical processes may result in poor growth and development. Abnormal development is characterised by embryos which die at an early stage or larvae which are developing but fail to reach the D stage. The protocol for the conduct of the OEL test (ICES 1991) is shown in Figure 2.1.

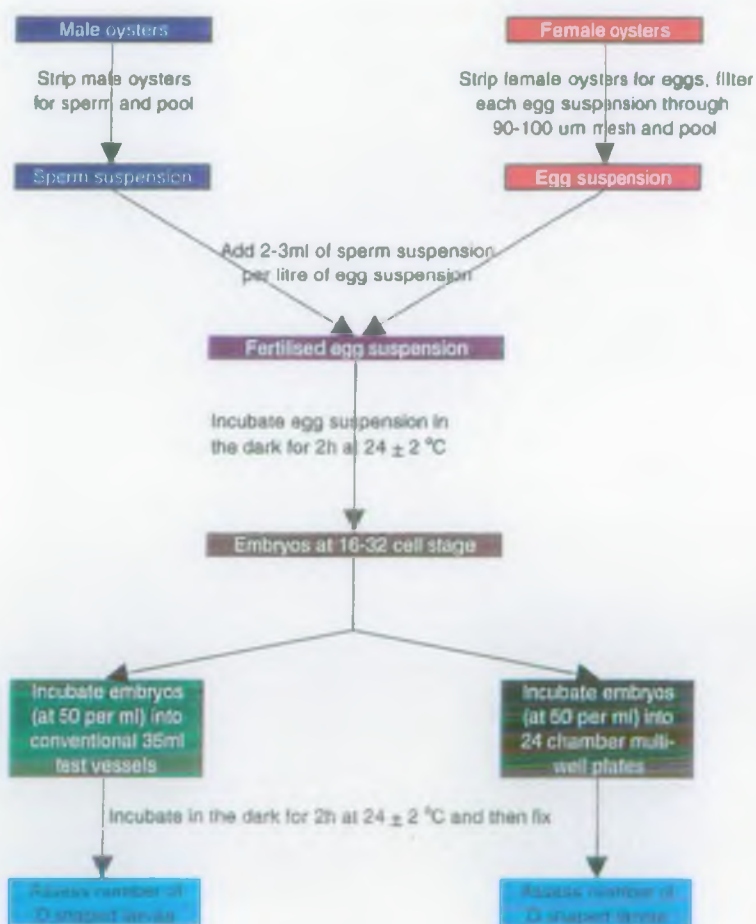


Figure 2.1 Summary of the oyster embryo-larval test procedure used in the study

However, the current procedure for conducting the oyster embryo-larval development test presented a number of issues which needed to be addressed before the image analysis technique could be used. Firstly it was necessary to establish the feasibility of conducting the OEL test in multiwell plates since it was not practical or cost-effective to use the current test vessels. The use of conventional 35 ml test vessels (with 30 ml of test solution) would require sub-sampling whereas the conduct of the test in the multiwell plates would allow the automation of the counting procedure using the image analysis software and a computer driven X-Y stage. Secondly it was necessary to identify a useful subset of the complete set of image parameters which would provide reliable and non-redundant data.

## 2.2 Image analysis equipment

The image analysis hardware (ELONEX 586 PC with SNAPPER 8 frame grabber card and Pulnix TM 1001 CCD camera) and software (Image Pro Plus) used to assess responses in the tests are shown in Figure 2.2.



**Figure 2.2** Photograph of the image analysis apparatus



The Image Pro Plus software is a Windows and Power Macintosh based application which is capable of taking information obtained by the frame grabber card and processing this in a variety of ways. Further information on the software is given in Appendix A. Additional details on the use of the equipment for the different tests is given in the relevant section of the report and in Appendix B.

### 2.3 Comparison of test results using conventional test vessels and multiwell plates

A series of four tests with the reference toxicant zinc were conducted in which responses observed in a conventional test using 35 ml vessels were compared with those found in multiwell plates (see Table 2.1). The embryos inoculated into the conventional test vessels and the multiwell plates were from the same pool on each test occasion, thereby eliminating the source of test organisms as a cause of test variability.

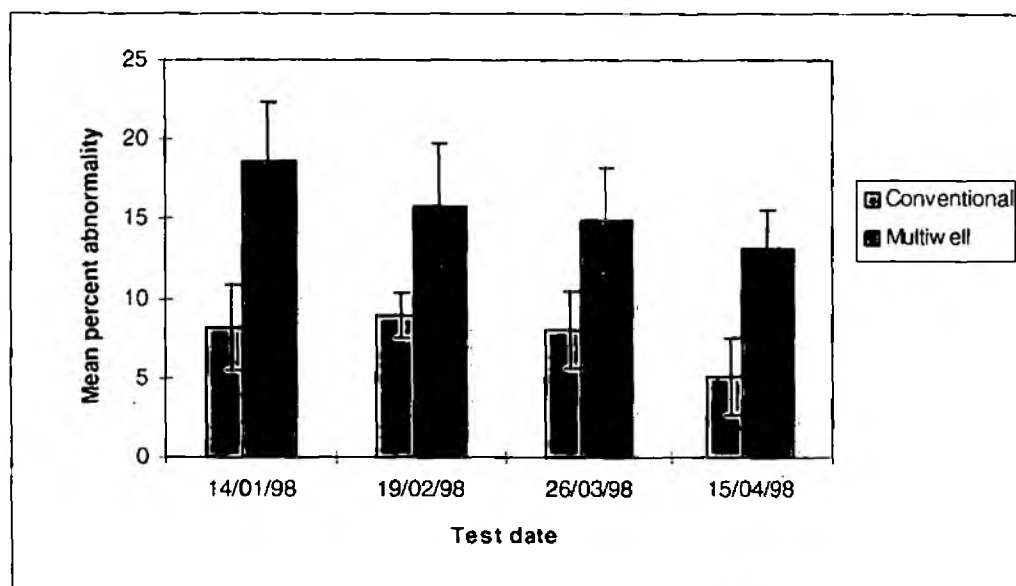
**Table 2.1 Summary of the tests carried out with the reference toxicant zinc**

Test date	Zinc concentration range (mg l <sup>-1</sup> ) used for conventional and multiwell plate tests
14.1.98	0 (Control), 0.032, 0.1, 0.32, 1.0 and 3.2
19.2.98	as above
26.3.98	0 (Control), 0.032, 0.1, 0.21, 0.32, 0.66, 1.0 and 3.2
15.4.98	as above

For each test the number of D and non-D shaped larvae in each replicate of an exposure concentration were counted visually using a Nikon Diaphot-TMD microscope. The proportions of abnormal larvae at each test concentration were then used to determine the EC<sub>50</sub> values.

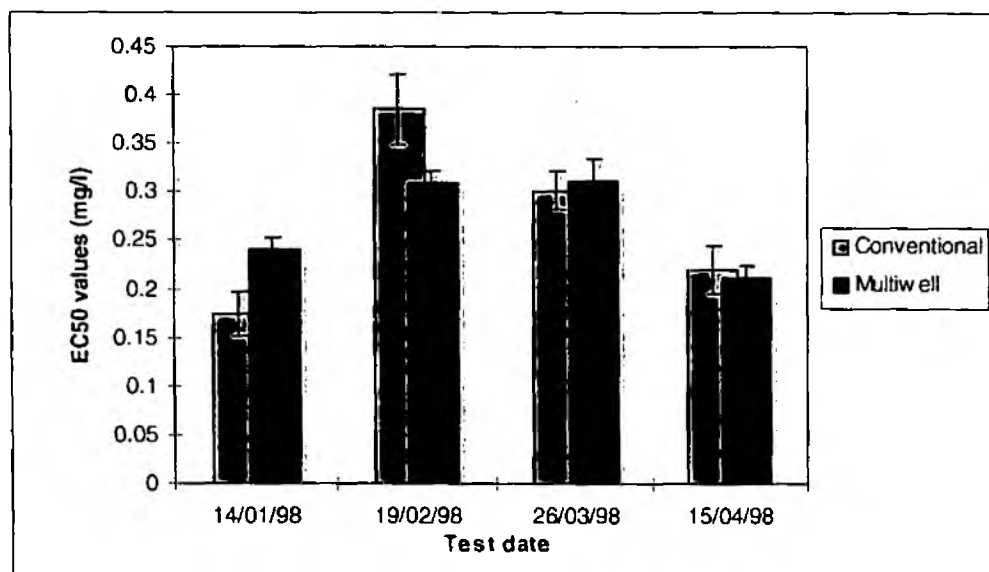
Figures 2.3 and 2.4 summarise the results for the tests carried out with the reference toxicant zinc in conventional 35 ml volume test vessels and also in 3 ml volume multiwell plates. The level of control abnormality was <20% for both procedures (Figure 2.3) and was markedly less than the 40% test validity criterion specified in the ICES test method. Typically the mean percent abnormality measured in the multiwell test vessels was in the range of 10-20%, which is consistent with historical levels in the conventional test controls. However, the mean level of abnormalities in the controls in the conventional test procedures in this study (<10% on all occasions) were lower than the average level found on previous occasions.

Two-way analysis of variance (ANOVA) showed that there was a statistically significant difference between levels of control abnormality in tests carried out in conventional test vessels and multiwell plates ( $F=92.9$ ,  $P<0.001$ ) with higher, but acceptable, values being found in the multiwell plates. There was also a statistically significant temporal effect in the level of control abnormalities in both test systems ( $F=4.54$ ,  $P=0.008$ ), that is the values varied over time. For the multiwell plates the mean level of abnormality decreased from 19% to 13% as the study progressed.



**Figure 2.3 Mean percent abnormality in the controls of OEL tests carried out in conventional vessels and multiwell plates**

In Figure 2.4 it is evident that the  $EC_{50}$  values derived using the conventional and multiwell test procedures showed differences of 25 to 39% on the first two occasions (14/1/98 and 19/2/98). However, on the third and fourth test occasions (26/3/98 and 15/4/98) the two procedures produced highly comparable  $EC_{50}$  values which only differed by 5%.



**Figure 2.4 Toxicity values for OEL tests with zinc carried out in conventional vessels and multiwell plates**

Two-way ANOVA showed that there was no statistically significant difference between the  $EC_{50}$  values derived for the tests carried out in conventional test vessels and multiwell plates ( $F=0.0045$ ,  $P>0.05$ ). Although the  $EC_{50}$  values derived by each system varied over time, this pattern was not statistically significant ( $F=5.33$ ,  $P>0.05$ ).

On the basis of the data generated for zinc reference toxicant tests it was concluded that the OEL method could be successfully carried out in the multiwell plates and would generate comparable results to those obtained with the conventional test procedure. The conduct of OEL tests in multiwell plates has significant advantages over the current glass vessels in terms of the use of image analysis since it obviates the need for sub-sampling and opens up the possibility of automating the measurement process.

