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A Novel Quantitative Method For The Validation Of The Dosage Of Amnesic Shellfish Poisoning Toxins In Bivalve Mollusks

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Abstract:

The intra-laboratory validation of an analytical method is defined as the action consisting in subjecting an analytical method to an intra-laboratory statistical study, based on a standardized and/or recognized protocol, and in providing proof that in its field of application, the analytical method meets the pre-established performance criteria. In this study, we used the DR-12-VMC protocol (Rev. 2012) and the Quilliam, M.A. protocol for the validation of a method of assaying toxins, in bivalve mollusks used internally, causing amnesic poisoning by mollusks (ASP). Thus, we proceeded to characterize the performance of the analytical method used by evaluating, through experimental analysis and statistical tests, several parameters among the most frequently recommended, namely: limit of detection, limit of quantification, compliance rate, linearity, precision, sensitivity, specificity, accuracy, and recovery.

Keywords: Amnesic shellfish poisoning, bivalve mollusks, quantification, validation

1 INTRODUCTION

Various research has emerged to take advantage of marine by-products, and others discuss the evaluation of their applications in various products (Bachra et al., 2020; Damiri et al., 2020; Fouad et al., 2020; Maaghloud et al., 2020). However, this does not impact research on the properties and consumption of marine products such as determining the dose of toxins which has prompted many researchers to focus on amnesic toxins in shellfish (Jeffery et al., 2004; Mauriz and Blanco, 2010; Johnson, Harrison and Turner, 2016; Soliño et al., 2019). It is indeed the control of toxin doses that has become an absolute necessity everywhere, especially in the context of the post-COVID-19 pandemic, where certification authorities are claiming enhanced regulation of marine organisms(Bachra et al., 2021; Huveneers et al., 2021). The principle of the validation of quantitative analytical procedures is in great demand today in all fields of activity where

measurements are carried out. The scope of analytical validation extends to any analytical procedure used, in particular in the control and monitoring of the quality and safety of products intended for human consumption. In this context, we were interested in making a synthesis of the main steps of the validation of analytical methods for the quantification of heavy metals in fishery products. Indeed, we have internally validated a method for determining lead in bivalve mollusks (DR-12-VMC, 2009). The main toxin responsible for amnesic shellfish poisoning (ASP) is Domoic acid (Figure 1), an amino acid belonging to the class of kainoid compounds. Domoic acid is produced by several marine organisms, and ten Domoic acid isomers have been identified in marine samples (Jeffery et al., 2004).

In the present work, a statistical approach was undertaken for the in-house validation of a method for quantifying ASP in bivalve mollusks, following the Quilliam, M.A. (Quilliam, Xie, and Hardstaff, 1995) and DR-12-VMC protocols (Rev. 2012) (DR-12-VMC, 2009)]. The method establishes the following parameters: limit of detection, limit of quantification, linearity, precision (repeatability and reproducibility), accuracy, sensitivity, specificity, and recovery. The results were analyzed and reported in tables and figures.

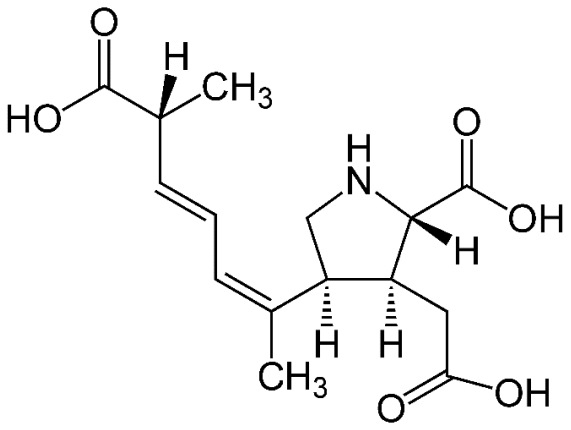


Figure 1: Chemical structure of Domoic acid

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

The chemicals used are all of the analytical purity, including 65% Nitric acid (Merck) and ultrapure water (MilliQ). Acetonitrile and Methanol were used as chromatographic grade solvents. Trifluoroacetic acid (TFA), concentrated Ammonium Hydroxide (NH_4OH), and Citric acid monohydrate were analytical grade. The reference material used in Domoic acid MUS-1B, is a homogenate of mussel meat containing approximately 38 µg of Domoic acid / g of meat.

