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# Novel method for detecting and quantifying phenol with transient response of glycolytic oscillations of synchronised yeast cells

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ABSTRACT

Yeast displays cyclic changes of autofluorescence of NADH in living, metabolising cells, known as glycolytic oscillations. In this study, we have demonstrated the proof of concept for exploiting this mechanism for a novel oscillation-based biosensor. The metabolism of *Sacharomyces cerevisae* BY4742 cells were synchronised and treated with various concentrations of phenol and with glucose and nitrobenzene additions for comparison. The established method allowed detection of phenol within 0.6–26.6 mM concentration range resulting in signal frequency of 0.29–2.03*$10^{-2}$ Hz, correspondingly. The analysed frequency was negatively and linearly correlated with phenol concentration ($R$ = 0.98). Positive (glucose) and negative (nitrobenzene) control did not caused significant oscillations indicating selectivity of the proposed mechanism. The proposed method is fast (27 min), easy to conduct and can be performed also with other types of yeast strains which makes it accessible for application with local strains and in developing countries. This is the first time, when the natural oscillatory mechanism of yeasts was demonstrated for the biosensing purposes.

## 1. Introduction

The role of oscillatory behaviour in biology ranges from periodic cell growth and division to pattern formation in embryogenesis. Oscillations may also play a role in cancer dynamics and control [1,2]. Oscillatory behaviour is abundant in biochemical and biological systems [3,4] and is indicative of far from equilibrium dissipative systems, such as living cells. Impressive demonstrations of biochemical oscillations have also been shown *in vitro* using the Min system of *E. coli* responsible for septum localization prior to cell division [5]. Although simple models may capture the qualitative oscillations of cellular metabolism [6,7], this mechanism has never been exploited for the biosensing purposes.

One of the longest studied systems of biological oscillation is the glycolytic metabolism of yeast, known since the pioneering work in 1950s and 1960s [8–10]. In particular it was shown that fluorescence spectrophotometry can be used to monitor the fluctuations of NADH in intact yeast cells [11]. This is typically done by adding glucose to starved yeast cells. Then respiration is inhibited and the oscillatory response measured. The oscillations depend highly on cell density with macroscopic oscillations more apparent at higher cell densities indicating mutual interactions among the cells [12,13]. Below a certain cell density the cells continue to oscillate but are desynchronized [14]. A downstream membrane-permeable metabolite, acetaldehyde, is thought to the coordinating agent [15–18] along with a role for glucose as well [19]. Using such cell density threshold dynamics, yeast cells exchange information of their metabolic state [20].

Yeasts are widely used as a model system for developing various types of biosensors with a separate class of biosensors dedicated to the environmental pollutants [21]. Additionally, yeast strains remain as good model organisms for genetic manipulations. Apart from the versatility as a model system, yeast cells offer other advantages such as increased tolerance to the chemical stress when compared to bacteria [21]. These advantages led to a development of multiple types of biosensors for biological oxygen demand (BOD) detection [22], endocrine-disrupting compounds (EDCs) as well as other organic compounds [23,24] and toxic metals, in particular $Cu^{2+}$ [25]. Applying genetic alterations allows for the detection of toxic copper ions by either introducing the lux operon and recording the luminescence signal [26], or modifying the ADE2 and ADE5,7 genes along with CUP1 gene which

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result in secretion of a red pigment in presence of $Cu^{2+}$. In addition, high throughput screening of yeast cell factories is possible through the use of transcription factor-based biosensors [27]. A vast majority of the yeast-based biosensors are based on luminescence and fluorescence signals [21,24,26,28–30] which offer the advantages of miniaturisation of the final device and the simplicity of the signal processing.

Biosensors may be an alternative to conventional chromatographic methods for a rapid detection and quantification of phenol in environmental samples. Phenol and its derivatives are known for their toxic properties. When released to the soil or aquatic environment, it affects vast majority of the organisms living in these ecosystems. It is also harmful for humans – it can be quickly adsorbed through skin or by inhalation and long term exposure can be fatal [31,32]. Phenol is released to the environment due to anthropogenic activity such as industrial processes. It was shown, that the toxicity of groundwater samples was correlated to phenol quantity. The samples were contaminated with up to 195 $mg/m^3$ of phenol and up to 92 $g/m^3$ of phenolic compounds [33]. Developing rapid detection systems for phenol is important for minimising the exposure for this toxic compound as well as its monitoring in the environment, particularly when bioremediation techniques are implemented and on-site methods are required.

