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Influence of storage methods, refrigeration or freezing, on the toxicity of wastewater samples to oyster embryos

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One of the main concerns in wastewater whole effluent assessment is the sampling phase and the sample chain of custody before any toxicity evaluation. The major problem is related to establishing the correct method for sample storage in order to perform toxicity bioassays. The toxicity of some domestic and glass factory industrial wastewater samples stored both by refrigeration at 4 ± 1 °C for no more than three days, and freezing at –18 ± 1 °C for no more than one month was compared via the embryo larval development bioassay with the oyster *Crassostrea gigas*. The results showed no significant differences between the toxicities of refrigerated and frozen wastewater samples. The wastewater classification, according to a score based on four toxicity classes, showed that the preservation methods did not alter the toxicity classification of the samples. In particular, it was demonstrated that the samples considered as 'not acutely toxic' after refrigeration were also found to have this classification after freezing.

Keywords: wastewater; refrigeration; freezing; toxicity bioassay; *Crassostrea gigas*

Introduction

A major problem in wastewater monitoring programmes is related to sampling, sample handling, preservation and storage. Although specific guidelines have been developed over time for this purpose and are well established [1–6], some gaps still remain in the knowledge. The physical and chemical characteristics of wastewater samples tend to change rapidly after sampling. It is not always possible to process them in a short time, especially for large numbers of samples. Indeed, the cost effectiveness of the analysis could be affected, and methodological problems such as sub-chronic and chronic toxicity testing should be taken into account.

The US EPA guidelines suggest storing wastewater samples in darkness at 4 °C after sampling and maintaining this until samples are delivered to the laboratory for analysis. Samples should be assessed no later than 36 h after sampling, avoiding any other form of storage than that mentioned [6,7–9].

The main problems that could affect wastewater sample preservation are related to its biological activity, such as biodegradation processes by microorganisms, volatilization of pollutants, especially of organic compounds, and physico-chemical reactions related to pH change, dissolved oxygen concentration and redox potential, apart from all the factors related to a possible misuse of sampling apparatus [10].

Some studies tried to assess the best sample preservation mode to allow longer storage after sampling [11–13]. In particular, the influence of industrial effluent preservation modes was investigated by Naudin *et al*. [12], considering samples refrigerated in darkness at 4 ± 1 °C, frozen in a deep freezer at –26 ± 3 °C and freeze-dried. The authors finally concluded that samples need to be frozen if toxicity tests are not performed within a week after sampling, and that more studies were necessary on other wastewaters before definitive assumptions could be made. Other authors allowed samples for freezing when toxicity testing cannot be performed in 48 h after sampling [14], but a period of longer than two months must not be exceeded [15].

The aim of this study was to investigate refrigeration and freezing as two potential methods for storage of wastewater samples for ecotoxicological surveys. In particular, the toxicity of some domestic and glass factory industrial wastewater samples was determined, using both refrigerated and frozen samples, via the embryotoxicity test with the oyster *Crassostrea gigas* [16–18]. Finally, toxicity data from refrigerated and frozen samples were compared in order to highlight

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potential similarities between the two wastewater preservation techniques.

Materials and methods

Sample collection and storage

National Pollutant Discharge Elimination System general guidelines [6] were followed for sampling and sample handling. Well-mixed influent samples were collected from a storage tank of 7 m^3 for domestic wastewater and of 1 m^3 for glass factory industrial wastewater after physico-chemical treatment, whereas effluent samples were taken at the end of the whole treatment just at the end of the discharge pipe. In total, three specimens of domestic and three specimens of industrial wastewater were collected, considering one influent and two effluent samples, respectively. Samples were named A, B and C for domestic wastewaters and D, E and F for the glass factory industrial ones. Samples A and D were influents whilst all others were effluents.

In order to avoid 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. Non-reactive polyethylene sample containers were pre-cleaned (hot water and detergent, rinsed with acid, rinsed six times with deionized water and dried in a contaminant-free area) and provided with a closure to protect the sample from contamination. The containers were completely filled. An appropriate preservation of wastewater samples was supplied during their transfer from the sampling site to the laboratory (cooling at 4 °C in a refrigerated holding container) to minimise physico-chemical and biological changes. The transport of the wastewater samples from the sampling site to the laboratory facilities lasted no more than three hours.

