

Investigation of the toxicity of bromate
on the fertilization success of the Sea
Urchin *Paracentrotus lividus*



Kalundborg Forsyning/VUDP

Final report

October 2022

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Approved by

07/11/2022

X *Anja Kamper*

Approved by

Signed by: Anja Kamper

Investigation of the toxicity of bromate on the fertilization success of the Sea Urchin *Paracentrotus lividus*

Prepared for Kalundborg Forsyning, VUDP project
Represented by Preben Thisgaard



Project manager	Anja Kamper
Quality supervisor	Connie Seierø

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1 Introduction

DHI has conducted a toxicity test assessing the effects of bromate to the fertilisation success of the sea urchin, *Paracentrotus lividus*. The test method is found in Section 15 of EPA's Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition /1/.

Sperm cells were exposed to a dilution series of the test item concentrations for 1 hour. The eggs were then introduced to the test chambers which contain the sperm cells. After 20 minutes, the test was ended and the effects on exposed gametes were compared to controls to determine if the test concentrations had any effect on fertilization.

The test aimed at determining the No Effect Concentration (NOEC) level and if possible, the EC50 concentration, which results in inhibition of the fertilisation success of 50 %.

2 Test item

The purpose of the test was to investigate the toxicity of bromate (BrO_3^-), which can be formed if ozone treatment is installed at a waste water treatment plant. DHI has therefore purchased 99.5% pure Sodium Bromate (NaBrO_3) from Sigma-Aldrich (Product No. 71325, Batch No. BCBX9200) for use in the test. The certificate of analysis and the safety data sheet is attached in Annex C.

Table 2.1 Information regarding the test item

Product Name:	Sodium Bromate
Purity	>= 99.5 % RT
Product Number:	71325
Batch Number:	BCBX9200
Brand:	Sigma-Aldrich
CAS Number:	7789-38-0
Formula:	NaBrO_3
Formula Weight:	150.89
Quality Release Date:	03 AUG 2018
Recommended Retest Date:	JAN 2023

3 Test method

3.1 Test solution

A stock solution of sodium bromate (NaBrO_3) was prepared by dissolving 1180 mg in 1000 mL of filtered natural seawater with a salinity of 32 PSU. This stock solution

corresponds to 1000 mg bromate/L. The dilution series was prepared by adding the appropriate amounts of stock solution to 300 mL of filtered sea water. No adjustment of stock solution or dilution series was necessary.

3.2 Test organism

The species in the EPA Method is not available in the EU and therefore the species *Paracentrotus lividus* was used instead. Sexually mature adult echinoids (12 males and 12 females) were delivered by Ecimat, University of Vigo, Spain. The sea urchins were shipped cold and arrived within 24 hours from shipment.

The adults were shipped “dry” (wrapped in moist paper towel) and a gradually shift of adults from shipping conditions to test conditions was started just after arrival at DHI facilities. First the sea urchins were gradually acclimatised to the holding temperature and then after achieving the temperature, they were added to the tanks with filtered sea water with heavy aeration and seaweed.

Conditions for culturing were: 16-hour light:8-hour dark photoperiod, salinity 32 psu. The water in tanks holding adults was renewed every third day to prevent a build-up of metabolic wastes. Feeding with seaweed ad libitum.

The recommendation from the supplier is to use the sea urchins as soon as possible (the first 48 hours) to have good quality eggs and sperm and guarantee the viability of the individuals.

Table 3.1 Summary of test conditions

Test guideline	US EPA fertilization test method 1008.0
Test type	Static, non-renewal
Salinity	30‰ ± 2‰
Temperature (C°)	20°C ± 1°C Test temperatures must not deviate by more than 3°C during the test (i.e., max. temp – min. temp ≤ 3°C) (required)
Light quality	Ambient laboratory light during test preparation
Light intensity	10 – 20 µE/m ² /s (ambient laboratory levels)
Test chamber size	Disposable (glass) liquid scintillation vials (20 mL capacity)
Test solution volume	5 mL
Number of eggs and sperm cells per chamber	About 2,000 eggs and 5,000,000 sperm cells per vial
Number of replicate chambers per concentration	4 replicates of each test concentration 8 control
Dilution water	Uncontaminated source of filtered natural seawater
Test concentrations	0; 240; 340; 490; 700 and 1,000 mg bromate/L