Although fungi species are known as organisms able to biotransform and biodegrade phenolic compounds in the environment [34,35], their features are rarely exploited to design novel biosensors for detection of these compounds. Conventional methods for detection of phenolic compounds include spectrophotometry and chromatography, but in principle biosensing technologies offer faster response [36]. Vast majority of the biosensing principles for such detection systems are based on electrochemical activity of laccase derived from *Trametes*, *Aspergillus* and *Ganoderma* genera [37,38]. Amperometric biosensors are relatively matured and have been successfully developed for other types of analytes [39,40]. More recently, determination of phenol was carried out in a microfluidic system using tyrosinase activity – an enzyme which may be present in fungal cells [41] and reveals high sensitivity when immobilised on highly conductive materials [42]. In recent years advanced materials and biosensor designs were employed for detection of phenolic compounds such as photocatalytic systems [43], molecularly imprinted polymers [44] or modified carbon materials for electrodes [45,46].

Using the autofluorescence of natural yeast offers a great advantage due to the simplicity of detection system. In addition, detailed analysis of yeast oscillations can reveal different types of oscillations highlighting the sensitivity of the yeast metabolism to environmental conditions. For example, different oscillations in nicotinamide adenine dinucleotide (NADH) occur when the yeast are fed with different hexoses. The period and amplitude of the oscillations are different when the cells are fed with either D-glucose or D-fructose [47]. The oscillation-based piezoelectric biosensor were demonstrated in the past for *Pleurotus ostreatus* extracts, which exhibited high selectivity and sensitivity towards phenol [48], and more recently, an oscillating mechanisms are exploited in microcantilever-based systems as biosensing platforms [49,50]. In a recent study Pasternak et al. successfully demonstrated detection of organic contaminants in water by an autonomous biosensor where signal frequency is dependent on analyte concentration [51].

The mechanism of glycolytic oscillations of yeast has never been used for developing the biosensor platform. This is the first time, when such novel principle is being proposed and successfully demonstrated towards selective detection of phenol. We show that the frequency and amplitude of glycolytic oscillations in yeast cells can be sensitive to potential pollutants and toxins in the environment. We also show that the oscillation patterns may be distinct for different pollutant triggers and that macroscopic fluorescence signal can be quantified and correlated with the concentration of a target compound. We discuss how such natural living systems may be used as sophisticated biosensors in environmental applications.

## 2. Materials and methods

### 2.1. Yeast culture

The yeast culture protocol was similar to the method described by Richard et al. [52]. *Saccharomyces cerevisiae* BY4742 haploid strain cells were incubated in semiaerobic conditions in SD medium containing 6.7 g/L Yeast nitrogen base (Sigma-Aldrich, Germany), 10 g/L glucose (Sigma-Aldrich, Germany) supplemented with 1 g/L of Bacto-Peptone (Sigma-Aldrich, Germany) as a source of amino-acids. The growth was carried out using rotary shaker at 30 °C and 170 rpm until the glucose depletion point as measured with a glucose oxidase activity assay kit (Abcam, USA). Subsequently, the cells were washed twice in 0.1 M potassium phosphate buffer (pH 6.8) and resuspended using the same buffer to a biomass concentration of 5%. Afterwards yeast cells were starved for 3–5 h at 30 °C and 170 rpm and then stored on ice.

### 2.2. Fluorescence signal measurements

The fluorescence signal of NADH was measured using PTI Quantamaster Fluorimeter (Horiba, Japan). All measurements were carried out in an incubation chamber at 25 °C, continuously agitated using a magnetic stirrer in a 1 cm cuvette. The fluorescence signal was measured using excitation wavelength of 351 nm (10 nm slit) and emission wavelength of 450 nm (10 nm slit). The signal was measured every second, for approximately 2000 s (33 min).

### 2.3. Addition of phenol

The yeast suspension (2 mL) was added to pre-heated cuvette and placed into the incubation chamber to stabilise its temperature. Synchronising the yeast metabolism was achieved by pulse addition of 20 mM glucose and (after 200 s) 3 mM potassium cyanide (KCN) to the cell suspension. This caused inhibition of the aerobic respiration and initiation of macroscopic oscillations of NADH. Once the KCN-mediated oscillations were damped (after 500 s in each test), phenol solution was added to the sample in three separate pulses with 400 s interval. The procedure was repeated for each individual final concentration of phenol ranging from 0.6 to 26.6 mM, meaning that each single concentration was introduced repeatedly to the same yeast sample. Glucose was used as a control and added to the same suspension of yeast at increasing concentrations in range of 4.5 to 19.9 mM.

