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Innovative Biosensors and Analytical Methods for The Analysis and Valorization of Agri-Food and Agro-Industrial Waste

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Problem Statement

Agro-industrial activities generate vast volumes of nutrient-rich wastewater, organic matter, nitrogen, phosphorus, micronutrients, as well as contaminants such as heavy metals, pathogens, and xenobiotics. Inefficient and inadequate management of these streams lead to uncontrolled discharge that causes soil and water contamination, greenhouse gas emissions, and the dissipation of valuable nutrients¹⁻³. Circular bioeconomy promotes the recovery and reuse of water nutrients. However, due to safety concerns and insufficient process control, the implementation stays constrained^{1,4,5}. It is required that treated water be used safely and effectively, and for this purpose, consistent and thorough monitoring of pollutants and nutrients throughout the entire treatment and reuse system is required^{3,5,6}.

Conventional methods for wastewater characterization rely on centralized laboratories, complex instrumentation, and trained personnel. This makes monitoring costly, time-consuming, and limited in spatial coverage^{7,8}. These approaches provide low-frequency measurements and do not support control of treatment units and real-time certification for reuse. As a result, low-resource settings do not operate, and full monitoring capacity undermines environmental protection and resource recovery^{4,7-9}.

There is an urgent need for real-time, decentralized, and sustainable monitoring tools that are deployable at the point of waste generation and reuse to support waste management approaches. This shall eventually contribute to Sustainable Development Goal 6 on clean water and sanitation. Biosensors such as low-cost paper-based, optical, and smartphone-integrated systems are promising to bridge the gap between labs and analysis and continuous decision-making, enabling rapid, on-site quantification^{2,5,6,8-11}. However, biosensors' potential for coupling monitoring with active control, optimization, and valorization in wastewater streams and agro-industrials is underexplored relative to the urgency and scale of the challenge. Addressing these gaps and challenges will operationalize circular economy principles in agro-industrial systems, transforming wastewater from an environmental liability into a managed resource^{4,7,9,10}.

Research Aim

This PhD research aims to design, develop, and validate a new generation of sustainable, paper-based optical biosensing platforms, integrated with smartphones and artificial intelligence. These analytical platforms could enable decentralized environmental monitoring and support the valorization of agro-industrial waste and wastewater streams. Building on recent advances in bioluminescent smartphone paper biosensors with embedded AI-driven image analysis for water toxicity assessment, the aim is to develop origami-inspired colorimetric paper devices for instrument-free pollutant detection. These devices provide a contribution of bioluminescent sensing to Sustainable Development Goal 6 (Clean Water and Sanitation); the project seeks to consolidate these paradigms into modular platforms capable of on-site, low-cost, and user-friendly quantification of both contaminants and value-added components in complex agro-industrial matrices.

Within this broader vision, the specific objective is to engineer eco-designed, disposable paper and origami-based biosensors. That employs bioluminescent and colorimetric transduction, coupled with smartphone imaging and AI algorithms, to transform qualitative optical readouts into robust quantitative information in real time, independent of device variability or field conditions. By deploying these platforms at points of waste generation and reuse, the research aims to advance data-enabled circular-economy interventions, helping to manage process control, risk assessment, and decisions on safe reuse, recovery of nutrients, and bioactive compounds for the valorization of agro-industrial valorization chains. In doing so, the dissertation positions AI-assisted paper biosensors as key digital-analytical interfaces that reconcile sustainability, decentralization, and circular resource management in environmental and agri-food systems.

Abstract

The presence of toxic contaminants is still a large analytical and environmental concern. This is especially true in areas with limited resources, like decentralized facilities. Reference laboratory analytical methods like chromatography and mass spectrometry provide exceptional sensitivity and selectivity for contaminant analysis, but the degree to which they rely on sophisticated instrumentation, trained personnel, and centralized infrastructure also limits their real-time and/or in situ applicability. The development and analytical validation of two sustainable, paper-based biosensing platforms for quantitative measurement of water quality are presented in this thesis.

