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Attn: TSCA 8(e) Coordinator  
Re: TSCA 8(e) Document Control Number 8EHQ-0497-13581

TSCA 8(e) Substantial Risk  
Bisphenol A  
CAS No. 80-05-7

This submission is made by:  
The Society of the Plastics Industry, (SPI), Inc.  
SPI Bisphenol A Global Industry Group  
1801 K Street, NW, Suite 600K  
Washington, DC 20006-1301

SPI is submitting the final report for a study sponsored by the Bisphenol A Global Industry Group entitled *Bisphenol A: Determination of effects on larval growth, development and sexual differentiation of the African clawed frog (Xenopus laevis)*. SPI is submitting these results pursuant to current guidance issued by the Environmental Protection Agency (EPA) indicating EPA's interpretation of section 8(e) of the Toxic Substances Control Act. Neither SPI nor any of its members has determined that the final results contained within this report indicate any potential risk of injury to human health or the environment.

Please do not hesitate to contact me if you have any questions at 202-974-5217.

Sincerely,

Lynne R. Harris  
Director  
SPI Bisphenol A Global Industry Group

Contain NO CBI



8EHQ-96-13581



89010000042

Performing laboratory project identification AH0072/A

RECEIVED  
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2000 NOV 22 AM 11:17**BL6906/B****Bisphenol A: Determination of effects on larval growth,  
development and sexual differentiation of the African clawed frog  
(*Xenopus laevis*)**

Data requirements	GLP-compliant repeat of study by Kloas <i>et al</i> (1999)
Study completed	8 June 2000
Performing laboratory	Brixham Environmental Laboratory AstraZeneca UK Limited Brixham Devon TQ5 8BA UK
Sponsor Corresponding Address	Bisphenol A Global Industry Group The Society of the Plastics Industry, Inc. Suite 600 K 1801 K Street NW Washington DC, USA
Authors	D B Pickford J E Caunter M J Hetheridge T H Hutchinson
Approved by	J F Tapp October 2000

Bisphenol-A: Determination of effects on larval growth, development and sexual differentiation of the African clawed frog (*Xenopus laevis*)

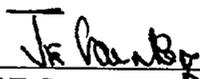
Brixham study number: AH0072/A

## GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with the UK Principles of Good Laboratory Practice (United Kingdom GLP Regulations 1999). These principles are in accordance with the OECD Principles of Good Laboratory Practice, revised 1997 (ENV/MC/CHEM(98)17).

These international standards are acceptable to the United States Environmental Protection Agency and this study, therefore, satisfies the requirements of 40 CFR Part 160 and 40 CFR Part 792.

This study is valid for the purpose for which it was conducted and this report is a true reflection of the raw data generated.



\_\_\_\_\_  
J E Caunter  
Study Director  
Brixham Environmental Laboratory

04 Oct. 2000

Date

Brixham study number: AH0072/A

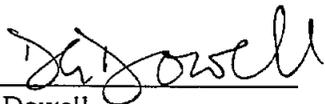
## QUALITY ASSURANCE STATEMENT

This study has been inspected/audited in accordance with AstraZeneca UK Limited's policies and procedures for Good Laboratory Practice, as follows

Date	Inspection/audit	Date of QA report
21 February 2000	Draft Study Plan	21 February 2000
28 February 2000	Study conduct	2 March 2000
10 March 2000	Study conduct	17 March 2000
11 April 2000	Study conduct	11 April 2000
9 May 2000	Study Conduct	10 May 2000
13 September 2000	Draft report	15 September 2000
2 October 2000	2 <sup>nd</sup> draft report	2 October 2000
5 October 2000	Final report	5 October 2000

Facilities and procedures associated with this type of study are periodically inspected in accordance with QA Standard Operating Procedures.

So far as can be established, the methods described and the results incorporated in the final report accurately reflect the raw data produced during the study.

  
\_\_\_\_\_  
D G Dowell  
Quality Assurance Unit

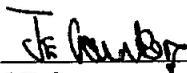
5.10.2000.  
Date

Bisphenol-A: Determination of effects on larval growth, development and sexual differentiation of the African clawed frog (*Xenopus laevis*)

Brixham study number: AH0072/A

### AUTHENTICATION STATEMENT

I, the undersigned, hereby declare that this study was performed under my direction according to the principles of Good Laboratory Practice and that this report represents a true and accurate record of results obtained.

Study Director  04 Oct. 2000  
J E Caunter Date

The following personnel carried out work on this study:

Principal Scientist, Ecotoxicology: D B Pickford

Principal Scientist, Chemistry Support (LC): C B Woods

Report approved by  4 Oct 2000  
Business Manager J F Tapp Date

Brixham study number: AH0072/A

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## 1 SUMMARY

**Sponsor** Bisphenol A Global Industry Group  
**Corresponding Address** The Society of the Plastics Industry, Inc. Suite 600 K, 1801 K Street, NW Washington, DC 20006-1301, USA  
**Contact** Dr. A T Hall International telephone: (001) 913 433 5362  
 Dr. D B Pickford International telephone: (44) 1803 882882  
**Location of study, raw data and final report** Brixham Environmental Laboratory, AstraZeneca UK Limited, Brixham, Devon, TQ5 8BA, UK  
**Test substance common name** Bisphenol A (BPA)  
**Chemical** 4,4'-(1-methylethylidene)bis-phenol  
**Abstracts name**  
**Subject** Bisphenol A: Determination of effects on larval growth, development and sexual differentiation of the African clawed frog (*Xenopus laevis*)  
**Test guideline** Non-standard method requested by Client  
**Test concentrations** Four replicates of each were employed

Nominal conc of Bisphenol A ( $\mu\text{g l}^{-1}$ )	Mean, time-weighted, measured conc of Bisphenol A ( $\mu\text{g l}^{-1}$ )	Percentage of nominal conc
Dilution water control	<0.5 <sup>a</sup>	N/A
1.0	0.83	83
2.3	2.1	90
10	9.5	95
23	23.8	103
100	100	100
500	497	99
Positive Control <sup>b</sup>	2.0	75

<sup>a</sup> all measured concentrations <0.5  $\mu\text{g l}^{-1}$  except replicate B on exposure day 61, 0.79  $\mu\text{g l}^{-1}$ .

<sup>b</sup> The positive control (PC) substance used was 17 $\beta$ -estradiol (E<sub>2</sub>) at a nominal concentration of 2.7  $\mu\text{g l}^{-1}$

**Test dates** Fertilisation (Exposure day -4) 6 March 2000  
 Initiation (Exposure day 0) 10 March 2000  
 Termination (Exposure day 90) 8 June 2000  
**Length of test** 90 days  
**Test species** African clawed frog (*Xenopus laevis*) larvae (4 days old)

**Source of organisms** The brood stock frogs used were purchased from Blades Biological, Cowden, Kent (individuals BBF 01, 02, BBM 01, 02) or were bred at University of Manchester, School of Biological Sciences, Oxford Road, Manchester, UK, by D B Pickford (individuals MUF 01, 02, 03, 04, MUM 01, 02, 03, 04). The frogs were induced to spawn by injection of exogenous gonadotropins. The larvae were examined macroscopically at the initiation of the study and were considered to be viable and in good condition.

**Results** All values are based on the nominal concentrations of BPA ( $\mu\text{g l}^{-1}$ )

Parameter	Treatment Group	Range (individual replicates)	mean replicates pooled	No observed effect concentration (NOEC) <sup>a</sup> ( $\mu\text{g l}^{-1}$ )
Percentage survival at 90 days	DWC	82.9 - 92.5%	87.1%	500
	BPA treatments	67.5 - 96.7%	81.4 - 90.7%	
	PC	77.5 - 97.5%	87.6%	
Sex ratio (% male, stage 66 froglets only)	DWC	43.8 - 54.1%	47.7%	500
	BPA treatments	32.0 - 74.2%	45.2 - 53.7%	
	PC	24.2 - 37.5%	30.8% <sup>c</sup>	
Mean days to completion of metamorphosis	DWC	61.0		500
	BPA treatments	59.1 - 62.5		
	PC	68.2 <sup>c</sup>		
Mean froglet total length <sup>b</sup>	DWC	36.4 mm		500
	BPA treatments	35.6 - 36.9 mm		
	PC	39.0 <sup>d</sup> mm		
Mean froglet snout-vent length <sup>b</sup>	DWC	17.0 mm		500
	BPA treatments	16.5 - 16.9 mm		
	PC	17.9 <sup>e</sup> mm		
Mean froglet wet weight <sup>b</sup>	DWC	541.3 mg		500
	BPA treatments	502.2 - 541.4 mg		
	PC	610.8 <sup>f</sup> mg		

DWC dilution water control

PC positive control (17 $\beta$ -estradiol, 2.7  $\mu\text{g l}^{-1}$ )

<sup>a</sup> BPA test concentrations compared to DWC

<sup>b</sup> at completion of metamorphosis

<sup>c</sup> PC group significantly different to DWC ( $p < 0.05$ )

<sup>d</sup> PC group significantly different to DWC, for males and pooled data ( $p < 0.05$ )

<sup>e</sup> PC group significantly different to DWC, for males only ( $p < 0.01$ )

<sup>f</sup> PC group significantly different to DWC, for males only ( $p < 0.05$ )

Overall No Observed Effect Concentration (NOEC) for BPA on survival, sex ratio, length and weight = 500  $\mu\text{g l}^{-1}$

Overall Lowest Observed Effect Concentration (LOEC) for BPA on survival, sex ratio, length and weight = >500  $\mu\text{g l}^{-1}$

## 2 INTRODUCTION

At the request of the Bisphenol A Global Industry Group (representing The Society for the Plastics Industry and CEFIC), a study was undertaken to determine the effect of chronic exposure to Bisphenol A (BPA) on sex differentiation in larvae of the African clawed frog (*Xenopus laevis*). This request was in response to a study published by Kloas *et al.* (1999) (Ref 1) which reported alteration of sex ratio (feminisation) in *Xenopus* larvae exposed to BPA at a nominal concentration of 23 µg l<sup>-1</sup>. The current study incorporated a number of improvements in study design, including greater replication, increased statistical power, analytical monitoring of exposure concentrations and use of a flow-through exposure system to maintain steady concentrations of the test substance in the test vessels. Other changes to the conduct of the study included constant monitoring of temperatures in two of the test vessels, regular measurements of temperature, pH and dissolved oxygen concentration in all test vessels, *in situ* fixation of gonadal tissue to aid in assessment of gross gonadal morphology, and use of different statistical methodology in analysis of sex ratios. We maintain that these alterations constituted improvements in terms of better water quality data, more reliable assessment of gonadal sex, and more appropriate treatment of sex ratio data, enabling a more robust interpretation of the results. Moreover, these improvements did not result in changes to the fundamental design and endpoints of the study, and comparisons of the findings of this report to those of the original study reported by Kloas (Ref 1) are therefore valid.

The definitive study was run between 10 March and 8 June 2000. The study number was AF0072/A and the Brixham test substance number was AF0072. The sample was supplied by the Shell Research and Technical Centre and the reference number was ST98148. The sample of 17β-estradiol, used as a positive control (PC) for oestrogen agonist effects, was obtained from Sigma Chemicals (Lot # 77HO666) and the Brixham test substance reference number was AF0314. All original data, together with other relevant records, are filed in the Brixham Environmental Laboratory archive.

## 3 MATERIALS

### 3.1 Test substance

The test substance, Bisphenol A (BPA), was supplied by Shell Research and Technology Centre, Thornton, PO Box 1, Chester, CH1 3SH.

Chemical Abstracts name: 4,4'-(1-methylethylidene)bis-Phenol

Chemical Abstract Service  
Registry Number (CAS) 80-05-7

Molecular Formula C<sub>15</sub>H<sub>16</sub>O<sub>2</sub>

The batch of BPA (Lot no. DS11518BS, SRTC-T Ref. ST98148) was received at Brixham Environmental Laboratory on 23 October 1998 and assigned the Brixham test

substance number AF0567. A sub-sample of this batch, for use in this study, was assigned the number AH0072, and was used throughout this study. The substance was a white crystalline powder and the certificate of analysis for the batch of BPA stated that its purity was 99+% mm and that it was stable until 19 October 2000 (analytical Ref 6552/1/99, certificate authorised 15 September 1999). The solubility of BPA in dechlorinated water at pH 7 is quoted to be 120 - 300 mg l<sup>-1</sup> (Ref 2).

During the definitive run of the study (including test system equilibration) 55.9 g of BPA was used to prepare the stock solutions. The sample of BPA was stored in the dark, at ambient temperature, in the container in which it was received until required for testing, when appropriate subsamples were provided for the test operators.

### 3.2 Positive control

The positive control (PC) substance, 17 $\beta$ -estradiol, was supplied by Sigma-Aldrich Ltd, Fancy Road, Poole, Dorset, BH12 4QH (Sigma Lot # 77HO666).

Chemical Abstracts name: (17.Beta.)-Estra-1,3,5(10)-Triene-3,17-Diol

Chemical Abstract Service  
Registry Number (CAS) 50-28-2

Molecular Formula C<sub>18</sub>H<sub>24</sub>O<sub>2</sub>

The batch of 17 $\beta$ -estradiol was received at Brixham Environmental Laboratory on 17 June 1998 and assigned the Brixham test substance number AF0314. The substance was a white powder with a quoted solubility of 13 mg l<sup>-1</sup> (Ref 3). The substance is considered to be stable until 1 December 2000.

During the definitive run of the study (including test system equilibration), 468.2 mg of 17 $\beta$ -estradiol was used to prepare the stock solutions.

The sample of 17 $\beta$ -estradiol was stored in the dark, at ambient temperature, in the container in which it was received until required for testing, when appropriate subsamples were provided for the test operators.

### 3.3 Test species

The African clawed frog (*Xenopus laevis*) larvae used in this study were obtained by induced spawning of 6 male/female pairs of adult *Xenopus* from the broodstock maintained at Brixham Environmental Laboratory.

The adult brood stock (individuals BBF 01, 02 and BBM 01, 02) were purchased from Blades Biological, Cowden, Kent TN8 7DX, UK and have been maintained at Brixham Environmental Laboratory since 7 July 1999. The females obtained from Blades Biological (BBF 01, 02) were laboratory-bred individuals imported from a US breeding

establishment. The frogs showed no evidence of disease, therefore, no treatment was necessary.

The adult brood stock (individuals MUF 01, 02, 03, 04, MUM 01, 02, 03, 04) were bred at Manchester University, School of Biological Sciences, Oxford Road, Manchester, M13 9PT by D B Pickford (25 November 1997), from adults purchased from Blades Biological. The frogs were transferred to Brixham Environmental Laboratory on 25 May 1999. These frogs have been disease free and therefore have not been treated.

The Brixham Environmental Laboratory adult brood stock are maintained in opaque 200 l tanks (working volume 50 l) in dechlorinated water (as described for the test dilution water) at  $22 \pm 2^\circ\text{C}$ . Frogs are fed twice-weekly on a complete pellet diet purchased from Blades Biological. Tank water is replaced twice-weekly, the morning after feeding. Water temperatures in the holding tanks are monitored daily. The pH, conductivity, alkalinity, hardness, free and residual chlorine and total ammonia concentration of the Laboratory dechlorinated water supply are monitored regularly (Table 30).

The broodstock individuals used for the study were induced to spawn by injection of exogenous gonadotropins (pregnant mare's serum gonadotropin and human chorionic gonadotropin). *Xenopus* eggs/embryos/larvae resulting from this spawning were maintained in general accordance with the Standard guide for conducting Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX; Ref 4) throughout the pre-test period.

The test was initiated with 4 day old larvae, which were at Nieuwkoop and Faber developmental stages 43 - 45, approximately (Nieuwkoop and Faber 1967, Ref 5). As hatching occurred principally on day 2 post-fertilisation, exposure to the test substance therefore commenced approximately 2 days post-hatching. At this time the larvae were attaining free-swimming capability and beginning to feed independently.

### 3.4 Dilution water

The dilution water was dechlorinated tap water supplied from a 100 m<sup>3</sup> reservoir with an average retention time of 24 hours. It was passed through activated carbon, coarsely filtered to remove particulate material, dechlorinated with sodium thiosulphate and salts added, as required, to maintain minimum hardness levels. The treated water was held in a secondary reservoir with a capacity of 36 m<sup>3</sup> and an average retention time of 8 hours. The water was passed through an ultra violet steriliser to a second set of filters (25 and 10 µm) and then to a third storage tank with a capacity of 13.5 m<sup>3</sup>. The treated water was delivered via a ring circuit to a temperature controlled header tank in the test laboratory set to a nominal temperature of  $22 \pm 1^\circ\text{C}$  and finally passed through a 5 µm filter before use.

## 4 METHODS

### 4.1 Apparatus

A dynamic (flow through) test system was used for this study. The test apparatus was constructed of glass with a minimum of other materials (PVC and silicone tubing and sealant) in contact with the test solutions.

The test vessels were of all glass construction, rectangular in shape with dimensions of 305 × 205 × 210 mm (length × width × height) and a maximum capacity of approximately 12 l. The test solution volume used was nominally 9.5 l. Four replicate test vessels (termed A, B, C and D) were employed for each test exposure concentration and the dilution water and positive controls.

The dilution water was fed from the header tank via flow control devices to glass mixing chambers. The nominal flow rate of the dilution water to each mixing chamber was 400 ml min<sup>-1</sup>. Each chamber also received the required amounts of BPA dissolved in basified dechlorinated water, supplied by a Watson-Marlow model 202U/AA10 peristaltic pump. The tubing used was 1.12 mm id and 1.52 - 2.05 mm id PVC tubing for the test substance and the PC, respectively, externally lubricated with a commercially available silicone gel. The mixing chambers were fitted with independent magnetic stirrers to ensure adequate mixing of the test solutions at all times. The chambers also acted as flow-splitting devices supplying approximately six tank volumes per day to each of four test vessels. The dosing system was designed so that each replicate tank received a nominal 40 ml min<sup>-1</sup> of the required test solution with a further 80 ml min<sup>-1</sup> running to waste. A diagram of the dosing system is shown in Figure 1.

### 4.2 Preparation of stock and test solutions

This study was run with a dilution water control (DWC) and positive control (PC) together with nominal BPA concentrations of 1.0, 2.3, 10, 23, 100 and 500 µg l<sup>-1</sup>. A 1000 mg l<sup>-1</sup> stock concentrate was prepared by dissolving a nominal 3.5 g of BPA in 3.5 l of dechlorinated water. This concentration of BPA is above the experimentally determined solubility limit of approximately 300 mg l<sup>-1</sup> (Section 3.1), but solubility of BPA is pH dependent. Therefore, to ensure complete solubilisation, the stock concentrate was basified during preparation. To achieve this basification, the BPA was added to 500 ml dechlorinated water in a 1 l conical flask, and 1 g of NaOH pellets was added to this stock concentrate, which was stirred on a magnetic stirrer/hotplate while being heated to approximately 40°C. The stock concentrate was then placed in an ultrasonic bath for 10 minutes. A further 500 ml of dechlorinated water was added to the stock concentrate, which was again stirred on a magnetic stirrer/hotplate while being heated to approximately 40°C. The stock concentrate was then transferred to a 5 l stock jar, and the volume made up to 3.5 l by rinsing out the flask with 2.5 l of dechlorinated water, with all rinsings going into the stock jar. Overhead mechanical stirring was introduced and left to run overnight. The resulting solution of the stock concentrate was clear and colourless. The pH of the stock concentrate ranged from 10.08 - 10.33 (over 11 measurements).

