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Influence of the salinity adjustment methods, salts and brine, on the toxicity of wastewater samples to mussel embryos

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One of the main problems of the Whole Effluent Toxicity is related to the use of bioindicator species representative of the target environment. Most wastewater discharges are of fresh water, so their salinity has to be adjusted when they are discharged to transitional and marine coastal waters, in order to perform toxicity bioassays with reliable organisms. At the moment, there is no optimum technique to allow sample salinity to be adjusted and no specific information regarding salinity adjustment when bivalves are being considered for toxicity test performance. This paper provides information on the potential use of different methods to adjust the salinity of hotel/domestic wastewater samples with different brands of natural and synthetic Dry Salts (DS) and HyperSaline Brine (HSB) for use in the embryo larval development bioassay with the mussel *Mytilus galloprovincialis*. HyperSaline Brine derived from reconstructed artificial seawater proved to be more viable for wastewater salinity adjustment than DS.

Keywords: *Mytilus galloprovincialis* bioassay; salinity adjustment; dry salts; brine; hotel/domestic wastewater

Introduction

One of the main points of interest in Whole Effluent Toxicity (WET) testing is the assessment of the potential impact of wastewater on the target environment using reliable species [1]. Thus, when wastewaters are discharged to transitional and marine coastal waters, euryhaline salt-water species are used for toxicity bioassays. The main problem with this is related to the choice of the best method to adjust wastewater salinity, which is generally lower than that of marine water. Most industrial and sewage treatment discharges entering estuarine and coastal waters have low salinity which needs to be increased to match that of the receiving water or, just to a sufficient salinity for performing toxicity tests [2]. Physiological differences can cause marine organisms to show toxicological responses unlike those of terrestrial and freshwater organisms, due to osmotic pressure and ways of exposure and interaction with the aquatic media [3, 4]. For example, chlorine can mitigate toxicity effects (e.g. of metals), creating compounds of relatively low toxicity.

Dry Salts (DS) and HyperSaline Brine (HSB) are the two potential sources for sample salinity adjustment [5]. Dry Salts and HSB can be either naturally or artificially derived. HyperSaline Brine can be made by concentrating natural seawater (NSW) or artificial seawater (ASW) by freezing or evaporation [6].

In Table 1, the major advantages, disadvantages and assumptions of different ways of adjusting wastewater salinity, according to the USEPA [5] and Jonczyk *et al*. [6], are summarized. Jonczyk *et al*. [6] discussed the use of a brand of DS (Instant Ocean®), and natural and synthetic HSB to adjust the salinity of wastewaters to be tested with the sea urchin *Lytechinus pictus* fertilisation assay. These experiments did not demonstrate any significant differences between the use of DS and HSB for sample salinity adjustment for domestic and industrial wastewaters.

However, no data are available for bivalve mollusc bioassays, in particular mussel toxicity tests, on the use of DS or HSB for salinity adjustment, and, at the moment, 'their use must be considered provisional', as stated by the USEPA [5]. Mussels, like oysters, are considered reliable and sensitive organisms in WET and whole effluent assessment, and are used in biomonitoring and bioaccumulation studies [5,7–10].

This paper provides basic and preliminary information on the potential use of some natural and synthetic DS and HSB to adjust the salinity of wastewater samples, originating from hotel/domestic activities, to be used in the embryo larval development bioassay with the mussel *Mytilus galloprovincialis*. The salinity of

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Table 1. Advantages and disadvantages when using Dry Salts (DS) and Hypersaline Brine (HSB) for adjusting salinity of wastewater samples. N = natural and S = synthetic.

