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Review of the Enhanced Chemiluminescence (ECL) Test

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Review of the Enhanced Chemiluminescence (ECL) Test

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EXECUTIVE SUMMARY

This review was produced to assess the current availability of information on the enhanced chemiluminescence test in relation to the criteria developed for method selection in Phase 1 of Environment Agency Project 494 (Ecotoxicology Method Development).

The enhanced chemiluminescence (ECL) test is available as test kits from Aztec Environmental (ECLOX™ system) and Randox Laboratories (AQUANOX™ system). Available information shows the test to be:

- easy to use, with staff capable of demonstrating proficiency after conducting 5 repeat reference toxicant tests;
- rapid, with single concentration tests taking about 10 minutes and full (5) concentration range tests taking about 30 minutes;
- cost-effective, with tests costing from £7 for a single concentration test to £21-22 for a full concentration range test;
- repeatable with coefficients of variation (CV's) for IC_{50} values from repeat concentration range tests with the reference toxicant phenol in a laboratory always being less than 17% and often less than 5%;
- readily available as reagents can be stored for 3 months in a refrigerator.

Further information is needed on the response of the system to a wider range of substances representative of different modes of toxic action including non-polar narcotics, uncouplers of oxidative phosphorylation, cholinesterase inhibitors, central nervous system (CNS) convulsants and photosynthetic inhibitors.

It is also important to clarify:

- the mechanisms by which toxicants with different modes of action affect the enhanced chemiluminescence reaction;
- the apparent incongruity between the high sensitivity of the test for environmental samples (relative to higher organism tests) with the low sensitivity shown for many pure substances (relative to higher organism tests).

There is a ultimately a need for a standardised generic guideline for the chemiluminescence test which considers issues relating to parameters such as test temperature, the effects of sample pH, salinity and turbidity and the most appropriate means of analysing data. This standard guideline can then be ring tested to provide information on test method performance, in terms of both repeatability (variability in responses to a test substance in a laboratory over time using the same equipment) and reproducibility (variability in responses to a test substance in different laboratories using the different equipment). The ring test will need to consider the issue of

sensitivity of different reagent formulations and the causes of any major differences will need to be addressed in the standard operating procedure to ensure that the reproducibility of the test meets defined acceptability criteria.

At present the ECL test can be used by the Environment Agency to identify pollution 'hotspots' requiring further investigation. The ECL test can apparently discriminate between stations of differing biological quality from good (clean) to poor/bad in a similar manner to higher organism tests. In this context the test may be measuring the effects of both toxicants and/or BOD but this is not a limitation if the test is used as a rapid and cost-effective screen of water column pollution. The ECL test can also be used to screen effluents for toxicity but in this role test responses have not yet been shown to be surrogates for those in higher organism tests. Further work is needed to establish whether for complex effluent samples the ECL test and higher organism tests are responding to the same parameters if the ECL test is to be used to set action levels to control discharge toxicity. This could be investigated by studying the relative responses of the ECL system to BOD and individual toxicants in mixtures and trying to separate out whether BOD or toxicity causes ECL response at different ratios.

KEY WORDS

Enhanced chemiluminescence, toxicity test, ECLOX™, Aquanox™.

1. INTRODUCTION

The Environment Agency in collaboration with the Scotland and Northern Ireland Forum for Environmental Research has instigated project EMA 003 'Ecotoxicological Method Development' which has the objective of identifying and developing ecotoxicological methods which are considered appropriate for use in particular regulatory operational roles. The initial output of this project was a criteria-based selection procedure (Johnson 1995). In this approach candidate methods for a particular operational role are scored against the criteria given below:

- ease of use
- cost of implementing a test
- cost of conducting the test
- rapidity of the test
- sensitivity
- graduation of response
- spectrum of response
- standard operating procedure
- test method precision (repeatability and reproducibility)
- test substrate or organism variability
- availability of test substrate and organisms
- indigenous test species
- importance of test species
- ecological relevance
- Home office regulations
- previous application to an operational role

In a review of methods for assessing the toxicity of effluent and leachate discharges the *E*nhanced *C*hemiluminescence (ECL) test was identified as a potentially useful method. Subsequently the test was used as a candidate rapid test in the Toxicity-Based Consents Pilot Study (Project 493) and the Toxicity-Based Criteria for Receiving Waters Study (Project 703). Further development work has also been carried out at the Wolfson Applied Technology Laboratory, University of Birmingham, where the test was developed.

The Environment Agency is now considering which methods to pursue for further development and, for these methods, to identify the work needed to achieve a robust test procedure which, if appropriate, can be included in the Direct Toxicity Assessment (DTA) Methods Guidelines. Therefore, a review of the current status of the method has been completed to assess how the method scores against the selection criteria and the areas of further work which need to be carried out to produce a standardised generic guideline for the chemiluminescent test.

The review has been prepared using all currently available information including data obtained from the Wolfson Laboratory, Aztec Environmental Ltd and Randox Ltd relating to responses for substances representative of different modes of toxic action. Commercially sensitive information has been coded where necessary.

2. INFORMATION ON THE TEST METHOD

2.1 Principle

Chemiluminescence results from a chemical reaction in which molecules undergo a transition from an excited state to a lower energy state (usually the ground state). The chemiluminescent molecule absorbs free energy released by the chemical reaction to form an excited intermediate product which then loses its energy as a photon of light.

In most aerobic biological systems which emit light, this occurs by bioluminescent reactions generally involving the action of a 'luciferase' enzyme on a 'luciferin' substrate. A range of chemical reactions also emit light. Typical of such chemiluminescent reactions is the oxidation of luminol by oxidants such as hydrogen peroxide or sodium perborate which can be catalysed by horseradish peroxidase (HRP). Such reactions can be performed under alkaline conditions (pH >10), however, light is emitted as a flash which is difficult to quantify.

Luminol + Oxidant + Catalyst -------> Light (Flash, pH > 10)

High intensity, prolonged and stable light emission can be obtained from the horseradish peroxidase chemiluminescent oxidation of luminol under milder conditions (pH 8.5) by the inclusion of an enhancer (such as para-iodophenol) (see Figure 2.1, after Whitehead *et al.* 1992).

Luminol + Oxidant + Catalyst + Enhancer -------> Light (Glow, pH 8.5)

The chemiluminescence test involves a free radical reaction between a hydrogen acceptor molecule (oxidant) and a hydrogen donor molecule (luminol) in the presence of the enhancer.