A series of stock solutions was prepared by addition of the required volume of stock concentrate (1000 mg l<sup>-1</sup>) to 5 l glass jars. The volumes used were as follows:

Nominal concentration of BPA (µg l <sup>-1</sup> )	Nominal stock solution concentration required (mg l <sup>-1</sup> )	Nominal volume of 1000 mg l <sup>-1</sup> stock concentrate (ml)	Nominal volume of dechlorinated water (ml)
DWC	-	-	-
1.0	2.0	5	2495
2.3	4.6	11.5	2488.5
10	20	50	2450
23	46	115	2385
100	200	500	2000
500	1000	2500	-

The PC stock solution was prepared by several methods throughout the study, in order to optimise delivery of the 17β-estradiol in the absence of solvent.

- 1 From 9 March 2000 to 27 March 2000: a nominal 13.5 mg of 17β-estradiol was weighed in a glass weighing boat and washed into a 5 l glass stock jar with 4 l of dechlorinated water. The solution was stirred with a magnetic stirrer and follower while being heated to approximately 40°C, followed by ultrasonic treatment for a minimum of 1 hour. The solution was then stirred overnight by mechanical overhead stirrer, after which an additional 1 l of dechlorinated water was added prior to placing the 5 l stock jar on the test apparatus. Nominal stock concentration 2.7 mg l<sup>-1</sup>, nominal toxin flow rate 0.4 ml min<sup>-1</sup>.
- 2 From 27 March 2000 to 15 May 2000: a nominal 40.5 mg of 17β-estradiol was weighed in a glass weighing boat and washed into a 5 l glass stock jar with 5 l of dechlorinated water. The solution was stirred with a magnetic stirrer and follower while being heated to approximately 40°C, followed by ultrasonic treatment for a minimum of 1 hour. The solution was transferred to a 20 l glass stock jar containing approximately 5 l dechlorinated water. The smaller stock jar was rinsed out with dechlorinated water, all rinsings going into the larger stock jar, which was then topped up with dechlorinated water to a final volume of 15 l. This solution was then stirred overnight with a large mechanical overhead stirrer. Prior to use, smaller volumes of approximately 5 l were transferred to a 5 l stock jar and placed on the test apparatus. Nominal stock concentration 2.7 mg l<sup>-1</sup>, nominal toxin flow rate 1.33 ml min<sup>-1</sup>.
- 3 From 15 May 2000 to 8 June 2000: a nominal 13.5 mg of 17β-estradiol was weighed in a glass weighing boat and washed into a 5 l glass stock jar with 1 l of dechlorinated water. A nominal 2.0 g NaOH was weighed out and added to the stock solution. An additional 3.5 l (approximately) of dechlorinated water was then

added to the stock jar, and the solution was stirred with a magnetic stirrer and follower while being heated to approximately 40°C, followed by ultrasonic treatment for a minimum of 1 hour. The solution was then stirred overnight by mechanical overhead stirrer, after which an additional 0.5 l of dechlorinated water was added prior to placing the 5 l stock jar on the test apparatus. Nominal stock concentration 2.7 mg l<sup>-1</sup>, nominal toxin flow rate 0.4 ml min<sup>-1</sup>. The pH of the PC stock concentrate prepared by this method was 11.93 (one measurement), but when diluted 1000:1 (nominal dilution ratio for this method) with dilution water the pH was 7.80.

These stock solutions were continuously stirred during the test and were delivered to the mixing chambers at nominal flow rates of 0.2 ml min<sup>-1</sup> and 1.33 or 0.4 ml min<sup>-1</sup>, for BPA stocks and 17β-estradiol stocks, respectively. The flow rates of the stock solutions, and of the dilution water, were measured on day 0 and twice per week thereafter. The nominal dilution achieved at this stage, immediately before delivery to the exposure concentrations and solvent control test tanks, was 2000 × for BPA stocks, and 1000 × for the 17β-estradiol stock (except between 27 March 2000 and 15 May 2000, when the nominal dilution ratio was 300 ×). The stock solutions were replenished weekly (each Tuesday) except for the 2.0 mg l<sup>-1</sup> stock (for test concentration 1.0 µg l<sup>-1</sup>), and the PC stock which were replenished twice per week (Tuesdays and Fridays) from 7 April 2000 onwards.

### 4.3 Test conditions

The test was conducted at a temperature of 22 ± 1°C with a photoperiod of 12 hours light, followed by 12 hours of dark with 20 minute dawn/dusk transition periods. The light intensity was measured by cosine receptor during the study. These measurements were made midway between the front and back of the apparatus, using a Skye Instruments unit SKL300 photometer with a matching sensor head type SKL310. Readings were taken at the centre and near the two ends of the test rig to determine the range of values along the entire length.

### 4.4 Pre-test procedure

On exposure day -8 adult male and female frogs were primed with PMSG (pregnant mare's serum gonadotropin). Three days later, (exposure day -5) male and female frogs were injected with hCG (human chorionic gonadotropin) and then placed, one male:female pair per tank, in spawning tanks containing FETAX solution, a reconstituted water medium (Ref 4). The following day (day of fertilisation, exposure day -4), eggs were collected from the spawning tanks and de-jellied by a short incubation in a solution of L-cysteine in FETAX solution, to facilitate selection of healthy embryos. *Xenopus* embryos were incubated in a static renewal system in 200 ml evaporating dishes containing FETAX solution (approximately 100 ml) at ambient temperature (room temperature 22°C) under 12 hour light:12 hour dark photoperiod. On each day subsequent to day of fertilisation (exposure day -4), dead or visibly abnormal/unhealthy embryos were removed and the incubation medium was replaced (approximately 90% replacement).

On day 1 post-fertilisation (exposure day -3), FETAX solution was replaced with 50% FETAX solution diluted with dilution water. On day 2 post-fertilisation (exposure day -2) 50% FETAX solution was replaced with 25% FETAX solution, and on day 3 post-fertilisation (exposure day -1) 25% FETAX solution was replaced with 10% FETAX solution. Batches of embryos from each spawning pair ( $n = 6$ ) were kept separate during the pre-test period (ie exposure days -4 to 0).

#### 4.5 Test procedure

A dynamic (flow through) test system was used to provide a continuous supply of test solutions. The test apparatus was started on 28 February 2000 when the tanks were first filled with dilution water. BPA was first dosed into the test system on 29 February 2000 (exposure day -10).

On day 4 post-fertilisation (exposure day 0) all healthy normal larvae were selected from the six batches of spawn and pooled in a single 9.5 l working volume vessel containing dilution water. Larvae were then transferred by wide-bore glass pipette in groups of 5 to glass pots which had been randomly assigned to test vessels and labelled accordingly. Groups of 5 larvae were transferred to all pots until each pot contained 40 larvae. Each pot was then inspected and any abnormal or unhealthy looking larvae were removed and replaced with larvae selected from the pooling tank.

To initiate the test, exposure was commenced on 10 March 2000 by gently decanting the contents of each glass pot, each containing a nominal 40 larvae, into each of the test vessels (giving a total of 40 larvae per replicate and 160 larvae per test concentration). This gave a nominal loading of 4.21 larvae per litre of test solution on a static basis and a nominal flow loading of 60 ml per larva per hour.

Daily observation of larval mortality, behaviour and appearance was made and any abnormal effects recorded. Numbers of dead larvae in each tank were recorded daily with any dead larvae being discarded.

Growth and development assessments were performed on all larvae from one replicate (tank A) per treatment group on exposure days 32 and 62 (36 and 66 days post-fertilisation respectively). Individual larvae were removed from the test vessel using a fine-mesh dip net and lightly anaesthetised in a solution of MS222 (tricaine, 3-aminobenzoic acid ethyl ester) in dilution water ( $200 \text{ mg l}^{-1}$ , buffered with  $\text{NaHCO}_3$ ). Each larva was measured for total length and snout-vent length using dial calipers (Fig 2). Developmental stage according to Nieuwkoop and Faber (1967) (Ref 5), was assessed by visual inspection under a stereo dissecting microscope. Larvae were then allowed to recover from anaesthesia in an aerated vessel containing the appropriate test solution before being returned to the test vessel.

From exposure day 41 onwards, individual larvae reached developmental stage 66 and completion of metamorphosis (as froglets). From exposure day 41 onwards, as and when froglets at stage 66 were observed they were removed from the test vessels and killed by terminal anaesthesia in a solution of MS222 ( $2 \text{ g l}^{-1}$ , buffered to pH 7.5 with  $\text{NaHCO}_3$ ),

followed by destruction of the brain stem with a penetrating probe. Wet weight, total length and snout-vent length of the froglets were then measured. Total length of froglets was measured from the tip of the snout to the tip of the longest toe on the fully extended right hind-limb (not shown in Fig 2).

Froglets were then dissected to assess gross gonadal morphology under a stereo-dissecting microscope. An assessment of gonadal sex, male, female, or intersex, was made based on presence on testes, ovaries, or mixed tissue types, respectively. Assessments were made and recorded before (as per Kloas *et al*, Ref 1) and after *in situ* fixation of the gonadal tissue with Bouin's fixative. *In situ* fixation renders the translucent gonadal tissue more opaque and makes the overall structure of the gonad, as well as any gross morphological abnormalities, more visible. Gonads were then excised as part of a gonadal-adrenal-mesonephros complex and fixed in Bouin's solution.

Terminations were performed at least twice per week from exposure day 46 through to termination of the test.

The test was terminated on exposure day 90 (94 days after fertilisation). Surviving larvae at developmental stage <58 were terminally anaesthetised, weighed (wet weight) and measured for total and snout-vent length before whole fixation in 10% neutral buffered formalin. Surviving larvae at developmental stages 58 - 66 inclusive were terminally anaesthetised, weighed (wet weight) and measured for total and snout-vent length before dissection to assess gross gonadal morphology under stereo-dissecting microscope. Gonads were then excised as part of a gonadal-adrenal-mesonephros complex and fixed in Bouin's solution.

Bouin's was cleared from fixed gonads by rinsing with tap water then 70% ethanol between 3 and 6 hours after fixation. Fixed tissue was then stored in 70% ethanol. Details of Bouin's solution are contained in Roberts 1989 (Ref 6).

#### 4.6 Physical and chemical parameters

Dissolved oxygen, pH and temperature measurements were made in each test vessel on day 0 and then once weekly throughout the study. pH measurements were carried out with a Corning Model 240 pH meter. Dissolved oxygen concentrations were measured with a Yellow Springs Instruments dissolved oxygen meter, Model 51B. Temperatures were measured using a mercury-in-glass thermometer calibrated to 0.1°C and conforming to BS593. The salinity of the dilution water was measured in 1 replicate tank of the DWC and in 1 replicate tank of the 23 µg l<sup>-1</sup> test concentration on exposure day 0 and then once per week until the end of the study. A continuous record of the temperature was kept in one replicate of the DWC and one replicate of the 23 µg l<sup>-1</sup> using an electronic recording system (platinum resistance sensor). In addition, the pH, conductivity, alkalinity and total hardness of one replicate of the control and one replicate of the 23 µg l<sup>-1</sup> test concentration were determined once per week. pH measurements were carried out with a Corning Model 240 pH meter. Conductivity was measured with a Jenway 4010 conductivity meter. Alkalinity was measured using an electrometric

method. Hardness was determined by titration with standard EDTA solution using a proprietary total hardness indicator.

In addition to the above, the pH, conductivity, alkalinity, total hardness, total ammonia, total filterable solids and dissolved non-purgeable organic carbon of the dechlorinated water supply were determined periodically. Conductivity, hardness, alkalinity and pH were measured as described above. Total ammonia was determined using a colorimetric technique. Dissolved non-purgeable organic carbon was measured using a Dohrman DC190 carbon analyser.

The free available chlorine and total residual chlorine of the dechlorinated water supply was determined daily (Monday - Friday) using a colorimetric method, with a Lovibond comparator, based upon the reaction between the various chlorine species and N,N-diethyl-p-phenylene diamine (DPD) to form a stable pink complex. Interference from metal ions was suppressed using EDTA as a chelating agent. The combined available residual chlorine was calculated by subtracting the free available chlorine from the total available residual chlorine.

Representative samples of the Laboratory freshwater supply were also analysed for trace metals and pesticides on a periodic basis.

Appendix 1 details the sampling schedule for all of the dechlorinated water supply analyses.

#### 4.7 Feeding regime

The larvae were fed a proprietary fry food (Sera Micron®; Sera GmbH, Heinsberg, Germany) throughout the study. A suspension of Sera Micron in dilution water was prepared in 1.7 l batches at a nominal concentration of 10 mg ml<sup>-1</sup> (a nominal 17 g of Sera Micron powder was added to 1700 ml dilution water, and stirred first with a glass rod, then by magnetic stirrer and follower). The larvae were fed 5 ml suspension per test vessel once on day 0 of the study. On exposure days 1 - 6 inclusive, larvae were fed twice with 5 ml suspension per test vessel. From exposure day 7 onwards, larvae were fed three times per day on weekdays and twice per day on weekends. Total daily food ration increased from 15 ml on exposure day 7 (ie 3 × 5 ml per test vessel) to 39 ml (3 × 13 ml per test vessel) on exposure day 32. Feeding rate remained constant at 39 ml per test vessel per day from exposure day 32 through exposure day 90 (termination).

From exposure day 42 (21 April 2000), a proprietary pellet food for juvenile *Xenopus* (purchased from Blades Biological, Cowden, Kent, TN8 7DX, UK) was fed to froglets approaching completion of metamorphosis (developmental stages 62 - 66). This was to try to avoid cannibalism of smaller less developed larvae by froglets. Once per day, larvae in each test vessel were assessed and one pellet of food per larvae at developmental stage 62 - 66 was added to each test vessel.

Each batch of food was analysed for pesticides (including alpha, beta and gamma HCH, Heptachlor, Aldrin, Heptachlor epoxide, Dieldrin, DDE, DDD, DDT, Chlordane, Endrin,

Endosulfan, Quintozene, Technazene and HCB). The pesticide analysis was carried out by Aspland and James (Medcalfe Way, Bridge Street, Chatteris, Cambs PE16 6QZ, UK). The reports of their analytical results are archived at Brixham Environmental Laboratory.

Each batch of food was analysed for trace metals at Brixham Environmental Laboratory.

#### 4.8 Analytical method

The analytical method employed to measure the concentrations of both BPA and 17 $\beta$ -estradiol in the test solutions is summarised below. The method, for the analysis of BPA, followed Brixham Environmental Laboratory Standard Operating Procedure EM024, and is shown in Appendix 2.

Samples of each test concentration were taken from each of the four replicate vessels on exposure days -3, -1, 0, 7, 10, 19, and at weekly intervals (sampling day Wednesday) after that until the end of the study. Several additional samplings were conducted in response to unexpectedly low analytical results.

Water samples were preserved by addition of mercuric chloride (50  $\mu$ l of a 30% solution) and diluted as required prior to analysis by liquid chromatography. Separation was achieved using a 150 mm  $\times$  4.6 mm (id) H50DS (Hypersil) column, operated isocratically with 35% water 65% methanol at a flow rate of 1 ml min<sup>-1</sup>. Peak detection was obtained using fluorescence with excitation and emission wavelengths of 230 and 308 nm respectively.

#### 4.9 Statistical analysis

Data were entered into electronic data files and analysed using statistical procedures in the Brixham Environmental Laboratory computer program 'LIFESTATS' (programme version 3.3).

The percentage survival data were analysed by 2  $\times$  2 contingency table tests to compare the exposure concentrations and PC against the DWC, looking for differences at the 5% significance level (Ref 7).

Data from the growth and development assessments on exposure days 32 and 62 (developmental stage composition of larvae in replicate A) and data collected at termination of stage 66 froglets (time taken to complete metamorphosis, total/snout-vent length, and wet weight) were tested for normality (Ref 8) and homogeneity of variance (Ref 9). If the data had a normal distribution and the variance was homogeneous then the data were analysed using analysis of variance techniques. If the data did not meet the assumptions for analysis for variance, then the data were analysed using a non-parametric procedure such as Wilcoxon's Rank Sum Test (Ref 10).

Sex ratio data were analysed by replicated Goodness of fit test (G-test), as described by Sokal and Rohlf (Ref 11), to compare sex ratios with the expected sex ratio of 50:50 male:female. G values, degrees of freedom and attendant probability values from the

$\chi^2$ -distribution were calculated for each individual replicate, and pooled data, for each test concentration. G values for individual replicates are additive to give a total G value ( $G_T$ ), from which the pooled G value ( $G_P$ ) value can be subtracted to give a G value for heterogeneity among replicates ( $G_H$ ).

Analysis was performed on data collected from a) stage 66 individuals only, and b) all sampled individuals at developmental stages 58 - 66 inclusive.

## 5 RESULTS

### 5.1 LC Analytical results

Limit of quantification was determined as the concentration of the lowest calibration standard (ie  $0.5 \mu\text{g l}^{-1}$ ). Measured concentrations of BPA and the PC substance,  $17\beta$ -estradiol, for each replicate test vessel in each test concentration are presented in Table 1. Time-weighted mean measured concentrations for the test concentrations of BPA and  $17\beta$ -estradiol are summarised in Table 2.

Following failure to detect BPA in samples from all replicates in the  $1.0 \mu\text{g l}^{-1}$  test concentration on exposure day 10, additional sampling of this test concentration was conducted on exposure day 12. On this additional sampling day concentrations of BPA in all four replicate test vessels were in the range  $0.9$ - $1.5 \mu\text{g l}^{-1}$  (mean 104% of nominal). In light of this anomalous result, in the following week additional samplings of the  $1.0 \mu\text{g l}^{-1}$  were conducted to determine if test concentrations were declining during the period of use of the stock concentrate. Mean concentrations of BPA were 93% of nominal on exposure day 19 (first day of use of stock concentrate), but declined to 77% of nominal by exposure day 21, and 18% of nominal on exposure day 24 (last day of use of stock concentrate). Chemical analysis of all stock solutions confirmed decline of BPA in the  $2 \text{ mg l}^{-1}$  stock concentrate (for the  $1.0 \mu\text{g l}^{-1}$  test concentration), but not in other stock concentrates. Consequently, the  $2.0 \text{ mg l}^{-1}$  stock concentrate was replenished twice per week from this point onwards. Given that mean exposure concentration in the  $1.0 \mu\text{g l}^{-1}$  test concentration was 83% of nominal, and fell between mean measured concentrations of the DWC and the  $2.3 \mu\text{g l}^{-1}$  test concentration, this temporary fluctuation in exposure concentration in the  $1.0 \mu\text{g l}^{-1}$  test concentration was deemed to be inconsequential to the results of the test.