#### **2.4     Assessment of the feasibility of using image analysis in the OEL test**

Table 2.2 summarises the different parameters of an object which could be measured using the Image Pro Plus software. In the case of the OEL test the 31 potential candidate measurement parameters were reduced down to 7 which had the greatest relevance to the circular (eggs) and D-shaped objects which were to be measured in the test. These eight parameters are identified in Table 2.2 with an asterix.

Since the study used a monochrome camera, the colour dependent density (blue, green and red) parameters were not appropriate. In addition, the objects do not have holes, therefore, the holes, hole area and hole ratio parameters were not appropriate. Because of the shape of the oyster larvae the size (length) and size (width) parameters were used and were considered more appropriate than axis (minor or major), radius (minimum or maximum) or diameter (average, minimum or maximum).

**Table 2.2 Measurement parameters available in the Image Pro Plus software**

Parameter	Description
Angle	Angle between the major axis of the object and the vertical
Area *	Area of the object
Area/Box	Ratio between the area of the object and the area of its boundary box
Aspect *	Ratio between the major axis and minor axis of an ellipse equivalent to the object
Axis (major)	Length of the major axis of an ellipse with the same moments of order 0, 1, 2 as the object
Axis (minor)	Length of the minor axis of an ellipse with the same moments of order 0, 1, 2 as the object
Box X/Y	Ratio between the width and height of the objects bounding box
Centre X	X coordinate of the object's centroid
Centre Y	Y coordinate of the object's centroid
Class	Class to which the object belongs
Cluster	Estimated number of individual objects contained within the outline
Density - Blue	Objects mean blue value (for colour images only)
Density - Green	Objects mean green value (for colour images only)
Density - Lum	Average optical density (or intensity) of object
Density - Red	Objects mean red value (for colour images only)
Diameter (Ave)	Average length of diameter measured at 2 degree intervals and passing through object's centroid
Diameter (Max)	Length of longest line joining two points of objects outline and passing through the centroid
Diameter (Min)	Length of shortest line joining two points of objects outline and passing through the centroid
Hole area	Area of holes within the object
Hole ratio	Ratio of object area excluding holes to total area of object
Holes	Number of holes within the object
IOD	Integrated Optical Density (or integral) = Area x Average density (or intensity)
Per Area	Ratio of area of object to total area of image or ADI
Perimeter *	Length of objects outline
Perimeter <sup>2</sup>	Chain code length of the outline including any hole outlines
Radius (max)	Maximum distance between objects centroid and outline
Radius (min)	Minimum distance between objects centroid and outline
Radius ratio *	Ratio between maximum radius and minimum radius
Roundness *	$(\text{Perimeter})^2 / 4\pi \times \text{area}$
Size (length) *	Feret diameter (that is caliper length) along major axis of object
Size (width) *	Feret diameter (that is caliper length) along minor axis of object

Initially groups of D larvae were measured using the 7 identified parameters to provide an indication of the range of values which could be expected for each parameter. Table 2.3 summarises the minimum and maximum values for each parameter measured in a group of fifty D larvae for each of the measurement parameters (see Table C1 in Appendix C). This data was used to define the upper and lower boundaries of what constituted D larvae for each of the different measurement parameters.

**Table 2.3** Maximum and minimum values recorded for D larvae for each measurement parameter

Parameter	Minimum value for D larvae	Maximum value for D larvae
Area	2960	4922
Aspect ratio	1.06	1.37
Perimeter	192	238
Radius ratio	1.34	1.87
Roundness	1.00	1.06
Size (length)	63	86
Size (width)	54	70

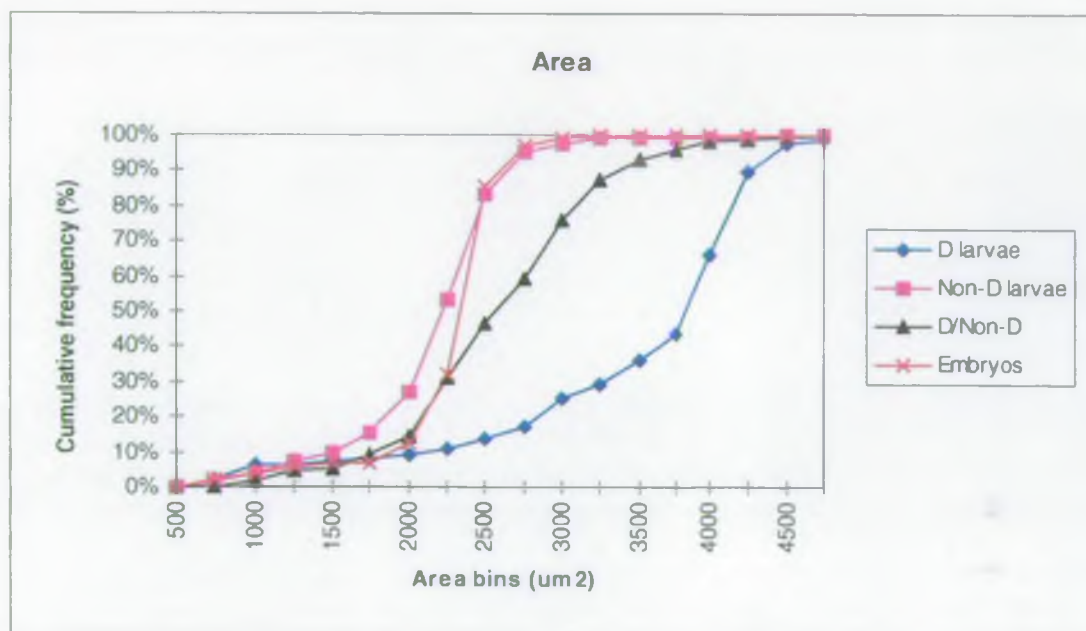
Having established the potential ranges within which D larvae would fall for the different measurement parameters (Table 2.3) it was then necessary to determine whether the 7 parameters in Table 2.3 were capable of discriminating between groups of D larvae and non-D larvae (that is inoculated embryos and abnormally developed larvae).

To assess discrimination, four groups of objects which would be encountered in the OEL tests; three homogeneous groups comprising inoculated embryos, non-D (undeveloped) larvae, D (developed) larvae and a heterogeneous group containing larvae at different stages of development (that is a combination of non-D larvae and D larvae) were assessed using all the seven measurement parameters. For each group the measurements of each parameter for the objects were separated into a series of bins and cumulative frequency curves were plotted. If a measurement parameter was capable of discriminating between the different object groups then there should be a distinct separation of the frequency curves.

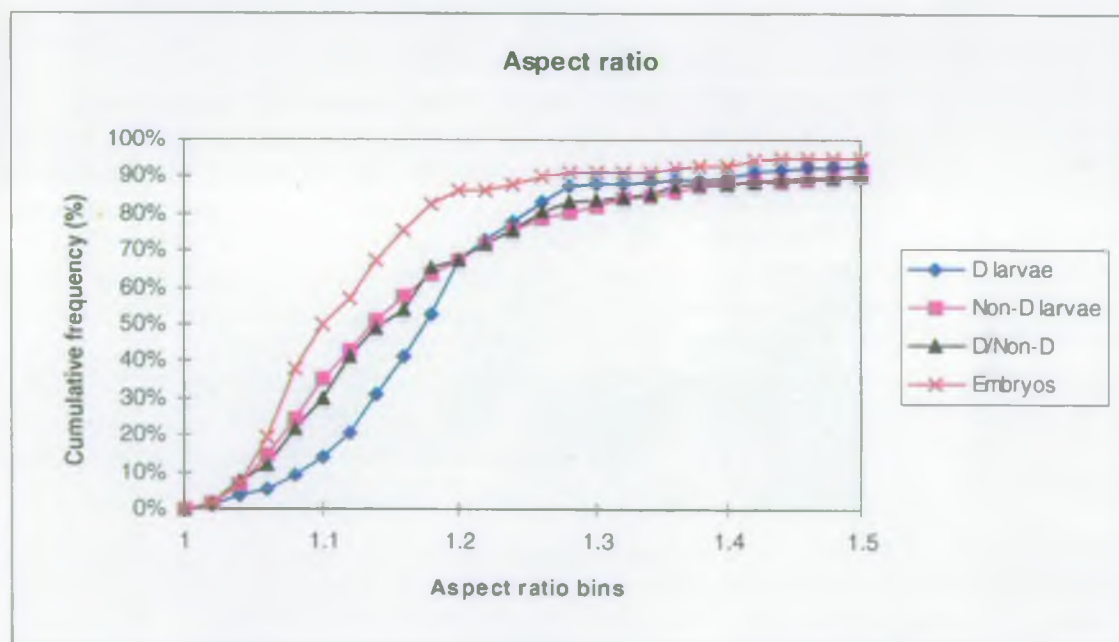
Figures 2.5 - 2.11 show the cumulative frequency curves for the different object groups for the individual measurement parameters (area, aspect ratio, perimeter, radius ratio, roundness, size-length and size-width). From the data it was evident that only the measurement parameters of area, perimeter, size (length) and size (width) showed discrimination between the object groups whereas for aspect ratio, radius ratio and roundness discrimination was limited. The extent of the discrimination between D larvae and non-D larvae for the measurement parameters of area, perimeter, size (length) and size (width) is shown in Table 2.4, where the cumulative frequencies of objects in each group at the minimum threshold values for D larvae given in Table 2.3 are summarised. For example, for the measurement parameter area at the threshold value of 2960  $\mu\text{m}^2$  the cumulative frequency of objects in the D larvae group was only 25%, whereas for the inoculated embryos and non-D larvae the cumulative frequencies were 99 and 98% respectively. In the combined D/non-D larvae group the cumulative frequency of objects at 2960  $\mu\text{m}^2$  was 76%.

However, this is not to say that the other parameters, which could be included in a 'macro', may not be useful in discriminating between true test objects and extraneous material or light artefacts.

