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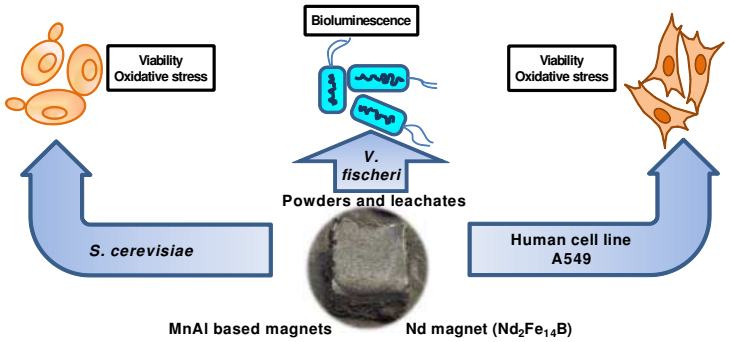

Toxicological evaluation of MnAl based permanent magnets using different *in vitro* models.

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HIGHLIGHTS

Different organisms were used to study the toxicity of MnAl permanent magnets.

MnAl based magnets induced oxidative stress on A549 cells at short exposure times.

MnAl based magnets significantly affected yeast viability at 160 mg/L.

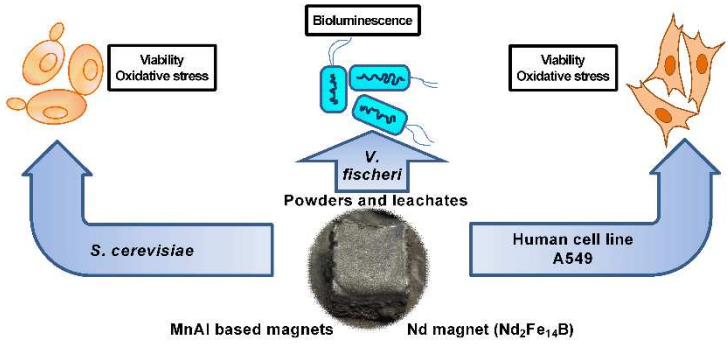
MnAl leachates showed to be safe for the organisms exposed.

Overall, MnAl(C) magnets showed a similar biological impact to that of a Nd magnet.

Abstract

Due to economic, environmental and geopolitical issues, the development of permanent magnets with a composition free of rare earth elements and with acceptable magnetic properties has been considered a priority by the international community, being MnAl based alloys amongst the most promising candidates. The aim of this work was to evaluate the toxicity of powders of two forms of newly developed MnAl(C) permanent magnets through exposure experiments applying three model organisms, using as a benchmark powders of a commercial rare-earth-containing magnet ($Nd_2Fe_{14}B$). For this purpose, the direct exposure to the different particles suspensions as well as to magnets leachates was evaluated. Both viability and oxidative stress assays were applied in an adenocarcinomic human alveolar basal epithelial cell line (A549) and in the yeast *Saccharomyces cerevisiae*, together with the bioluminescent inhibition assay in the Gram negative bacterium *Vibrio fischeri*. The obtained results indicate that MnAl(C) permanent magnets, in general terms, presented similar toxicity than the Nd magnet for the selected biological models under the studied conditions. Overall, the presented data provide, for the first time, an *in vitro* toxicity analysis of MnAl based magnets.

Graphical Abstract



Keywords

Magnetic alloys; Toxicity assays; Cell lines; Yeast; Bacteria.

1. Introduction

Due to their vital role in several critical technologies, permanent magnets have become indispensable in modern world, being applied in different fields such as communications or the fabrication of motors, generators and medical equipment (Coey, 2010; Faiz *et al*., 2017; Gutfleisch *et al*., 2011; Lewis and Jiménez-Villacorta, 2013). The unique physico-chemical properties that rare earths present, as well as their exceptional magnetic characteristics, make them the most frequently used elements in the manufacturing of permanent magnets and, among them, particularly the neodymium. Thus, the NdFeB magnets, a neodymium, iron and boron alloy, is the strongest permanent magnet known, presenting exceptional characteristics such as its superior induction and coercive force (Brown, 2016), which make them very demanded and preferred for a variety of applications ranging from the automotive sector (Nguyen *et al.*, 2019) to dentistry (Hahn *et al.*, 2008; Mancini *et al.*, 1999).

However, in spite of the excellent properties exhibited for their use in the fabrication of permanent magnets, the difficulties associated to the obtainment of rare earth elements in the market, such as their high cost and their scarcity, have led the scientific community to invest great efforts in the development of new alternatives composed of rare-earth-free materials. These factors, combined with the looming shortage of their availability and the environmental impact that their extraction involves (Lee and Wen, 2017) have prompted the search for new candidates with acceptable characteristics, suitable to be introduced into permanent magnets manufacturing processes. Mn-based alloys are among the best alternative candidates to be used as neodymium magnets substitutes, with potential to reach the market (Patel *et al.*, 2018). Many studies have been conducted in these materials, revealing MnAl

alloys as those with most outstanding characteristics. Firstly reported by Kōno (Kōno, 1958) and Koch *et al.* (Koch *et al.*, 1960), the low cost of this alloy, along with its superior characteristics, such as its high modulus of elasticity, or the large anisotropy observed in the $L1_0$ phase (also known as τ-MnAl) (Patel *et al.*, 2018), makes it one of the most promising alternatives to the currently commercialized permanent magnet compositions.

Toxicological studies are essential to determine the human and environmental risk of new materials before they can enter the market. *In vitro* test systems use simple biological models that bypass ethical concerns, presenting additional advantages, since the approaches used are cost-effective and easy to apply, allowing a great control over the variables under study. These approaches are in line with the replacement, reduction, and refinement of animal testing (3Rs) policies (Russell W. M. and Burch, 1959), one of the basis of the good laboratory practices, and have been applied before to assess the toxicological potential of different materials, such as nanoparticles or metal alloys using different model organisms (Dalal *et al.*, 2012; Lanone *et al.*, 2009; Wu *et al.*, 2019; Yang *et al.*, 2018; Yu *et al.*, 2017). With regards to rare-earth-containing permanent magnets, despite their widespread use, to our knowledge there are only few studies focusing on the toxicity of the metal alloys used in these materials. Although the effects of rare earths have been addressed in several works (Rim *et al.*, 2013), the impact of these alloys on health remains unclear, and was their use in dentistry which made the appearance of some studies where their toxicity was assessed. The toxicity of SmCo and/or $Nd_2Fe_{14}B$ alloys, both the most widely used magnetic alloys in dentistry, as well as of leachable products derived from their corrosion, was described in different works, where the results and conclusions reached by them are apparently opposite (Bondemark *et al.*, 1994; Donohue *et al.*, 1995; Hopp *et al.*, 2003; Rogero *et al.*, 2003). This underlines the importance of carrying out toxicological analysis using different models to characterize and clarify the safety of these materials.

