Assessment of the environmental acceptability of potential artificial reef materials using two ecotoxicity tests: Luminescent bacteria and sea urchin embryogenesis

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HIGHLIGHTS
• Geopolymer mortars show mobility of As and Mo due to alkaline activation.
• Geopolymer mortars do not present toxicity effect on bioluminescence bacteria.
• Mortars with shell sand as aggregate present the greatest effect on bioassays.
• Sea urchin embryogenesis is a sensitive bioassay to the assess mortars.
• Bioassays results support the use recycled glass sand as aggregate in ARs.

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ABSTRACT
Ecotoxicological analysis of construction products is a relatively unexplored area at international level. Aquatic toxicity tests on construction products has been recommended recently for freshwater environment. However, the biological effects of alternative materials on marine ecosystem are still not considered. In this study, the main aim was to assess the environmental impact of alternative mortars proposed as artificial reefs (ARs) materials. The ARs specimens were developed by 3D printing, based on cement and geopolymer mortars using recycled sands of glass and seashells. For this purpose, a leaching test and two different toxicity bioassays, luminosity reduction of marine bacteria Vibrio fischeri (Microtox®) and the success of embryo-larval development of sea-urchin Paracentrotus lividus, were conducted. From the leaching results it should be noted that the mobility of all trace elements considered in both, raw materials and mortars, meet the inert landfill limits, except As, Mo, Se or Sb in the leachates geopolymer mortars. However, the results obtained from the both bioassays show low environmental acceptability for those mortars containing shell sand, probably due to the degradation of the organic matter adhered to the shells. On the other hand, cement mortars obtain better results than geopolymer mortars, regardless of the aggregate used, showing certain consistency with the leaching behaviour, since they

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1. Introduction

Marine ecosystems are high sensitivity to the impact of external factors, including climate change and marine pollution. These marine pollutants exist in many forms like solid waste (plastics), nutrient enrichment (eutrophication), toxic chemicals, untreated sewage discharge, oil spills, discarded fishing nets, and more recently the COVID-19 pandemic face mask waste around the world (Li et al., 2016). These pollutants threaten the marine diversity of coastal and oceanic areas, and consequently affect their productivity (Dharmaraj et al., 2021). In this line, Artificial reefs (ARs) are created to improve the environment, increase the productivity of marine ecosystems and their diversity. These structures set up in the sea have the intended purpose of generating artificial habitats of aquatic organisms. The positive environmental impact of ARs mainly depend on design factors such as depth, water circulation and materials used (Carral et al., 2020).

On the other hand, within the context of international conventions for the protection of the marine environment, it is considered that ARs “set of elements, made up of various inert materials and with various shapes, which are distributed over a delimited surface of the seabed in order to protect, regenerate and develop populations of species of fishing interest” (Lima et al., 2019; Reis et al., 2021; Suzdaleva and Beznosov, 2021). In any case, it is necessary to emphasize that any inert material that is to be used for the creation of an artificial reef must be previously evaluated in accordance with the criteria of the “Specific Guidelines for Assessment of Inert, Inorganic Geological Material” developed by the London Convention (London Convention and Protocol/UNEP, 2009). For the purpose of these guidelines, inert materials are those that do not cause contamination through leaching, physical and chemical deterioration, and/or biological activity (UNEP/MAP, 2005).

Of the different environmental effects that an artificial reef can cause in the marine environment, it is highlighted the effect on biological communities, species that will colonize the reefs (Rouse et al., 2020). Therefore, it is necessary to address the study of these alterations derived from the installation of the structures, as well as from the presence of reefs in the marine environment, since ARs in general can provide shelter and food for many benthic and pelagic species, in addition to breeding areas, spawning, etc. (London Convention and Protocol/UNEP, 2012).