The first platform (elaborated in Chapter 2) is an integrated bioluminescent toxicity biosensor using entrapped *Aliivibrio fischeri* cells immobilised over a paper matrix. This system combines the use of smartphone imaging with an associated artificial intelligence application (*Scentinel*) and an on-board calibration model developed to compensate for variability due to different smartphone devices; this allows for model-independent quantitative analysis. The biosensor achieved a detection limit of 0.23 ppb for microcystin-LR and demonstrated high analytical sensitivity to environmentally relevant toxins.

The second platform (elaborated in Chapter 3), NitriPad, is a ready-to-use origami colorimetric device based on the Griess diazotization reaction for nitrite (NO_2^-) determination. The rationally engineered folding architecture allows for controlled mixing of reagents and initiation of reactions without the need for additional equipment or pipetting steps, thereby improving the reproducibility and robustness of the operational process. Analytical characterization of NitriPad yielded a limit of detection (LOD) of 0.27 mg L^{-1} and a limit of quantification (LOQ) of 1.11 mg L^{-1} when fortified drinking water samples with recoveries ranging from 87% to 110%.

Furthermore, suitable linearity was established across environmentally relevant concentration ranges, and the platform was found to be amenable to smartphone imaging for quantification of results. White Analytical Chemistry (WAC) metrics were used to evaluate the sustainability of the two methods, confirming that both biosensing platforms were more sustainable than reference methods, which provides the potential for developing a scalable analytical infrastructure for decentralized environmental monitoring and rapid assessment of wastewater and screening.

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Chapter 1 – Introduction

This chapter introduces the foundational concepts and emerging trends in sustainable biosensing platforms for point-of-need applications. Biosensors are classified based on their transduction mechanisms, such as optical, electrochemical, and piezoelectric, among others. Moreover, the bioreceptor (enzymes, antibodies, nucleic acids, cells, or biomimetic materials) based details are included. Optical biosensors enable visual detection and include colorimetric systems (chromogenic reactions), chemiluminescent/electrochemiluminescent platforms (light emission upon chemical or electrochemical stimulation), fluorescent sensors (fluorophore-tagged interactions), and bioluminescence approaches (enzymatic light generation). Biosensing systems can use different methods, living-cell-based platforms for complex analytes, cell-free constructs for enhanced specificity and stability, or hybrid configurations combining both modalities for optimization in a monitoring context.

Integrating biosensors into environmental and waste management facilities offers real-time data acquisition, sensitive contaminant tracing, affordability through miniaturization, and mass production. These features make them aligned with sustainability principles, enabling circular economy practices such as waste valorization. Paper-based substrates provide biodegradability, low cost, ease of preparation, microfluidic systems, and origami designs, allowing multiplexing and minimal reagent consumption, making them sustainable platforms. For painting biosensor's stability and reproducibility, effective immobilization strategies for biological and chemical recognition elements in paper are critical.

Despite these advances and the availability of technological advancements, the deployment of biosensors in field applications is challenging: sample matrices, bioreceptor's stability issues in harsh environments, and biosensors' lifetime are major stumbling blocks that hinder deployment outside of the lab. Smartphone availability has catalyzed the development of portable analytical detection systems. It allows image-based quantitative analysis through cameras and provides computational resources. Coupling it with artificial intelligence (AI) for image interpretation, pattern recognition, and machine learning based estimation has enhanced sensitivity and enabled decentralized monitoring.

Finally, in the transition from environmental monitoring to valorization of agri-food and agro-industrial waste streams, biosensors provide not only the contaminant detection but

also facilitate resource recovery processes. This holistic approach underscores the convergence of smart diagnostics, AI-driven analysis, and circular economy principles within agro-industrial systems aimed at fostering resilient and sustainable food production networks^{12–16}.

1.1 Definition and Classification of Biosensors

A chemical sensor is a device that detects the presence of an analyte and produces a quantifiable value. It consists of three main components: the receptor, which interacts with the analyte; the transducer, which converts the recognition event into a measurable signal; and the readout system, which processes and displays the signal. In the case of a biosensor (Figure 1.1), the receptor is a biological component, such as an enzyme, cells, tissue, antibodies, or DNA/RNA^{17,18}. The performance of biosensors is evaluated based on biorecognition events and the effectiveness of signal transduction¹⁹.

