

1 **Indicator of harmful cyanobacteria using an odor-binding protein-**
2 **based biosensor for β -cyclocitral detection**

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Abstract:

β -Cyclocitral, a compound that causes a hay/woody/tobacco-like odor, is closely associated with cyanobacterial cell density and can serve as a biomarker for cyanobacterial blooms. Biosensors utilizing odor-binding proteins (OBPs) have been developed for detecting chemicals exhibiting odors; however, their performance in environmental water samples has been rarely reported. In this study, an OBP-based electrochemical biosensor was developed for the detection of β -cyclocitral by immobilizing human OBP2a on a gold screen-printed electrode via nitrocellulose membrane. 1:5 mm²/μL nitrocellulose membrane/methanol mixture solution and 100 μg/mL OBP2a were selected as the optimal concentrations for OBP2a immobilization. The optimized sensor showed concentration-dependent characteristics in the range of 0.1-1000 μg/L. The performance of OBP2a-based biosensor was first comprehensively evaluated. Compared to 10 typical odorants in drinking water, the biosensor showed a relatively higher affinity for β -cyclocitral. The biosensor had a good detection accuracy and precision in real water samples, with recoveries of 119.86% and 115.31% in dechlorinated tap water and raw water samples, respectively. It maintains good stability when stored at -20 °C, with a current decline of 5.3% on the 6th day. These results demonstrate that OBP2a-based biosensors show promising potential for detecting β -cyclocitral, providing a useful tool for early warning of cyanobacterial blooms.

Keywords: Drinking water; Odor-causing compounds; Odor-binding proteins; Biosensors; Cyanobacterial bloom

1 Introduction

Harmful cyanobacterial blooms have become increasingly prevalent in recent decades, posing significant threats to aquatic ecosystems and human health. Cyanobacterial outbreaks cause a sharp decline in dissolved oxygen levels, disrupting the balance of aquatic ecosystems [1], but also release a large number of secondary metabolites, e.g., microcystins, odor-causing compounds [2, 3]. The rapid rise of harmful cyanobacterial blooms could raise serious alarms in communities [4, 5]. For example, an outbreak of blue-green algae in Lake Erie, United States (2014), has led to high levels of microcystin toxins preventing approximately 500,000 residents from accessing safe drinking water for two days [6]. In China, cyanobacterial bloom-induced odor incidents in Lake Taihu in 2007 and Lake Chaohu in 2015 affected the quality of local drinking water and even forced temporary shutdowns of water supplies [7, 8]. Similar incidents have been reported in Ontario, Canada [9], the Mississippi River in the U.S. [10], and Setumo Reservoir in South Africa [11].

According to the World Health Organization (WHO) guidelines, cyanobacterial cell densities below 20,000 cells/mL ($\sim 10 \mu\text{g/L}$ chlorophyll a) and above 100,000 cells/mL ($\sim 50 \mu\text{g/L}$ chlorophyll a) are classified as low and high risk for recreational water, respectively [12]. In addition, the concentration of chlorophyll a in slightly eutrophic surface waters is less than $10 \mu\text{g/L}$ based on the *Environmental Quality Standards for Surface Water* (GB 3838–2002) of China. Regarding drinking water safety, the WHO has established a maximum permissible limit of $1 \mu\text{g/L}$ for

61 microcystin-LR (MC-LR) [13]. To effectively monitor harmful cyanobacterial blooms,
62 detection modalities including cell counting, chlorophyll a measurement,
63 Phycobiliprotein detection, qPCR, remote sensing, and semi-quantitative enzyme-
64 linked immunosorbent assay (ELISA) were developed for algal monitoring [14, 15].
65 These techniques are typically limited by the timeliness of the data and the complexity
66 of disturbances in the water [16]. Chlorophyll a primarily reflects total algal biomass
67 and is prone to interference from non-cyanobacterial species [17]. Phycobiliprotein
68 detection often requires complex extraction procedures and is susceptible to
69 degradation, making it less suitable for rapid, non-destructive analysis [18, 19].
70 Cyanotoxins such as Microcystis are often released predominantly during the later
71 stages of bloom development or cell lysis, which may constrain their effectiveness as
72 early-warning indicators [20]. The development of rapid and sensitive detection
73 techniques for monitoring cyanobacteria is of great practical significance.

74 β -Cyclocitral (2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde), a compound
75 characterized by its distinctive hay/woody/tobacco-like odor, has been widely detected
76 as a taste and odor (T&O) compound in eutrophic water sources and drinking water
77 [21]. As a key allelopathic agent, β -cyclocitral is reported to be significantly associated
78 with the growth of Microcystis, and the production by *Microcystis* sp. was 2-10 fg/cell
79 [22, 23]. The emission of β -cyclocitral can reach a high level during cyanobacterial
80 blooms in summer. For example, β -cyclocitral was detected at about 284 ng/L in Lake
81 Taihu, and 538 ng/L in Western Lake Chaohu, China [23, 24]. The elevated

concentrations, coupled with its cyanobacteria-specific production pathway, position β -cyclocitral as a promising indicator compound for early warning and monitoring of harmful cyanobacterial blooms.

Gas chromatography-mass spectrometry (GC-MS) and gas chromatography-triple quadrupole tandem mass spectrometry (GC-MS/MS) have been widely used for the analysis of β -cyclocitral in drinking water [25, 26], which offer high sensitivity, high resolution, and broad applicability for qualitative and quantitative detection [27]. However, their use for rapid on-site testing is challenging due to the complex sample preparation and equipment requirements. In contrast, electrochemical sensors have been recognized as a successful tool for the detection of whole bacteria, bacterial metabolites, and various chemical compounds, owing to their simplicity, low cost, and short analysis time compared to classical methods [28, 29]. Recently, based on biosensing elements such as olfactory cells, olfactory receptors (ORs), and odor-binding proteins (OBPs), types of electrochemical biosensors including field effect transistor (FET) [30], and electrochemistry (EC) [31] have been developed, enabling rapid, sensitive detection of various odorous compounds [32]. Among various bio-sensitive elements, OBPs are an ideal sensor material for the detection of chemicals exhibiting odors due to their advantages of easy production, sufficient stability, and high affinity to specific odorants [33].