2.2 Instrumentation

Liquid chromatography system composed of stainless steel columns (25 cm and 4.6 mm) filled with C18 particles of 5 µm in diameter (VYDAC 201TP54), pre-column with the same characteristics (C18, 5 µm), isocratic pump, automatic injector, column oven, UV detector, data acquisition, and processing system.

2.3. Equipment decontamination

The material used was cleaned separately, before use. The material was kept overnight (12 hours) in a special detergent (micro 90) at 20%, then it was rinsed with tap water and then with distilled water, followed by another 24-hour stay in 10% nitric acid then rinsing with distilled water and afterward with ultra-pure water. Finally, the material was allowed to dry in a laminar flow hood.

2.4. Preparation of samples

The grinding and homogenization were carried out by drawing off the flesh of the shells and rinsing them well to remove the saltwater and then leave to drain. Then, 100 g of whole shells were crushed to obtain a representative sample. If the analysis to be performed was on a reduced amount of sample, it was weighed directly into the centrifuge tube. For the analysis of the diluted extracts, in the case of the screening of strongly contaminated and/or salted samples, 1 mL of the filtrate obtained after draining was diluted with water to a final volume of 5 mL. Then, the sample was mixed and analyzed directly by HPLC without going to the step of cleaning the SAX cartridge.

2.5 Principle of the analytical method

The principle of the analytical method is based on a process divided into four main parts which are a test and conditioning of the cartridges, followed by cleaning and pre-concentration on a SAX cartridge, and finally an extraction blank. For the SAX cartridge test, it is necessary to test the Domoic acid recovery rate for each new lot of SAX cartridges. Then, the duplicate determination of the Domoic acid concentration was carried out by HPLC of a filtered extract containing 10 to 30 µg / mL of Domoic acid in the extraction solvent from the previous step. Where possible, an extract from a naturally contaminated sample or a supplemented sample was used. From the same extract, the determination in duplicate of the concentration of Domoic acid was carried out by HPLC of the three eluates. The recovery rate was calculated for each eluate using the appropriate dilution factor and the average level of Domoic acid determined in the crude extract.

The three recoveries should be between 85 and 115% and the average recovery should be greater than 90%. Otherwise, another type of SAX cartridge has been tried. For the conditioning of the SAX cartridges, 3 mL of water and 3 mL of extraction solvent (methanol-water, 1: 1) were successively passed through the 6 mL of methanol cartridge. During cleaning and pre-concentration on SAX cartridges, the elution rate of the various solvents must be regular, of the order of 1 drop / s. 5 mL of filtered supernatant (b) was added to the cartridge and eluted. The elution sample was discarded and the cartridge was washed with 5 mL of washing solution (Acetonitrile - water 1: 9). The eluate has been discarded

0.5 mL of citrate buffer was added and the eluate was discarded. A clean 5 or 10 mL vial was placed for collection of the eluate. The tube was weighed and Domoic acid and its derivatives were eluted with 2 mL of citrate buffer. The collection of the eluate was stopped at the 2 mL mark on the vial. During elution, a few drops of eluate may be deposited along the walls of the tube. To make this volume into account, the tube was weighed after collecting the eluate. The density of the citrate buffer is similar to that of water. Then the tube was capped and shaken to collect the drops deposited on the walls. The solution was stirred before taking an aliquot for HPLC analysis. The samples should be analyzed, as soon as possible, after preparation. It should be noted that the extracts should be placed in tightly closed vials and stored in the refrigerator. Also, the extracts should not be frozen because Domoic acid decomposes into a frozen acidic solution. Also, the extracts should not be frozen because Domoic acid breaks down into a frozen acidic solution. Concerning the extraction blank, the previous steps were carried out by replacing the test portion of 4 g of a sample with 4 g of water. The following steps were performed on one aliquot of the filtered sample and the following steps on another aliquot. Finally, these samples were analyzed. Additionally, it should be noted that the chromatograms would be better not to have peaks at a retention time close to that of Domoic acid and that the baseline should not be subject to drift.

2.6 Validation protocol

The study of the validation parameters was carried out under the specifications of the DR-12-VMC (Rev. 2012) (DR-12-VMC, 2009) protocol and the requirements of Regulation (EU) No. 836/2011 (DR-12-VAL, 2009). This is done according to the parameter/matrix plan (table 1) specified in the directive DR-12-VAL on the validation of analytical methods (DR-12-VAL, 2009). This study was carried out in 5 phases.