### 2.4. Data reproducibility and response to nitrobenzene and glucose

The same procedure for phenol addition was used to verify the reproducibility of the signal. Phenol solution (6.6 mM) was added to a population of yeast prepared as above in two separate pulses with 400 s interval. Similar procedure was applied for detecting glucose as a control (20 mM) and nitrobenzene as a compound chemically similar to phenol. Due to unexpected drop of fluorescence intensity when nitrobenzene (0.12 mM) was used, further experiments were carried out by increasing the nitrobenzene concentration to 1.22 and 12.2 mM.

### 2.5. Data analysis

Real time fluorescence data recorded during assay was processed by normalising the signal to the moving average. The normalised signal was processed by Fast Fourier Transformation algorithm (FFT). Frequency spectra of the transformed data were used to determine the frequency and amplitude of the signal for individual pulses of compounds being detected.

## 3. Results and discussion

Subculturing *S. cerevisiae* BY4742 and subsequent starvation of the

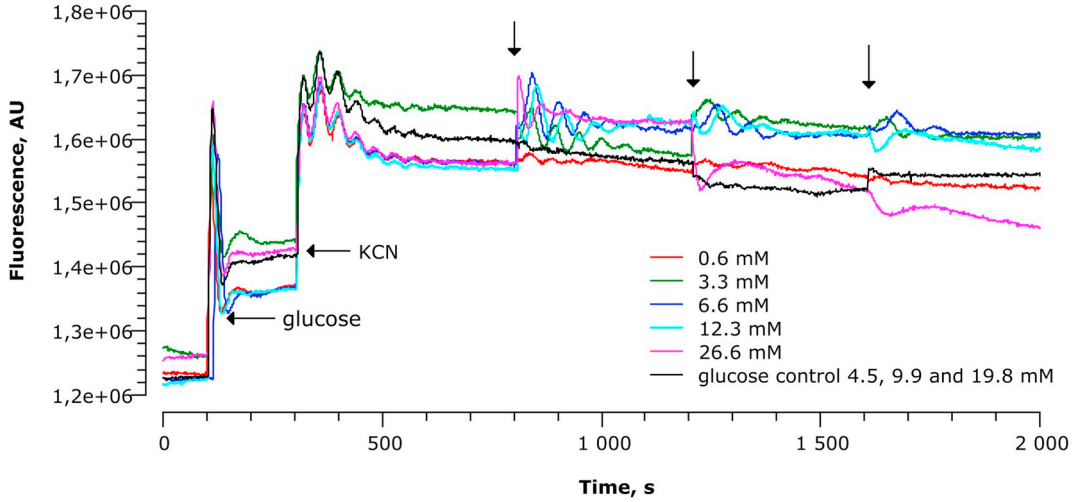


**Fig. 1.** Real time raw NADH fluorescence signal of yeast suspension, showing glycolytic oscillations. Arrows indicate the introduction of different concentrations of phenol and glucose (control).

cells have been conducted within 24 h prior synchronisation. Glucose and KCN addition resulted in synchronisation with consistent NADH oscillations (Fig. 1). KCN was added as an inhibitor of aerobic respiration. Once the KCN-oscillations damped and the signal decayed, the addition of phenol caused an immediate transient response as reflected presence of the oscillations. *Re*-supplementing the phenol to the yeast suspension in 400 s interval caused the renewal of oscillations, and with each phenol pulse the oscillatory signal was decaying. This phenomenon was observed for all of the tested phenol concentrations. When the concentration was equal to 26.6 mM, the second and third pulse caused significant drop of the fluorescence intensity. It is assumed, that reaching this concentration triggered a different type of response of yeast metabolism to a toxic environmental factor. Introducing glucose (4.5 mM) resulted only in negligible oscillations, while increasing its concentration to 9.9 and 19.8 mM did not result in improvement of the signal.