The use of metallic powders in several industrial applications may have negative consequences derived from the handling and the subsequent management of the powder used and the wastes generated, since small particles can involve serious risks to human health and the environment (Arrizubieta *et al*., 2020). Moreover, as a result of their deterioration, metals can release several substances such as metal ions to the surrounding environment. In the specific case of the magnets, some authors suggested that the corrosion products of NdFeTi magnets can involve a negative effect on the viability of fibroblasts (Donohue *et al*., 1995). On the other hand, metallic ions may represent an important environmental impact (Vardhan *et al*., 2019), being the contamination of water with heavy metal ions one of the most serious problems, even when these particles are present at low trace levels. This, together with the rapid development of industries such as the additive manufacturing or the metal plating facilities, which can lead to an increase in the amount of metal residues or metals discharge in the environment, make the availability of information about the safety of these materials crucial.

With the aim to provide new knowledge related to the possible hazard of MnAl based magnets, the toxicological potential of powders of two types of newly developed MnAl(C) demagnetized magnets, as well as their leachates, was analyzed in this work performing different *in vitro* assays in three model organisms that were selected as representatives of human (A549 cell line) and environmental exposures (*S. cerevisiae* and *V. fischeri*). A commercial Nd demagnetized magnet was used as benchmark to establish reference toxicity values in the conditions of study. The effects of different concentrations of the materials and their leachable products on the viability of A549 cells and the yeast *Saccharomyces cerevisiae* were assessed, as well as the oxidative stress caused in both organisms. In addition, the potential toxicity of the magnet leachates was analyzed through the bioluminescent inhibition assay using the Gram negative bacterium *Vibrio fischeri*. All together, the results obtained provide a preliminary *in vitro* toxicity evaluation of MnAl based magnets.

2. Materials and Methods

2.1 Synthesis and description of the magnets

All the materials used in this study were used demagnetized. In table 1, their crystal structure and their suppliers are specified.

Samples	Crystal structure	Supplier
$Nd_2Fe_{14}B$	$P4_2/mnm$, space group 136	Vacuumschmelze GmbH & Co. KG
MnAl(C) S1	P4/mmm, τ-phase, space group 123	Technischen Universität Darmstadt
MnAl(C) S2	P4/mmm, τ-phase, space group 123	Technion – Israel Institute of Technology

Table 1: Composition, crystal structure and suppliers of the materials used in this study.

For the preparation of MnAl(C) S1 samples, $Mn_{53.3}Al_{45}C_{1.7}$ alloy ingots were prepared by arc melting pure Mn, Al and C under argon atmosphere for 5 times to ensure homogeneity. 3 wt.% of Mn in excess was added to compensate evaporation during melting. The ingot was annealed at 1373 K for 24 h and then quenched in water to eliminate the non-magnetic γ_2 and β phases. The ingot was then pulverized and milled in a planetary ball mill at a rotation speed of 250 rpm with a powder to ball mass ratio of 1:10. 10mm hardened steel balls were used and milling time was varied from 2-12 h. Ethanol was used as the milling media. The as-milled powders were used in the experiments.

MnAl(C) S2 samples were produced using the ball-milled powder by additive manufacturing (Radulov *et al.*, 2019). The bulk ingot of $Mn_{53}Al_{47}$ composition was produced using vacuum induction melting of pure Mn and Al under a protective argon atmosphere. The as-cast bulk Mn-Al ingot with addition of pure Carbon powder was ball-milled by crashing it into 2–5 mm chips. After sieving, the ball-milled powder had a fraction size below 100 µm according to requirements to the EBM process. Cubic 10x10x10 mm MnAl(C) samples were additively

manufactured from the powder using the GE-Arcam EBM A2 machine (Radulov *et al.*, 2019) modified for small amounts of powder. Finally, samples were smashed in a mortar further to obtain uniform powder, which was used in the experiments.

As reference, a commercial Nd magnet ($Nd_2Fe_{14}B$) from Vacuumschmelze GmbH & Co. KG(VACODYM 238 TP) was used. Previous to study its toxicological potential in the selected biological models, a piece of the magnet was smashed in a mortar as explained above with MnAl(C) S2 magnet.

2.2 Sample preparation

Previous to perform the experiments, powders of the different materials were resuspended in water to prepare stocks at 10 mg/mL. Before being used in the different direct contact tests, samples were vortexed at full speed for 1 min and then placed in an ultrasonic bath and sonicated for 20 min at low power intensity. Finally, an additional vortex step just before adding the materials to the organisms was performed. To carry out the toxicity assays using the leachates, the samples were prepared as follows: powders of the different magnets were resuspended in water at 10 mg/mL, and stored for 3 months at 4 °C. After this time, samples were centrifuged (1600 rpm, 10 min), and the supernatants containing magnet leachates free of powders were recovered and filtered using 0,22 µM filters to be used in the experiments. Model organisms were exposed to leachate dilutions equivalent to the same concentrations of the materials used in the direct contact tests.

2.3 XPS Analysis

X-ray photoelectron spectroscopy (XPS) was done by the SGIker unit at the University of the Basque Country (UPV/EHU), using a SPECS system equipped with a Phoibos 150 on powders deposited into glass slides.

2.4 ICP-MS

Filtered powder-free water samples containing the leachates that were obtained from magnet powders suspensions (10 mg/mL) as previously described (Section 2.2; Sample preparation) where analyzed by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 8900 ICP-QQQ instrument at the University of Burgos. For data acquisition, 5 replicates were used.

2.5 SEM analysis

The morphology and the size of the powders particles were analyzed by Scanning Electron Microscopy. A small quantity of each magnet powder was directly examined using JEOL JSM-6460LV at the Microscopy Facility of the University of Burgos.

2.6 AFM analysis

The nano-sized fraction of the magnet particles was analyzed by Atomic Force Microscopy at the Microscopy Unit from the University of Valladolid. In brief, 10 µL from an aqueous solution of the powders, obtained excluding the biggest particles, were acquired on a mica surface to prepare samples by droplet evaporation. Images were recorded in AC mode (tapping mode) with a MFP3D-BIO instrument from Asylum Research (Oxford Instruments) using silicon cantilevers AC160TS-R3. AR 16.10.208 and Gwyddion 2.56 software were utilized for all the images processing.

2.7 Organisms and culture conditions

A549 lung cancer cell line was cultured in commercial Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and 100 U/mL penicillin and 100 mg/L streptomycin. Cells were kept in a thermostatic incubator in saturated humid air with 5% CO_2 at 37 °C.

Saccharomyces cerevisiae was maintained in YPD (Yeast Extract (1%) - Peptone (1%) – Dextrose (2%)) broth or agar at 30 °C.

The Gram negative bacterium *V. fischeri* NRRL B-11177 was maintained at room temperature in culture Marine Broth or Agar 2216 (BD Difco™).