In the last decades, innovative uses of residual materials in ARs construction have increased in the framework of the Sustainable Development Goals, reducing the resources and energy consumption, and consequently, greenhouse gas emissions (Xu et al., 2019; Carral et al., 2020; Goelz et al., 2020; Kong et al., 2022). In this context, these structures are called green artificial reefs, that is, environmentally friendly artificial reefs adapted to the main principle of the circular economy, which consists of closing and reducing the flows of energy and materials (Carral et al., 2020; Ellen MacArthur Foundation, 2022; Galdo et al., 2022). In relation to verify its repercussion on the biosphere, it is necessary to assess the environmental impact at all stages of its life cycle. Since in practice, only the positive aspects of ARs consider in the short term while the negative aspects that usually appear in the long term are ignored (Nagalakshmi et al., 2020; Suzdaleva and Beznosov, 2021).

Ecotoxicity is one of the indicators that can show the extent to which living organisms or the entire ecosystem can be affected (Brás et al., 2020; Mariakóva et al., 2021). Ecotoxicity tests have the advantage of integrating the effects of all hazardous substances including additive, synergistic and antagonistic effects (EC, 2013). Although many ecotoxicological methods are reported in the scientific literature that can be used to assess potential effects to chemical stress in benthic organisms (Rodrigues et al., 2017; Guo et al., 2021), there is no organism to test all the possible effects on the ecosystem. In practice, only a few (“model organism” or reference) species representing relevant ecological functions can be tested (CEN/TR 17105:2017). However, to ensure the ecological relevance, in addition to a suitable species selection, other aspects such as the selection of appropriate endpoint responses which allow us to know also relevant sublethal changes (e.g. biomarkers responses) should be considered (Hook et al., 2014). Many studies applying a test strategy that combines leaching with ecotoxicity tests have been carried out to characterize soils, sediments or contaminated sites, as well as to classify waste according to the hazard property H14 (ecotoxic waste) in the European regulatory framework (Directive, 2008/98/EC) and recently to assess the environmental impact of construction products (Steinröm et al., 2014; Bandarra et al., 2020). For the purpose of having a clear understanding of the biological assessment of materials prior to reef deployment makes it easier to assess post deployment impacts, two aquatic bioassays on different marine model organisms have been considered.

First, the marine Luminescent Bacteria Test with Vibrio fischeri obtained as freeze-dried bacteria. It is a representative microorganism of the marine aquatic environment, used mainly for a rapid determination of the toxicity of different waste materials (Schiavo et al., 2018; Bandarra et al., 2019). Due to its high sensitivity for toxicity in organic extracts, this test is a good candidate for the screening step (Weltens et al., 2014; Manzano et al., 2017). For this reason, it is widely used in biological hazard assessment batteries on complex matrices such as soils and sediments, and recently proposed for construction products for outdoor use (Gartiser et al., 2017a) in the framework of Assessment of release of dangerous substances from Construction products (CEN/TR 17105:2017).

Second, sea urchin embryo development test with specie Paracentrotus lividus. Bioassays with embryonic and larval stages of marine invertebrates are widely used to assess the quality of the marine environment (His et al., 1999; Gopalakrishnan et al., 2008; Achiorno et al., 2010; Olivierio et al., 2019; Beiras et al., 2021). The sea urchin is one of the organisms that is most used for this purpose due to its availability along coasts and its important role in coastal ecosystem maintenance (Steneck, 2013; Labbé-Bellas et al., 2016; Cirino et al., 2017) as well as for the simplicity and standardization of the tests using this species (Garmendia et al., 2009). The sea urchin Paracentrotus lividus has been used as a particular ecological indicator of marine pollution because its first stages of embryonic development are very sensitive to a variety of pollutants, both specific pollutants and mixtures of these (Vereira et al., 2018; Morrone et al., 2018, 2019; Rendell-Bhatti et al., 2021), specially for metal contamination (Cruz et al., 2019). Besides, this test has been regularly used to assess complex environmental matrices such as seawater and sediments (Carballeira et al., 2012; Khosrovyan et al., 2013).