Domestic and glass factory industrial effluents were sampled after treating via ultra-filtration membrane biological reactor (UF–MBR) technology in two distinct wastewater treatment plants located in Venice (Italy). Only glass factory industrial wastewaters were characterised by a physico-chemical pretreatment before UF–MBR, which consisted of the addition of lime and $FeCl_3$ to enhance the removal of heavy metals. 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 [19].

Sample salinity was adjusted with HyperSaline Brine (HSB, 110‰) prepared with salts for artificial seawater (ASW) according to [20,21], in order to have a final salinity equal to that of the receiving water body (34‰) – the Venice lagoon (Italy). Actually, effluents are considered as a potential direct threat for salt water receiving environments [2].

Samples were stored both by refrigeration for no more than three days at 4 ± 1 °C in darkness and by freezing at −18 ± 1 °C for no more than one month for ecotoxicological assessments [12,21,22], while physical and chemical analyses were performed on fresh samples. Frozen wastewater samples were allowed to slowly defrost at room temperature, 20 ± 2 °C, and then ecotoxicological analyses were performed.

Physico-chemical analysis

A basic knowledge of physical and chemical parameters of fresh wastewater samples was provided to allow their essential characterization and to help in the interpretation of ecotoxicological data.

The chemical oxygen demand (COD) was determined according to APAT 5130 procedure, ionized ammonia ($N\text{-}NH_4^+$) according to APAT 4030/C procedure, SS according to APAT 2090 procedure, total kjeldhal nitrogen (TKN) according to APAT 5030 procedure and total phosphorus (P_{TOT}) according to APAT 4060 procedure [22]. Un-ionized ammonia ($N\text{-}NH_3$) was determined as a function of pH, salinity and temperature in accordance with [5] on the basis of total ammonia concentration. The pH was measured with a HI 9025 microprocessor-based pH meter from Hanna Instruments (Beverly, MA, USA). Anions (chloride, nitrite, nitrate, sulphate and phosphate) were determined by ion chromatography (IC) after filtering at 0.45 μm (Metrohm 761 Compact IC Column Metrohm Metrosep A Supp 5, 150 × 4 mm). When concentrations were not measurable, detection limits were provided in accordance with Metrohm IC Systems, (Herisau, Switzerland). Salinity was checked with a refractometer and dissolved oxygen (DO) by a WTW (multiparametric device Nova Analytics, Weilheim, Germany). The analysis of total heavy metals in the wastewater samples (As, Cd, Cr, Cu, Ni and Zn) was performed by inductively coupled plasma emission spectroscopy (Spectro Flame Compact E, Analytical Instruments, Kleve, Germany) in accordance with [6]; detection limit values were in accordance with [23].

Toxicity tests

The oysters for the embryo toxicity test were purchased ready to spawn from an English hatchery, Guernsey Sea Farm Ltd, Vale, Guernsey. Living oysters were delivered within 24 h after collection in a refrigerated holding container. The bioassay was performed in accordance with His *et al*. [24], modified for gamete pools according to Libralato *et al*. [21] to avoid the

limits relating to the use of the best-spawning male and female. After thermal stimulation of organisms (alternating cycles at 18 ± 1 °C and 28 ± 1 °C), good quality male and female gametes (high motility sperm cells and eggs with homogeneous dimensions and regular shape) were collected and filtered at 32 μm and at 100 μm, respectively, to remove impurities. The eggs, resuspended in a 1000 mL glass cylinder, were fertilised by injecting 10 mL of sperm cell suspension. Once the fertilisation had been checked, zygote suspension was adjusted in order to obtain a final density of around 200 zygotes per 3 mL of wastewater dilution. Zygotes were incubated for 24 h at 24 °C in the darkness. At the end of the test, samples were fixed with buffered formalin and 100 larvae were observed, 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 equal to or higher than 70% [24–25]. Sterile, capped, polystyrene 24-well microplates (Iwaki brand, Asahi Techno Glass Corporation, Tokyo, Japan) were used as test chambers for the toxicity test. Dilution water (for test solutions and gametes) was ASW reconstituted according to [20] at a salinity of 34‰.

Wastewater samples were tested in three replicates per dilution concentration. A minimum of six concentrations per wastewater sample, a negative control per fresh, refrigerated and frozen ASW and a reference toxicant were considered. Sample concentrations were assayed according to a geometric scaling. The reference toxicant, a copper solution prepared from copper nitrate standard solution for atomic absorption spectroscopy, was only performed with fresh ASW [20]. Moreover, in order to reduce any variability among test media, a number of quality assurance/quality control measures were implemented throughout the study. The same genetic pool of gametes was used for assessing the fertilisation rate in each testing series, and the same technician conducted all tests.