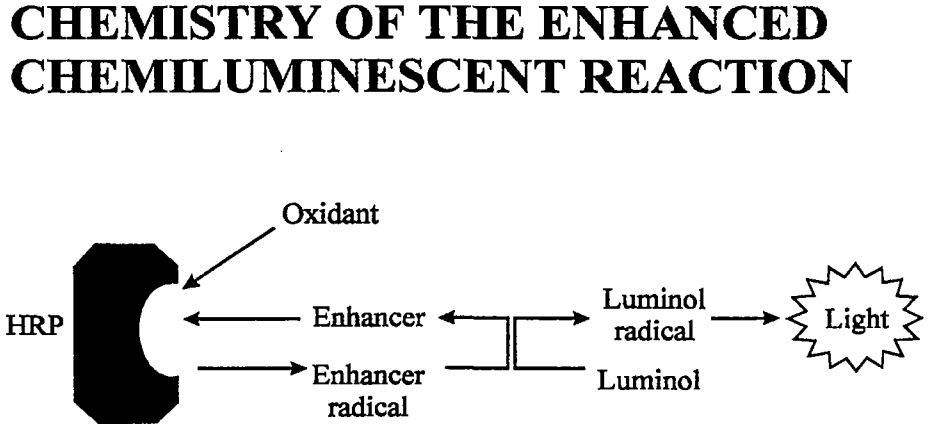

Figure 2.1 Schematic representation of an ECL reaction (after Whitehead *et al.* 1992)

Contaminants which would be expected to interfere with the ECL reaction include:

- radical scavengers (such as antioxidants which remove oxygen from water and scavenge free radical molecules)
- competitive enzyme inhibitors
- non-competitive enzyme inhibitors
- miscellaneous influences on general chemiluminescent reactions

The HRP enzyme has an 'oily' active site and numerous compounds (such as phenols, amines and benzothiozoles) can 'fit' into this site and alter the activity of the enzyme (Sawcer Pers. Comm.).

2.2 Test procedure

2.2.1 Reagents

The ECL test can be carried out using reagents supplied by two licensed organisations, Aztec Environmental Ltd and Randox Laboratories Ltd. The reagents are provided in different ways as shown below:

Supplier	Reagents	
	Component	Form
Aztec	1. Luminol and p-iodophenol	Liquid
	2. Sodium perborate	Liquid
	3. Horse radish peroxidase	Liquid
Randox	1. Luminol, sodium perborate and p-iodophenol	Liquid
	2. Horse radish peroxidase	Liquid

The different test systems use different volumes of reagents and potentially different ratios of reagents which may have implications for the sensitivity of the test.

2.2.2 Luminometers

The test can be carried out using both portable and laboratory-based luminometers including:

- the ECLOX™ system (Aztec Environmental Ltd);
- the Aquanox™ system (Randox Laboratories Ltd);

- the BioOrbit 1250 luminometer;
- the BioOrbit 1251 luminometer.

The ECLOX™ and Aquanox™ systems and the BioOrbit 1250 luminometer require all the reagents to be dispensed manually. The BioOrbit 1251 luminometer allows all the reagents to be added automatically.

2.2.3 Test procedure

In the test procedure a reference water blank is initially measured by adding a volume of a de-ionised or HPLC water to a sample cuvette and then adding appropriate volumes of luminol, sodium perborate and para-iodophenol. Finally an appropriate aliquot of the enzyme reagent is added to the cuvette, which is then agitated and introduced into the luminometer. The light output over time (usually 4 minutes) is then displayed and stored. The process is then repeated for each sample and the data analysed.

2.2.4 Data analysis

In the test the response measured for each sample (whether a control, solution of a pure substance or environmental sample) is the integral of the area under the light emission curve over a 4 minute period.

The percentage inhibition in light emission of a sample can be calculated using the formula:

% Inhibition = [(Integral of control - Integral of sample)/Integral of control] x 100

This approach is appropriate for developing concentration-response curves for tests of pure substances or effluents where a dilution series may be used.

For receiving waters, where only the undiluted sample may be tested ECLOX Units can be calculated using the formula:

ECLOX Unit = (% Light inhibition/100) x (Total volume/Sample volume) x Dilution x 10

The total volume is the total working volume including reagents while the sample volume is the actual volume of sample tested. The dilution factor takes into account any modification of the sample before it is tested. ECLOX Units can be most representatively calculated using % inhibition data which falls between 20 and 80%. The shape of the response curves may also provide diagnostic information on the type of substances present in a sample. Curves for pure substances can be classified into four types (Thorpe, Pers. Comm.): convex, concave, s-shaped and square wave (Figure 2.2). Further details on the types of pure chemicals and effluents producing each shape of curve are given in Section 4.5.