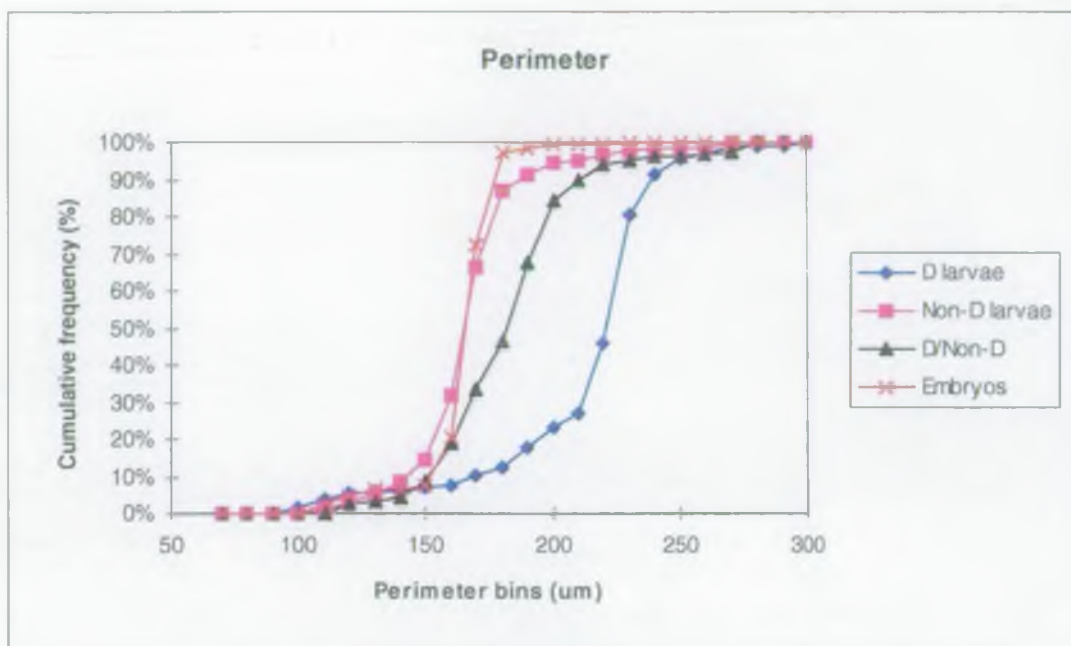




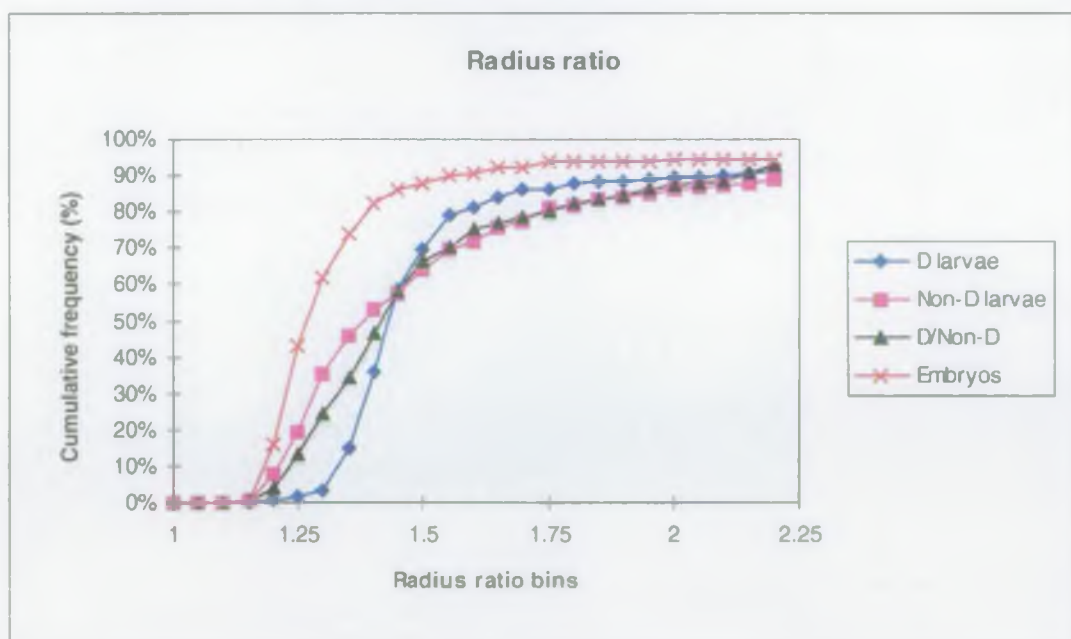
**Figure 2.5** Cumulative frequency curves for objects in the D larvae, non-D larvae, D/non-D larvae and inoculated embryos groups as measured by image analysis using the area parameter



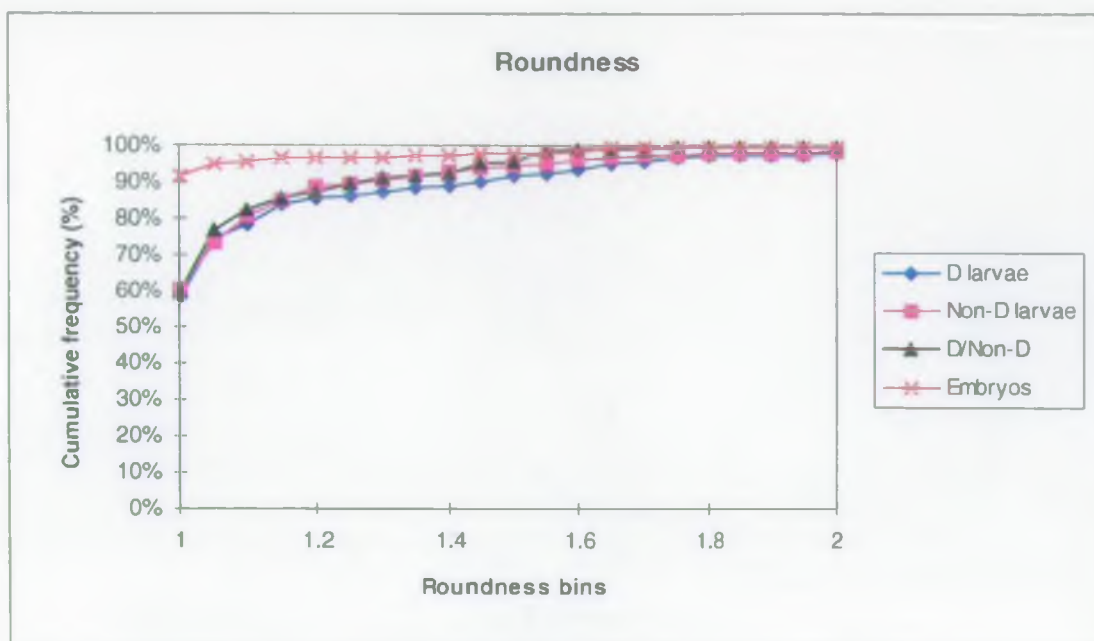
**Figure 2.6** Cumulative frequency curves for objects in the D larvae, non-D larvae, D/non-D larvae and inoculated embryos groups as measured by image analysis using the aspect ratio parameter



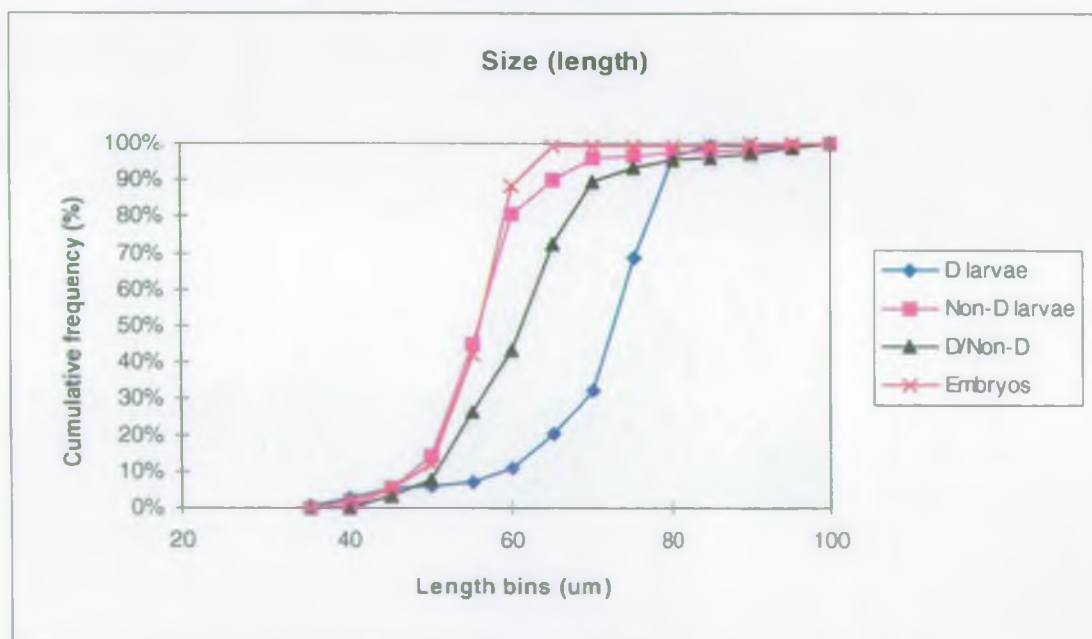
**Figure 2.7** Cumulative frequency curves for objects in the D larvae, non-D larvae, D/non-D larvae and inoculated embryos groups as measured by image analysis using the perimeter parameter



**Figure 2.8** Cumulative frequency curves for objects in the D larvae, non-D larvae, D/non-D larvae and inoculated embryos groups as measured by image analysis using the radius ratio parameter

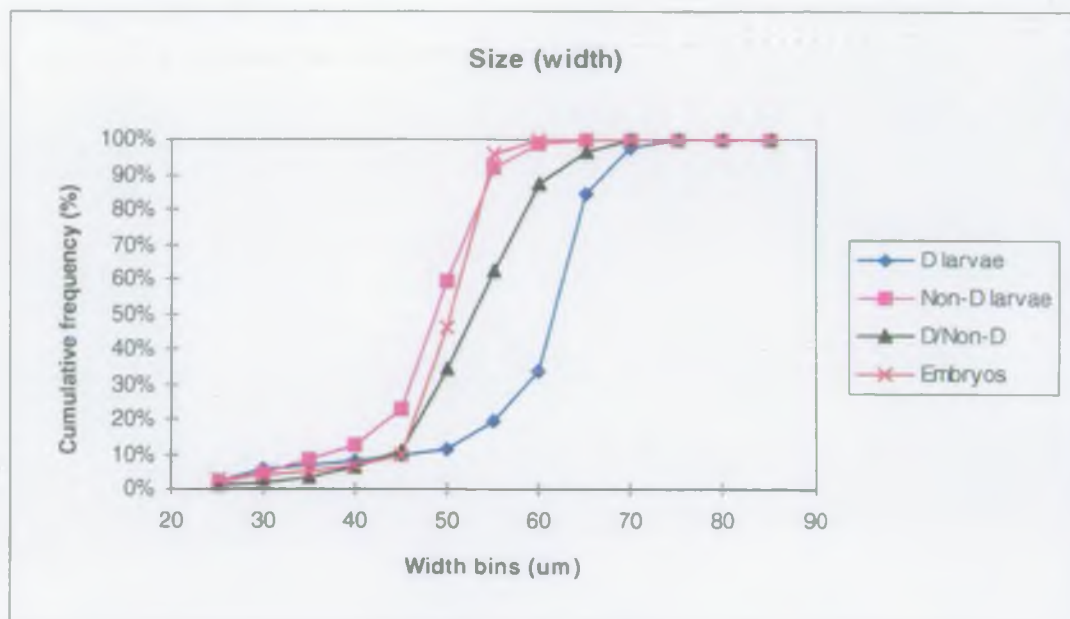


**Figure 2.9** Cumulative frequency curves for objects in the D larvae, non-D larvae, D/non-D larvae and inoculated embryos groups as measured by image analysis using the roundness parameter