Table 1: Parameter/matrix plan.

Parameters	Material to be used
LOD/LOQ	Reel sample
Accuracy	Reel sample
Sensitivity	Standard
Exactitude	Reference material
Recovery	Reel sample

2.6.1 Determination of the limit of detection (LOD), limit of quantification (LOQ), and compliance report

First, the limit of detection (LOD) must be performed. LOD is evaluated at the concentration that corresponds to the instrumental limit of detection, which is the lowest concentration that the

analytical instrument can accurately detect. The LOD was established by adding the compound analyzed to the standard solution to obtain a final concentration corresponding to approximately 5 times the estimated LOD (i.e. 0.3 µg / l) (AFNOR, 2019). This solution was analyzed (ten replicas) directly in the instrumental system. When establishing the LOD which was three times the standard deviation of 10 replicate samples (spiked with a concentration between 5 and 7 times the estimated LOD) analyzed over a short period. On the other hand, the calculation of the compliance report validated the approach used to establish the LOD. In addition, the limit of quantification (LOQ) corresponds to ten times the standard deviation of the series studied for the establishment of the LOD. The results of the ten replicates were reported in Table 2, which summarizes the statistical study carried out.

Table 2: Limit of detection, limit of quantification, and compliance report.

LOD (µg/l)	LOQ (µg/l)	R
0,2193	0,7311	8,6866

2.6.2 Characterization of linearity

Linearity is used to define and validate the linearity domain of the method. For this, the approach of ISO 11095 was used (ISO 11095, 1996), which consists of comparing the residual error with the experimental error using the Fischer test. This approach requires good homogeneity of the number of measurements over the entire range studied. Thus, nine concentrations of the reference material were analyzed five times; to cover the whole range of the concentrations studied (0.5, 1, 2, and 5 mg/kg). The results obtained for each series are presented in figure 2 and linearity domain validation results have been summarized in Table 3.

Table 3: Variation's table.

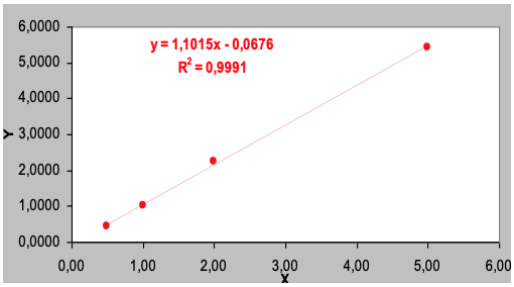
Variation	Inter	Intra	Total
SS	0,059	68,32	68,3228
df	4	15	19
MS	0,01493	4,55	
F	0,0033		
Fcritical	3,0556		
p-value	1,000		
α	0,05		

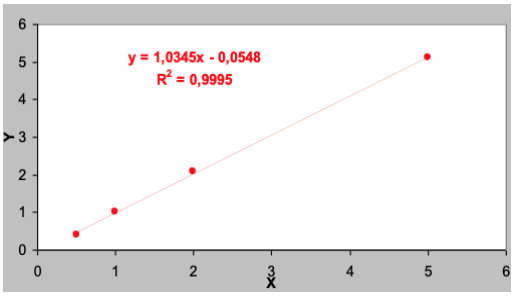
2.6.3 Determination of fidelity and sensitivity

The Accuracy at a given level is the closeness of agreement between the results obtained by applying the experimental procedure several times (ten repetitions) under specified conditions. A

distinction has been made between replicability, repeatability, and reproducibility. Replicability at a certain level refers to the closeness of agreement between successive individual results obtained, within a short period of time, on the same test sample, in the same laboratory, and under conditions such as same analyst, same apparatus, and same day. Although repeatability is the closeness of agreement between individual results obtained, in a short period of time, on the same test sample in the same laboratory and in which at least one of the following is different: analyst, analysis device, or day. However, reproducibility at a specified level is the closeness of agreement between individual results obtained, in a short period of time, on the same sample tested in different laboratories and under conditions such as different analysts, different apparatus, and different days or the same day. Table 4 summarizes the results for replicability, repeatability, and reproducibility(EURACHEM / CITAC, 2016).