OBPs function by providing hydrophobic pockets to selectively bind odorant molecules and mediating their delivery to olfactory receptors [34]. Previous studies

have demonstrated that OBP2a exhibits remarkable binding affinity for aldehydes through hydrogen bonds between Lys112 and the carbonyl groups of aldehydes [35]. According to this characteristic, various OBP2a-based biosensors have been developed. Lu et al. (2015) constructed a nanopore array electrochemical sensor using OBP2a to realize the detection of benzaldehyde and docosahexaenoic acid at the ng/L level in ultrapure water [36]. Li et al. (2024) reported OBP2a combined with electrochemical impedance detection for the sensitive and selective detection of aldehyde in ultrapure water with detection limits of $<10^{-11}$ mol/L [37]. However, critical knowledge gaps persist regarding the applicability of these biosensors in environmental water samples, particularly in terms of measurement precision and accuracy when analyzing complex aqueous matrices.

This study describes the construction of an electrochemical biosensor using OBP2a for the detection of β -cyclocitral to rapidly evaluate the progress of cyanobacterial blooms. The biosensor fabrication involved immobilization of OBP2a onto screen-printed gold electrodes through electrostatic adsorption. Detection was achieved via cyclic voltammetry, capitalizing on the high binding specificity of OBP2a for aldehyde groups in β -cyclocitral molecules. In addition, the selectivity, precision and accuracy, and method stability of the established biosensor were first systematically evaluated. This study provides a novel approach for real-time monitoring of β -cyclocitral in environmental water samples, which could serve as a promising tool for the early warning of harmful cyanobacteria blooms.

2 Materials and methods

2.1 Expression and purification of OBP2a

The active recombinant OBP2a was cloned from the full-length cDNA of humans in Cellregen Biotech Co., Ltd (China). Briefly, a DNA sequence encoding the OBP2a was cloned into the expression vector pET30a (+) and transferred to the Escherichia coli strain BL21 (DE3) [38]. The culture was induced for expression with Isopropyl- β -D-thiogalactopyranoside (IPTG), and the supernatant was obtained after ultrasound and centrifugation [39]. Supernatant was purified by Ni affinity chromatography, and the final product was obtained by dialysis. The purity of OBP2a was greater than 90% by SDS-PAGE electrophoresis (Fig. S.1). The molecular weight of the OBP2a was predicted to be 19 kDa, and the concentration was assayed to be 400 μ g/mL by BCA. The protein was suspended in a buffer of NaHCO₃ (20 mM, pH 8.3) and stored at -20 °C.

2.2 Chemicals and materials

All odorant standards were obtained from Beijing Ruizhihanxing Technology Co. (China) at a minimum purity of 95% (most > 99%, see detailed information in Table S.1). Due to the low solubility of the odorant in water, the odorant standard was prepared as a 100 mg/L stock solution using a 1% aqueous methanol solution (chromatographic-grade, Fisher Scientific Co. (China)) and stored at 4 °C. Nitrocellulose membrane (NC) for protein immobilization, with a membrane pore size of 0.45 μ m and a diameter of 47 mm, was purchased from Whatman (UK). Phosphate

buffer (PBS, 50 mmol/L, pH = 7.4) and Bicinchoninic Acid Assay (BCA) protein assay kit were purchased from Beijing Solarbio Science & Technology Co. Ltd (China). Analytical Grade Potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium chloride (KCl), and ethanol absolute were purchased from Sinopharm Chemical Reagent Co., Ltd. Ultrapure water ($> 18 M\Omega\text{ cm}$) was produced with a Milli-Q water purification system.

Screen-printed electrodes (SPE) provided by Poten Technology Co., Ltd. (Weihai, China), were used for all the electrochemical experiments. SPE was prepared by printing conductive ink on a $34\text{ mm} \times 12\text{ mm}$ polyimide substrate. The electrode system consisted of a silver electrode as the reference electrode, a carbon electrode as the counter electrode, and a gold electrode (4 mm in diameter) as the working electrode.

2.3 Preparation of biosensor immobilized with OBP2a

Prior to surface modification, the electrodes were cleaned with anhydrous ethanol and ultrapure water to eliminate organic residues and then blown dry using nitrogen. NC membrane solution was prepared by dissolving the membrane in chromatographic-grade methanol. 5 μL of NC membrane/methanol mixture was added dropwise to the surface of the working electrode. Because of the volatility of methanol, a thin layer of NC membrane was left on the working electrodes after 10 min at room temperature. The thawed OBP2a was dissolved in NaHCO_3 , and 15 μL of OBP2a solution was injected uniformly on the NC membrane and incubated at 25 $^\circ\text{C}$ for 2 h [40]. The immobilization of OBP2a on the electrode surface was achieved by electrostatic and

hydrophobic interactions of the NC membrane [41, 42]. Finally, unbound OBP2a was removed by rinsing with PBS, and the electrode with OBP2a immobilization was stored at 4 °C. The schematic diagram of OBP2a immobilized on SPE by NC membrane was shown in Fig. 1a. The preparation of biosensors was optimized by the changes in the ratio of NC membrane area to methanol volume (1:10, 1:5, 1:3 mm²/μL), and the amount of protein loaded at the electrode depends (OBP2a concentrations of 50 μg/mL, 100 μg/mL, and 200 μg/mL).

The surface morphologies of the electrode before and after OBP2a immobilization were evaluated by Scanning electron microscope SU8000 (SEM, Hitachi, Japan) in secondary electron mode at an accelerating voltage of 3 kV. The chemical composition of the micro-regions on the electrode surface was analyzed by an Energy Dispersive Spectrometer QUANTAX XFlash 6|100 (EDS, Bruker Nano GmbH Berlin, Germany) at an accelerating voltage of 15 kV.

2.4 Sample analysis

All the electrochemical experiments were performed on an electrochemical workstation (PGSTAT302N, Metrohm Autolab). Cyclic voltammetry was employed to record the redox response at the electrode surface during a single complete triangular potential scan [43-45]. The peak current (I_p) was extracted using the 'Peak Search' within the NOVA software (version 2.1.7), where a linear baseline mode (free cursor) was applied to manually define the onset and offset points of each redox peak, ensuring the effective subtraction of the non-Faradaic capacitive current. The potential range was

set from 0.7 V to -0.3 V, and the scanning rate was 50 mV/s. A 5 mmol/L mixed solution of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ (1:1, containing 0.1 mol/L KCl) was configured as a redox pair for electrochemical measurements [43]. For electrochemical measurements, 100 μ L of water sample (or odorant standard solutions) was thoroughly mixed with 100 μ L of electrolyte solution and then added dropwise to the surface of the SPE for cyclic voltammetry detection. After each measurement, the residual electrolyte solution was rinsed with ultrapure water, and the electrode was immersed in PBS for 5 min to remove residual odorants. The electrochemical measurements were performed at room temperature (25 ± 2 °C) with three replicates for all odorants.