On exposure day 10 test concentrations in the  $500 \mu\text{g l}^{-1}$  test vessels averaged 49% of nominal. This was found on exposure day 11 to be due to a blocked stock solution delivery line, which was then cleared. Additional sampling of the  $500 \mu\text{g l}^{-1}$  test vessels on exposure day 12 indicated mean concentrations of BPA at 100% of nominal.

Chemical analysis of the PC test solution on exposure days -3 to 10 indicated that concentrations of  $17\beta$ -estradiol were only 44% of nominal. Subsequent additional sampling of the PC test solutions on exposure day 12 indicated mean concentrations of  $17\beta$ -estradiol at 32% of nominal. The method of preparation of the PC stock concentrate

was therefore altered from exposure day 17 (27/3/2000), and this was reflected in the increased concentrations of 17 $\beta$ -estradiol in the PC test vessels on exposure day 19 (mean concentrations 126% of nominal). However, by exposure day 61, concentrations of 17 $\beta$ -estradiol again appeared to have declined to sub-optimal levels (61% of nominal) and the method of PC stock concentrate preparation was again altered (stock concentrate basified by addition of NaOH), with a subsequent increase in exposure concentrations (Table 1).

## 5.2 Larval survival

Cumulative recorded mortality data for pooled replicates, summarised at 5 day intervals, are presented in Table 3. Overall summary survival data for the 90 day exposure period are presented in Table 4. This table indicates, for each test vessel, nominal starting number of larvae (40), numbers of larvae sampled by the end of the study (ie surviving larvae) and numbers of recorded mortalities (Table 3). In a number of test vessels there is a discrepancy between the nominal starting number of larvae and the sum of recorded mortalities plus sampled (ie surviving) larvae. This is attributed to mortalities that were not recorded because they went unnoticed. Mortalities of some small early stage larvae may have gone unnoticed due to rapid decomposition in the test vessel before the next mortality check, or because the dead larvae was concealed in faecal debris at the bottom of the test vessel prior to cleaning. Additionally, some smaller larvae may have been cannibalised by individuals at late metamorphic climax (ie froglets at developmental stages 63 - 66). This is a phenomenon which has been observed in other studies with *Xenopus laevis* at Brixham, and is corroborated by the experience of other groups (Kloas, personal communication). These mortalities would obviously also have gone unrecorded. Pellet food was fed to froglets at developmental stages 62 - 66, but this may not have completely precluded cannibalism of smaller larvae by froglets. In one test vessel (1.0  $\mu\text{g l}^{-1}$ , C rep) there was a discrepancy of 10 larvae. It is thought unlikely that there would be so many unrecorded mortalities in tank this alone, and the discrepancy is more reasonably attributed to an error in counting larvae into the tank at the start of the study (larvae were counted in multiples of 5). Statistical analysis of survival data utilised the total number of mortalities in each test vessel (ie recorded and unrecorded mortalities).

The percent survival in the individual replicates ranged from 67.5% to 97.5%. The percent survival in the pooled replicates ranged from 81.4% to 90.7% with an overall mean of 85.9%.

Results of pair-wise comparisons of survival data from individual replicates are shown in Table 5. When comparing treatment groups to the DWC, survival was significantly lower in the 500  $\mu\text{g l}^{-1}$  D rep ( $p=0.037$ ) than in the DWC A and C reps.

Results of pair-wise comparisons of treatment groups, with data replicates pooled, are shown in Table 6. No significant differences (at  $p=0.05$  level) were detected in pooled survival data for any of the exposure concentrations or PC, when compared to the DWC. Survival in the 500  $\mu\text{g l}^{-1}$  group was significantly lower than in the 100  $\mu\text{g l}^{-1}$  group ( $p=0.017$ ).

Therefore, in considering the pooled survival data, the overall LOEC of BPA for survival was  $>500 \mu\text{g l}^{-1}$  and the NOEC was  $500 \mu\text{g l}^{-1}$ .

### **5.3 Larval growth and development**

#### **5.3.1 Growth and Development Assessment 1 (Exposure Day 32)**

Developmental stages of larvae sampled from replicate tanks A on exposure day 32 are shown in Table 7. The percentage proportion of larvae at given developmental stages (Nieuwkoop and Faber Stages 48 - 66, Ref 5) for DWC, BPA test concentrations, and PC are presented in Table 8.

The day 32 developmental stage composition data did not have a normal distribution, however Bartlett's test indicated that the data were homoscedastic (ie displaying homogeneity of variance). Therefore, both parametric (analysis of variance) and non-parametric (Wilcoxon's Rank Sum Test) statistical procedures were used for comparison of the test exposure concentrations and PC with the DWC stage data (A replicates only). No significant differences in stage composition ( $p=0.05$ ) were detected in any of the test concentrations or the PC, when compared to the DWC, using either statistical method.

Total length and snout-vent length data for all A replicate larvae measured at Growth and Development Assessment 1, are presented in Tables 9 and 11 respectively. These data are also summarised in Table 10 (total length) and Table 12 (snout-vent length). In the summary tables, for each test concentration, mean lengths are given for each developmental stage represented in the test vessel. The number of larvae at the same developmental stage represented by each mean value range from 1 to 12. Mean values are not provided for all developmental stages, as not all stages are represented in each test vessel. Statistical analysis was not performed on total or snout-vent length data due to variability of stage of development of the larvae, resulting in insufficient numbers of larvae at any particular developmental stage in each test concentration for valid statistical comparison between treatment groups.

#### **5.3.2 Growth and Development Assessment 2 (Exposure day 62)**

Developmental stages of larvae sampled from replicate tanks A on exposure day 62 are shown in Table 13. The percentage proportion of larvae at given developmental stages (Nieuwkoop and Faber Stages 48 - 66, Ref 5) for DWC, test concentrations of BPA, and the PC are presented in Table 14. Figures for percentage of larvae at stage 66 (ie completion of metamorphosis) include froglets which reached this stage and were removed from the tank for sampling prior to day 62.

The day 62 developmental stage composition data were normally distributed and homoscedastic, and therefore Analysis of Variance was used for comparison of the test exposure concentrations and PC with the DWC stage data (A replicates only). No significant differences in stage composition ( $p=0.05$ ) were detected in any of the test concentrations or the PC, when compared to the DWC.

Total length and snout-vent length data for all A replicate larvae measured at Growth and Development Assessment 2, are presented in Tables 15 and 17 respectively. These data are also summarised in Table 16 (total length) and Table 18 (snout-vent length). In the summary tables, for each test concentration, mean lengths are given for each developmental stage represented in the test vessel. The number of larvae at the same developmental stage represented by each mean value range from 1 to 6. Mean values are not provided for all developmental stages, as not all stages are represented in each test vessel. As for Growth and Development Assessment 1, total and snout-vent length data were not subjected to statistical analysis.

#### 5.4 Termination data

The number of larvae that survived to completion of metamorphosis (stage 66) and were sampled, for each test vessel and for each treatment group, is represented in the 'total' column of Table 19. Similarly, the total number of larvae sampled (developmental stages 58 - 66 inclusive), for each test vessel and for each treatment group, are represented in the total column of Table 20.

##### 5.4.1 Sex ratio

Phenotypic sex was assessed in stage 66 froglets based on gross gonadal morphology, both before and after *in situ* fixation with Bouin's fluid. Sex ratio data summarised in Tables 19 and 20 represent post-fixation assessments of gonadal morphology. Pre-fixation assessment was also performed, in order to provide data that were directly comparable in method to the report by Kloas *et al.* (Ref 1), and to enable comparison of the methods. There were a total of 19 cases where assessment of gonadal sex differed pre- and post- fixation, 15 cases relating to froglets sampled at stage 66, and a further 4 relating to froglets sampled at developmental stages prior to stage 66 on day 90 of the study. These cases represent 1.51% and 1.77% of the total number of gonadal sex assessments performed on froglets at stage 66 only, and stages 58 - 66 inclusive, respectively. These cases of miss-assignment of sex are summarised in Table 21. It can be seen from these data that there was no overall bias in the direction of miss-assignments, as there were 8 male/female (pre-fix/post-fix) inconsistencies and 7 female/male inconsistencies. The remaining 4 cases comprised two cases where gonads had indeterminate structure pre-fixation which was clarified by fixation, and two cases where intersex condition was diagnosed after fixation of gonads that had been assigned as female pre-fixation. Sex ratio data presented in Tables 19 and 20, and discussed below, relate to gonadal sex assessed post-fixation.

Table 19 shows number of males, number of females, total number of froglets sampled at stage 66 (completion of metamorphosis), and sex ratio as percentage male, for each replicate tank in each test concentration. Table 20 shows the same data, but includes all larvae/froglets (developmental stages 58 - 66 inclusive) for which gonadal morphology was assessed at termination. Pooled data from all replicates are also shown.

Tables 19 and 20 also present statistical analysis of the sex ratio by replicated Goodness of fit test (G-test), as described by Sokal and Rohlf (Ref 11).

When considering pooled sex ratio data from either stage 66 individuals only (Table 19), or all sampled individuals, stages 58 - 66 inclusive (Table 20), there were no significant departures from the expected 50:50 sex ratio in the DWC or any of the test concentrations. There was a significant male bias (74.2% male) in replicate B of the  $1.0 \mu\text{g l}^{-1}$  BPA treatment, and this departure from the expected ratio in this replicate alone was reflected in a significant  $G_H$  value ( $p=0.031$ ). This result indicates that the slight departure from expected values overall ( $G_T$ ,  $p < 0.05$ ) is a result of heterogeneity among replicates, rather than a consistent bias away from the expected ratio. This is supported by the absence of a significant departure of the pooled sex ratio in this test concentration ( $G_p$ ,  $p > 0.05$ ). Moreover, the male bias in sex ratio in the B replicate is not consistent with an oestrogenic effect of the test substance, nor with the apparent feminising effect of BPA on sex ratio reported by Kloas *et al.* (Ref 1). The departure of sex ratio to a male bias in one replicate alone of the lowest test concentration presumably represents random variability in sex ratio, and does not indicate a significant effect of BPA on sex ratio.

The sex ratios of all replicates in the PC treatment are female biased, indicating a feminising effect of the  $17\beta$ -estradiol on larval sex differentiation. While sex ratios in only replicates C (24.2% males) and D (29.6% males) depart significantly from expected values ( $p < 0.05$ ),  $G_T$  and  $G_p$  values are both significant indicating a significant and consistent feminisation. The insignificant  $G_H$  value confirms that the deviations of the sex ratios in the individual replicates were in the same direction and were not significantly different from each other.

The statistical analysis of sex ratios including all sampled individuals (developmental stages 58 - 66 inclusive, Table 20) gave results that were essentially the same as those for stage 66 individuals only. There were no significant deviations from the expected 50:50 sex ratio in the DWC or any of the test concentrations of BPA, while there was a significant feminisation in the PC group exposed to  $17\beta$ -estradiol.

#### 5.4.2 Gonadal abnormalities

Incidence of testicular abnormalities noted at time of assessment of gross gonadal morphology are tabulated in Table 22. Types of abnormalities recorded were asymmetry of the testes, unusual presence of melanocytes, irregular shape, overt segmentation or fragmentation of the testes, presence of fluid filled vacuoles in the testes, suspected intersex condition (ambiguous gonadal morphology with ovarian and testicular tissue types apparent), and complete absence of the visible gonad. Incidence of testicular abnormalities, all types pooled for all replicates, was markedly greater in the PC group (37) than in DWC (8) or BPA test concentrations (range 5 - 12).

Incidence of ovarian abnormalities noted at time of assessment of gross gonadal morphology are tabulated in Table 23. Abnormalities recorded were asymmetry of ovaries (including cases where one ovary was missing), presence of very few or no melanocytes in the ovaries, abnormally shaped ovaries (including ovaries which presented with swollen and unpigmented sections), and thin ovaries with little or no segmented (lobular) structure. Complete absence of visible gonads and intersex

condition are not recorded in this table as these cases are recorded with testicular abnormalities in Table 22. Incidence of ovarian abnormalities, all types pooled for all replicates, was markedly greater in the PC group (14) than in DWC (8) or BPA test concentrations (range 2 - 8).

In a number of individuals, testes or ovaries exhibited more than one type of abnormality. In such cases, one entry only was entered for the most extreme type of abnormality exhibited. This was so that numbers of abnormalities recorded on the tables were representative of numbers of affected individuals (which would not be the case were some individuals entered more than once on the table). It is clear from Tables 22 and 23 that some 'abnormalities' occurred in all treatment groups, including the DWC. Gonadal abnormalities, as recorded, only indicate deviations from the typical appearance of a testis or ovary, and do not necessarily imply that the observed abnormality would persist to sexual maturity or that the gonad would be functionally impaired. Given the background level of gonadal abnormalities in the DWC, it does not appear that exposure of larvae to BPA resulted in an increase in gross gonadal abnormalities evident in stage 66 froglets.

#### 5.4.3 Time to metamorphosis

Time (days) taken for larvae to complete metamorphosis (ie reach developmental stage 66) was recorded. These data, pooled for all replicates in each test concentration, are summarised in Table 24. Mean time to metamorphosis is given for all individuals, and for males and females.

Analysis of variance indicated no significant difference in time to completion of metamorphosis in any of the test concentrations of BPA, compared to DWC, when considering all froglets together, or considering males and females separately. However, time to completion of metamorphosis was significantly increased in the PC, when considering all froglets together ( $p < 0.01$ ) or males and females separately ( $p < 0.01$  and  $p < 0.05$ , respectively).

#### 5.4.4 Total length

Total lengths of stage 66 froglets (pooled for all replicates in each test concentration) are summarised in Table 25. Mean total length is given for all individuals, and for males and females separately.

Analysis of variance indicated no significant difference in total lengths in any of the test concentrations of BPA, compared to DWC, when considering all froglets together, or considering males and females separately. However, total length was significantly greater in the PC, when considering all froglets together ( $p < 0.01$ ).

Male total length data were not normally distributed, but was homoscedastic. Wilcoxon's Rank Sum Test indicated no significant differences among any of the BPA test concentrations or PC, when compared to the DWC. Analysis of variance indicated no differences in total length in any of the test concentrations of BPA, compared to

DWC. However, total length of PC males was significantly greater than that of DWC males ( $p < 0.01$ ).

Female total length data were normally distributed and homoscedastic, and were therefore analysed by analysis of variance. There were no significant differences in total length of females among any of the test concentrations or PC, compared to DWC.

#### 5.4.5 Snout-vent length

Snout-vent lengths of stage 66 froglets (pooled for all replicates in each test concentration) are summarised in Table 26. Mean snout-vent length is given for all individuals, and for males and females separately.

Snout-vent length data for all individuals, or for females alone, were not normally distributed and were heteroscedastic, and were therefore analysed by non-parametric methods. For either all individuals, or females only, there were no significant differences in snout-vent length in any of the test concentrations or PC, when compared to the DWC.

Male snout-vent length data were normally distributed and homoscedastic. Analysis of variance indicated no significant differences in snout-vent length among test concentrations of BPA, when compared to DWC, while male snout-vent length was significantly greater in the PC than in DWC ( $p < 0.05$ ).

#### 5.4.6 Wet weight

Wet weights of stage 66 froglets (pooled for all replicates in each test concentration) are summarised in Table 27. Mean wet weight is given for all individuals, and for males and females separately.

Wet weight data for all individuals, or for females alone, were not normally distributed and were heteroscedastic, and were therefore analysed by non-parametric methods. For either all individuals, or females only, there were no significant differences in wet weight in any of the test concentrations or PC, when compared to the DWC.

Male snout-vent length data were not normally distributed, but were homoscedastic. Wilcoxon's Rank Sum Test indicated no significant differences in wet weight of males among any of the test concentrations or PC, when compared to the DWC. Analysis of Variance indicated no significant differences in wet weight of males among test concentrations of BPA, when compared to DWC. However, wet weight of PC males was significantly greater ( $p < 0.05$ ) than that of DWC males.

#### 5.4.7 Additional termination data

Termination data from larvae sampled at stages 58 - 65 inclusive (for which gonad morphology was assessed), either during the exposure period or at termination of the study, are presented in Table 28.

Termination data from larvae at developmental stages <58, remaining at termination of the study, are presented in Table 29. Gonad morphology was not assessed at sampling of these larvae.

### 5.5 Water quality parameters monitored - dilution water and tadpole foods

Trace metals and pesticide concentrations, and water quality parameters in routine samples of the dechlorinated water supplied to the Laboratory are given in Table 30.

It is considered that none of the pesticide or trace metals were present in sufficient quantity to have adversely affected the validity of the study.

During the study, the pH of the dilution water varied from 7.25 to 7.81. Conductivity ranged from 204 to 233  $\mu\text{S cm}^{-1}$ . The alkalinity and hardness of the dilution water ranged from 12.8 to 22.8 and from 41.0 to 52.0  $\text{mg l}^{-1}$  as  $\text{CaCO}_3$ , respectively. The free available residual chlorine and the total available residual chlorine were each determined on 65 separate occasions during the study. All determinations of the free available residual chlorine and total available residual chlorine were 2  $\mu\text{g l}^{-1}$  or less, 2  $\mu\text{g l}^{-1}$  being the limit of detection.

Twenty six measurements of total ammonia were made. Values ranged from <0.01 to 0.05  $\text{mg l}^{-1}$ . The total filterable solids were measured on 13 occasions. Values ranged from 0.1 to 0.4  $\text{mg l}^{-1}$ . The dissolved non-purgeable organic carbon content of the dilution water ranged from 0.32 to 1.07  $\text{mg l}^{-1}$ . Full results of water quality analysis are presented in Table 30.

Water quality parameters sampled from test vessels (DWC rep A, and 10  $\mu\text{g l}^{-1}$  rep A) are presented in Table 31.

Pesticide and trace metal analyses of the tadpole and froglet diets are presented in Table 32.

### 5.6 Water quality parameters monitored - test solutions

The data obtained show little variation during the whole study period (Table 33a-d).