The sensitivity was expressed as the average slope of the calibration curves. It can also be expressed as the ratio between the signal obtained and the concentration of a standard. Data were obtained from the results of the linearity study. The standard ranges studied were then used as a matrix to evaluate the sensitivity of the method studied. The results obtained are shown in Figure 3. The sensitivity results were used to assess the specificity of the method by studying the regression, the slope of the line, and the intersection at the origin. Table 5 summarizes the results of the statistical test.





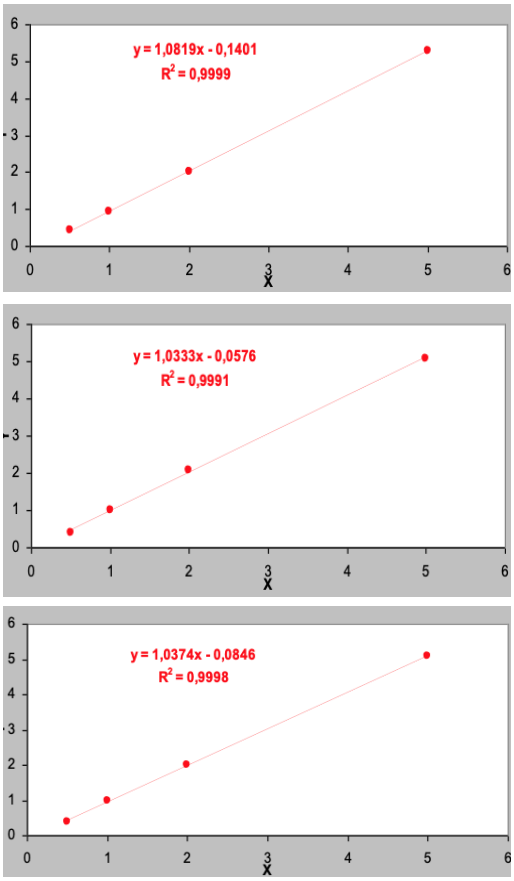


Figure 2: Calibration curves.

Table 4: Accuracy data.

	Repeatability	Reproducibility	Replicability
Number (n)	10	10	10
Average (d)	3,82	3,82	1,91
Standard deviation (Sd)	0,037	0,04	0,073
α	0,050		
t(crit)	1,833		
(tcrit x Sd)/√n	0,034	0,036	0,0522
$t_{observed}$	321,690	321,690	82,409
Limit	0,02685	0,02685	
Repeatabili	1		

ty limit/repro ducibility limit ratio	

Table 5: Regression characteristics.

Regressionequation (y = ax + b)	y = 0,9707 x + 0,1051
Slope (a)	0,9707
Intercept (b)	0,1051
Coefficient of determination (r^2)	0,9961
Standard deviation on slope (Sa)	0,1565
Standard deviation on intercept(Sb)	1,0426
Degree of freedom (DF)	7
$T_{obs}(a) = (1 - a)/Sa$	0,187220
$T_{obs}(b) = (0 - b)/Sb$	-0,10080
α	0,05
t(crit)	2,36

2.6.4 Determination of accuracy and Percentage recovery

Accuracy is the closeness of agreement between the value certified by a recognized body and the average result that would be obtained by applying ten times the experimental process (ten replicates) to the reference material. Table 6 summarizes the results obtained. The percentage recovery made it possible to identify, for a given sample or a given type of matrix and at a given concentration level, the presence of potential interferences during the analytical process. The recovery rate was the difference (in percentage) between the measured concentration of an enriched sample and the measured concentration of the same unenriched sample, divided by the concentration of the added substance(Way et al., 2022). This ratio takes into account the chemical transformation that has occurred. For this, five real samples were analyzed in a first step then a known concentration (between 50% and 100%) of the standard was added to the real concentration of the sample to be assayed in a second step. The addition was made directly, before mineralization on the test sample and after digestion. The samples were analyzed and the percent recovery was calculated and validated. The results of this test have been reported in Table 7.

Table 6: Exactitude data.

Number (n)	5
Average (d)	0,805
Standard deviation (Sd)	0,010
Concentration of reference material (C) (µg/g)	0,820
Uncertainty of reference material	0,160
Relative error(%)	1,841
Exactitude (%)	98,159
Exactitude error	0,094

Table7: Results of the recovery study.