Until to date, only few studies using an ecotoxicological approach to assess the potential toxicity of construction materials on aquatic environment have been conducted (e.g. Gartiser et al., 2017b; Bandow et al., 2018). However, to the best of our knowledge, there are no reports of existing studies over the toxicity effect of new construction products based on waste for use in marine ecosystem. In this study, the main aim was to assess the environmental impact of potential different mortars proposed as artificial reefs materials on the marine environment. The artificial reefs specimens were developed by 3D printing, based on cement mortars and coal fly ash geopolymer mortars and using recycled sands, from seashells and glass, to partially replace the limestone sand. To assess the potential toxicity effects of the final products developed, two different acute toxicity tests were conducted, first, a microbial test based on the luminosity reduction of the marine bacteria Vibrio Fischeri.

present the lowest mobility of trace chemical elements. Therefore, the results supporting the environmental acceptability of its potential use as alternative materials in the production of ARs.
2. Materials and methods

The methodology followed in this work is summarized in Fig. 1. First of all, the different samples of artificial reefs were developed, cement mortars and coal fly ash based geopolymer mortars using recycled sands, from seashells and glass, partially replacing the limestone sand. The mortar formulations were selected in previous studies according to characteristics such as rheology (printability), mechanical strength developed, costs, as well as the impacts associated with the life cycle of mortars (Yoris-Nobile et al., 2022). Second, the leaching test were developed, costs, as well as the impacts associated with the life cycle of mortars, due to high silicate and alumina content mainly in an amorphous or vitreous state (65% of the sample measured by X-Ray diffraction) that make it suitable to be alkaline activated, using as activator 14 M NaOH. While as additives modifying the mortars workability, the superplasticizer (SP) MasterEaser 3850, suspension of precipitated nanosilica (NS) MasterRoc MS 685 and densified microsilica (MS) MasterRoc MS 610, all from BASF Company, were used.

As fine aggregate, it was used limestone sand (LS) (53.81% CaO), and recycled fine aggregates, such as crushed glass sand (GLA), with a high SiO$_2$ content (70.61%) and sea shell sand (SHE), with significant content of SiO$_2$ (4.66%) and CaO (49.96%). The glass sand came from the smashing of windshields of cars and the sea shell sand was obtained from the recycled and smash of seashells, from the canning industry.

2.1. Raw materials description

The raw materials used to develop the six samples of cement and geopolymer mortars are described below. The composition of major, minor and trace elements of the raw materials was determined using X-ray fluorescent spectrometry (XRF) in Activation Laboratories in Ancaster, Canada and it is shown in Table 1.

The type of cement used (CEM) is Cem III/B 32.5 N-SR, with low clinker content (31%), blast furnace slag (66%), and with a high content of SiO$_2$ (29.66%) and CaO (50.15%). Coal Fly ash (FA), a by-product of thermal power plants, was used as fine material and Kaolin (KAO) was used as an additive, to improve the cohesiveness to the cement mortar mixes. Both materials contain significant amounts of SiO$_2$ and Al$_2$O$_3$, which are relevant in the formation of mortars. On the other hand, the low-calcium coal fly ash (FA) was also used as precursor in geopolymer mortars, due to high silicate and alumina content mainly in an amorphous or vitreous state (65% of the sample measured by X-Ray diffraction) that make it suitable to be alkaline activated, using as activator 14 M NaOH. While as additives modifying the mortars workability, the superplasticizer (SP) MasterEaser 3850, suspension of precipitated nanosilica (NS) MasterRoc MS 685 and densified microsilica (MS) MasterRoc MS 610, all from BASF Company, were used.

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2.2. Development of samples

Three different cement mortars and other three geopolymer mortars were developed (Table 2). The main difference in each set corresponding to the partial substitution of the aggregate, limestone sand, for recycled sands, the glass sand and shell sand.

For the preparation of the mortar, a planetary mixer with a capacity of 30 l was used. For cement mortars, first, dry materials were mixed;
then, tap water is included and homogenized and finally the additives to obtain the rheological characteristics needed. For geopolymeric mortar, first, dry materials were mixed, then the activator was included and homogenized the mixture and finally the water and additives.

The samples were fabricated using a 3D printer. The printing equipment used was based on the Extruded Material Systems (EMS) technology and corresponds to a printer type Delta of the brand Wasp. It is composed of a hopper, in which the mortar to be extruded is poured. By and endless screw the material is transferred to the print nozzle, with a circular section and 20 mm diameter. Once extruded, the mortar is able to bear its own weight and that of the upper layers that are added, without losing the shape. The formulation of the crushed seashell mortars for 3D printing is patented (Blanco-Fernandez et al., 2022).