The analysis of the organic matter composition of water samples was performed on a fluorescence spectrophotometer F-7000 (Hitachi, Japan) in a 1 cm light path quartz cuvette. The parameters were set as follows: excitation wavelength of 200-500 nm, emission wavelength of 250-550 nm, slit width used for excitation and emission were 10 nm, PMT voltage of 400 V, and scanning speed of 12000 nm/min. The results were analyzed using the regional integration method for data analysis [46]. The analysis of coexisting odorants in the environmental water samples by the GC-MS/MS method was described in detail in a previous study [25].

2.5 Assessment of biosensor performance

In the experiment, β -cyclocitral at concentrations of 0.1 μ g/L, 1 μ g/L, 10 μ g/L, 100 μ g/L, and 1000 μ g/L was detected with OBP-modified SPE, for recording the cyclic voltametric curves, respectively. The detection limit (LOD) and the quantification limit (LOQ) are calculated based on the following formulas: $LOD = 3\sigma/S$, $LOQ = 10\sigma/S$,

where σ represents the standard deviation of the current response for 11 consecutive blank measurements, and S is the slope of the calibration curve for the initial low-concentration range (0.01 $\mu\text{g/L}$ –0.1 $\mu\text{g/L}$) [47]. To prove that the current change during electrochemical detection was caused by the binding of β -cyclocitral to OBP2a, bare SPE unmodified by OBP2a was set up as a control group, and the detection was carried out under the same experimental conditions. A concentration-dependent curve was determined by a non-linear regression between odorant concentration and the corresponding change in peak current using Origin 2021 software. The fitting model is “Hill equation”, describing the relationship between the concentration of the compound adsorbed to the binding site and the occupied fraction of the binding site [48]. The equation is expressed as: $Y = V_{\max} * X^n / (K^n + X^n)$, where X is the detected ligand concentration, $V_{\max}$ represents the predicted maximum specific binding, K denotes the ligand concentration at which half occupancy is produced, and n is the Hill coefficient, which is used to describe the synergistic nature of receptor-ligand binding. When $n > 1$, it indicates a positive synergistic relationship between the two; when $n = 1$, it indicates a non-synergistic reaction, and when $n < 1$, it indicates a negative synergistic relationship [49].

To assess the specificity of the biosensor, we analyzed 10 odorants representing various odor types at a concentration of 100 $\mu\text{g/L}$ (Table S.1). β -Cyclocitral possesses both an aldehyde functional group and a terpenoid structure. Therefore, five structurally similar aldehydes (including hexanal, heptanal, 2-octenal, decanal, and undecanal) and two terpenoid compounds (2-MIB and geosmin) were selected as potential interferents. In addition, two thioethers, which are widely detected odor-causing compounds in drinking water sources in China, were also included, as listed in Table S.1. Pymol

software is used to characterize the binding mode of OBP2a and odorants.

β -Cyclocitral at three concentrations of 0.2 $\mu\text{g/L}$, 5 $\mu\text{g/L}$, and 50 $\mu\text{g/L}$ spiked in ultrapure water was detected three times to verify the reproducibility of the biosensor. The modified electrode could be reused for multiple measurements. After each measurement, the electrolyte was rinsed with ultrapure water and then immersed in PBS for 5 min to remove residual odorants. To evaluate the recoveries in different water backgrounds, β -cyclocitral was spiked into ultrapure water, tap water, dechlorinated tap water (obtained by sunlight exposure for 8 h to remove residual chlorine), and raw water (Taihu Lake, China) at a concentration of 10 $\mu\text{g/L}$. The recovery rate was calculated as: $\text{Recovery (\%)} = C/C_0 \times 100\%$, where C represents the measured concentration of the spiked sample (substitute the peak current (ΔI) variation value back into the standard curve to calculate the concentration of β -cyclocitral), and C_0 represents the theoretical spiked concentration.

The storage stability of the OBP2a-based biosensor when stored at 4 °C and -20 °C. Sensors prepared from the same batch were placed individually in sealed bags and tested on the 3rd day, 6th day, and 9th day. The sensor response was assessed by measuring ΔI toward β -cyclocitral at a concentration of 10 $\mu\text{g/L}$, with each measurement performed in triplicate ($n = 3$).

3 Results and discussion

3.1 The immobilization of OBP2a

SPE and glassy carbon electrode (GCE) are widely used as electrode materials for

constructing electrochemical biosensors due to their reproducibility and low cost [50]. Compared with traditional electrodes (e.g., GCE), SPE has the advantages of portability, low sample consumption, easy mass production, and avoidance of a time-consuming cleaning process [51-53]. Common protein immobilization techniques include physical adsorption, covalent cross-linking, and self-assembled monolayer. Physical adsorption relies on non-covalent interactions between the protein and the material surface [54]. Its main advantages are the simplicity and rapidity of the procedure, and the absence of chemical modification, which helps preserve the native structure and active sites of the protein. Covalent cross-linking and self-assembled monolayer approaches generally provide improved long-term stability [55]. However, these methods often involve multi-step chemical reactions, longer preparation times, and higher costs. Moreover, the introduction of chemical modifications may alter functional groups on the protein, potentially reducing its binding affinity or biological activity. Thus, physical adsorption onto a NC membrane was selected in this study for biosensors designed for rapid and low-cost detection.

Fig. 1b shows the ΔI for the detection of 10 $\mu\text{g/L}$ β -cyclocitral using SPE-based biosensors modified with different ratios of NC membrane area to methanol volume and varied OBP2a concentrations. Increasing the ratio of the NC membrane area/methanol volume produces greater potential for protein adsorption, which leads to more OBP2a binding to odorants and greater current changes [56]. The ΔI value was $7.79 \pm 0.93 \mu\text{A}$, at the highest ratio (1:3) and the concentration of OBP2a (200 $\mu\text{g/mL}$),

which was significantly lower compared to the other experimental groups. This could be explained that the electrode surface is loaded with more NC membrane and OBP2a, which showed poor conductivity, leading to hindered interaction between the sensing region and the external environment [57]. In contrast, the condition with 1:5 mm²/μL NC membrane/methanol mixture and 100 μg/mL OBP2a exhibited a better current response (ΔI , 12.00±0.21 μA), making it suitable as the optimal concentration for protein immobilization. Moreover, the relatively low standard deviations indicate that the optimization results are robust and reproducible.