C	C_f	C_f - C	C_a	Recovery (%)
2,870	4,490	1,720	1,5	114,67
2,870	5,230	2,360	1,8	131,11
2,870	5,690	2,820	2	141,00
2,870	6,950	4,080	2,5	163,20
2,870	8	5,130	2,8	183,21
			Average	146,64
			Sd	26,96
			α	0,05
			t(crit)	2,132
			t(obs)	12,163

C: Concentration before dosed additions

C_f: Concentration after dosed additions

C_a: Concentration of dosed additions

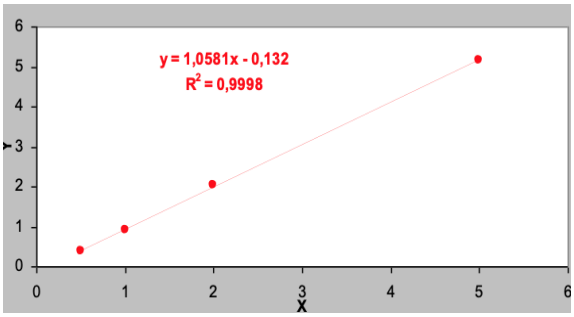


Figure 3: Regression curve.

3 RESULTS & DISCUSSION

The compliance rate (Table 2) is between 4 and 10 (ISO 11095, 1996), which validates the process used to establish the detection limit. The LOD was found to be 0.0088 mg/kg, as required by regulation (EU), it must be less than or equal to one-tenth of the maximum admissible content of ASP in bivalve mollusks, which gives a maximum level of 2 mg Domoic acid per kg for the LOD and therefore shows the validity of the calculated LOD (Table 2).

Regarding the LOQ, the LOQ must be less than or equal to one-fifth of the maximum content of ASP in bivalve mollusks(Compendium of international analysis of methods, 2005; National testing laboratory LNE/France, 2019), i.e. a maximum value of 4 mg/kg, thus demonstrating the validity of the calculated LOQ (table 2). The results of the correlation coefficients show values well above 0.99. The results of the correlation coefficients show values well above 0.99. This was correct for all the series studied, which attested to the linearity of the method (figure 2). Likewise, and to compare the results of the five replicates, the Fischer test was used to validate the domain of linearity (Table 3). This test verified that the part of the variance due to a model error was not greater than the variance of the experimental error. In this study, the ratio F was lower than the critical value F corresponding to a Fisher variable at risk α = 5%. The results obtained then showed that the linearity domain was validated and that the regression model was acceptable. The student's t-test validated the replicability, repeatability, and reproducibility (Table 4). All these results led to the conclusion that there was a close agreement between the results obtained by applying the experimental procedure several times under given conditions. The ranges of standards were studied during the linearity test (figure 2), which served as a matrix to assess the sensitivity of the method studied (figure 3).

All the regression curves, including that of the mean of the calibration curves, showed that an increase in concentration again results in the signal. This means that the method has been sensitive. The student's t-test (Table 5) concluded that the value of the slope of the regression line was not significantly different from the value of 1. Likewise, the value of the intersection at the origin was not significantly different from the value of 0. Therefore, the specificity was acceptable without interference. Since the calculated normalized deviation is less than 2 (Table 6), the precision error is considered to be insignificant [17]. The uncertainty associated with the accuracy of the method was therefore equal to the uncertainty of the reference material used for the accuracy test. Thus, the results obtained showed that the studied method had a good level of accuracy. This is explained by a close agreement between the certified value and the average result. The results reported in Table 7 showed that the calculated recovery percentages vary from 79.38% to 131.62%. These percentages were validated by the student test(NATA, 2012).

4 CONCLUSION

It appears from the results of this study that the method used is consistent with all the specific performances for the determination and quantification of ASP in bivalve mollusks. Thus, we obtained a linear response with correct regression, a limit of detection of the range of 10^{-3} mg/kg,

and a limit of quantification of the order of 4.10^{-3} mg/kg and proved the sensitivity, accuracy, and precision of the method. Nevertheless, participation in an external quality assessment program is essential to monitor the performance of this method and ensure the quality of the results.

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REFERENCES

AFNOR (2019) 'Synthesis report: study of the AFAQ AFNOR validation Certification of the REBECCA + EB test for the enumeration of Enterobacteriaceae according to the NF ISO16140 standard'.