The developed test samples were cured at room temperature for a period of 28 days, before performing the leaching test.

The mortars developed in this paper have been used to build 36 artificial reefs that have been immersed in Santander (Spain), Porto (Portugal), Poole Bay (United Kingdom) and Saint Malo (France), as part of the 3DPare project. The artificial reefs were immersed and have been monitored to follow up the colonisation process.

2.3. Leaching tests

The compliance leaching test EN 12457–2:2003 was used to determine the release of metals from the mortars in equilibrium conditions. According with this test, samples were milled to obtain a material with a grain size of at least 95% less than 4 mm, to promote contact between phases and consequent a maximum mobility of trace elements. In artificial reefs that have been immersed in Santander (Spain), Porto (Portugal), Poole Bay (United Kingdom) and Saint Malo (France), as part of the 3DPare project. The artificial reefs were immersed and have been monitored to follow up the colonisation process.

The leachates obtained with deionized water were divided into two aliquots. One of them was acidified with 2% HNO₃ and sent for chemical analysis, and the luminescence inhibition bioassay was performed on the second aliquot. On the other hand, the leachates obtained with sea water were used in the sea urchin embryogenesis test, The critical elements included in Decision (2003)/33/CE, establishing the criteria and procedures for admitting waste to landfills, As, Ba, Cd, Cr, Cu, Hg, Mo, Ni, Pb, Sb, Se, and Zn were analysed. For the analysis were used a Inductive Coupled Plasma Atomic Emission Spectrometry (ICP-AES) and a Inductively Coupled Plasma Spectrometer with Mass Detector ICP-MS (Agilent, 7700) in Central Analysis Service of the University of the Basque Country (Spain) according to ISO quality control standards. The limits of quantification are the following (in μg/L): As (0.01); Ba (0.1); Cd (0.01); Cr (0.1); Cu (0.1); Hg (0.07); Mo (0.1); Ni (0.01); Pb (0.1); Se (0.1); Sb (0.01); V (0.1); Zn (0.1).

The concentration of trace chemical elements in the leachates were obtained in triplicate and the mean values are shown.

2.4. Luminescence inhibition (Vibrio fischeri)

The bioluminescence test has been carried out according to the international standard ISO 11348–3:2007 using MICROTOX Lx model bioluminescence toxicity analyser, provided by the Modern Water laboratory. It is a highly sensitive photometer that measures, automatically and under controlled test conditions and temperature, the light emission by the microorganism, by the Microtox LX software. Vibrio fischeri bacteria (NRRL B-11177 strain) was supplied by Aqua Science, lyophilized and frozen in 1 mL vials (stored at a temperature between -18 °C and -20 °C). The bacteria were reconstituted by a commercial solution (sodium chloride, magnesium chloride hexahydrate and potassium chloride) to emit the light required in the test.

Four sample dilutions of each leachate and a blank were used: 45, 22.5, 11.25, 5.6 and 0%, and 3 replicates per dilution. The pH values were adjusted (6.5–7.5) by adding different aliquots of NaOH or HCl (0.1 N) and the osmotic adjustment (20 g/L of NaCl) as indicated by ISO 11348–3:2007. The luminescence emitted by the bacteria is measured before and 15 min after the bacteria have come into contact with the leachate dilutions. The ecotoxicity parameter obtained is the reduction in luminosity between both measurements.

LID or threshold concentration test is defined as the concentration which produces the slightest significantly measurable and reproducible decrease of light emission under the experimental conditions, in Microtox® the classic option to calculate the LID for this test is to associate it with the EC20 (Garito et al., 2017a). The toxicity criteria for this bioassay proposed by the framework of “Assessment of release of dangerous substances from Construction products” (CEN/TR 17105:2017) and used in the German legislative framework in the construction sector (German Institute for Building Technology, 2017), is a luminescence inhibition of 20%.