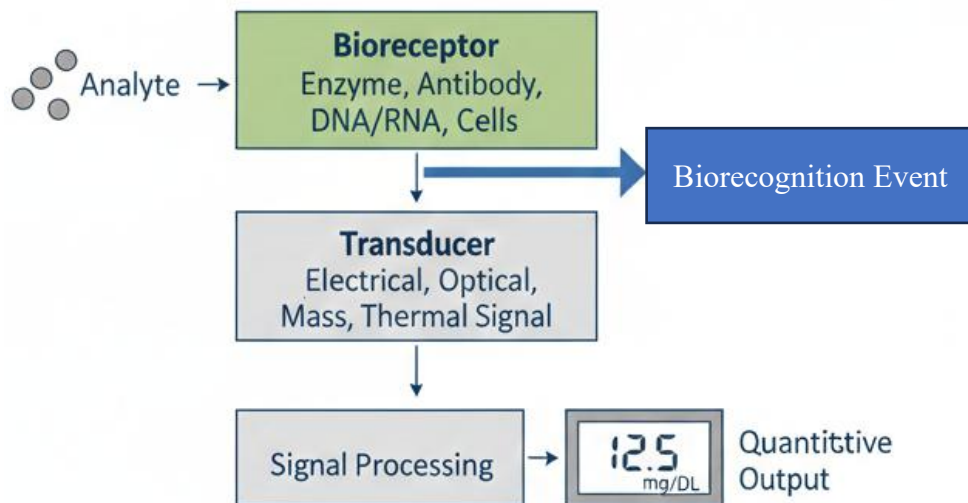


Figure 1.1 Biosensor: Basic working principle.

1.1.1 Classification Based on Transduction Mechanisms

Biosensors can be classified according to their transduction mechanisms, such as electrochemical biosensors (including amperometric, potentiometric, and impedimetric), optical biosensors (including fluorescence, surface plasmon resonance, and colorimetric), and other types (such as piezoelectric and thermometric)^{20–22}. Schematic of classification of biosensors is shown Figure 1.2. Electrochemical biosensors measure the electrical activity of chemical change in terms of current (amperometry), potential (potentiometry), or resistance (impedimetric)²³. Optical biosensors produce measurable light signals to indicate biorecognition events by altering the amplitude, polarization, and frequency of light. Common

types include signal forms with labels such as luminescent and colorimetric, as well as label-free methods like SPR ¹⁸.