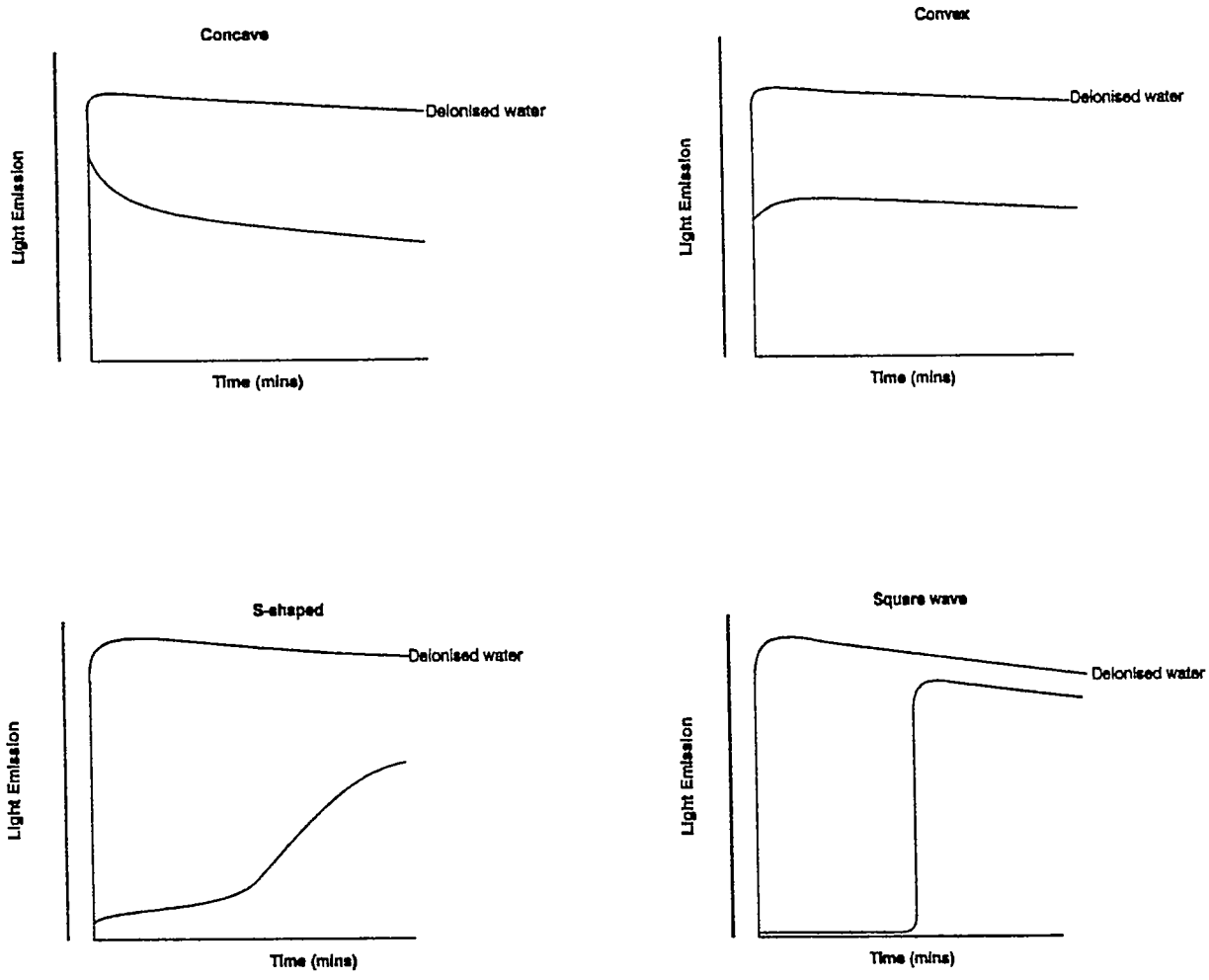

Figure 2.2 Shapes of curves recorded for different types of toxicants in the ECL test

Data analysis represents an area where additional research may be required particularly with regard to:

- how the integral of the control is used in the calculation of IC_{50} values;
- whether a consistent ECLOX Unit is derived if different ratios of sample to clean water are used.

2.3 Impact of environmental conditions

2.3.1 Dissolved oxygen

Chappell and Wright (1995) investigated whether the level of dissolved oxygen in a sample has an important effect on the ECL reaction by testing tap water and river water samples at different percent oxygen saturations (see Table 2.1). These were prepared by bubbling the samples with different oxygen/nitrogen mixtures. The data showed no measurable differences in mean 4 minute integrals at the different O_2 levels for either the tap water or river water samples.

Table 2.1 Mean 4 minute integrals of reference water samples of different dissolved oxygen concentrations using Aztec reagents (SD = Standard deviation)

Dissolved oxygen (% saturation)	4 minute integrals for tap water		4 minute integrals for river water	
	Mean	SD	Mean	SD
2 ± 2	9.3	6.0	78.4	3.2
42 ± 2	10.1	4.8	76.7	3.4
71 ± 4	7.9	7.2	73.2	3.0
100 ± 5	13.7	5.6	74.4	2.5
152 ± 5	7.7	4.6	75.1	0.9
194 ± 10	13.2	6.4	73.9	3.3

2.3.2 Temperature

Experiments carried out by Hall (1995) with Aztec reagents have shown that light output in reference water samples increases with temperature from 4.0 to 14.5 °C after which there was a plateau to 25 °C (see Table 2.2). The tests indicated that sample temperature can have an important influence on light output and should, therefore, be standardised as far as practical to avoid variations between results. However, by performing the test and reference method reasonably close together in time and at the same temperature any effect due to temperature should be further minimised. If the reference reaction and sample reaction are carried out

between 15 and 20 °C there would appear to be no requirement for involved methods of temperature control (Thorpe, Pers. Comm.). Temperature effects may be most problematic where measurements are made in the field and samples are subsequently re-analysed in the laboratory.

Table 2.2 Mean 4 minute integrals of reference water samples at different temperatures using Aztec reagents

Sample temperature (°C)	4 minute integral	
	Mean	Standard deviation
4.0	235	3.5
7.0	248	3.4
14.5	276	3.9
25.0	277	5.9

2.3.3 pH

Thorpe *et al.* (1985) investigated pH effects on the ECL reaction and showed that the enhancement of the chemiluminescence reaction by para-iodophenol is markedly pH dependent with significant increases in light emission occurring between pH 7.0 and 9.5. The maximum light intensity was recorded at approximately pH 8.6. The Aztec and Randox reagents both contain buffers to ensure that the pH is optimum for the enhanced reaction and to maintain the pH when the sample is added. In addition to the buffering capacity of the buffer used in the reagent the initial pH of the sample and the volume added will determine the pH of the final mixture when the sample has been added. The Aztec and Randox systems are designed to maintain test pH at an optimum level of around 8.6 when sample pH's range from 3 to 11.

2.3.4 Salinity

Experiments carried out by Hall (1995) with Aztec reagents have shown that light output increases with increasing conductivity (see Table 2.3). As conductivity affects light output it was recommended that the conductivity of the controls is adjusted to that of the samples (by the addition of Sigma sea salt) to prevent conductivity-influenced sample effects.

Table 2.3 Mean 4 minute integrals of reference water samples of different conductivities using Aztec reagents

Sample conductivity (mS)	4 minute integral	
	Mean	Standard deviation
0	879	15.6
10	1018	12.1
20	1118	6.8
30	1233	16.7
40	1314	7.5

2.3.5 Turbidity

Testing of the Aquanox system carried out by Randox has indicated that the test is not significantly affected by turbid samples. Sewage samples up to 800 FTU were tested with no significant interference of the test response.

2.3.6 Coloured samples

Testing of the Aquanox system carried out by Randox has indicated that the test is not significantly affected by non-toxic coloured samples. Samples of yellow, green and red dye did not cause significant interference with the Aquanox response due to their colour. The coloured dyes were introduced into the reaction vessel but they were physically isolated from the ECL reaction by enclosure in a separate vessel.

3. PREVIOUS APPLICATION TO OPERATIONAL ROLES

3.1 Effluents

The chemiluminescence test was used in the battery of methods to characterise the 12 sewage and industrial discharges released to fresh and marine waters which were investigated as case study discharges in the Environment Agency/SNIFFER funded Toxicity-Based Consents Pilot Study. The other tests used were the rapid Microtox test as well as conventional algal growth inhibition and lethality tests, *D. magna* immobilisation tests, oyster embryo-larval development tests, *A. tonsa* lethality tests and fish lethality tests. The ECL test consistently identified all case study effluents as toxic (see Table 3.1) but discriminated between the different discharges. The sensitivity of the ECLOX test ranged from IC_{50} values of 0.007-0.029% effluent for discharge 7 to 35->80% effluent for discharge 38. For all effluents, except number 38, ECLOX IC_{50} values were comparable to NOEC values in the most sensitive of the higher organism tests. The ECL test generally showed greater sensitivity relative to the Microtox test which may reflect the easier accessibility of toxicants to target sites in the ECL test due to the absence of a membrane wall in the test system.