Cyclic voltammetry was used to characterize the changes in dielectric properties at the electrode/electrolyte interface of OBP2a-modified SPE. Compared with the bare SPE, the OBP2a-modified SPE showed a significant decrease in the peak current and an increase in the potential difference between the two peaks (Fig. 1c). The OBPs are bio-materials with low conductivity, which inhibits electron transmission at the electrode surface [57, 58]. With the impedance increased, peak currents for oxidation and reduction were reduced by approximately 50 μA compared to bare SPE.

As indicated by SEM (Fig. 1d), the surface of the bare gold electrode was dense and flat gold particles. After volatilization of the methanol, the NC membrane-modified electrode surface becomes rough, and porous channels consisting of randomly oriented fibers can be observed (Fig. 1e), which is similar to the morphology in the previous study [56]. After the protein solution was added to the electrode surface, OBP2a was embedded into the pore by physical adsorption (Fig. 1f). EDS spectroscopy was

performed to analyze the mass percentage of each element before and after the protein immobilization (Fig. 1g and Fig. 1h). The results showed that the bare electrode surface was mainly composed of Au and C elements, while the proportions of C, N and O increased significantly after immobilizing OBP2a. The above results confirm that OBP2a has been successfully immobilized on the electrode surface.

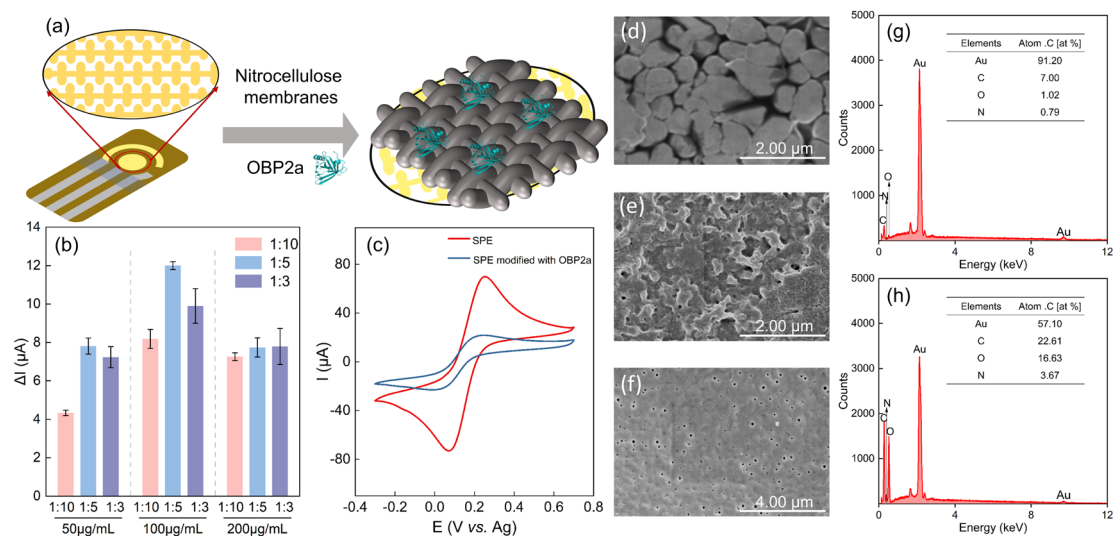


Fig. 1. OBP2a immobilized on SPE by NC membranes (a); The ΔI detected by biosensors modified with different ratios of NC membrane area to methanol volume (1:3, 1:5, 1:10) and different concentrations of OBP2a (50 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$, 200 $\mu\text{g/mL}$) ($n=3$) (b); Cyclic voltammograms of OBP2a before and after immobilization at the SPE (c); SEM images of SPE (d) modified with NC membrane (e) and OBP2a (f); EDS energy spectroscopy of bare electrode (g) and OBP2a immobilized electrode (h).

3.2 Electrochemical sensing of β -cyclocitral

Fig. 2a demonstrates the cyclic voltammetry curves of β -cyclocitral using the OBP2a-based biosensor at concentrations from 0.1 $\mu\text{g/L}$ to 1000 $\mu\text{g/L}$. The current values of the oxidation and reduction peaks decreased with the increase of the

concentration of β -cyclocitral. In contrast, the bare SPE showed no obvious concentration-related trend, as shown in Fig. S.2. The binding of β -cyclocitral to OBP2a was reversible, and the fitted Hill equation was shown as follows:

$$\Delta I = \frac{20.65 * X^{0.18}}{(2.36 * 10^{-7})^{0.18} + X^{0.18}}$$

The fitting results showed that the amount of the change in the peak current rose with the increase of odorant concentrations (Fig. 2b). The slope (S) of the low-concentration calibration curve is 16.65, and the standard deviation (σ) of the blank values is 0.19, the LOD and LOQ were calculated to be 0.03 $\mu\text{g/L}$ and 0.10 $\mu\text{g/L}$, respectively. The Hill coefficient of 0.18 < 1, characterizes a negative synergistic relationship of receptor-ligand binding. Physically, the exceptionally low Hill coefficient reflects the inherent heterogeneity of the biosensing interface. Protein immobilization on the electrode surface could lead to non-uniform orientation, resulting in a heterogeneous population of binding sites [59]. Furthermore, the conformational change of OBP2a upon binding with β -cyclocitral may sterically hinder adjacent immobilized proteins, thereby reducing the accessibility of neighboring binding sites. Consistent with our findings, Lu et al. (2017) reported a Hill coefficient of 0.27 in a similar immobilized protein system [57].