2.5. Sea urchin embryo-larval assay

First, the pH values of the leachates obtained (pH > 8.5 or pH < 7) was adjusted to meet the suitable seawater pH (7–8.5) conditions according to CEN/TR 17105:2017 by adding 1 M NaOH or 1 M HCl and not exceeding 5% of the total volume of the leachate. Five different dilutions of each leachate with seawater were used: control (only clean filtered seawater), 25%, 50%, 75% and 100%.

Sea urchins of the species Paracentrotus lividus were collected by hand at low tide from a rocky intertidal coastal and clean area located near Santander (NW Spain) and transported in a cooler to the laboratory. Once at the laboratory, gametes were obtained by dissecting mature organisms (three males and three females) and their maturity (sperm mobility and egg sphericity) was checked with a microscope (Rial et al., 2017). Prior embryogenesis test, the fertilization procedure was performed according to Volpi Ghirardini et al. (2005). Briefly, eggs were transferred to a 100 mL graduated cylinder containing clean filtered seawater and subsequently, 10 μL of the sperm were added. The mixture was shaken gently to facilitate fertilization. Aliquots of 20 μL were taken and the total number of eggs and fertilized eggs were counted in a Neubauer counting chamber under inverted microscope (Motic AE 2000). Fertilization success, those eggs with surrounded fully by a fertilization membrane, was approximately 95%.

The embryogenesis assay was performed according to the procedure described by Garminida et al. (2009). Briefly, vials (20 mL) were filled with the different selected dilutions (5 replicates per dilution) of each leachate and approximately 500 fertilized eggs were placed in each vial. The fertilized eggs were incubated for 48 h at 20 °C under dark conditions. After the incubation period, the larvae were fixed by adding 1 mL of 40% formalin and were then observed under inverted microscope. The percentage of normally developed pluteus larvae (those having four well-developed arms), per 100 organisms were recorded in each replicate as endpoint (Khosrovyan et al., 2013). The control treatment was used to ensure the acceptability of the tests (>90% normal larval development). Furthermore, the recorded larvae were classified according to degree of development stabilized by Carballoa et al. (2012) with some modifications. Fig. 2 shows the toxicity level classification proposed for assessment of construction products. Three toxicity levels were established: Level 0 (Normal development pluteus stage), level 1 (prepluteus stage), level 2 (undevolved stages).

2.6. Statistical analysis

Data obtained from the ecotoxicological tests and trace elements analysis in leachates were expressed as means ± standard deviations and were calculated using Microsoft Office Excel 2016. The data collected from the Microtox® bioassay are obtained directly from the
For its dose-response modelling, the parameter light variation emitted by bacteria I was used. Data obtained from embryogenesis bioassay were tested for normality and homogeneity by using the Shapiro–Wilk and Levene Tests, respectively. One-way analysis of variance (ANOVA) with Tukey’s HSD Post Hoc Test was performed to determine for significant differences (α = 0.05) of larval development between the different leachates obtained from the mortars and regarding to the control treatment (only clean and filtered seawater). When normality and homogeneity assumptions to satisfy ANOVA requirements were not met, the data were transformed accordingly to Tukey’s transformation ladder. Embryogenesis bioassay modelling dose-response curve was adjusted by carrying out a non-linear regression analysis using MOSAIC (Charles et al., 2017).

MOSAIC (Modelling and Statitical tools for ecotoxicology) is an online linear regression analysis using MOSAIC (Delignette-Muller et al., 2016). Two parameters have been used as expression of the bioassays results: (i) EC50 value, calculated using dose-response curves, was defined as the concentration of the leachate that cause a 50% of inhibition of luminescence on the bacteria, or induces negative effects on the 50% of sea urchin larvae; and (ii) LID value, was the Lowest Ineffective Dilution or the highest concentration of the leachate that not cause significant effect or only effects not exceeding the test-specific variability. In Microtox® results, it corresponds to EC20 (Gartiser et al., 2017a). Statistical analyses were performed with the aid of the statistical software packages SPSS 21.

3. Results and discussion

3.1. Leaching behaviour

For the evaluation and understanding of the leaching behaviour of the artificial reefs materials, the leaching test (EN 12457-2 standard) was performed for the cement mortars, CL, CG and CS, the geopolymer mortars, GL, GG and GS, and their main raw materials, Cement (CEM), Fly Ash (FA), Kaolin (KAO) and, the sands as aggregate, Limestone (LS), Glass (GLA), and Shells (SHE). The results are shown in Table 3.