Table 3.1 Range of toxicity values measured for different case study effluents with the ECLOX test

Effluent number	Effluent type	Range of ECL IC_{50} values (% effluent)
8	Sewage treatment works	0.48-1.85 (n=8)
12	Chemical manufacturing	1.0-1.6 (4)
14	Sewage treatment works	5.2-9.0 (8)
60	Sewage treatment works	4.9-8.6 (8)
1	Chemical manufacturing	0.57-2.9 (8)
5	Chemical manufacturing	2.7-12.5 (8)
7	Chemical manufacturing	0.007-0.029 (8)
19	Chemical manufacturing	0.22-0.59 (8)
31	Sewage treatment works	1.05-1.85 (8)
38	Chemical manufacturing	35->80 (8)
47	Plastic manufacturing	0.36-1.55 (8)
50	Gas separation	0.72-5.9 (8)

Hayes and Smith (1996) described a toxicity tracing exercise using the ECLOX system which was carried out at four sewage treatment works representing a broad diversity of treatment processes and receiving different levels of domestic and industrial inputs (see Table 3.2). The chemiluminescence test tracked toxicity removal throughout each works and in all cases the results were correlated with BOD_5 values ($r^2 > 0.85$) . This finding is consistent with a previous study using the ECLOX system carried out by Billings *et al.* (1993) which showed a correlation coefficient of 0.91 between chemiluminescence data (as the 4 minute integral) and BOD_5 values of 0 to 200 mg l^{-1} for a range of water samples and a correlation coefficient of 0.96 between chemiluminescent data and COD values over the range 20 to 600 mg l^{-1}.

Table 3.2 Toxicity tracing through treatment works using the ECLOX system

Works	Description	Sample	ECLOX data			BOD_5 ($mg\ l^{-1}$)	COD ($mg\ l^{-1}$)	Ammonia as N ($mg\ l^{-1}$)
			% Area inhibition	ECLOX number	% Toxicity reduction			
A	Large manned works treating both domestic and industrial waste from a large W. Midlands city	Crude sewage	89	445	-	227	583	29
		Settled sewage	84	418	6	144	351	34
		Humus tank effluent	46	228	49	18	99	12
		Final effluent	27	134	70	7	57	4
B	Large manned works treating both domestic and industrial waste with a significant proportion of textile effluent	Crude sewage	94	471	-	263	839	72
		Settled sewage	87	436	7.3	157	403	37
		Activated sludge plant effluent	45	224	53	6	57	14
		Final effluent	32	158	67	4	55	0.5
C	Small unmanned works treating only domestic waste from a small number of villages	Crude sewage	94	470	-	291	701	32
		Settled sewage	78	392	17	255	627	41
		Humus tank effluent	20	102	78	7	56	5
		Final effluent	20	98	79	2	8	0.7
D	Medium sized works with a major discharge from food processing	Crude sewage	97	486	-	738	2143	49
		Settled sewage	96	482	1.0	305	637	42
		Humus tank effluent	30	152	69	14	88	3
		Nutrient removal plant effluent	26	128	74	2	40	0.3
		Final effluent	31	157	68	6	56	0.7

Studies carried out with the AQUANOX system on seawater samples contaminated with sewage have also shown correlations between the ECL test response and BOD_5 (r = 0.977) and COD (r = 0.975) values.

Environment Agency (Thames Region) are carrying out an operational investigation using the ECL test in which the effects of sewage works effluent discharges on receiving water quality are being investigated. Table 3.3 shows the level of light inhibition in effluent and receiving water samples taken at 18 locations. The level of inhibition found in effluent samples (using a standard 200 µl aliquot) varied from 31 to 90%. At eight locations these sewage treatment works discharges were identified as causing a downstream impact as shown by increased light inhibition relative to the upstream location.

Table 3.3 Light inhibition measured in the ECL test in effluent and receiving water samples taken at 18 locations in Environment Agency - Thames Region

Sewage Treatment Works	Receiving water	NGR	Sampling date	% inhibition		
				River u/s	Effluent	River d/s
Barkway	Quin	TL38793477	8.6.95	-	71	-
Braughing	Rib	TL39402680	8.6.95	-	57	-
Buntingford	Rib	TL36402865	7.6.95	23	31	23
Chapman End	Rib	TL33201671	28.2.95	13	43	23
Clavering	Stort	TL47683168	28.2.95	16	48	18
Deephams	Salmon Bk	TQ35669317	27.2.95	44	64	60
Epping (Fiddlers	Brookhouse	TL47790038	21.3.95	33	66	61
Hamlet)	Bk		13.6.95	25	62	63
			15.2.96	22	74	73
Great Gaddesdon	Gade	TL03101120	26.6.95	16	43	20
Gerrards Cross	Misbourne	TQ01908750	17.7.95	20	51	26
Hatfield (Mill Green)	Lee (Tributary)	TL25000970	30.10.95	50	54	50
Luton (East Hyde)	Lee	TL12301780	7.8.95	12	39	33
Manuden	Stort	TL49322642	28.2.95	18	33	17
Maple Lodge	Colne	TQ04209200	5.7.95	11	83	64
North Weald	N.Weald Bk	TL49600460	15.2.96	21	85	20
Rye Meads	Tollhouse	TL39250975	28.2.95	43	79	-
	stream		16.10.95	-	80	-
			12.12.95	-	90*	-
			13.2.96	-	84*	-
Therfield	Rib	TL34283469	28.2.95	18	31	18
Thornwood	Cripsey Bk	TL47700500	15.2.96	22	57	44
Basingstoke	Loddon	SU68005520	27.9.95	5	63	36
			14.11.95	27	86	62
			22.1.96	17	69*	39*

All samples volumes 200 µl except * where 100 µl used

3.2 Receiving waters

The portable ECLOX test system has been used to assess the quality of a number of river systems in the United Kingdom and the Republic of Ireland. Receiving waters monitored include Letcombe Brook (Oxfordshire), the Rivers Avon. Blythe, Cole, Sowe and Tame (Warwickshire) and the Rivers Barrow, Nore and Suir (Republic of Ireland). The test discriminated between sites with 'good' quality receiving waters (that is those which are unimpacted sites) having ECLOX Units (see page 7) of <5 and 'bad' quality sites (that is those that are impacted) having ECLOX Units >40.

A study of 18 sites on the River Avon showed that ECLOX responses for receiving water samples (as ECLOX numbers) were not correlated with dissolved oxygen concentrations, which is consistent with data obtained in the laboratory by Chappell and Wright (1995).

The ECL test was one of a large battery of tests used in the Toxicity-based Criteria for Receiving Waters Study (Project 703) to assess receiving water toxicity at freshwater and estuarine sites. The objective of the study was to identify a cost-effective battery of tests which could be used as part of a general quality assessment scheme and also in local environmental impact assessments. Laboratory-based and *in situ* water column and sediment tests were used at four case study sites which showed a gradation of biological quality from good to poor/bad (see Table 3.4). The two freshwater sites used were the River Aire (Yorkshire) and Willow Brook (Northamptonshire) while the estuarine sites used were the Tees and Mersey. At each site eight sampling/deployment stations were used and the tests were deployed between two and four times during a July-October period.