OBP2a binds to β -cyclocitral molecule via hydrophobic interactions and hydrogen bonding, causing conformational changes in the odor-binding proteins [35]. This molecular recognition process causes the amino acids in the protein backbone to adjust their positions and achieve a new equilibrium, ultimately modifying both the

333 polypeptide topology and ligand-binding cavity characteristics [60, 61]. This leads to
334 an increase in impedance at the electrode surface, which hinders the rate of electron
335 transfer and reduces the peak current [34]. Although the calculated LOD (0.03 $\mu\text{g/L}$)
336 for the biosensor is slightly higher than the high sensitivity offered by GC-MS/MS
337 (typically below 0.01 $\mu\text{g/L}$), it remains well below the observed β -cyclocitral levels
338 during cyanobacterial bloom events as well as the reported odor threshold concentration.
339 To further improve sensitivity, strategies such as electrode surface modification, signal
340 amplification, and sample pre-concentration can be employed to reliably detect β -
341 cyclocitral at sub-100 ng/L levels, thereby enhancing early-warning performance. In
342 contrast to GC-MS/MS, which necessitates cost-intensive instrumentation, tedious
343 sample pre-treatment, and specialized personnel, the developed biosensor offers a short
344 analytical turnaround time (minutes vs. hours) and minimal operational costs. Table 1
345 summarizes the different types of biosensors for the detection of odorants in water
346 samples. As indicated, similar responses were reported for the binding of OBPs from
347 honeybees to pheromones and OBPs from oriental fruit fly to semiochemicals, such as
348 isoamyl acetate, β -ionone, and benzaldehyde. The biosensor in this study showed a
349 relatively higher sensitivity compared to other OBP-based biosensors, but a lower
350 sensitivity (LOD 0.03 $\mu\text{g/L}$) compared to olfactory protein-based biosensors (≤ 0.01
351 $\mu\text{g/L}$). The stability of these reported olfactory protein-based biosensors in
352 environmental water matrices remains unverified.

353 Table 1 Summary of different types of biosensors for the detection of odorants in

354 ultrapure water

Type of biosensor	Biosensing elements	Target	Linear range (µg/L)	Detection limit (µg/L)	References
Electrochemical (CV)	Human OBP2a	β-cyclocitral	0.1-1000	0.03	This study
Localized surface plasmon resonance (LSPR)	Acer-ASP2 from honeybee	β-ionone	1.92-192300	0.005	[62]
Surface Acoustic Wave (SAW)	Bovine OBPs	Octenol	68-320	14.7	[63]
		carvone	55-491	11.1	
		Isoamyl acetate	13-13000	4.30	
Electrochemical impedance spectroscopy (EIS)	BdorOBP2 from oriental fruit flies	β-ionone,	19.2-19200	11.9	[64]
		benzaldehyde	10.6-10600	8.91	
Single Walled Carbon Nanotube (SWCNT)	probe-DNA	Oxytetracycline	10-75	1.13	[65]
Quartz crystal microbalance (QCM)	peptide	3-Methyl-1-butanol	0-361	7.2	[66]
		1-hexanol	0-418	7.5	
Field-effect transistor (FET)	hOR51S1, hOR3A4	Geosmin	0.01-100	0.01	[30]
		2-MIB	0.01-100	0.01	
Electrophysiology	GPROR2	Benzaldehyde	10.6-106120	Not estimated	[67]

355 Note: SWCNT sensors have recoveries of 86.4%- 95.8% for spiked ambient water

356 samples (wastewater, tap water). Recoveries and storage stability for other types of

biosensors are not reported in the literature.

3.3 Analytical performance of biosensor

Specificity. Fig. 2c shows the ΔI of the OBP2a-based biosensor for the detection of 10 odorants. A larger ΔI indicates a stronger binding affinity between OBP2a and the corresponding odorant [57]. As indicated, the ΔI for the 10 odorants ranged from 4.03 μA to 15.41 μA , among which four aldehydes, including β -cyclocitral, heptanal, hexanal, and 2-octenal, showed ΔI higher than 10 μA . In particular, aldehydes had overall higher ΔI than other types of odorants, with the largest ΔI of aldehydes being for β -cyclocitral (ΔI , $15.41 \pm 0.34 \mu A$), followed by heptanal (ΔI , $13.68 \pm 0.35 \mu A$) and 2-Octenal (ΔI , $12.74 \pm 1.06 \mu A$). Despite the highest response observed for β -cyclocitral, interference from structurally similar aldehydes can occur, potentially affecting its selective detection. 2-MIB, geosmin, and the two thioethers have been widely detected in major watersheds in China. Although 2-MIB and geosmin belong to the same terpenoid class as β -cyclocitral, their interference was substantially lower.

Our previous studies suggest that Lys112 within the hydrophobic pocket of OBP2a showed good binding affinity by interacting with aldehydes through hydrogen bonding (see the binding mechanism depicted in Fig. 2c (embedded plot)) [35]. Meanwhile, the hydrophobicity of aldehydes appears to significantly influence their interaction with OBP2a, exhibiting a positive trend that aligns with the biosensor responses. Table S.2 summarizes the calculated dissociation constants (K_i , calculated by Fluorescent Competitive Binding Assays) and molecular docking results with 10 odorants [30].

While lower K_i values and docking scores theoretically indicate stronger binding potential, correlation analysis was conducted to link these computational predictions to the sensor responses. The experimental ΔI showed a moderate correlation with the calculated K_i values ($n = 10$, $r = -0.64$, $p < 0.05$, Fig. S.3a), whereas a stronger negative correlation was observed with the docking scores for aldehydes ($n = 6$, $r = -0.75$, Fig. S.3b). These findings suggest that integrating Fluorescent Competitive Binding Assays with molecular docking could serve as a supportive predictive framework for rationalizing the specificity of odorant-binding proteins and assisting in the structural screening of candidate proteins for biosensor development.

Although OBP2a exhibits binding specificity for aliphatic aldehydes, it still has limitations when co-occurring with other odorants. This might be closely related to the structural fitness of the OBP2a binding pocket and the target molecule. For example, Lu et al. (2017) found that OBP2a showed excellent sensitivity to long-chain fatty acids [57]. Peng et al. (2024) also showed that OBP2a is more sensitive to citronellal and lily aldehydes than to aliphatic aldehydes, probably due to their relatively large molecular weight and heterocyclic molecular structure [37]. In contrast, Briand et al. (2002) revealed the reverse mechanism through experiments with brominated fatty acids: the spatial site-blocking effect of bromine atoms or electrostatic repulsion with lysine residues at the edge of the pocket significantly weakens the binding capacity to OBP2a [68]. Therefore, modification of the binding pocket by genetic engineering, such as introducing amino acid mutations to enhance the binding force to the target molecule

or interfering with the binding of the protein to the impurity molecule, might become an effective way to improve protein selectivity in the future. Besides, the development of multiplexed biosensor arrays incorporating heterologous OBPs enables systematic optimization of detection specificity through cross-reactive element coordination and multivariate signal processing algorithms.