Dissolved oxygen levels ranged from	7.0 to 9.0 $\text{mg l}^{-1}$
pH values ranged from	7.19 to 7.79
Temperature values ranged from	21.2 to 22.4°C
Salinity values ranged from	0.5 to 1.5‰

The test solution flow rates (Table 34a) to the individual tanks ranged from 26 to 37  $\text{ml min}^{-1}$  between exposure day 0 and exposure day 10 (nominal 30  $\text{ml min}^{-1}$ ), and from 36 to 47  $\text{ml min}^{-1}$  between exposure day 11 and exposure day 90 (nominal 40  $\text{ml min}^{-1}$ ). The dilution water flow rates to the mixing chambers (Table 34b) ranged from 380 to 440  $\text{ml min}^{-1}$  (nominal 400  $\text{ml min}^{-1}$ ). The test substance stock solution flow rates to the mixing chambers (Table 34c) ranged from 0.16 to 0.26  $\text{ml min}^{-1}$  (nominal

0.2 ml min<sup>-1</sup>) for test substance concentrations, and from 0.37 to 1.3 ml min<sup>-1</sup> (nominal either 0.4 or 1.3 ml min<sup>-1</sup>) for PC. The test substance stock solution dilution ratios (Table 34d) ranged from 1461 to 2500 for test substance concentrations (nominal 2000 × dilution ratio), and from 308 to 1081 for PC (nominal dilution ratio either 300 × or 1000 ×).

### 5.7 Light intensity

The light intensities over the test rig were recorded as 500, 600 and 580 lux on 14 March 2000, and 480, 590, and 570 lux on 18 May 2000.

## 6 SUMMARY AND DISCUSSION OF RESULTS

There were no significant differences in survival or growth and development in larvae exposed to the test substance, BPA, when compared against those in the DWC.

Larval exposure to BPA at any of the test concentrations did not cause significant deviations from the expected sex ratio of 50:50, when considering data pooled for all replicates, as assessed at completion of metamorphosis by inspection of gross gonadal morphology under stereo dissecting microscope. In contrast, exposure of larvae to the Positive Control (PC) substance, 17β-estradiol, resulted in a feminised sex ratio (30% male) that deviated significantly from the expected 50:50 sex ratio.

This is supported by the fact that the PC substance had a similar, statistically significant, feminising effect on post-metamorphic sex ratio in this study, as in the Kloas study.

There were no significant differences in time taken to complete metamorphosis, total length, snout-vent length, and wet weight, in froglets exposed to any of the test concentrations of BPA during larval development, when compared to the DWC. In contrast, time taken to complete metamorphosis was significantly increased in the PC group, (in both males and females). This effect is consistent with an inhibitory effect of 17β-estradiol on development and/or metamorphosis, potentially through negative feedback on the hypothalamic-pituitary regulation of the thyroid axis. Additionally, total length, snout vent length and wet weight were significantly increased in males exposed to 17β-estradiol.

## 7 OVERALL NO OBSERVED EFFECT CONCENTRATION

Based on mean measured concentrations the overall NOEC of BPA for larval growth, development and sexual differentiation (as assessed by gross gonadal morphology) of *Xenopus laevis* was 500 µg l<sup>-1</sup> and therefore the overall LOEC for these endpoints was >500 µg l<sup>-1</sup>.

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TABLE 1

**MEASURED CONCENTRATIONS OF BPA AND E<sub>2</sub> BY LIQUID CHROMATOGRAPHY AS PERCENTAGE OF NOMINAL TEST CONCENTRATIONS**

Exposure day (date)	rep	Percentage of Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )							PC <sup>b</sup> E <sub>2</sub> 2.7 $\mu\text{g l}^{-1}$
		DWC <sup>a</sup>	1.0	2.3	10	23	100	500	
-3 (7/3/2000)	A	<0.5	37	87	110	109	100	78	33
	B	<0.5	43	91	110	109	92	76	36
	C	<0.5	45	91	110	109	98	78	35
	D	<0.5	46	91	110	109	97	76	33
-1 (9/3/2000)	A	<0.5	34	74	140	139	90	68	30
	B	<0.5	33	78	140	139	86	66	29
	C	<0.5	36	74	140	139	90	68	30
	D	<0.5	36	74	140	139	86	70	28
0 (10/3/2000)	A	<0.5	<0.5 <sup>a</sup>	83	160	148	76	70	59
	B	<0.5	<0.5 <sup>a</sup>	74	160	143	86	68	59
	C	<0.5	<0.5 <sup>a</sup>	74	160	143	79	70	59
	D	<0.5	<0.5 <sup>a</sup>	70	160	143	86	68	59
7 (17/3/2000)	A	<0.5	45	91	102	115	117	59	44
	B	<0.5	41	91	101	117	117	75	41
	C	<0.5	45	87	100	116	117	76	41
	D	<0.5	41	89	101	117	117	74	44
10 (20/3/2000)	A	<0.5	<0.5 <sup>a</sup>	88	97	112	107	46	44
	B	<0.5	<0.5 <sup>a</sup>	88	99	114	104	49	44
	C	<0.5	<0.5 <sup>a</sup>	87	98	112	103	54	41
	D	<0.5	<0.5 <sup>a</sup>	89	99	112	104	47	0
12 (22/3/2000)	A		86					100	41
	B		150					100	29
	C		91					111	29
	D		87					90	31
13 (23/3/2000)	A								126
	B								126
	C								122
	D								122
14 (24/3/2000)	A								<0.5
	B								<0.5
	C								<0.5
	D								<0.5

<sup>a</sup> expressed as actual concentrations, not percentage of nominal

<sup>b</sup> expressed as percentage of nominal test concentration

TABLE 1 CONTD

**MEASURED CONCENTRATIONS OF BPA AND E<sub>2</sub> BY LIQUID CHROMATOGRAPHY AS PERCENTAGE OF NOMINAL TEST CONCENTRATIONS**

Exposure day (date)	rep	Percentage of Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )							PC <sup>b</sup> E <sub>2</sub> 2.7 $\mu\text{g l}^{-1}$
		DWC <sup>a</sup>	1.0	2.3	10	23	100	500	
17 (27/3/2000)	A								104
	B								104
	C								104
	D								100
19 (29/3/2000)	A	<0.5	94	96	99	107	105	103	130
	B	<0.5	92	87	104	110	90	98	130
	C	<0.5	95	91	96	97	84	103	122
	D	<0.5	92	91	96	97	89	104	122
21 (31/3/2000)	A		70						
	B		74						
	C		89						
	D		75						
24 (3/4/2000)	A		21						
	B		14						
	C		18						
	D		19						
26 (5/4/2000)	A	<0.5	96	91	97	103	105	108	78
	B	<0.5	91	96	102	113	105	107	74
	C	<0.5	92	91	92	110	99	94	74
	D	<0.5	100	91	90	119	98	107	70
33 (12/4/2000)	A	<0.5	99	91	81	97	105	90	93
	B	<0.5	89	96	97	112	99	79	81
	C	<0.5	86	83	86	100	84	82	81
	D	<0.5	86	83	85	105	90	88	74
40 (19/4/2000)	A	<0.5	91	91	94	113	106	106	67
	B	<0.5	86	96	98	111	106	106	70
	C	<0.5	93	91	90	112	93	112	67
	D	<0.5	83	83	82	104	102	108	63
47 (26/4/2000)	A	<0.5	95	100	89	104	105	114	63
	B	<0.5	92	96	100	113	103	114	56
	C	<0.5	98	96	97	111	94	101	56
	D	<0.5	99	96	86	99	107	113	52

<sup>a</sup> expressed as actual concentrations, not percentage of nominal

<sup>b</sup> expressed as percentage of nominal test concentration

TABLE 1 CONTD

**MEASURED CONCENTRATIONS OF BPA AND E<sub>2</sub> BY LIQUID CHROMATOGRAPHY AS PERCENTAGE OF NOMINAL TEST CONCENTRATIONS**

Exposure day (date)	rep	Percentage of Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )							PC <sup>b</sup> E <sub>2</sub> 2.7 $\mu\text{g l}^{-1}$
		DWC <sup>a</sup>	1.0	2.3	10	23	100	500	
54 (3/5/2000)	A	<0.5	96	100	87	98	109	143	63
	B	<0.5	90	104	110	109	109	132	56
	C	<0.5	96	91	105	105	95	136	56
	D	<0.5	92	96	89	96	106	135	56
61 (10/5/2000)	A	<0.5	120	96	84	83	99	106	63
	B	0.79	140	96	99	98	105	96	63
	C	<0.5	100	91	97	93	94	90	59
	D	<0.5	110	91	85	82	99	98	59
62 (11/5/2000)	A	<0.5							
	B	<0.5	84						
	C	<0.5							
	D	<0.5							
68 (17/5/2000)	A	<0.5	81	87	73	88	97	108	163
	B	<0.5	87	91	94	97	98	97	163
	C	<0.5	83	83	91	97	91	88	159
	D	<0.5	88	87	79	85	96	104	170
75 (24/5/2000)	A	<0.5	88	100	83	93	107	113	85
	B	<0.5	97	100	100	101	108	109	78
	C	<0.5	102	96	99	101	109	82	74
	D	<0.5	109	96	87	100	109	105	78
82 (31/5/2000)	A	<0.5	91	87	81	83	97	111	67
	B	<0.5	91	87	92	95	95	95	67
	C	<0.5	89	87	87	94	92	91	67
	D	<0.5	84	83	72	91	95	108	70
89 7/6/2000)	A	<0.5	87	63	79	77	100	116	72
	B	<0.5	91	67	94	88	95	110	75
	C	<0.5	91	66	82	94	94	83	73
	D	<0.5	89	61	70	95	114	107	76

<sup>a</sup> expressed as actual concentrations, not percentage of nominal

<sup>b</sup> expressed as percentage of nominal test concentration

Bisphenol-A: Determination of effects on larval growth, development and sexual differentiation of the African clawed frog (*Xenopus laevis*)

TABLE 2

**TIME-WEIGHTED MEAN MEASURED CONCENTRATIONS OF BPA AND E<sub>2</sub> BY LIQUID CHROMATOGRAPHY**

	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )							PC E <sub>2</sub> ( $\mu\text{g l}^{-1}$ )
	DWC	1.0	2.3	10	23	100	500	2.7
time-weighted mean ( $\mu\text{g l}^{-1}$ )	N/A	0.83	2.1	9.5	23.8	100	497	2.0
as percentage of nominal conc	N/A	83	90	95	103	100	99	75

TABLE 3

CUMULATIVE RECORDED MORTALITIES (REPLICATES POOLED)

exposure day	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )							
	DWC	1.0	2.3	10	23	100	500	PC
5	11	10	9	12	9	4	19	6
10	13	15	11	15	14	6	22	7
15	14	15	12	16	14	6	23	7
20	15	15	12	16	14	7	23	7
25	15	15	12	17	14	8	23	7
30	15	16	12	17	14	8	23	7
35	15	16	12	17	15	8	23	7
40	15	16	12	17	15	8	25	7
45	15	16	12	17	18	8	25	7
50	16	17	12	17	18	8	25	8
55	16	17	12	17	18	8	25	8
60	16	17	12	17	18	8	25	8
65	17	17	12	17	19	8	25	8
70	17	17	12	18	19	8	25	8
75	18	17	12	18	19	8	25	8
80	18	17	12	18	19	8	25	8
85	18	17	13	18	19	8	25	8
90	18	17	13	18	20	8	27	8

TABLE 4  
SUMMARY SURVIVAL DATA

Nominal conc of BPA ( $\mu\text{g l}^{-1}$ )	rep.	Nominal starting number of larvae	Number of larvae sampled by end of study	Number of recorded mortalities	Calculated number of unrecorded mortalities	Revised starting number	Percentage survival of larvae from exposure day 0 to exposure day 90	
							Individual replicates	Pooled replicates
DWC	A	40	37	3	2	42 <sup>†</sup>	88.1	87.1
	B	40	34	6		40	85.0	
	C	40	37	2	1	40	92.5	
	D	40	34	7		41	82.9	
1.0	A	40	37	4		41	90.2	89.0
	B	40	32	9		41	78.0	
	C	40	29	1		30 <sup>‡</sup>	96.7	
	D	40	39	3		42	92.9	
2.3	A	40	38	1	1	40	95.0	87.5
	B	40	33	5	2	40	82.5	
	C	40	34	4	2	40	85.0	
	D	40	35	3	2	40	87.5	
10	A	40	34	5	1	40	85.0	85.7
	B	40	36	1	3	40	90.0	
	C	40	33	6	1	40	82.5	
	D	40	35	6		41	85.4	
23	A	40	33	4	3	40	82.5	83.8
	B	40	35	5		40	87.5	
	C	40	30	7	3	40	75.0	
	D	40	36	4		40	90.0	
100	A	40	34	3	3	40	85.0	90.7
	B	40	38	3		41	92.7	
	C	40	38	1	1	40	95.0	
	D	40	36	1	3	40	90.0	
500	A	40	32	7	1	40	80.0	81.4 <sup>b</sup>
	B	40	37	3		40	92.5	
	C	40	35	6		41	85.4	
	D	40	27	11	2	40	67.5 <sup>a</sup>	
PC	A	40	31	1	8	40	77.5	87.6
	B	40	39	1		40	97.5	
	C	40	39	2		41	95.1	
	D	40	32	4	4	40	80.0	
overall		1280	1109	129	53	1291		85.9

<sup>†</sup> extra larvae evident at growth and development assessment 1

<sup>‡</sup> large discrepancy of (10) larvae attributed to error in counting larvae at start of study

<sup>a</sup> percentage survival significantly lower than DWC A and C reps ( $p < 0.05$ )

<sup>b</sup> pooled percentage survival significantly lower than in 100  $\mu\text{g l}^{-1}$  treatment group

TABLE 5

**SURVIVAL DATA CONTINGENCY TABLE  
- PAIRWISE COMPARISONS OF INDIVIDUAL REPLICATES**

	rep.	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )															
		DWC	DWC	DWC	DWC	1.0	1.0	1.0	1.0	2.3	2.3	2.3	2.3	10	10	10	10
		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
DWC	A	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	75	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C	71 <sup>#</sup>	48 <sup>#</sup>	100	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	55	100	31	100	-	-	-	-	-	-	-	-	-	-	-	-
1.0	A	100	52 <sup>#</sup>	100	36 <sup>#</sup>	100	-	-	-	-	-	-	-	-	-	-	-
	B	25	57	12	78	23	100	-	-	-	-	-	-	-	-	-	-
	C	39 <sup>#</sup>	23 <sup>#</sup>	63 <sup>#</sup>	13 <sup>#</sup>	39 <sup>#</sup>	3.7 <sup>#</sup>	100	-	-	-	-	-	-	-	-	-
	D	71 <sup>#</sup>	31 <sup>#</sup>	100	19 <sup>#</sup>	71 <sup>#</sup>	6.7 <sup>#</sup>	64	100	-	-	-	-	-	-	-	-
2.3	A	43 <sup>#</sup>	26 <sup>#</sup>	100	15 <sup>#</sup>	68 <sup>#</sup>	4.8 <sup>#</sup>	100	100	100	-	-	-	-	-	-	-
	B	54	100	31	100	35	78 <sup>#</sup>	13	19	15	100	-	-	-	-	-	-
	C	75	100	48	100	52	57 <sup>#</sup>	23	31	26	77 <sup>#</sup>	100	-	-	-	-	-
	D	100	100	71	76 <sup>#</sup>	74	38 <sup>#</sup>	23	48	43	55 <sup>#</sup>	100	100	-	-	-	-
10	A	75	100	48	100	52	57 <sup>#</sup>	23	31	26	77 <sup>#</sup>	100	100	100	-	-	-
	B	100	74 <sup>#</sup>	100	52 <sup>#</sup>	100	23 <sup>#</sup>	38	71	68	36 <sup>#</sup>	74 <sup>#</sup>	100	74 <sup>#</sup>	100	-	-
	C	54	100	31	100	100	78 <sup>#</sup>	13	19	15	100	100	76	100	52	100	-
	D	76	100	48	77 <sup>#</sup>	74	41 <sup>#</sup>	23	31	26	77 <sup>#</sup>	100	100	100	74	77 <sup>#</sup>	100
23	A	54	100	31	100	35	78 <sup>#</sup>	13	19	15	100	100	76	100	52	100	77
	B	100	100	71	76 <sup>#</sup>	74	38 <sup>#</sup>	23	48	43	55 <sup>#</sup>	100	100	100	100	55 <sup>#</sup>	100
	C	16	40	6.6	42	8.4	80	1.9	3.5	2.5	59	40	25	40	14	59	28
	D	100	74	100	52 <sup>#</sup>	100	23 <sup>#</sup>	38	71	68	36 <sup>#</sup>	74 <sup>#</sup>	100	74 <sup>#</sup>	100	36 <sup>#</sup>	74 <sup>#</sup>
100	A	75	100	48	100	52	57 <sup>#</sup>	23	31	26	77 <sup>#</sup>	100	100	100	74	77 <sup>#</sup>	100
	B	71 <sup>#</sup>	31 <sup>#</sup>	100	20 <sup>#</sup>	100	7.1 <sup>#</sup>	63	100	100	19 <sup>#</sup>	31 <sup>#</sup>	48 <sup>#</sup>	31 <sup>#</sup>	71 <sup>#</sup>	19 <sup>#</sup>	48 <sup>#</sup>
	C	43 <sup>#</sup>	26 <sup>#</sup>	100	15 <sup>#</sup>	68 <sup>#</sup>	4.8 <sup>#</sup>	100	100	100	9.2 <sup>#</sup>	26 <sup>#</sup>	43 <sup>#</sup>	26 <sup>#</sup>	68 <sup>#</sup>	9.2 <sup>#</sup>	26 <sup>#</sup>
	D	100	74 <sup>#</sup>	100	52 <sup>#</sup>	100	23 <sup>#</sup>	38	71	68	36 <sup>#</sup>	74 <sup>#</sup>	100	74 <sup>#</sup>	100	36 <sup>#</sup>	74 <sup>#</sup>
500	A	37	77	19	78	23	100	6.8	11	8.7	100	77	55	77	35	100	57
	B	71 <sup>#</sup>	48 <sup>#</sup>	100	31 <sup>#</sup>	100	12 <sup>#</sup>	63	100	100	20 <sup>#</sup>	48 <sup>#</sup>	71 <sup>#</sup>	48 <sup>#</sup>	100	20 <sup>#</sup>	48 <sup>#</sup>
	C	76	100	48	77 <sup>#</sup>	74	41 <sup>#</sup>	23	31	26	77 <sup>#</sup>	100	100	100	74	77 <sup>#</sup>	100
	D	3.3	11	1.0	13	1.5	33	0.2	0.5	0.3	20	11	5.9	11	2.7	20	7.0
PC	A	25	57	11	59	14	100	3.6	6.4	4.8	78	57	38	57	22	78	40
	B	20 <sup>#</sup>	11 <sup>#</sup>	62 <sup>#</sup>	5.7 <sup>#</sup>	36 <sup>#</sup>	1.4 <sup>#</sup>	100	62 <sup>#</sup>	100	3.1 <sup>#</sup>	11 <sup>#</sup>	20 <sup>#</sup>	11 <sup>#</sup>	36 <sup>#</sup>	3.1 <sup>#</sup>	11 <sup>#</sup>
	C	43 <sup>#</sup>	15 <sup>#</sup>	68 <sup>#</sup>	9.2 <sup>#</sup>	68 <sup>#</sup>	2.8 <sup>#</sup>	100	100	100	8.8 <sup>#</sup>	15 <sup>#</sup>	26 <sup>#</sup>	15 <sup>#</sup>	43 <sup>#</sup>	8.8 <sup>#</sup>	26 <sup>#</sup>
	D	37	77	19	78	23	100	6.8	11	8.7	100	77	55	77	35	100	57