Table 3.4 Biological survey data for the station used at each of the four receiving water study sites (ND = No data)

Site	Biological index	Biological survey data at each station							
		1	2	3	4	5	6	7	8
River Aire	BMWP	149	68	27	ND	47	42	ND	22
Willow Brook	BMWP	19	12	55	49	60	83	28	136
Tees Estuary	No. of taxa	12	11.4	ND	15.4	7.4	ND	14.4	25.2
Mersey Estuary	No. of taxa	ND	ND	12.8	6.8	ND	5.8	ND	5

Samples from the eight locations on the River Aire and Tees Estuary were analysed on four occasions while for the Willow Brook and Mersey Estuary samples were tested on three occasions. Other water column tests carried out at the freshwater sites were the 7 day *L. minor* growth test and the 10 day *D. magna* reproduction test while at the estuarine sites the oyster embryo-larval development test and the *T. battagliai* reproduction test were used. The Microtox acute test was used alongside the ECL test to assess whether rapid tests discriminated between stations in a similar way to higher organism tests.

4. ASSESSMENT OF THE METHOD AGAINST SELECTION CRITERIA

4.1 Ease of use

In the selection/development procedure ease of use is assessed as the time taken for staff to become proficient with a test. Proficiency is considered to be achieved when testing an appropriate reference substance in a series of tests (usually 5 or 6 as a minimum) leads to similar test results (IC_{50}, EC_{50} or LC_{50} values) and a threshold coefficient of variation which does not get markedly lower with further testing. The coefficient of variation for each substance is calculated using the equation:

Coefficient of variation = (Standard deviation/Mean) x 100

Information is available for repeat testing with the reference substance phenol using Aztec reagents and both BioOrbit 1250 and 1251 luminometers (see Table 4.1). It is evident that the coefficients of variation for the both the BioOrbit 1250 and 1251 luminometers were in the same range after 5 tests. The CV's obtained by different operators after 5 tests were always less than 20% irrespective of the luminometer used and could be as low as 4-6%. The data indicate that operators can become proficient in the use of the chemiluminescence method after conducting 5 repeat reference toxicant tests, which would take approximately 3 hours.

Table 4.1 Summary of the IC_{50} data generated by different operators with the reference toxicant phenol using Aztec reagents and BioOrbit 1250 or 1251 luminometers

Test number	IC_{50} data for phenol using BioOrbit 1250		IC_{50} data for phenol using BioOrbit 1251		
	Operator 1	Operator 2	Operator 1	Operator 2	Operator 3
Mean for 5 tests	0.96	0.93	1.08	0.98	1.05
SD for 5 tests	0.055	0.18	0.12	0.04	0.11
CV (%) for 5 tests	5.7	19.4	11.1	4.1	10.5

4.2 Rapidity of the test

The time involved in conducting the procedure is essentially the same irrespective of the system (reagents and luminometer) used, being approximately 10 minutes for a single concentration test (that is a control and one treatment) and 30 minutes for a toxicity test consisting of a control and 5 treatment concentrations).

4.3 Cost of implementing the test

Table 4.2 shows the cost of purchasing the different luminometers which can be used with the method. The costs of the portable ECLOX and Aquanox luminometers and the laboratory BioOrbit 1250 luminometer which allow a single sample to be measured at one time are similar at £4250-6400, whereas the cost of the automated multi-sample BioOrbit 1251 luminometer is approximately 2.5-3.7 times higher.

Table 4.2 Costs of the different luminometers

Type of luminometer	Cost of luminometer (£)
ECLOX	5 000
Aquanox	6 400
BioOrbit 1250	4 250
BioOrbit 1251	15 800

4.4 Cost of conducting the test

Table 4.3 shows the cost of conducting both single concentration tests (with an accompanying control) and a toxicity test (that is with a control and five treatment concentrations) using the different reagents. The staff time in conducting a particular procedure was calculated using a rate of £30/hour. The costs for conducting tests are not markedly different for the two sources of reagents.

Table 4.3 Cost of conducting the ECLOX test using Aztec and Randox reagents

Supplier	Cost for a single concentration test (£)			Cost for a toxicity test (£)		
	Staff	Materials	TOTAL	Staff	Materials	TOTAL
Aztec	5	2	7	15	6	21
Randox	5	2.4	7.4	15	7.2	22.2

4.5 Sensitivity, spectrum of response and graduation of response

In the selection procedure the sensitivity, spectrum of response and graduation of response criteria are assessed using data on test responses for substances representative of different modes of toxic action (such as non-polar narcotics, polar narcotics, uncouplers of oxidative phosphorylation, cholinesterase inhibitors, membrane irritants, CNS convulsants, respiratory

blockers, cell division inhibitors, photosynthetic inhibitors and heavy metals). Sensitivity of a test is assessed by calculating a sensitivity index (SI) for each test substance representative of a mode of toxic action using the equation:

SI = Toxicity value/Lowest algal growth, invertebrate reproduction or fish growth NOEC

The sensitivity of a test is judged as the lowest sensitivity index for any of the representative substances (that is the lowest values show the highest sensitivity). Spectrum of response is judged by the number of specific modes of toxic action which show a sensitivity index of ≤100. Graduation of response reflects the number of representative substances with different modes of toxic action which cause intermediate rather than all or nothing responses.

4.5.1 Sensitivity to substances representative of different modes of toxic action

Table 4.4 shows the toxicity test data for substances representative of different modes of toxic action generated using the BioOrbit 1250 luminometer with Aztec reagents. The test showed greater sensitivity (that is a sensitivity index of <100) for the polar narcotic phenol, the membrane irritant chlorine and the respiratory blocker cyanide. The lowest SI (0.13) was recorded for phenol. Sensitivity indices higher than 100 were found for the heavy metals mercury and zinc and the cell division inhibitor trifluralin indicating lower sensitivity of the test to these substances. Information on substances other than those representative of different modes of toxic action is given in Table A1 of Appendix A.

Paterson (1996) showed that for three metal cations (copper, magnesium and zinc) the type of anion (chloride, nitrate or sulphate) was found to affect the response of the ECLOX test to each of the metals. This showed that chloride compounds tended to be the most toxic, with nitrate compounds of intermediate toxicity and sulphate compounds being the least toxic.

Table 4.5 shows the sensitivity of the ECL test to substances representative of different modes of toxic action using three different reagent formulations. The formulations have been coded for confidentiality but the point of interest relates to the difference in sensitivity of the different formulations. From the IC_{50} data the extent of differences ranged from 1.8x for phenol to 59x for cyanide, whereas using the LOD (IC_5) data differences ranged from 1.1x for chromium to 1429x for phenol. Differences for the metals aluminium, copper and lead also fall within these ranges. The differences in sensitivity between the LOD and IC_{50} values for a given formulation reflect the slope of the concentration-response curve. Although different reagent formulations can show large differences in response to pure chemicals these differences are not as marked for effluents. The extent of differences in sensitivity to pure substances between reagent formulations may have implications for the reproducibility of the test using a generic standard operating procedure (see Section 4.7).

4.5.2 Spectrum of response to substances representative of different modes of toxic action

The data for the ECLOX test using Aztec reagents indicate that it meets the test criterion for 3 of the 6 modes of toxic action for which information is available. Information is not available for substances representative of a further 5 modes of toxic action and for the heavy metal chromium.

