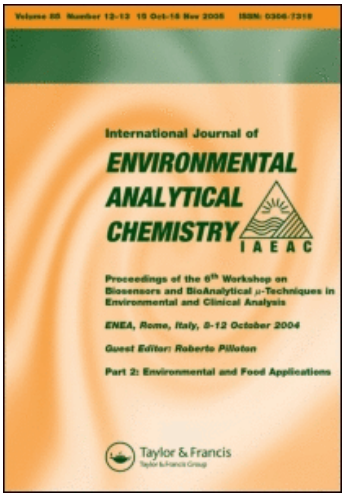

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

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Online publication date: 22 September 2010

To cite this Article Saavedra, Yolanda , González, Aurora and Blanco, Juan(2009) 'Inter-individual distribution of metal concentrations in four marine bioindicator organisms and its use for optimal sampling design of a monitoring system', International Journal of Environmental Analytical Chemistry, 89: 8, 559 — 568
To link to this Article: DOI: 10.1080/03067310802553146
URL: http://dx.doi.org/10.1080/03067310802553146

Intern. J. Environ. Anal. Chem.
Vol. 89, Nos. 8–12, 15 July–15 October 2009, 559–568

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Inter-individual distribution of metal concentrations in four marine bioindicator organisms and its use for optimal sampling design of a monitoring system

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(*Received 18 July 2008; final version received 13 October 2008*)

The monitoring of heavy metals in the marine environment is often carried out by using bioindicator organisms. In most cases metal concentrations in an area are evaluated using pooled samples from a single sampling site. Thus, a large number of individuals are analysed per site, but neither the statistical distribution of the data nor the intra-site variability are known. In order to optimise the monitoring of heavy metals using this kind of samples, some authors have suggested that the variability among individuals should be studied at least in one site. This work was designed to know the frequency distribution and the inter-individual variability of Hg, Cd, Pb, Cr, Ni, As, Ag, Cu and Zn in four bioindicator organisms (the blue mussel *Mytilus galloprovincialis*, the clam *Venerupis pullastra*, the king scallop *Pecten maximus* and the cockle *Cerastoderma edule*). In most cases metals in one-individual samples were shown to follow a log-normal distribution. As the pooled samples included more individuals they approached the normal distribution but still being closer to the log-normal one, suggesting that, in all cases, a logarithmic transformation should be used to normalise the data. The inter-individual variability observed indicated that at least two pooled samples of 30 individuals (a hundred in few cases) must be analysed to detect differences of 25% (both between sites and with time).

Keywords: metals; molluscs; inter-individual variability; frequency distributions and sample size

1. Introduction

The success of the initial US 'Mussel Watch' program and its suggestions [1] lead many countries to implement monitoring systems of contaminants that use bivalves as bioindicator organisms. As a consequence of these programmes, temporal and spatial trends in metal concentration of some bivalves – mostly, but not only, mussels of different species – have been reported throughout the world.

Nevertheless, in many cases (especially when the number of temporal observations is not large), the statistical significance of those trends is difficult to evaluate. Apart from the magnitude of the change in the metal concentration found, the significance depends

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ISSN 0306–7319 print/ISSN 1029–0397 online

DOI: 10.1080/03067310802553146
http://www.informaworld.com

on the inter-sample variability and on the shape of the distribution (when parametric tests are used). None of these two are usually analysed, mostly because of the need of keeping monitoring costs to an affordable budget.

The most frequent way of dealing with the inter-sample variability is to reduce it by means of the use of composite samples that comprise a number of individuals. The number of pooled individuals varies with monitoring and species. Neither the inter-individual nor the inter-sample variability is usually reported. Notwithstanding, some studies have shown a substantial variation in metal concentration between individuals of the same species from a single site, even if the influence of physiological factors (size, reproductive state) was minimised, which suggests that the reduction of the variability obtained by pooling is unlikely to be large enough to make it negligible.

As an excessive variability would prevent from detection of even moderate changes in metal concentrations, using the adequate number of individuals or replicate samples is an absolute requirement. In this direction, some authors have suggested that an estimate of the inter-individual variability must be obtained for at least one bioindicator population even if a pooling strategy has been adopted in the monitoring programme.

Additionally, when parametric tests are used, the frequency distribution of the metal concentration is critical but is not usually checked or, at least, reported. Most of studies involving monitoring of metal concentrations in molluscs assumed a normal distribution of data for statistical considerations but this distribution might be not suitable for some cases [2,3]. Assuming an incorrect distribution can have important consequences for the interpretation of the results as Lobel and Wright [4] showed in their study of Zn in *Mytilus edulis*. It is, therefore, necessary to know the frequency distribution of the data, and to decide if they must be transformed, before comparing temporal or spatial means.