Values given in the table are the percentage significance of pair-wise comparisons (values <5% represent statistically significant differences)

# percent survival in the treatment described in the left-hand column is greater than that in the treatment described in the row across the top of the table

- redundant comparison

TABLE 5 CONTD

**SURVIVAL DATA CONTINGENCY TABLE  
- PAIRWISE COMPARISONS OF INDIVIDUAL REPLICATES**

	rep	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )															
		23 A	23 B	23 C	23 D	100 A	100 B	100 C	100 D	500 A	500 B	500 C	500 D	PC A	PC B	PC C	PC D
DWC	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1.0	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.3	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	A	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	55 <sup>#</sup>	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C	59	25	100	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	36 <sup>#</sup>	100	8.8 <sup>#</sup>	100	-	-	-	-	-	-	-	-	-	-	-	-
100	A	77 <sup>#</sup>	100	28 <sup>#</sup>	74	100	-	-	-	-	-	-	-	-	-	-	-
	B	19 <sup>#</sup>	48 <sup>#</sup>	3.7 <sup>#</sup>	71 <sup>#</sup>	31 <sup>#</sup>	100	-	-	-	-	-	-	-	-	-	-
	C	9.2 <sup>#</sup>	43 <sup>#</sup>	1.4 <sup>#</sup>	68 <sup>#</sup>	26 <sup>#</sup>	100	100	-	-	-	-	-	-	-	-	-
	D	36 <sup>#</sup>	100	8.8 <sup>#</sup>	100	74 <sup>#</sup>	71	68	100	-	-	-	-	-	-	-	-
500	A	100	55	79 <sup>#</sup>	35	77	12	8.7	35	100	-	-	-	-	-	-	-
	B	20 <sup>#</sup>	71 <sup>#</sup>	4.0 <sup>#</sup>	100	48 <sup>#</sup>	100	100	100	12 <sup>#</sup>	100	-	-	-	-	-	-
	C	77 <sup>#</sup>	100	28 <sup>#</sup>	74	100	48	26	74	57 <sup>#</sup>	48	100	-	-	-	-	-
	D	20	5.9	62	2.7	11	0.5	0.3	2.7	31	1.0	7.0	100	-	-	-	-
PC	A	78	38	100	22	57	6.7	4.8	22	100	11	40	33 <sup>#</sup>	100	-	-	-
	B	3.1 <sup>#</sup>	20 <sup>#</sup>	0.4 <sup>#</sup>	36 <sup>#</sup>	11 <sup>#</sup>	62 <sup>#</sup>	100	36 <sup>#</sup>	1.6 <sup>#</sup>	62 <sup>#</sup>	11 <sup>#</sup>	0.0 <sup>#</sup>	0.8 <sup>#</sup>	100	-	-
	C	8.8 <sup>#</sup>	26 <sup>#</sup>	1.3 <sup>#</sup>	43 <sup>#</sup>	15 <sup>#</sup>	100	100	43 <sup>#</sup>	4.8 <sup>#</sup>	68 <sup>#</sup>	26 <sup>#</sup>	0.2 <sup>#</sup>	2.6 <sup>#</sup>	100	100	-
	D	100	55	79 <sup>#</sup>	35	77	12	8.7	35	100	19	57	22 <sup>#</sup>	100	2.9	4.8	100

Values given in the table are the percentage significance of pair-wise comparisons (values <5% represent statistically significant differences)

# percent survival in the treatment described in the left-hand column is greater than that in the treatment described in the row across the top of the table

- redundant comparison

TABLE 6

## SURVIVAL DATA CONTINGENCY TABLE - DATA REPLICATES POOLED

	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )							
	DWC	1.0	2.3	10	23	100	500	PC
DWC	100	-	-	-	-	-	-	-
1.0	73 <sup>#</sup>	100	-	-	-	-	-	-
2.3	100	73	100	-	-	-	-	-
10	75	40	74	100	-	-	-	-
23	43	19	43	64	100	-	-	-
100	38 <sup>#</sup>	71 <sup>#</sup>	38 <sup>#</sup>	23 <sup>#</sup>	6.8 <sup>#</sup>	100	-	-
500	17	8.1	17	37	66	1.7	100	-
PC	100	73	100	63 <sup>#</sup>	34 <sup>#</sup>	38	17 <sup>#</sup>	100

Values given in the table are the percentage significance of pair-wise comparisons (values <5% represent statistically significant differences)

# percent survival in the treatment described in the left-hand column is greater than that in the treatment described in the row across the top of the table

- redundant comparison

TABLE 7

**DEVELOPMENTAL STAGES OF LARVAE AT GROWTH AND DEVELOPMENT ASSESSMENT 1 (EXPOSURE DAY 32)**

larvae number	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )							
	DWC	1.0 <sup>a</sup>	2.3	10	23	100	500	PC
1	51	50	51	50	49	49	50	49
2	50	51	50	51	50	50	51	48
3	53	50	50	53	51	50	52	52
4	50	52	52	51	50	51	52	53
5	51	50	54	53	53	51	54	53
6	52	52	54	54	52	50	54	53
7	51	54	50	55	52	49	54	51
8	51	51	52	55	51	51	54	54
9	52	51	51	54	50	48	55	54
10	52	53	53	55	53	52	56	54
11	55	51	52	56	52	53	55	53
12	55	51	51	52	53	54	54	54
13	55	54	53	55	55	55	55	54
14	54	53	53	55	54	54	55	55
15	54	55	55	55	55	55	56	55
16	55	55	52	56	55	54	56	56
17	54	55	54	57	55	55	56	55
18	54	56	54	56	55	54	55	54
19	54	53	55	56	54	55	56	55
20	56	56	55	56	56	54	56	55
21	54	56	55	56	55	55	57	53
22	56	55	55	56	55	56	57	54
23	56	57	56	56	55	55	57	56
24	56	55	55	56	56	55	57	55
25	57	57	54	57	56	56	56	56
26	56	57	55	56	56	56	55	55
27	56	56	55	59	56	55	57	55
28	55	57	56	57	55	56	57	55
29	56	57	55	57	55	56	57	56
30	56	57	56	56	56	57	57	57
31	56	57	56	57	56	56	57	57
32	56	57	55	57	55	56	58	56
33	57	57	56	57	56	57	58	56
34	56	57	56	57	57	57	-	57
35	57	59	56	-	58	58	-	56
36	57	-	57	-	57	57	-	57
37	57	-	56	-	62	59	-	57
38	57	-	57	-	-	-	-	57
39	56	-	57	-	-	-	-	57

- no larvae

<sup>a</sup> two larvae apparently missed during growth and development assessment.



TABLE 9

**TOTAL LENGTHS (mm) OF LARVAE AT GROWTH AND DEVELOPMENT  
ASSESSMENT 1 (EXPOSURE DAY 32)**

larvae number	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ ) (A replicates)							
	DWC	1.0	2.3	10	23	100	500	PC
1	24.8	22.4	22.5	26.4	20.9	22.6	23.3	19.8
2	19.5	25.6	24.4	29.4	19.5	23.4	28.6	17.7
3	25.5	25.5	27.4	26.9	22.2	20	30.8	26.6
4	27.3	24.4	28.6	27.5	25.5	27.5	31.2	25.1
5	26.7	28.5	33.2	35.1	25.0	22.4	35.4	32.4
6	25.8	21.8	33.6	35.4	27.8	23.5	38.4	27.6
7	22.3	30.5	23.7	36.8	31.7	22.4	38.8	26.6
8	26.7	29.8	31.6	35.6	20.6	28.9	36.6	31.7
9	30.7	28.6	27.8	38.7	27.8	17.8	37.9	33.0
10	28.9	26.7	32.7	38.8	26.8	23.8	38.3	34.6
11	35.8	27.5	29.8	40.4	27.1	28.9	35.8	27.8
12	38.8	31.2	29.7	38.1	35.4	28.7	37.3	35.3
13	38.4	36.7	32.6	35.7	35.7	34.3	43.7	31.2
14	30.1	34.7	36.6	45.3	35.5	33.8	39.7	34.8
15	42.1	35.9	30.8	40.9	36.6	35.7	48.6	41.8
16	36.0	35.8	34.4	47.7	36.7	32.5	38.1	47.6
17	38.4	39.5	36.4	47.5	37.6	36.4	41.9	41.2
18	37.0	42.9	34.4	42.9	39.6	32.3	37.8	42.5
19	37.2	35.2	33.8	44.5	34.4	37.7	41.6	42.9
20	34.4	42.5	36.2	47.6	45.0	37.9	42.3	44.5
21	39.4	42.4	40.1	44.0	44.3	41.4	41.6	36.0
22	40.5	42.7	41.7	41.9	42.7	46.3	45.8	42.3
23	40.5	40.8	40.5	45.4	44.5	46.7	47.0	44.7
24	40.0	43.5	36.6	43.6	38.9	42.6	52.7	44.9
25	44.2	50.4	39.8	49.5	41.6	45.8	47.9	45.2
26	43.8	46.8	43.7	43.0	45.5	49.5	45.4	44.5
27	40.4	50.0	41.4	55.0	42.4	41.2	42.8	45.1
28	39.5	46.5	43.4	45.6	42.3	44.1	48.5	44.4
29	46.4	44.5	44.5	48.4	40.8	49.7	48.5	43.5
30	39.9	45.7	47.1	49.4	46.8	46.5	52.5	40.7
31	50.8	43.6	44.8	42.8	41.0	41.6	50.8	51.3
32	43.5	49.8	40.3	47.4	43.8	45.4	48.8	50.0
33	47.9	45.9	49.7	50.2	45.9	44.5	47.4	46.8
34	44.7	51.7	39.7	49.1	50.1	52.7	-	48.9
35	47.9	58.6	42.9	-	54.8	52.6	-	48.3
36	52.4	-	50.7	-	57.8	56.7	-	48.6
37	50.0	-	48.4	-	39.4	54.9	-	53.2
38	54.1	-	46.8	-	-	-	-	53.5
39	47.5	-	54.5	-	-	-	-	47.1

- no larvae

TABLE 10

**MEAN TOTAL LENGTHS BY DEVELOPMENTAL STAGES AT GROWTH AND DEVELOPMENT ASSESSMENT 1 (EXPOSURE DAY 32)**

Dev. stage	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ ) (A replicates)							
	DWC	1.0	2.3	10	23	100	500	PC
48	-	-	-	-	-	17.8	-	17.7
49	-	-	-	-	20.9	22.5	-	19.8
50	23.4	25.5	25.2	26.4	24.3	22.3	23.3	-
51	25.1	28.5	26.7	28.5	21.4	26.3	28.6	26.6
52	28.5	23.1	31.1	38.1	28.9	23.8	31.0	26.6
53	25.5	32.2	34.0	31.0	29.1	28.9	-	31.8
54	37.4	33.6	35.5	37.1	35.0	33.0	37.3	35.8
55	37.7	39.5	38.9	38.9	40.4	39.5	40.1	42.7
56	42.7	44.5	44.6	44.6	43.4	46.1	42.7	46.6
57	49.4	46.6	50.7	47.6	54.0	50.1	47.8	49.0
58	-	-	-	-	54.8	52.6	48.1	-
59	-	58.6	-	55.0	-	54.9	-	-
60	-	-	-	-	-	-	-	-
61	-	-	-	-	-	-	-	-
62	-	-	-	-	39.4	-	-	-
63	-	-	-	-	-	-	-	-
64	-	-	-	-	-	-	-	-
65	-	-	-	-	-	-	-	-
66	-	-	-	-	-	-	-	-

- no larvae at that stage

Bisphenol-A: Determination of effects on larval growth, development and sexual differentiation of the African clawed frog (*Xenopus laevis*)

TABLE 11

**SNOUT-VENT LENGTHS (mm) OF LARVAE AT GROWTH AND DEVELOPMENT ASSESSMENT 1 (EXPOSURE DAY 32)**

larvae number	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )							
	DWC	1.0	2.3	10	23	100	500	PC
1	9.3	8.3	7.3	8.9	7.6	9.0	8.6	7.7
2	7.5	8.7	9.2	10.3	7.2	9.5	11.1	9.2
3	9.0	9.8	10.2	10.1	8.5	7.2	12.1	10.0
4	9.5	9.7	10.7	10.0	10.0	10.3	12.5	9.0
5	9.6	9.3	11.2	13.5	10.1	8.1	12.9	11.6
6	9.9	8.3	12.4	12.1	9.9	9.0	14.1	10.0
7	7.9	10.0	9.5	13.3	12.1	7.7	13.0	11.4
8	9.6	10.5	12.6	13.4	7.9	10.9	13.2	11.6
9	10.9	11.1	10.9	13.9	10.8	7.8	13.5	13.4
10	10.2	9.6	11.2	14.5	11.5	9.4	14.7	12.9
11	12.5	10.0	11.4	15.2	10.3	10.4	12.7	11.0
12	14.0	11.9	10.3	13.7	13.5	11.7	15.8	13.4
13	13.6	12.6	12.7	15.6	12.4	11.9	15.7	12.8
14	11.1	13.5	13.4	16.0	12.9	12.9	14.8	14.0
15	15.0	13.6	12.2	15.8	14.4	12.6	17.5	15.2
16	12.0	13.6	12.1	16.5	14.4	12.0	14.0	16.4
17	14.1	15.4	13.4	17.4	14.2	12.0	15.3	14.6
18	13.3	15.1	12.5	15.5	13.6	11.3	14.9	14.2
19	14.2	13.8	12.7	14.8	12.9	11.9	15.0	14.5
20	13.6	15.0	13.8	16.5	16.4	13.6	15.3	14.8
21	13.9	16.0	15.7	16.8	16.0	16.6	15.4	13.2
22	14.7	15.7	15.6	15.9	14.4	15.6	18.5	14.9
23	12.8	14.9	14.8	15.5	15.7	15.8	16.6	16.2
24	15.7	16.1	13.7	15.5	15.2	16	19.6	15.5
25	17.1	18.2	16.2	18.5	15.8	15.9	17.3	14.8
26	15.1	16.0	14.9	16.6	15.7	17.7	16.9	15.8
27	14.6	18.0	15.8	15.8	14.6	15.6	15.5	15.8
28	14.0	16.2	16.7	16.8	16.8	16.4	16.7	17.3
29	16.5	16.9	16.0	16.1	15.5	16.9	16.7	16.3
30	14.2	18.1	17.2	16.7	17.4	14.5	18.9	15.3
31	18.6	18.2	17.4	16.8	16.5	16.8	17.0	17.1
32	15.9	17.8	15.7	17.9	16.0	17.1	16.7	17.2
33	16.2	17.9	17.0	17.9	17.6	15.9	16.5	17.7
34	15.9	17.2	15.5	18.6	18.5	18.0	-	16.8
35	15.8	19.9	16.3	-	17.8	18.0	-	15.6
36	19.5	-	17.0	-	18.7	19.0	-	16.9
37	17.5	-	16.9	-	14.5	15.9	-	18.6
38	18.2	-	18.2	-	-	-	-	18.5
39	16.6	-	18.8	-	-	-	-	17.4

- no larvae

TABLE 12

**MEAN SNOUT-VENT LENGTH BY DEVELOPMENTAL STAGES AT GROWTH  
AND DEVELOPMENT ASSESSMENT 1 (EXPOSURE DAY 32)**

Dev. stage	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ ) (A replicates)							
	DWC	1.0	2.3	10	23	100	500	PC
48	-	-	-	-	-	7.8	-	9.2
49	-	-	-	-	7.6	8.4	-	7.7
50	8.5	9.1	9.6	8.9	9.3	8.6	8.6	-
51	9.1	10.4	9.5	10.2	8.2	9.8	11.1	11.4
52	10.3	9.0	11.7	13.7	10.8	9.4	12.3	10.0
53	9.0	12.3	12.4	11.8	11.7	10.4	-	11.0
54	13.6	11.3	13.1	13.0	12.9	12.3	13.8	13.3
55	13.2	14.9	14.6	14.8	14.9	14.1	14.8	15.3
56	15.4	16.0	16.5	16.0	16.2	16.6	15.6	16.3
57	17.4	17.1	18.0	17.5	18.6	16.9	17.2	17.2
58	-	-	-	-	17.8	18.0	16.6	-
59	-	19.9	-	15.8	-	15.9	-	-
60	-	-	-	-	-	-	-	-
61	-	-	-	-	-	-	-	-
62	-	-	-	-	14.5	-	-	-
63	-	-	-	-	-	-	-	-
64	-	-	-	-	-	-	-	-
65	-	-	-	-	-	-	-	-
66	-	-	-	-	-	-	-	-

- no larvae at that stage

TABLE 13

**DEVELOPMENTAL STAGES OF LARVAE AT GROWTH AND DEVELOPMENT  
ASSESSMENT 2 (EXPOSURE DAY 62)**

larvae number	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )							
	DWC	1.0	2.3	10	23	100	500	PC
1	54	54	51	53	52	49	57	49
2	53	55	53	51	55	50	57	54
3	53	53	54	57	56	50	57	55
4	54	55	54	58	54	54	57	52
5	56	56	54	59	56	50	60	54
6	56	56	54	61	54	54	62	55
7	56	54	57	63	55	54	63	55
8	57	56	55	63	55	55	64	56
9	59	57	56	64	56	56	64	56
10	58	58	57	65	58	56	66	57
11	57	61	57	-	58	56	66	59
12	59	61	57	-	59	57	-	58
13	59	62	57	-	63	58	-	57
14	58	62	59	-	64	59	-	57
15	61	61	57	-	65	59	-	60
16	62	64	59	-	66	64	-	60
17	63	65	62	-	66	64	-	60
18	64	66	64	-	66	65	-	63
19	66	-	65	-	66	-	-	63
20	66	-	66	-	-	-	-	64
21	-	-	65	-	-	-	-	64
22	-	-	66	-	-	-	-	66
23	-	-	66	-	-	-	-	65
24	-	-	-	-	-	-	-	66
25	-	-	-	-	-	-	-	66
26	-	-	-	-	-	-	-	66