2.8 Toxicology assays

2.8.1 Experiments using A549 cell line

Viability assay: The viability of A549 cell line after 24 h of exposure to the different magnet suspensions was determined by using the neutral red uptake assay. Cells were seeded in 96 well plates at 3×10^4 cells per well. Twenty-four hours after seeding, cells were washed once with Dulbecco's Phosphate-Buffered Saline (DPBS), and 200 µl of different concentrations of the materials resuspended in fresh medium supplemented with 1% of FCS (treatment media) were added to each well, including the controls. Treatment medium consisted in DMEM with low percentage of FCS and without antibiotics, which was used to avoid interactions between these components and the materials that could interfere in the results. With the aim to cover a huge range of concentrations, 160 mg/L was selected as the highest dose to be tested, together with two serial 1:5 dilutions (32 mg/L and 6.4 mg/L). Cells were incubated for 24 h with treatment medium alone (control cells) or in presence of magnets. Cells treated 5 min with formaldehyde 4% and cells incubated 24 h in water were used as controls for cell death in the experiments. After exposure, cell culture medium was discarded, and wells were washed with DPBS. Cells were then incubated for 2.5 h at 37 °C with 100 µl of neutral red solution. This solution was prepared as follows: Neutral red powder was suspended at 4 mg/mL in DPBS, further diluted at 1/100 in treatment media, and incubated in the dark for 24 h at 37 °C before use. At that time, the solution was centrifuged to remove debris from neutral red powder. After 2.5 hour incubation, neutral red solution was discarded, cells were washed once with DPBS and fixed with formaldehyde 4% for 2 min. Cells were washed again with DPBS and 150

µl of a dye release solution (50% ethanol 96°, 49% distilled H_2O and 1% acetic acid) were added to each well. After 10 min of gentle shaking, 100 µl of the supernatant of each well were transferred to a new opaque 96-well plate, and fluorescence was measured with a microplate reader (BioTek Synergy HT, excitation wavelength, 530/25; emission wavelength 645/40). Results were expressed as percentage of control (fluorescence of cells in absence of materials). Each assay included three independent replicates. To test the toxicity of leachates, different dilutions prepared in treatment medium were added to the cells. The viability was tested using the above-explained protocol.

Oxidative stress assay: A549 cells were seeded in 96 well plates at 3×10^4 cells per well. Twenty-four hours after seeding, cells were washed 1 time with Hank's Balanced Salt Solution (HBSS) without phenol red, and incubated with DCFH-DA 50 µM for 30 min at 37 °C in the dark. After incubation, each well was washed once with HBSS, and 200 µl of the same concentrations of materials used in the viability experiments (6.4, 32 and 160 mg/L) resuspended in HBSS were added to each well. Cells incubated with HBSS alone were used as control, while cells treated with H_2O_2 20 µM were used as positive control. Fluorescence was monitored at 0, 30 and 60 min of exposure with a microplate reader (BioTek Synergy HT, excitation wavelength, 485/20; emission wavelength 528/20). Each assay included three independent replicates. To test the toxicity of leachates, different dilutions prepared in HBSS were added to the cells. The oxidative stress was tested using the above-explained protocol.

2.8.2 Experiments using *S. cerevisiae*

Viability assay: Yeast cells in exponential growth phase (OD_{600} = 1) were exposed to 160 and 800 mg/L of the materials in microcultures using 24 well plates within an orbital shaker (180 rpm), for 2 and 24 h. After exposure, suspensions were serially diluted and plated to determine the number of viable cells on solid YPD medium (6% agar) and incubated at 30°C. Results were expressed as percentage of control (CFUs grown in absence of materials). Each assay included

three independent replicates. The toxicity of different dilutions of leachates in YPD was also tested using this method.

Oxidative stress assay: Intracellular reactive oxygen species levels in *S. cerevisiae* cells were determined following the protocol described by Domi *et al.* (Domi *et al.*, 2020). Yeast cells growing in exponential phase were pelleted, washed and incubated with CM-H2DCFDA (7 μM) in DPBS for 60 min at 30 °C and 185 rpm. Subsequently, yeast cells were washed again, resuspended in YPD and exposed to the selected magnets for 2 h. Yeast cells treated with H_2O_2 10 mM were used as positive control. Afterwards, yeast cells were washed two times with DPBS, incubated 2 min in a solution containing Lithium Acetate 2M, and washed and incubated again for 2 min in a solution containing SDS (0.01%) and chloroform (0.4%), which were added to facilitate the exclusion of the dye from the cells. Finally, 150 μL of each sample were transferred to a black opaque 96 micro-well plate and the fluorescence was measured using a microplate reader (BioTek Synergy HT, excitation wavelength, 485/20; emission wavelength 528/20). Each assay included three independent replicates. Leachates from magnets diluted in YPD were evaluated using the same protocol.

2.8.3 *V. fischeri* bioluminescence inhibition assay

The effect of the magnet leachates over the bioluminescence produced by *V. fischeri* was studied applying the following protocol: One luminescent colony was selected in a petri dish, and resuspended in 5 mL of Marine Broth 2216 for 48 h. After this time, the bacterial suspension was pelleted, resuspended in 5 mL of NaCl 2% (w/v) at 15 °C and maintained at 10 °C for 30 min. A 96 well opaque microplates containing 90 μL of leachates in a water suspension with 2% of NaCl (at concentrations equivalent to 160 and 800 mg/L of the magnets), positive controls ($ZnSO_4.7H_2O$, 219.8 mg/L of 2% NaCl) and negative controls (2% w/v NaCl) were prepared. 10 μL of the bacterial suspension were added into each well of the microplates with the different samples, and the luminescence was immediately measured

(initial peak value) using a microplate reader BioTek Synergy HT. The microplate was then incubated in a Thermomixer at 800 rpm and 15 °C, and *V. fischeri* luminescence was recorded each 5 min throughout 30 min in the microplate reader. The inhibition of luminescence (percentage of control) was calculated using the values obtained at 30 min (*M30*-value) applying the following formula, adapted from Jarque *et al.* (Jarque *et al.*, 2016):

$$INH\% = 100 - \frac{M30}{CF \times peak} \times 100$$

where CF is a correction factor (the *M30*/peak ratio in negative controls) reflecting natural attenuation of bacterial luminescence after 30 min of incubation.

2.9 Statistical analysis

Statistical analysis data are presented as means ± SD. The one-way analysis of variance (ANOVA) was used for multiple comparisons, followed by Dunnett *post hoc* test to compare every mean with the control. Statistical tests were carried out using Prism 6.0 (GraphPad Prism, GraphPad Software, Inc.). Differences were considered significant at $P \leq 0.05$.