	Advantages	Diasdvantages
DS	Presence of natural microelements (N)	Collected from an uncontaminated site (could be uneasy to find) (N)
	Ecologically relevant (N)	Conditioning procedures are needed (N, S)
	Full sample can be tested (N, S)	Interactions between salts and toxicant in wastewater are not understood (N, S)
	Broad selection of brands (S)	Potentially not feasible for the receiving environment (N, S)
	Not limited by geographical location (N, S)	
	Limited contamination due to standardization (S)	
HSB	Stored for long-term period (N, S)	Collected from an uncontaminated site (could be uneasy to find) (N)
	Presence of natural microelements (N)	Highest exposure concentration as a function of sample initial salinity and the required salinity (N, S)
	Cost-effective when no clean seawater is naturally available (S)	Interactions between salts and toxicant in wastewater are not understood (N, S)
	Ecologically relevant (N)	Costs related to seawater sampling (N)
	Quality constancy (S)	Quality variability over time (N)
		Potential presence of pathogens (N)

four hotel/domestic wastewater samples was adjusted in different ways using natural and artificial salts as dry salts and brine solutions. Influent and effluent samples were collected from two hotels (A and B) in the city of Venice (Italy) and the treated wastewater was allowed to discharge into the Lagoon of Venice. Hotel A uses Ultra-Filtration Membrane Biological Reactor (UF-MBR) technology for wastewater treatment and hotel B has Activated Sludge Sequencing Batch Reactor (AS-SBR) technology. The UF-MBR is an alternative to traditional biological treatment plants, with the secondary clarifier replaced by membrane filtration; a higher quality effluent is generally achieved than with conventional wastewater treatment technologies, as suspended solids (SS) and high-weight molecular compounds can be completely removed [11]. The AS-SBR is a conventional treatment technology without primary clarification where bio-oxidation and secondary clarification can take place in the same basin [12].

Materials and methods

Sampling and salinity adjustment methods

The US National Pollutant Discharge Elimination System guidelines [7] were followed for sampling and sample handling. Two wastewater samples were collected from hotel A and two from hotel B in the spring (April) (two influents and two effluents). Influents were sampled from the untreated wastewater storage tank and effluents from the end of the discharge pipe. In order to limit wastewater toxicity variability, three grab samples were collected over a period of time not exceeding six hours and combined to create composite samples representing the average characteristics of the waste stream during the compositing period. Samples were not chlorinated and were stored in darkness at 4 ± 1 °C for 12 h before and after salinity adjustment [13]. An aliquot of wastewater samples without adjusted salinity was stored for physical and chemical analyses. Samples from hotel A and B were called 1 and 2, respectively, and influent samples were denoted by an *a* and effluent samples, by a *b* (e.g. 1a).

Three dry-salt brands were tested: natural sea salts from Salt Works (Porto Viro, Italy) (SW), Prodac Ocean Fish® (Prodac International, Cittadella, Italy) (Prodac) and Instant Ocean® (Aquantium Systems, Mentor, OH, USA) (IsOc). The wastewater salinity adjustment procedure suggested by ASTM as DS [14] was not taken into consideration, because it was shown to be too time-consuming and not cost-effective. Indeed, a series of ten DSs must be added in a specific order, and their amounts can sometimes be very small depending on the wastewater sample volume. Moreover, the previous salt has to be dissolved before adding the next one and a 24 h aeration period is necessary for sample pH and salinity adjustment. It is evident that the last procedure could significantly change the sample characteristics.

In addition, six HSB solutions were checked, three were produced from the above-mentioned DS (SW, Prodac and IsOc) and two from NSW collected at two sites – an incoming tide of the Atlantic Ocean (at Guernsey Sea Farm Ltd, Vale, Guernsey, United Kingdom (NSW(Gu)) and at the Bacino of San Marco, Lagoon of Venice, Italy (NSW(Ve)) – in order to compare potential differences between an uncontaminated site where bivalve sea-farming is practiced and the actual wastewater-receiving environment (Lagoon of Venice). Another HSB was produced from ASW prepared according to the ASTM protocol for ASW [14].

Synthetic brine solutions were obtained from ASW by dissolving SW, Prodac and IsOc dry salts (about 34 g per litre of deionised water) and DS according to ASTM [14] in deionized water. Before HSB production the ASW was aerated for 24 h to thoroughly solubilise all the added salts and stabilise salinity at 34 ppt (salinity of the Lagoon of Venice) and pH 8.0–8.2. The salinity concentration was reached by slowly evaporating ASWs at 40 °C in the dark for about 24 h. To prevent temperature stratification and increase water evaporation the solution was stirred by a magnetic stirrer. Natural brine solutions were obtained in the same way, but NSW was previously filtered at 2 μm. The final HSB salinity was 110 ppt. The HSBs were prepared at least three days prior to toxicity testing, and were stored in portable capped containers in the dark at 4 °C. When DSs were added in the crystalline form to the whole effluent sample to achieve 34 ppt salinity, the solution was allowed to age for at least four hours and stored in the dark at 4 °C. When HSB was added to wastewater samples to adjust salinity a minimum period of four hours was still required, to equilibrate the pH of the solutions.