- no larvae

TABLE 14

**PERCENTAGE OF LARVAE AT DEVELOPMENTAL STAGES (48-66) AT GROWTH AND DEVELOPMENT ASSESSMENT 2 (EXPOSURE DAY 62)**

Dev. stage	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ ) (A replicates)							
	DWC	1.0	2.3	10	23	100	500	PC
48	0	0	0	0	0	0	0	0
49	0	0	0	0	0	3	0	3
50	0	0	0	0	0	8	0	0
51	0	0	3	3	0	0	0	0
52	0	0	0	0	3	0	0	3
53	5	3	3	3	0	0	0	0
54	5	6	11	0	6	8	0	6
55	0	6	3	0	9	3	0	9
56	8	9	3	0	9	8	0	6
57	5	3	16	3	0	3	13	9
58	5	3	0	3	6	3	0	3
59	8	0	5	3	3	6	0	3
60	0	0	0	0	0	0	3	9
61	3	9	0	3	0	0	0	0
62	3	6	3	0	0	0	3	0
63	3	0	0	6	3	0	3	6
64	3	3	3	3	3	6	6	6
65	0	3	5	3	3	3	0	3
66 <sup>a</sup>	54	51	47	71	55	50	72	37

<sup>a</sup> figures for stage 66 include froglets terminally sampled at completion of metamorphosis prior to exposure day 62

TABLE 15

**TOTAL LENGTHS (mm) OF LARVAE AT GROWTH AND DEVELOPMENT  
ASSESSMENT 2 (EXPOSURE DAY 62)**

larvae number	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )							
	DWC	1.0	2.3	10	23	100	500	PC
1	32.4	32.5	35.1	43.7	26.9	18.2	56.4	25.1
2	32.8	37.5	31.7	34.7	34.6	26.4	59.6	30.7
3	30.7	37.4	41.4	56.7	47.8	27.6	63.5	40.8
4	39.9	49.6	44.7	70.3	41.3	36.5	69	32.4
5	46.9	41.5	43.2	78.2	44.6	25.4	65.8	44.6
6	43.6	50.8	46.9	69.4	40	29	64.8	44.9
7	58.4	38.8	52.5	60.4	46.5	37.6	44.7	43.8
8	51.8	50.4	54.3	51.4	52.3	45.2	32.8	50
9	58.5	65.6	57	29.8	58.2	45.8	21.5	52.4
10	67.7	73.5	51.9	17.6	67.4	58.8	nm	54.5
11	65.1	56.9	57.2	-	76.1	53.6	nm	51.9
12	60	57.8	60.2	-	71.3	57.5	-	58.4
13	69.4	56.2	63.6	-	47.9	61.4	-	50.8
14	64.9	47.2	53.8	-	26.3	58.6	-	67.1
15	55.9	62.5	63.4	-	19.2	58.6	-	60.8
16	49	21.4	63.6	-	nm	23.4	-	67.6
17	32.7	16.7	42.2	-	nm	22.6	-	53.9
18	17.9	nm	23.8	-	nm	nm <sup>a</sup>	-	55.2
19	nm	-	17.1	-	nm	-	-	48.2
20	nm	-	nm	-	-	-	-	27.7
21	-	-	nm <sup>a</sup>	-	-	-	-	21.9
22	-	-	nm	-	-	-	-	nm
23	-	-	nm	-	-	-	-	nm <sup>a</sup>
24	-	-	-	-	-	-	-	nm
25	-	-	-	-	-	-	-	nm
26	-	-	-	-	-	-	-	nm

- no larvae

nm not measured for stage 66 individuals

<sup>a</sup> not measured for these stage 65 individuals as tail did not project posterior to vent

TABLE 16

**MEAN TOTAL LENGTH BY DEVELOPMENTAL STAGES AT GROWTH AND DEVELOPMENT ASSESSMENT 2 (EXPOSURE DAY 62)**

Dev. stage	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ ) (A replicates)							
	DWC	1.0	2.3	10	23	100	500	PC
48	-	-	-	-	-	-	-	-
49	-	-	-	-	-	18.2	-	25.1
50	-	-	-	-	-	26.5	-	-
51	-	-	35.1	34.7	-	-	-	-
52	-	-	-	-	26.9	-	-	32.4
53	31.8	37.4	31.7	43.7	-	-	-	-
54	36.2	35.7	44.1	-	40.7	34.4	-	37.7
55	-	43.6	54.3	-	44.5	45.2	-	43.2
56	49.6	47.6	57.0	-	50.2	56.2	-	51.2
57	58.5	65.6	58.1	56.7	-	57.5	57.0	57.5
58	66.3	73.5	-	70.3	71.8	61.4	-	58.4
59	62.6	-	58.7	78.2	71.3	58.6	-	51.9
60	-	-	-	-	-	-	65.8	60.8
61	55.9	59.1	-	69.4	-	-	-	-
62	49.0	51.7	42.2	-	-	-	64.8	-
63	32.7	-	-	51.4	47.9	-	44.7	51.7
64	17.9	21.4	23.8	29.8	26.3	23.0	27.2	24.8
65	-	16.7	17.1	17.6	19.2	-	-	-
66	nd	nd	nd	nd	nd	nd	nd	nd

- no larvae at that stage

nd not determined (total length not measured)

TABLE 17

**SNOUT-VENT LENGTHS (mm) OF LARVAE AT GROWTH AND DEVELOPMENT  
ASSESSMENT 2 (EXPOSURE DAY 62)**

larvae number	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )							
	DWC	1.0	2.3	10	23	100	500	PC
1	11.5	12.9	12.7	14.6	9.5	7.0	19.5	8.4
2	12.0	12.5	11.1	12.4	12.5	8.9	20.5	11.3
3	11.5	13.5	15.4	19.3	15.5	9.4	21.6	13.7
4	13.5	16.4	14.4	21.1	14.0	11.6	22.6	11.3
5	16.4	15.6	14.6	27.1	14.8	9.6	20.6	14.6
6	16.8	17.3	16.5	22.7	14.8	10.5	20.5	15.8
7	18.0	15.7	17.5	20.3	16.7	13.3	18.8	17.1
8	17.4	16.6	19.4	18.0	18.2	15.5	18.7	19.6
9	20.1	23.8	20.5	16.8	21.3	16.0	17.4	18.5
10	20.9	25.4	19.5	15.5	23.7	19.7	18.4	19.9
11	21.5	18.5	20.5	-	22.8	17.9	19.5	18.7
12	20.2	18.5	20.7	-	21.7	17.2	-	21.3
13	23.0	18.7	21.6	-	17.3	22.8	-	19.8
14	21.6	16.4	19.5	-	17.5	18.7	-	24.0
15	17.4	17.7	21.2	-	17.9	18.6	-	21.6
16	16.4	16.5	20.5	-	17.3	19.5	-	22.5
17	18.9	15.4	15.4	-	17.4	16.7	-	19.4
18	15.1	14.7	17.7	-	14.5	19.6	-	19.7
19	16.6	-	16.1	-	20.2	-	-	18.4
20	20.7	-	15.6	-	-	-	-	17.4
21	-	-	19.2	-	-	-	-	17.7
22	-	-	14.5	-	-	-	-	17.3
23	-	-	18.6	-	-	-	-	16.8
24	-	-	-	-	-	-	-	17.1
25	-	-	-	-	-	-	-	15.2
26	-	-	-	-	-	-	-	18.9

- no larvae

TABLE 18

**MEAN SNOUT-VENT LENGTH BY DEVELOPMENTAL STAGES AT GROWTH AND DEVELOPMENT ASSESSMENT 2 (EXPOSURE DAY 62)**

Dev. stage.	Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ ) (A replicates)							
	DWC	1.0	2.3	10	23	100	500	PC
48	-	-	-	-	-	-	-	-
49	-	-	-	-	-	7.0	-	8.4
50	-	-	-	-	-	9.3	-	-
51	-	-	12.7	12.4	-	-	-	-
52	-	-	-	-	9.5	-	-	11.3
53	11.8	13.5	11.1	14.6	-	-	-	-
54	12.5	14.3	15.2	-	14.4	11.8	-	13.0
55	-	14.5	19.4	-	15.8	15.5	-	15.5
56	17.1	16.5	20.5	-	17.2	18.8	-	19.1
57	19.5	23.8	20.2	19.3	-	17.2	21.1	21.2
58	21.3	25.4	-	21.1	23.3	22.8	-	21.3
59	21.1	-	20.0	27.1	21.7	18.7	-	18.7
60	-	-	-	-	-	-	20.6	21.2
61	17.4	18.2	-	22.7	-	-	-	-
62	16.4	17.6	15.4	-	-	-	20.5	-
63	18.9	-	-	18.0	17.3	-	18.8	19.1
64	15.1	16.5	17.7	16.8	17.5	18.1	18.1	17.6
65	-	15.4	17.7	15.5	17.9	19.6	-	16.8
66 <sup>a</sup>	18.7	14.7	16.2	-	17.4	-	19.0	17.1

- no larvae at that stage

<sup>a</sup> mean SV lengths representing only larvae in test vessel at time of assessment

TABLE 19

SEX RATIO DATA AS DETERMINED BY GROSS GONADAL MORPHOLOGY AT TERMINATION (DEVELOPMENTAL STAGE 66 ONLY)

Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )	rep	male	female	total	sex ratio (% male)	G-statistic	degrees of freedom	p value
DWC	A	14	18	32	43.8	0.50	1	0.479
	B	12	15	27	44.4	0.33	1	0.563
	C	20	17	37	54.1	0.24	1	0.622
	D	15	17	32	46.9	0.13	1	0.724
	total					1.20	4	0.877
	pooled heterogeneity	61	67	128	47.7	0.28	1	0.596
						0.92	3	0.820
1.0	A	15	15	30	50.0	0.00	1	1.000
	B	23	8	31	74.2	7.57	1	0.006**
	C	14	12	26	53.8	0.15	1	0.695
	D	14	22	36	38.9	1.79	1	0.181
	total					9.52	4	0.049
	pooled heterogeneity	66	57	123	53.7	0.66	1	0.417
						8.86	3	0.031*
2.3	A	15	17	32	46.9	0.13	1	0.724
	B	14	15	29 <sup>†</sup>	48.3	0.03	1	0.853
	C	15	16	31	48.4	0.03	1	0.857
	D	16	17	33	48.5	0.03	1	0.862
	total					0.22	4	0.994
	pooled heterogeneity	60	65	125 <sup>†</sup>	48.0	0.20	1	0.655
						0.02	3	0.999
10	A	16	16	32	50.0	0.00	1	1.000
	B	16	14	30	53.3	0.13	1	0.715
	C	12	18	30	40.0	1.21	1	0.272
	D	15	17	32	46.9	0.13	1	0.724
	total					1.47	4	0.833
	pooled heterogeneity	59	65	124	47.6	0.29	1	0.590
						1.18	3	0.759
23	A	14	13	27 <sup>†</sup>	51.9	0.04	1	0.847
	B	16	18	34	47.1	0.12	1	0.732
	C	8	17	25	32.0	3.31	1	0.069
	D	19	17	36	52.8	0.11	1	0.739
	total					3.58	4	0.466
	pooled heterogeneity	57	65	122 <sup>†</sup>	46.7	0.52	1	0.469
						3.05	3	0.383

\* significant departure from the expected 50:50 sex ratio at the p<0.05 level

\*\* significant departure from the expected 50:50 sex ratio at the p<0.01 level

<sup>†</sup> plus one intersex individual

TABLE 19 CONTD

SEX RATIO DATA AS DETERMINED BY GROSS GONADAL MORPHOLOGY AT TERMINATION (DEVELOPMENTAL STAGE 66 ONLY)

Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )	rep	male	female	total	sex ratio (% male)	G-statistic	degrees of freedom	p value
100	A	15	12	27	55.6	0.33	1	0.563
	B	16	18	34	47.1	0.12	1	0.732
	C	21	15	36	58.3	1.00	1	0.316
	D	14	17	31	45.2	0.29	1	0.590
	total					1.75	4	0.782
	pooled heterogeneity	66	62	128	51.6	0.13	1	0.724
						1.62	3	0.654
500	A	13	19	32	40.6	1.13	1	0.287
	B	18	16	34	52.9	0.12	1	0.732
	C	14	18	32	43.8	0.50	1	0.479
	D	11	15	26	42.3	0.62	1	0.432
	total					2.37	4	0.668
	pooled heterogeneity	56	68	124	45.2	1.16	1	0.281
						1.21	3	0.752
PC	A	9	15	24	37.5	1.52	1	0.218
	B	11	22	33 <sup>‡</sup>	33.3	3.74	1	0.053
	C	8	25	33	24.2	9.19	1	0.002**
	D	8	19	27	29.6	4.61	1	0.032*
	total					19.06	4	0.001**
	pooled heterogeneity	36	81	117 <sup>‡</sup>	30.8	17.76	1	<0.0001***
						1.30	3	0.729

- \* significant departure from the expected 50:50 sex ratio at the  $p < 0.05$  level
- \*\* significant departure from the expected 50:50 sex ratio at the  $p < 0.01$  level
- \*\*\* significant departure from the expected 50:50 sex ratio at the  $p < 0.0001$  level
- ‡ plus 3 intersex individuals

TABLE 20

**SEX RATIO DATA AS DETERMINED BY GROSS GONADAL MORPHOLOGY AT TERMINATION (DEVELOPMENTAL STAGES 58 - 66 INCLUSIVE)**

Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )	rep	male	female	total	sex ratio (% male)	G-statistic	degrees of freedom	p value
DWC	A	16	20	36	44.4	0.45	1	0.505
	B	16	17	33	48.5	0.03	1	0.862
	C	20	17	37	54.1	0.24	1	0.622
	D	15	19	34	44.1	0.47	1	0.492
	total					1.191	4	0.880
	pooled heterogeneity	67	73	140	47.9	0.257	1	0.612
						0.93	3	0.817
1.0	A	18	18	36	50.0	0.00	1	1.000
	B	23	9	32	71.9	6.34	1	0.012*
	C	16	12	28	57.1	0.57	1	0.449
	D	16	22	38	42.1	0.95	1	0.329
	total					7.862	4	0.097
	pooled heterogeneity	73	61	134	54.5	1.076	1	0.300
						6.79	3	0.079
2.3	A	16	19	35	45.7	0.26	1	0.612
	B	14	15	29 <sup>†</sup>	48.3	0.03	1	0.853
	C	16	17	33	48.5	0.03	1	0.862
	D	17	18	35	48.6	0.03	1	0.866
	total					0.351	4	0.986
	pooled heterogeneity	63	69	132	47.7	0.273	1	0.601
						0.08	3	0.994
10	A	16	16	32	50.0	0.00	1	1.000
	B	20	16	36	55.6	0.45	1	0.505
	C	13	18	31	41.9	0.81	1	0.368
	D	17	17	34	50.0	0.00	1	1.000
	total					1.255	4	0.869
	pooled heterogeneity	66	67	133	49.6	0.008	1	0.931
						1.25	3	0.742
23	A	16	13	29 <sup>†</sup>	55.2	0.31	1	0.577
	B	16	18	34	47.1	0.12	1	0.732
	C	12	18	30	40.0	1.21	1	0.272
	D	19	17	36	52.8	0.11	1	0.739
	total					1.748	4	0.782
	pooled heterogeneity	63	66	129	48.8	0.070	1	0.792
						1.68	3	0.642

\* significant departure from the expected 50:50 sex ratio at the  $p < 0.05$  level

\*\* significant departure from the expected 50:50 sex ratio at the  $p < 0.01$  level

† plus one intersex individual

TABLE 20 CONTD

## SEX RATIO DATA AS DETERMINED BY GROSS GONADAL MORPHOLOGY AT TERMINATION (DEVELOPMENTAL STAGES 58 - 66 INCLUSIVE)

Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )	rep	male	female	total	sex ratio (% male)	G-statistic	degrees of freedom	p value
100	A	17	15	32	53.1	0.13	1	0.724
	B	17	19	36	47.2	0.11	1	0.739
	C	23	15	38	60.5	1.70	1	0.193
	D	14	18	32	43.8	0.50	1	0.479
	total					2.434	4	0.656
	pooled	71	67	138	51.4	0.116	1	0.733
	heterogeneity					2.32	3	0.509
500	A	13	19	32	40.6	1.13	1	0.287
	B	18	17	35	51.4	0.03	1	0.866
	C	15	20	35	42.9	0.72	1	0.397
	D	11	16	27	40.7	0.93	1	0.335
	total					2.808	4	0.590
	pooled	57	72	129	44.2	1.748	1	0.186
	heterogeneity					1.06	3	0.787
PC	A	10	18	28	35.7	2.32	1	0.128
	B	11	24	35 <sup>‡</sup>	31.4	4.95	1	0.026
	C	9	28	37	24.3	10.24	1	0.001**
	D	8	23	31	25.8	7.57	1	0.006**
	total					25.074	4	<0.0001***
	pooled	38	93	131	29.0	23.823	1	<0.0001***
	heterogeneity					1.25	3	0.741

\* significant departure from the expected 50:50 sex ratio at the  $p < 0.05$  level

\*\* significant departure from the expected 50:50 sex ratio at the  $p < 0.01$  level

\*\*\* significant departure from the expected 50:50 sex ratio at the  $p < 0.0001$  level

‡ plus 3 intersex individuals

TABLE 21

**SUMMARY OF CASES WHERE ASSESSMENT OF SEX BASED ON GONADAL MORPHOLOGY PRE-FIXATION AND POST-FIXATION DID NOT AGREE**

Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )	rep	direction of mis-assignment <sup>1</sup>	developmental stage
DWC	A	MF	61
	A	FM	58
	B	MF	66
	B	FM	66
1	B	FM	66
	B	?M	66
2.3	C	MF	66
	C	FM	59
	D	MF	66
	D	FM	66
23	A	FI	66
100	B	MF	66
500	A	?F	66
	A	FM	66
	B	MF	66
	B	MF	66
	C	MF	66
PC	B	FI	66
	C	FM	62

<sup>1</sup> first letter indicates pre-fixation assessment, second letter indicates post-fixation assessment.