3. Results and Discussion

3.1 Synthesis and characterization of selected permanent magnets

Two MnAl based magnets with the same crystal structure (MnAl(C) S1 and MnAl(C) S2) were used in this work. The methodology used for their preparation has been detailed at the Materials and Methods section. Additionally, a commercial Nd magnet ($Nd_2Fe_{14}B$) was selected to be used as reference material. To obtain insights into the elemental composition of both MnAl alloys, their surface chemistry was studied through high-resolution X-ray photoelectron spectroscopy (XPS). Both MnAl(C) S1 and MnAl(C) S2 showed similar Al/Mn ratios (1.6 and 2.0, respectively) and C composition, while no significant contaminant elements were detected in any of the materials.

Since the potential toxicity induced by the different magnets could be also produced by dissolved metal ions originated from alloy powders present in water suspensions, ICP-MS analysis was performed on the magnets leachates obtained after the incubation of the different powders at 10 mg/mL in water during 3 months. Thus, both Mn and Al concentrations, in case of the MnAl(C) magnets, and Nd, Fe and B, in case of the commercial Nd magnet, were quantified. The obtained results revealed that MnAl(C) S1 and MnAl(C) S2 leachates contained different Mn (0.36 and 15.74 ppb respectively) and Al (100.32 and 21.52 ppb respectively) concentrations, while Nd (11.50 ppb), Fe (1.24 ppb) and B (388.59 ppb) could also be quantified in the reference magnet aqueous leachate. Interestingly, both MnAl magnets showed differences in the leachability of their elements. Since no coatings were applied in any of the materials, and in the absence of a further analysis, it could be suggested that the observed differences were the result of the inherent characteristics of the elements applied in the magnets manufacturing, being some of them more susceptible to metal leaching than the others.

3.2 Particle size and morphology analysis

To study the morphology and the size of the particles from the magnet powders, SEM and AFM techniques were performed. A small amount of powders from each magnet was directly observed by SEM. Figure 1 A displays the appearance of the particles in $Nd_2Fe_{14}B$ (a, d) and in both MnAl(C) samples (b and e; c and f). All of them showed to be formed from particles with dimensions ranging from a few micrometers to a few hundred micrometers, and presenting a variety of irregular morphologies, appearing polygonal and round shapes. Moreover, in some of the images, particles in the nanoscale range were distinguished. Their presence was confirmed by AFM analysis (Figure 1B) in all the powders analyzing aliquots (10 µL) from aqueous solutions obtained excluding the biggest particles. Altogether, these results confirm

that the powders studied in this work consisted in a heterogeneous population of particles with a variety of morphologies and sizes (micro and nano-sized particulates).

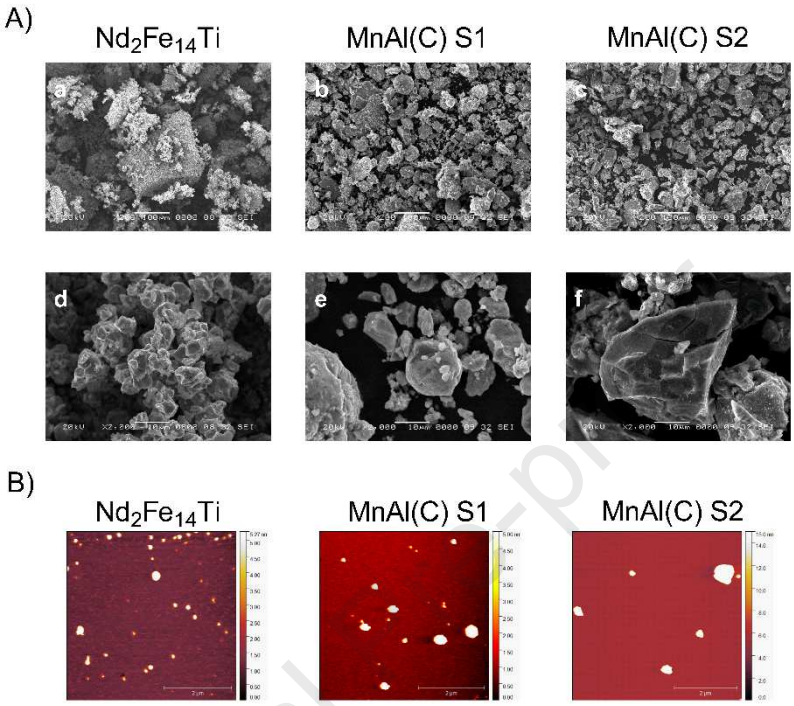


Figure 1: A) SEM images showing the morphology of the magnet powders. NdFeTi (a, d); MnAl(C) S1 (b, e); MnAl(C) S2 (c, f). Images a, b and c: Original magnification ×200 (Scale bar =100 µm); Images d, e and f: Original magnification ×2000 (Scale bar =10 µm). AFM images of the nano-fraction of the magnet powders. (Scale bar =2 µm).

3.3 Determination of magnets toxicity using A549 human cell line

The viability of A549 cells after being directly exposed to different concentrations of the magnets suspensions and their associated leachates was determined by using the neutral red uptake assay, a widely applied cytotoxicity test which is based on the ability of viable cells to incorporate the neutral red dye and retain it in their lysosomes. As controls of death, cells exposed to water for 24 h or formaldehyde for 5 min were used (Supplementary material, Figure S1). In Figure 2, the results obtained in the neutral red assay after direct cell exposition to the materials are presented. No negative effect on cell viability was observed in any of the concentrations tested, showing all the studied conditions and controls a similar percentage of

viable cells (Figure 2A, B and C). By the same token, the viability of the cells was not affected after being exposed to leachates at dilutions equivalent to the different concentrations of the magnets suspensions used in the direct contact tests (Figure 2C, D and F).