Normalising the signal and fitting the peaks to the point in time where the target compound was introduced to the suspension revealed different oscillatory behaviour and transient responses of yeast metabolism when the cells were treated with toxic phenol (Fig. 2). The most distinctive oscillations and the highest number of individual peaks occurred after the first pulse of phenol, while the least distinctive signal with shorter settling time was observed for the third pulse. Therefore, the most underdamped signal was observed for the first pulse of target compound, where the longest settling time of the signal was observed. Similarly, the overshoot of normalised signal was much higher for the first pulse and decreasing with each pulse. As an example the highest tested concentration of phenol (26.6 mM) caused the overshoot of fluorescence to $7.42*10^4$, $3.56*10^4$ and $7.81*10^3$ for first, second and third phenol pulse, respectively. Similar behaviour was observed when lower phenol quantities were tested.

Furthermore, the real time data suggested that subsequent pulses could have affected the amplitude and frequency of the signal when compared to the first pulse. Signal processing with FFT as shown in Fig. 2 confirmed these findings. The FFT analysis revealed that the highest amplitude of the signal was observed when phenol within 3.3–12.3 mM range was detected, reaching maximum values of $2.36*10^6$ and $9.24*10^5$ observed for 6.6 mM phenol for the first and second pulse, respectively. The highest amplitude values reported for the third pulse were also observed for 6.6 mM ($4.57*10^5$), as well as 3.3 mM ($4.72*10^5$). Due to negligible effect of glucose (control) on oscillations, its concentration was gradually increased with each pulse. For all glucose concentrations (4.5–19.8 mM), the maximum value of oscillation amplitude reported was lower than amplitude observed for target compound. Therefore, 0.6 mM of phenol was determined as the limit of detection (LoD) of the oscillation biosensor. The signal to noise ratio for 0.6 mM (LoD) was calculated as 7.2 and the response of yeast was immediate (below 1 s) after the addition of KCN.

In order to determine, whether the frequency and amplitude of the oscillations can be used to quantify the tested compounds, maximal values of amplitude and the corresponding frequencies were chosen to establish the calibration curve (Fig. 3). Introducing the first pulse of phenol resulted in nearly constant frequency (Fig. 3A), which was observed for the particular maximal amplitude values (Fig. 3B). The highest frequency was observed for 0.6 mM (0.021 Hz), while within the 3.3–26.6 mM range, the frequency was constant and equal to 0.017 Hz. The second pulse of phenol, caused linear response of signal frequency. The highest frequency – 0.020 Hz was observed for the lowest (0.6 mM) phenol concentration. Increasing the concentration resulted in linear decrease of the frequency down to $0.029*10^{-1}$ Hz, when 26.6 mM phenol was used. The regression analysis revealed a very good correlation between frequency and phenol concentration. Determination coefficient reached $R^2 = 0.98$. The data obtained for the third pulse suggested rather polynominal dependence of frequency on concentration, although linear dependence was observed within the lower concentration range (0.6–6.6 mM).

The frequency observed for the glucose control did not seem to be affected by glucose concentration in a predictable manner. Following the fact, that introducing glucose resulted in a very low amplitude of the signal: $9.90*10^4$–$2.08*10^5$, the observed frequencies, which were the highest in this study, were rather the result of steady state error. This behaviour was also observed for the third pulse of phenol, when higher concentration range (12.3–26.6 mM) was used. Therefore it is assumed, that frequency data acquired from signal with amplitude below the certain threshold may be considered as a negative response, where the signal oscillates only within steady state error of transient response. In our study this threshold was determined as $1.39*10^5$.

The oscillatory responses by the synchronised yeast to successive pulses of phenol reveal distinct characteristics in amplitude and frequency. The first pulse of phenol resulted in the highest amplitudes within the whole concentration range in parallel with constant frequency. Therefore, the combined approach of analysing the signal amplitude and frequency would be an appropriate strategy to quantify the target chemical using oscillatory response of yeast autofluorescence. The second pulse resulted in the best quantification of the signal based on frequency. Therefore, the time required to quantify the target chemical was approximately 27 min. The proposed technique is faster and easier to conduct for inexperienced user when compared to