M male, F female, I intersex, ? indeterminate pre-fixation

Bisphenol-A: Determination of effects on larval growth, development and sexual differentiation of the African clawed frog (*Xenopus laevis*)

TABLE 22

## INCIDENCE OF TESTICULAR ABNORMALITIES

Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )	rep.	asymmetry	melano-cytes	irregular	seg-mented	frag-mented	vacuoles	intersex	no gonad	total
DWC	A	1	1							2
	B			1	1	1				3
	C					1				1
	D		2							2
	total	1	3	1	1	2	0	0	0	8
1.0	A		1	2		2				5
	B		1	1		1				3
	C				1	1				2
	D		2							2
	total	0	4	3	1	4	0	0	0	12
2.3	A			1	1					2
	B					1		1	1	3
	C		1			2				3
	D					1				1
	total	0	1	1	1	4	0	1	1	9
10	A		1	1		1				3
	B	1		2		1				4
	C									0
	D					2				2
	total	1	1	3	0	4	0	0	0	9
23	A							1		1
	B			2		1	1			4
	C									0
	D	1		2	1					4
	total	1	0	4	1	1	1	1	0	9
100	A			1		1				2
	B			1	1					2
	C		1		1					2
	D				1	1				2
	total	0	1	2	3	2	0	0	0	8
500	A									0
	B			1					1	2
	C						1			1
	D				1	1				2
	total	0	0	1	1	1	1	0	1	5
PC	A			3		2	1			6
	B		1	1		4	5	3	2	16
	C			1		4	2			7
	D			3		3	2			8
	total	0	1	8	0	13	10	3	2	37

Bisphenol-A: Determination of effects on larval growth, development and sexual differentiation of the African clawed frog (*Xenopus laevis*)

TABLE 23

## INCIDENCE OF OVARIAN ABNORMALITIES

Nominal concentration of BPA ( $\mu\text{g l}^{-1}$ )	rep.	asymetry or one ovary missing	few/no melanocytes	abnormal shape	thin ovaries with little segmentation	total
DWC	A		2			2
	B		1		2	3
	C			1		1
	D		1		1	2
	total	0	4	1	3	8
1.0	A			1	1	2
	B					0
	C		1			1
	D			1	2	3
	total	0	1	2	3	6
2.3	A				1	1
	B			1		1
	C		1		1	2
	D		1		1	2
	total	0	2	1	3	6
10	A					0
	B	1	1		1	3
	C				1	1
	D				2	2
	total	1	1	0	4	6
23	A					0
	B	1				1
	C					0
	D				1	1
	total	1	0	0	1	2
100	A	1				1
	B					0
	C					0
	D				1	1
	total	1	0	0	1	2
500	A				1	1
	B	1		1	2	4
	C			1	1	2
	D	1				1
	total	2	0	2	4	8
PC	A			1	2	3
	B			1	2	3
	C	1		2	1	4
	D			2	2	4
	total	1	0	6	7	14

TABLE 24

**SUMMARY OF TIME (DAYS) TO COMPLETION OF METAMORPHOSIS  
(DEVELOPMENTAL STAGE 66)**

Nominal conc. of BPA ( $\mu\text{g l}^{-1}$ )	Number of froglets (stage 66)	Minimum days to completion of metamorphosis	Mean days to completion of metamorphosis			Standard deviation (all data)
			male	female	all	
DWC	128	46	59.46	62.37	60.98	12.08
1.0	123	41	62.91	60.11	61.61	12.37
2.3	126	41	59.78	62.14	60.91	12.30
10	124	41	57.41	60.65	59.10	12.15
23	123	41	59.72	62.27	61.09	12.57
100	128	41	63.30	61.66	62.51	12.82
500	124	41	59.30	59.29	59.30	12.00
PC	120	47	69.89**	67.88*	68.15**	12.17

replicates pooled

\* significantly different from DWC at the  $p < 0.05$  level

\*\* significantly different from DWC at the  $p < 0.01$  level

TABLE 25

**SUMMARY OF TOTAL LENGTH DATA (mm) AT COMPLETION OF  
METAMORPHOSIS (DEVELOPMENTAL STAGE 66)**

Nominal conc of BPA ( $\mu\text{g l}^{-1}$ )	Number of froglets (stage 66)	Min.	Max.	Mean			Standard deviation (all data)
				male	female	all	
DWC	128	25.1	51.5	34.90	37.73	36.38	5.82
1.0	123	20.6	51.2	36.80	36.22	36.53	5.82
2.3	126	26.0	46.3	35.92	36.17	36.00	4.89
10	124	27.5	50.6	34.44	37.91	36.26	5.84
23	123	24.9	48.9	34.78	36.94	35.94	5.82
100	128	25.3	49.7	37.20	36.63	36.92	5.44
500	124	24.6	48.4	35.21	35.95	35.62	5.78
PC	120	28.9	64.2	38.11**	39.51	38.98**	5.36

replicates pooled

\*\* significantly different from DWC at the  $p < 0.01$  level

TABLE 26

**SUMMARY OF SNOUT-VENT LENGTH DATA (mm) AT COMPLETION OF METAMORPHOSIS (DEVELOPMENTAL STAGE 66)**

Nominal conc of BPA ( $\mu\text{g l}^{-1}$ )	Number of froglets (stage 66)	Min.	Max.	Mean			Standard deviation (all data)
				male	female	all	
DWC	128	11.6	38.0	16.28	17.61	16.98	3.00
1.0	123	11.0	23.0	16.95	16.70	16.84	2.50
2.3	126	12.3	20.5	16.53	16.57	16.52	2.09
10	124	12.2	23.8	15.91	17.23	16.60	2.59
23	123	11.5	27.1	16.15	17.08	16.65	2.75
100	128	11.8	23.6	16.97	16.78	16.88	2.42
500	124	11.2	23.9	16.48	16.88	16.70	2.66
PC	120	13.4	25.3	17.65*	18.11	17.92	2.13

replicates pooled

\* significantly different from DWC at the  $p < 0.01$  level

TABLE 27

**SUMMARY OF WET WEIGHT (mg) DATA AT COMPLETION OF METAMORPHOSIS (DEVELOPMENTAL STAGE 66)**

Nominal conc of BPA ( $\mu\text{g l}^{-1}$ )	Number of froglets (stage 66)	Min.	Max.	Mean			Standard deviation (all data)
				male	female	all	
DWC	128	143.5	1296.7	479.1	597.9	541.3	264.3
1.0	123	139.7	1245.4	554.5	526.2	541.4	255.4
2.3	126	187.7	1013.3	504.0	505.4	502.2	202.5
10	124	210.8	1533.9	444.4	611.8	532.2	280.3
23	123	144.8	1246.8	462.3	562.9	516.3	263.6
100	128	150.6	1242.9	544.8	524.1	534.8	246.9
500	124	130.6	1299.9	503.5	546.1	526.9	261.5
PC	120	237.9	1593.8	594.4*	625.5	610.8	240.8

replicates pooled

\* significantly different from DWC at the  $p < 0.05$  level

TABLE 28  
 TERMINATION DATA FROM LARVAE AT DEVELOPMENTAL STAGES 58 - 65  
 (GONAD MORPHOLOGY ASSESSED)

Nominal conc of BPA ( $\mu\text{g l}^{-1}$ )	Rep.	Larvae number	Terminated on exposure day	Total length (mm)	SV length (mm)	Wet weight (mg)	Gonadal sex (post-fix)	Developmental stage
DWC	A	34	90	56.4	18.0	614	female	58-
	A	36	90	50.1	18.9	820	male	58
	A	33	90	59.2	17.3	671	female	61
	A	35	90	44.9	13.9	369	male	62
	B	33	90	59.1	19.3	787	female	60
	B	31	90	44.3	17.2	786	male	63
	B	25	74 <sup>1</sup>	23.7	11.0	164	male	64
	B	26	74 <sup>1</sup>	27.3	12.3	210	male	64
	B	24	73 <sup>2</sup>	28.4	12.1	238	female	64
	B	30	90	29.9	13.3	249	male	65
	D	34	90	67.0	16.8	689	female	62
	D	33	90	18.4	11.5	200	female	64
1.0	A	35	90	45.8	14.9	387	female	59
	A	36	90	75.4	26.2	1920	male	60
	A	33	90	45.7	14.1	405	female	62
	A	34	90	63.2	20.4	963	female	62
	A	31	88 <sup>1</sup>	36.1	15.0	523	male	64
	A	32	90	30.0	13.4	234	male	65
	B	32	90	57.5	20.2	985	female	58
	C	27	90	51.0	16.1	510	male	62
	C	28	90	57.2	21.0	1140	male	63
	D	38	90	53.9	17.7	619	male	59
2.3	D	37	90	43.9	20.1	930	male	65
	A	33	90	57.3	17.0	617	female	59
	A	34	90	57.0	15.8	657	female	61
	A	35	90	48.4	19.4	851	male	63
	C	33	90	63.3	19.7	878	male	59
	C	32	90	62.5	19.9	871	female	59
	D	33	90	50.4	13.8	427	female	61
	D	34	90	52.6	14.6	478	male	62
10	B	31	90	61.0	20.1	906	male	59
	B	33	90	52.9	17.5	536	male	59
	B	32	90	57.8	16.0	622	male	60
	B	34	90	27.9	14.4	353	female	64

<sup>1</sup> terminated early as froglets appeared unhealthy, emaciated, and abnormal (limp forelimbs)

<sup>2</sup> found dead in tank on day of sampling. Gonad morphology assessed at post mortem.

Bisphenol-A: Determination of effects on larval growth, development and sexual differentiation of the African clawed frog (*Xenopus laevis*)

TABLE 28 CONTD

Nominal conc of BPA ( $\mu\text{g l}^{-1}$ )	Rep.	Larvae number	Terminated on exposure day	Total length (mm)	SV length (mm)	Wet weight (mg)	Gonadal sex (post fix)	Developmental stage
10 (cntd)	B	36	90	18.6	12.9	239	female	64
	B	35	90	18.6	17.0	498	male	65
	C	31	90	57.6	18.5	659	male	60
	D	33	90	59.1	17.7	1093	male	62
	D	34	90	29.2	12.7	249	male	65
	A	29	90	33.9	12.5	271	male	63
23	A	28	90	19.2	12.0	207	male	64
	C	26	90	42.1	12.9	282	male	63
	C	28	90	28.4	19.2	855	male	64
	C	27	90	22.7	13.6	288	female	64
	C	29	90	14.7	12.8	206	male	65
	C	30	90	42.8	18.7	774	male	65
	A	28	90	51.5	18.0	492	male	59
100	A	29	90	54.3	21.1	983	male	59
	A	30	90	60.3	20.2	782	female	59
	A	32	90	56.9	18.8	718	female	62
	A	31	90	37.4	15.8	417	female	64
	B	35	90	42.0	14.8	340	male	63
	B	36	90	50.5	14.3	452	female	63
	C	1	46 <sup>3</sup>	30.6	13.9	395	male	62
	C	38	90	32.1	16.2	383	male	65
	D	30	90	55.4	18.9	1000	female	60
	B	35	90	62.7	17.5	833	female	62
500	B	36	90	30.6	12.8	236	no gonad	65
	C	34	90	53.0	18.0	641	male	60
	C	35	90	31.7	12.8	271	female	63
	C	32	88 <sup>4</sup>	28.1	12.7	247	female	65
	D	26	90	29.5	13.2	226	female	65
PC	A	26	90	57.6	18.6	745	female	60
	A	27	90	49.8	14.1	399	female	62
	A	28	90	37.2	19.5	867	male	63
	A	25	90	59.2	21.9	1250	female	63
	B	37	90	53.8	21.3	1460	female	63
	B	38	90	42.3	18.6	837	female	65
	C	34	90	56.3	18.0	721	female	59
	C	33	90	62.3	18.9	854	female	60
	C	32	90	49.6	18.2	858	male	62
	C	35	90	21.0	14.2	316	female	64

<sup>3</sup> terminated prematurely - operator error

<sup>4</sup> found dead in tank on day of sampling. Gonad morphology assessed at post mortem.

TABLE 29

DATA FROM LARVAE TERMINATED ON EXPOSURE DAY 90 AT DEVELOPMENTAL STAGES < 58 (GONAD MORPHOLOGY NOT ASSESSED)

Treatment	Rep.	Larvae number	Total length (mm)	SV length (mm)	Wet weight (mg)	Developmental stage
DWC	A	37	58.5	18.8	736	57
	B	34	55.2	17.8	686	57
10	A	29	49.4	15.5	531	57
	C	29	56.8	19.3	822	57
	D	39	52.3	17.2	713	56
2.3	A	36	57.5	18.2	814	57
	A	37	48.6	15.9	470	56
	A	38	53.8	17.3	649	57
	B	31	54.1	15.6	510	57
	B	32	51.0	15.6	478	57
	B	33	48.7	15.1	397	57
	C	34	44.2	15.5	357	56
10	A	33	51.1	16.2	521	56
	A	34	36.9	13.3	254	53
	C	32	56.9	18.9	790	57
	C	33	38.9	13.7	280	54
	D	35	51.0	16.7	531	56
23	A	31	52.2	17.0	610	57
	A	32	39.4	12.4	245	56
	A	33	38.9	12.4	238	56
	B	35	47.2	16.5	517	57
100	A	33	40.7	12.9	279	55
	A	34	52.9	16.8	572	57
	B	37	54.3	15.8	554	57
	B	38	47.4	15.8	482	56
	D	33	53.6	17.3	730	57
	D	34	51.4	17.1	657	57
	D	35	60.5	19.0	986	57
	D	36	54.9	16.5	683	57
500	B	37	55.7	18.2	859	57
PC	A	30	56.2	16.7	768	57
	A	31	35.5	13.3	295	53
	B	39	51.6	16.9	592	56
	C	38	57.3	17.8	870	57
	C	39	34.6	12.3	255	50
	D	32	50.4	16.0	527	56
	A	37	47.6	15.0	379	56

TABLE 30

**WATER QUALITY - ANALYSIS OF TOTAL TRACE METALS, PESTICIDES AND OTHER PARAMETERS IN REPRESENTATIVE SAMPLES OF BRIXHAM DECHLORINATED WATER SUPPLY**

(a) **Total trace metals** - sampled during 2000. Values are reported as mg l<sup>-1</sup>

Sample date	Cd	Co	Cu	Pb	Mn	Ni	Zn	Fe
28.02.2000	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	0.004	0.001
29.05.2000	<0.001	<0.001	0.001	0.001	<0.001	<0.001	0.003	0.003

(b) **Pesticides** - sampled during 2000. Values are reported as µg l<sup>-1</sup>

Analysis completed	Organo-chlorine pesticides (total)	PCBs as Arochlor 1254	Organo-phosphorus pesticides (total)
30.03.2000	<0.01	<0.01	no result
07.06.2000	<0.01	<0.01	<0.01

Analysis of pesticides in water was carried out by Severn Trent Laboratories, STL Business Centre, Torrington Avenue, Coventry, CV4 9GU, UK. Severn Trent Laboratories are a non-GLP compliant laboratory, but are registered to NAMAS 1314.



Bisphenol-A: Determination of effects on larval growth, development and sexual differentiation of the African clawed frog (*Xenopus laevis*)

TABLE 30 CONTD

**WATER QUALITY - ANALYSIS OF TOTAL TRACE METALS, PESTICIDES AND OTHER PARAMETERS IN REPRESENTATIVE SAMPLES OF BRIXHAM DECHLORINATED WATER SUPPLY**

## (c) Other parameters measured

Sample date	pH	Conductivity @ 25°C ( $\mu\text{S cm}^{-1}$ )	Alkalinity as $\text{CaCO}_3$ ( $\text{mg l}^{-1}$ )	Total hardness as $\text{CaCO}_3$ ( $\text{mg l}^{-1}$ )	Chlorine as $\text{Cl}_2$ ( $\mu\text{g l}^{-1}$ )		Total ammonia as $\text{NH}_4\text{-N}$ ( $\text{mg l}^{-1}$ )	Total filterable solids ( $\text{mg l}^{-1}$ )	Dissolved non purgeable organic carbon ( $\text{mg l}^{-1}$ )
					Free residual	Combined residual			
					10.04.2000	7.54			
11.04.2000	7.57	229		47.7	<2	<2			
12.04.2000	7.67	230		47.0	<2	<2			0.43
13.04.2000	7.69	228	21.0	46.3	<2	<2		<0.2	
14.04.2000	7.72	226		46.7	<2	<2	<0.01		
15.04.2000									
16.04.2000									
17.04.2000	7.50	225	20.0	46.3	<2	<2	0.03		
18.04.2000	7.69	225		47.0	<2	<2			
19.04.2000	7.41	223		45.7	<2	<2			0.32
20.04.2000	7.63	213	19.8	45.3	<2	<2		<0.2	
21.04.2000	7.46	223		48.0	<2	<2	0.05		
22.04.2000									
23.04.2000									
24.04.2000	7.46	211	18.8	47.3	<2	<2	0.02		
25.04.2000	7.56	213		46.7	<2	<2			
26.04.2000	7.64	217		48.3	<2	<2			0.44
27.04.2000	7.78	216	20.8	49.0	<2	<2		0.2	
28.04.2000	7.68	224		52.0	<2	<2	<0.01		
29.04.2000									
30.04.2000									
01.05.2000	7.71	223	21.0	47.0	<2	<2	0.04		
02.05.2000	7.61	222		43.3	<2	<2			
03.05.2000	7.81	226		44.3	<2	<2			0.69
04.05.2000	7.78	231	22.8	44.7	<2	<2		0.3	
05.05.2000	7.73	229		46.0	<2	<2	0.01		
06.05.2000									
07.05.2000									
08.05.2000	7.62	233	21.4	46.0	<2	<2	0.01		
09.05.2000	7.58	233		45.3	<2	<2			
10.05.2000	7.50	228		45.3	<2	<2			0.84
11.05.2000	7.62	222	20.4	44.7	<2	<2		<0.2	

Bisphenol-A: Determination of effects on larval growth, development and sexual differentiation of the African clawed frog (*Xenopus laevis*)

TABLE 30 CONTD

## (c) Other parameters measured

Sample date	pH	Conductivity @ 25°C ( $\mu\text{S cm}^{-1}$ )	Alkalinity as $\text{CaCO}_3$ ( $\text{mg l}^{-1}$ )	Total hardness as $\text{CaCO}_3$ ( $\text{mg l}^{-1}$ )	Chlorine as $\text{Cl}_2$ ( $\mu\text{g l}^{-1}$ )		Total ammonia as $\text{NH}_4\text{-N}$ ( $\text{mg l}^{-1}$ )	Total filterable solids ( $\text{mg l}^{-1}$ )	Dissolved non purgeable organic carbon ( $\text{mg l}^{-1}$ )
					Free residual	Combined residual			
12.05.2000	7.54	221	21.0	45.0	<2	<2	0.05		
13.05.2000									
14.05.2000									
15.05.2000	7.47	224	21.2	45.3	<2	<2	<0.01		
16.05.2000	7.64	223		45.0	<2	<2			
17.05.2000	7.65	218		45.0	<2	<2			0.82
18.05.2000	7.54	225	20.4	44.3	<2	<2		<0.2	
19.05.2000	7.53	222		44.3	<2	<2	<0.01		
20.05.2000									
21.05.2000									
22.05.2000	7.55	215	20.6	43.7	<2	<2	<0.01		
23.05.2000	7.56	219		43.3	<2	<2			
24.05.2000	7.60	224		42.0	<2	<2			0.88
25.05.2000	7.52	218	19.6	43.3	<2	<2		<0.2	
26.05.2000	7.55	215		45.3	<2	<2	0.02		
27.05.2000									
28.05.2000									
29.05.2000	7.52	210	20.0	44.6	<2	<2	0.02		
30.05.2000	7.52	204		41.0	<2	<2			
31.05.2000	7.58	207		41.3	<2	<2			0.94
01.06.2000	7.46	212	18.0	43.3	<2	<2		0.4	
02.06.2000	7.51	212		45.0	<2	<2	0.03		
03.06.2000									
04.06.2000									
05.06.2000	7.52	209	17.4	44.0	<2	<2	0.02		
06.06.2000	7.59	210		43.7	<2	<2			
07.06.2000	7.62	214		44.7	<2	<2			1.07
08.06.2000	7.63	214	18.8	44.3	<2	<2		<0.2	