Physico-chemical analysis

A basic physico-chemical characterization of the wastewater samples was provided to integrate toxicity data and facilitate its interpretation. Chemical oxygen demand (COD) was determined according to procedure 5130, $N\text{-}NH_4^+$ according to procedure 4030/C, SS according to procedure 2090 and total Kjeldahl nitrogen (TKN) according to procedure 5030, of IRSA/CNR [15]. The pH was measured with a pH meter HI 9025 Microcomputer (HANNA Instrument®, Woonsocket, USA). Un-ionized ammonia ($N\text{-}NH_3$) was determined as a function of pH, salinity and temperature according to the method of the USEPA [1] on the basis of total ammonia concentrations. Anions (nitrite, nitrate, sulphate and phosphate) were determined by an ion chromatograph system after filtering at 0.45 μm (Metrohm 761 Compact IC (Herison, Switzerland), column Metrohm Metrosep A Supp 5 150 × 4 mm). Salinity was measured with a refractometer and dissolved oxygen (DO), with a WTW multi-parametric device. The pH, salinity and DO were monitored and maintained in the best ranges throughout the test.

Test species and protocols

Samples of *M. galloprovincialis* were collected along the Adriatic coast (Italy) during the breeding season (March). The embryo toxicity test was performed according to the method proposed by His *et al*. [16] modified for gametes pools. Adults were induced to spawn by thermal stimulation (temperature cycles at 18 ± 1 °C and 28 ± 1 °C). Artificial seawater for gamete collection was ASTM ASW [14] at 34 ppt. Gametes of good quality, derived from the best three males and females, were selected and filtered at 32 μm (sperm cells) and 100 μm (eggs) to remove impurities. Eggs (1000 mL) were fertilized by injecting 10 mL of sperm suspension; fertilization was checked by microscopy. Egg density was determined by counting four subsamples of known volume. Fertilized eggs, added to the test solutions in order to obtain a density of 60–70 eggs mL^{-1}, were incubated for 48 h at 18 ± 1 °C. At the end of the test, samples were fixed with buffered formalin (4%) and 100 larvae were counted, distinguishing between normal larvae (D-shaped) and abnormalities (malformed larvae and pre-larval stages). The acceptability of test results was based on negative control for a percentage of normal D-shaped larvae > 70% [17]. Sterile, capped polystyrene 24-well microplates (Iwaki brand, Asahi Techno Glass Corporation, Tokyo, Japan) were used as test chambers for the toxicity test.

Hotel/domestic wastewater samples were tested in three replicates per dilution concentration. A minimum of six concentrations per wastewater sample (dilution water was in accordance with the specific salinity adjustment method under study); a negative control per salinity adjustment method, consisting of deionized water with salinity adjusted using all the different methods applied, and a reference toxicant in ASTM ASW alone [14] were considered. A negative control was also performed on ASTM ASW. Sample concentrations were assayed according to a geometric scaling. The reference toxicant, Cu as copper solution prepared from copper nitrate standard solution for atomic absorption spectroscopy, was only performed with ASTM ASW.

Data analysis

Data are expressed as EC50 values based on the percentages of abnormal larvae. EC50 values with 95% confidence limits were calculated by the Trimmed Spearman-Karber method. The responses for each treatment as percentage of effect (per cent of effect or per cent of abnormalities) were corrected for effects in the negative control by applying Abbott's formula [14]. The percentages of effect were displayed at the lowest available wastewater dilution volume (w/v) tested in order to compare also the toxicity of those samples with no quantifiable EC50.

Results and discussion

Physical and chemical analyses

Physical and chemical data are reported in Table 2. The wastewater treatment facility from hotel A

Table 2. Physical and chemical parameters of hotel/domestic wastewaters. 1 and 2 are the samples collected from hotels A and B, respectively. a = influent and b = effluent.