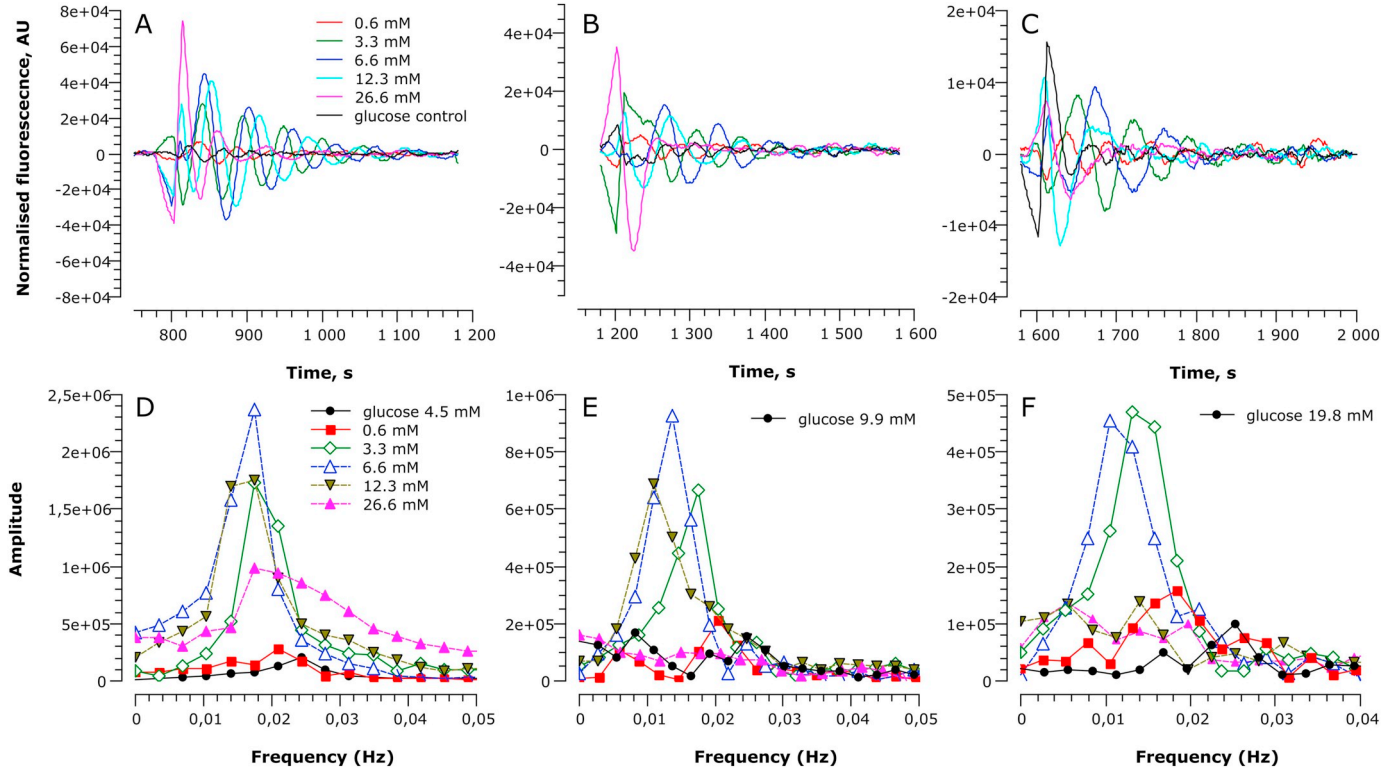


**Fig. 2.** Overlapping normalised fluorescence signal indicating amplitude, frequency and phase shift occurrence (A, B, C) and corresponding FFT frequency spectrum (D, E, F) observed during first (A, D) second (B, E) and third (C, F) pulse of phenol and glucose additions.

the conventional methods, such as high pressure liquid chromatography or gas chromatography. Depending on the preparatory steps these techniques require from 1 to several hours and maintaining large expensive apparatus [53,54].

Validating the reproducibility of the data was carried out using separate batch of yeast (Fig. 4). The control experiments confirmed that glucose had only negligible effect on fluorescence changes. Using higher (20 mM) glucose solution for both pulses revealed only low-amplitude signal in one of the replicates for pulse 2. By using higher glucose concentration, we have confirmed that this principal compound in glycolysis pathway [55] does not give a false-positive biosensor response.

