USE OF AUTOMATED IMAGE ANALYSIS TO DETERMINE ENDPOINTS IN THE OYSTER EMBRYO LARVAL DEVELOPMENT TEST

by W. Reynolds[1] and D. Leverett[2]

Introduction

The oyster-embryo larval development test (OEL) has been used for a number of years for direct toxicity evaluations of compounds and effluents, and in monitoring the marine environment.

The conventional method of assessing larval development, involving the visual assessment of normal to abnormal ratios, is inevitably subjective and relies on an accurate definition of what constitutes abnormality.

Imaging systems have now been developed which are capable of detecting and counting normal ('D' shaped) embryos based on a series of defining measurements (Johnson, *et. al*, 2001). The use of automated technologies in tandem with imaging facilities can provide accurate and repeatable observations that reduce the need for human input and minimise observational error.

The Environment Agency and CEFAS have applied this automated imaging technology to the OEL test and addressed a series of technical issues that have enabled the development of a robust, consistent technique. Recent work has focussed on the validation of the methodology within the two laboratories for use within routine testing programmes, while a limited intra-laboratory validation exercise has also been initiated.

Methods

The OEL test was performed according to the Ecotoxicity Test Methods for Effluent and Receiving Water Assessment – Comprehensive Guidance (Environment Agency 2001). Test replicates contained a 3ml dilution / sample within 12 well multiwell plates. Zinc sulphate was used as a reference toxicant in a standard logarithmic concentration series (expressed as Zn).

Preserved larvae were scored, *in situ*, using imaging methodologies and by conventional manual assessment. Results were analysed by initially calculating a Percentage Net Response (PNR) value for each test replicate. Individual PNR values were then used to calculate point estimates in the reference tests.

Four intra-laboratory reference tests were performed at the Environment Agency Biological Effects Laboratory to compare and validate the two assessment methods. These were supported by a series of environmental samples (seawater) which were tested without dilution. An inter-laboratory validation reference test was also carried out at the Agency Laboratory. Identical samples from this test were scored by the Agency and CEFAS laboratories and EC_{50} values calculated.

The collection of robust data suitable for statistical analysis using imaging systems follows a 2 step process of image acquisition and subsequent extraction of image information (Figure 1).

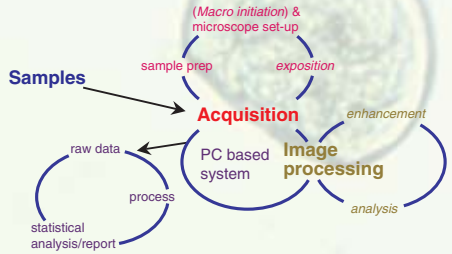


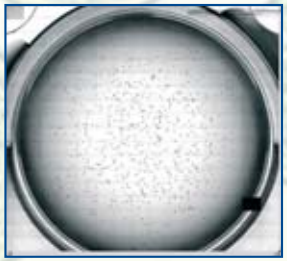
Figure 1: Acquisition of data for the OEL test (modified from Pontual et. al, 1997)

Image analysis methodology

Observations were made using an inverted microscope with motorised x-y stage using brightfield illumination. Image capture was provided by a digital camera, with live feed, using a PC based image capture and processing facility (Scope Pro, Media Cybernetics, USA).

Image analysis system

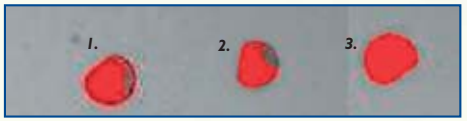
The use of a motorised stage permits the movement of the sample, under an objective, in an orderly, accurate and repeatable manner, such that specific areas of interest are covered. A macro (computer script containing a repeatable set of instructions) was written to fully automate the procedure of scanning and photographing the wells of a 12 well multiwell plate and to tile individual images together to form high magnification composite images.



Composite well plate image

The composite images were manipulated in the following way to extract the 'D-shaped' embryos from the sample:

1) Segmentation – this is the level of definition between light and dark pixels within a gray scale image and is the process by which objects in an image are identified and isolated from the image as a whole. Uncontrollable variation in the levels of light and dark within and between composite images (e.g. caused by slightly differing ambient light conditions or test solution volume) can excerpt a detrimental effect on the analysis of an image and cause counts to be inaccurate. Even very small changes in the segmentation value of an image can be the source of significant inaccuracies in analysis owing to erosion or expansion of objects within the image beyond their natural boundaries (See Figure 2a). A suitable procedure for selecting and calibrating the correct segmentation value for each image is essential.