In this work, the frequency distribution and the inter-individual variability of Hg, Cd, Pb, Cr, Ni, As, Ag, Cu and Zn in four bio-indicator organisms (the blue mussel *Mytilus galloprovincialis*, the clam *Venerupis pullastra*, the king scallop *Pecten maximus* and the cockle *Cerastoderma edule*) have been described and their consequences for the usefulness of pooled samples were studied.

2. Experimental

2.1 *Biological sampling*

Sampling consisted of the selection of 26 similar-sized individuals among commercial-size individuals of *P. maximus* (100 ± 6 mm width, 90 ± 5 mm long), and 30 of *M. edulis* (82 ± 1.4 mm long), *V. pullastra* (46 ± 1.1 mm width) and *C. edule* (34 ± 1.1 mm width). All samples were collected from the Galician Rías (Northwest Spain).

2.2 *Sample preparation and metal quantification*

The soft tissues of each collected individual were separated from the shells and freeze-dried. The scallop soft tissues were triturated after freeze-drying by means of a mixer mill of zirconium oxide (M.M.). The clam, cockle and mussel wet soft tissues were homogenised with an Ultra-Turrax (U.T.) (IKA) because of their small weight. A subsample of the mollusc dry homogenates was digested with nitric acid by heating in a microwave. The analytical determination of copper and zinc concentrations in the digest was carried out by flame atomic absorption spectrometry. Cadmium, lead,

chromium, nickel, arsenic and silver were analysed by electrothermal atomic absorption spectrometry with Zeeman background correction and STPF (Stabilised Temperature Platform Furnace) conditions. Mercury was determined by flow injection system-cold vapour atomic absorption spectrometry. Details of the methodology can be obtained from a previous paper [5].

The validation of the techniques used was performed by intra-laboratory quality control using two certified reference materials: CRM 278R-trace metals (except Ni and Ag) in mussel tissue from BCR (Community Bureau Reference), and NIST 1566 b-trace metals (Ni and Ag) in oyster tissue from NIST (National Institute of Standards and Technology). The results obtained for certified materials were in close agreement with the certified values, with metal concentration recoveries of between 90 and 110% for all metals.

2.3 *Data processing*

Minitab 15 statistical package was used for all the statistical procedures used. The procedure 'Individual Distribution Identification' was used for fitting normal, log-normal and exponential distributions to data. The Anderson–Darling statistic was used to evaluate the goodness-of-fit.

In order to know how the sample pooling affects the metal distribution in bivalve samples, we have used the fitted distributions to generate 10,000 random data that followed it. Then, group means of 5, 10, 20, 30 and 100 individuals –simulating pooled samples – were computed from the random data obtained and finally the resulting frequency distributions were studied.

As a practical mean for quantifying the usefulness of using composite samples, the number of samples required to discriminate real differences of 10 and 25% of the mean in metal content by an one-way ANOVA were computed (with an $\alpha = 0.05$ and a power of 0.80).

3. Results and discussion

3.1 *Inter-individual variability*

The coefficients of variation of metal concentration and contents were, in general, similar for all metals and species (Table 1). This agrees with the results found by Daskalakis in an oyster population [6]. Nevertheless, the coefficients of variations were found to be dependent on both, species and metal.

In general, scallop population showed the lowest variability and clam population the highest ones. Among metals, the highest coefficients of variation were observed for Cr and Ag.

The observed variability for Cr, and to a less extent Ni, in samples of clam, cockle and mussel, that were homogenised with an U.T. is probably poorly representative of the actual variability as the U.T. probe seems to have contaminated the samples (Table 2). The systematic contamination of the samples could have produced an increase in the average level of Cr and Ni, making that the contribution of the inter-individual variability was smaller in relation those increased levels. This would explain why the coefficients of variation observed in this study are slightly lower than those reported for *M. galloprovincialis* [7] and for *M. edulis* [8,9].

The inter-individual variability Ag was high in all the species studied. This, being the level in the studied samples (especially mussel and cockle) close to the limit of

Table 1. Descriptive statistics of the mollusc populations.