**Figure 2.10** Cumulative frequency curves for objects in the D larvae, non-D larvae, D/non-D larvae and inoculated embryos groups as measured by image analysis using the size (length) parameter





**Figure 2.11** Cumulative frequency curves for objects in the D larvae, non-D larvae, D/non-D larvae and inoculated embryos groups as measured by image analysis using the size (width) parameter

**Table 2.4** Summary information on the discriminatory capability of the area, perimeter, size (length) and size (width) measurement parameters

Parameter	Threshold value	Cumulative frequency of objects at threshold value in different groups			
		D larvae	D/non-D larvae	Non-D larvae	Inoculated embryos
Area	2960 $\mu\text{m}^2$	25	76	98	99
Perimeter	192 $\mu\text{m}$	20	77	94	99
Size (length)	63 $\mu\text{m}$	18	64	88	96
Size (width)	54 $\mu\text{m}$	19	59	90	96

Although there were four measurement parameters that could be potentially used to discriminate between D larvae and non-D larvae it was decided to proceed with the use of area and size (length) individually and in combination in subsequent comparisons of the effect of counting procedure. Given that the objects of interest (oyster embryos) are broadly circular in outline there is an inevitably strong auto-correlation between area and perimeter and for this reason perimeter, as an independent parameter, was deemed redundant. Size (width) was not used since this parameter showed a similar pattern of response to that for size (length) and would also represent redundancy in the measurement suite for test objects.

In the D larvae group the proportion of objects measured as D larvae by visual observation corresponded closely to the proportion of D larvae identified in the group by area and length using the threshold criteria of 2960  $\mu\text{m}^2$  and 63  $\mu\text{m}$  (see Table 2.5).

**Table 2.5** Proportion of D larvae in the 'D larvae' group counted by visual observation and image analysis using area and length

Counting method	Number of objects		Proportion of D larvae (%)
	D larvae	Non-D larvae	
Visual	165	33	83.3
Image analysis - Area	151	31	83
Image analysis - Length	171	24	87.7

### 3. APPLICATION OF THE IMAGE ANALYSIS TECHNIQUE TO TOXICITY TESTS

#### 3.1 Oyster embryo-larval development test

##### 3.1.1 Introduction

The assessment of the feasibility of using image analysis involved comparing the results of toxicity tests carried out in multiwell plates where the D and non-D larvae counts using the Image Pro Plus software were compared against the data from conventional tests where visual counts were made.

##### 3.1.2 Materials and Methods

The comparability between OEL test results derived from visual observations or image analysis measurements of multiwell plates was assessed on the basis of concentration-response data obtained from tests carried out with the reference toxicant zinc on two occasions (26/3/98 and 15/4/98). The zinc concentrations used in the tests are shown in Table 2.1.

Visual observations of the number of D and non-D shaped larvae in each well were made using a Nikon Diaphot-TMD microscope. All objects in the well were counted, rather than a sub-sample, to minimise potential differences in the numbers of identified objects measured by the visual method and image analysis.

When assessing the status of objects in each well using the image analysis software the criteria given in Table 3.1 were applied based on the work described in Section 2. When the parameters of area and length alone were used objects less than  $1580 \mu\text{m}^2$  and  $38 \mu\text{m}$  respectively were considered to be spurious based on the preliminary work described in Section 2.

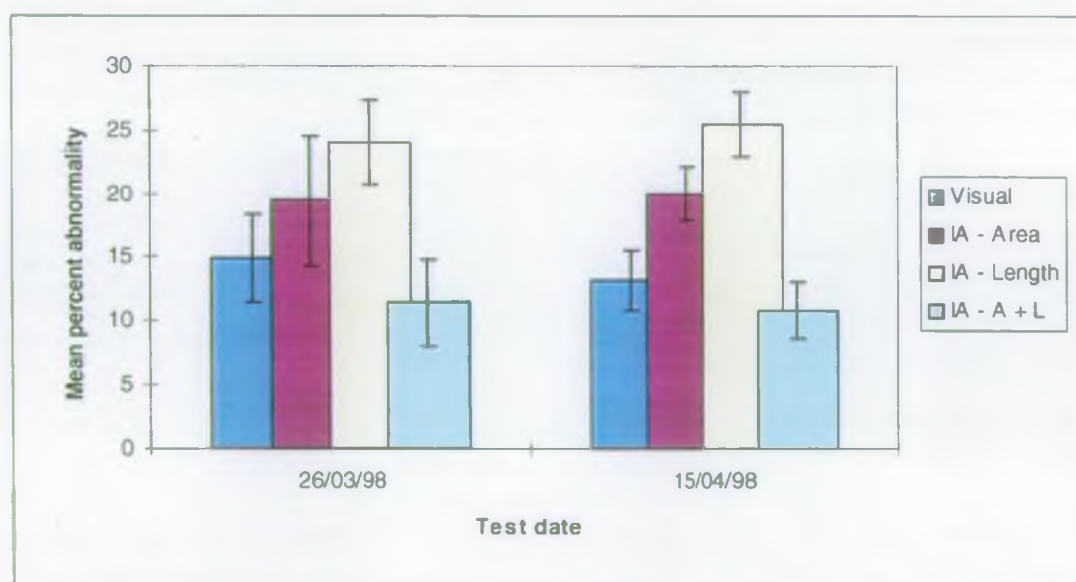
**Table 3.1 Criteria used to determine the status of objects in multiwell plates using the image analysis software**

Parameter used	Object type	Criteria
Area alone	D larvae	$2960-4922 \mu\text{m}^2$
	Non-D larvae	$1548-2959 \mu\text{m}^2$
Length alone	D larvae	$63-86 \mu\text{m}$
	Non-D larvae	$38-62 \mu\text{m}$
Area + Length	D larvae	$2960-4922 \mu\text{m}^2$ and $63-86 \mu\text{m}$
	Non-D larvae	$2960-4922 \mu\text{m}^2$ and $38-62 \mu\text{m}$ or $1548-2959 \mu\text{m}^2$ and $63-86 \mu\text{m}$
	Spurious objects	$1548-2959 \mu\text{m}^2$ and $38-62 \mu\text{m}$



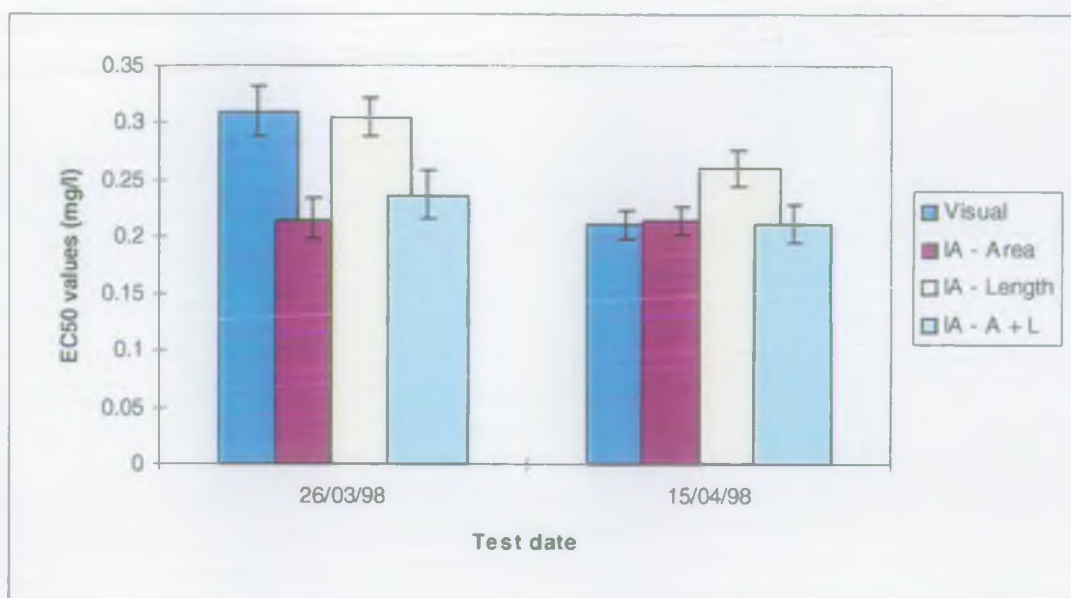
### 3.1.3 Results and Discussion

Figures 3.1 and 3.2 summarise the results for the tests carried out in the 3 ml volume multiwell plates and measured both visually and by image analysis using the area and length parameters individually and together. The level of abnormalities measured in the controls increased in the order Image analysis-Area + Length < Visual < Image analysis-Area < Image analysis-Length (Figure 3.1). The level of control abnormality was <25% for all the procedures used and was markedly less than the ICES test validity criterion. Two-way analysis of variance (ANOVA) showed that there was a statistically significant effect of counting method on the level of control abnormality in a test ( $F=43.7$ ,  $P<0.001$ ). The results show that the mean abnormalities in the controls measured using image analysis with both the area and length parameters were not significantly different from those measured by visual observations. In contrast, the mean abnormalities by image analysis using the area and length parameters individually were significantly different on both occasions from those obtained by visual observation ( $P<0.05$  in all cases). There was no statistically significant temporal effect on control abnormalities ( $F=0.02$ ,  $P>0.05$ ), that is control abnormalities for a given procedure were similar on the different test occasions.



**Figure 3.1** Mean percent abnormality in the controls of OEL tests carried out in multiwell plates and measured visually and by image analysis

The  $EC_{50}$  values derived using the different measurement procedures are shown in Figure 3.2. Two-way ANOVA showed that there was no statistically significant difference between  $EC_{50}$  values derived from data collected by the different counting procedures ( $F=2.3$ ,  $P>0.05$ ). In addition, there was no statistically significant temporal effect on  $EC_{50}$  values ( $F=4.1$ ,  $P>0.05$ ), that is the  $EC_{50}$  values for a procedure were similar on the different test occasions.



**Figure 3.2** Toxicity values for OEL tests with zinc carried out in multiwell plates and measured visually and by image analysis

Table 3.2 shows the NOEC and LOEC values obtained for each test using the different counting methods and indicates that these values were the same in all cases (NOEC = 0.1 mg Zn l<sup>-1</sup> and LOEC = 0.21 mg Zn l<sup>-1</sup>), except for the lower values obtained using the area measurement parameter on the 26/3/98.