Data analysis

Whenever possible, EC50 values based on the percentages of abnormal larvae (percentage of effect, PE) were calculated with 95% confidence limits by the Trimmed Spearman–Karber method [20]. The toxic unit at 50% of the population exhibiting a response (TU50) was determined as 100/EC50 to provide values directly correlated to the toxicity magnitude. The responses for each treatment were corrected for the effects in the negative control by applying Abbott's formula [20]. Whenever toxicity could not be expressed as EC50, it was provided by only the PE value.

Results

The wastewater physico-chemical data are reported in Table 1. Samples A, B and D presented 115 mg L^{-1}, 100 mg L^{-1} and 250 mg L^{-1} of SS, respectively, to which part of the contamination could potentially be bound. Samples A and D showed 352 mg L^{-1} and 216 mg L^{-1} of COD, whereas all other samples presented COD concentrations lower than 42 mg L^{-1}. Ionized ammonia concentrations ranged from 1.64 to 34.98 mg L^{-1} and un-ionized ammonia from 0.03 to 0.58 mg L^{-1}.

All samples showed arsenic concentrations below the detection limit (<10 μg L^{-1}). Samples A, B, C and D presented cadmium concentrations below the detection limit (< 2 μg L^{-1}), whereas samples E and F displayed similar values: 11 μg L^{-1} and 15 μg Ll^{-1}, respectively. All samples showed a chromium concentration lower than the detection limit (< 10 μg L^{-1}). Just sample F presented a copper concentration lower than the detection limit (< 5 μg L^{-1}), with the other samples ranging from 5 to 9 μg L^{-1}. Samples A, B, D and E displayed a concentration of nickel lower than the detection limit (< 15 μg L^{-1}), while sample F showed a concentration of 151 μg L^{-1}. Regarding zinc, samples A, B and C presented concentrations ranging from 4 to 5 μg L^{-1}, but higher concentrations were registered for samples D, E and F, with a hot spot concentration of 6969 μg L^{-1} for sample F.

Regarding toxicity data, it was highlighted that the negative controls are all acceptable, reporting a mean value of 83% ± 2% for fresh ASW for gamete collection and fertilisation, 83% ± 4% for refrigerated ASW and 82% ± 2% for frozen ASW. The reference toxicant in fresh ASW displayed an EC50 value of 12.50 μg Cu L^{-1} (11.40–13.70 μg Cu L^{-1}), being in line with those reported by [24,26]. No significant differences ($p < 0.01$) were found between the effects of fresh, refrigerated and frozen ASW in control treatments. Salinity was 34‰, DO 6 mg L^{-1} and pH values remained around 8.2.

The toxicity data are reported in Table 2 both as PE on the whole sample and TU50 whenever possible (i.e. samples B and C showed no calculable EC50s). The percentage variation of toxicity and the coefficient of variation were also calculated. Refrigerated domestic wastewater samples A, B and C showed the lowest toxicity values: 14.33 TU50, PE = 52% and PE = 20%, respectively. Refrigerated glass factory industrial wastewater samples D, E and F showed toxicity varying from 16.67 TU50 for sample D to higher values for samples E and F, 49.26 TU50 and 27.17 TU50, in that order. The general trend indicated that the sample freezing procedure tended to vary the toxicity of refrigerated samples, but in different ways. The mean variation value is about 19% and the mean coefficient of

Table 1. Physical and chemical data measured on fresh wastewater samples; when concentrations were not measurable the detection limits are provided.