Table 4.4 Toxicity data for substances representative of different modes of toxic action to the Aztec reagents measured using a BioOrbit 1250 luminometer (ND = No data)

Substance	Toxicity test data		Sensitivity index
	LOD/IC_5 (mg l^{-1})	IC_{50} (mg l^{-1})	
Heavy metals			
Cadmium	247	1114	1 856 667
Chromium	0.32	ND	-
Mercury	0.3	15.6	4 588
Zinc	287	630	5 250-12 600
Non-polar narcotics			
Toluene	ND	ND	-
Polar narcotics			
Phenol	0.056	0.5	0.13
3,5-Dichlorophenol	1.3	37	>17.6
Uncouplers of oxidative phosphorylation			
Pentachlorophenol	ND	ND	-
Cholinesterase inhibitors			
Azinphos-methyl	ND	ND	-
Malathion	25	ND	-
Membrane irritants			
Chlorine	0.004	0.032	2.91
CNS convulsants			
Endosulphan	1.2	ND	-
Endrin	ND	ND	-
Respiratory blockers			
Cyanide	0.0052	0.38	38
Cell division inhibitors			
Trifluralin	2.7	11.7	4 875
Photosynthetic inhibitors			
Atrazine	13	ND	-

Table 4.5 Summary of the sensitivity of the ECL test using different reagent formulations (A, B and C)

Substance	LOD for different formulations (mg l^{-1})			IC_{50} for different formulations (mg l^{-1})		
	A	B	C	A	B	C
Cadmium	247	2.75	27.5	1114	562	343
Chromium	0.32	0.36	0.36	-	-	230
Mercury	0.3	0.032	0.32	15.6	1.5	18
Phenol	0.06	0.000042	0.0084	0.5	0.92	0.78
Cyanide	0.0052	0.0058	0.000058	0.38	2.9	0.049

4.5.3 Graduation of response to substances representative of different modes of toxic action

The ECLOX test using Aztec reagents indicates that graduated responses were evident for the substances representative of the 7 modes of toxic action for which information is available.

4.5.4 Classification of substances representative of different modes of toxic action by curve shapes

The shape of the response curve varies depending on the toxicant and these can be classified into four types (Thorpe, Pers. Comm.): convex, concave, s-shaped and square wave (see Figure 2.2). Table 4.6 shows some of the pure substances associated with each shape of curve.

S-shaped curves are typical of sewage treatment works effluents while square wave curves have been found for food processing effluents.

Table 4.6 Pure chemicals associated with each curve shape

Shape of curve	Pure chemicals typical of curve
Convex	Chromium, zinc, phenol, 3,5-dichlorophenol
Concave	Cadmium, mercury, cyanide, trifluralin
S-shaped	Tin
Square wave	Uric acid

4.6 Standard operating procedure

A draft standard operating procedure for the test using the BioOrbit 1250 has been prepared by the Wolfson Laboratory, while Aztec and Randox provide operating instructions for tests using their portable luminometers. A standardised generic guideline for the chemiluminescent test needs to be prepared.

4.7 Test method precision

4.7.1 Repeatability

Table 4.7 shows the data for repeat testing of the ECLOX test using Aztec reagents and the reference substance phenol. The CV's for the test using either BioOrbit 1250 or 1251 luminometers were always less than 17%, irrespective of the test operator, and could be as low as 7.3%.

Table 4.7 Summary of the IC_{50} data (mg l^{-1}) generated by different operators with phenol using Aztec reagents and BioOrbit 1250 or 1251 luminometers

Parameter	IC_{50} data for phenol using BioOrbit 1250		IC_{50} data for phenol using BioOrbit 1251		
	Operator 1	Operator 2	Operator 1	Operator 2	Operator 3
Mean	0.93	1.01	1.07	1.03	1.07
SD	0.068	0.15	0.13	0.17	0.11
CV (%)	7.3	14.9	12.1	16.5	10.3
n	10	13	37	17	8

4.7.2 Reproducibility

At present there are no ring test data on the reproducibility of the chemiluminescence test to pure chemicals. However, the extent of the differences in sensitivity to pure substances between reagent formulations may have implications for the reproducibility of the test using a generic standard operating procedure. Furthermore, comparative testing of effluents in the Toxicity-Based Consents Pilot Study with the ECL test showed systematic differences in responses when samples were measured with Aztec reagents using the BioOrbit 1250 and 1251 luminometers.

Any ring test carried out for the ECL procedure will need to consider the issue of sensitivity of different reagent formulations and the use of different luminometers to ensure that the reproducibility of the test meets defined acceptability criteria.

4.8 Test substrate variability

As no organisms are used in the test genotypic variability is not a problem. However, there is still a potential problem for variability between batches of reagents.

4.9 Availability of reagents

Both Aztec and Randox reagents can be stored in a refrigerator for 3 months meaning availability of reagents is not a problem.

4.10 Indigenous test species and importance of test species

Since the enhanced chemiluminescent test is an *in vitro* enzyme-based assay in which no organisms are used the criteria for indigenous test species and importance of test species are not applicable.

4.11 Ecological relevance of the test

As no organisms are used in the test it cannot be considered to have any direct ecological relevance. However, indirect ecological relevance can be demonstrated by establishing correlations between the enhanced chemiluminescence test response and effects on either the growth, reproduction and survival of higher organisms or community structure (as measured by biological surveys).

4.11.1 Pure substances

Table 4.8 shows toxicity data on substances representative of different modes of action for the ECLOX, Microtox and *D. magna* immobilisation tests. The table also includes a comparison of the sensitivity between the ECL test and these other toxicity tests.

From the data the ECL test is more sensitive than the *D. magna* immobilisation test to phenol (20x) and chlorine (1969x), but less sensitive to cadmium (8233x), mercury (39.5x), zinc (573x) and trifluralin (61.6x). In comparison to the Microtox test, the ECL test is more sensitive to phenol (40x), cyanide (7.4x), but less sensitive to cadmium (17.6x), mercury (30x), zinc (3150x) and 3,5-dichlorophenol (12.8x).

The use of the ECL and Microtox tests as a rapid test battery would be as sensitive as the *D. magna* immobilisation test (that is within a factor of 2) to mercury, zinc, phenol, 3,5-dichlorophenol, pentachlorophenol and chlorine but not cadmium, chromium, toluene, azinphos methyl and malathion.