Samples	SS mg l^{-1}	pH	COD mg l^{-1}	TKN mg l^{-1}	$N–NH_4^+$ mg l^{-1}	$N–NH_3$ mg l^{-1}	$N–NO_2^-$ mg l^{-1}	$N–NO_3^-$ mg l^{-1}	P_{TOT} mg l^{-1}	$P–PO_4^{3-}$ mg l^{-1}	$S–SO_4^{2-}$ mg l^{-1}
Ai	304	8.32	500	30	24	1.536	0.4	< 0.01	8	2.4	9.8
Bi	115	7.40	352	36	20	1.708	0.8	< 0.01	4	1.2	1.2
Ae	0	7.45	9	8	2.4	0.025	< 0.01	18	4	1.9	11
Be	100	7.45	42	38	4.0	0.042	< 0.01	15	4	4.5	12

completely removed SS from the final discharge (1b), whereas there was inconsistent SS removal in sample 2b. This might mean that the 1b discharge could be less toxic than the 2b discharge because the absence of suspended particulate matter reduces the potential presence of heavy metals, P- and N-based compounds. All pHs remained in the best range for toxicity testing (7.40–8.32) without any substantial variation during the test. Chemical oxygen demand was greatly reduced by the wastewater treatment process in both wastewater treatment plants (WWTPs), as was both $N\text{-}NH_4^+$ and $N\text{-}NH_3$ concentrations. Indeed, ammonia represents one of the major hazards for the receiving-water environment. In particular, it is expected that all samples, except 1b, would be toxic to a different extent because the $N\text{-}NH_3$ EC50 for *M. galloprovincialis* is 0.036 mg L^{-1} [18].

Both WWTPs showed the presence of nitrification processes (1b and 2b). A decrease in total phosphorus and TKN was only observed for sample 1, principally due to the absence of suspended solids.

Toxicity bioassays data

Data from negative controls are reported in Table 3. It is evident that SW and Prodac DS and SW HSB are not at all suitable for salinity adjustment procedures (100% effect in the control water solutions). The toxicity results from sample salinity adjustment are reported, nonetheless. The best performances in the negative controls were shown in decreasing order by ASTM test water, ASTM HSB, IsOc HSB, NSW(Gu) HSB and NSW(Ve) HSB with 6%, 11%, 14%, 16% and 18% effect. IsOc DS and Prodac HSB had similar effects, 20% and 25%, respectively. Although all these values are acceptable (< 30% effect as stated by His *et al*. [17]), ASTM reconstituted seawater [14] from HSB was shown to be the most suitable for salinity adjustment purposes. The two NSW HSB reconstructed seawaters displayed very similar outcomes, with no substantial differences between the water quality in the two sampling sites.

The positive control (Cu) gave an EC50 value of 17.63 µg l^{-1} (16.58 µg l^{-1} – 18.75 µg l^{-1}), which is in line with that reported by His *et al*. [16] and by the research group reference toxicant control chart (15.60 µg l^{-1} (9.47 µg l^{-1}–21.72 µg l^{-1}), n = 9, CV = 19.63%).

Table 3. Toxicity results with whole dilution waters prepared according to different Dry Salts (DS) and Hypersaline Brine (HSB) methods and brands. SW = Salt Works, IsOc = Instant Ocean®, Prodac = Prodac Ocean Fish®, ASTM = artificial seawater prepared according to ASTM (2004), NSW(Gu) = natural seawater collected from the Guernsey Sea Farm (UK) sampling site, NSW(Ve) = natural seawater collected from the Lagoon of Venice (Italy) sampling site. Data are expressed as percentage of effect (%) ± standard deviation.