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_3^- mg L^{-1}	cl^- mg L^{-1}	S-SO_2^- mg L^{-1}	As µg L^{-1}	Cd µg L^{-1}	Cr µg L^{-1}	Cu µg L^{-1}	Ni µg L^{-1}	Zn µg L^{-1}
A	115	7.40	352	36.59	19.50	0.26	0.77	<0.01	3.90	1.2	31	9	< 10	<2	<10	6	<15	5
B	100	7.32	42	3.80	3.67	0.04	<0.01	15.08	4.00	1.9	93	11	< 10	<2	<10	5	<15	4
C	0	7.45	9	2.44	1.64	0.03	<0.01	18.44	4.20	4.6	120	11	< 10	<2	<10	<5	<15	4
D	250	7.45	216	26.18	34.98	0.58	<0.01	0.66	–	30	818	1	< 10	<2	<10	5	<15	99
E	0	7.57	24	10.80	17.85	0.29	<0.01	13.01	–	85	1341	175	< 10	11	<10	9	<15	250
F	0	7.84	11	11.46	13.00	0.42	<0.01	20.60	–	147	2042	<0.01	< 10	15	<10	<5	151	6969

– = not analysed

Table 2. Toxicity data expressed as PE (%) and TU50 for both refrigerated and frozen samples. Percentage of toxicity variation (% var R-F) between refrigerated (R) and frozen (F) wastewater samples and the respective coefficient of variation (CV,%) are provided.

	Refrigerated		Frozen			
Samples	PE (%)	TU50	PE (%)	TU50	% var R-F	CV
A	100	14.33 (13.00 – 15.77)	100	59.52 (53.48 – 66.23)	75.92	86.54
B	52±4	n.a.	53±4	n.a.	1.89	1.35
C	20±1	n.a.	20±1	n.a.	0.00	0.00
D	100	16.67 (15.06 - 18.42)	100	15.02 (13.40 – 16.81)	–10.99	7.36
E	100	49.26 (44.25 - 54.95)	100	78.74 (68.49 – 90.09)	37.44	32.57
F	100	27.17 (24.81 - 29.67)	100	29.67 (26.67–33.11)	8.43	6.22

n.a. = not available

variation is about 22%. The toxicity increased after freezing for A, E and F and decreased for D, but when little toxicity (expressed as percentage of effect) was shown after refrigeration, the same toxicity was found after freezing (B and C). A maximum variation in toxicity between the two preservation methods was found for sample A (75.92%) and a minimum for sample C (0%). In any case, the effects of these variations did not affect the toxicity of samples by more than one order of magnitude. Analogously, the coefficient of variation ranged between 0% and ~87%.

A Student's *t*-test for comparing the means of sample toxicities, stored both by refrigeration and freezing, accepted the null hypothesis at a significance level of 0.05 ($P < 0.05$), and the Pearson's correlation coefficient was 0.62.

Discussion

The ionized and un-ionized ammonia concentrations in samples A, D, E and F exceeded the No Observed Effect Concentration (NOEC) level for *C. gigas* embryotoxicity test according to [20], i.e. 4.68 mg L^{-1} of total ammonia (0.08 mg L^{-1} for un-ionized ammonia) at a test pH range of 7.8–8.1 and a salinity range of 27–28‰. Thus the toxicity of samples A, D, E and F could be partly explained as an ammonia concentration consequence, but not that of samples B and C.

In addition, the comparison between metal concentrations in wastewater samples extrapolated at dilution concentrations causing EC50 (shown in Table 3) and EC50 values from the literature for the embryotoxicity test with oysters, towards metals as pure substances, could help data interpretation. For example, it could be assumed that if a metal concentration is up to the EC50 value for the metal itself as a pure substance, the effluent toxicity could be potentially affected. In particular, copper was not directly involved in the toxicity definition of all samples, given that EC50 values for *C. gigas* bioassay are in the range 0.005–0.023 mg L^{-1} [24,27], nor was nickel due to an EC50 value for *C. gigas* ranging from 0.039 mg L^{-1} to 0.250 mg L^{-1} [28–29]. Only zinc showed a potential toxic influence for sample F due to an EC50 value for *C. gigas* in the range 0.119–0.250 mg L^{-1} [28, 30]. On the other hand, neither arsenic, cadmium nor chromium seemed to contribute to wastewater toxicity. Indeed, the EC50 values for *C. gigas* ranged between 0.326 and 0.920 mg L^{-1} for arsenic [28,31], 0.050 and 0.611 mg L^{-1} for cadmium [28–29], and for chromium (VI) the EC50 was 4.5 mg L^{-1} [28]. Nevertheless, these

Table 3. Metal concentrations extrapolated at EC50 values.