The biosensor specificity was further tested with another derivative of benzene – nitrobenzene. Although nitrobenzene is known for its toxic properties, no oscillations were reported within a concentration range of 0.12 to 12.2 mM. Nevertheless, significant drop of the fluorescence from its baseline was detected, demonstrating another type of yeast response, which is known for other types of luminometric and fluorimetric methods [56,57]. The change in signal intensity was stronger when the highest nitrobenzene concentration was used. It is worth to note that this toxic response was observed for the strain in which no additional genetic alterations have been made to induce such a response. This is in contrast to other yeast-based methods as described by Garcia-Alonso [24]. It opens another way of signal analysis and potential application of the yeast-based biosensor.

The data suggest that NADH oscillation-based biosensor may be

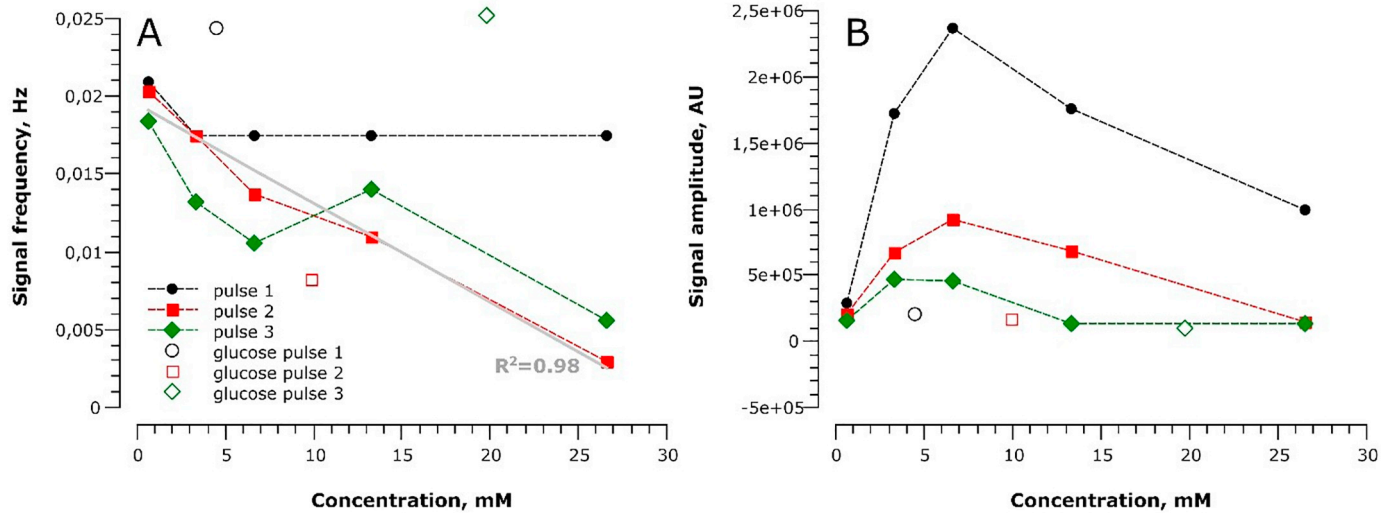


**Fig. 3.** The dependence of frequency (A) and amplitude (B) of glycolytic oscillations on phenol concentration.

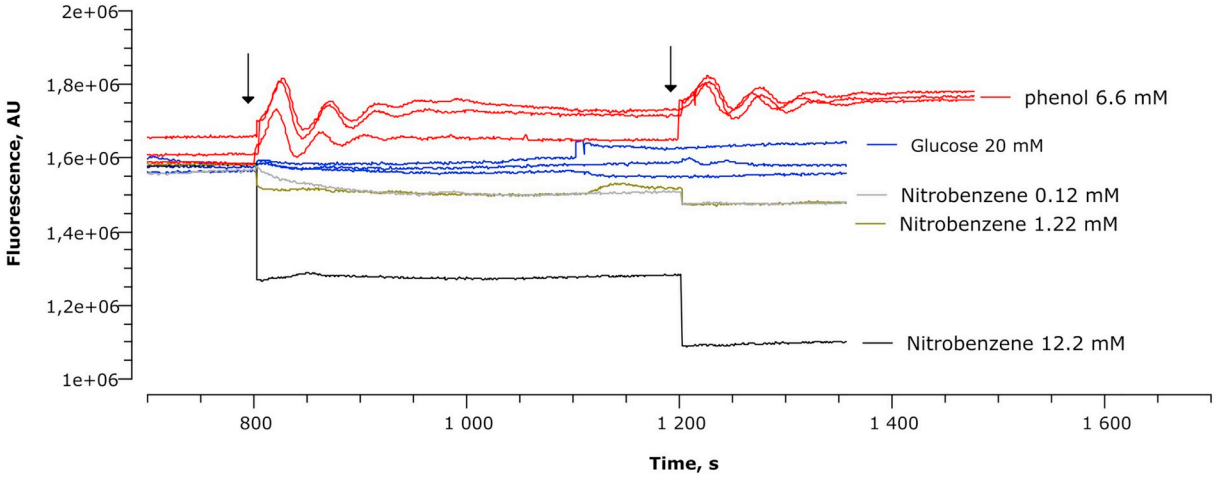


**Fig. 4.** Real time response of yeast cells when detecting various compounds. Arrows indicate addition of particular compound in concentrations as indicated in plotting area.

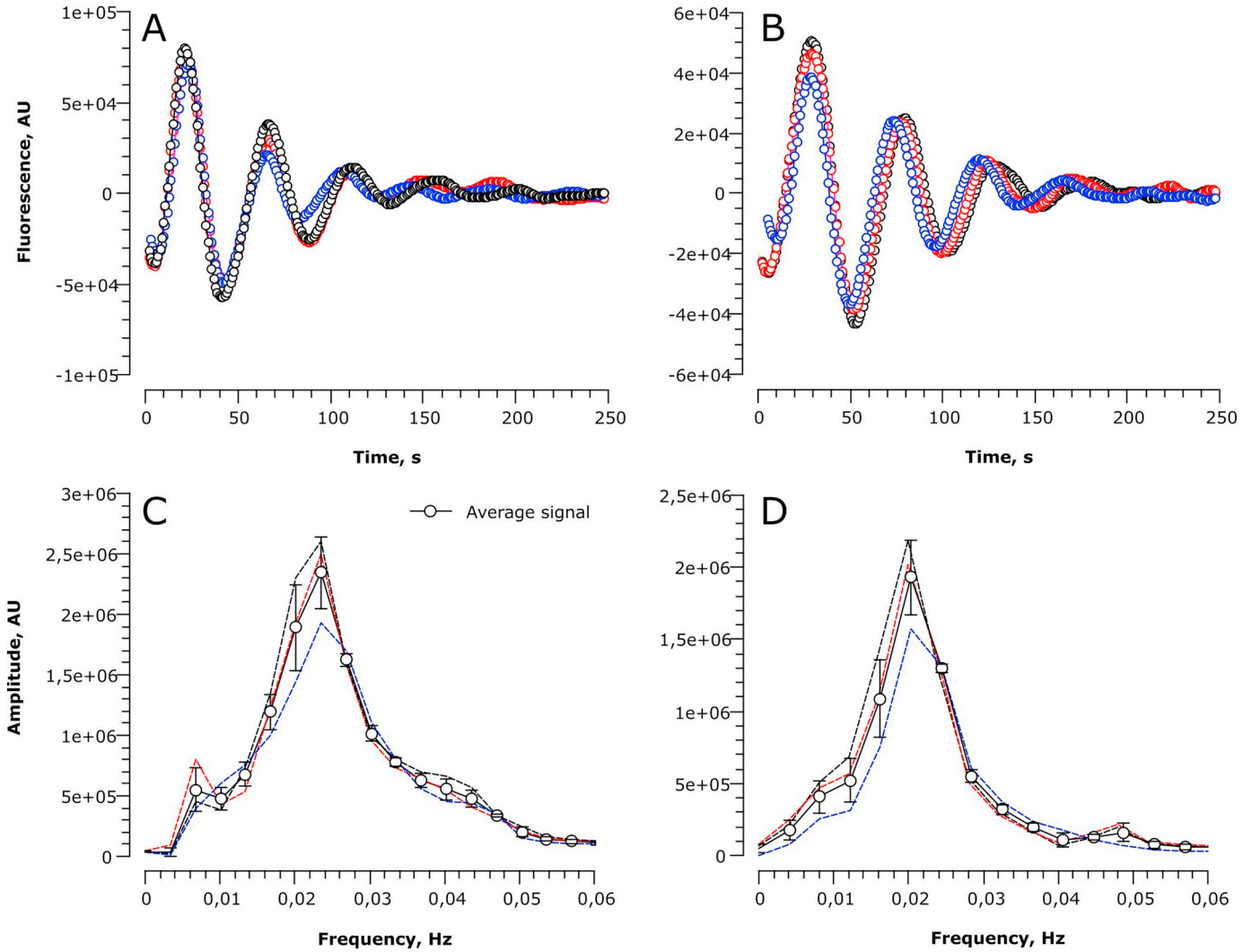


**Fig. 5.** Overlapping normalised fluorescence signal (A, B) and FFT frequency spectrum (C, D) of first (A, C) and second pulse (B, D) of 6.6 mM phenol indicating data reproducibility when using single batch of yeast. Each line represents individual replicate, averaged signal on plots C and D represents mean ± SD.