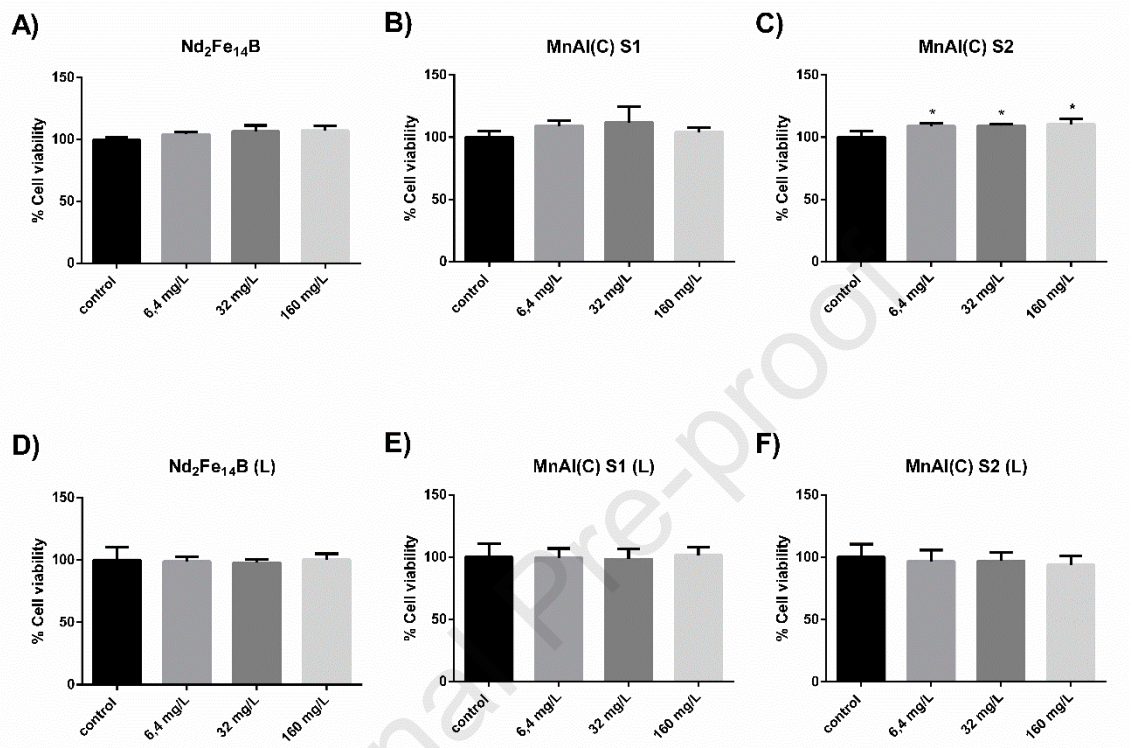


Figure 2: Viability of A549 cells (Neutral Red assay) after direct exposure to magnet suspensions (A, B, C) and to different concentrations of the magnet leachates (D, E, F). Results are expressed as % of control (untreated cells). Data represent the mean of 3 independent replicates (± standard deviation, SD). Differences were established using a One-way ANOVA followed by Dunnett *post hoc* test to compare every mean with the control, and considered significant at $P \leq 0.05$. * $P \leq 0.05$.

Regarding the induction of oxidative stress after direct contact exposure of A549 cells to different concentrations of the magnets, the DCFH-DA assay was applied to measure the levels of reactive oxygen species (ROS) at different time points (0, 30 and 60 min), and using H_2O_2 as positive control (Supplementary material, Figure S2). Figure 3 shows the results obtained after a 1-hour incubation. ROS levels were increased in A549 cells after being exposed to the three magnets, being statistically significant from concentrations of 32 mg/L in the case of $Nd_2Fe_{14}B$ and MnAl(C) S1 (Figure 3A, B), and from 6.4 mg/L in the case of MnAl(C) S2 (Figure 3C). This induction was much higher in the case of the cells incubated with MnAl(C) S2, where besides causing significant oxidative stress at the lowest concentration tested, the levels of ROS produced at 160 mg/L were more than double the levels produced by $Nd_2Fe_{14}B$ at the same

concentration (Figure 3C). On the other hand, leachates of magnets showed no induction of oxidative stress at any of the equivalent concentrations tested (Figure 3D, E, F).

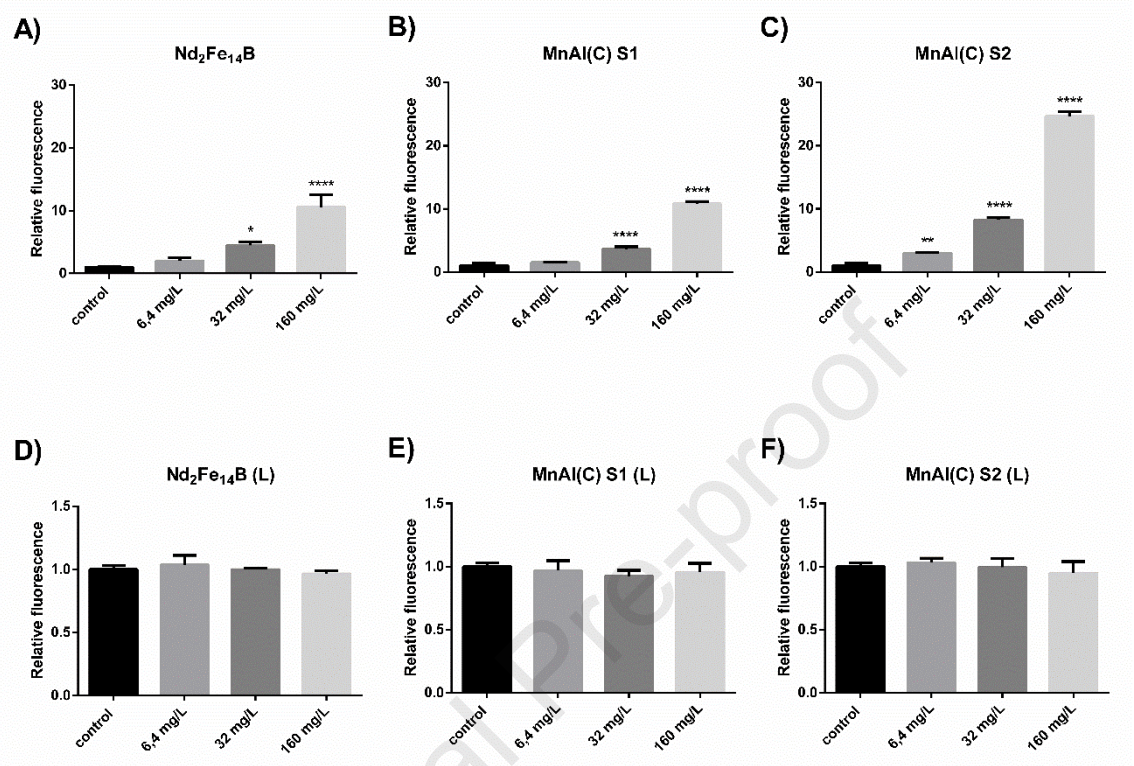


Figure 3: Oxidative stress of A549 cells after direct exposure to magnet suspensions (A, B, C) and to different dilutions of the magnet leachates (D, E, F) for 1 h. Results are expressed as the relative fluorescence value to the control (untreated cells) which was assigned a value of 1. Data represent the mean of 3 replicates (± standard deviation, SD). Differences were established using a One-way ANOVA followed by Dunnett *post hoc* test to compare every mean with the control, and considered significant at $P \leq 0.05$. *$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$.