Oxygen, pH, temp and salinity	Measured at the test start in test solutions
Test duration	1 hour and 20 minutes
Endpoint	Fertilization of sea urchin eggs
Test validity criteria	70% – 90% egg fertilization in controls
Reference test	Copper test solutions were prepared with copper sulphate. Copper concentrations: 6.25, 12.5, 25.0, 50.0, and 100 µg Cu/L.

3.3 Test procedure

3.3.1 Sperm collection

Sperm was collected from 2 males. 1 mL of 0.5 M KCl solution was injected by the mouth with a thin needle (26 G) distributed over 3 sites, and then the animals were gently turned a few times. The sperm was sucked up with a pipette into a vial. The sperm was kept on ice until use and was used within approx. 1 hour. The sperm suspension was counted in a Neubauer hemacytometer.

3.3.2 Egg collection

Eggs were collected from 2-4 females. 1 mL of 0.5 M KCl solution was injected by the mouth with a thin needle (26 G) distributed over 3 sites, then animals were gently turned a few times. The eggs were rinsed into a beaker with sea water, with a disposable pipette. Eggs were transferred to a plastic centrifuge tube (15 ml). The eggs were washed 3 times with seawater, by centrifuging at speed: 850 for 3 min, removing the above water and putting clean seawater on. The washed egg suspension can last for a few hours at room temperature.

The egg stock solution containing approx. 2000 eggs/mL (counted in a Sedgwick-Rafter chamber). In order to keep the eggs in suspension in the egg stock solution, a weak aeration was present in the beaker.

3.3.3 Test set-up

At the time where the sperm solution was ready to use, 100 µL of the sperm solution was added to each test vials with 5 ml test solution and then mixed by gentle circulation of the rack. The exposure conditions were as described in Table 3.1.

After 1 hour of exposure of the sperm cells, 1 mL of egg solution was added to all vials, with a pipette where the tip is cut off, the rack was mixed again by circulation. After 20 minutes of fertilisation time the test was stopped by adding 1 mL of 1% glutaraldehyde to all vials and the rack was circulated again.

Within 48 hours, all vials were counted: 100-200 eggs per vial. During counting it was noted how many eggs were fertilized and unfertilized. A Sedgwick-Rafter chamber and a stereo microscope was used for counting.

4 Statistical analysis

The statistical analyses were performed using the free software R /1/. The corresponding packages used for the different analysis are described in Section 7, References.

The LOEC/NOEC values were determined by use of the Dunnett's test /3/. The NOEC values ($p > 0.05$) were determined as the highest tested concentration, at which no significant negative effect was observed compared with the control. The LOEC ($t < 0$ and $p < 0.05$) is the concentration just above the NOEC.

To comply with the conditions of the Dunnett's test, the normality of the data was assessed but only by a visual description using a boxplot /4/. The variance was analysed with a Levene's test /5/.

5 Results

5.1 Results from chemical analysis

Duplicate samples for chemical analysis were collected from each test concentration and the control at the initiation of the test, and at the termination of the test.

The samples were collected in 60-mL PE bottles containing NaOH and EDTA for conservation and stored at $4 \pm 2.0^\circ\text{C}$. The samples were shipped cold to the analytical laboratory, ALS Scandinavia AB, Danderyd, Sweden.

Verification of the test item stability in the test system was verified by analysing the lowest and the highest test concentration at the test start (Table 5.1 and Annex B). The stability was satisfactory and therefore no more samples were analysed.

