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# Intracellular free $\text{Ca}^{2+}$ signals antibiotic exposure in cyanobacteria†

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Intracellular free  $\text{Ca}^{2+}$ ,  $[\text{Ca}^{2+}]_i$ , is a key element of the cellular response to many abiotic and biotic stresses. Cyanobacteria are ancient organisms which have evolved signal transduction systems in response to an ever changing environment. Cyanobacteria have been found to respond to specific and quick  $\text{Ca}^{2+}$  signatures (unique combinations of changes in  $[\text{Ca}^{2+}]_i$ ) when exposed to environmental stress. Antibiotics are bioactive molecules which are being considered as pseudopersistent contaminants as they are being continuously released to the environment; although non-target organisms, cyanobacteria as prokaryotes are sensitive to antibiotics. In order to check whether  $[\text{Ca}^{2+}]_i$  could be involved in sensing antibiotic exposure, we have recorded the  $\text{Ca}^{2+}$  signatures induced by antibiotics belonging to different classes at two effective toxicity concentrations, the  $\text{EC}_{10}$  and the  $\text{EC}_{50}$ . For that, we have used a recombinant bioluminescent strain of the cyanobacterium *Anabaena* sp. PCC7120 that constitutively expresses the calcium-binding indicator protein, apoaequorin. We found that  $\text{Ca}^{2+}$  signatures upon exposure to antibiotics were induced quickly (2–3 seconds) and that they differed between antibiotics and antibiotic concentration. As different classes of antibiotics have been detected simultaneously in the aquatic environment, we also recorded and analyzed the  $\text{Ca}^{2+}$  signatures induced by binary mixtures and a multiantibiotic mixture; we found that these  $\text{Ca}^{2+}$  signatures could determine the nature of the interaction between antibiotics in the mixture, whether synergistic or antagonistic, well before toxicity was evident. To our knowledge, this study is the first report of intracellular free  $\text{Ca}^{2+}$  as an early biomarker of antibiotic exposure in the environment.

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## Introduction

Cyanobacteria are ancient organisms dating from the Precambrian era which constitute a phylogenetically diverse group of photosynthetic prokaryotes.<sup>1,2</sup> Increasing evidence indicates that they are the ancestors of chloroplasts of photoautotrophic eukaryotes.<sup>3</sup> They are ubiquitous organisms occupying a diverse range of habitats;<sup>2</sup> they have a crucial role both in the N and C cycles and as primary producers; and any negative impact on this group may also negatively affect organisms of higher trophic levels.

As any other living being, cyanobacteria possess signal transduction systems to sense and respond to changes in their environment. Intracellular messengers are basic components of the cellular signal transduction toolkits. Intracellular free  $\text{Ca}^{2+}$ ,  $[\text{Ca}^{2+}]_i$ , is probably the most universal of second messengers in living organisms including prokaryotes.<sup>4–6</sup> A prerequisite for  $[\text{Ca}^{2+}]_i$  to act as a second messenger is a tight homeostasis of its basal levels which is regulated by  $\text{Ca}^{2+}$ -binding proteins,  $\text{Ca}^{2+}$

ATPases,  $\text{Ca}^{2+}/\text{H}^+$  antiporters and  $\text{Ca}^{2+}$  pumps in order to avoid the formation of toxic intracellular calcium phosphate precipitates.

Our group was the first to construct a recombinant strain of the freshwater nitrogen-fixing cyanobacterium *Anabaena* sp. PCC7120 constitutively expressing apoaequorin, *Anabaena* sp. PCC7120 (pBG2001a), which allows continuous and *in vivo* monitoring of  $[\text{Ca}^{2+}]_i$ ; with this strain we demonstrated that cyanobacteria were also able to tightly regulate their basal  $[\text{Ca}^{2+}]_i$  levels with values in the range of 100–200 nM;<sup>7</sup> similar to the levels found in other bacteria<sup>5,6</sup> and eukaryotic cells.<sup>8–11</sup> Cells respond to different abiotic and biotic stimuli by transient changes in  $[\text{Ca}^{2+}]_i$ , the specificity of the induced  $\text{Ca}^{2+}$  signals relies not only in the change of the intracellular calcium concentration. A combination of changes in all  $\text{Ca}^{2+}$  parameters of the signal such as amplitude, duration, frequency, rise time, final  $\text{Ca}^{2+}$  resting levels, recovery time and source of the signal induced by a specific stimulus is referred to as a “ $\text{Ca}^{2+}$  signature”.<sup>10–14</sup> The  $\text{Ca}^{2+}$  signatures encode information relating to the nature and strength of stimuli in their spatio-temporal dynamics.<sup>10,11</sup> In cyanobacteria, we have been able to record and analyse a variety of calcium signatures induced by specific environmental stimuli in this strain as well as in a unicellular cyanobacterium also expressing apoaequorin.<sup>7,15–18</sup>

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There are numerous studies reporting that a wide range of pollutants affect  $\text{Ca}^{2+}$  homeostasis/signalling in a number of organisms<sup>19–23</sup> with microorganisms being under represented.<sup>24–30</sup> Regarding specifically antibiotics, there are few studies reporting that antibiotics (mostly aminoglycosides and tetracycline) significantly alter  $\text{Ca}^{2+}$  homeostasis in animal and human cell lines.<sup>31–35</sup>

Our group using the apoaequorin expressing cyanobacterial *Anabaena* strain, *Anabaena* sp. PCC7120 (pBG2001a), has recorded and analysed  $\text{Ca}^{2+}$  signatures triggered by a variety of pollutants: cationic an anionic heavy metals, the metalloid As, naphthalene, organic solvents and some pharmaceuticals; we also recorded  $\text{Ca}^{2+}$  signatures induced by some binary mixtures of the pollutants as well as that induced by a real wastewater sample.<sup>24</sup> It was found that, in general, each group of tested pollutants induced a specific  $\text{Ca}^{2+}$  signature in a reproducible and dose-dependent way. We hypothesized that the recorded  $\text{Ca}^{2+}$  signatures might be early biomarkers of exposure to pollutants.

In the present study, we wish to deepen this hypothesis by recording  $\text{Ca}^{2+}$  signatures induced by antibiotics both applied singly and in combination to the apoaequorin expressing *Anabaena* strain, *Anabaena* sp. PCC7120 (pBG2001a). The applied antibiotics were from different classes and mode of action: amoxicillin ( $\beta$ -lactam, AMO); erythromycin (macrolide, ERY); tetracycline (tetracycline, TET) and the quinolones norfloxacin (NOR) and levofloxacin (LEV). All of them highly used in human and veterinary medicine. In a previous study, we investigated the individual and combined toxicity of these five antibiotics in the transgenic bioluminescent cyanobacterium *Anabaena* sp. PCC7120 CPB4337.<sup>36</sup> The Combination Index (CI) method was used to identify and quantify the toxicological interactions between the antibiotics in the mixtures finding a heterogeneous patterns of interactions whether synergistic or antagonistic. Thus, in the present study, we aimed at determining the role of  $[\text{Ca}^{2+}]_i$  in the cellular perception of the presence of antibiotics both singly and combined in the external medium and whether the recorded  $\text{Ca}^{2+}$  signatures are related with the toxicity and pattern of interactions observed in our previous toxicological study.

## Materials and methods

### Chemicals

Amoxicillin (AMO), erythromycin (ERY) and levofloxacin (LEV) were purchased from TCI Chemicals; tetracycline (TET) was purchased from Sigma-Aldrich; and norfloxacin (NOR) purchased from Fluka.

The purity of each antibiotic was AMO  $\geq 90\%$ , ERY  $\geq 98\%$ , LEV  $\geq 98\%$ , TET  $\geq 98\%$ , NOR  $\geq 98\%$ . Except ERY, which was dissolved in methanol, stock solutions of antibiotics were made in high purity water obtained from a Millipore Milli-Q system with a resistivity of at least 18 M $\Omega$  cm at 25 °C. The antibiotics were stored in the dark and –20 °C.

The dilutions of each antibiotic and their corresponding mixtures were freshly prepared. For the case of ERY, the final concentration of methanol in the assay media was always below

0.005% (v/v) which did not affect the performance of *Anabaena* sp. PCC7120 (pBG2001a) (ESI Fig. S1†).

Injection of culture medium induced a quite smaller  $\text{Ca}^{2+}$  transient in amplitude (peak height of  $0.30 \pm 0.05 \mu\text{M}$ ) and duration ( $11 \pm 1$  s) as shown in ESI Fig. S1† which has been attributed to a small mechanically induced  $\text{Ca}^{2+}$  increase in *Anabaena* sp. PCC7120 (pBG2001a).<sup>7</sup> Native coelenterazine, the apoaequorin cofactor, was purchased from Biotium.

### Organism and growth conditions

*Anabaena* sp. PCC7120 (pBG2001a) expressing apoaequorin was routinely grown in 250 mL conical flasks containing 100 mL of BG11 medium with 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH, pH 7 and 2.5  $\mu\text{g mL}^{-1}$  spectinomycin, with the standard calcium concentration (0.25 mM). Cell cultures were incubated on a rotatory shaker at 28 °C under 65  $\mu\text{E m}^{-2} \text{s}^{-1}$  fluorescent white light.

### *In vivo* aequorin reconstitution and luminescence measurements

For aequorin luminescence measurements, *in vivo* reconstitution of aequorin was performed by the addition of 2.5  $\mu\text{M}$  coelenterazine to cell suspensions (15  $\mu\text{g mL}^{-1}$  chlorophyll) and incubation for 4 h in darkness and shaking at 28 °C. Before  $\text{Ca}^{2+}$  measurements were made, cells were washed twice, firstly in  $\text{Ca}^{2+}$ -depleted BG11 medium buffered with 2 mM HEPES, pH 7.5, containing 0.5 mM EGTA, and later in  $\text{Ca}^{2+}$ -depleted BG11 medium to remove excess coelenterazine.<sup>7</sup> Cells were finally resuspended in BG11 medium (calcium concentration of 0.25 mM) to carry out the bioassays.

Luminescence measurements were made using a digital luminometer with a photomultiplier (BioOrbit 1250). Reconstituted cell suspensions (0.5 mL) in a transparent polystyrene cuvette were placed in the luminometer and luminescence was recorded every 1 s over the duration of the experiment.

Calibration of the  $[\text{Ca}^{2+}]_i$  changes requires knowledge of the total available amount of reconstituted aequorin in cell suspensions ( $L_{\text{max}}$ ) at any one point in time of the experiment, as well as the running luminescence ( $L_0$ ). For estimation of total aequorin luminescence, the remaining aequorin was discharged at the end of the experiment by the addition of 0.3 mL of 1 M  $\text{CaCl}_2$  and 10% (v/v) Triton X-100. Rate constants of luminescence ( $L_0 \times L_{\text{max}}^{-1}$ ), and  $[\text{Ca}^{2+}]_i$  were calculated by using calibration curves obtained for aequorin extracted from the recombinant strain of *Anabaena* sp. PCC7120 (pBG2001a) according to Torrecilla *et al.*<sup>7</sup>

### Cell lysis check

For each of the treatments used in the present study, the occurrence of cell lysis was checked by the following methods: (a) examination by optical microscopy; (b) measurement of luminescence after addition of  $\text{Ca}^{2+}$  to the medium in which reconstituted cells were present after removing the cells by centrifugation at 23 000g at room temperature for 15 min; (c) measurements of phycobiliproteins in the medium in which



reconstituted cells were present after removing the cells by centrifugation at 23 000g at room temperature for 15 min.

### Statistical analysis

All data were obtained from a minimum of three replicates for each assay situation. The  $\text{Ca}^{2+}$  traces represented in the figures represent the mean  $\pm$  S.D. from at least three independent experiments.

## Results and discussion

### $\text{Ca}^{2+}$ signatures induced in *Anabaena* sp. PCC7120 (pBG2001a) in response to exposure to antibiotics applied singly

*Anabaena* sp. PCC7120 (pBG2001a) cells expressing apo-aequorin were incubated with the cofactor coelenterazine in order to reconstitute a functional aequorin. Then, the cells were placed in a luminometer cuvette, the selected antibiotics were injected and subsequent changes (if any) in  $[\text{Ca}^{2+}]_i$  were recorded. Antibiotics were tested at their  $\text{EC}_{10}$  (as a surrogate of the NOEC: very low or negligible effect on growth) and  $\text{EC}_{50}$  (as sublethal concentration: 50% growth inhibition) according to our previous 72 h toxicity study.<sup>36</sup> All the antibiotics at the tested concentrations induced a calcium transient (Table 1). The  $\text{Ca}^{2+}$  signal in all cases was very quick, within seconds of injection. Transient shape, amplitude, rise time (time from application of the stimulus to maximum amplitude of the response), total transient duration (length of the transient from zero time to the recovery of the resting  $[\text{Ca}^{2+}]_i$ ) were the characteristics of the  $\text{Ca}^{2+}$  signatures recorded.

As shown in Table 1, all tested antibiotics triggered quick  $\text{Ca}^{2+}$  transients with the same rise time (2–3 s) but which differed in the amplitude (ranging from  $0.36 \pm 0.06 \mu\text{M}$  for ERY at its  $\text{EC}_{10}$  to  $2.80 \pm 0.15 \mu\text{M}$  for NOR at its  $\text{EC}_{50}$ ) and total transient duration (from  $32 \pm 5$  s for AMO at its  $\text{EC}_{10}$  to  $>4200$  s for TET at its  $\text{EC}_{50}$ ). Regarding shape, in most cases it was characterized by a quick burst of  $[\text{Ca}^{2+}]_i$ , followed by a quick decline reaching a plateau that restored the level of  $[\text{Ca}^{2+}]_i$  back to resting values (Table 1); in the case of TET applied at both concentrations, the  $\text{Ca}^{2+}$  signature consisted of two consecutive  $\text{Ca}^{2+}$  transients: one quick and steep spike followed by a bell-shaped transient with lower amplitude but higher duration than the first transient; it should be noted that the amplitude of the second transient was higher for the  $\text{EC}_{50}$  than for the  $\text{EC}_{10}$ . In the case of ERY applied at its  $\text{EC}_{50}$ , there were also two consecutive transients; the second transient had slightly lower amplitude but also higher duration than the first transient.

Although no correlation was found between any of the characteristics of the recorded  $\text{Ca}^{2+}$  signature and the order of toxicity of the tested antibiotics which was: ERY > LEV > NOR > TET > AMO.<sup>36</sup> It should be noted that antibiotics at the higher concentration tested,  $\text{EC}_{50}$ , which already inhibited growth by 50%, triggered  $\text{Ca}^{2+}$  signatures with higher amplitudes, longer transient duration (meaning a longer time to restore  $[\text{Ca}^{2+}]_i$  basal levels) and the appearance of secondary bell-shaped transients with much longer duration times (case of TET and ERY). This means that

$[\text{Ca}^{2+}]_i$  may sense differences in antibiotic concentrations even at concentrations causing negligible or very low toxicity (*i.e.*  $\text{EC}_{10}$ ); this could be essential for the cellular response to the pollutant.

Taken altogether these results show that  $[\text{Ca}^{2+}]_i$  responds very quickly, within seconds, to antibiotic exposure, the  $\text{Ca}^{2+}$  signature of each antibiotic differs in amplitude, shape and total transient duration and within each antibiotic also differs with concentration; this suggests that the presence of antibiotics is signalled through immediate  $[\text{Ca}^{2+}]_i$  transients in the cyanobacterial cytoplasm that may trigger the subsequent cellular response to the pollutant.

In a previous work,<sup>24</sup> our group recorded the  $\text{Ca}^{2+}$  signatures triggered in *Anabaena* sp. PCC7120 (pBG2001a) when exposed to 17 chemicals grouped in cations ( $\text{Zn}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Hg}^{2+}$ ), anions (arsenate and chromate), pharmaceuticals such as lipid regulators (bezafibrate, gemfibrozil, fenofibric acid and clofibrac acid) and quinolones (ciprofloxacin and ofloxacin), the PAH naphthalene and the organic solvents acetone, ethanol and toluene.<sup>24</sup> For easy comparison, chemicals were tested at the same concentration,  $5 \text{ mg L}^{-1}$ , finding that, in general, each group of chemicals triggered a specific  $\text{Ca}^{2+}$  signature; in fact the two tested quinolones ciprofloxacin and ofloxacin showed a signature very similar to the quinolones assayed in the present study: LEV and NOR; in fact, chemicals of the same group induced very similar  $\text{Ca}^{2+}$  signatures, it was proposed that the observed similarity might correlate with a similar perception by the organism of the pollutants and eventually similar toxic mode of action. In the present study, five antibiotics belonging to four different classes [ $\beta$ -lactam (AMO); macrolide (ERY); tetracycline (TET) and quinolone (LEV and NOR)] have been used, the four classes differ in their mechanisms of toxic action:  $\beta$ -lactams inhibit bacterial cell wall synthesis; macrolides irreversibly bind to the 50S subunit of the bacterial ribosome impairing protein synthesis; tetracycline also inhibits bacterial protein synthesis but by a different mechanism as it prevents the association of aminoacyl-tRNAs with the bacterial ribosome; quinolones act at the level of DNA replication by inhibiting bacterial gyrase. The lack of correlation between the order of toxicity of the tested antibiotics and the specific features of  $\text{Ca}^{2+}$  signatures might then be due to  $\text{Ca}^{2+}$  signalling discriminating between chemicals or pollutants with different mode of toxic action and not the intensity of the toxicity which is mostly related with the concentration of the pollutants used. In this context, in our previous study as well as in this one, chemicals were tested at higher concentrations (shown to be toxic) and they induced  $\text{Ca}^{2+}$  signatures characterized by broad bell-shaped transients (incapacity to rapidly restore basal cytosolic  $[\text{Ca}^{2+}]_i$ ) or by two consecutive transients being the second also broad and bell-shaped. Besides, it was found that  $\text{Ca}^{2+}$  signatures preceded toxicity in the lights-off bioreporter *Anabaena* CPB4337 as  $\text{Ca}^{2+}$  signatures were induced within seconds at concentrations much lower than their  $\text{EC}_{50}$  values. It was concluded that the specific  $\text{Ca}^{2+}$  signature subsequently induces a signaling pathway which results in an appropriate cellular response to the pollutant which may be adaptation and survival or, when the pollutant is at high concentration, or, the exposure is long, may cause deregulation of  $\text{Ca}^{2+}$  homeostasis leading to



**Table 1** Properties of calcium signatures induced by antibiotics ( $EC_{10}$  and  $EC_{50}$ ) in *Anabaena* sp. PCC7120 (pBG2001a). The traces represent the mean  $\pm$  SD of three independent experiments

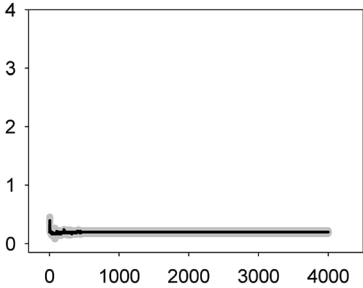
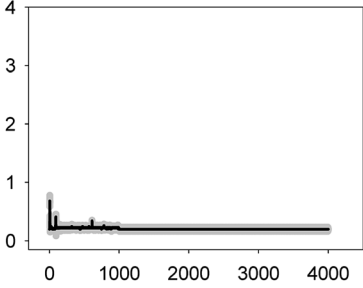
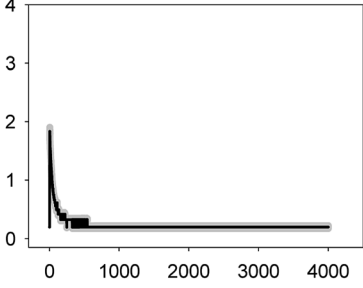
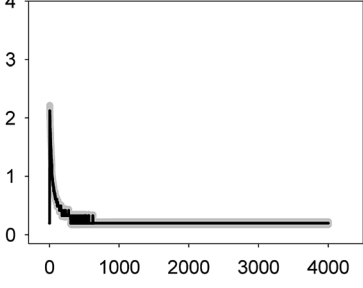
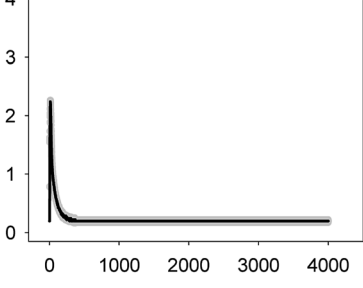
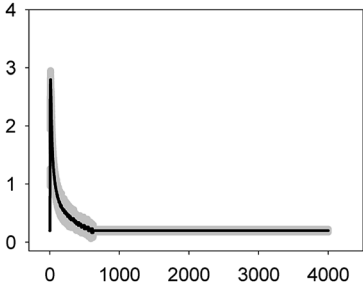
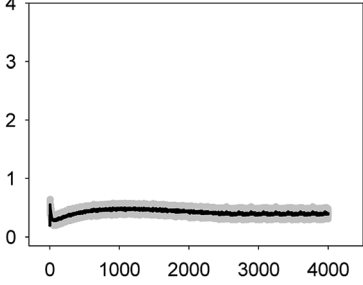
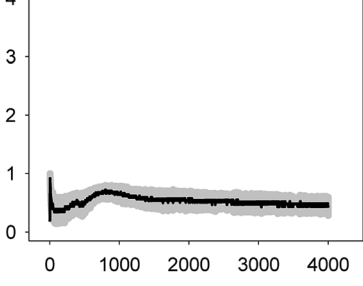
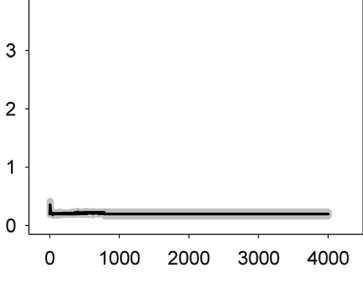
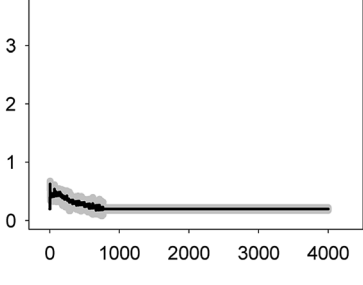
Toxic	Transient shape (x-axis: time (s); y-axis: $[Ca^{2+}]$ ( $\mu M$ ))	Amplitude $[Ca^{2+}]$ ( $\mu M$ )	Rise time (s)	Total transient duration (s)
AMO 6.2 mg L <sup>-1</sup> ( $EC_{10}$ )		0.40 $\pm$ 0.05	2–3	32 $\pm$ 5
AMO 56 mg L <sup>-1</sup> ( $EC_{50}$ )		0.59 $\pm$ 0.09	2–3	98 $\pm$ 11
LEV 1.1 mg L <sup>-1</sup> ( $EC_{10}$ )		1.84 $\pm$ 0.07	2–3	249 $\pm$ 77
LEV 4.8 mg L <sup>-1</sup> ( $EC_{50}$ )		2.13 $\pm$ 0.09	2–3	304 $\pm$ 59
NOR 2.5 mg L <sup>-1</sup> ( $EC_{10}$ )		2.24 $\pm$ 0.01	2–3	288 $\pm$ 22



Table 1 (Contd.)

Toxic	Transient shape (x-axis: time (s); y-axis: $[Ca^{2+}]$ ( $\mu M$ ))	Amplitude $[Ca^{2+}]$ ( $\mu M$ )	Rise time (s)	Total transient duration (s)
NOR 6.2 mg L <sup>-1</sup> (EC <sub>50</sub> )		2.80 ± 0.15	2–3	557 ± 142
TET 2.5 mg L <sup>-1</sup> (EC <sub>10</sub> )		1.55 ± 0.09 0.46 ± 0.08	2–3	>4000
TET 6.2 mg L <sup>-1</sup> (EC <sub>50</sub> )		0.92 ± 0.08 0.70 ± 0.12	2–3	>4000
ERY 0.005 mg L <sup>-1</sup> (EC <sub>10</sub> )		0.36 ± 0.06	2–3	44 ± 12
ERY 0.022 mg L <sup>-1</sup> (EC <sub>50</sub> )		0.63 ± 0.05 0.54 ± 0.08	2–3	664 ± 54



cellular toxicity. In this regard, Ruta *et al.*<sup>28,29</sup> have suggested that the features of the  $\text{Ca}^{2+}$  signatures may have a significant role in the final cellular response with sudden and sharp  $\text{Ca}^{2+}$  transients allowing the adaptation to high concentrations of Cd and Cu in apoaequorin-expressing *Saccharomyces cerevisiae* cells while the absence of  $\text{Ca}^{2+}$  signals or broad ones lead the cells to hypersensitivity and toxicity. They concluded that cells prepared to face high and rapid  $\text{Ca}^{2+}$  cytosolic waves were fitter for survival. Kozlova *et al.*<sup>26</sup> also tried to correlate  $\text{Ca}^{2+}$  signatures triggered by  $\text{Cr}^{6+}$ ,  $\text{Zn}^{2+}$  and 3,5-dichlorophenol in apoaequorin-expressing fungus *Aspergillus awamori* with toxicity; for this, they chose length of the  $\text{Ca}^{2+}$  transients ( $\text{LT}_{50}$ ) to compare with the  $\text{EC}_{50}$  at 5 and 30 min of the *Aliivibrio fischeri* classical bioluminescent toxicity assay. All toxicants provoked a response in one or more of the studied parameters, but  $\text{LT}_{50}$  was not the best parameter to compare with the *A. fischeri* bioassay. Therefore, the authors concluded that assessment of a different parameter was needed.

All these results suggest that  $\text{Ca}^{2+}$  signatures between different pollutants may not correlate with their order of toxicity but that specific  $\text{Ca}^{2+}$  signatures may serve to discriminate between pollutants and that, within a single pollutant and depending on the concentration, they may serve to tune the cellular response, whether tolerant or hypersensitive.

#### $\text{Ca}^{2+}$ signatures induced by selected mixtures of antibiotics in *Anabaena* sp. PCC7120 (pBG2001a)

Different classes of antibiotics co-occur in environmental compartments.<sup>37–40</sup> Aquatic organisms are thus exposed to the

combined action of antibiotics. An important issue of chemical mixtures is the determination of potential interactions between pollutants in a mixture; in this regard, antibiotics in a mixture may not interact (zero interaction or additive effect) or may interact synergistically when the effect of the mixture is greater than that expected from the sum of their individual effects (more-than-additive effects) or antagonistically when the effect of the mixture is less than expected from the sum of individual effects (less-than-additive effect).

In our previous toxicity study, we determined the nature of the interactions between the five antibiotics in binary mixtures and in a multiantibiotic mixture in the bioluminescent strain *Anabaena* sp. PCC7120 CPB4337 after 72 h of exposure.<sup>36</sup> As  $[\text{Ca}^{2+}]_i$  responds very quickly to individual antibiotics (as shown in Table 1) we evaluated whether  $[\text{Ca}^{2+}]_i$  could also respond to antibiotic binary mixtures and a multiantibiotic mixture so that by comparing the  $\text{Ca}^{2+}$  signature of the mixture with those of antibiotics when applied singly, patterns of interactions might become evident. We also wanted to assess whether  $\text{Ca}^{2+}$  signatures of the mixtures could anticipate/predict the toxicological interactions observed at longer times of exposure.

For this set of experiments, we selected four binary mixtures that represented different types of toxicological interactions and the five-antibiotic or multiantibiotic mixture:<sup>36</sup> two synergistic mixtures at both very low (10%) and median effect levels (50%) which were AMO–TET and LEV–TET; TET–ERY which was synergistic at very low effect level (10%) but turned into antagonistic at effect levels higher than 60% and AMO–ERY which

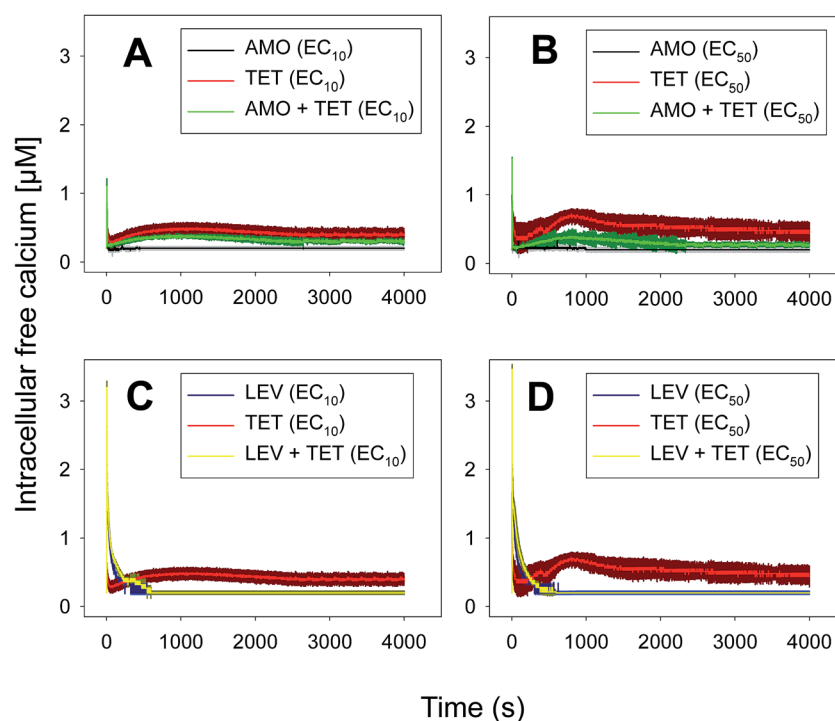


Fig. 1  $\text{Ca}^{2+}$  signatures induced in *Anabaena* sp. PCC7120 (pBG2001a) in response to antibiotics AMO and TET applied individually and in binary mixture (AMO + TET) at constant ratio 1 : 1 based on either the  $\text{EC}_{10}$ s (A) or  $\text{EC}_{50}$ s (B) and antibiotics LEV and TET applied individually and in binary mixture (LEV + TET) at constant ratio 1 : 1 based on either the  $\text{EC}_{10}$ s (C) or  $\text{EC}_{50}$ s (D). The traces represent the mean  $\pm$  SD of three independent experiments.



was antagonistic at both very low (10%) and median effect levels (50%). The multiantibiotic mixture was antagonistic at very low (10%) effect levels, nearly additive at 20–30% effect level, slightly synergistic at 50% effect level and clearly synergistic above this level. For comparison, the  $\text{Ca}^{2+}$  signatures induced by the antibiotics when applied individually at the same concentrations ( $\text{EC}_{10}$  and  $\text{EC}_{50}$ ) as those in the mixtures are shown in Fig. 1 and 2. Amplitude of the first transient (some but not all tested antibiotics/concentrations induced a second transient; see Table 1) was chosen as the most representative parameter to discern potential interactions between antibiotics in the binary mixtures. Both, the AMO–TET and LEV–TET mixtures, tested at the  $\text{EC}_{10}$  and  $\text{EC}_{50}$ , induced a  $\text{Ca}^{2+}$  signature whose amplitude was higher than the sum of the  $\text{Ca}^{2+}$  signatures when both pollutants were applied individually, suggesting a more-than-additive effect or synergism; synergism was clearer in the case of the LEV–TET combination (Fig. 1).

In the case of the TET–ERY combination, the mixture tested at  $\text{EC}_{10}$  induced a  $\text{Ca}^{2+}$  signature with an amplitude higher than the sum of the individual  $\text{Ca}^{2+}$  signatures suggesting synergism; however, the interaction was clearly antagonistic when tested at the  $\text{EC}_{50}$ . The AMO–ERY combination, tested at the  $\text{EC}_{10}$  and  $\text{EC}_{50}$ , induced a  $\text{Ca}^{2+}$  signature whose amplitude was lower than the sum of the individual  $\text{Ca}^{2+}$  signatures suggesting a less-than-additive effect or antagonism (Fig. 2).

Although amplitude was chosen as the reference parameter, it should be noted that the shape of some binary mixtures clearly changed with respect to the individual  $\text{Ca}^{2+}$  signatures; in the AMO–TET combination, the amplitude of the second TET

transient clearly diminished when mixed at their  $\text{EC}_{50}$ s; in the LEV–TET mixture, the second TET transients disappeared in both combination concentrations (Fig. 1). Interestingly, in the TET–ERY combination, the second TET transient was clearly sharper and shorter in both combination concentrations and in the AMO–ERY mixture, a second transient appeared at both combination concentrations that was larger and with lower amplitude than that of ERY applied at its  $\text{EC}_{50}$ . The found differences did not clearly correlate with the differences seen when considering the amplitude as reference parameter and, in some cases, they were independent of the tested concentration; so, these differences in shape could indicate that cells are just detecting the specific mixture.

The multiantibiotic mixture, tested at the  $\text{EC}_{10}$  and  $\text{EC}_{50}$ , induced  $\text{Ca}^{2+}$  signatures whose amplitudes were lower than the sum of the individual  $\text{Ca}^{2+}$  signatures suggesting a less-than-additive effect or antagonism (Fig. 3 and Table 1). Regarding the shape and for both concentrations, only one quick transient which rapidly returned to basal levels was recorded.

As thoroughly discussed by many scientists, the toxicity and, thereby, the risk associated with environmental pollutants is the result of the many interactions of co-occurring pollutants in a given environmental compartment.<sup>41,42</sup>  $\text{Ca}^{2+}$  signatures in response to mixtures of pollutants could serve as an excellent biomarker for prediction of potential types of interaction, whether synergistic or antagonistic, in exposed organisms. Our group previously reported that the analysis of the  $\text{Ca}^{2+}$  signatures induced by binary mixtures of  $\text{Zn}^{2+}$  plus  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  plus the lipid regulator fenofibric acid,  $\text{Zn}^{2+}$  plus arsenate and the

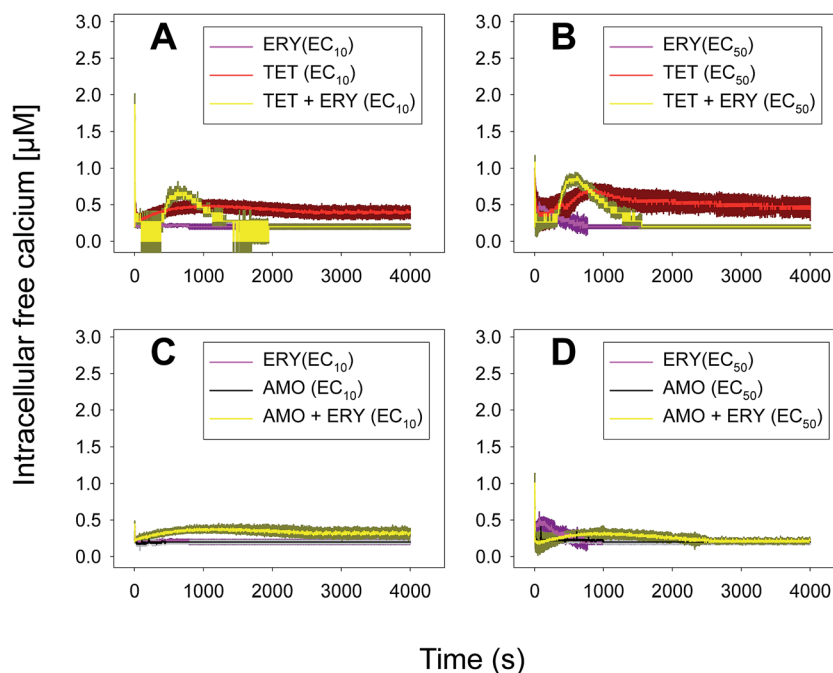
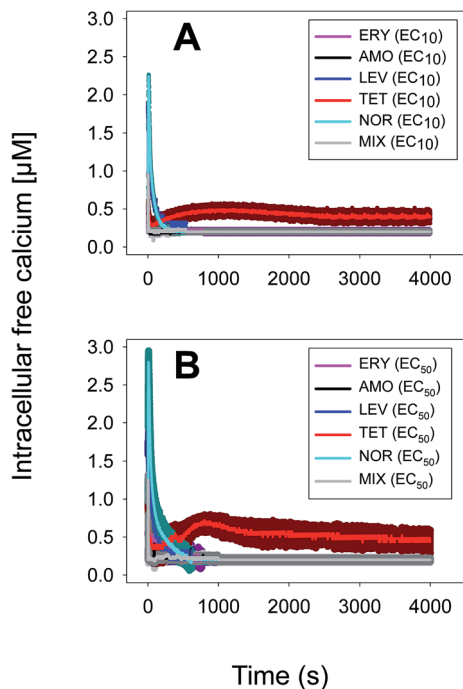


Fig. 2  $\text{Ca}^{2+}$  signatures induced in *Anabaena* sp. PCC7120 (pBG2001a) in response to antibiotics TET and ERY applied individually and in binary mixture (TET + ERY) at constant ratio 1 : 1 based on either the  $\text{EC}_{10}$ s (A) or  $\text{EC}_{50}$ s (B) and antibiotics AMO and ERY applied individually and in binary mixture (AMO + ERY) at constant ratio 1 : 1 based on either the  $\text{EC}_{10}$ s (C) or  $\text{EC}_{50}$ s (D). The traces represent the mean  $\pm$  SD of three independent experiments.





**Fig. 3**  $\text{Ca}^{2+}$  signatures induced in *Anabaena* sp. PCC7120 (pBG2001a) in response to the multiantibiotic (five antibiotic) mixture applied at constant ratio 1 : 1 based on either the  $\text{EC}_{10}$ s (A) or  $\text{EC}_{50}$ s (B). The traces represent the mean  $\pm$  SD of three independent experiments.