The implementation of rare earth permanent magnets in dentistry implied the emergence of some works addressing the toxicity of these materials. Factors such as the magnet composition (Bondemark *et al.*, 1994), the presence of film layers coating their surface (Donohue *et al.*, 1995; Périgo *et al.*, 2012) or even the magnetic field may directly affect their interactions with cells and, subsequently, their inherent toxicity (Ghodbane *et al.*, 2013; Vergallo *et al.*, 2014). With the aim to analyse the exclusive effect of the magnet composition, in the present study the materials were used demagnetized to carry out the different assays. The toxicity of Nd magnets using cell lines as model organisms have been investigated in different studies, with fibroblasts mainly being the selected cell line to study their biocompatibility performing

viability assays and presenting, in some cases, apparently opposite results. Thus, some works showed that the cytotoxicity of these materials and their corrosion products are negligible, regardless of whether or not they have been coated (Bondemark *et al*., 1994; Hopp *et al.*, 2003; Rogero *et al*., 2003). However, Donohue *et al.* (Donohue *et al.*, 1995) described that human oral mucosal fibroblasts were sensitive to the effects of these rare earth magnets without coatings, being possibly the corrosion products and the magnetism the cause of the cytotoxicity described. Likewise, other work suggested that the surface coating of nanoparticles prepared from NdFeB compound with oleic acid, which was applied during milling process, is related with the acceptable cytotoxicity (≥ 80% of viability) that these nanoparticles presented in MRC-5 cell line over a wide range of concentration (0.1–100 µg/ml) (Périgo *et al.*, 2012). In our experiments, the lung A549 cell line was used to carry out the different assays. In spite of the fact that the risk of incidental exposure by powders inhalation is not totally determined, being the nano-sized fraction particularly relevant, respiratory exposure is likely to occur in workplaces during their manipulation, which could be an important threat to the health of vulnerable groups such as individuals with pulmonary diseases (Geiser *et al*., 2017). For this reason, this lung cell line was selected to perform the assays, and the materials were used in powder form, thereby also facilitating their distribution in the direct exposure experiments. In the studies cited above, contact tests were performed using directly pieces of magnets in the millimetre range. In the specific case of $Nd_2Fe_{14}B$, our results are in concordance with those that concluded that Nd magnets and its leachable products are safe in terms of cell viability. However, oxidative stress induction, parameter that was not addressed by any study, was observed after an acute direct exposure. No data are available for the toxicity of MnAl based magnets in the current bibliography. Our findings revealed that, as with $Nd_2Fe_{14}B$, both MnAl(C) magnets did not have any negative effect on A549 cells viability under the conditions tested. Regarding oxidative stress, cells exposed to MnAl(C) S1 presented similar ROS levels than cells exposed to the $Nd_2Fe_{14}B$, whereas cells

exposed to MnAl(C) S2 showed higher levels of ROS production (significant ROS levels at 6.4 mg/L, and more than doubled at 160 mg/L). It is worth mentioning that, in spite of the fact that A549 cells after 1 hour-exposure to the materials showed signs of oxidative stress, a relationship between cell viability and ROS production was not observed in the conditions tested. It has been proved that high levels of ROS can cause cell damage. Thus, different biomolecules of the cells, including proteins or nucleic acids, can be affected, leading to the activation of cell death processes such as apoptosis (Redza-Dutordoir and Averill-Bates, 2016). However, it is known that eukaryotic cells have the ability to overcome oxidative stress through the activation of autophagy (Filomeni *et al*., 2015). Considering this, there are two circumstances that could explain this results. On the one hand, it would be possible that the damage caused by the generated oxidative stress was not enough to result in cell death after 24 hour-exposure, but being critical in longer incubation times. On the other hand, the damage levels caused by ROS could be low enough to be overcome by the cells through some mechanism such as autophagy. In any case, further research is needed to properly clarify this issue. Regarding the experiments carried out using the magnet leachates, unlike what has been observed in direct contact tests, they were not able to produce significant levels of ROS. Since it has been described that transition metal ions, such as Fe or Al, can induce oxidative stress (Snezhkina *et al*., 2020), the absence of ROS is indicative that their concentrations are lower than toxic levels in the magnet leachates.

3.4 Determination of magnets toxicity using the yeast *Saccharomyces cerevisiae*

To determine the toxicological potential of MnAl based magnets for the fungal genetic model *S. cerevisiae*, two different exposure times (2 and 24 h) and two materials concentrations (160 and 800 mg/L) were studied (Figure 4; Supplementary material, Figure S3A). At the shorter exposure time, no significant differences in *S. cerevisiae* viability could be observed between the control condition (non-exposed cells) and any of the samples tested. However, after 24 h

of exposure, some viability differences could be observed between the studied conditions. In the presence of 160 mg/L, the viability of *S. cerevisiae* cells exposed to both MnAl(C) magnets was found to be significantly lower (around 35% in average; $P \leq 0.01$) than that in the control condition, while in the presence of the Nd reference magnet, no significant differences in viability were observed between the exposed cells and the negative control at the lower concentration tested. In case of the yeast cells exposed to the higher concentration of the different magnets for 24 hours, a higher decrease in CFUs was observed, indicative of a dose-response relationship. The percentage of viability was similar in the three conditions tested (around 60% in average; $P \leq 0.001$).