		Scallop		Mussel		Clam		Cockle	
Metal	Data	Mean	Coefficient of variation	Mean	Coefficient of variation	Mean	Coefficient of variation	Mean	Coefficient of variation
Hg	Concentration	0.085	11.4	0.16	15.0	0.24	23.7	0.10	15.39
	Content	2.56	16.0	1.85	15.7	1.64	21.2	0.29	15.17
Cd	Concentration	8.29	18.7	0.80	26.3	0.51	46.75	0.36	20.78
	Content	248.3	21.5	9.40	27.9	3.41	43.99	1.02	23.53
Pb	Concentration	2.25	20.0	2.20	41.1	0.85	33.88	2.18	52.1
	Content	67.63	23.1	25.9	40.6	5.77	34.32	6.14	55.3
Cr	Concentration	4.34	34.2	3.42	39.4	22.83	66.92	22.45	61.9
	Content	131.1	39.2	40.59	44.8	156.2	67.85	62.11	60.3
Ni	Concentration	2.54	24.8	2.15	23.0	13.72	50.43	36.55	28.2
	Content	76.69	32.6	25.66	28.8	94.68	53.01	101.3	27.0
As	Concentration	12.24	8.9	18.87	15.6	19.11	27.09	9.90	13.34
	Content	367.6	15.3	223.7	19.5	130.1	25.77	27.95	17.88
Ag	Concentration	0.50	26.4	0.021	52.4	0.87	22.2	0.067	37.31
	Content	15.07	28.9	0.24	50.0	6.04	23.7	0.19	43.16
Cu	Concentration	7.97	8.8	8.25	19.4	12.83	18.85	6.45	16.70
	Content	240.0	16.8	96.62	15.4	88.10	23.24	18.27	23.36
Zn	Concentration	177.7	20.3	285.2	54.2	65.21	17.41	75.04	15.39
	Content	5534.9	23.5	3403.1	56.7	450.6	23.64	211.9	19.46

Table 2. Metal concentrations of two batches of the same mollusc homogenate. One homogenised with U.T. and the other one grinded with a mill of M.M.

Mollusc		Hg	Cd	Pb	Cr	Ni	As	Ag	Cu	Zn
Mussel	M.M.	0.16	0.75	2.21	1.05	1.19	16.8	0.036	6.75	267.3
	U.T.	0.16	0.85	2.05	2.52	1.70	19.9	0.021	7.69	238.3
Clam	M.M.	0.28	0.38	0.85	0.99	2.96	18.5	0.83	10.95	69.6
	U.T.	0.32	0.42	0.75	7.94	4.99	19.9	0.67	10.57	65.5
Cockle	M.M.	0.090	0.42	1.94	2.28	21.9	8.89	0.057	6.15	75.7
	U.T.	0.092	0.39	1.92	15.0	28.4	9.47	0.067	6.40	79.4

quantification of the technique used, could be attributed to a high analytical variability. Notwithstanding, in other studies in which *M. edulis* contained a concentration three times higher than that found here, coefficients of variation of 43% have been observed [10], suggesting that most of that variation should be true inter-individual variation.

A high variability was also found in particular cases like Zn in mussels, Pb in mussel and cockle and Cd in clam. The coefficient of variation of Zn concentrations in mussels (*M. galloprovincialis*) (50%) is higher than that previously reported for this species [7] but it agrees with previous studies in *M. edulis* [11]. The high Pb variability was similar than that reported for this species [7]. In relation to Pb in cockle and Cd in clam, no other studies are known about it.

3.2 *Frequency distribution of individual and pooled samples*

Frequency distributions of one-individual samples of the metal concentrations and metal contents in the species studied were approximately log-normal in most cases. Notwithstanding, in some particular metal-species combinations, normal and reciprocal distributions described better the actual distribution found (Table 3).

Very little work about frequency distributions of metals has been carried out up to date. Skewed distributions for Zn, Cr and Ni in mussels *M. edulis* have been observed [4,8,11,12], which agrees with our observations in *M. galloprovincialis*. Additionally, the existence of 'superaccumulators' – individuals with an accumulation capability exceptionally high – was proposed as an universal characteristic of Zn accumulation in *M. edulis* [10], which seems to indicate, if this observations are interpreted from a continuous distribution point of view, that a skewed distribution with a heavy tail (as log-normal) is usually found for this metal/species combination. From our data, its seems to be also true for *M. galloprovincialis*.

Pooling a number of individuals into samples, which is the usual procedure in monitoring systems, affects the inter-sample distribution substantially. In most of the cases studied here (except when original data fits to a reciprocal distribution), pooled samples follow a distribution that is approximately normal when 30 or more individuals are pooled (Table 4). In those cases, the use of pooled samples is very convenient because the process yields two desirable effects: (a) reduces the inter-sample variability and (b) normalises the distribution.