Table 5.1 Results from chemical analysis

Nominal concentration		Actual measured concentration of bromate
NaBrO ₃ mg/L	BrO ₃ ⁻ mg/L	Test day
283	240	218
1180	1000	976

5.2 Ecotoxicological results

The reference substance was tested in the concentration series of 6.25, 12.5, 25.0, 50.0, and 100 µg Cu/L. The sensitivity of the sea urchin to the reference substance was comparable to results in the US EPA guideline /1/ in which an average EC50 of 29.9 µg Cu/L was reported. This result was an average of 5 tests performed with a different sea urchin species in natural sea water. As the results in the present reference test are comparable to results from the guideline and no validity criteria in this respect is present, the results from the reference test are accepted.

Table 5.2 Results of the ecotoxicological test with the reference item

NOEC ($\mu\text{g Cu/L}$)	LOEC ($\mu\text{g Cu/L}$)	EC10 ($\mu\text{g Cu/L}$)	EC50 ($\mu\text{g Cu/L}$)
12.5	25	20.3 (16.6-24.2)	36.0 (32.8-39.3)

The primary data are presented in Appendix A. The results of this test are summarized below.

As no significant effect or any dose-response relationship was observed, the EC10 and EC50 for the endpoint fertilisation success were estimated >1000 mg bromate/L.

Table 5.3 Sea Urchin fertilisation success after exposure to sodium bromate.

Concentration BrO_3^- mg/L	Average number of fertilised eggs (%)	Standard deviation
Control	75	5.9
240	73	1.5
340	71	1.9
490	67	6.0
700	74	4.3
1000	69	3.4

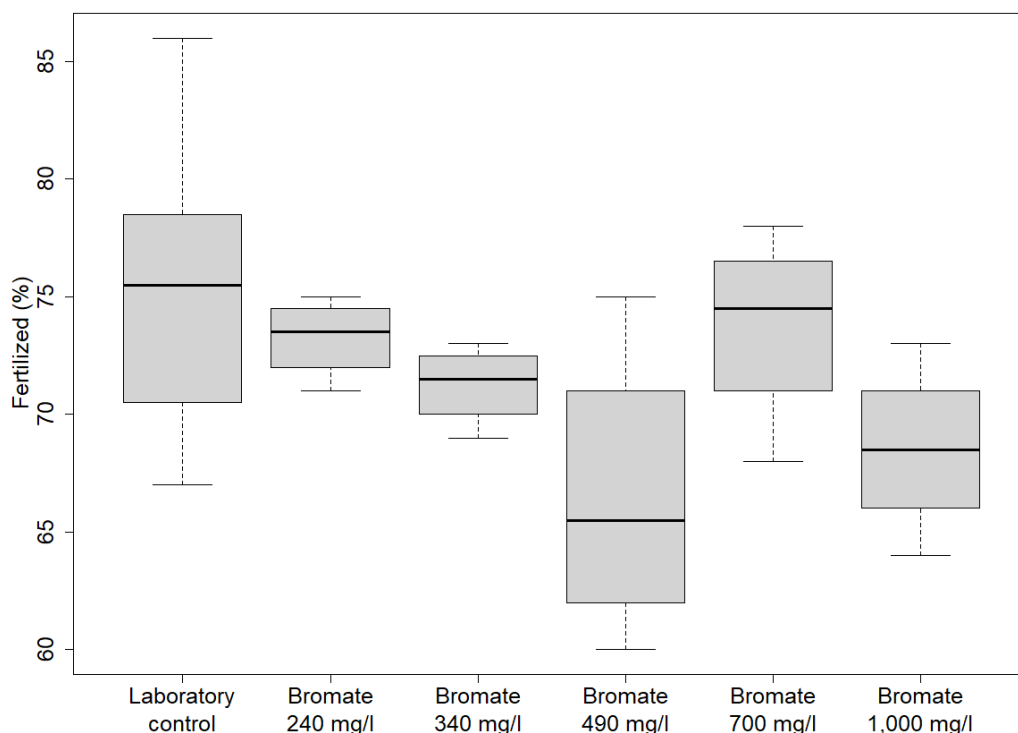


Figure 1 Observed percentage of fertilised eggs after exposure to sodium bromate

6 Conclusion

No significant observed effect on the fertilisation success of sea urchin was observed and therefore all effect concentrations were above the highest tested nominal concentration of 1000 mg bromate/L.