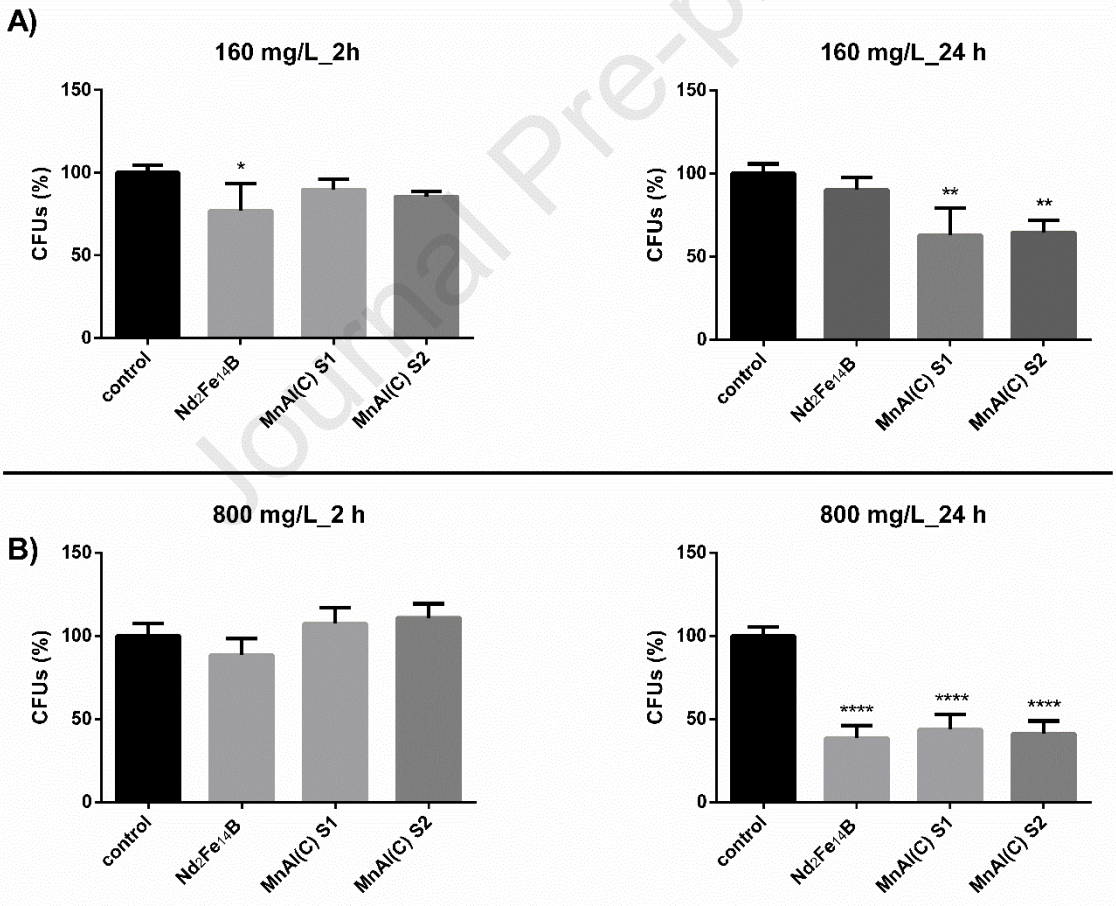


Figure 4: Colony forming units (CFUs) of *S. cerevisiae* cells exposed to different magnets suspensions at two exposure times (2 and 24 h) and two concentrations: 160 (A) and 800 mg/L (B). Results are expressed as the percentage (%) of CFUs determined for each exposure condition using as reference value the non-exposed cells condition, which was assigned a value of 100%. Data represent the mean of 3 replicates (± standard deviation, SD). Differences were established using a One-way ANOVA followed by Dunnett *post hoc* test to compare every mean with the control, and considered significant at $P \leq 0.05$. * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$.

In addition to the toxicological potential of the magnets after direct contact exposure, the ability of the magnets leachates to reduce the viability of *S. cerevisiae* was studied as well. In this case, a leachate equivalent to a concentration of 800 mg/L of each of the magnets was used to expose *S. cerevisiae* cells for 24 h (Figure 5; Supplementary material, Figure S3B).

Differently to what was observed in case of the exposure experiments done with the three magnets suspensions, leachates showed to be safe for this model organism. Only a statistically significant but very slight decrease on viability could be observed in cells exposed to MnAl(C) S1 leachates in the studied conditions after 24 h exposure, showing ≈ 95% of viability, which, in any case, is a very high value. In the rest of the samples, no significant differences in this parameter were observed between them and the control.

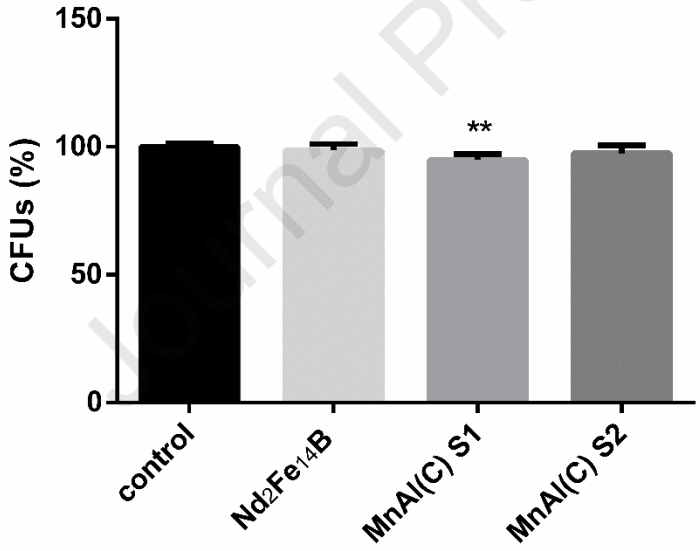


Figure 5: CFUs of *S. cerevisiae* cells exposed to magnets leachates equivalent to 800 mg/L during 24 h. Results are expressed as the percentage (%) of CFUs determined for each exposure condition using as reference value the non-exposed cells condition, which was assigned a value of 100%. Data represent the mean of 6 replicates (± standard deviation, SD). Differences were established using a One-way ANOVA followed by Dunnett *post hoc* test to compare every mean with the control, and considered significant at $P \leq 0.05$. $^{**}P \leq 0.01$.

The toxicological potential for *S. cerevisiae* of the magnets present in liquid suspensions was as well determined by investigating their ability to induce the formation of ROS. As in the viability experiments, concentrations of 160 and 800 mg/L were used to expose yeast cells for 2 h

(Figure 6). Some differences in fluorescence levels could be observed between the *S. cerevisiae* cells exposed to the distinct magnets. At the lowest concentration, the highest average ROS levels were observed for both MnAl(C) magnets, being significantly different in the case of MnAl(C) S2. Similar results were obtained for the higher concentration of the materials tested, but in this case, slightly higher ROS levels were observed in the presence of MnAl(C) S1. The Nd magnet, for its part, showed to produce not significantly higher levels of ROS at both concentrations tested. Either way, the observed values were close to those presented by the unexposed cells, and also very low, specially taking into account the levels of ROS presented by the positive control (yeast exposed to H_2O_2, Supplementary material, Figure S4). Thus, the observed differences could be considered the result of the variability between the samples, which explains the absence of a dose-response and indicates the inability of the different magnets to induce oxidative stress in the conditions tested.

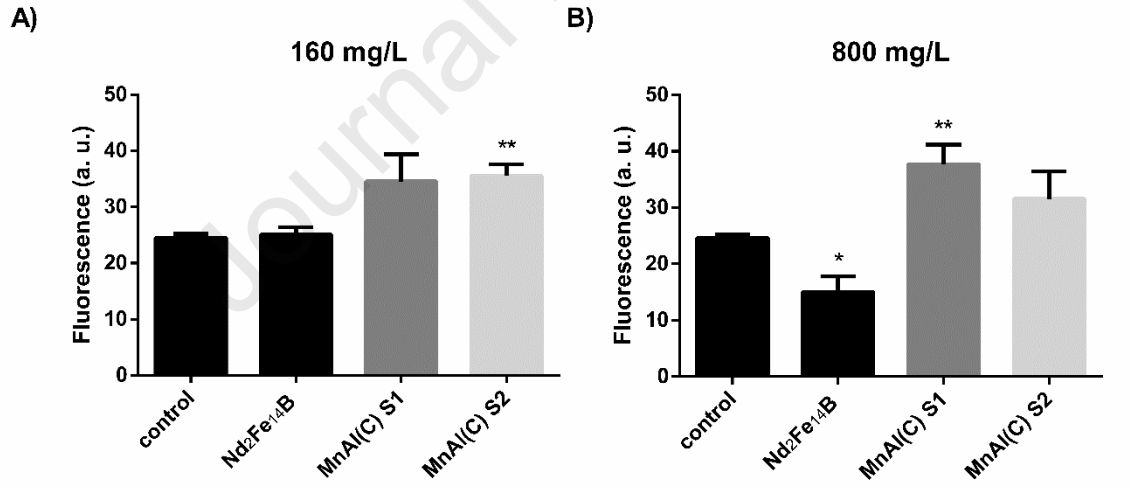


Figure 6: ROS induction analysis of *S. cerevisiae* cells exposed to different magnets suspensions during 2 h at two different concentrations (160 and 800 mg/L). Results are expressed as arbitrary fluorescence values. Data represent the mean of 3 replicates (± standard deviation, SD). Differences were established using a One-way ANOVA followed by Dunnett *post hoc* test to compare every mean with the control, and considered significant at $P \leq 0.05$. * $P \leq 0.05$ ** $P \leq 0.01$.

