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Longitudinal Study of the Effects of Environmental pH on the Mechanical Properties of *Aspergillus niger*

Wenjun Zheng,[†,‡,§] Hua Yang,[†,⊥] Guanghui Xuan,[‡,§] Letian Dai,[⊥] Yunxiao Hu,[⊥] Shuijin Hu,[⊥,∥] Shengkui Zhong,[#] Zhen Li,[*,⊥] Mingyuan Gao,[‡] Shimei Wang,[⊥] and Yuan Feng[*,‡,§]

[‡]Center for Molecular Imaging and Nuclear Medicine, School of Radiological and Interdisciplinary Sciences (RAD-X), Collaborative Innovation Center of Radiological Medicine of Jiangsu Higher Education Institutions, Soochow University, Suzhou, Jiangsu 215123, China

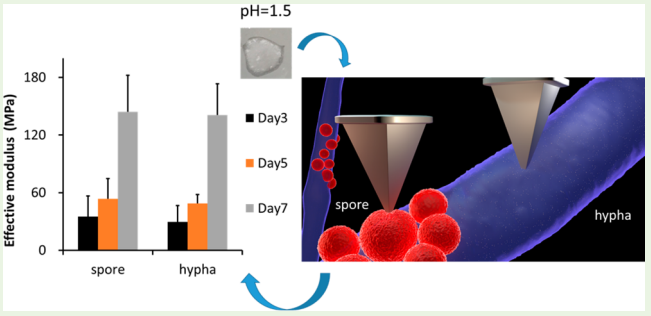
[§]School of Mechanical and Electronic Engineering, Soochow University, Suzhou, Jiangsu 215021, China

[⊥]College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China

[∥]Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina 27695, United States

[#]School of Iron and Steel, Soochow University, Suzhou, Jiangsu 215021, China

ABSTRACT: The regulation of environmental pH is key to the health of an ecosystem, influencing the metabolic activity, growth, and development of organisms within it. Although pH values can be measured by a wide range of readily available technologies ranging from fluorescent dyes and nanosensors, these cannot reveal the history of environmental pH from before monitoring begins. This information is sometimes crucial for piecing together what has happened to an ecosystem, and our long-term goal is therefore to develop technologies capable of obtaining it. Here, we propose monitoring environmental pH over time by tracking mechanical properties of a common fungus. As a first step toward obtaining a time history of pH, we evaluate the effect of pH upon the effective indentation modulus of spores and hyphae of *Aspergillus niger*. We report that the indentation modulus of this phosphorus-solubilizing fungus, obtained through atomic force microscopy and nanoindentation, correlated with environmental acidity. We observed a significant, monotonic increase in moduli over the course of incubation in an acidic environment, but no change in moduli over time for incubation in a neutral environment. Results show promise for using our scheme to detect and track environmental pH over time, and more broadly for using a microorganism's mechanical properties as a biomarker for environmental detection.



KEYWORDS: *nano indentation, Young's modulus, environment, pH, biomarker*

1. INTRODUCTION

Environmental pH affects cells and tissues in ways including metabolism, growth, and development.[1−4] The historical time course of environmental pH is of interest when designing remediation of an ecosystem, but this is difficult to obtain. Strategies such as magnetic resonance imaging,[5] fluorescent dyes,[6,7] and particle-based nanosensors[8,9] are fully capable of measuring pH at the time of inquiry, but cannot trace back the history of the environmental pH. Therefore, a method that can be used to measure and track the pH before a period of time is needed.

Given the broad range of effects of pH on cells, we investigated via atomic force microscopy (AFM) whether the biological changes related to pH shifts could affect the indentation modulus of a common fungus. Indeed, pH changes can be expected to affect the surface structure of a microorganism, especially one with a cell wall, and therefore, affect the measured stiffness using AFM. The very first AFM-like instruments were invented for the purpose of measuring cell mechanical properties.[10] Although true mechanical moduli of structured materials are nearly impossible to identify using AFM outside of the most idealized circumstances,[11] AFM has been used to obtain much useful information about the mechanics of cells.[12−14] With respect to microorganisms, AFM has been used to study the adhesion of spores of *Bacillus thuringiensis* (Bt).[15] Studies with respect to *Escherichia coli* have shown that *E. coli* is less rigid after filamentous phage M13 infection.[16] A surface modification related stiffness change was also observed between the *E. coli* with and without exposure to ampicillin.[17] In this study, we used AFM and nano indentation techniques to measure the mechanical properties of micro-

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organisms, and to explore the correlation between the environmental pH and effective mechanical properties over time.