Precision and accuracy. In Fig. 2d, β -cyclocitral was detected at three concentration levels in ultrapure water. The recoveries were in the range of 91.69%-116.82% for with RSDs of 6.29% to 13.54%, which met the general chemical analysis acceptable values of 70%-120% for recovery and < 20% for RSD (Table S.3). The performance variability is primarily attributed to the non-uniform orientation of immobilized OBP2a proteins and the cumulative errors inherent in the multi-step electrode modification process. To further enhance reproducibility, future efforts could focus on integrating automated fabrication techniques, such as inkjet printing, to ensure a more homogeneous and standardized sensing interface. The detection of β -cyclocitral in different water matrix backgrounds is shown in Fig. 2e. The ΔI detected in tap water, dechlorinated tap water, and raw water were $19.18 \pm 1.55 \mu A$, $13.83 \pm 0.95 \mu A$, and $13.80 \pm 0.92 \mu A$. The ΔI in tap water increased by 140% relative to that measured in ultrapure water ($13.67 \pm 0.47 \mu A$), likely due to the presence of free chlorine. Kongkaew et al. (2024) found that free chlorine can undergo a reduction reaction on SPE surfaces with high sensitivity [69]. The reduction peak of free chlorine and the signal of the target odorant might be superimposed, resulting in an amplified detection current. The

significant reduction of the matrix effect in the dechlorinated tap water samples confirmed this conjecture.

The calculated recoveries of dechlorinated tap water and source water samples were 119.86% and 115.31% with RSDs of 8.38% and 10.62%, respectively. The slightly higher recoveries observed in the actual samples suggest the presence of matrix interference, which can be attributed to two main factors. First, the presence of proteinaceous substances and microbial metabolites (see organic matter fractions in Fig. S.4) in the matrix [46] may contribute to redox peaks [70]; Second, coexisting odorants such as benzaldehyde (82.80 ng/L), bis(2-chloro-1-methylethyl) ether (81.57 ng/L), and decanal (52.68 ng/L) were detected in the raw water samples (Table S.4), and their synergistic effect may slightly enhance the current signal [70]. Although competitive binding may occur among coexisting aldehydes, β -cyclocitral was still the predominantly detected compound with stronger binding affinity relative to other aldehydes. For trace analysis of volatile or semi-volatile odorants, a recovery rate of <120% and an RSD of <20% are considered acceptable for methods such as GC-MS [71]. To mitigate these effects, residual chlorine in chlorinated drinking water can be quenched by sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) [72]. For raw water with high NOM levels, the standard addition method can be applied to compensate for matrix-induced signal suppression or enhancement [73]. In addition, dilution with ultrapure water can also reduce matrix strength and associated interferences.

It is noted that pH and ionic strength can also impact sensor performance. The OBP2a-based biosensor has been shown in previous studies to maintain stable structural integrity and binding activity under moderate environmental variations, owing to its compact eight-stranded antiparallel β -barrel and C-terminal α -helix, which form a typical hydrophobic calyx structure [74]. This folded architecture endows the protein with natural resilience against thermal denaturation, organic solvents, and a broad range of pH values. In this study, measurements were performed under environmentally relevant conditions, including tap water, dechlorinated tap water, and raw water samples, which encompassed typical variations in pH and conductivity. The sensor exhibited generally consistent responses across these samples, suggesting that its performance is not severely affected by such environmental factors. Similarly, Low et al. (2013) investigated the adsorption behavior of proteins onto nitrocellulose membranes over a pH range of 2.0-8.0. They reported that when the pH was below the protein isoelectric point (pI), electrostatic attraction facilitated adsorption, whereas at pH values above the pI, proteins could still attach to the membrane via hydrophobic interactions [56]. Future research is needed to conduct a systematic evaluation of the sensor's robustness across a broader range of pH and ionic strength conditions.

Storage stability. Fig. 2f shows the detection of β -cyclocitral at the concentration of 10 $\mu\text{g/L}$ by the OBP2a-based biosensor when stored at 4 °C and -20 °C up to the 3rd day, 6th day, and 9th day, respectively. The results showed that the OBP2a biosensor was more stable when stored at -20 °C than at 4 °C, suggesting a more rapid decrease

in the capacity of OBP2a on the electrode surface under the condition of 4 °C [75]. At -20 °C, ΔI decreased to $97.3\% \pm 0.91$ and $93.9\% \pm 0.46$ on the 3rd day and 6th day, while it declined to $76\% \pm 1.43$ on the 9th day, indicating that the biosensor could remain relatively stable at -20 °C for about 1 week. Therefore, -20 °C is recommended for the storage of the biosensor.

Among the various bio-sensitive materials, olfactory tissues are easily affected by the in vitro environment (temperature, carbon dioxide content, etc.), which makes it difficult to maintain tissue activity. Olfactory receptors, as seven-transmembrane proteins, have a lipid membrane dependence that makes the receptors susceptible to conformational changes or inactivation [76]. In contrast, OBP2a exhibits relatively excellent stability due to its unique structure [74]. This compact hydrophobic folded structure has good stability and prevents hydrolysis effectively [77]. Sui et al. (2025) developed a *Drosophila* OBP-based biosensor that showed good storage stability with only a 10.2% decrease in response value after ten days of storage [78]. El Kazzy et al. (2025) reported that biosensors developed using rat OBP and pig OBP showed stable assay responses after one week of storage at 4 °C [79]. Additionally, the construction of organic-inorganic hybrid nanoflowers using metal ions can enhance protein activity and stability by providing a high specific surface area and a confinement effect [80]. Previous studies have demonstrated that such three-dimensional nanostructures can improve protein stability by 3-5-fold through confinement effects and protective

mineralization. As a result, OBP has the potential to overcome temperature limitations in outdoor detection and cold-chain transportation scenarios.