**Table 3.2** Summary of the NOEC and LOEC values obtained by the different counting procedures on the different test occasions

Test date	Parameter	Toxicity data (mg Zn l <sup>-1</sup> ) obtained using different counting methods			
		Visual	IA - Area	IA - Length	IA - Area + Length
26/3/98	NOEC	0.1	0.032	0.1	0.1
	LOEC	0.21	0.1	0.21	0.21
15/4/98	NOEC	0.1	0.1	0.1	0.1
	LOEC	0.21	0.21	0.21	0.21

Consequently, the use of the area and length parameters together in the image analysis software can apparently provide data comparable to that generated by visual observations.

Table 3.3 shows the EC<sub>50</sub> values derived from data obtained by visual observations of the same sample by four different operators and also the corresponding value obtained using image analysis with the area and length parameters. It is evident that the sub-sampling and counting elements of the current OEL test will result in variability (up to 32%) in the derived EC<sub>50</sub> values for different operators. This source of variability can be eliminated using multiwell plates (as there is no sub-sampling) and image analysis counting of objects (as there are no inter-operator differences in the counts of D and non-D larvae).



**Table 3.3**  $EC_{50}$  (mg l<sup>-1</sup>) values derived from data obtained by visual observations of the same samples by different operators and by image analysis using area and size (length)

Test date	EC <sub>50</sub> values derived from data obtained by visual observations of the same sample by different operators				Mean (SD)	EC <sub>50</sub> derived by image analysis
	Operator 1	Operator 2	Operator 3	Operator 4		
26/3/98	0.31	0.33	0.32	0.35	0.33 (0.02)	0.24
15/4/98	0.21	0.44	0.23	0.32	0.30 (0.1)	0.21

Table 3.4 summarises the time implications of the multiwell image analysis approach compared to the conventional glass vessel visual observation approach. Although the use of the multiwell image analysis approach used in this study would not result in a marked saving in the time taken to count the test vessels compared to the conventional approach, the implementation of a partially or fully automated approach utilising an X-Y stage would result in marked savings for counting time. The use of a partially automated system in which the multiwell plates have to be loaded onto the X-Y stage would result in a considerable time saving over the fully manual approach. However, a fully automated system would allow a greater volume of samples to be processed in a unit time since the automated system could operate outside of normal working hours and at weekends. There would obviously be capital cost implications in moving from the partially automated approach (estimated cost = £10000) to a fully automated system (estimated cost = £20000) and the decision would need to be made on the basis of current and predicted workloads associated with the OEL test and others which would benefit from the application of image analysis.

**Table 3.4** Summary of the time implications of the different study approaches used in the study (for a 10 concentration test and controls)

Issue	Approach	
	Conventional vessel: visual observation	Multiwell plates: image analysis
Test time (h)		
- Conduct	7.0 h	7.0 h
- Counting (Manual)	3.0 h	2.0 h
- Counting (Partially automated)	3.0 h	1.0 h
- Counting (Fully automated)	3.0 h	1.0 h*

\* - Counting can be carried outside of normal working hours

## 3.2 *Daphnia magna* growth test

### 3.2.1 Introduction

This study was conducted to ascertain whether image analysis could provide a more cost-effective way of assessing growth than methods which require test operators to assess the length of organisms microscopically using an eyepiece graticule or estimating growth using dry weight.

Microscopic examination is time-consuming and laborious compared to the use of image analysis. Measurement of growth using dry weight terminates the test and means only one set of data can be obtained from experimental animals during the exposure period unlike the non-invasive image analysis technique which means repeat measurements can be made throughout the test. This work builds on an initial study investigating the use of image analysis to measure growth in *Daphnia magna* described in Johnson and Delaney (1998).

Initial assessments of the length of *Daphnia* of different sizes by both microscopic examination and image analysis showed that there was a highly significant correlation between the data obtained by the two methods ( $r=0.97$ ,  $P<0.01$ ).

The measurements of length in the growth test were combined with measurements of mobility after 48 h and juvenile production (that is whether juveniles had been released by the adults after a 10 day exposure period). The successful application of image analysis to all these endpoints would provide the opportunity to use an integrated *Daphnia magna* test with immobilisation, growth and reproduction endpoints.

### 3.2.2 Materials and Methods

Tests were performed using juvenile (<24-hr old) *Daphnia magna* which were cultured in hard (250 mg  $\text{CaCO}_3 \text{ l}^{-1}$ ) groundwater at the Medmenham laboratory. The *Daphnia* had been typed as IRCHA clone 5 and had been cultured at the laboratory since 1985.

The zinc test solutions were prepared on the day of the test using freshly made stock solutions. A 10 mg  $\text{l}^{-1}$  zinc stock solution was prepared by dissolving 44 mg of zinc sulphate heptahydrate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) in 1 litre of groundwater in a volumetric flask. The nominal test concentrations of zinc used were 0 (Control), 100, 200, 400, and 800  $\mu\text{g Zn l}^{-1}$ .

Test concentrations of zinc were prepared in 1 litre volumetric flasks by adding the appropriate volumes of the relevant stock solution and then diluting these with groundwater.

Ten animals were used at each test concentration and these were exposed individually to 40 ml of test solution in 50 ml plastic containers (cell culture flasks). The *Daphnia* were randomly added to each vessel from the isolation vessel. The transfer of daphnids was carried out rapidly and carefully whilst minimising the stress to the organisms. Animals were transferred using a Pasteur pipette and were released under the surface of the test solution. Any organisms which were floating or injured following transfer were replaced.

An image of each daphnid was captured using the image analysis equipment and the length of each animal was recorded. A Canon FD 28-55 mm zoom lens attached to the CCD camera with a C mount adapter was used to measure the daphnids. The lens was fixed at 35 mm and the distance between the centre of the vessel and the focal plane of the CCD chip was 292 mm.

In the integrated study an organism was considered mobile if it was possible to obtain an image within the allotted time frame for length measurements. As the whole test vessel was captured in the frame of the CCD camera animals which were immobile or dead on the bottom of the test vessel were not captured as images. The absence of an image for the daphnids was confirmed by visual observations. In terms of making measurements of the presence/absence and number of juveniles produced in the later stages of the test, images

taken on Days 7 to 10 were examined for the presence of the larger adult female and smaller juveniles in the water column.

After starting a test, the temperature, pH, and dissolved oxygen of the solutions in each preparation flask were measured. Duplicate samples from each test concentration were taken for chemical analysis after the test vessels had been filled.

The test vessels were observed each day and any anomalies in the behaviour of the *Daphnia* or mortalities were noted. The body length of each daphnid was measured on days 2, 3, 4 and 7. Temperature, pH, and dissolved oxygen of the old and new test dilutions were measured. The *Daphnia* were fed *Chlorella vulgaris* var. *viridis* at 0.2 mg C l<sup>-1</sup> daily. At the end of the test, the temperature, pH, and DO in the controls and test concentrations were measured.

The resulting regression equations of length against day were compared by analysis of covariance (ANCOVA) and treatment groups different from the control in each experiment were identified by the Dunnett's test (Zar 1984).

### 3.2.3 Results and Discussion

Table 3.5 shows the regression equations (and correlation coefficients) of growth against exposure time in *Daphnia magna* exposed to zinc. In the control vessels, growth was linear over the 7 day period. Growth was not significantly inhibited by zinc concentrations of 100-200 µg Zn l<sup>-1</sup>. However, there was significant inhibition of growth in organisms exposed to 400-800 µg Zn l<sup>-1</sup>.

ANCOVA showed that both the slope and elevation of the regression equation for the *D. magna* exposed to 800 µg Zn l<sup>-1</sup> were significantly different from those of the control indicating that the effect of zinc increased with exposure duration.

**Table 3.5 Regression equations of *Daphnia magna* growth during zinc exposure**

Zinc conc. (µg l <sup>-1</sup> )	Regression equation	r <sup>2</sup> value	Significant difference from control	
			Slope	Elevation
0	Length = 0.96 + 0.225 Day	0.573	-	-
100	Length = 1.13 + 0.257 Day	0.876	NS	P<0.01
200	Length = 1.00 + 0.246 Day	0.730	NS	NS
400	Length = 1.17 + 0.164 Day	0.725	P<0.05	NS
800	Length = 1.10 + 0.104 Day	0.493	P<0.01	P<0.01

For the 400 µg Zn l<sup>-1</sup> treatment the slope, but not the elevation, differed from that of the control, indicating the effect on organism growth only became marked at the end of the exposure period. These conclusions are evident from Table 3.6 which shows the lengths of zinc-exposed *D. magna* after 4 and 7 days estimated from the regression equations in Table 3.5.

At the lower exposure concentrations there was evidence of slight stimulation of growth, as shown by the significantly different elevation of the 100 µg Zn l<sup>-1</sup> regression equation

compared to the control. The results for zinc are directly comparable with those of a previous study at WRc (Johnson and Delaney 1998) and are also consistent with those of Berglund (1986) who showed that zinc exerted no inhibitory effects on the growth of *Daphnia magna* at concentrations up to 200 µg l<sup>-1</sup> after 8 days exposure. In that study, measurements of organism length (from the top of the head to the base of the dorsal spine) were made from photographs taken through a dissection microscope.

**Table 3.6 Estimated lengths of zinc-exposed *Daphnia magna* after 4 and 7 days and the % change in length from controls**

Zinc level (µg l <sup>-1</sup> )	Estimated length (mm) after given exposure time (% change from controls)	
	4 days	7 days
0	1.86	2.54
100	2.16 (+16.1)	2.93 (+15.4)
200	1.98 (+6.5)	2.72 (+7.1)
400	1.83 (-1.6)	2.32 (-8.7)
800	1.52 (-18.3)	1.83 (-27.9)

For zinc the threshold value of 400 µg l<sup>-1</sup> for growth effects after 7 days occurred at a higher concentration than that the threshold EC<sub>20</sub> values of 25-101 µg l<sup>-1</sup> for juvenile production after 21 days (Environment Agency 1998), but a lower concentration than that causing effects on immobilisation after 48-hr. The 48-hr EC<sub>50</sub> value for *D. magna* immobilisation measured at WRc was 1570 µg l<sup>-1</sup> for zinc (WRc unpublished observations).

These results indicate that growth is a useful short-term indicator of chronic toxicity. Indeed Winner (1981) showed that the body size of primiparous *Daphnia magna* was predictive of lifetime exposure effects of copper and zinc.

Further investigations are currently being conducted at the WRc laboratory to assess responses of the *D. magna* growth assay to a wider range of substances with different modes of toxic action. Available data from the literature indicates that growth in *Daphnia sp.* is impaired following exposure to sub-lethal concentrations of atrazine (Schober and Lampert 1977), carbaryl (Hanazato 1991), and dithiocarbamates and related compounds (van Leeuwen *et al.* 1985).