Phosphorus solubilizing fungi (PSF) are common microorganisms. They can withstand pH values in their culture medium as low as 1−2.[18] Therefore, the PSF has a stronger adaptability to the acidic environment. Because the acidity is a critical factor in evaluating the living of PSF, possible relationships exist between the environmental pH and the physical properties of PSF.

In this study, we used *Aspergillus niger* (*A. niger*), which is better at secreting organic acids compared to other PSF, to explore the potential of using AFM nano indentation to correlate the mechanical properties of the fungi with the culturing environment over time. For the first time, we investigated the longitudinal change of the mechanical properties of *A. niger* over time in environments with different pH values. Properties of both the spores and hyphae of *A. niger* were tested. Our goal was to explore the potential of using mechanical properties as biomarkers for uncovering a history of environmental pH. The method not only provides a non-chemical way for measuring the local pH values of the microorganism, but also provides important indications for longitudinal tracking of environmental pH with potential applications in waste management[19] and pollution detection.[20,21]

2. METHODS

2.1. Sample Preparation. *A. niger* strain (NJDL-12)[22] was cultured in acidic medium with an initial pH value of 1.5 (pH 1.5). Potato dextrose agar (PDA) for culturing the strain was composed of potato (200 g/L), glucose (20 g/L), agar (20g/L), and water (1L). The liquid PDA was prepared without agar. We used 1 mol/L HCL and 1 mol/L NaOH to modulate the pH value of the solution. To compare with the mechanical responses of the *A. niger* in the acidic environment, we also cultured the strain with a pH value of 6.5 (pH 6.5).

The spores of the *A. niger* cultured in the PDA with different pH values were placed in a rotary shaker (rotation speed 170 r/min) at an ambient temperature of 28 °C. A drop of the medium was deposited on a silicon wafer and was then air-dried for the AFM analysis. To investigate the longitudinal effects of the culturing time, the spore and hypha samples were also tested after 3, 5, and 7 days of incubation. All of the sample preparations were in a clean bench.

2.2. AFM Measurement. A commercial AFM device (Dimension Icon, Bruker) was used for the nanoindentation tests at room temperature. To measure the elastic modulus of the sample, an aluminum reflex coated silicon cantilever probe with a cone tip was used (Tap 150AI-G, Budget Sensors, Innovative Solutions Bulgaria Ltd., Sofia, Bulgaria). The tip has a half cone angle of 10 degrees, a radius of 10 nm, and a resonance frequency of 150 kHz. The nominal force constant is 5 N/m and the actual spring constant of the probe was calibrated before each test. A tapping mode (PeakForce Tapping) was employed to measure the force−displacement curve. The indentation depth for all the samples was between 100 to 300 nm.

The spores and hyphae of *A. niger* were identified under the microscope of the AFM system. Each specific indentation position was selected based on a 50 × 50 μm^2 field of view (FOV). To avoid edge effects and tip slippage,[23] the circular-shaped spore was indented close to the center and the bar shaped hypha was indented at a position close to the middle line. To see the intersample and intrasample variations, we selected at least 5 different testing locations for each spore and hypha during the measurement. Each position was indented three times and an average value was reported. The force and displacement information from the nanoindentations was measured and analyzed.

The indentation process can be separated into the approach phase and retract phase. In the approach phase, the AFM probe approached the sample surface until the touching and bending of the tip cantilever. The retract phase was when the AFM probe was retracted back from the sample (Figure 1a). The surface structure of the sample was also acquired for comparison between different culturing environments.