The pH values obtained in the leachates of cement, fly ash and kaolin, are within the usual range for this type of material in the literature. While the pH values of the sands used as aggregates, are in a range of 9–10. Cement mortars have slightly higher pH values (12.23–12.39) than geopolymer mortars (11.83–11.88), this difference may be due to the chemistry of the cement versus the amorphous structure of the geopolymer matrix. On the other hand, the conductivity gives information on the saline content of the leachate, presents higher values in geopolymer mortars (8.02–9.28 mS/cm) than in cement mortars (3.36–4.4 mS/cm), due to the alkaline activation process with NaOH.

In relation to the leaching behaviour of the raw materials, only the fly ash presents a mobility of Cr, Mo and Sb exceed the inert landfill limit and Se the non-hazardous one. The cement (CEM) shows great mobility of Ba, exceeding the inert limit.

ARs materials present a very different leaching behaviour between
both types of mortars. Mo and Se mobility from geopolymer mortars, exceeded the inert landfill limit, and As, the non-hazardous one. Some authors have reported that the geopolymerization mobilizes more arsenic than that available from untreated coal fly ash (Alvarez-Ayuso et al., 2008). This could be due to the presence of an excess of alkali that can solubilize arsenic (III) as arsenic acid or sodium arsenate. Mo also exhibits greater leaching from geopolymer samples than untreated ashes (Provis and van Deventer, 2009). In the same way, geopolymer mortars mobilize a greater amount of V compared to cement-based construction products or the raw materials. On the other hand, although shells sand does not leach Ni, when it is introduced into both types of mortars, especially geopolymer, appear to mobilize Ni above the inert limit. In the literature there are very few studies on the immobilization of transition metals in geopolymers and specifically there are no one on coal fly ash based geopolymers.

The rest of trace elements studied of the raw materials and mortars present concentration values under the inert landfill limits. In any case, the landfill waste acceptance criteria used for comparison, serving as a guide, do not form a complete or adjusted framework for the case of ARs materials.

3.2. Luminescence inhibition bioassay

Bioassay results expressed as percentage of light emission reduction of *Vibrio fischeri* after 15 min exposure as a function of leachate concentration of raw materials and mortars, are shown in Fig. 3. Each figure contains a table with the EC$_{50}$ and LID results for each sample obtained from the dose-response curves model, expresses as concentration of leachate.

The raw material that produces the greatest reduction in bioluminescence and, therefore, the greatest polluting effect, is limestone aggregate, reducing light intensity by more than 50%, while the shell sand, reached a percentage of reduction of 40% in luminescence. On the contrary, cement is the raw material that produced the least effect on the bacteria. None of the other raw materials exceed the luminescence inhibition toxicity criteria of 20% proposed by the framework of construction products.

Regarding the inhibition of luminescence of the ARs materials, the mortar that produced the greatest inhibition was luminescence is the CS sample, producing a 99% of reduction. This effect is much higher than that shown by the raw materials of which it is made. The following samples that produce the most effect are those corresponding to CL (28%) and CG (28%). In the limit of 20% luminescence inhibition, the corresponding samples with GG (20%) and GS (18%) are found. Finally, the sample corresponding to GL does not cause any effect on the bacteria.

For the raw materials, in descending order, according to the values of EC$_{50}$, the affectation to the luminosity reduction was as follows: LS $\gg$ SHE $>$ GLA $>$ FA, KAO, CEM. For the case of the mortars, the toxicity in descending order was as follows: CS $\gg$ GS $\gg$ CG $>$ GC $>$ CL $>$ GL.