Table 5.4 Results of the sea urchin fertilisation success toxicity test after exposure to sodium bromate

Endpoint	NOEC	LOEC	EC10	EC50
Fertilisation success	1000 mg/L	>1000 mg/L	>1000 mg/L	>1000 mg/L

7 References

- /1/ US EPA. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, Third Edition, October 2002
- /2/ R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>.
- /3/ Frank Bretz, Torsten Hothorn and Peter Westfall (2010) (Dunnett's test), Multiple Comparisons Using R, CRC Press, Boca Raton.

- /4/ Fox, J. (2005) The R Commander: A Basic Statistics Graphical User Interface to R. *Journal of Statistical Software*, 14(9): 1–42
- /5/ Fox, J. and Weisberg, S. (2011) *An R Companion to Applied Regression*, Second Edition, Sage.
- /6/ Environment Canada (2011). Biological Test Method: Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars) EPS 1/RM/27 Second Edition.

APPENDICES

APPENDIX A

Raw data and statistical evaluation

A Raw data toxicity test

A.1.1 Primary data toxicity test

Table A.1 Raw data generated in the toxicity test with NaBrO₃.

Concentration BrO ₃ ⁻ (mg/l)		Non-fertilised eggs	Fertilised eggs	Percent eggs fertilised
0	A	41	122	75
0	B	35	124	78
0	C	52	117	69
0	D	34	108	76
0	E	41	106	72
0	F	26	95	79
0	G	48	97	67
0	H	23	138	86
240	A	44	124	74
240	B	46	114	71
240	C	38	112	75
240	D	37	99	73
340	A	38	103	73
340	B	42	107	72
340	C	36	89	71
340	D	39	85	69
490	A	44	91	67
490	B	49	75	60
490	C	40	72	64
490	D	47	138	75
700	A	39	116	75
700	B	44	92	68
700	C	36	105	74
700	D	35	123	78
1000	A	46	100	68

Concentration BrO ₃ ⁻ (mg/l)		Non-fertilised eggs	Fertilised eggs	Percent eggs fertilised
1000	B	47	106	69
1000	C	47	85	64
1000	D	43	115	73

Tabel A.2 pH, oxygen and salinity

Test concentration BrO ₃ ⁻ (mg/l)	Date: 24.01.2022 t = 0		
	% O ₂	‰ sal.	pH
Control	100	31.7	7.9
240	94	31.6	8.0
340	92	31.6	8.1
490	91	31.7	8.1
700	90	31.9	8.1
1000	88	31.9	8.1

A.1.2 Statistical analysis

A.1.2.1 Determination of NOEC and LOEC for the effect on the fertilisation success

Table A.2 Experimental results, fertilisation (%)

Replicate No.	Laboratory control	240 mg/L (BrO ₃ ⁻)	340 mg/L (BrO ₃ ⁻)	490 mg/L (BrO ₃ ⁻)	700 mg/L (BrO ₃ ⁻)	1000 mg/L (BrO ₃ ⁻)
1	75	74	73	67	75	68
2	78	71	72	60	68	69
3	69	75	71	64	74	64
4	76	73	69	75	78	73
5	72	-	-	-	-	-
6	79	-	-	-	-	-
7	67	-	-	-	-	-
8	86	-	-	-	-	-
Count	8	4	4	4	4	4
Mean	75	73	71	67	74	69

- Dunnett's test

Dunnett's test tests the null hypothesis that the averages of each test group are not different from the control group (Table A.3).

Table A.3 Multiple comparisons of means: Dunnett's contrasts.