Bachra, Y. et al. (2020) 'A new approach for assessing the absorption of disposable baby diapers and superabsorbent polymers: A comparative study', Results in Materials. Elsevier, 8, p. 14. doi: 10.1016/j.rinma.2020.100156.

Bachra, Y. et al. (2021) 'A Novel Superabsorbent Polymer from Crosslinked Carboxymethyl Tragacanth Gum with Glutaraldehyde: Synthesis, Characterization, and Swelling Properties', International Journal of Biomaterials, 2021, pp. 1–14. doi: 10.1155/2021/5008833.

Compendium of international analysis of methods (2005) Practical guide for the validation, quality control, and uncertainty estimation of an alternative oenological method of analysis; (Oeno Resolution).

Damiri, F. et al. (2020) 'Synthesis and Characterization of Lyophilized Chitosan-Based Hydrogels Cross-Linked with Benzaldehyde for Controlled Drug Release', Journal of Chemistry, 2020, pp. 1–10. doi: 10.1155/2020/8747639.

DR-12-VAL (2009) 'Guideline for the validation of analytical methods in chemistry'.

DR-12-VMC (2009) Protocol for the Validation of an Analytical Method in Chemistry, Centre d'expertise en analyse environnementale, edition: June 9, 2009.

EURACHEM / CITAC (2016) Guide to Quality in Analytical Chemistry Eurachem/CITAC Guide Guide to Quality in Analytical Chemistry An Aid to Accreditation. 3rd edn. Edited by Vicki Barwick. Available at: www.eurachem.org. (Accessed: 30 November 2021).

Fouad, D. et al. (2020) 'A Novel Drug Delivery System Based on Nanoparticles of Magnetite Fe3O4 Embedded in an Auto Cross-Linked Chitosan', Chitin and Chitosan - Physicochemical Properties and Industrial Applications. IntechOpen. doi: http://dx.doi.org/10.5772/intechopen.94873.

Huveneers, C. et al.(2021) 'The power of national acoustic tracking networks to assess the impacts of human activity on marine organisms during the COVID-19 pandemic', Biological Conservation. Elsevier, 256, p. 108995. doi: 10.1016/J.BIOCON.2021.108995.

ISO 11095 (1996) Linear calibration using reference materials.

Jeffery, B. et al. (2004) 'Amnesic shellfish poison', Food and Chemical Toxicology. Pergamon, pp. 545–557. doi: 10.1016/j.fct.2003.11.010.

Johnson, S., Harrison, K. and Turner, A. D. (2016) 'Application of rapid test kits for the determination of Amnesic Shellfish Poisoning in bivalve molluscs from Great Britain', Toxicon. Elsevier Ltd, 117, pp. 76–83. doi: 10.1016/j.toxicon.2016.03.017.

Maaghloud, H. et al.(2020) 'Ingestion of microplastics by pelagic fish from the Moroccan Central Atlantic coast', Environmental Pollution. Elsevier Ltd, 261, p. 114194. doi: 10.1016/j.envpol.2020.114194.

Mauriz, A. and Blanco, J. (2010) 'Distribution and linkage of domoic acid (amnesic shellfish poisoning toxins) in subcellular fractions of the digestive gland of the scallop Pecten maximus', Toxicon. Pergamon, 55(2–3), pp. 606–611. doi: 10.1016/j.toxicon.2009.10.017.

NATA (2012) Guidelines for the validation and verification of quantitative and qualitative test methods.

National testing laboratory LNE/France (2019) Methodological guide for the estimation of uncertainties in chemical analysis.

Quilliam, M. A., Xie, M. and Hardstaff, W. R. (1995) 'Rapid Extraction and Cleanup for Liquid Chromatographic Determination of Domoic Acid in Unsalted Seafood', Journal of AOAC INTERNATIONAL. Oxford University Press (OUP), 78(2), pp. 543–554. doi: 10.1093/jaoac/78.2.543.

Soliño, L. et al. (2019) 'Are pelagic seabirds exposed to amnesic shellfish poisoning toxins?', Harmful Algae. Elsevier B.V., 84, pp. 172–180. doi: 10.1016/j.hal.2019.03.014.

Way, C. et al. (2022) 'Evidence of underestimation in microplastic research: A meta-analysis of recovery rate studies', Science of The Total Environment. Elsevier, 805, p. 150227. doi: 10.1016/J.SCITOTENV.2021.150227.