TABLE 30 CONTD

**WATER QUALITY - ANALYSIS OF TOTAL TRACE METALS, PESTICIDES AND OTHER PARAMETERS IN REPRESENTATIVE SAMPLES OF BRIXHAM DECHLORINATED WATER SUPPLY**

**(c) Other Parameters Measured - summary data**

Sample date	pH	Conductivity @ 25°C ( $\mu\text{S cm}^{-1}$ )	Alkalinity as $\text{CaCO}_3$ ( $\text{mg l}^{-1}$ )	Total hardness as $\text{CaCO}_3$ ( $\text{mg l}^{-1}$ )	Chlorine as $\text{Cl}_2$ ( $\mu\text{g l}^{-1}$ )		Total ammonia as $\text{NH}_4\text{-N}$ ( $\text{mg l}^{-1}$ )	Total filterable solids ( $\text{mg l}^{-1}$ )	Dissolved non purgeable organic carbon ( $\text{mg l}^{-1}$ )
					Free residual	Combined residual			
Mean	-	219.1	18.7	45.2	-	-	-	-	0.66
Number of samples	65	65	27	65	65	65	26	13	13
Standard deviation	-	6.64	2.57	2.02	-	-	-	-	0.26
Minimum	7.25	204	12.8	41.0	<2	<2	<0.01	0.1	0.32
Maximum	7.81	233	22.8	52.0	2	2	0.05	0.4	1.07

- not determined

TABLE 31

**WATER QUALITY - ANALYSIS OF PARAMETERS SAMPLED FROM TEST VESSELS DURING THE STUDY**

Sample date	Tank 8A (DWC)				Tank 6A (10 µg l <sup>-1</sup> )			
	pH	Conductivity @ 25°C (µS cm <sup>-1</sup> )	Alkalinity as CaCO <sub>3</sub> (mg l <sup>-1</sup> )	Total hardness as CaCO <sub>3</sub> (mg l <sup>-1</sup> )	pH	Conductivity @ 25°C (µS cm <sup>-1</sup> )	Alkalinity as CaCO <sub>3</sub> (mg l <sup>-1</sup> )	Total hardness as CaCO <sub>3</sub> (mg l <sup>-1</sup> )
09.03.2000	7.62	226	18.6	45.3	7.65	222	19.0	46.3
16.03.2000	7.51	220	15.8	43.7	7.42	219	15.6	42.3
23.03.2000	7.26	222	15.6	45.3	7.29	225	15.4	45.3
30.03.2000	7.44	227	16.6	46.0	7.43	226	16.6	45.0
06.04.2000	7.44	233	18.4	48.3	7.40	237	17.8	49.0
13.04.2000	7.50	235	20.8	46.7	7.45	232	21.0	46.0
20.04.2000	7.31	222	19.8	46.0	7.34	223	20.2	45.7
27.04.2000	7.46	222	22.8	50.3	7.46	221	21.8	53.0
04.05.2000	7.33	232	23.0	46.0	7.32	236	23.0	46.0
11.05.2000	7.29	227	22.2	46.7	7.34	226	22.0	46.7
18.05.2000	7.43	226	21.6	45.0	7.41	224	21.6	44.7
25.05.2000	7.48	220	18.0	42.7	7.42	222	18.0	44.3
01.06.2000	7.21	215	17.2	44.3	7.39	212	18.0	45.0
08.06.2000	7.57	213	19.2	44.0	7.56	212	18.6	43.7
Mean	-	224	19.3	45.7	-	224	19.2	45.9
Number of samples	14	14	14	14	14	14	14	14
Minimum	7.21	213	15.6	42.7	7.29	212	15.4	42.3
Maximum	7.62	235	23.0	50.3	7.65	237	23.0	53.0

- not determined

TABLE 32

**PESTICIDE AND TRACE METAL ANALYSIS OF TADPOLE AND FROGLET DIETS**

**(a) Pesticide results in mg kg<sup>-1</sup> (wet weight)**

Date of analysis	19.04.2000	05.05.2000
Food type	Sera Micron Powder	<i>Xenopus</i> pellet food
Food batch no.	FF448	FF450
Alpha HCH	<0.02	<0.02
Beta HCH	<0.02	<0.02
Gamma HCH	<0.02	<0.02
Heptachlor	<0.01	<0.01
Aldrin	<0.02	<0.02
Heptachlor expoxide	<0.01	<0.01
Dieldrin	<0.02	<0.02
DDD	<0.01	<0.01
DDE	<0.02	<0.02
DDT	<0.03	<0.03
Chlordane	<0.02	<0.02
Endrin	<0.01	<0.01
Endosulfan	<0.02	<0.02
Quintozene	<0.01	<0.01
Tecnazene	<0.02	<0.02
HCB	<0.01	<0.02

Analysis carried out by Aspland and James, Medcalfe Way, Bridge Street, Chatteris. Cambs., PE16 6QZ, UK. Original data filed in their archive.

**(b) Metals values reported as mg kg<sup>-1</sup> dry weight**

Food batch no	Food type	Cd	Co	Cr	Cu	Pb	Mn	Ni	Zn	Fe
FF448	Sera Micron Powder	0.15	0.16	1.5	9	2.1	27	1.8	97	700
FF450	<i>Xenopus</i> pellet food	0.14	0.14	0.73	7.8	0.41	51	1.4	160	98

Original data for the metals results, along with copies of the pesticide results, are held in the Brixham Environmental Laboratory archive under reference FD01.

TABLE 33

## SUMMARY OF WATER QUALITY MEASUREMENTS

## a) Dissolved oxygen

Nominal conc of BPA ( $\mu\text{g l}^{-1}$ )	Range ( $\text{mg l}^{-1}$ )	Mean ( $\text{mg l}^{-1}$ )	Standard deviation	Number of samples
DWC	7.0 - 9.0	8.12	0.401	56
1.0	7.2 - 8.8	8.03	0.395	56
2.3	7.6 - 8.8	8.09	0.346	56
10	7.2 - 8.8	8.01	0.381	56
23	7.6 - 8.8	8.09	0.343	56
100	7.6 - 9.0	8.09	0.347	56
500	7.4 - 9.0	8.01	0.462	56
PC	7.2 - 9.0	7.96	0.415	56

## b) pH

Nominal conc of BPA ( $\mu\text{g l}^{-1}$ )	Range	Number of samples
DWC	7.28 - 7.79	56
1.0	7.19 - 7.78	56
2.3	7.25 - 7.78	56
10	7.22 - 7.79	56
23	7.25 - 7.79	56
100	7.27 - 7.78	56
500	7.19 - 7.78	56
PC	7.21 - 7.79	56

TABLE 33 CONTD

SUMMARY OF WATER QUALITY MEASUREMENTS

c) Temperature

Nominal conc of BPA ( $\mu\text{g l}^{-1}$ )	Range ( $^{\circ}\text{C}$ )	Mean ( $^{\circ}\text{C}$ )	Standard deviation	Number of samples
DWC	21.2 - 22.0	21.65	0.159	108
1.0	21.6 - 22.2	21.86	0.128	108
2.3	21.6 - 22.4	21.92	0.191	108
10	21.5 - 22.1	21.80	0.135	108
23	21.5 - 22.2	21.81	0.161	108
100	21.3 - 22.1	21.69	0.192	108
500	21.6 - 22.2	21.87	0.132	108
PC	21.5 - 22.2	21.74	0.150	108

TABLE 33 CONTD

## SUMMARY OF WATER QUALITY MEASUREMENTS

## d) Salinity

Exposure day (date)	Salinity(‰)	
	DWC (tank 8B)	23 µg l <sup>-1</sup> (tank 2B)
0 (10.03.2000)	0.5	0.5
7 (17.03.2000)	0.5	0.5
14 (24.03.2000)	0.5	0.5
21 (31.03.2000)	1.0	1.0
28 (07.04.2000)	1.0	1.0
35 (14.04.2000)	0.5	0.5
42 (21.04.2000)	0.5	0.5
49 (28.04.2000)	0.5	0.5
56 (05.05.2000)	<1.0	<1.5
63 (12.05.2000)	<1.0	<1.0
70 (19.05.2000)	0.5	0.5
77 (26.05.2000)	0.5	0.5
84 (02.06.2000)	0.5	0.5



TABLE 34 CONTD.

## SUMMARY OF FLOW DATA (EXPOSURE DAYS 0 - 90)

## c) Test substance stock solution flow rates

Stock solution flow rates (ml min <sup>-1</sup> )	Nominal conc of BPA ( $\mu\text{g l}^{-1}$ )							PC <sup>†</sup>
	DWC	1.0	2.3	10	23	100	500	
Minimum	-	0.20	0.20	0.22	0.22	0.20	0.16	0.37
Maximum	-	0.24	0.24	0.26	0.26	0.26	0.26	1.30
Mean	-	0.22	0.21	0.23	0.23	0.23	0.21	-
SD	-	0.010	0.012	0.012	0.011	0.013	0.030	-
no. of samples	-	27	27	27	27	27	27	28

- not applicable

† 17 $\beta$ -estradiol stock solution flow rate was altered during the course of the study (Section 4.2)

## d) Test substance stock solution dilution ratios

Dilution ratios	Nominal conc of BPA ( $\mu\text{g l}^{-1}$ )							PC <sup>†</sup>
	DWC	1.0	2.3	10	23	100	500	
Minimum	-	1792	1792	1539	1461	1539	1539	308
Maximum	-	2150	2150	1955	1818	1950	2500	1081
Mean	-	1977	2000	1770	1690	1723	1888	-
SD	-	100	105	96	86	93	300	-
no. of samples	-	27	27	27	27	27	27	28

- not applicable

† 17 $\beta$ -estradiol stock solution flow rate was altered during the course of the study (Section 4.2)

FIGURE 1  
DOSING SYSTEM

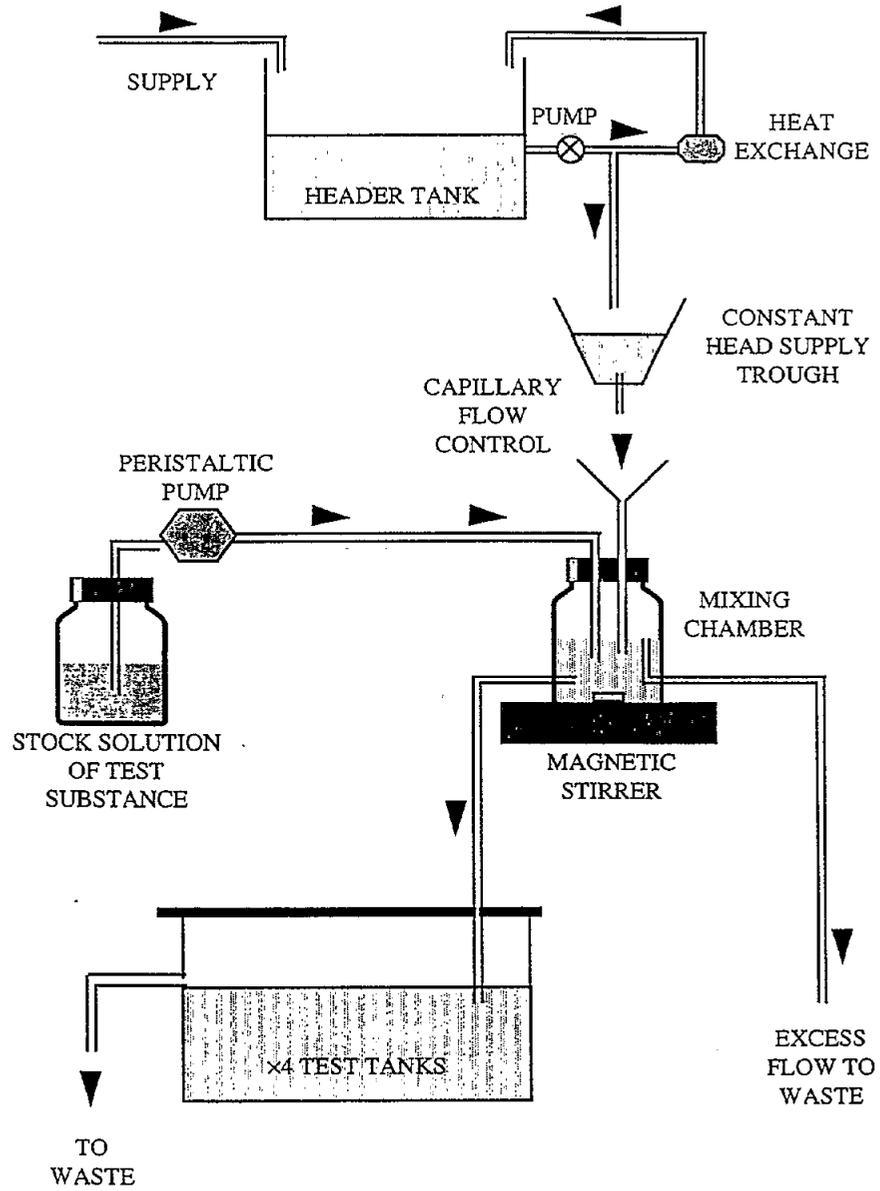
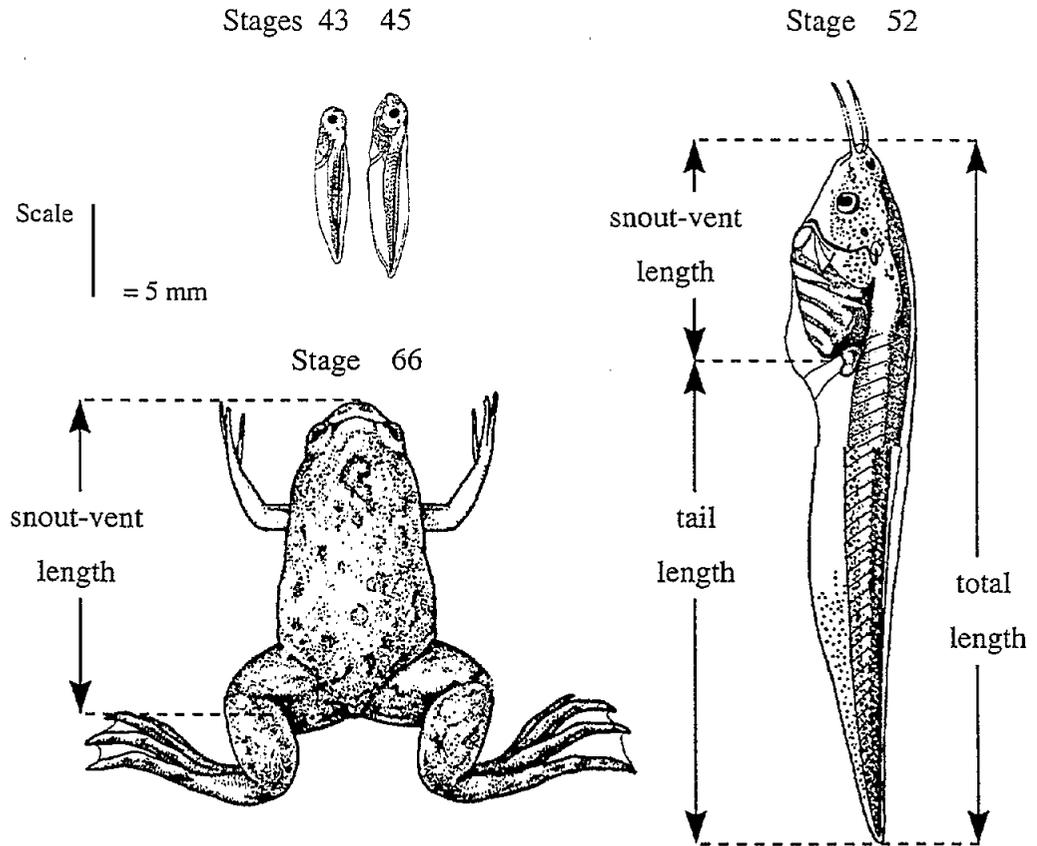


FIGURE 2

**XENOPUS LAEVIS LARVAE AT NIEUWKOOP AND FABER STAGES 43/45 (START OF EXPOSURE), 52 (BEGINNING OF SEXUAL DIFFERENTIATION OF GONADS) AND 66 (COMPLETION OF METAMORPHOSIS; END OF EXPOSURE)**



Figures adapted from Nieuwkoop and Faber (1967).

## APPENDIX 1

## SCHEDULE FOR WATER QUALITY ANALYSIS OF FRESHWATER

Parameter	Sampling frequency
pH	Daily (Mon-Fri)
Total hardness	
Conductivity	
Free available residual chlorine	
Total available residual chlorine	
Total ammonia	Twice weekly
Alkalinity	
Total filterable solids	Weekly
Dissolved non purgeable organic carbon	
Trace metals	Quarterly
Total organo-chlorine pesticides	Quarterly
Total organo-phosphorus pesticides	
Total PCBs	

## APPENDIX 2

### DETERMINATION OF BPA IN AQUEOUS SAMPLES BY HPLC WITH FLUORESCENCE DETECTION

#### 1 SUMMARY

Aqueous samples are analysed by HPLC using a fluorescence detector and quantified by comparison against known standards of the test substance. Using the given conditions a linear response is obtained up to  $50 \mu\text{g l}^{-1}$ , the limit of detection being approximately  $1 \mu\text{g l}^{-1}$  and no interferences are found in the media used for fathead minnow studies.

#### 2 MATERIALS AND REAGENTS

- A sample of test substance of known purity
- HPLC grade methanol
- HPLC grade water
- Mercuric chloride

#### 3 PREPARATION OF STOCKS AND STANDARDS

Prepare a  $1000 \text{ mg l}^{-1}$  stock of the test substance in methanol. From this stock prepare a series of standard solutions of the test substance in deionised water to cover the expected concentration range. The test substance stock solution will be stable for at least 6 months. The standards will be stable for at least 6 weeks.

Prepare a stock solution of mercuric chloride in methanol by dissolving 3 g of mercuric chloride in 10 ml methanol.

#### 4 OPERATING CONDITIONS FOR THE LIQUID CHROMATOGRAPH

The analysis of the samples for the test substance will depend upon the performance of the instrumentation and the SOPs concerning the operation of these instruments should be consulted to ensure optimum response. The following conditions are suitable for the analysis of the test substance:

column	150 mm × 4.6 mm (id)
column packing	Hypersil H5ODS
injection volume	200 $\mu\text{l}$
eluent	65:35 methanol:water
eluent flow rate	1 $\text{ml min}^{-1}$
detection	Excitation @ 230 nm

## CIRCULATION

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