Mean Comparisons	Estimate	Std. Error	t value	Pr(> t)	Significance ¹⁾
1-Bromate-240mg/L - 0-Bromate-0mg/L == 0	-2	2.883	-0.694	0.9497	
2-Bromate-340mg/L - 0-Bromate-0mg/L == 0	-4	2.883	-1.387	0.5624	
3-Bromate-490mg/L - 0-Bromate-0mg/L == 0	-8.75	2.883	-3.035	0.0274	*
4-Bromate-700mg/L - 0-Bromate-0mg/L == 0	-1.5	2.883	-0.52	0.9848	
5-Bromate-1000mg/L - 0-Bromate-0mg/L == 0	-6.75	2.883	-2.341	0.1188	

1) Signif. codes: 0 '****' 0.001***' 0.01 '**' 0.05 '.'0.1 '.'1 (Adjusted p values reported - single-step method)

None of the exposure groups was significantly different from the control group. Table A.3

Table A.4 Endpoint: estimations of NOEC and LOEC, Dunnett's test.

Endpoint	NOEC	LOEC	Unit
Fertilisation	1000	>1000	mg/L (BrO ₃)

A.1.2.2 Results of the Probit analysis

As no difference was observed among the test groups, the data to estimate the ECX on the fertilisation success were not computed.

Table A.5 Hatching success endpoint, estimations of EC10, EC50, Probit analysis.

Endpoint	EC10	EC50	Unit
Hatching success	>1000	>1000	mg NaBrO ₃ /L

A.1.3 Validity criterion

The average fertilisation in the control group was 75% which is within the range of 70% – 90% egg fertilization in controls, which is given as a test validity criteria in the guideline.

APPENDIX B– Chemical analysis

Results from chemical analyses

B Results from chemical analyses



This certificate replaces any previous certificate with the same number.

CERTIFICATE OF ANALYSIS

Work Order : ST2203688 Amendment : 1	Page : 1 of 2
Client : Niras Sweden AB Contact : Anders Sjölin Address : External office	Project : Project 11827338 Purchase Number : Anders Sjölin / VA syd Sampler : JAB Site : ---- Date Samples Received : 2022-02-10 11:00 Date Analysis Commenced : 2022-02-15 Issue Date : 2022-02-23 15:42 No. of samples received : 2 No. of samples analysed : 2
E-mail : anders.sjolin@niras.se Telephone : ---- C-O-C number : ---- Quote number : ST2021SE-NIR-SWE0001 (OF210090)	

General Comments

This report supersedes any previous report(s) with this reference. Results apply to the sample(s) as submitted. All pages of this report have been checked and approved for release.

This certificate represents the original certificate and may not be modified or reproduced other than in full, except with the prior written approval of the issuing lab. The results apply only to the material that has been identified, received, and tested.

Regarding the laboratory's liability in relation to assignment, please refer to our website <http://www.alsglobal.se>

Signatories	Position
Niels-Kristian Terkildsen	Laboratory Manager



Laboratory : ALS Scandinavia AB Danderyd Address : Rinkebyvägen 19C 182 36 Danderyd Sweden	Webpage : www.alsglobal.com E-mail : info.ta@alsglobal.com Telephone : +46 8 5277 5200
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Page : 2 of 2
 Work Order : ST2203688 Amendment 1
 Client : Niras Sweden AB



Analytical Results

Sub-Matrix: SEAWATER		Client sample ID		VUDP Bromate, 1000 A mg/L, Sampling 24/1-2022				
		Laboratory sample ID		IAB				
		Client sampling date / time		ST2203688-001				
				2022-01-24				
Parameter	Result	MU	Unit	LOR	Package	Method	Issuer	
Nonmetallic Inorganic Parameters								
Bromates	976000	± 195000	µg/L	5.0	Bromat	W-OXY-IC	PR	

Sub-Matrix: SEAWATER		Client sample ID		VUDP Bromate, 240 A mg/L, Sampling 24/2-2022				
		Laboratory sample ID		IAB				
		Client sampling date / time		ST2203688-002				
				2022-01-24				
Parameter	Result	MU	Unit	LOR	Package	Method	Issuer	
Nonmetallic Inorganic Parameters								
Bromates	218000	± 43700	µg/L	5.0	Bromat	W-OXY-IC	PR	

The end of result part of the certificate of analysis

Brief Method Summaries

Analytical Methods	Method Reference
W-OXY-IC	CZ_SOP_D06_02_098 - Determination of dissolved bromate, chlorate and chlorite by ion liquid chromatography method and calculation of the sum of chlorate and chlorite from measured values. (CSN EN ISO 15061, CSN EN ISO 10304-4)

Key: LOR = Limit of reporting represents the standard LOR for the respective parameters in each method. Note that limits of reporting may be affected if, e.g. additional dilution was required because of matrix effects, or the sample quantity was limited.
 MU = Measurement Uncertainty
 * = Symbol succeeding any result indicates laboratory or subcontractor non-accredited test.