Negative controls		
		% effect
ASTM	HSB	11 ± 1
ASTM	Test water	6 ± 2
IsOc	DS	20 ± 3
	HSB	14 ± 2
NSW(Gu)	HSB	16 ± 4
NSW(Ve)	HSB	18 ± 3
Prodac	DS	100 ± 0
	HSB	25 ± 3
SW	DS	100 ± 0
	HSB	100 ± 0

The toxicity data with *M. galloprovincialis* are shown in Table 4. Toxicity effects for the same sample seemed to vary according to the salinity adjustment method used, considering the fact that the maximum percentage of wastewater after salinity adjustment was equal to 69.64%. The greater toxicity effects came from influent samples 1a and 2a. As shown by the negative controls, SW and Prodac DS and SW HSB salinity adjustment methods did not allow any substantial distinction between samples due to the adverse effects produced by the same method. Anyway, a certain per cent of normally developed larvae were found at the lower dilutions, probably due to hormetic effects induced by the diluted wastewater (e.g. 1b SW HSB produced 82% effect at 8.36% w/v, and 1b Prodac DS produced 81% effect at 69.64% w/v).

Table 4. Toxicity effects of wastewater samples with salinity adjusted according to different Dry Salts (DS) and Hypersaline Brine (HSB) methods assessed via *M. galloprovincialis* embryo larval development test. Results are shown as percentage of effect at the lowest available wastewater dilution concentration tested per sample and per salt brand (% effect at% w/v) and EC50 values (as per cent of the whole effluent) (when quantifiable). 1 and 2 are the samples collected from hotels A and B, respectively. a = influent, b = effluent, SW = Salt Works, IsOc = Instant Ocean®, Prodac = Prodac Ocean Fish®, ASTM = artificial seawater prepared according to ASTM (2004), NSW(Gu) = natural seawater collected from the Guernsey Sea Farm (UK) sampling site, NSW(Ve) = natural seawater collected from the Lagoon of Venice (Italy) sampling site, * = EC50 not determined because the negative control registered 100% effect,–= EC50 not quantifiable.

		Mytilus galloprovincialis							
Salt brands	Salinity adjustment method	Samples							
		1a		2a		1b		2b	
		% effect at% w/v	EC50	% effect at% w/v	EC50	% effect at% w/v	EC50	% effect at% w/v	EC50
ASTM	HSB	84 at 4.18	2.33 (2.11–2.57)	84 at 4.22	2.91 (2.67–3.16)	0 at 69.64	–	57 at 69.64	68.37 (59.75–78.24)
IsOc	DS	40 at 2.09	–	78 at 8.36	5.05 (4.04–6.32)	23 at 69.64	–	78 at 69.64	14.04 (10.64–18.53)
	HSB	100 at 1.04	–	100 at 1.04	–	10 at 69.64	–	83 at 69.64	33.53 (27.85–40.36)
NSW(Gu)	HSB	78 at 4.18	2.61 (2.26–3.01)	71 at 4.18	2.40 (2.11–2.74)	2 at 69.64	–	59 at 69.64	67.57 (53.08–86.10)
NSW(Ve)	HSB	65 at 4.18	3.25 (2.91–3.63)	63 at 4.18	2.99 (2.26–3.36)	2 at 69.64	–	55 at 69.64	60.55 (57.09–63.44)
Prodac	DS	100 at 2.13	–	100 at 2.09	–	81 at 69.64	*	100 at 2.09	–
	HSB	100 at 2.09	–	100 at 2.09	–	48 at 69.64	–	83 at 69.64	22.34 (18.23–27.38)
SW	DS	100 at 1.04	–	100 at 1.04	–	100 at 1.04	–	100 at 1.04	–
	HSB	100 at 1.04	–	100 at 1.04	–	82 at 8.36	–	100 at 1.04	–

The ASTM HSB and both NSW HSB salinity adjustment methods allowed clear distinction between sample toxicities, whereas IsOc DS and HSB adjusted samples showed intermediate characteristics.