From literature a great sensitivity of Microtox® to the toxicity of some metals is expected (Hsieh et al., 2004), but in this study is not observed. In the case of raw materials, fly ash was the material that showed the greatest leaching of metals and metalloids and, however, it did not present a significant effect on the luminosity of the bacteria. In contrast, limestone and shells, which do not have high levels of metals and metalloids in leachate, showed a high light-reducing effect on bacteria. This may be related to the organic load in the leachate, because it is very important in the toxic effect on bacteria (Welten et al., 2014; Abbas et al., 2018; Teodorovic et al., 2009). Both raw materials, limestone and shells present in their composition (Table 1) a high proportion of LOI, greater than 43%, which is attributable to inorganic carbon, but also to organic carbon. In different studies, the environmental impacts of sea shells are analysed, and indicate that due to the decomposition of organic compounds adhered to the shells, can give rise to toxic compounds such as amines (Carral et al., 2020).

It is important to remark that, while the mortars that present the greatest leaching, especially of arsenic are the geopolymers, the greatest toxic effect in the bioassay appears in the mortars containing shells, both cement (CS) and geopolymer (GS). Probably, it is due to the organic matter, from the remains of the organisms associated with the shells. These results are in line with the work of Ishaque et al. (2006), who concludes that after studying the toxicity of various metals, arsenic is the one that presents the least effect on bacteria luminescence. Furthermore, different studies that analyse the toxicity of metals in synthetic waste through the use of Microtox® have shown that there are synergistic effects when several elements are present, varying the level of toxicity (Fulladosa et al., 2005; Tsiridis et al., 2006, 2012). Moreover, the addition of organic carbon reduces the toxicity of some metals, like copper, but enhances the toxicity of others, like lead (Abbas et al., 2018).

3.3. Sea urchin embryo-development test

Sea urchin embryos were exposed to different concentrations of each eluate to assess the potential effects of them on their development. The results are shown as a percentage of the population of embryos that reached the two endpoints, and therefore, considering three levels: level
0 (green) those that developed four legs; level 1 (orange) the larvae that began their development and; level 2 (red) those that remained in undeveloped stages (Fig. 4c–h). Control replicates show success in the development of the four arms >90% validating the experiment. Statistical analysis shows significant differences with the control from 50% concentration for cement mortars, CL, 50%; CG, 10%; CS, 25%, and for geopolymer mortars, GL, 75%; GG, 10%; GS, the 4 legs development. From this statistical analysis, the LID values are defined, which are the highest concentrations at which no significant effect is observed (Fig. 4b).

Besides endpoint recorded, a wide variety of teratogenic effects such as incomplete, absent skeleton; or prepluteus stages were observed. The teratogenic effects on sea urchin embryo development are well documented along the literature. More specifically, the effects of metals from synthetic samples have been studied, only one metal, Ni (Bonaventura et al., 2018), Cu (Morroni et al., 2018) or their combined toxicity, Cd, Pb and Cu (Manzo et al., 2010), Hg with Cd, Pb and Cu (Fernández and Beiras, 2001), and also between an insecticide component, deltamethrin and Cu (Ghared et al., 2015).

The results showed that leachates from mortars using shell sands as aggregate, CS and GS tend to, not only affect negatively in the developing, but inhibit the organisms to develop even the first stages, at very low concentrations. Similar responses have been reported in the literature when testing microplastics or plastic leachates (Oliviero et al., 2019; Trifuoggi et al., 2019; Rendell-Bhatti et al., 2021) or associated to the presence of high Ni concentrations in synthetic samples (Bonaventura et al., 2018).

The abnormalities found in the rest of the leachates from the mortars CL, CG, GL and GG, are mainly in the developing legs stages. These kind of effects (i.e. lack of skeleton, not four legs development and leg to short) have been previously reported in the literature as the consequence of nickel (100 µg/l), lead (500 µg/l), zinc (14–58 µg/l) or copper (128 µg/l) concentration in seawater (Fichet and Miramand, 1998; Fernández and Beiras, 2001; Kobayashi and Okamura, 2004; Camacho et al., 2018) or in different vanadium concentrations (Chiarelli et al., 2018).
2021) or glyphosate-based herbicides (100 μg/l) (Anicar et al., 2020).

On the other hand, in the modelling of the dose-response curves (Fig. 5), it can be observed that the samples that affect the population of sea urchin embryos at lower concentrations are those from GS and CS, materials in which part of the aggregate has been replaced by shells sand. This result agrees with those observed when applying the comparison of means of the values. Furthermore, the curves corresponding to the geopolymer mortars (GG and GL) decline before than those based on cement mortars (CL and CG), showing that a smaller dose (leachate concentration) is necessary to affect the embryonic development.