Table 4.8 Comparison of ECL, Microtox and *D. magna* immobilisation test data for substances representative of different modes of toxic action

Substance	Toxicity data for different test methods				
	ECL	Microtox		*D. magna* immobilisation	
	IC_{50} (mg l^{-1})	IC_{50} (mg l^{-1})	Relative sensitivity of ECL test	EC_{50} (mg l^{-1})	Relative sensitivity of ECL test
Cadmium	247	14	17.6x l	0.03	8233x l
Chromium	ND	13	-	0.02-0.086	-
Mercury	1.5	0.05	30x l	0.038	39.5x l
Zinc	630	0.2	3150x l	1.1	573x l
Toluene	ND	49.5	-	14.9	-
Phenol	0.5	20	40x g	10	20x g
3,5-Dichlorophenol	37	2.9	12.8x l	2.1	17.6x l
Pentachlorophenol	ND	0.52	-	0.55	-
Azinphos methyl	ND	0.35	-	0.0016	-
Malathion	ND	3	-	0.001	-
Chlorine	0.032	ND	-	63	1969x g
Endosulphan	ND	ND	-	0.31	-
Endrin	ND	ND	-	0.059	-
Cyanide	0.38	2.8	7.4x g	ND	-
Trifluralin	11.7	ND	-	0.19	61.6x l
Atrazine	ND	ND	-	>39	-

ND = No data, l = less sensitive, g = more sensitive

4.11.2 Effluents

Data from the Environment Agency/SNIFFER Toxicity-Based Consents Pilot Study (Project 493) (see Section 3.1) showed a statistically significant correlation between the ECLOX test (using IC_{50} values) and oyster embryo-larval development test NOEC (r=0.905) or EC_{10} values (r=0.83) for one of eight discharges to marine waters (Environment Agency 1996). No statistically significant correlations were evident between ECLOX responses and *Daphnia magna* immobilisation test NOEC or EC_{10} values for four discharges to riverine systems. In some instances the absence of correlations between ECLOX and higher organism test data may have been due to the limited response ranges measured over time.

4.11.3 Receiving waters

The ECL data generated in the Toxicity-based Criteria for Receiving Waters Study (Project 703) showed that the responses measured in freshwater and estuarine receiving waters samples (see Section 3.2) were correlated with those of higher organism tests. Table 4.9 shows the correlations obtained between ECL test data and responses in the 10 day *D. magna* reproduction test for freshwater samples and the oyster embryo-larval development test and *T. battagliai* reproduction test for estuarine samples. The ECL test data were significantly correlated with lethality in the *D. magna* reproduction test for the River Aire samples and the oyster embryo-larval development and *T. battagliai* reproduction test data for the Tees and Mersey samples.

Table 4.9 Correlations between ECL test responses and those responses in higher organism tests for freshwater and estuarine receiving water samples

Higher organism test	Test endpoint	Correlation between responses of rapid tests and higher organism tests (r value)	
		ECL	Microtox
D. magna reproduction	Juvenile production	R.Aire: 0.001 (NS) W.Brook: 0.122 (NS)	R.Aire: 0.026 (NS) W.Brook: 0.193 (NS)
	Lethality	R.Aire: 0.488 (P<0.01) W.Brook: 0.254 (NS)	R.Aire: 0.102 (NS) W.Brook: 0.103 (NS)
Oyster embryo-larval development	Embryo abnormality	Tees: 0.485 (P<0.01) Mersey: 0.961 (P<0.001)	Tees: 0.735 (P<0.001) Mersey: 0.409 (P<0.05)
T. battagliai reproduction	Juvenile production	Tees: 0.363 (P<0.05) Mersey: 0.396 (NS)	Tees: 0.602 (P<0.001) Mersey: 0.76 (P<0.001)
	Lethality	Tees: 0.13 (NS) Mersey: 0.831 (P<0.001)	Tees: 0.735 (P<0.001) Mersey: 0.439 (P<0.05)

NS - Not significant

German (1996) assessed the extent of correlations between chemiluminescence test responses (as IC_{50} values derived from field measurements using the ECLOX™ system) and biological survey data for 27 receiving water sites in Environment Agency Southern Region. The biological survey information used was Biological Monitoring Working Party (BMWP) and Average Score Per Taxa (ASPT) scores, Number of taxa and Environmental Quality Indices (EQI's) derived from BMWP and ASPT scores. Analysis using the Spearman Rank Correlation Coefficient showed no statistically significant correlations between ECLOX IC_{50} values and any biological survey index (see Table 4.10).

The establishment of a statistically significant correlation between data from a water column toxicity test and biological survey data at different sites will depend on the type and source of contaminants responsible for the differences in biological quality at those sites.

Table 4.10 Correlation coefficient between ECLOX IC_{50} values and biological survey indices for 27 receiving water sites

Relationship	Spearman Rank Correlation Coefficient (r)
ECLOX IC_{50} v BMWP	-0.05
ECLOX IC_{50} v ASPT	-0.13
ECLOX IC_{50} v Number of taxa	-0.13
ECLOX IC_{50} v EQI (BMWP)	0.1
ECLOX IC_{50} v EQI (ASPT)	-0.09

4.12 Home Office regulations

Home Office regulations do not currently apply to the test and are not likely to be applicable in the future.

4.13 Summary of data relating to selection criteria

Table 4.11 summarises the available data for the chemiluminescence test in relation to the selection criteria. The main data gaps relate to information on substances representative of different modes of toxic action (such as non-polar narcotics, uncouplers of oxidative phosphorylation, cholinesterase inhibitors, central nervous system (CNS) convulsants and photosynthetic inhibitors) which is needed to assess sensitivity, spectrum of response and graduation of response. Information is also needed on the mechanisms by which toxicants with different modes of toxic action act on the enhanced chemiluminescence test.

A standardised guideline is also needed which accounts for the effects of different physico-chemical parameters such as temperature, pH, salinity and turbidity. This guideline can then be ring tested to provide information on test method reproducibility.

Table 4.11 Summary of available data on the chemiluminescence test

Criteria	Available data
Ease of use	Proficiency achieved after 5 repeat tests, that is approximately 3 hours
Cost of implementing a test	£5000-7000
Cost of conducting a test	£7 for a single concentration test and £21-22 for a full (5) concentration range test
Rapidity of test	10 minutes for a single concentration test and 30 minutes for a full concentration range test
Sensitivity	SI = 0.13
Spectrum of response	3 modes of toxic action
Graduation of response	7 modes of toxic action
Standard operating procedure	Basic procedure available
Test precision - repeatability	<20% for the reference substance phenol
Test precision - reproducibility	No published data
Test substrate/organism variability	Control over the substrate used in the method
Availability of substrate/organisms	Commercial reagents available throughout the year
Indigenous test species	Not applicable
Importance of test species	Not applicable
Ecological relevance	Test response has been correlated with lethality, though only in limited instances
Home Office regulations	Method not covered by present regulations or likely to be covered by future regulations
Previous application to operational role	Method has identified toxicity in effluents and receiving water samples

5. CONCLUSIONS

The enhanced chemiluminescence test is available as test kits from Aztec Environmental (ECLOX™ system) and Randox Laboratories (AQUANOX™ system). Available information shows the test to be:

- easy to use, with staff capable of demonstrating proficiency after conducting 5 repeat reference toxicant tests;
- rapid, with single concentration tests taking about 10 minutes and full (5) concentration range tests taking about 30 minutes;
- cost-effective, with tests costing from £7 for a single concentration test to £21-22 for a full concentration range test;
- repeatable with coefficients of variation (CV's) for IC_{50} values from repeat concentration range tests with the reference toxicant phenol in a laboratory always being less than 17% and often less than 5%;
- readily available as reagents can be stored for 3 months in a refrigerator.