	Refrigerated							Frozen						
		As	Cd	Cr	Cu	Ni	Zn		As	Cd	Cr	Cu	Ni	Zn
Samples	EC50	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}	EC50	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}
A	6.98	< 10	<2	<10	<5	<15	<2	1.68	< 10	<2	<10	<5	<15	<2
6	n.a.	< 10	<2	<10	<5	<15	<2	n.a.	< 10	<2	<10	<5	<15	<2
C	n.a.	< 10	<2	<10	<5	<15	<2	n.a.	< 10	<2	<10	<5	<15	<2
D	6.00	< 10	<2	<10	<5	<15	6	6.66	< 10	<2	<10	<5	<15	2
E	2.03	< 10	<2	<10	<5	<15	5	1.27	< 10	<2	<10	<5	<15	4
F	3.68	< 10	<2	<10	<5	<15	256	3.37	< 10	<2	<10	<5	<15	117

n.a. = not available

metals on their own seemed not to be directly involved in wastewater toxicity, but more complex response patterns, such as synergism and antagonism, cannot be excluded as potential contributors to the final toxicity of the samples.

It might be suspected that the freezing procedure could have contributed to an increase in the release of metals bound to wastewater SS for samples A, B and D, suggesting a potentially higher toxicity for frozen samples, as indicated by Geffard *et al*. [32] for sediment samples where toxicities increased after freeze-drying and freezing procedures because of an increase in the concentration of bioavailable contaminants. Anyway, in this study this might be suspected only for sample A with a SS content of 115 mg L^{-1}, but it might be not for samples B and D that presented no SS at all because of the ultra-filtration treatment (0.12 μm particle cut-off).

Nevertheless, sample toxicities were shown to be statistically comparable; their specific singular difference could be a problem for sample ranking, for example when a toxicity score is considered, potentially changing their final classification and the compliance to regulatory requirements. A toxicity score based on an order-of-magnitude ranking scheme for the assessment of complex industrial effluents using a whole effluent toxicity (WET) approach was introduced by Tonkes *et al*. [33] to facilitate toxicity data comprehension and to rank data providing a simple classification. The score is composed of four ranks and the classification is related to EC50 values. A wastewater sample according to that ranking could be classified as *not acutely toxic* ($X < 1$ TU50), *minor acutely toxic* ($1 \text{ TU50} \leq X < 10$ TU50), *moderately acutely toxic* ($10 \text{ TU50} \leq X < 100$ TU50) and *very acutely toxic* ($X \geq 100$ TU50). For easy data interpretation, an integer number from 1 to 4 was attributed to each rank from the lower to the higher toxicity level [34]. In Table 4, wastewater samples are classified according to Tonkes *et al*. [33]. It can be noted that, in this case, the preservation methods did not alter the toxicity classification of the samples, although this could be confuted by the implementation of other toxicity scores or by improvement of the classification under study (e.g. increasing the number of ranks). Anyway, these outcomes are closely related to that found by Naudin *et al*. [12] where refrigeration and freezing maintained industrial effluents in a similar way, according to an entire battery of biotests composed by Microtox using *Pseudokirchneriella subcapitata*, *Brachydanio rerio* and *Ceriodaphnia dubia* as testing species.

Table 4. Toxicity classification of samples according to Tonkes *et al.* [33] (1 = not acutely toxic, 2 = minor acutely toxic, 3 = moderately acutely toxic and 4 = very acutely toxic).

Samples	Refrigerated Tonkes' score	Frozen
A	3	3
B	1	1
C	1	1
D	3	3
E	3	3
F	3	3

Conclusions

Close attention should be paid in monitoring programmes to the handling and storage of wastewater samples, whatever their origin and composition. The best solution would be to perform all kinds of analyses as soon as possible, especially ecotoxicological ones, in accordance with the general requirements of US EPA guidelines. Nevertheless, alternative scenarios need to be considered, perhaps relating to hostile geographical sampling locations or to the availability and readiness of toxicity test organisms.

The comparison between the toxicities of domestic and glass factory industrial wastewater samples showed no significant differences between refrigeration and freezing storing methods, maintaining their classification within the same rank according to the Tonkes *et al*. [33] scoring system. In conclusion, it can be stated that the wastewater ranking showed that the considered preservation methods did not alter the toxicity classification of the wastewater samples. Therefore, it might be suggested that wastewater samples could be stored by freezing if toxicity tests are not to be performed within a short time period (no more than three days). Further studies should be conducted to explore for how long wastewater samples can be stored by freezing without compromising toxicity results, to assess new wastewater selections and to increase the data set for comparability of responses. It is recommendated that a battery of toxicity tests with different endpoints and sensitivities be used.

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