Due to their widespread presence in nature, as well as to their ability to affect the different biological systems, the effect of different metals and metalloids on *S. cerevisiae* has been a topic of interest for the scientific community, and many research studies have been published

in this field generating knowledge in different aspects of metal biology (Wysocki and Tamás, 2010). However, little is known about the effect on microbial systems of specific metal combinations, and in particular of those present in permanent magnets, providing this work new insights about the potential effects of MnAl based magnets and also Nd magnets on *S. cerevisiae.*

3.5 Determination of *Vibrio fischeri* bioluminescence inhibition in presence of magnet leachates

The toxicity of the magnets was also assessed incubating the bioluminescent bacteria *V. fischeri* with different concentrations of their correspondent leachates. Results showed that none of the leachates presented any negative impact on the light intensity at the concentrations tested. Figure 7 represents the evolution of the bioluminescence produced by the bacteria monitored over the 30-minute period with intervals of 5 min. Curves showed that all the leachates caused a drop of ≈ 18% in the initial bioluminescence peak, similar to the drop observed in the negative control ($ZnSO_4.7H_2O$). From this point on, the levels of bioluminescence attenuation were smaller than in the control, being the light intensity practically the same in all samples between 10 and 15 min of incubation, and slightly higher in the bacteria exposed to the different leachates from this point. Table 2 represents the inhibition percentages of the emitted light obtained in this experiment, which confirms that the drop in light intensity after the 30-minute incubation was smaller than the natural attenuation of the bacterium (negative values on the table).

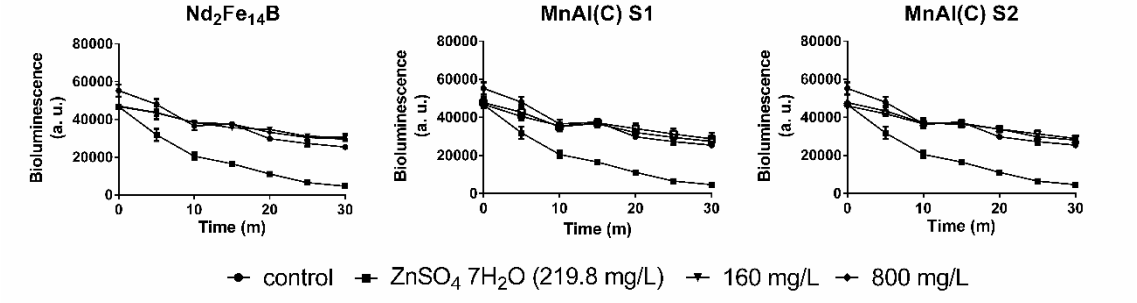


Figure 7: Evolution of *V. fischeri* luminescence in presence of magnet leachates for 30 min.

Sample	% of bioluminescence inhibition
$ZnSO_4 \cdot 6H_2O$, 219.8 mg/L	78.458 ± 6.827
$Nd_2Fe_{14}B$ 160 mg/L	-42.080 ± 8.169
$Nd_2Fe_{14}B$ 800 mg/L	-37.171 ± 4.795
MnAl(C) S1 160 mg/L	-27.378 ± 12.214
MnAl(C) S1 800 mg/L	-31.857 ± 20.275
MnAl(C) S2 160 mg/L	-28.218 ± 9.220
MnAl(C) S2 800 mg/L	-36.657 ± 6.102

Table 2: Bioluminescence inhibition of *V. fischeri* cells exposed to different concentrations of leachates during 30 min (percentage of control).

In a previous study, Kurvet *et al.* evaluated the toxicity of rare earth elements and rare earth oxides in *V. fischeri*, including Nd (Kurvet *et al.*, 2017). These authors found that Nd in their soluble form was toxic to this bacterium, describing that $Nd(NO_3)_3$.6H2O presented an EC50 of 6.87 mg/L. However, the toxicity of the leachates of Nd permanent magnet alloys in this organism, as well as that of MnAl based alloys, was not previously evaluated, being described in this work for the first time.

4. Conclusions

In the present work, the potential toxicity of two newly developed MnAl based magnets was studied when interacting with distinct model organisms representative of human and environmental exposures, determining the doses that result in a harmful effect and providing a general overview of their possible adverse consequences. In general terms, the results obtained were similar to those observed using a commercial rare-earth-containing magnet ($Nd_2Fe_{14}B$) in the same conditions. Thus, direct exposure to human cells showed that all the materials have the capacity to induce oxidative stress at short exposure times, being the levels of ROS much higher in cells exposed to MnAl(C) S2. In *S. cerevisiae*, the viability of this organism was affected by all the magnets after long exposure times, being this effect higher in MnAl based magnets at the lowest concentrations tested (160 mg/L). On the other hand, and

in spite of the fact that yeast cells exposed to leachates of MnAl(C) S1 showed statistically significant decrease in its viability (≈5%), it can be stated that the leachates released by these materials are safe for the selected organisms. In summary, the doses of MnAl magnets with potential negative effects are presented here. Although further research is needed to determine the toxicity of these materials in more realistic exposure scenarios, these results can be considered as a preliminary study of their potential impact for human health and for the environment.

Author contributions

Carlos Rumbo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Supervision.

Cristina Cancho Espina: Investigation, Formal analysis.

Vladimir V. Popov: Resources, Writing - Review & Editing.

Konstantin Skokov: Resources, Writing - Review & Editing.

Juan Antonio Tamayo-Ramos: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Review & Editing, Supervision.

Conflict of interest

The authors declare no conflict of interest.

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HIGHLIGHTS

Different organisms were used to study the toxicity of MnAl permanent magnets.

MnAl based magnets induced oxidative stress on A549 cells at short exposure times.

MnAl based magnets significantly affected yeast viability at 160 mg/L.

MnAl leachates showed to be safe for the organisms exposed.

Overall, MnAl(C) magnets showed a similar biological impact to that of a Nd magnet.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: