Science of the Total Environment 771 (2021) 144823

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Development of a living mammalian cell-based biosensor for the monitoring and evaluation of synergetic toxicity of cadmium and deoxynivalenol

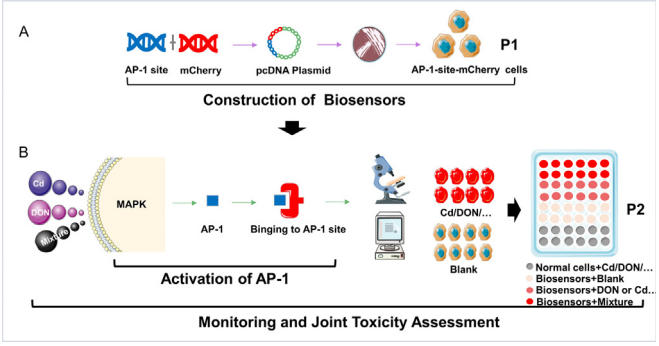
Hongyan Guo, Jian Ji, Jiadi Sun, Yinzhi Zhang, Xiulan Sun *

State Key Laboratory of Food Science and Technology, School of Food Science and Technology, National Engineering Research Center for Functional Food, Synergetic Innovation Center of Food Safety and Quality Control, Jiangnan University, Wuxi, Jiangsu 214122, PR China

HIGHLIGHTS

- Humans may be simultaneously exposed to DON and Cd.
- AP-1 participated in the interaction of DON and Cd.
- DON+Cd^{2+}, DON, and Cd^{2+} induced dose-dependent fluorescence signal in the biosensors.
- Enhanced fluorescence signals were observed in the combined groups compared with DON and Cd^{2+}.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 22 October 2020
Received in revised form 15 December 2020
Accepted 21 December 2020
Available online 26 January 2021

Editor: Henner Hollert

Keywords:
Cadmium
Deoxynivalenol
Joint toxicity
Mammalian cell-based biosensor
Optical

ABSTRACT

With increased interest in the toxic interactions of multiple toxins, biotoxicity models have to be urgently developed for joint toxicity evaluation. This study aimed to develop an optical biosensor based on living mammary cells for monitoring of cadmium (Cd)/deoxynivalenol (DON) in water and evaluating their combined toxicity. Our previous survey found that DON and Cd appeared simultaneously in various products, and RNA seq revealed that AP-1 participated in combined toxicity of DON+Cd in HT-29 cells. Thus AP-1 site-mCherry-based biosensors were constructed, optimized, and then tested for their applicability and stable fluorescence response activities. DON+Cd^{2+}, DON, and Cd^{2+} induced dose-dependent fluorescence signal in the biosensors (at environmental exposure levels). The enhanced fluorescence signal suggested that the toxicity of DON+Cd^{2+} was enhanced compared with that of single toxin. The advantages of the biosensors include: I) The easy and visual screening of multiple toxins on the basis of environmental exposure levels; II) Potential as a broad-spectrum tool for joint toxicity evaluation of DON+Cd; III) Pollution-free and stable fluorescence response; IV) A slight effect on viability.

1. Introduction

Heavy metals such as cadmium (Cd) are toxicants commonly found in food, feed, and water. Owing to their nonbiodegradable properties, toxic metals in food present a serious concern (Saha et al., 2016). Heavy metals enter the air, water, and soil, and soil and water are the largest contributors of toxicity to humans and plants. Rhizome crops

* Corresponding author at: State Key Laboratory of Food Science and Technology, School of Food Science and Technology, National Engineering Research Center for Functional Food, Synergetic Innovation Center of Food Safety and Quality Control, Jiangnan University, Lihu Road 1800, Jiangsu 214122, PR China.
E-mail address: sxlzzz@jiangnan.edu.cn (X. Sun).

https://doi.org/10.1016/j.scitotenv.2020.144823
0048-9697/

may absorb Cd, and aquatic animals contain high levels of Cd, damaging the liver, kidneys, brain, lungs, and bones, leading to osteoporosis, renal insufficiency, cardiovascular diseases, and cancer in humans (Satarug et al., 2010). In addition, low environmental exposure to Cd was associated with adverse health effects, such as renal tubular damage (Wang et al., 2016). Toxicology screening of this hazard need to be conducted for water or food quality assessment.

Notably, humans may be at the risk of co-exposure to multiple kinds of toxins, including heavy metals, pesticides, mycotoxins, and environmental estrogens. Several recent studies have focused on the co-exposure risks of different types of toxins, such as deoxynivalenol (DON) and Cd (Guo et al., 2020a: Luo et al., 2019; Le et al., 2018). DON is the most prevalent trichothecene mycotoxin and occurs worldwide (Zhou et al., 2020a). Air, water, and food are the sources of DON. DON causes acute toxicity, gastrointestinal toxicity, immunotoxicity, hepatotoxicity, and developmental toxicity, and affects the functions of brain (Guo et al., 2020b; Maresca, 2013). Acute DON exposure leads to vomiting, anorexia, diarrhea, malnutrition, etc. In intestine, DON inhibits nutrients absorption, alters permeability, affects cell viability and gut microflora, etc. (Maresca, 2013). In addition, DON consumption may be related with the chronic intestinal inflammatory diseases in certain patients (Maresca and Fantini, 2010). Humans can be exposed to Cd and DON simultaneously by water or food consumption, and inhalation. DON and Cd were found in spices, herbs, traditional and organic wheat, and other grains in Greece, Turkey, Belgium, Romania, France, Vietnam, Indonesia, China, Iran, Italy and other countries (Guo et al., 2020a). In Europe, humans are exposed to DON and Cd with an average ratio of 1 μM: 1 μM (Le et al., 2018). Numerous microbial biosensors or models have been developed for the screening or toxicity evaluation of toxins (Singh and Kumar, 2020), whereas none of them can realize the joint toxicity evaluation of DON and Cd.

Various biosensors have been employed for measurement of heavy metals, toxins, pesticides, and so on (Antonacci and Scognamiglio, 2020; Qi et al., 2020). Based on the biorecognition elements, biosensors can be divided into DNA biosensors, enzymatic biosensors, cell-based biosensors, immunosensors and biomimetic biosensors (Jayesh et al., 2020). According to the signal transduction principle, biosensors mainly include optical biosensors, electrochemical biosensors, thermal biosensors, etc. (Wang et al., 2014). Among the above biosensors, enzymatic biosensors are of great significance because of their high selectivity, whereas cell-based biosensors are deemed as the best replacement to overcome enzyme's weakness (Park et al., 2013; Wang et al., 2014). In addition, sensors are evolving from offline monitoring systems to online monitoring systems, such as microbial fuel cell biosensors (Do et al., 2020; Li et al., 2014). Biological toxicity tests are powerful tools for assessing contaminants because living cells possess the innate ability to sense toxic stimuli and response in the manner of genetic transcription, physiological change, etc. Living cell-based methods use organisms and determine toxicity on the basis of the response of living organisms (Eom et al., 2020; Gupta et al., 2019). Microorganisms have been widely used in whole-cell optical biosensing for toxicity testing of heavy metals (Yu et al., 2019). Compared with that of microbes, the response of mammalian cells is closer to the effects of toxicity on human and animals. In addition, mammalian cell sensors do not need to introduce harmful substances, which is environmentally friendly. However, few studies have employed mammalian cells as biosensors for evaluating heavy metal toxicity. Moreover, with increased interest in the toxic interactions and co-contamination of multiple toxins (Kopp et al., 2018; Luo et al., 2019; Le et al., 2018), biotoxicity test models have to be urgently developed for screening or joint toxicity evaluation of multiple toxins.

Our previous work found that AP-1 can be activated by DON, Cd, and DON+Cd, and AP-1 participated in the interaction of DON and Cd, resulting in increased toxicity in HT-29 cells (. Activator protein-1 (AP-1) activity has vital effects on cellular processes such as apoptosis and growth (Whitmarsh and Davis, 1996). Stimulation of the transactivation potential of AP-1 was associated with AP-1 binding activity. In the current study, we used the AP-1 binding site to construct a mammalian cell-based fluorescence biosensor for joint toxicity evaluating of DON and Cd, and monitoring their concentration in water. The constructed models provide a simple and stable method for monitoring heavy metals and mycotoxins. This study is the first to investigate visual intracellular sensors for multiple toxins screening and joint toxicity evaluation of DON and Cd.

2. Materials and methods

2.1. Cell culture and treatment

HEK293 and HT-29 cells (Chinese Academy of Science Cell Bank, China) were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) with 10% fetal bovine serum (LONSERA) at 37 °C. A 5% CO_2 air incubator was used to control humidity. Cadmium chloride, lead nitrate, mercuric chloride, DON, ochratoxins (OTA), zearalenone (ZEN), aflatoxin B1 (AFB_1), and fumonisin B1(FB_1) were purchased from Sigma-Aldrich Inc. (USA). DON, Cd^{2+}, mercury (Hg^{2+}) and lead (Pb^{2+}) solutions were prepared by water. OTA, ZEN, AFB_1, and FB_1 were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Inc., USA). All the stock solutions were stored at −20 °C. Toxic detection solutions were filtered, and dissolved in DMEM (DMSO<0.001%, water<5%, *v*/v). Cells were passaged every 3 d.

2.2. AP-1 activity induced by DON, cd and their mixture

HT-29 cells were exposed to DON (33.78 μM), Cd^{2+} (33.78 μM), and DON+Cd^{2+} (33.78 + 33.78 μM) for 24 h in logarithmic phase respectively. RNA was extracted based on operations manual of TRIzol reagent (Thermo Scientific, China). Corresponsing RNA sequence library was generated by Illumina HiSeq 2000 (Illumina, China), and then inspected by Agilent 2100 Bioanalyzer. Next-Generation Sequencing was conducted based on Illumina sequencing platform. Genes those with fragments per kilo bases per million fragments >1 was definied as expressed genes.

2.3. Construction of AP-1 site-mCherry biosensors

The vector pcDNA™ 3.1 (+) contained the T7 promoter and a multiple-cloning site (MCS). An AP-1 recognition site (5′-TGAC/GTCA-3′) was reconstructed into an 8-tandem repeat sequence structure (5′-TGACTCATGACTCATGACTCATGACTCATGACTCATGACTCATGACTCA TG AC-3′). The obtained tandem sequence was linked to the promoter of the fluorescent protein mCherry to obtain the target gene sequence. The designed target gene sequence was inserted into the MCS of pcDNA to construct a recombinant plasmid. AmpR was designed for screening of prokaryotic resistance and NeoR for eukaryotic resistance. The recombinant plasmid was amplified and expressed in competent *Escherichia coli* (screened for ampicillin resistance). The plasmid was extracted using the EndoFree Plasmid Kit (TIANGEN Biotech, China). The concentration and purity of the plasmid were assessed by nucleic acid electrophoresis and absorbance value.

HEK 293 cells of 90% confluence were transfected with Lipofectamine 2000 (Invitrogen, CA) as stated in the operations manual. After incubation for 24 h, the transfected cells were screened using neomycin. The screened cells (with the passage number P1) were passaged into 96-well plates and exposed to toxins and then stored in darkness before detection.

2.4. Applicability and verification of AP-1 site-mCherry biosensors

To assess viability, subculture biosensors were cultivated in 6-well plates at a concentration of 4×10^5 cells/well. The apoptosis of the collected cells was analyzed by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (Beyotime, China), and the ROS level was

assessed using the ROS Assay Kit (Beyotime, China). For apoptosis analysis, 8 μL of Annexin V-FITC and 7.5 μL of propidium iodide (PI) were mixed with the cells successively and then incubated for 10 min. For ROS detection, the cells were dissolved in 10 μM DCFH-DA and then incubated for 30 min. The cells were washed with phosphate buffer solution 3 times.

To demonstrate the fluorescence response of the biosensor, biosensors were seeded in a 96-well plate and treated with Cd^{2+} during the logarithmic phase. After the biosensors were incubated at 37 °C for 5 h, their fluorescence intensity was determined using the ImageXpress Micro XLS high-content screening (HCS) system (Molecular Devices, USA) or the SpectraMAX 340 (Molecular Devices, USA) microplate reader. The sensors in the DMEM medium were designated as the blank group and the untransfected cells as negative controls. The subculture cells (P2–P5) were placed under same conditions to evaluate the stability of the fluorescence response of the biosensors. The DNA-to-liposome ratio (2:1 to 1:4) was optimized to achieve the best response.

2.5. Optimization of screening time, monitoring of DON and Cd, and joint toxicity evaluation

Biosensors were seeded in a 96-well plate at a concentration of 3×10^4 cells/well. Cd^{2+} (0.5/5 μM) working solutions were exposed to biosensors incubated in the dark. Fluorescence intensity was measured hourly to obtain the best monitor time. Optimization of screening time for DON was evaluated using the similar method. Then water containing various levels of Cd^{2+} (0.001, 0.05, 0.5, 1, 5, and 10 μM) or DON (0.03, 0.17, 0.34, 1.69, 3.36, and 6.76 μM) was exposed to the biosensor (water<5% of DMEM, *v*/v), and the levels of Cd^{2+} and DON were monitored at the optimized screening time. For joint toxicity evaluation, the mixtures of DON and Cd^{2+} (1:1, μM: μM) with increasing concentration were incubated with the biosensors, and the fluorescence signal were detected at the optimized screening time.

2.6. Fluorescence response induced by other major heavy metals and mycotoxins

Biosensors were seeded in a 96-well plate at a concentration of 3×10^4 cells/well. Pb^{2+} (0.001–10 μM), Hg^{2+} (0.001–10 μM), ZEN (0.1–10 μM), AFB_1 (0.001–0.05 μM, 100 μM), FB_1 (0.1–10 μM), DON (0.1–10 μM), and OTA (0.05–10 μM, 100 μM) were screened using the biosensors for up to 24 h to identify the specificity of the biosensors.

2.7. Statistical analysis

All experiments were repeated individually at least three times. Significant differences were determined using the *t*-test. $P < 0.05$ indicated a significant difference. $^{**}P < 0.01$, $^{*}P < 0.05$. All statistical analyses were conducted using the software FlowJo (Tree Star, OR, USA) and SPSS. Relative fluorescence = Fluorescence $_{toxins}$- Fluorescence $_{blank.}$ Fluorescence = Fluorescence $_{toxins}$/Fluorescence $_{blank}$.

3. Results and discussion

3.1. DON+Cd induced enhanced AP-1 activity than DON or Cd

Intake of food or water is one of the main exposure routes of mycotoxin and heavy metals. Intestinal tract is the first barrier for human body to protect poison invasion. DON mainly targets intestinal, and Cd impairs the intestinal barrier function, and causes inflammation of the intestines (Tinkov et al., 2018; Xie et al., 2020). Inflammatory reaction and oxidative damage are important causes of Cd and DON toxicity (Xie et al., 2020; Zhou et al., 2020b). Therefore, HT-29 cells, one of the classical intestinal cell models, were applied for RNA sequence. KEGG analysis demonstrated MAPK as key mechanism involved in 24 h interaction of DON+Cd (Table S1). AP-1 is downstream of MAPK pathway and specifically binds to the TPA response element (AP-1 site), causing inflammatory reaction. Therefore, we tested activity of AP-1 under toxin exposure (24 h) in HT-29 cells.

As shown in Table 1, six and two kinds of AP-1 transcription factor subunits were significantly upregulated by DON ($P < 0.001$) and Cd^{2+} ($P < 0.001$) respectively, with fold changes ranging from 2.67 to 65.78. When DON and Cd^{2+} were jointly exposed, transcription levels of AP-1 members were promoted to a greater extent ($P < 0.001$). FOSB was upregulated by 124 times in the mixture group, being the most up-regulated transcription factor subunit. The data in Table 1 suggested that joint toxicity of DON and Cd^{2+} (33.78 + 33.78 μM) may be stronger than that of single toxin. AP-1 played an important role. This conclusion was in according with our previous study for 12 h treatment of DON+Cd (Guo et al., 2020c). To verify this conclusion, we constructed AP-1 site-based biosensors for further analysis.

3.2. Construction of AP-1 site-mCherry biosensors

The fluorescent protein mCherry is one of the commonly used tracers and is superior to other fluorescent protein labels because of its color and the photostability of monomer molecules. HEK-293 cells are easy to transfect, which are commonly used cells expressing foreign genes. Pre-experiment in our lab found that biosensors constructed by HEK293 cells showed a better fluorescence response than that constructed by HT-29 cells. Thus, mCherry protein and HEK293 cells were applied in the biosensor. In the construction of the biosensors, the promoter sequence of mCherry was linked to the sequence of the AP-1 site, with the reporter gene mCherry as the output signal in the sensor and the AP-1 site as the "promoter" (Fig. 1A). The transfected cells were assigned as AP-1 site-mCherry-based biosensors and those that were not successfully transfected were killed by neomycin. If the biosensors ($P \geq 2$) are treated with the AP-1 substrate, the activated AP-1 binds to the AP-1 site, inducing the activation of the mCherry protein. Enhanced fluorescence intensity indicates the presence of hazardous chemicals that activate AP-1 (Fig. 1B).

3.3. Practicability and efficiency of the fluorescence response of AP-1 site-mCherry biosensors

3.3.1. Viability status

Liposome may confer a certain degree of cytotoxicity to cells; thus, we tested the cytotoxicity parameters of the constructed biosensors with respect to apoptosis, calcium concentration, and ROS level to determine its applicability. We observed apparent cytotoxic effects during the first passage of the transfected cells. By contrast, no such effects were observed in the subculture owing to their capacity for self-repair. We only evaluated the viability of the subculture cells ($P \geq 2$), which were the only cells used for chemical screening. The results were presented in Fig. 2. The degree of toxicity was considerably low despite the increase in the toxicity of subculture cells. The living normal cells comprised more than 90% of the subculture, suggesting that the cells were in good condition (Fig. 2A). Moreover, no apparent damage was observed in ROS and calcium level (Fig. 2B, C). These findings indicated the viability and practicability of the biosensors.

3.3.2. Fluorescence response and optimization

We subsequently determined the fluorescent response of the constructed biosensors ($P \geq 2$) (Fig. 3). The constructed plasmid was first identified by gel electrophoresis as digested with *Sac*I-*Xho*I (Fig. 3A). Compared with those of the negative control group or the blank group, the red fluorescence intensity of the biosensors ($P = 2$) treated with Cd^{2+} was significantly enhanced, suggesting the stimulation of AP-1 activity and mCherry protein. When no toxins were added, the fluorescence was very weak up to 5 h. To further evaluate the visual applicability of the biosensors, the fluorescence activation was tested in

Table 1
Activation of AP-1 transcription factor subunits by DON, Cd^{2+}, and DON+Cd^{2+} (24 h) in HT-29 cells.

Toxins	Name	ID	Fold change	*P* value	Description
DON (Compare with control)	FOSL2	ENSG00000075426	10.86	<0.001	FOS like 2, AP-1 transcription factor subunit
	FOSB	ENSG00000125740	16.05	<0.001	FOSB proto-oncogene, AP-1 transcription factor subunit
	FOS	ENSG00000170345	5.67	<0.001	FOS proto-oncogene, AP-1 transcription factor subunit
	FOSL1	ENSG00000175592	65.78	<0.001	FOS like 1, AP-1 transcription factor subunit
	JUN	ENSG00000177606	9.57	<0.001	JUN proto-oncogene, AP-1 transcription factor subunit
	JUNB	ENSG00000171223	2.98	<0.001	JUN B proto-oncogene, AP-1 transcription factor subunit
Cd^{2+} (Compare with control)	FOSL1	ENSG00000175592	5.12	<0.001	FOS like 1, AP-1 transcription factor subunit
	FOSB	ENSG00000125740	2.67	<0.001	FOSB proto-oncogene, AP-1 transcription factor subunit
DON+Cd^{2+} (Compare with control)	FOSL2	ENSG00000075426	9.42	<0.001	FOS like 2, AP-1 transcription factor subunit
	FOSB	ENSG00000125740	124.05	<0.001	FOSB proto-oncogene, AP-1 transcription factor subunit
	FOS	ENSG00000170345	20.26	<0.001	FOS proto-oncogene, AP-1 transcription factor subunit
	FOSL1	ENSG00000175592	88.02	<0.001	FOS like 1, AP-1 transcription factor subunit
	JUN	ENSG00000177606	15.28	<0.001	JUN proto-oncogene, AP-1 transcription factor subunit
	JUNB	ENSG00000171223	4.39	<0.001	JUN B proto-oncogene, AP-1 transcription factor subunit
DON+Cd^{2+} (Compare with DON)	FOS	ENSG00000170345	3.43	<0.001	FOS proto-oncogene, AP-1 transcription factor subunit
	FOSB	ENSG00000125740	7.43	<0.001	FOSB proto-oncogene, AP-1 transcription factor subunit
Cd^{2+} (Compare with Cd^{2+})	FOSL2	ENSG00000075426	5.38	<0.001	FOS like 2, AP-1 transcription factor subunit
	FOSB	ENSG00000125740	45.49	<0.001	FOSB proto-oncogene, AP-1 transcription factor subunit
	FOS	ENSG00000170345	17.40	<0.001	FOS proto-oncogene, AP-1 transcription factor subunit
	FOSL1	ENSG00000175592	16.86	<0.001	FOS like 1, AP-1 transcription factor subunit
	JUN	ENSG00000177606	8.51	<0.001	JUN proto-oncogene, AP-1 transcription factor subunit
	JUNB	ENSG00000171223	3.14	<0.001	JUN B proto-oncogene, AP-1 transcription factor subunit

the subculture cells. With Cd^{2+} stimulation, fluorescence response was successfully activated in the third-, fourth-, and fifth-generation biosensors (Fig. 3B, C). Subculture cells grew efficiently in the 96-well plates (Fig. 3D). Owing to the good viability and stable activation of mCherry protein in the subculture cells, we proposed that the constructed biosensors could be used as a visualization tool for AP-1 substrate screening.

To acquire the best visualization signal, the plasmid-to-liposome applied for biosensor construction was optimized. When the ratio changed from 2:1 to 1:4 (μg:μL, DNA: liposome), the fluorescence intensity first increased and then decreased (Fig. 3E, F). Thus, the ratio of 1:3 (μg: μL, DNA: liposome) was chosen for further analysis.

3.4. Screening and joint toxicity evaluation of DON and Cd

3.4.1. Dose-dependent enhancement of fluorescence induced by Cd

Toxic metals interact with thiol groups of phosphatases, leading to protein conformational changes and induction of redox-regulated transcription factors (Poli et al., 2004; Thannickal and Fanburg, 2000). Some transcription factors can potentially be used as biomarkers of toxic metals (Koedrith and Seo, 2011). Upon stimulation, the transcription factor AP-1 specificity binds to AP-1 site. In this regard, the present study tried to use AP-1 as a biomarker and tested the screening capability of the optimized AP-1 site-biosensors for Cd^{2+}. All monitored toxins in the experiment were at the levels shown in Table S2.

Consistent with our assumptions, the fluorescence signal of the biosensors appeared after 3 h and intensified up to 6 h with Cd^{2+} stimulation. The fluorescence of low and high levels of Cd^{2+} showed the same law of fluorescence, which were markedly enhanced and became moderately stable at 5 h (Fig. 4A, B). We selected 5 h for further toxicity screening. Significantly increased fluorescence signal emerged as Cd^{2+} concentration increased (0.05–10 μM). A negligible change in fluorescence intensity was observed in the control group or the blank group (Fig. 4C). The biosensors achieved successful monitoring of Cd in water solutions. The test concentrations were among the real contamination levels of Cd in food and feed samples (Table 2).

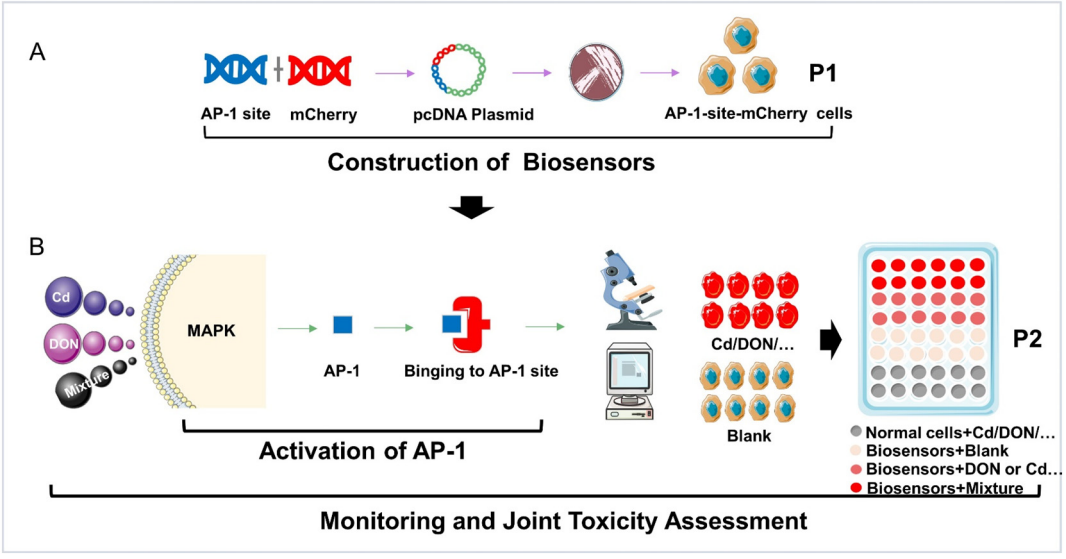


Fig. 1. Design and fluorescence analysis of the biosensors. A: Construction of AP-1 site-based biosensors. B: Visual screening and joint toxicity assessment of DON, Cd, and other toxic chemicals by AP-1 site-based biosensors. P: Passage number. Blank: Biosensors treated without toxins.

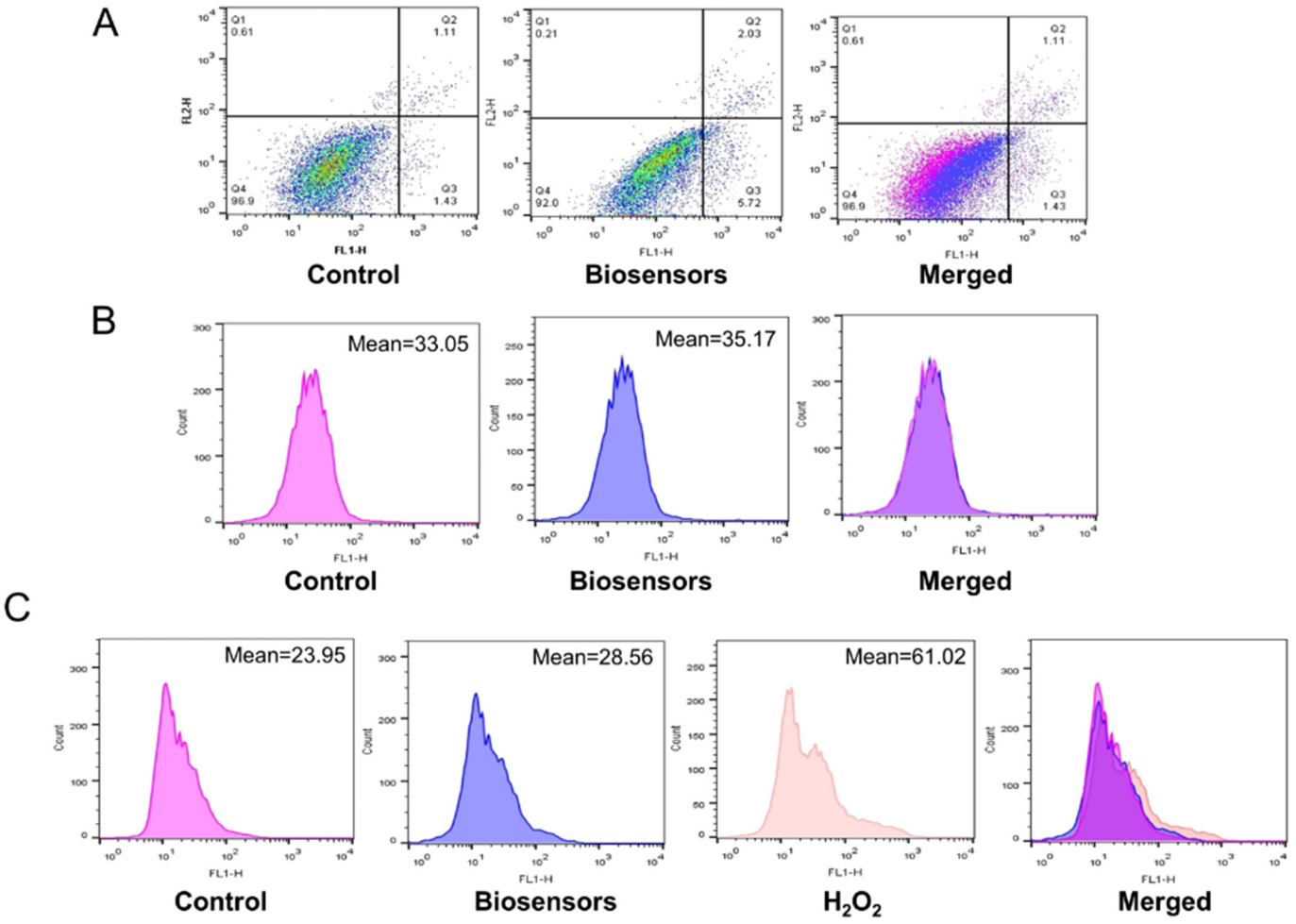


Fig. 2. Results showing the viability of the biosensors. A: Apoptosis as determined by biosensors ($P = 2$). B: Calcium level of biosensors ($P = 2$). C: ROS level of biosensors ($P = 2$). Untransfected cells as the negative control and H_2O_2 as the positive control. P: Passage number of biosensors.

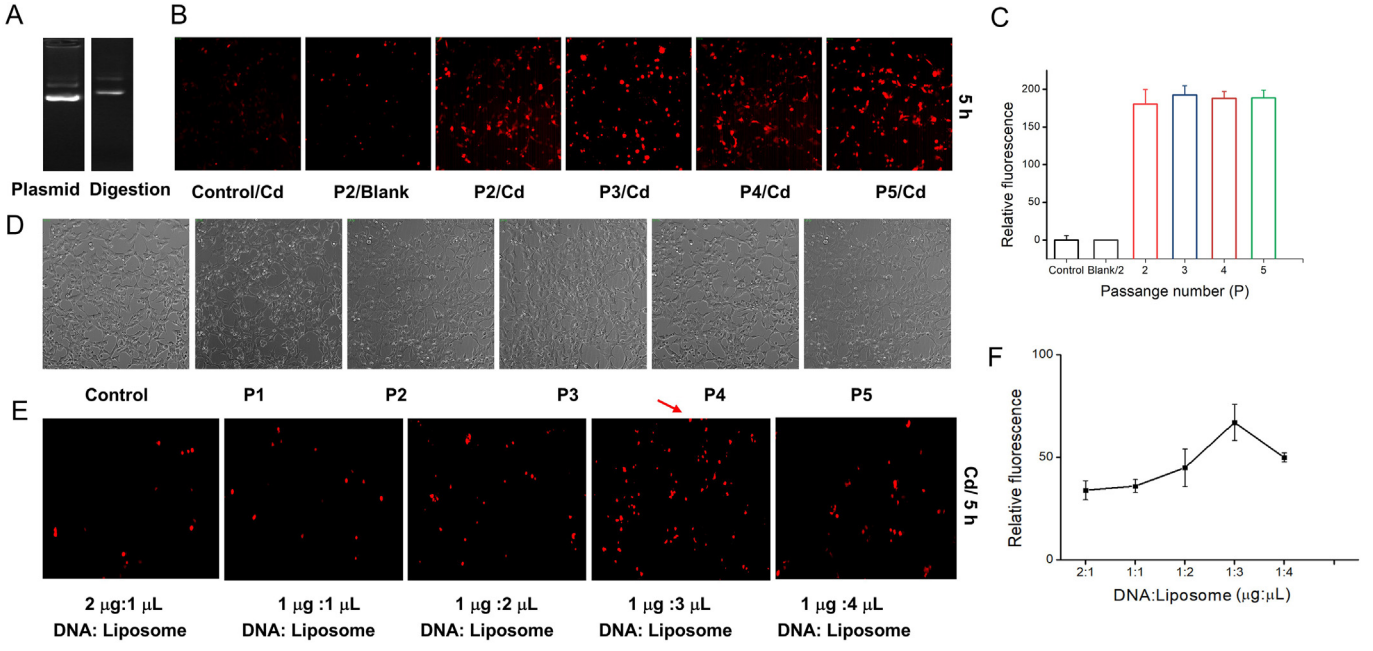


Fig. 3. Verification and optimization of the biosensors. A: Digested map of AP-1 site-mCherry plasmid. B, C: Stable fluorescence response of biosensors ($P \geq 2$) exposed to water containing Cd^{2+}. Normal cells treated with Cd^{2+} (1 μM) as the negative control group. Biosensors without Cd^{2+} treatment as the blank group. D: Brightfield diagram of biosensor offspring, with normal cells as the control group. E, F: Optimization of the plasmid-to-liposome 2000 ratio used during transfection. Corresponding biosensors ($P = 2$) exposed to water containing Cd^{2+} (0.05 μM) for 5 h. P: Passage number of biosensors.

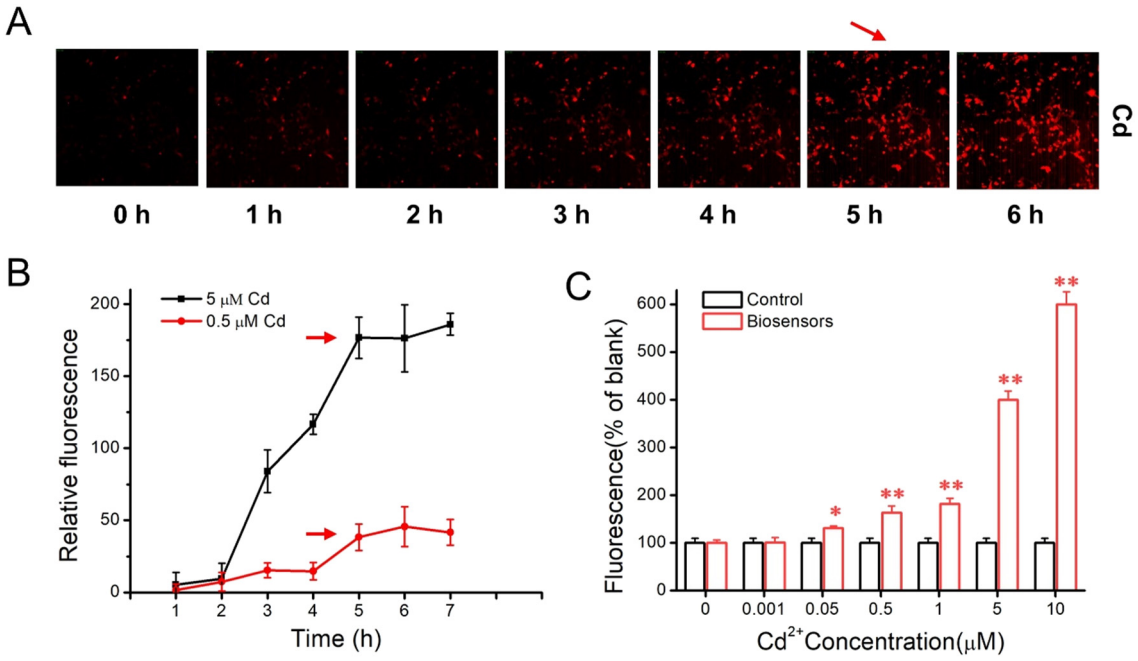


Fig. 4. Screening of Cd^{2+} by the biosensors. A, B: Fluorescence response of biosensors stimulated by Cd^{2+} (0.5/5 μM) over time. Fluorescence intensity was detected every hour. C: Fluorescence response of biosensors to various level of Cd^{2+} (0.001, 0.05, 0.5, 1, 5, and 10 μM) after incubation for 5 h. Biosensors with no added toxins were the blank group. Normal cells were the control group.

3.4.2. Dose-dependent enhancement of fluorescence induced by DON

DON widely exists in various food and feed samples, as shown in Table 2. In addition, DON level in water is efficient biomarker for estimating human exposure to DON and its masked forms (Gracia-Lor et al., 2019). Therefore, it is important to monitor DON. We further used the biosensor for screening of DON in water solution.

As presented in Fig. 5A and B, fluorescence intensity increased in the biosensors treated with DON. The visual fluorescence signal induced by low or high levels of DON increasingly intensified with exposure time. With the time consumption and fluorescence signal considered, 9 h was ultimately selected as exposure time for DON screening. Consequently, a dose-dependent increase in fluorescence was observed in DON group (0.03–6.76 μM), but not in the control group (Fig. 5C). Significantly increased signal appeared at 0.17–6.76 μM, which was in the real contamination level of DON in food and feed samples (Table 2).

3.4.3. Screening of joint toxicity of DON and Cd

In food or feed samples, DON was found to have a pollution level ranging from 0.04 ppm to 2 ppm (0.14–6.76 μM) and a Cd level ranging from 0.001 ppm to 2.5 ppm (0.01–22.25 μM) (Table 2). In part 3.1, we identified AP-1 pathways as key mechanisms underlying the toxicity of DON and Cd. Prompted by these findings, we further used the biosensors to evaluate the joint toxicity of DON and Cd (at environmental exposure levels and exposure ratio, 1 μM: 1 μM) (Luo et al., 2019).

Obviously enhanced fluorescence signals were observed in the combined groups compared with DON and Cd^{2+} (1 μM:1 μM, 0.5–10 μM) (Fig. 6A). These enhanced signals indicated synergism effects of DON and Cd on AP-1 toxicity pathway. However, no apparent change in fluorescence signal was found in the low level of combined group (0.05 + 0.05 μM) (Fig. 6B), suggesting that low levels of DON+Cd^{2+} induced no significant increases in toxicity compared with individual toxins. This finding was consistent with earlier research (Luo et al., 2019; Le et al., 2018). With combined treatment (1:1, 0.05–10 μM) for

Table 2
Contamination level of Cd and DON in various products.

Toxins	Samples	Level	Country	Reference
Cd	Black pepper, nutmeg, chilli	<0.005–0.15 ppm	India, Turkey, Poland, Brazil, Vietnam, Indonesia, China	Reinholds et al., 2017
	Basil, oregano, thyme	0.03–0.19 ppm	India, Turkey, Poland, Brazil, Vietnam, Indonesia, China	Reinholds et al., 2017
	Feed	≤2.5 ppm	Iran	Eskandari and Pakfetrat, 2014
	Breast milk	0.12–0.80 ppb	Cyprus	Kunter et al., 2017
	Pearled wheat fraction	ND-0.07 ppm	Romania	Sovrani et al., 2012
	Wheat and milling fractions	0.01–0.17 ppm	France, Greece, Iran, Italy, Mexico, Syria, Turkey, US	Cheli et al., 2010
	Traditional wheat	Mean 61.7 ppb	Belgium	Alkhalaf et al., 2010
	Organic wheat	Mean 66.8 ppb	Belgium	Alkhalaf et al., 2010
	Milk	Median 0.092–0.250 ppm	Pakistan	Younus et al., 2013
	Wheat, rye, barley, oat, rice, maize	≤199 ppb	Greece	Skendi et al., 2020
	Poultry feeds	0.004–0.249 ppm	Saudi Arabia	Alkhalaf et al., 2010
	Corn grains	0.02–0.08 ppm	Turkey	Pekel et al., 2018
DON	Basil, oregano, thyme	40–209 ppb	India, Turkey, Poland, Brazil, Vietnam, Indonesia, China	Dagaşan and Ozen, 2011
	Pearled wheat fraction	≤1789 ppb	Romania	Al-Seeni, 2012
	Corn grains	0.040–0.725 ppm	Turkey	Pekel et al., 2018
	Traditional wheat	Mean 354 ppb, ≤ 2824 ppb	Belgium	Alkhalaf et al., 2010

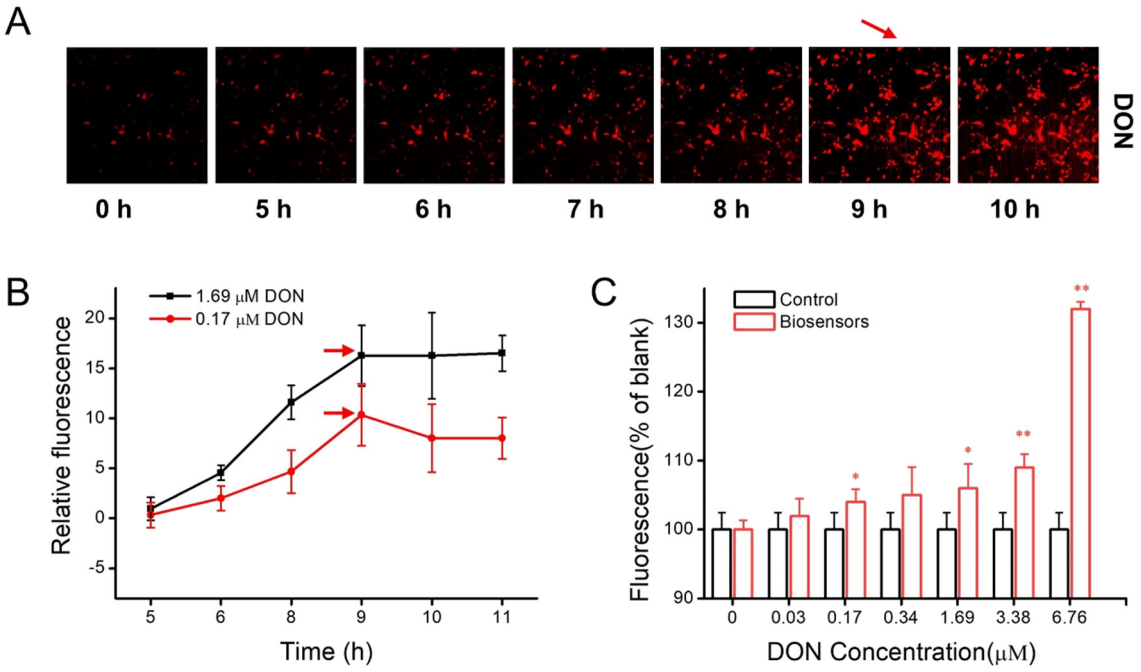


Fig. 5. Screening of DON by the biosensors. A, B: Fluorescence response of biosensors induced by DON (0.17/1.69 μM) over time. Fluorescence intensity was detected every hour. C: Toxicity screening of DON at varying levels (0.03, 0.17, 0.34, 1.69, 3.36, and 6.76 μM) by the biosensors after incubation for 9 h. Biosensors with no toxins added were the blank group. Normal cells were the control group.

9 h, a significantly dose-dependent increase in fluorescence signal was observed in the biosensors (Fig. 6B). All aforementioned findings suggested that the transfected cells could be used as biosensors for joint toxicity evaluation of DON and Cd^{2+}.

In order to further explore the specificity of the biosensor, we tested its screening activity to three major heavy metals and five major mycotoxins correspond to environmental level. Cd, Hg, Pb are all important toxic heavy metals. In addition to Cd, Pb^{2+} and Hg^{2+} have been shown to stimulate AP-1 activity in a short time manner (Korashy and El-Kadi, 2008). Thus, Pb^{2+} and Hg^{2+} (on the basis of environmental concentration levels, Table S3) water solutions were screened by the biosensor. We found that Hg^{2+} and Pb^{2+} (0.001–10 μM) induced fluorescence signal in the biosensor at 5 h incubation (Fig. S1A). Among the five major mycotoxins (DON, ZEN, AFB_1, OTA, FB_1) correspond to environmental level, the biosensor realized specific screening of DON (Fig. S1B). However, very high level of AFB_1 (100 μM) weakly activated fluorescence signal (Fig. S2). Data were shown in Supplementary materials. These tests and data showed that the biosensor constructed at present belonged to broad-spectrum biosensor. Other members of trichothecene family such as T-2 toxin, and degradation products of DON should be tested in further program. We think it will be an interesting work. For further analysis, more advanced engineered plasmids and more specific toxicity pathways should be designed for rapid and specific screening of DON and Cd.

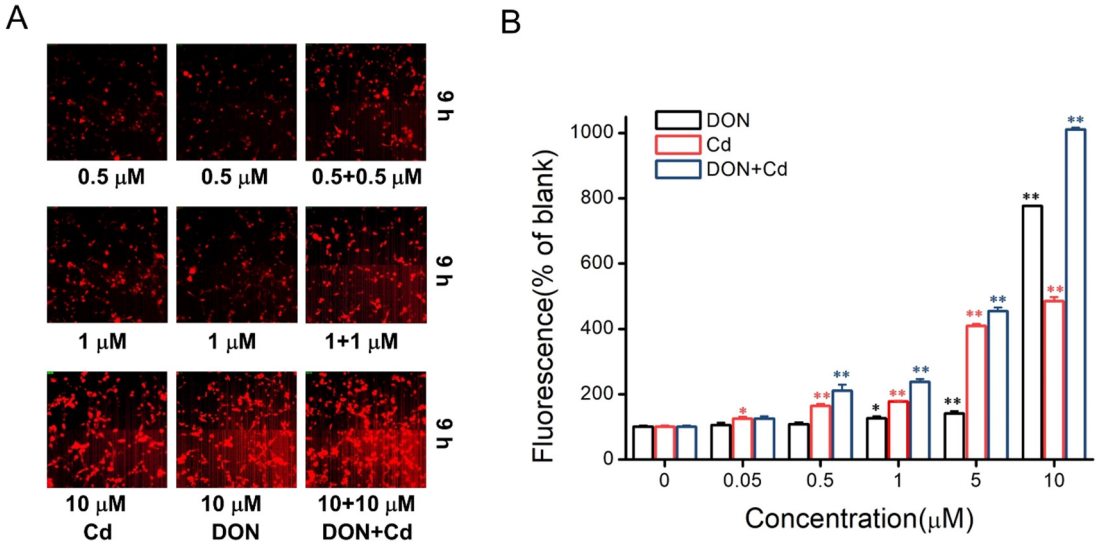


Fig. 6. Joint toxicity evaluation of DON and Cd^{2+} by the biosensor. Addition of DON and Cd^{2+} (0.5–2 μM) at a constant ratio (1:1) into the biosensors. Biosensors with no toxins added were the blank group. Normal cells were the control group.

4. Conclusions

AP-1 site-mCherry biosensors were constructed to screen heavy metals and DON in water, and evaluate joint toxicity of DON and Cd^{2+}. The transfected cells exhibited efficient growth and stable fluorescence response. DON, Cd^{2+}, and DON+Cd^{2+} (on the basis of environmental exposure levels) induced dose-dependent fluorescence in the biosensors. Since AP-1 is one of the downstream of MAPK, and plays vital role in cellular apoptosis and oxidative stress, we reasonably speculate that the AP-1 mCherry biosensor is a suitable tool for joint toxicity evaluation of heavy metals and mycotoxins. In addition, this biosensor may be further used for joint toxicity evaluation of DON and other heavy metals (such as DON+Pb^{2+}) or joint toxicity of heavy metals (such as Hg^{2+}+Pb^{2+}). More advanced and specific toxicity pathways should be designed for rapid and specific screening of DON and Cd in future research.

Declaration of competing interest

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

Acknowledgements

The work was supported by the National Natural Science Foundation of China (31772069, 31801660), National Key Research and Development Program of China (2018YFC1602800), Postgraduate Research and Practice Innovation Program of Jiangsu Provience (KYCX20_1855), Jiangsu Province Agricultural Science and Technology Independent Innovation Fund (CX (17)1003), National first-class discipline program of Food Science and Technology (JUFSTR20180303), Collaborative Innovation Center for Food Safety and Quality Control.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.144823.

References

Alkhalaf, N.A., Osman, A.K., Salama, K.A., 2010. Monitoring of aflatoxins and heavy metals in some poultry feeds. Afr. J. Food Sci. 4 (4), 192–199.
Al-Seeni, M.N., 2012. Natural occurrence of heavy metal, Fungi and Mycotoxins in soybean meal samples used in animal feeding in Saudi Arabia. Afr. J. Biotechnol. 11 (38), 9288–9294. https://doi.org/10.5897/AJB11.3080.
Antonacci, A., Scognamiglio, V., 2020. Biotechnological advances in the Design of Algae-Based Biosensors. Trends Biotechnol. 38 (3), 334–347. https://doi.org/10.1016/j.tibtech.2019.10.005.
Cheli, F., Campagnoli, A., Ventura, V., Brera, C., Berdini, C., Palmaccio, E., 2010. Effects of industrial processing on the distributions of Deoxynivalenol, cadmium and Lead in durum wheat milling fractions. LWT – Food Sci. Technol. 43 (7), 1050–1057. https://doi.org/10.1016/j.lwt.2010.01.024.
Dagaşan, O., Ozen, N., 2011. Aflatoxin, heavy metal and pesticide residue contents of some compound feeds produced in Turkey. Ziraat Fakültesi Dergisi, Akdeniz Üniversitesi *24* (1), 9–13.
Do, M.H., Ngo, H.H., Guo, W., Chang, S., Nguyen, D.D., Liu, Y., Varjani, S., Kumar, M., 2020. Microbial fuel cell-based biosensor for online monitoring wastewater quality: a critical review. Sci. Total Environ. 712, 135612. https://doi.org/10.1016/j.scitotenv.2019.135612.
Eom, H., Kang, W., Kim, S., Chon, K., Oh, S.E., 2020. Improved toxicity analysis of heavy metal-contaminated water via a novel fermentative bacteria-based test kit. Chemosphere. 285, 127412. https://doi.org/10.1016/j.chemosphere.2020.127412.
Eskandari, M., Pakfetrat, S., 2014. Aflatoxins and heavy metals in animal feed in Iran. Food Addit. Contam. Part B 7 (3), 202–207. https://doi.org/10.1080/19393210.2013.876675.
Gracia-Lor, E., Zuccato, E., Hernández, Félix, Castiglioni, S., 2019. Wastewater-based epidemiology for tracking human exposure to mycotoxins. J. Hazard. Mater. 382, 121108. https://doi.org/10.1016/j.jhazmat.2019.121108.
Guo, H., Ji, J., Wang, J.S., Sun, X., 2020a. Co-contamination and interaction of fungal toxins and other environmental toxins. Trends Food Sci. Technol. 103, 162–178. https://doi.org/10.1111/1541-4337.12545.
Guo, H., Ji, J., Wang, J.S., Sun, X., 2020b. Deoxynivalenol: masked forms, fate during food processing, and potential biological remedies. Compr. Rev. Food Sci. Food Saf. 19 (2), 895–926. https://doi.org/10.1016/j.tifs.2020.06.021.
Guo, H., Ji, J., Wei, K., Sun, J., Zhang, Y., Sun, X., 2020c. MAPK/AP-1 and ROS participated in ratio- and time-dependent interaction effects of deoxynivalenol and cadmium on HT-29 cells. Food Chem. Toxicol. 111921. https://doi.org/10.1016/j.fct.2020.111921.
Gupta, N., Renugopalakrishnan, V., Liepmann, D., Paulmurugan, R., Malhotra, B.D., 2019. Cell-based biosensors: recent trends, challenges and future perspectives. Biosens. Bioelectron. 141, 111435. https://doi.org/10.1016/j.bios.2019.111435.
Jayesh, M., Sonawane, C.I., Ezugwu, Prakash, C.G., 2020. Microbial fuel cell-based biological oxygen demand sensors for monitoring wastewater: state-of-the-art and practical applications. ACS Sensors. 5 (8), 2297–2316. https://doi.org/10.1021/acssensors.0c01299.
Koedrith, P., Seo, Y.R., 2011. Advances in carcinogenic metal toxicity and potential molecular markers. Int. J. Mol. Sci.. 12 (12), 9576–9595. https://doi.org/10.3390/ijms12129576.
Kopp, B., Vignard, J., Mirey, G., Fessard, V., Zalko, D., Le Hgarat, L., Audebert, M., 2018. Genotoxicity and mutagenicity assessment of food contaminant mixtures present in the French diet. Environ. Mol. Mutagen. 59 (8), 742–754. https://doi.org/10.1002/em.22214.
Korashy, H.M., El-Kadi, A.O.S., 2008. NF-κB and AP-1 are key signaling pathways in the modulation of NAD(P)H:Quinone Oxidoreductase 1 gene by mercury, Lead, and copper. J. Biochem. Mol. Toxicol. 22 (4), 274–283. https://doi.org/10.1002/jbt.20238.
Kunter, I., Hürer, N., Gülcan, H.O., Oztürk, B., Dogan, I., Sahin, G., 2017. Assessment of Aflatoxin M1 and heavy metal levels in mothers breast Milk in Famagusta, Cyprus. Biol. Trace Elem. Res. 175 (1), 42–49. https://doi.org/10.1007/s12011- 016-0750-z.
Le, T.H., Alassane-Kpembi, I., Oswald, I.P., Pinton, P., 2018. Analysis of the interactions between environmental and food contaminants, cadmium and deoxynivalenol, in different target organs. Sci. Total Environ. 622, 841–848. https://doi.org/10.1016/j.scitotenv.2017.12.014.
Li, W., Yu, H., He, Z., 2014. Towards sustainable wastewater treatment by using microbial fuel cells-centered technologies. Energy Environ. Sci. 7 (3), 911–924. https://doi.org/10.1039/C3EE43106A.
Luo, S., Terciolo, C., Bracarense, A.P.F.L., Payros, D., Pinton, P., Oswald, I.P., 2019. *In vitro* and *in vivo* effects of a mycotoxin, deoxynivalenol, and a trace metal, cadmium, alone or in a mixture on the intestinal barrier. Environ. Int. 132, 105082. https://doi.org/10.1016/j.envint.2019.105082.
Maresca, M., 2013. From the gut to the brain: journey and pathophysiological effects of the food-associated Trichothecene Mycotoxin Deoxynivalenol. Toxins. 5 (4), 784–820. https://doi.org/10.3390/toxins5040784.
Maresca, M., Fantini, J., 2010. Some food-associated Mycotoxins as potential risk factors in humans predisposed to chronic intestinal inflammatory diseases. Toxicon. 56 (3), 282–294. https://doi.org/10.1016/j.toxicon.2010.04.016.
Park, M., Tsai, S.L., Chen, W., 2013. Microbial biosensors: engineered microorganisms as the sensing machinery. Sensors. 13, 5777–5795. https://doi.org/10.3390/s130505777.
Pekel, A.Y., Çalık, A., Alatas, M.S., Kuter, E., Cengiz, O., Omurtag, G.Z., 2018. Evaluation of correlations between nutrients, fatty acids, heavy metals, and deoxynivalenol in corn. J. Appl. Poult. Res. 28 (1), 94–107. https://doi.org/10.3382/japr/pfy023.
Poli, G., Leonarduzzi, G., Biasi, F., Chiarpotto, E., 2004. Chiarpotto oxidative stress and cell signaling. Curr. Med. Chem. 11 (9), 1163–1182. https://doi.org/10.2174/0929867043365323.
Qi, X., Wang, S., Li, T., Wang, X., Liang, P., 2020. An electroactive biofilm-based biosensor for water safety: pollutants detection and early-warning. Biosens. Bioelectron. 173, 112822. https://doi.org/10.1016/j.bios.2020.112822.
Reinholds, I., Pugajeva, I., Bavrins, K., Kuckovska, G., Bartkevics, V., 2017. Mycotoxins, pesticides and toxic metals in commercial spices and herbs. Food Addit. Contam. Part B 10 (1), 5–14. https://doi.org/10.1080/19393210.2016.1210244.
Saha, N., Mollah, M.Z.I., Alam, M.F., Rahman, M.S., 2016. Seasonal investigation of heavy metals in marine fishes captured from the bay of Bengal and the implications for human health risk assessment. Food Control 70, 110–118. https://doi.org/10.1016/j.foodcont.2016.05.040.
Satarug, S., Garrett, S.H., Sens, M.A., Sens, D.A., 2010. Cadmium, environmental exposure, and health outcomes. Environ. Health Perspect. 118 (2), 182–190. https://doi.org/10.1289/ehp.0901234.
Singh, A., Kumar, V., 2020. Recent advances in synthetic biology-enabled and natural whole-cell optical biosensing of heavy metals. Anal. Bioanal. Chem. https://doi.org/10.1007/s00216-020-02953-6.
Skendi, A., Papageorgiou, M., Irakli, M., Katsantonis, D., 2020. Presence of mycotoxins, heavy metals and nitrate residues in organic commercial cereal-based foods sold in the Greek market. J. Consum. Prot. Food Safety 15, 109–119. https://doi.org/10.1007/s00003-019-01231-7.
Sovrani, V., Blandino, M., Scarpino, V., Reyneri, A., Coïsson, J.D., Travaglia, F., Arlorio, M., 2012. Bioactive compound content, antioxidant activity, deoxynivalenol and heavy metal contamination of pearled wheat fractions. Food Chem. 135 (1), 39–46. https://doi.org/10.1016/j.foodchem.2012.04.045.
Thannickal, V.J., Fanburg, B.L., 2000. Reactive oxygen species in cell signaling. Am. J. Physiol. Lung Cell. Mol. Physiol. 279, L1005–L1028. https://doi.org/10.1080/09540120020014336.
Tinkov, A.A., Gritsenko, V.A., Skalnaya, M.G., Cherkasov, S.V., Aaseth, J., Skalny, A.V., 2018. Gut as a target for cadmium toxicity. Environ. Pollut. 235, 429–434. https://doi.org/10.1016/j.envpol.2017.12.114.
Wang, X., Lu, X., Chen, J., 2014. Development of biosensor Technologies for Analysis of environmental contaminants. Trends Environ. Anal. Chem. 2, 25–32. https://doi.org/10.1016/j.teac.2014.04.001.
Wang, D., Sun, H., Wu, Y., Zhou, Z., Ding, Z., Chen, X., Xu, Y., 2016. Tubular and glomerular kidney effects in the Chinese general population with low environmental cadmium exposure. Chemosphere Environ. Toxicol. Risk Assess. 147, 3–8. https://doi.org/10.1016/j.chemosphere.2015.11.069.
Whitmarsh, A.J., Davis, R.J., 1996. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J. Mol. Med. 74, 589–607. https://doi.org/10.1007/s001090050063.