Available data indicate that the ECL test responds to a wide range of effluents and can be used to screen effluents for toxicity. However, in this role, test responses have not yet been shown to be surrogates for those in higher organism tests. The absence of correlations may be because the ECL test is responding to both BOD and COD as well as toxicants (see Section 3.1) while higher organism tests are responding primarily to the toxicants present.

On the basis of comparative data for receiving water samples the ECL test can be used by the Environment Agency to identify pollution 'hotspots' requiring further investigation. The ECL test can apparently act as a surrogate for endpoints in higher organism tests such as the 10 day *D. magna* reproduction test, the oyster embryo-larval development test and the 9 day *T. battagliai* reproduction test (number of live young and lethality) in certain situations. The test discriminated between stations of differing biological quality from good (clean) to poor/bad in a similar manner to the higher organism tests. In this context the test may be measuring the effects of both toxicants and/or BOD but this is not a limitation if the test is used as a rapid and cost-effective screen of water column pollution.

6. RECOMMENDATIONS

Further information is needed on the response of the system to a wider range of substances representative of different modes of toxic action including non-polar narcotics, uncouplers of oxidative phosphorylation, cholinesterase inhibitors, central nervous system (CNS) convulsants and photosynthetic inhibitors.

It is also important to clarify:

- the mechanisms by which toxicants with different modes of action affect the enhanced chemiluminescence reaction;

- the apparent incongruity between the high sensitivity of the test for environmental samples (relative to higher organism tests) with the low sensitivity shown for pure substances (relative to higher organism tests).

There is ultimately a need for a standardised generic guidelines for the chemiluminescence test which considers issues relating to parameters such as test temperature, the effects of sample pH, salinity and turbidity and the most appropriate means of analysing data. This standard guideline can then be ring tested to provide information on test method reproducibility. Any ring test carried out for the ECL procedure will need to consider the issue of sensitivity of different reagent formulations and the use of different luminometers to ensure that the reproducibility of the test meets defined acceptability criteria.

Further work is also needed to establish whether for complex effluent samples the ECL test and higher organism tests are responding to the same parameters if the ECL test is to be used to set action levels to control discharge toxicity. This could be investigated by studying the relative responses of the ECL system to BOD and individual toxicants in mixtures and trying to separate out whether BOD or toxicity causes ECL response at different ratios.

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APPENDIX A INFORMATION ON CHEMILUMINESCENT TEST RESPONSES TO DIFFERENT TOXICANTS

Table A1 Response of the ECLOX system to substances other than those representative of different modes of toxic action

Inhibitor type	Substance	LOD/IC_5 (mg l^{-1})	IC_{50} (mg l^{-1})
Metal (cation) inhibitors	Aluminium	750	1 651
	Antimony	50	-
	Arsenic	10	250
	Barium	523	2 039
	Bismuth	4	-
	Cobalt	2	116
	Copper	15	329
	Iron (III)	9	921
	Lanthanum	3	20
	Lead	26.5	323
	Manganese	0.003	0.1
	Nickel	2.3	1 038
	Selenium	0.6	-
	Silver	340	-
	Thallium	2	1 591
	Tin	0.2	10
	Vanadium	0.1	-
Organic	Acetic acid	36	-
	2 Amino-6-methoxy-benzothiazole	1	40
	Aniline	0.037	3.4
	1,2-Benzendiamine	0.001	0.02
	1,3-Benzendiamine	0.0007	0.04
	1,4-Benzendiamine	0.004	0.05
	1,2,3-Benzenetriol	0.001	0.06
	1,2,4-Benzenetriol	0.001	0.06
	4-Bromophenol	-	103
	Butyric acid	705	-
	Carbofuran	0.5	-
	4-Chloro-1-napthol	0.007	0.1
	2-Chlorophenol	-	10
	3-Chlorophenol	0.005	0.1
	4-Chlorophenol	-	37
	Dehydroascorbic acid	1	10
	Diethyldiothiocarbamic acid	0.1	0.8
	2,4-Dinitrophenol	1.5	33
	Ethanol	3686	70 634

Inhibitor type	Substance	LOD/IC_5 (mg l^{-1})	IC_{50} (mg l^{-1})
	4-Fluorophenol	0.009	0.9
	2-Hydroxy-6-methoxy-benzothiazole	13	120
	4-Iodophenol	-	70
	2-Methoxyphenol	0.0001	0.001
	3-Methoxyphenol	0.0009	0.05
	4-Methoxyphenol	0.001	0.02
	Nonylphenol	0.3	13.5
	Sulphanilic acid	156	4 703
	2-Sulphobenzoic acid	162	1 406
	4-Sulphobenzoic acid	192	2 026
	Tetramethylene sulphone	10	866
	Thionin		
	Thiosemicarbazide	0.007	0.3
	Thiourea	0.06	5
	3,4,5-Trihydroxybenzoic acid	0.03	0.4
	Urea	5	59
	Uric acid	0.001	0.03
Detergents	Aerosol OT (anionic)	356	2 501
	Brij 35 (non-ionic)	10	329
	CPC	29	121
Miscellaneous	Ammonia	1.4	106
	Azide	520	4 958
	Borate	40	4 111
	Cysteine	0.00048	0.097
	Ferricyanide (II)	0.03	0.3
	Ferricyanide (III)	3	18
	Hydrazine	0.009	0.14
	Iodine	0.3	4
	Metabisulphate	0.002	0.07
	L-Methionine	12	828
	Nitrite	552	39 417
	Phosphate	96	1 381
	Thiocyanate	32	443
	Thiosulphate	15	218

Table A2 Response of the Aquanox system to toxicants

Type of substance	Test substance	LOD (mg l^{-1})
Metals and metal compounds	Aluminium chloride	1 000
	Cadmium	1
	Chromium chloride	50
	Copper	1
	Copper chloride	100
	Iron	0.1
	Lead acetate	10
	Lead nitrate	1
	Magnesium chloride	1 000
	Manganese sulphate	0.1
	Nickel chloride	1
	Potassium chloride	10
	Vanadium chloride	1 000
	Zinc	0.1
	Zinc sulphate	1 000
Detergents	Brij-25 (25%)	1 000
	Brij-30	1 000
	Tween 80	100
	Tween 20	1 000
	Triton X-114	100
	Dobanol	100
	Lipoclear	100
	Nonidet	100
	Teepol HB6	10 000
	Dispenol green C-6B	1
Dyes	Solophenyl Yellow AGL	10
	Remazol brilliant violet	10
Pesticides	Chlortoluron	1 000
	Diuron	1 000
	Glyphosine	500
	Isoproturon	1 000
	Linuron	1 000
	Monuron	1 000
	Pentachlorophenol	0.5
	Permethrin	1
Miscellaneous	Acetic acid	1 000
	Ammonia	1
	Aniline	1 000
	Butyric acid	10
	Creosote	0.2
	Nonylphenol	5
	Phenol	100
	Tetrachloroethylene	1 000