From the comparison of the EC₅₀ values obtained from the dose-response models it is observed that the worst values were obtained for GS (10.6%), CS (10.8%) and GL (11.8%) followed by GG (21.9%). The samples that obtained the best values were CG (32.8%) and CL (52.3%).

These results indicate that the mortars with the worst environmental acceptability from the point of view of sea urchin embryogenesis are those with shells in their composition. On the other hand, cement-based mortars obtain better results than those based on geopolymers, with CL and CG showing the best environmental acceptability in this bioassay. The toxicity in descending order was as follows: GS, CS, GL > GG > CG > CL.

The results obtained from sea urchin embryogenesis assay show some correspondence with the leaching behaviour (leaching test EN 12457–2) of the ARs products; even though the leaching agents used were seawater and deionized water, respectively.

However, it is compared the results of EC₅₀ and LID parameters of embryogenesis test (Fig. 5) with those obtained in the luminescence test (Fig. 3), the correspondence is not clear.

For EC₅₀ parameter, the concentrations of leachate that generate the effect studied (no development of the four legs of the sea urchin in embryogenesis or inhibition of luminescence in Microtox®), are much higher for Microtox®, being therefore, the embryogenesis assay more sensitive to the potential toxicity of these AR materials. However, if the LID parameters are analysed, the concentration values are more similar from both assays in most samples. The differences in sensitivity of tests for construction materials is widely described in the literature (Heist-erkamp et al., 2021; Gartiser et al., 2017a, 2017b).

Regarding the materials, both bioassays indicate the low environmental acceptability of mortars with shells in their composition; furthermore, geopolymer mortars seem to be a better option if Microtox® results are considered; while mortars based on cement show the best results from the sea urchin embryogenesis assay.

4. Conclusions

An evaluation of potential mortars, cements and geopolymers, incorporating recycled sands, glass and sea shells for the manufacture of artificial reefs (ARs) and its raw materials has been carried out based on leaching tests and two different bioassays. The coal dry ash as secondary raw material showed the highest element mobility (Cr, Mo, Ni, Se and Sb), while ARs materials present a different leaching behaviour between both types of mortars. The cement mortars fulfil the inert landfill limit, and As, the non-hazardous one, which could be due to the presence of an excess of alkali in the matrix.

In both bioassays, inhibition of luminescence and sea urchin embryogenesis, the ARs materials incorporating glass sand as aggregate show the best results and mortars containing shell sand the greatest toxic effect. It is probably due to the degradation of the organic matter adhered to the shells. It is also important to note that while the leaching behaviour, specifically the presence of As, is not appreciated on the luminescence bacteria. However, the results of the embryogenesis bioassay show a certain consistency with the mobility of the trace chemical elements studied. Therefore, these results supporting the potential use of glass sand as an alternative raw material in the production of ARs from the environmental acceptability point of view.

The comparison of the results obtained shows that the Microtox bioassay could be considered as a rapid and economical screening method, while the use of sea urchin embryogenesis bioassay is validated to assess the ecotoxicity of ARs materials, due to its great robustness in the results, its greater sensitivity to the potential effects in the environment because the possibility of collecting more levels (endpoints) and, consequently much more reliable results are achieved statistically.

Author contribution statements

J. Santos (JS), E. Cifrian (EC), A. Rodriguez-Romero (AR), Adrian I. Yoris-Nobile (AY), Elena Blanco-Fernandez (EB), Daniel Castro-Fresno (DC), A. Andres (AA). Conceptualization JS, EC, AR, AA; Data curation JS EC, AR, AA; Formal analysis JS, EC, AA; Funding acquisition AA, EB, DC; Investigation JS, EC, AR, Methodology JS, EC, Project administration AA, EB, DC; Resources EC, AA; Software JS, AR, AY; Supervision EC, EB, DC, AA; Roles/Writing – original draft JS, EC, AA; Writing – review & editing AR, AE, EB.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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