Table 3. Best fitting distributions for concentration and content data in the four mollusc populations studied.

	Scallop		Mussel		Clam		Cockle	
Metal	Concentration	Content	Concentration	Content	Concentration	Content	Concentration	Content
Hg	Normal	Normal	Log-normal	Normal	Reciprocal	Normal	Normal	Normal
Cd	Normal	Normal	Log-normal	Log-normal	Log-normal	Log-normal	Normal	Normal
Pb	Log-normal	Log-normal	Log-normal	Reciprocal	Normal	Normal	Reciprocal	Reciprocal
Cr	Log-normal	Reciprocal	Log-normal	Reciprocal	Log-normal	Log-normal	Normal	Log-normal
Ni	Log-normal	Reciprocal	Reciprocal	Reciprocal	Log-normal	Log-normal	Normal	Reciprocal
As	Log-normal	Normal	Normal	Log-normal	Reciprocal	Reciprocal	Reciprocal	Normal
Ag	Log-normal	Log-normal	Normal	Normal	Reciprocal	Log-normal	Log-normal	Log-normal
Cu	Normal	Log-normal	Log-normal	Log-normal	Log-normal	Log-normal	Log-normal	Log-normal
Zn	Log-normal	Normal	Log-normal	Log-normal	Normal	Normal	Log-normal	Normal

Notwithstanding, when the original data followed a reciprocal distribution, the pooling of individuals neither reduce noticeably the original variability of data, nor have any effect in normalising the distribution. Moreover, the new distributions do not seem normalisable. Consequently, in those cases, the use of pooled samples cannot be recommended, but if they are used in any case, then non-parametric statistic tests, for which no assumption of normality is required, must be used.

3.3 *Sample size and resolution with pooled samples*

The shape of the frequency distribution of the metal content affects the ability of any parametric statistical test to detect actual differences between population means (power). The following discussion is focussed on two types of frequency distributions, normal and log-normal. The reciprocal distribution was excluded because of the reasons explained in the previous section.

To detect a difference of 10% of the mean, it is unlikely that less than 3 samples are needed (Table 4) even if 100 individuals were pooled in each sample. Notwithstanding, a difference of 25% can usually be detected with two or three samples provided the number of pooled individuals were high (30–100).

In any of those cases, the number of samples needed is higher when the samples do not follow a normal distribution and therefore the data have to be transformed to normalise them. This fact is especially true for samples in which a small number of individuals have been pooled (Table 4).

If these conclusions are extrapolated to cover most monitoring programmes, that do not use more than two samples of 30 individuals [6], then, in most cases (78%) they will be able to discriminate a difference of 25% of the mean if the samples follow a normal distributions but only in the 57% of the cases if they follow a log-normal.

Increasing the number of samples allows to decrease the mean difference between populations or dates that can be detected, but, in terms of being able to maintain a monitoring programme, the cost and the analytical load must to be maintained to an affordable level. To reach an equilibrium between the sample size required (and therefore the economical effort) and the desired resolution, it is very important to take into account the feasibility of increasing the number of individuals per pool (maximising the individual number will minimise variance) instead of the number of samples. The use of replicate samples, for example, would double the analytical cost, but, doubling the number of individuals, it would increase the cost by only 25%, approximately (Table 5). Assuming that most monitoring programmes cannot support more than two pools per site/date, the number of individuals to be pooled in each sample should be chosen depending on the magnitude of the change that must be detected and on the studied metal species. From our data it can be observed that differences about 30% of the mean can be detected for most metals examined in the studied species (except clam population) when two replicates of 20 individuals are used (Table 4). Notwithstanding, 100 individuals are necessary for 2 metals in cockles (Ag and Cr) and mussels (Ag and Zn).

4. Conclusions

Frequency distributions of one-individual samples of the metal contents in the species studied were approximately log-normal in most cases. Notwithstanding, in those cases

Table 4. Sample size needed to detect differences of 10 and 25% of the metal concentrations, and maximum difference (M.D., expressed as %) that can be detected using two replicates. *N*; individual number per pool. *; normal frequency distributions.