Observations of the mean percentage immobilisation of *Daphnia magna* in each concentration after 48 h exposure showed that no animals in any of the test vessels were immobile after 48 h, a finding confirmed by visual observations. The absence of immobile organisms at the highest exposure concentration is consistent with the results of previous studies with zinc at WRc where limited immobilisation (≤ 10%) was recorded at concentrations up to 800 µg l<sup>-1</sup> (Johnson unpublished observations).

In terms of the reproduction of the test organisms the data showed that juveniles were produced in all the test vessels of the control and 100 µg l<sup>-1</sup> treatments by Day 10, whereas in the 200, 400 and 800 µg l<sup>-1</sup> treatments no or limited numbers of juveniles were released (see Table 3.7). That juvenile production in animals exposed to zinc concentrations ≥ 200 µg l<sup>-1</sup> was limited or absent is consistent with the results obtained in an investigation into the development of a short-term *D. magna* reproduction test (Environment Agency 1999). In that

study juvenile production was usually reduced considerably after 10 days exposure to 200 µg Zn l<sup>-1</sup> compared to the controls. Exposure of test animals to 100 µg l<sup>-1</sup> for 10 days apparently resulted in a delay in the pattern of release of juveniles with zinc exposed animals releasing offspring on Days 9 and 10 compared to Days 8 and 9 in the controls. Furthermore, while all the animals in the control vessels produced offspring by Day 10 only 80 % of animals in the 100 µg Zn l<sup>-1</sup> treatment had released juveniles.

**Table 3.7** Summary information on the release of juveniles on days 7 to 10 in the *Daphnia magna* growth test

Zinc conc. (µg l <sup>-1</sup> )	Proportion of animals releasing juveniles on a given day				
	Day 7	Day 8	Day 9	Day 10	Total
Control	0	40	50	10	100
100	0	10	40	30	80
200	0	0	0	40	40
400	0	0	0	10	10
800	0	0	0	0	0

An integrated test (combining immobilisation, growth and short-term reproduction endpoints) would take approximately 30-35 h to carry out for a control and four treatments each consisting of 10 vessels. This timeframe comprises the period from obtaining test organisms, to preparing and conducting the test and analysing the data.

## 4. CONCLUSIONS

On the basis of the zinc reference toxicant test data derived in this study the following conclusions can be drawn:

- the OEL test can be effectively carried out in multiwell plates which provides the opportunity to count objects using image analysis software;
- the use of the image analysis parameters area and size (length) in combination results in mean control abnormalities and  $EC_{50}$  values which are not significantly different statistically from corresponding values derived using visual observations. Discrimination using the area and length parameters may be improved by the inclusion of other parameters into a measurement macro which would eliminate extraneous material or lighting artefacts;
- the use of multiwell plates and image analysis can eliminate the variability associated with sub-sampling and inter-operator differences in the counts of D and non-D larvae from the procedure. Image analysis also opens up the possibility of assessing egg quality at the start of the test by comparing measured values for parameters such as length and area against defined acceptability criteria. It also allows the initial inoculum number to be determined as a quality check of the numbers of objects measured at the end of the test;
- in terms of cost-benefit using image analysis the current system only resulted in a small time saving. However, there could be major saving in staff time if the an automated X-Y stage was added to the system and test replicates could be counted without the requirement for the presence of a human operator;
- growth of *D.magna*, as measured by image analysis, can be a sensitive sub-lethal indicator of toxicity and can be integrated with existing 48 h immobilisation and a short-term or 21 d juvenile production. This would result in a more cost-effective test method which would yield greater amounts of information on the toxicity of test substances than the existing approaches and would remove the need to conduct a suite of tests, thereby removing inter-test variability as a problem in data interpretation.



## 5. ISSUES

The DTA Methods Guidelines procedure for the oyster embryo-larval development test (Environment Agency 1997), which is based on the International Council for the Exploration of the Seas protocol (ICES 1991), is not overly prescriptive in terms of the counting method to be used. Therefore, this allows the multiwell plate approach using image analysis to be implemented under the DTA protocol without compromising the acceptability of the test. However, for the multiwell plate test to be valid it will be necessary for the mean control abnormality level of  $\leq 40\%$  to be achieved at all times. The potential advantages of using the multiwell plate - image analysis approach over the existing approach can be summarised as:

- a reduction in the costs of the test which will vary depending on whether a partial or fully automated system is used;
- the elimination in the variability associated with the subjectivity inherent in the counting of D larvae by different operators;
- improved standardisation of the test by making measurements of the inoculum added to the wells. In this way the actual number added would be known rather than assuming a value of 100 or inferring the value from the total number of normal and abnormal larvae measured at the end of the test.

In contrast, there are requirements for the ratio of test animals to test medium volume in the *Daphnia magna* immobilisation and juvenile production tests (10 and 50-100 ml per animal respectively). Therefore, it is not possible to implement the integrated *D.magna* test using multiwell plates without modifying existing test guidelines. If the guidelines are not to be modified it is necessary to use an appropriate test vessel which allows measurements to be made of the motility of the organisms, their length for growth assessment and latterly in an integrated test the presence/absence and numbers of juveniles.





## 6. RECOMMENDATIONS

On the basis of the results obtained in the study a multiwell plate based procedure using image analysis could be applied to the oyster embryo-larval development test if it is believed that the technical and cost benefits justify this strategy. If the approach is to be adopted then further work should be carried out to establish:

- the validity of the approach for environmental samples which are coloured or contain particulates. WRc is investigating these issues as part of an on-going programme;
- a standard operating procedure including the use of image analysis to assess the quality of eggs and measure the inoculum added to the test vessels. Standardisation of the quality of eggs used in the test could be achieved by comparing egg parameters such as length and area against defined acceptability criteria. In this way only good quality eggs will be used to provide embryos, thereby improving the likelihood of achieving the test validity criterion of  $\leq 40\%$  mean control abnormality.

Although the feasibility of carrying out an integrated immobilisation, growth and reproduction test with *Daphnia magna* has been established, further work needs to be carried out to identify the most practical and cost-effective test system.



## REFERENCES

- Berglind R (1986) Combined and separate effects of cadmium, lead and zinc on ALA- D activity, growth and hemoglobin content in *Daphnia magna*. *Environ Toxicol Chem* 5: 989-995.
- Environment Agency (1997) Methods Guidelines for Effluent and Receiving Water Assessment. Direct Toxicity Assessment (DTA) Demonstration Programme Report.
- Environment Agency (1999) Development of a short-term *Daphnia magna* reproduction test. WRc Report CO 4567.
- Hanazato T (1991) Effects of long and short term exposure to carbaryl on survival, growth and reproduction of *Daphnia ambigua*. *Environ Pollut* 74: 139-148.
- Johnson, I. and Delaney, P. (1998) Development of a 7 day *Daphnia magna* growth test using image analysis. *Bull Environ Contam Toxicol* 61: 355-363.
- Schober U, Lampert W (1977) Effects of sublethal concentrations of the herbicide atrazin on growth and reproduction of *Daphnia pulex*. *Bull Environ Contam Toxicol* 17: 269-277.
- van Leeuwen C J, Moberts F, Niebeek G (1985) Aquatic toxicological aspects of dithiocarbamates and related compounds - II. Effects on survival, reproduction and growth of *Daphnia magna*. *Aquat Toxicol* 7: 165-175.
- Winner R W (1981) A comparison of body length, brood size and longevity as indices of chronic copper and zinc stresses in *Daphnia magna*. *Environ Pollut* 26: 33-37.
- Zar J H (1984) Biostatistical Analysis, 2nd Edition. Prentice Hall, New Jersey.



**APPENDIX A      INFORMATION ON THE IMAGE PRO PLUS  
SOFTWARE**



## IMAGING SOFTWARE

# Image-Pro PLUS

## The Proven Solution

### FEATURES

#### ACQUISITION

- ▶ Read files from camera, scanner, disk, CD-ROM
- ▶ Capture multiple images after user-specified delay time
- ▶ Ability to acquire, create, and playback a sequence of images
- ▶ Average or Sum multiple images (may require special hardware)
- ▶ Hardware supported (on certain boards) gain and offset, fast frame averaging, logical and arithmetic operations, convolution filtering, histogram analysis, bitwise operations
- ▶ Support for 12-bit and 16-bit gray scale input devices

#### ENHANCEMENT

- ▶ Enhance color and contrast using equalization, gamma correction, contouring, thresholding and background subtraction
- ▶ Improved filter dialogs including filter descriptions and preview window
- ▶ Use Top-hat, Well, hi-pass, low-pass, Sobel, edge detection, Laplace, Roberts, erosion, dilation, opening, closing, thinning, watershed and user-defined kernels
- ▶ User-selectable kernel shapes and sizes for morphological filters
- ▶ User-selectable scale and boost
- ▶ Set number of filter passes and filter strength

#### CALIBRATION

- ▶ Work with intensity or spatial measurements
- ▶ Create and display spatial calibration markers
- ▶ Pre-defined spatial calibration units available
- ▶ Save and recall all calibrations

#### FAST FOURIER TRANSFORM (FFT)

- ▶ Create direct and inverse transforms
- ▶ Edit spectrum including low pass, high pass, unsharpen, spike cut, spike boost
- ▶ Save and re-load data

#### COLOR CHANNEL PROCESSING

- ▶ Extract red, green, and blue or hue saturation and intensity channels for processing
- ▶ New color processing tools to extract multiple color channels simultaneously
- ▶ Merge channels for processing
- ▶ Switch or copy channels within an image
- ▶ Use color information to separate objects from background

- ▶ Isolate a group of colors for processing and analysis
- ▶ New Pseudocolor tool allows setting pre-defined and custom color spreads (ranges)

#### OPERATIONS

- ▶ Perform spatial (bi-linear scale, decimation, rotate, warp, transpose, reflect) logical arithmetic, image alignment, and background correction operations
- ▶ Image registration

#### COUNTING AND SIZING

- ▶ Count and size objects automatically
- ▶ Measure areas, perimeters, lengths, roundness, major and minor axes, aspects, angles, centroids, holes, population density - over 30 measurements
- ▶ Set ranges for measurements to filter unwanted objects
- ▶ Group objects in classes based on measurements
- ▶ Watershed, Auto-Split and Cluster analysis tools to resolve clustered objects
- ▶ Use colors to separate and measure objects in color images
- ▶ Export measurements to statistical and spreadsheet package via DDE
- ▶ Display measurements as histograms, and scattergrams
- ▶ Auto Threshold
- ▶ Option to manually tag, count and classify objects