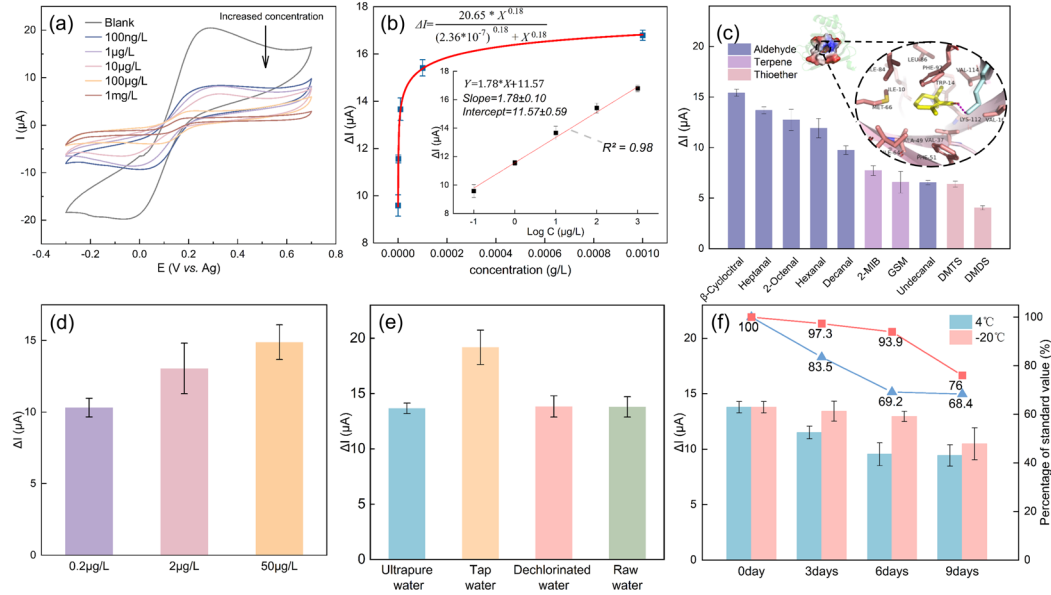


Fig. 2. Cyclic voltammetry detection results of β-cyclocitral in the concentration range of 0.1 μg/L to 1000 μg/L by the OBP2a-based biosensor (a); Concentration-dependent curves for the detection of β-cyclocitral (b); The ΔI of the OBP2a-based biosensor for the detection of 10 odorants at the 100 μg/L concentration (c) and analysis of binding mechanism of OBP2a to aldehyde (embedded plot); Reproducibility for the detection of β-cyclocitral at the concentration of 0.2 μg/L, 5 μg/L and 50 μg/L (d); Detection of the β-cyclocitral in different matrix samples (e); Storage stability of β-cyclocitral at concentration of 100 μg/L for 3, 6, and 9 days (f).

3.4 Environmental Implications.

The proliferation of cyanobacterial blooms poses significant threats to aquatic ecosystems and public health globally, necessitating reliable monitoring strategies. In eutrophic waters, microbial activity and algal growth are accompanied by the release of

large quantities of volatile organic compounds (VOCs), such as β -cyclocitral. This study introduces a novel biosensor based on an odor-binding protein for detecting β -cyclocitral, a volatile organic compound closely associated with cyanobacterial cell density and serving as a biomarker for cyanobacterial bloom detection. The biosensor utilized odorant-binding protein OBP2a as the biorecognition element, which enables rapid quantification of β -cyclocitral within the linear range of 0.1-1000 $\mu\text{g/L}$. During early stages of cyanobacterial blooms, β -cyclocitral levels generally rise from background to tens or hundreds of ng/L [22]. The biosensor showed acceptable linear responses above 100 ng/L (LOD 30 ng/L), indicating its suitability for early-warning monitoring of cyanobacterial activity. Moreover, the odor threshold of β -cyclocitral is typically in the $\mu\text{g/L}$ range, and the detection range of the biosensor covers both environmentally and sensorially relevant concentrations, thereby demonstrating the practical applicability of the sensor for both water quality monitoring and early-warning purposes.

We systematically evaluate the sensor performance, especially in environmental water samples. The biosensor maintained 80%-120% recovery rates in spiked lake water matrices, with relative standard deviations below 20%, demonstrating its detection capability against common environmental interferents such as natural organic matter and the co-occurrence of odor compounds. The study could promote a new paradigm for in situ monitoring of cyanobacterial dynamics through their metabolic signatures. Several critical challenges need further investigation:

First, the observed weak cross-reactivity with structurally analogous aldehydes and other odorants indicates room for specificity enhancement to mitigate interference from coexisting aldehydes. Future efforts could focus on protein engineering of binding pocket through site-directed mutagenesis to refine ligand discrimination, thereby reducing competitive binding among multiple aldehydes. Developing sensor arrays that integrate multiple OBPs with distinct binding profiles might enable pattern recognition of volatile fingerprints, thereby allowing the deconvolution of mixed aldehyde signals. In addition, the stability of the biosensor requires improvement for field application. The current biosensor retained >80% of its initial response after 7-day storage at -20 °C, suggesting that protein denaturation remains a limiting factor. Encapsulation of OBP2a as protein-inorganic hybrid nanoflowers may substantially enhance structural integrity. Furthermore, water quality monitoring that combines physicochemical parameters, biological indicators, and emerging chemical markers is also needed to provide a more comprehensive assessment of aquatic systems [81]. Future research could explore the integration of real-time monitoring of β -cyclocitral into existing systems (e.g., chlorophyll a, total phosphorus, total nitrogen, dissolved oxygen, and turbidity) and integrating biosensor output data with machine learning to establish a more comprehensive intelligent monitoring framework. This integration could enhance real-time monitoring accuracy, and increase the reliability of early warning and intervention in dynamic aquatic environments [82].

4 Conclusions

In this study, an OBP2a-based biosensor was developed for the detection of β -cyclocitral within the concentration range of 0.1-1000 $\mu\text{g/L}$ by immobilizing OBP2a on SPE with the cyclic voltammetry detection technique. Comparison of the detection of 10 odorants demonstrated that the OBP2a-based biosensor was more selective for β -cyclocitral. The integration of dissociation constant analysis with molecular docking enables the prediction of odor-binding protein specificity, thus concurrently facilitating structural screening of candidate proteins for the design of biosensors. In addition, the OBP2a-based biosensor showed good reproducibility and accuracy in real water samples and could remain relatively stable at $-20\text{ }^{\circ}\text{C}$ for 6 days storage. The developed OBP2a-based biosensors could realize the rapid detection of β -cyclocitral in source and drinking water samples, providing a promising means for early warning and monitoring of harmful algal blooms.