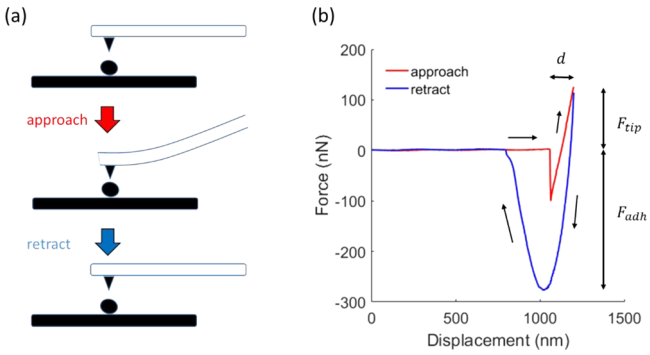

Figure 1. (a) Approach and retract phases of the AFM nanoindenation. (b) Typical indentation force−displacement curves during the approach and retract phases.

2.3. Effective Modulus Estimation. The estimation of true mechanical properties of an anisotropic, irregularly shaped organism are essentially impossible to capture using indentation methods. However, although the actual mechanical properties of the wall, periplasm, and other structures of a microorganism cannot be estimated, the effective indentation modulus can be evaluated as a metric for comparison between different cells. Here, for convenience of comparison, we used an effective isotropic elastic modulus (E) estimated from the indentation force−displacement curves using models for estimating moduli from idealized testing of homogeneous, isotropic, planar, linear, semi-infinite substrata.

The simplest of these methods simply apply linear elasticity solutions for frictionless indentation of semi-infinite elastic continua with no surface energy. Such classical solutions include the Hertz model[24−26] and Sneddon model.[27,28] These are inappropriate for biological tissues and nanoindentation for a broad range of reasons, including surface energy, surface irregularities, finite dimensions, surface adhesion, inhomogeneity, viscoelasticity, and anisotropy. Various adjustments have been made to these that account for some of these factors, (e.g., pericellular brush model[29−31]), but interpretation is in general tenuous.

Here, recognizing the limitations of indentation, we calculated only an effective modulus. Because significant adhesion effects were observed during indentation (Figure 1), as observed in many other microorganism studies,[32,33] we used an adjustment of the classical linear elastic treatment of indentation that accounted for adhesion, the DMT model.[34,35] The DMT model provides an effective modulus by assuming a spherical object indenting a flat surface of an linear elastic, homogeneous, isotropic, semi-infinite half-space. Although the radius of the AFM tip is at least one magnitude smaller than the thickness of the microorganism, we emphasize that, even here, the ability to estimate a modulus is questionable because the thickness of indented features within the cell wall might not always be small compared to this radius.

Using the DMT model to calculate the effective modulus for indentation by a probe with a conical tip, the effective modulus E can be calculated by[34]

$$E = \frac{3}{4}\frac{(F_{tip} - F_{adh})}{4\sqrt{Rd^3}}(1 - \nu^2) \quad (1)$$

where F_{tip} is peak force of the AFM probe in the approach phase (Figure 1b), F_{adh} is the adhesive force between the AFM tip and the sample during the retract phase, R is the tip radius, d is the indentation depth, and ν is Poisson's ratio. As a further caveat, we note that we

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treated the organism as incompressible; because isotropy was also assumed, this was achieved by choosing $\nu = 0.5$.

2.4. Data Analysis. To exclude the influences of the intersample variations, we compared the E values between different samples for both hyphae and spores. We also compared the E values between different testing locations within one sample for both hypha and spore samples.

The total numbers of the tested sample points for the spore group of pH 1.5 and pH 6.5 at 3, 5, and 7 incubation days were 30, 28, 30, and 30, 30, 30, respectively. The total numbers of the tested sample points for the hypha group of pH 1.5 and pH 6.5 at 3, 5, and 7 incubation days were 30, 30, 30, and 20, 30, 30, respectively. A student t-test of each of the three-repeated measurements at the same location showed that there was no significant difference ($p < 0.001$). Therefore, the three-repeated indentation results from the same location were averaged and counted as one test point. Student t-tests with a significance level of 5% were carried out to see the influences of the environment with different pH values and the incubation days.

3. RESULTS

Typical height images showing the surface structure of the spore and hypha cultured in different environment are shown in Figure 2 and Figure 3. No significant differences were observed topologically for different culturing environment and at different incubation days for the spore samples. The surface of the hypha appeared to be relatively smooth when cultured at pH 1.5, while relatively rough surfaces were observed for the hypha for a culturing environment of pH 6.5, 3 days of incubation. No significant topological differences were observed for other tested samples.

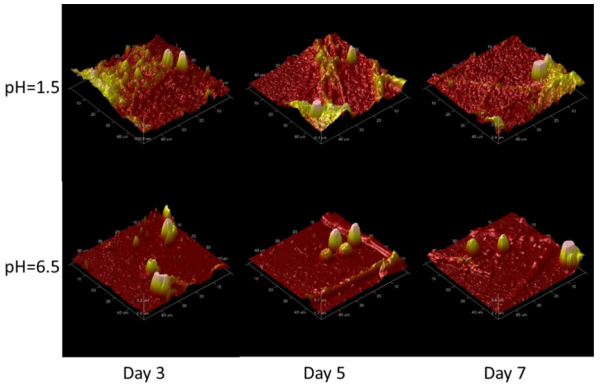

Figure 2. Typical AFM height images of the spores in an environment with pH 1.5 and pH 6.5 cultured after 3, 5, and 7 days of incubation. The diameter of the spores varied between 1.5 and 5 μm.

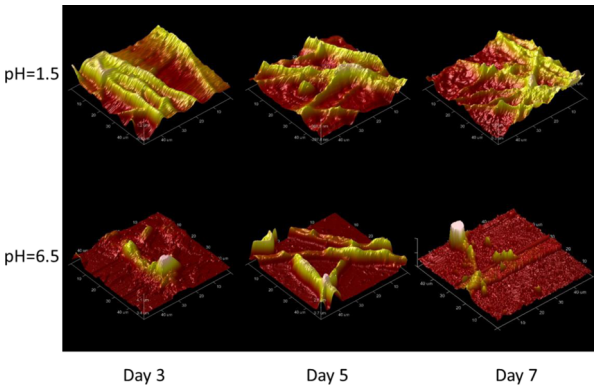

Figure 3. Typical AFM height images of the hyphae in an environment with pH 1.5 and pH 6.5 cultured after 3, 5, and 7 days of incubation. The diameter of the hyphae varied between 5 and 8 μm.

To show the intersample differences, we selected two different spores and two different hyphae under the same culturing condition (pH 1.5, Day 3) for a comparison (Figure 4). The numbers of testing points for the two spore samples were 4 and 5. For the two hyphae samples, the numbers were 12 and 5. The p-value of the measured E values between the two spores was 0.76, and for the two hyphae was 0.67. Within one sample, the standard deviation of the measured E values at different testing locations was about 1/3 to 1/10 of that of the mean value. Similar intersample and intrasample differences were also observed for other testing groups. Therefore, we summarized all the measured E values from each sample for the analysis.

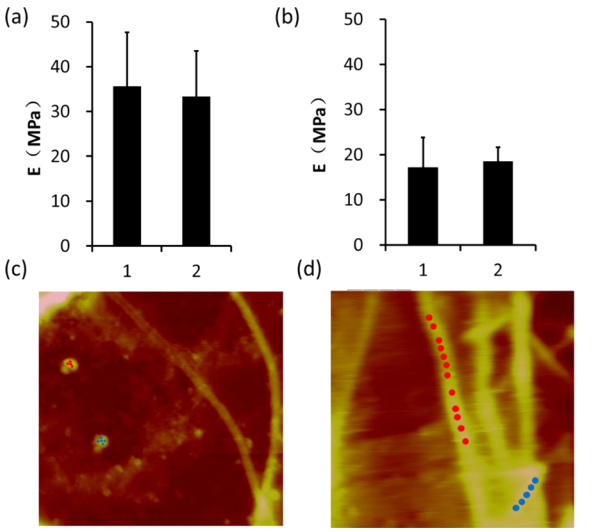

Figure 4. Intersample differences for the (a) spore and (b) hypha samples. The samples were tested in a culturing environment with pH 1.5 after 3 days of incubation. Student t-tests showed no significant differences between the two spore ($p = 0.76$) or hypha ($p = 0.67$) samples. The red and blue dots represent the indentation locations for the two different (c) spore and (d) hypha samples.

The mean and standard deviation values of the Young's modulus for the spores and hyphae cultured in different environment at each incubation day are summarized in Table 1. The maximum and minimum average E values for the spores were from the culturing medium with pH 1.5 after 7 and 3 days of incubation, respectively. For the hyphae, the maximum and minimum average E values were at pH 6.5 with 7 days of incubation and pH 1.5 with 3 days of incubation. We observed

Table 1. Mean and Standard Deviation of the E Values for the Spores and Hyphae Cultured in an Environment with pH 1.5 and pH 6.5 after 3, 5, and 7 Days of Incubation

		E (MPa)	
		pH 1.5	pH 6.5
day 3	spore	34.96 ± 21.73	85.75 ± 43.69
	hypha	29.48 ± 17.14	235.49 ± 93.26
day 5	spore	53.66 ± 20.98	103.54 ± 32.97
	hypha	48.87 ± 9.25	225.75 ± 106.06
day 7	spore	144.21 ± 37.91	59.75 ± 34.82
	hypha	140.92 ± 32.23	256.34 ± 46.56

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a monotonic increase of the E values with respect to the incubation days in an environment with pH 1.5 for both the spores and hyphae (Figure 5). Moreover, significant differences

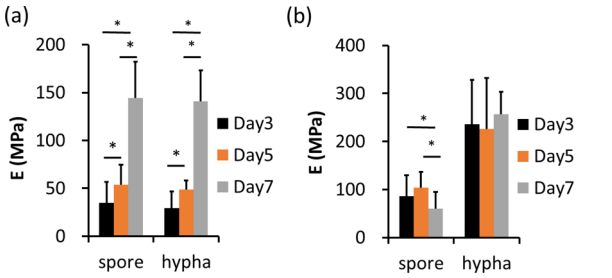

Figure 5. A comparison of the *E* values measured for the spores and hyphae at different incubation days in an environment with (a) pH 1.5 and (b) pH 6.5. The * symbol indicates a significant differences ($p < 0.05$) between the two sample groups.

were observed between the *E* values at each incubation days, for both the spores and hyphae. However, for culturing environment with pH 6.5, we did not observe a monotonic increase or decrease of the *E* value for either spores or hyphae. In addition, at pH 6.5, no significant differences were observed for the hyphae between each incubation days. Although no significant differences were observed between 3 and 5 incubation days for the spores, significant differences of the *E* values were observed between 7 days of incubation and the other two incubation time points.

By comparing the E values measured in a culturing environment with pH 1.5 and pH 6.5, we observed significant differences at each incubation time points for both spores and hyphae (Figure 6). All the *E* values measured at pH 1.5 environment were lower than that at pH 6.5 environment, for all of the incubation time points and for both spores and hyphae, except the spores after 7 days of incubation. No significant differences were observed for the *E* values between the spores and hyphae at each incubation time point with pH 1.5. However, when environment pH 6.5, the *E* values of the spores were significantly lower than that of the hyphae.

4. DISCUSSION

Growth and development of cells and tissues are closely related to the mechanical properties.[36,37] Longitudinal changes of mechanical properties have been observed in many different biological identities.[38,39] In this study, we observed a significant increase of the E value for both spores and hyphae in the acid environment over time. We postulate that in an acidic environment, *A. niger* tends to develop a stiffer cell wall over time for protection. However, no monotonic increase or decrease was observed in the neutral environment, showing that *A. niger* did not have a tendency to grow a stiffer cell wall. Therefore, these results indicate that the mechanical property could be used as a biomarker for tracking the growth of the microorganism, showing the infection days of certain fungi. Also, by correlating with the change of mechanical properties over time for both spores and hyphae, we could also estimate the environment pH values.

Throughout the observed incubation days, we observed that the acidity of the environment had significant influences of the mechanical properties of the fungi. While all the spores and hyphae appeared to be softer in the acidic environment than that of neutral environment, the spores incubated after 7 days was stiffer. This indicated that using a combination of information from both the spores and hyphae, it is possible to interpret the acidity of the environment using the mechanical properties of microorganisms. Also, the results had interesting implications from a biology perspective. Similar mechanical properties of the spores and hyphae in acid environment may indicate that the hyphae are in the process of generating spores. However, compared with neutral environment, mechanically softer hyphae in the acid environment may indicate a correlation with its high ability to generate organic acid.