#### ANALYSIS

- ▶ Calculate straight line, circle, irregular line or area histograms
- ▶ New thickness measurement options
- ▶ Percent area of multiple threshold levels
- ▶ Display data or histograms
- ▶ Define and manage multiple areas of interest in a single image
- ▶ Calculate statistics
- ▶ Analyze RGB, HSI, HSV or YIQ content of color images
- ▶ Combine image with background correction for precise intensity or optical density measurements
- ▶ Background corrected line profiles
- ▶ Output intensity map in ASCII format

#### MEASUREMENT

- ▶ Measure lengths, areas, perimeters, and angles
- ▶ Automatically outline objects with Auto-Trace feature
- ▶ Quickly calculate max, min, and average Thickness between lines

#### ANNOTATOR

- ▶ Add text, simple graphics, and arrows
- ▶ Modify palette or colors

#### IMAGE DATABASE

- ▶ Provides all the functionality to organize, store, and retrieve images
- ▶ Images can be located through key word searches or viewed in a thumbnail gallery view
- ▶ Print full size or image galleries from inside the database



#### REPORT GENERATOR

- ▶ Create customized reports with images, data, and text
- ▶ Images, data, and text can be formatted and sized to user's specifications
- ▶ Report templates can be created and saved

#### INTERNET

- ▶ Compose and send e-mail messages within Image-Pro
- ▶ Review information and images over the Internet using IRC (Internet Relay Chat)
- ▶ Support for FTP Protocols

#### PRINTING AND PUBLISHING

- ▶ Output high quality gray scale and color images to Windows printers
- ▶ Complete control over image position on page
- ▶ Print titles to make poster size prints
- ▶ Test Strip feature selects best output
- ▶ Screen Capture individual workspaces or entire screen

#### MACRO PROGRAMMING LANGUAGE (AUTO-PRO)

- ▶ Record sessions and play them back in Basic Syntax
- ▶ Assign scripts to files and function keys
- ▶ Add Basic loops and conditionals
- ▶ Integrate and customize macros with Visual Basic or Visual C++

#### IMAGE AND DATA FILE FORMAT SUPPORT

- ▶ Read and write files in TIFF, HPP, BMP, CUT, EPS, GIF, Photo CD, PCT, PCX, TGA and FLAT (Binary)
- ▶ Convert files to and from all formats
- ▶ Supports 1,8,12,16, 24 and 32 bit floating point images
- ▶ JPEG, LZW, and RLE compression supported
- ▶ Batch convert files
- ▶ Output Data files to ASCII, WK1, or XLS for input for spreadsheets
- ▶ Transfer images, data graphs and data files via "DDE" or "Clipboard"

#### LARGE IMAGES

- ▶ Make best use of memory to create space for images larger than the display resolution
- ▶ Store images on capture boards or in memory

#### MULTI-PLATFORM SUPPORT GIVES YOU CURRENT AND FUTURE COMPATIBILITY

With solutions for Microsoft Windows 3.1, Windows 95, Windows NT 4.0, and Power Macintosh under development, we understand that as imaging requirements grow in their complexity, so will the requirements for more powerful operating systems. Image-Pro Plus is the only imaging software solution giving you the flexibility and freedom to run on the platform of your choice.

#### SYSTEM REQUIREMENTS

##### IBM Compatible PC

- ▶ 80486 (or better) CPU, running at 33 MHz or higher
- ▶ Microsoft Windows 3.1, 3.11, Windows 95 or Windows NT 4.00
- ▶ A minimum of 16 Mbytes of RAM
- ▶ A VGA (or better) resolution display device
- ▶ Windows-compatible mouse, keyboard, and CD-ROM drive

##### Macintosh

- ▶ Power Macintosh
- ▶ System 7.5 or later
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**APPENDIX B      IMAGE ANALYSIS PROTOCOLS USED FOR EACH  
TEST IN THE STUDY**



## **Appendix B1 Protocol for use of Image Pro Plus Application for Oyster Embryo-larval Development Test**

In this protocol a reasonable degree of familiarity with the use of microscopes, Microsoft Windows and Microsoft Office (Word, Excel ) has been assumed.

### **B1.1 Apparatus**

1. Pentium PC and peripherals the following specification or higher:
  - 233 MHz CPU
  - 64 Mb RAM
  - 2.1 Gb SCSI Hard Disk
  - 17-inch SVGA Monitor
  - 3.5-inch floppy drive
  - Ink-jet Printer (optional)
  - Mouse
  - Analogue or digital image capture card ('Frame Grabber').
  - Installed software: Microsoft Windows 95/NT, Excel, Image Pro Plus with Data Base and Report Generator
  - Image Pro Plus Licence 'dongle'
2. Monochrome CCD camera (for example Pulnix TM1001 Frame Transfer Digital Camera).
3. Nikon Diaphot Inverted Microscope, including x4, x10 objectives, C-mount adapter and x1 magnification transfer (relay) lens.
4. Zero to 1000  $\mu\text{m}$  calibrated graticule

### **B1.2 Setting up and Optimization**

#### **B1.2.1 Microscope**

If the microscope is being set up for the first time the user should refer to the manufacturer's documentation for instructions on the correct adjustment of the optics for example collimation of the light source, centering the above-stage condenser etc.

1. Switch on the microscope and set the illumination to a comfortable level for direct observation.
2. Select the 10 times objective;
3. Remove the phase-contrast stop from the condenser assembly to allow a wide field of illumination over the stage;
4. Adjust the condenser diaphragm to fully open;
5. Place a diffuser above the condenser assembly e.g. 80 mm square of tracing paper or drafting film;
6. Adjust the x-y stage to the 'central' position (i.e. objective in the centre of the stage aperture);
7. Position a calibrated graticule on the microscope stage and focus accurately through eyepieces.
8. Set the camera port levers to allow direct observation via the eyepieces whilst the camera port (CCD camera) is open.

### **B1.2.2 Imaging Apparatus and Software**

The following lists describe the sequence of operations required for setting up the Image Pro Plus application with a microscope and CCD camera, to make measurements of oyster embryo-larvae, which may be used to define the 24-h  $EC_{50}$  value.

The items incorporated in bold text refer to specific named functions and menu item options within the Image Pro Plus software. The following stages cover;

- setting up illumination;
- calibration of the image in engineering units;
- defining object measurement parameters;
- automatic counting of objects;
- export of data to Microsoft Excel spreadsheets.

### **Setting Microscope Illumination and Image greyscale**

The following sequence of operations is carried out to ensure that the image brightness falls within the dynamic range of the CCD camera and frame grabber card so that detail is not lost, and that the image is adjusted to reduce the effect of an unevenly illuminated background. This work is best carried out using an actual Pacific oyster test sample containing a wide range of oyster embryo-larvae sub-types.

1. Switch on the PC and CCD camera
2. Start **Image Pro Plus Application** from within Windows 95;

3. Place a sample of normal oyster embryo-larvae (OELs) into a counting chamber on the stage; select the 10 times objective, and focus the image via the eyepieces;
4. Select the **Live Preview** option to acquire a 'live' image of the field on the PC monitor;
5. If necessary, adjust the focus of the image on the monitor by adjusting the position of the C-mount adapter tube on the microscope: this will ensure that the CCD camera and the eyepieces share a common focal plane;
6. Select the **Snap** option in the **Active Imaging Snapper** utility to store an image of the OELs;
7. Select the **Best fit equalization** option from the main menu level to optimize the image greyscale;
8. Open the **Measure** option from the main menu level; then open sub-menu items **Count Size; Image; Flatten Background**, and set the **Zone** value to 20 pixels;
9. Open the **Measure** option from the main menu level and check the greyscale optimization using the **Line profile** icon, to extend a transect through the image; to encompass the complete range of pixel levels (i.e. across objects and background);
10. View the greyscale histogram to check that the upper greyscale range (the brightest part of the image) lies above approximately 200, and below 255 pixels;
11. If the maximum greyscale value lies outside the above range, readjust the microscope illumination and repeat from step 9.

### Calibration

It is important to ensure that accurate calibration of the images is made where data collected using different optical systems (microscopes, CCD cameras etc.) may subsequently be compared. The following sequence of operations define the actual dimensions of the objects within the image. An accurately calibrated graticule, such as those intended for direct measurement of specimens on the microscope stage should be used. Calibration data may be stored as a configuration data-set used by Image Pro Plus (for example "OEL\_SIZE" ). This particular calibration data-set may then be recalled (or re-edited) and used within Image Pro Plus for the automatic measurement of objects.

**Note:** this calibration data relates only to the magnification ratio in use at the time the calibration was made. Where a different microscope objective (or CCD camera) is used a new calibration must be undertaken.

Following initial calibration, further refinement of the calibration can be made to account for geometrical aberrations in the image by using a cross-hatch graticule. The magnitude of any aberrations is not likely to be large (less than 4%), however, without such correction determination of a linear dimension will depend on the orientation of the object being measured. Measurements of the cross-hatch graticule made on both the x and y axes provides data which may be entered into the **Aspect Ratio** field of the **Calibration** option.

1. Place the graticule on the microscope stage, select the 10 times objective, and focus the image via the eyepieces;
2. Select the **Live Preview** option to acquire a 'live' image of the graticle on the PC monitor;
3. Select the **Snap** option in the **Active Imaging Snapper** utility to store an image of the graticule
4. Open the **Measure** option from the main menu level and then select sub-menu items **Calibration; Spatial; then 'New'**;
5. Select **Image** box for 'pixels/unit': Using the mouse place the Scaling Bar cursor at one end of the graticule scale and extend it across the image.
6. Enter the calibration units ( $\mu\text{m}$ ) into the appropriate field;
7. Enter the number of units over which the cursor was extended (for example 1000) into the appropriate field;
8. Ensure that the new calibration data (for example 0.370 pixels/ $\mu\text{m}$ ) is associated with a unique name (for example OEL\_SIZE) and saved before leaving the calibration routine;
9. Before proceeding with a long series of actual sample measurements check that the Image Pro Plus software correctly establishes the dimensions of the graticule, or a sample of OELs: Typical normal D-shaped OELs should be in region of 60 to 80  $\mu\text{m}$  in length.

### Setting Object Parameters

The following list details the setting up of appropriate object parameters: that is characteristics which serve to define the OELs and allow discrimination from other extraneous material and artifacts within the captured image field. The range values of the object parameters shown below serve as thresholds within which an object is deemed to be 'real'. All chosen object parameters act inclusively such that an object must fall within all the ranges to become a 'thresholded' object. Thus, if a object lies within only six of the seven range values, it is not included with the other thresholded objects. The data listed in Table B1 is intended as a starting point only, to allow the user to become familiar with the range of values typically encountered. More precise values are given within the main report.

1. Open the **Measure** option from the main menu level; then open the sub-menu item **Count Size**; then select option **Auto Dark Objects**;
2. At the **Count Size** sub-menu item select **File** then **Load Settings**. If the object parameters shown in Table B1 have been previously saved as an 'Environment' file (\*.ENV) they may be reloaded by selecting the file;
3. Where the object parameters have not previously been assigned the user must use the **Select Measurements** option and enter the parameters and values required using the **Filter Ranges** fields;
4. Save the file as a unique name (for example OYSTERS.ENV).