According to ASTM HSB, NSW(Gu) and NSW(Ve) HSBs, sample 1a showed a toxicity quantifiable as EC50 of 2.33%, 2.61% and 3.25%, respectively. Similarly, sample 2a had EC50 values of 2.91%, 2.40% and 2.99%, respectively, and sample 2b had EC50 values of 68.37%, 67.57% and 60.55%, respectively. For sample 2a, an EC50 value was also determined with another salinity adjustment method, IsOc DS (EC50 = 5.05%). The same was found for sample 2b with IsOc DS (EC50 = 14.04%), IsOc HSB (EC50 = 33.53%) and Prodac HSB (EC50 = 22.34%), but, for this specimen only, higher toxicity levels were evidenced with the ASTM HSB, NSW(Gu) and NSW(Ve) salinity adjustment procedures. Sample 1b appeared to be the only one with no quantifiable EC50, but just characterized by a percentage of effect. The ASTM HSB, NSW(Gu) and NSW(Ve) had low percentages of effect: 0%, 2% and 2%, repectively, at 69.64% w/v. Slightly greater effects were displayed by IsOc DS and HSB: 23% and 10% as percentage of effect, respectively, at 69.64% w/v. Moreover, Prodac DS and HSB showed 81% and 48% effect at 69.64% w/v.

After checking and comparing toxicity data, it can be stated that, of those studied, ASTM HSB, NSW(Gu) and NSW(Ve) are the best methods for wastewater salinity adjustment. In particular, the ASW brine generated the same results as the NSW brines. The salinity adjustment methods can be ranked according to increasing toxicity effects, as EC50, in wastewater samples for specimen 1a as NSW(Ve) < NSW(Gu) < ASTM HSB (all other samples had no quantifiable EC50 value at the same dilution concentrations), The ranking for 2a is IsOc DS < NSW(Ve) HSB < ASTM HSB < NSW(Gu) HSB, for 1b as ASTM HSB < NSW(Gu) ≈ NSW(Ve) < IsOc HSB < IsOc DS < Prodac HSB, for 2b as ASTM HSB < NSW(Gu) ≈ NSW(Ve) < IsOc HSB < Prodac HSB < IsOc DS.

The toxicity results can also be interpreted by taking into account physical and chemical results. In Table 2, SS, COD and un-ionized ammonia concentrations suggest that sample 1b would have potentially produced low toxicity phenomena, whilst samples 1a and 2a would have produced the highest ones for the same reasons. Sample 2b presented intermediate physical and chemical characteristics between sample 1b on the one side and samples 1a and 2a on the other, so its toxicity outcomes should be intermediate between those samples. The comparison between physico-chemical and ecotoxicological characteristics exactly confirmed this situation.

This preliminary study has demonstrated, by checking the effects on dilution waters, that the adverse effects of salinity on test organisms are partially directly related to the kind of natural/synthetic salts used. The *M. galloprovincialis* embryo larval development test indicated that HSB, especially that obtained from ASTM and NSWs, is the best way to adjust wastewater sample salinity and is better than DSs in general, allowing, in most cases, the determination of wastewater toxicity as EC50 in the range of the first six concentrations in geometric scaling whenever a toxic sample is checked. However, this is not in agreement with Jonczyk *et al*. [6], who found no significant differences in toxicity effects with sea urchins when DS or HSB were used for the adjustment of sample salinity. This might well be because the fertilization assay with sea urchins is generally about one order of magnitude less sensitive than embryo toxicity bioassays with bivalves and/or sea urchins [9,19,20].

Conclusions

Careful attention should be paid when selecting the method to adjust salinity of hotel/domestic wastewater samples for whole effluent toxicity testing when *M. galloprovincialis* embryo toxicity bioassay is to be used. This study compared different salinity adjustment methods using the direct addition of natural and synthetic dry salts or hypersaline brine of some commercial brands. Toxicity effects were shown to vary in relation to the selected salinity adjustment method and salt brand according to the negative controls performed on dilution waters. In general, dry salts proved to be the worst choice for salinity adjustment, especially for natural dry salts from salt works, but also for the synthetic salts such as Prodac Ocean Fish® brand. In contrast, the hypersaline brine obtained by evaporation appeared to be the most viable method for adjusting the salinity of wastewater samples to be tested with mussels, especially that originating from ASTM ASW [14]. Only the hypersaline brines prepared from ASTM ASW and NSWs allowed the determination of EC50s in all influent samples and showed that only the discharge from the UF-MBR had relatively no toxicity, as supposed from the chemical analyses. Based on the results of this study, hypersaline brine, especially that from ASTM ASW, should be preferred for hotel/domestic wastewater salinity adjustment when mussel and, potentially, oyster toxicity tests must be performed. Further research is still required to verify these results with oysters and check their reliability with industrial wastewaters.

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