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Conflict of Interest statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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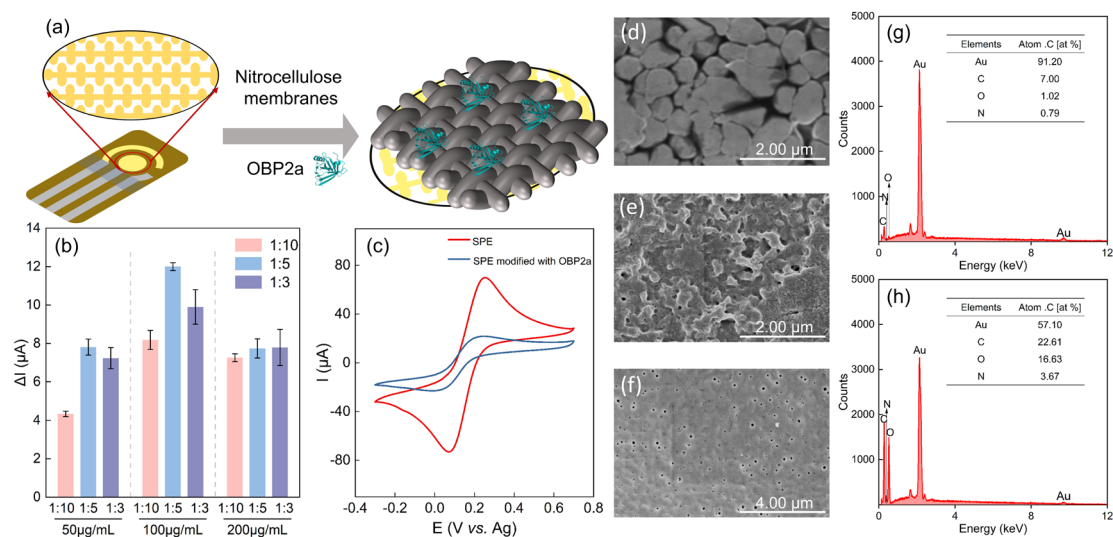


Fig. 1. OBP2a immobilized on SPE by NC membranes (a); The ΔI detected by biosensors modified with different ratios of NC membrane area to methanol volume (1:3, 1:5, 1:10) and different concentrations of OBP2a (50 $\mu g/mL$, 100 $\mu g/mL$, 200 $\mu g/mL$) ($n=3$) (b); Cyclic voltammograms of OBP2a before and after immobilization at the SPE (c); SEM images of SPE (d) modified with NC membrane (e) and OBP2a (f); EDS energy spectroscopy of bare electrode (g) and OBP2a immobilized electrode (h).

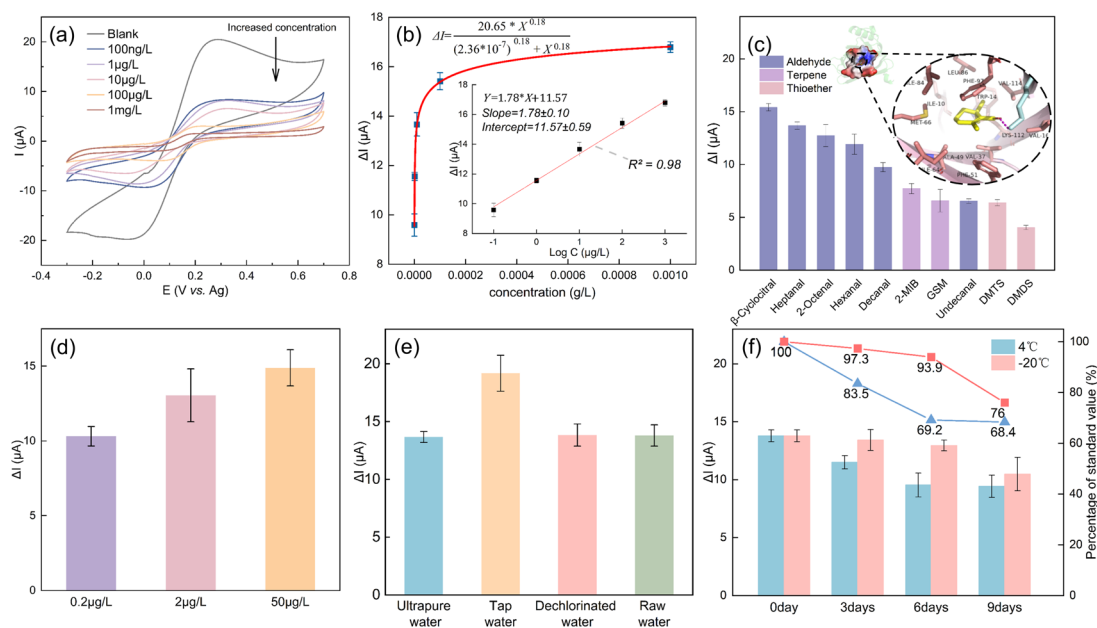


Fig. 2. Cyclic voltammetry detection results of β-cyclocitral in the concentration range of 0.1 μg/L to 1000 μg/L by the OBP2a-based biosensor (a); Concentration-dependent curves for the detection of β-cyclocitral (b); The ΔI of the OBP2a-based biosensor for the detection of 10 odorants at the 100 μg/L concentration (c) and analysis of binding mechanism of OBP2a to aldehyde (embedded plot); Reproducibility for the detection of β-cyclocitral at the concentration of 0.2 μg/L, 5 μg/L and 50 μg/L (d); Detection of the β-cyclocitral in different matrix samples (e); Storage stability of β-cyclocitral at concentration of 100 μg/L for 3, 6, and 9 days (f).

832 **List of Tables**

833 Table 1 Summary of different types of biosensors for the detection of odorants in

834 ultrapure water

Type of biosensor	Biosensing elements	Target	Linear range (µg/L)	Detection limit (µg/L)	References
Electrochemical (CV)	Human OBP2a	β-cyclocitral	0.1-1000	0.03	This study
Localized surface plasmon resonance (LSPR)	Acer-ASP2 from honeybee	β-ionone	1.92-192300	0.005	[62]
Surface Acoustic Wave (SAW)	Bovine OBPs	Octenol	68-320	14.7	[63]
		carvone	55-491	11.1	
Electrochemical impedance spectroscopy (EIS)	BdorOBP2 from oriental fruit flies	Isoamyl acetate	13-13000	4.30	[64]
		β-ionone,	19.2-19200	11.9	
Single Walled Carbon Nanotube (SWCNT)	probe-DNA	benzaldehyde	10.6-10600	8.91	[65]
		Oxytetracycline	10-75	1.13	
Quartz crystal microbalance (QCM)	peptide	3-Methyl-1-butanol	0-361	7.2	[66]
		1-hexanol	0-418	7.5	
Field-effect transistor (FET)	hOR51S1, hOR3A4	Geosmin	0.01-100	0.01	[30]
		2-MIB	0.01-100	0.01	
Electrophysiology	GPROR2	Benzaldehyde	10.6-106120	Not estimated	[67]

835 Note: SWCNT sensors have recoveries of 86.4%- 95.8% for spiked ambient water
836 samples (wastewater, tap water). Recoveries and storage stability for other types of
837 biosensors are not reported in the literature.



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Indicator of harmful cyanobacteria using an odor-binding protein-based biosensor for -cyclocitral detection

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