**Table B1 Measurement parameters for Image Capture of all Credible Objects (entered into Environment File for example OYSTERS.ENV)**

Object Parameter	Lower Limit	Upper Limit
Area	500	5000
Aspect Ratio	1	5
Perimeter	95	300
Radius Ratio	1	5
Roundness	1	5
Size (Length)	15	120
Size (Width)	15	80

### Measurement of Thresholded Objects

This section refers to the enumeration of the thresholded objects, the inspection of sample values and the export of data to Microsoft Excel.

1. Select the **Live Preview** option to acquire a 'live' image of the OELs on the PC monitor;
2. Select the **Snap** option in the **Active Imaging Snapper** utility to store an image of the OELs;
3. Select the **Best fit equalization** option from the main menu level to optimize image greyscale;
4. Open the **Measure** option from the main menu level; then open sub-menu item **Count Size**; then select option **Count** (objects). The PC monitor will show a captured image of the field, and will number those objects which have been 'thresholded'. The measurements of thresholded objects in the image can be inspected using the **View** option with the **Measure** menu.
5. All thresholded object parameter data (from that captured image) may be stored as (or appended to) an Excel (\*.xls) file on the PC's hard disk drive.

### B1.3 Use of Macros

Image Pro Plus allows the user to define Macros (executable listed instructions) to assist in the collection of data. Once the user has become familiar with the selection of the various options described above they can record them as a specific sequence of individual steps, which will be invoked each time a user-defined key is pressed. Using this method the user can select a particular area of the microscope field of view, press one of the PC keyboard functions keys, to automatically collect the data and append it to an Excel file, and then move to the next microscope field of view, to collect further image data, and so on.

A range of different Macro files can be compiled for various different functions, and if necessary re-edited to make minor adjustments to the sequence of steps, saving the need to repeat long sequences of image capture and processing steps.



**Addresses**

Nikon UK Ltd.

380 Richmond Road

Kingston

Surrey KT2 5PR

## **Appendix B2 Guidance for use of the Image Pro Plus Application in the *Daphnia magna* growth test**

The current status of the work on defining a test system to carry out the *Daphnia magna* growth test individually or as part of an integrated test system with immobilisation and reproduction endpoints means that it is not possible to provide specific instructions about many of the areas involved in obtaining measurements. There is considerably more scope for implementing different solutions for the tests with *Daphnia magna* than with the OEL test. Therefore, this document outlines the approach taken in this study.

### **Apparatus**

The same apparatus as for the oyster embryo-larval development test is required with the exception of the Nikon Diaphot inverted microscope.

### **Imaging Apparatus and Software**

The sequence of operations required for setting up the Image Pro Plus application with a CCD camera, to make measurements of *Daphnia* length, motility and juveniles is largely similar to that for the OEL test.

**Note:** The type of test vessel used to carry out the test and the choice of CCD camera and lenses used to obtain a satisfactory image of the *Daphnia* are inextricably linked and govern factors such as focal distance and depth of field. For an integrated *Daphnia* test measuring immobilisation, growth and juvenile production it is important that an image can be obtained of the whole vessel and that the animal is in focus at any point in the vessel. The depth of field issue may restrict the width of the test vessel.

### **Setting Image greyscale**

Follow steps 1-11 in the relevant section of Appendix B1 on the basis that the image of the organism in the test vessel is focused using the lenses attached to the CCD camera.

### **Calibration**

Follow steps 1-9 in the relevant section of Appendix B1 on the basis that the image of the organism in the test vessel is focused using the lenses attached to the CCD camera. Typical <24 h old juveniles should be in the region of 0.9-1.1 mm in length.

### **Setting Object Parameters**

Follow steps 1-4 in the relevant section of Appendix B1 on the basis that the image of the organism in the test vessel is focused using the lenses attached to the CCD camera. For the assessment of *Daphnia* growth, length is typically used but parameters such as area may also yield valuable information.



## **APPENDIX C    IMAGE ANALYSIS DATA**



Table C1 Image analysis data for D larvae

Object No.	Area	Aspect	Perimeter	Radius Ratio	Roundness	Size (length)	Size (width)
1	4397	1.20	232	1.42	1.00	79	64
2	4178	1.16	227	1.35	1.00	81	66
3	4310	1.16	229	1.34	1.00	80	68
4	4551	1.18	238	1.37	1.00	82	70
5	3755	1.17	215	1.40	1.00	73	65
6	4492	1.20	237	1.40	1.00	79	65
7	4317	1.18	232	1.41	1.00	80	68
8	4171	1.19	228	1.39	1.00	77	65
9	4295	1.25	234	1.50	1.02	81	65
10	4244	1.14	231	1.37	1.00	77	68
11	4105	1.14	221	1.36	1.00	74	65
12	4463	1.17	235	1.39	1.00	77	67
13	4624	1.18	238	1.42	1.00	82	70
14	4083	1.14	225	1.39	1.00	71	65
15	3842	1.15	216	1.40	1.00	74	66
16	3674	1.16	208	1.36	1.00	71	61
17	4018	1.18	223	1.38	1.00	77	66
18	3835	1.21	217	1.52	1.00	76	60
19	4156	1.12	226	1.39	1.00	73	65
20	4922	1.12	251	1.44	1.01	81	70
21	3813	1.14	218	1.38	1.00	74	66
22	3733	1.22	215	1.38	1.00	75	60
23	3776	1.19	215	1.38	1.00	72	58
24	3959	1.16	222	1.39	1.00	77	65
25	3835	1.16	219	1.41	1.00	74	63
26	3097	1.18	197	1.45	1.00	63	55
27	3667	1.18	212	1.44	1.00	70	58
28	4069	1.16	223	1.42	1.00	75	63
29	4120	1.24	227	1.46	1.00	79	63
30	4237	1.17	230	1.40	1.00	75	67
31	3996	1.18	222	1.38	1.00	75	63
32	3966	1.17	222	1.40	1.00	77	62
33	3850	1.15	219	1.39	1.00	74	63
34	3477	1.37	213	1.65	1.04	72	58
35	4266	1.22	227	1.50	1.00	79	68
36	3769	1.15	215	1.39	1.00	70	63
37	3703	1.14	212	1.35	1.00	68	62
38	3492	1.17	207	1.45	1.00	74	59
39	3930	1.21	223	1.50	1.01	76	62
40	4500	1.25	242	1.53	1.04	86	68
41	3017	1.11	193	1.33	1.00	63	57
42	3966	1.18	222	1.34	1.00	75	63
43	3258	1.06	208	1.37	1.06	73	65
44	3718	1.15	211	1.37	1.00	71	65
45	3996	1.17	222	1.42	1.00	73	62
46	4127	1.15	227	1.44	1.00	76	65
47	3689	1.20	213	1.44	1.00	75	64
48	3828	1.24	221	1.44	1.02	74	63
49	2960	1.21	192	1.57	1.00	63	54
50	3024	1.16	195	1.41	1.00	65	57
Minimum	2960	1.06	192	1.34	1.00	63	54
Maximum	4922	1.37	251	1.87	1.06	86	70

**Table C2 Summary of the toxicity test data obtained by image analysis for the zinc reference toxicant tests**

Test date	Conc and replicate	Area			Length			Area + length		
		D's	Non-D's	Total	D's	Non-D's	Total	D's	Non-D's	Total
26/3/98	Control/1	74	18	92	93	29	122	74	13	87
	Control/2	81	18	99	89	35	124	81	10	91
	Control/3	95	24	119	116	29	145	95	11	106
	Control/4	70	8	78	73	19	92	70	4	74
	Control/5	99	29	128	115	41	156	100	14	114
	Control/6	91	31	122	107	37	144	91	15	106
	0.032/1	69	23	92	83	25	108	70	8	78
	0.032/2	75	24	99	84	32	116	76	15	91
	0.032/3	109	26	135	117	30	147	109	17	126
	0.1/1	61	26	87	79	23	102	61	9	70
	0.1/2	53	23	76	61	38	99	53	15	68
	0.1/3	60	23	83	72	33	105	59	12	71
	0.21/1	43	45	88	72	27	99	43	18	61
	0.21/2	92	66	158	114	68	182	90	44	134
	0.21/3	56	72	128	91	65	156	58	39	96
	0.32/1	29	135	164	56	129	185	25	108	133
	0.32/2	18	119	137	53	111	164	15	87	102
	0.32/3	10	111	121	24	113	137	9	100	109
	0.66/1	3	127	130	14	145	159	1	116	117
	0.66/2	1	84	85	15	96	111	1	70	71
	0.66/3	1	105	106	11	122	133	0	93	93
	1.0/1	1	104	105	13	131	144	1	94	95
	1.0/2	5	105	110	19	126	145	5	92	97
	1.0/3	2	82	84	14	98	112	1	72	73
	3.2/1	3	72	75	9	87	96	3	64	67
	3.2/2	2	120	122	17	136	153	1	103	104
	3.2/3	5	118	123	18	131	149	2	104	106
15/4/98	Control/1	80	19	99	89	33	122	79	12	91
	Control/2	91	24	115	108	39	147	92	9	101
	Control/3	83	19	102	90	32	122	83	12	95
	Control/4	114	34	148	131	46	177	114	16	130
	Control/5	102	21	123	113	29	142	102	9	111
	Control/6	106	28	134	124	44	168	106	11	117
	0.032/1	114	22	136	125	24	152	115	11	126
	0.032/2	115	19	134	127	27	154	115	7	122
	0.032/3	108	21	129	115	39	154	105	13	118
	0.1/1	102	33	135	115	37	152	101	19	120
	0.1/2	105	29	134	116	37	153	104	18	122
	0.1/3	105	24	129	117	28	145	105	13	118
	0.21/1	63	47	110	88	39	127	61	23	84
	0.21/2	78	69	147	99	72	171	75	45	120
	0.21/3	56	69	125	79	62	141	54	47	101
	0.32/1	5	75	80	17	79	96	3	63	66
	0.32/2	1	40	41	12	42	54	1	30	31
	0.32/3	8	92	100	20	84	104	6	80	86
	0.66/1	4	213	217	21	238	259	4	197	201
	0.66/2	2	103	105	10	115	125	1	93	94
	0.66/3	5	123	128	11	128	139	3	114	117
	1.0/1	6	110	116	19	127	146	4	96	100
	1.0/2	4	142	146	9	161	170	3	135	138
	1.0/3	1	122	123	10	139	149	1	113	114
	3.2/1	0	98	98	4	120	124	0	94	94
	3.2/2	1	81	82	10	92	102	1	72	73
	3.2/3	0	85	85	17	94	111	0	70	70