Panel A: Data from mussel population

N	Hg				Cd				As				Zn				Cu				Ag			
	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2
5	* 9	3	40		24	6	92		15	4	64		146	28	413		9	3	45	*	76	14	124	
10	* 6	3	28		13	4	58		8	3	40		76	15	223		6	3	31	*	39	8	87	
20	* 4	2	20	*	7	3	32		5	3	26		39	8	130	*	3	2	18	*	20	5	62	
30	* 3	2	16	*	5	3	26	*	4	2	20		24	6	92	*	3	2	15	*	14	4	50	
100	* 2	2	9	*	3	2	15	*	2	2	10	*	8	3	35	*	2	2	8	*	5	3	28	

Panel B: Data from cockle population

N	Hg				Cd				As				Zn				Cr				Cu				Ag			
	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2
5	* 9	3	39		* 19	5	60		* 12	3	45	*	13	4	49		210	39	612		17	5	70		38	8	127	
10	* 5	3	27		* 10	3	42		* 7	3	32	*	8	3	35		110	21	311		9	3	45		20	5	78	
20	* 4	2	19		* 6	3	30		* 4	2	23	*	5	2	25		56	12	173	*	5	3	26	*	9	3	40	
30	* 3	2	16		* 5	2	25		* 4	2	19	*	4	2	20		38	8	126	*	4	2	22	*	7	3	34	
100	* 2	2	9		* 3	2	13		* 3	2	10	*	3	2	11	*	11	3	45	*	3	2	12	*	3	2	18	

Panel C: Data from clam population

N	Hg				Pb				Cd				Zn				Cr				Ni				Cu			
	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2	10	25	M.D.	Size2
5	* 21	5	63		* 38	8	87		54	11	168	*	19	5	60		216	41	633		112	22	318		18	5	74	
10	* 11	3	44		* 20	5	61		28	6	102	*	10	3	42		118	23	334		57	12	176		10	3	48	
20	* 6	3	31		* 11	3	43		15	4	65	*	6	3	30		60	12	182		32	7	111	*	5	3	27	
30	* 5	3	26		* 8	3	35	*	10	3	41	*	5	2	24		40	9	133		22	5	84	*	4	2	23	
100	* 3	2	14		* 4	2	19	*	5	2	25	*	3	2	13	*	14	4	51	*	7	3	33.6	*	3	2	14	

(*Continued*)

Table 4. Continued.

	Panel D: Data from scallop population																											
N	Hg				Cd				As				Zn				Pb				Cu				Ag			
		10	25	M.D. Size2		10	25	M.D. Size2		10	25	M.D. Size2		10	25	M.D. Size2		10	25	M.D. Size2		10	25	M.D. Size2		10	25	M.D. Size2
5	*	10	3	40	*	16	4	54	*	8	3	37	*	20	5	61		18	5	75		12	4	54		28	6	101
10	*	6	3	29	*	9	3	38	*	5	3	26	*	11	3	43		10	3	49		7	3	35		14	4	63
20	*	4	2	20	*	5	3	27	*	3	2	18	*	6	3	31		6	3	32	*	4	2	21		9	3	43
30	*	3	2	17	*	4	2	22	*	3	2	15	*	5	3	25	*	4	2	23	*	3	2	17	*	6	3	30
100	*	2	2	9	*	3	2	12	*	2	2	8	*	3	2	14	*	3	2	13	*	2	2	10	*	3	2	15

Table 5. Cost differences of running the monitoring system of doubling the number of samples or the individuals in the pooled samples.

Forty samples			
Analytical determination		Sampling process	
Material and reagents of laboratory	Cost (€)	*Material and reagents of laboratory*	Cost (€)
Acid wash	150	Acid wash	100
Argon	60	Total	100
Graphite tubes (2)	260		
Reference material	30		
Total	500		
Personnel	Time/cost (hours/€)	*Personnel rank*	Time/cost (hours/€)
Laboratory assistant	20/150	Laboratory assistant	65/500
Technician	100/900	Total	500
Scientist	20/225		
Total	1275		
		Doubling individuals	Doubling pools
Analytical determination	1 * 1775	1 * 1775	2 * 1775
Sampling process	1 * 600	2 * 600	2 * 600
Total process	2375	2975	4750

normal distributions were obtained if pooled samples are used. Thus, where data transformation is not desirable, pooled samples must be used to normalise data. In most monitoring programmes, using two pooled samples, 100 individuals must be included per pool if differences of 30% want to be detected for all metals. On the other hand, special attention is required for some metals as Ni, Cr and Pb in some species, because the frequency distributions in one-individual data follow a reciprocal distribution. It implies data transformation even if pooled samples are used.

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