It has been shown that the surface structure of microorganism could affect the stiffness measured by AFM.[17] Chen et al. (2009) has shown that the smooth surface of *E. coli* was correlated with a lower Young's modulus. In our study, we also found that the surface structure of spore and hypha could affect the nanoindentation results by indenting at different locations (Figure 4). To view the surface structure in detail, scanning electron microscopy (SEM) images (5 kV, Hitachi SU-5000N, Japan) of the gold sputter coated spores and hyphae were also acquired (Figure 7). However, the electron images did not have observable differences between environments with different pH values. This showed that the intrinsic physical properties contributed to the different modulus measured.

Compared with other pH measurement methods, using mechanical properties as a biomarker is an interesting prospect. This study provides a nonchemical method free of agents or particles to measure the environment pH. The proposed method also do not have the potential toxicity problem to cells.[40−42] Compared with the MR scanner used for pH measurement,[5] the AFM device is relatively cost-effective and easy to implement. The correlation observed between the elastic modulus and the pH values over time showed promise for using mechanical properties as a biomarker to detect the

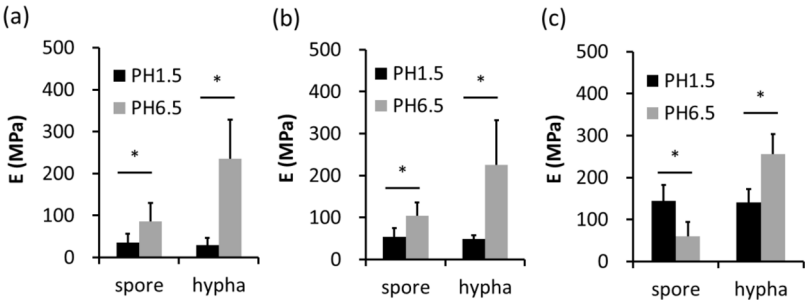

Figure 6. Comparisons of the *E* values of the spores and hyphae cultured in an environment with pH 1.5 and 6.5 after (a) 3, (b) 5, and (c) 7 days of incubation. The * symbol indicates a significant difference between the two groups ($p < 0.05$).

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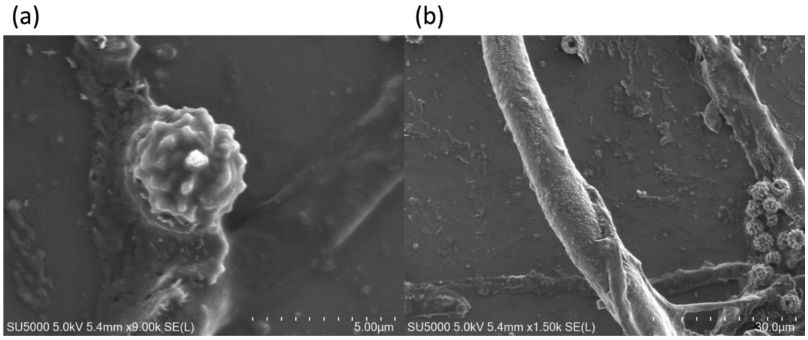

Figure 7. SEM images of the (a) spore and (b) hypha.

inter- and extra-cellular environment for a variety of biological identities not limited to the microorganism.

This study has many limitations. We showed only three incubation time points for the longitudinal study. Also the exact environment pH values may change in the process between sample handling and testing. Future studies include AFM measurement of other similar microorganisms.

5. CONCLUSION

In this study, we investigated the longitudinal change of the mechanical properties of microorganisms as a function of environmental pH values. By using nanoindentation techniques, we measured elastic moduli of a common PSF, *A. niger*. Results showed that the stiffness of both the spores and hyphae increased over the course of incubation time in an acidic environment. Also, significant differences of the stiffness were observed for both spores and hyphae as a function of environmental pH. As a preliminary study, these indicated that the mechanical properties of microorganisms can be used as biomarkers for detecting environment pH values. Results specifically demonstrated a strong correlation between mechanical properties and both the magnitude and duration of exposure to environmental acidity. More broadly, results demonstrated the feasibility of using mechanical properties to monitor both the growth conditions of a microorganism, and as a biomarker for environmental monitoring and detection.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: fengyuan@suda.edu.cn. Tel: +86-18625085336.
*E-mail: lizhen@njau.edu.cn.

ORCID

Yuan Feng: 0000-0003-1902-3408

Author Contributions

†W.Z. and H.Y. contributed equally in this work.

Notes

The authors declare no competing financial interest.

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