Xie, S., Jiang, L., Wang, M., Sun, W., Yu, S., Turner, J.R., Yu, Q., 2020. Cadmium ingestion exacerbates Salmonella infection, with a loss of goblet cells through activation of notch signaling pathways by ROS in the intestine. J. Hazard. Mater. 391, 122262. https://doi.org/10.1016/j.jhazmat.2020.122262.

Younus, M., Abbas, T., Rafique, K., Sajid, M., Aslam, M., Zafar, M., 2013. Analyses of selected heavy metals and aflatoxin M1 in milk for human consumption in Jhang City, Pakistan. International Food Policy Research Institute 1–17 (PSSP working papers 12).

Yu, D., Zhang, H., Bai, L., Fang, Y., Dong, S., 2019. Visual detection of the toxicity of wastewater containing heavy metal ions using a microbial fuel cell biosensor with a Prussian blue cathode. Sensors Actuators B Chem. 302, 127177. https://doi.org/10.1016/j.snb.2019.127177.

Zhou, Y., Li, Y., He, C., Lin, X., Li, C., Xu, W., Duan, N., Wu, S., Wang, Z., 2020a. Application of PEG-CdSe@ZnS quantum dots for ROS imaging and evaluation of deoxynivalenol-mediated oxidative stress in living cells. Food Chem. Toxicol. 146, 111834. https://doi.org/10.1016/j.fct.2020.111834.

Zhou, Y., Wu, S., Wang, F., Li, Q., He, C., Duan, N., Wang, Z., 2020b. Assessing the toxicity in vitro of degradation products from deoxynivalenol photocatalytic degradation by using Upconversion nanoparticles@Tio2 composite. Chemosphere. 238, 124648. https://doi.org/10.1016/j.chemosphere.2019.124648.

Hongyan Guo is a Ph.D. candidate in the School of Food Science and Technology at the Jiangnan University (China). Her research focuses on the toxicology study of deoxynivalenol and joint toxicity evaluation.

Jian Ji is a professor in the School of Food Science and Technology at the Jiangnan University (China). His research focuses on food safety, metabolic toxicology, mycotoxin joint toxicity, mass spectrometry metabonomics, and mycotoxin joint toxicity.

Jiadi Sun received her Ph.D in School of Food Science and Technology from Jiangnan University (China) in 2018. In the same year, he stayed in Jiangnan University (China), where she is currently a teacher at school of food science and technology. Her current research focuses on nano-sensing and food safety.

Yinzhi Zhang is an instrument enginner in the School of Food Science and Technology at the Jiangnan University (China).

Xiulan Sun is a professor in the School of Food Science and Technology at the Jiangnan University (China). Her research focuses on active intervention and prevention and control of food safety, including mycotoxin molecular immunization technology, antibody preparation of trace pollutants in food, biosensing detection and so on.