specific to phenol although further studies, incorporating wider range of chemicals are needed to confirm this statement. The observed selective response of the sensor could have been a result of the synergistic effect of inhibitory and stimulatory reactions that occur in presence of phenol. Phenol can be catabolised by *S. cerevisiae* and result in an increased ethanol yields, it may also induce the accumulation of acetic

acid [58–60]. Together with phenol oxidation which leads to formation of hydroxyl radicals, the acetic acid may be converted to acetaldehyde, which is suspected to be the synchronising agent for glycolytic oscillations [16].

Validation of the signal reproducibility using the same batch of yeast culture revealed that in two subsequent pulses of phenol, the first three peaks (with the highest amplitude) of the signal were almost identical (Fig. 5A, B). The third replicate in series was outstanding when compared to two first replicates. Although third replicate differed from two other replicates, the FFT analysis indicated that the spectrum of this signal observed for both pulses was almost identical (Fig. 5C, D). The only deviation of the spectrum shape was observed at 0.007 Hz. The maximum amplitude of all replicates was recorded at 0.023 Hz for the first pulse and 0.020 Hz for the second pulse reaching $2.34 \pm 0.30*10^6$ and $1.93 \pm 0.26*10^6$, respectively.

Therefore, although the standard deviation of signal amplitude reached 12.8% and 13.4% when comparing maximum values, the frequency at which the maximum amplitude was observed was not affected. Since the phenol quantity was linearly dependent on signal frequency (Fig. 3A), even high differences in amplitude between individual subsequent readings would not affect the sensor accuracy. Nevertheless, the amplitude profile is still important in order to validate the calibration curve and determine whether the amplitude is strong enough not to be considered as a steady state error or noise, as explained previously.

It is also worth to note that in this study, a fluorimeter with a single cuvette reader was used and therefore only one test was allowed at time. Prolonged time between individual readings may have affected the overall signal readings. Such an effect is commonly observed when whole cells are used as receptors. For example, Microtox™ test requires the user to discard the receptor cells after 1–2 h from reconstitution. It is assumed, that introducing high throughput approach, such as 96-weell microplate format to the methodology would have resulted in very consistent signal even when considering its amplitude and improved its overall accuracy. Further studies will also focus on determining the alternative (to cyanide) respiration inhibitor due to the environmental concerns. The earlier studies reported, that it is possible to obtain sustained glycolytic oscillations by sparging an inert gas through the yeast suspension without the need of using cyanide [17].

## 4. Conclusions

In this study, for the first time we have demonstrated the proof of concept of using natural yeast autofluorescence oscillations for biosensing purposes. By using this mechanism we were able to detect phenol with the response linearly correlated to the phenol concentration. The linear response was demonstrated using frequency of the signal, while amplitude was used to validate the signal strength. The oscillatory biosensing mechanism was not sensitive to other tested compounds – glucose and nitrobenzene. Nitrobenzene caused the decrease of fluorescence after each pulse, indicating another type of yeast response to the toxic compounds which could be exploited for the biosensing purposes. The time required to perform the test was estimated to 27 min, which makes it promising for the further development. The method is easy to conduct and may be possible using other types of yeast strains including locally sourced strains.

With increasing interest in exploiting the complexities of living systems for use in manufacture, there is also interest in expanding and integrating living systems into the built environment [61]. Living biosensors that could specifically detect certain hazardous toxins are consistent with this vision and expand what is possible using living cells as technology. However the proof of principle demonstration reported here is only the first step. For example, trace amounts of potassium cyanide are required for synchronisation of the cells, and therefore its use remains a main drawback of this new method. Further development of cell synchronisation will be required to replace the cyanide with better alternatives.

## Conflict of interest

None.

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