Measurement Uncertainty:

The uncertainty is given as extended uncertainty (according to the definition in "Guide to the Expression of Measurement", JCGM 100:2008 Corrected version 2010) calculated with a coverage factor of 2, which give level of approximately 95%.
 Measurement of uncertainty is reported only for detected substances with levels above the reporting limits.
 The uncertainty from subcontractors is often given as extended uncertainty calculated with a coverage factor of 2. Contact the laboratory for further information.

Issuing lab

	Issuer
PR	The analysis is provided by ALS Czech Republic, s.r.o., Na Harfe 336/9 Prague 9 - Vysocany Czech Republic 190 00 Accredited by: CAI Accreditation Number: 1163

APPENDIX C – CoA

Identification of test item (CoA)

C Identification of test item (CoA)

Certificate of Analysis

Product Name: SODIUM BROMATE
 puriss. p.a., >= 99.5 % RT
Product Number: 71325
Batch Number: BCBX9200
Brand: Sigma-Aldrich
CAS Number: 7789-38-0
Formula: NaBrO₃
Formula Weight: 150.89
Quality Release Date: 03 AUG 2018
Recommended Retest Date: JAN 2023

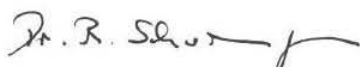
TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	COLORLESS OR WHITE	WHITE
APPEARANCE (FORM)	POWDER OR CRYSTALS	CRYSTALS
REDOX TITRATION	99.5 - 101.0 %	100.0 %
REDOXTITRATION (METHOD)	IODOMETRIC	IODOMETRIC
SOLUBILITY (COLOR)	COLORLESS	COLORLESS
SOLUBILITY (TURBIDITY)	CLEAR	CLEAR
SOLUBILITY (METHOD)	0.5G IN 10ML WATER	0.5G IN 10ML WATER
PH	5.0 - 9.0 (50 MG/ML H ₂ O, 25C)	6.3
METAL TRACE ANALYSIS (ICP)	CORRESPONDS TO REQUIREMENTS	PASSED
CALCIUM (ICP)	≤ 10 MG/KG	< 10 MG/KG
CADMIUM (ICP)	≤ 5 MG/KG	< 5 MG/KG
COBALT (ICP)	≤ 5 MG/KG	< 5 MG/KG
CHROMIUM (ICP)	≤ 5 MG/KG	< 5 MG/KG
COPPER (ICP)	≤ 5 MG/KG	< 5 MG/KG
IRON (ICP)	≤ 5 MG/KG	< 5 MG/KG
POTASSIUM (ICP)	≤ 100 MG/KG	< 100 MG/KG
MAGNESIUM (ICP)	≤ 5 MG/KG	< 5 MG/KG
MANGANESE (ICP)	≤ 5 MG/KG	< 5 MG/KG
NICKEL (ICP)	≤ 5 MG/KG	< 5 MG/KG
LEAD (ICP)	≤ 5 MG/KG	< 5 MG/KG
ZINC (ICP)	≤ 5 MG/KG	< 5 MG/KG

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Certificate of Analysis

BROMIDE (BR)	≤ 500 MG/KG	< 500 MG/KG
TOTAL NITROGEN	≤ 50 MG/KG	45 MG/KG
SULFATE (SO4)	≤ 500 MG/KG	< 500 MG/KG



Dr. Reinhold Schwenninger
Quality Assurance
Buchs, Switzerland

Sigma-Aldrich warrants that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.