1. *Larva showing erosion owing to low segmentation value.*
2. *Larva showing erosion after application of auto-segmentation tool.*
3. *Correctly segmented larva*

Figure 2a: Segmentation of 'D'-shaped larvae from background image

2) Application of parameters – measurements defining 'D' shape for oyster larvae were applied to the segmented image so that only 'normal' larvae were counted. These parameters (given below) have been developed from those used by Johnson *et. al* (2001) by measuring 2500 larvae (exposed only to seawater) and determining the ranges representing the most 'normal' larvae at a 0.1 critical level.

Figure 2b: Segmentation and extraction of 'D'-shaped larvae from image

Table 1: Parameters for extraction of 'D'- shaped oyster embryos from segmented image

Measurement Parameter	Range prescribed by Johnson *et al* (2001)	Range at 0.1 critical level (2500 larvae)
Area (um²)	2960-4922	2958-4700
Aspect	1.06-1.37	1.06-1.28
Perimeter (um)	192-238	153.3-250
Radius Ratio	1.34-1.87	1.27-1.78
Width (um)	54-70	45.6-75
Length (um)	63-86	53.33-85

Results

Table 2: Results of reference tests assessed using conventional and image analysis

Test	Visual EC_{50} (95% Fiducial Limits)	Image Analysis EC_{50} (95% Fiducial Limits)
1	0.114 mg/L (0.0905-0.132)	0.167 mg/L (0.146-0.190)
2	0.115 mg/L (0.113-0.117)	0.0523 mg/L (0.0482-0.0568)
3	0.0414 mg/L (0.0394-0.0432)	0.0802 mg/L (0.0724-0.0888)
4	0.141 mg/L (0.135-0.147)	0.225 mg/L (0.190-0.266)

Having established the equality of variance between the means of the EC_{50} values, and normality of distribution within the groups, a paired t test was applied to the data. No significant statistical difference ($p<0.05$) was detected between the results generated by visual and image analysis assessment ($p=0.547$).

The EC_{50} values were additionally plotted on the current Schewart Process Control Chart for the Environment Agency Biological Effects (Figure 3). All eight values lay within the warning and action limits of the chart (derived from 166 OEL tests performed between 1996-2003).

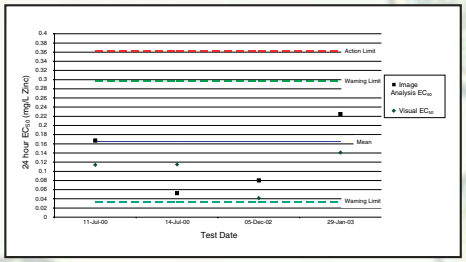


Figure 3: OEL Process Control Chart (Limits as on 18/02/03)

Table 3: Results of receiving water tests assessed using conventional and image analysis

Test	Visual PNR	Image Analysis PNR
1	1.12	13.5
2	0	9.43
3	88.9	73.9
4	61.5	40.8
5	99.2	70.6
6	30.8	18.7
7	12.4	29.8
8	12.9	5.23
9	3.75	12.8

As with the zinc results, the PNR values were checked for equality of variance and normality before applying a paired t test to investigate statistical differences. No significant statistical difference ($p<0.05$) was detected between PNR values generated from visual or image analysed raw data ($p=0.488$).

Table 4: Results of inter-laboratory reference OEL test using image analysis with automated x-y stage

Endpoint	EA	CEFAS
NOEC	0.1	0.1
LOEC	0.32	0.32
EC_{50}	0.18 (0.14-0.22)	0.23 (0.21-0.25)

Summary

- A technique has been developed that allows the automatic assessment of 'D' shaped larvae in the OEL test by image analysis using a motorised x-y stage to capture images. The use of macros in tandem with the x-y stage produces tiled photographs of entire test vessels. This provides an accurate and reproducible method of producing the highest quality images for subsequent analysis.
- The application of optimal defining measurement parameters and segmentation values to images prior to analysis is critical in obtaining accurate counts of normal 'D' shaped larvae.
- Using the procedures described, no significant statistical differences were found between test results (EC_{50} or PNR) derived from conventional assessment or image analysis of identical tests.
- Initial intra-laboratory validation of the techniques described has been undertaken and is on-going at the Environment Agency and CEFAS laboratories.

References

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Pontual, H., Robert, R. and Miner, P., 1998. Study of bivalve growth using image processing. *Aquacult. Eng.* **17**: 85-94.

Johnson, I., Harman, M., Forrow, D., Norris, M., 2001. An assessment of the feasibility of using image analysis in the oyster embryo-larval development test. Environ. Toxicol. **16**: 68-77

[1]The Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Burnham Laboratory, Remembrance Avenue, Burnham on Crouch, Essex CM0 8HA, UK. http://www.cefas.co.uk

[2]Environment Agency, Biological Effects Laboratory, 4 The Meadows, Waterberry Drive, Waterlooville, Hampshire PO7 7XX, UK.