two fibrates fenofibric acid plus bezafibrate could anticipate the toxicological interactions found in toxicity experiments.<sup>24,43,44</sup> Recently, Ruta *et al.*<sup>28</sup> reported, in apoaequorin-expressing *Saccharomyces cerevisiae*, that the binary mixture  $\text{Cu}^{2+}$  and  $\text{H}_2\text{O}_2$  induced a  $\text{Ca}^{2+}$  signature sharper and with an amplitude higher than that of individual  $\text{Ca}^{2+}$  signatures indicating a synergistic interactions between both chemicals in signaling the stress conditions; the authors indicated that the stronger and sharper  $\text{Ca}^{2+}$  signature induced by the mixture increased the cell chances of survival. In this study, we have found that binary mixtures of antibiotics triggered quick  $\text{Ca}^{2+}$  signatures that, based on amplitude as the reference parameter to compare with those of the individual  $\text{Ca}^{2+}$  signatures, could also predict the toxicological interactions found for these antibiotics.<sup>36</sup> In the case of the multiantibiotic mixture only antagonism was found (Fig. 3) although in the toxicological experiments a clear synergism was found at medium to high effect levels, this could be explained by the fact that the toxicological experiment was done after 72 h of exposure and cells might be already strongly affected by the antibiotic mixture.<sup>36</sup> This would imply that  $\text{Ca}^{2+}$  signatures are not only early biomarkers/sensors of antibiotic exposure both singly and in mixtures but also that they seem to anticipate the toxicological outcome of the mixture; besides, as suggested by Ruta *et al.*,<sup>28</sup> the specific features of the  $\text{Ca}^{2+}$  signatures induced by mixtures might perhaps signal whether the cells may survive or not; in this study, we also found that the  $\text{Ca}^{2+}$  signatures of the mixture were quite different from those of the individual antibiotics although at present we cannot give a clear explanation for these differences, it might occur that, in

order for the cell to distinguish between singly and combined antibiotic exposure, distinct  $\text{Ca}^{2+}$  influx or efflux systems might be involved in the generation of the recorded  $\text{Ca}^{2+}$  signatures. In most organisms,  $[\text{Ca}^{2+}]_i$  homeostasis is tightly regulated by the so-called  $\text{Ca}^{2+}$ -signalling toolkit which includes different  $\text{Ca}^{2+}$  transporters ( $\text{Ca}^{2+}$  channels, Ca-ATPases or  $\text{Ca}^{2+}$  exchangers such as  $\text{Ca}^{2+}/\text{H}^+$  antiporters) and  $\text{Ca}^{2+}$ -binding proteins (both intracellular  $\text{Ca}^{2+}$  buffers as well as  $\text{Ca}^{2+}$  sensors which activate downstream target proteins involved in the cellular response).<sup>6</sup> Many of these components are involved in the generation of the  $\text{Ca}^{2+}$  signals as well as in restoring the  $[\text{Ca}^{2+}]_i$  to resting values (around 100–200 nM in most organisms). In cyanobacteria, just a few proteins involved in  $\text{Ca}^{2+}$  transport have been characterized so far and all of them in unicellular cyanobacteria: the mechanosensitive channel encoded by the *mscL* gene in *Synechococcus* sp. PCC7942; the two  $\text{Ca}^{2+}/\text{H}^+$  antiporters ApCAX in *Aphanothece halophytica* and SynCAX in *Synechocystis* sp. Strain PCC6803 and two  $\text{Ca}^{2+}$ -ATPases, PMA1, an E1–E2 ATPase in *Synechocystis* sp. Strain PCC6803 and PaCL,  $\text{Ca}^{2+}$  transporting P-ATPase in *Synechococcus* sp. PCC7942; regarding  $\text{Ca}^{2+}$ -binding proteins, the only reported so far is CcbP from the filamentous *Anabaena* sp. PCC7120, the organism used in this study (reviewed in Dominguez *et al.*<sup>6</sup>). There is much to be done regarding the identification and characterization of the  $\text{Ca}^{2+}$ -signalling toolkit in cyanobacteria; this will aid in a better understanding of the  $\text{Ca}^{2+}$  signatures induced by different environmental pollutants and to decipher the signalling pathway between the perception of the pollutants by  $\text{Ca}^{2+}$  and the final cellular response whether tolerance or hypersensitivity to the pollutant. By using cyanobacterial strains mutated in these  $\text{Ca}^{2+}$  signalling components as done by Ruta *et al.*<sup>28,29</sup> in yeast, it might be determined whether the  $\text{Ca}^{2+}$ -mediated response to pollutants depends predominantly on extracellular  $\text{Ca}^{2+}$  influx or internal  $\text{Ca}^{2+}$  stores, how intracellular resting  $\text{Ca}^{2+}$  levels are restored and which role may have the  $\text{Ca}^{2+}$ -binding proteins in such response.

To gain insight into the correct interpretation of the  $\text{Ca}^{2+}$  signatures triggered by mixtures, a careful and thorough research is needed in more organisms and with more pollutants mixed in realistic scenarios; in this regard, in our previous study by Barran-Berdon *et al.*,<sup>24</sup> we recorded a  $\text{Ca}^{2+}$  signature triggered by a real wastewater sample that could be mimicked by mixing its main components at their measured environmental concentration.

## Conclusions

To our knowledge, this is the first report on intracellular free  $\text{Ca}^{2+}$ ,  $[\text{Ca}^{2+}]_i$ , as an early biomarker of antibiotic exposure in the environment. Upon antibiotic exposure, quick (within 2–3 s) and specific  $\text{Ca}^{2+}$  signatures were recorded. Signatures induced by the different antibiotics differed in amplitude, shape and total transient duration and within the same antibiotic also with the tested concentration with higher amplitude, longer transient duration and in some case the appearance of a second bell-shaped  $\text{Ca}^{2+}$  signal at the higher concentration tested,  $\text{EC}_{50}$ .  $\text{Ca}^{2+}$  signatures induced by binary mixtures and a multiantibiotic mixture were clearly different from those of the antibiotics applied singly and,



using amplitude as a reference parameter, potential interactions between antibiotics in the mixtures could be determined; the Ca<sup>2+</sup> signatures induced by the mixtures seemed to anticipate the toxicological interactions found at larger times of exposure; thus, well before toxicity is evident.

## Conflict of interest

There are no conflicts to declare.

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