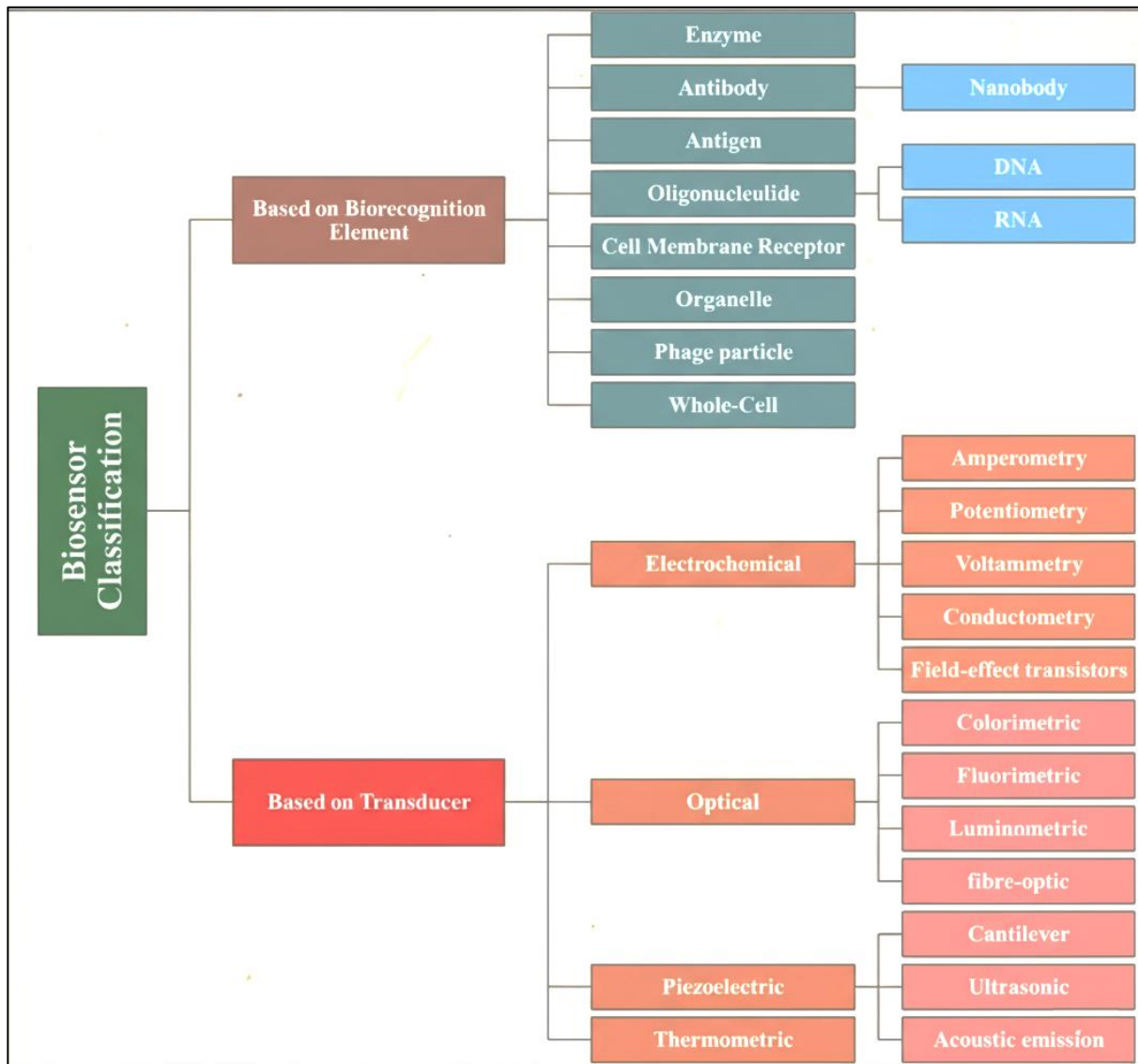


Figure 1.2 Biosensor and biochip classification²⁰. Reproduced from Huang et al., Journal of Materials Research and Technology, Vol. 12, 2021, under CC BY 4.0.

1.1.2 Bioreceptors for Biosensing: Types and Key Features and Contrasts

Selectivity and sensitivity are determined by the bioreceptor. Biosensors based on enzymes have high specificity and catalytic activity; however, factors such as temperature, pH, and concentration of substrate affect the activity of enzymes. Affinity-based biosensors utilize antibodies/antigens and DNA fragments for biochemical processes to form complementary complexes. The stability, receptor isolation, and purification are easy. However,

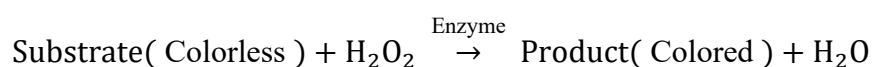
reproducibility of results and multi-stage procedures to produce results are common issues. Cell/ cell organelles-based biosensors used whole cells (bacteria, yeast) or organelles (mitochondria, membranes) as bioreceptors. They provide ease in immobilization; however, short shelf life, strict storage conditions, and biosafety are common issues with them. Other artificial receptors, such as aptamers and Molecularly Imprinted Polymers (MIPs), are chemically and thermally stable, have high shelf life, and are tolerant to environmental conditions. However, non-specific adsorption and lower catalysis efficiency are limiting their applicability^{18,21}.

1.2 Optical Biosensors: Colorimetric, Fluorescent, and Bioluminescent Approaches

From a sustainability viewpoint, optical sensors are highly significant. Optical biosensors merge biorecognition with optical transduction mechanisms.

1.2.1 Colorimetric Approaches

Colorimetric biosensors are based on the visible color change of dyes, nanoparticles, or nanozymes when they interact with a specific analyte. These sensors are used in applications that require quick detection visible to the naked eye, such as testing for aflatoxin B₁ and atrazine in food, as well as for environmental monitoring of toxic gases, heavy metals, and explosives. Reports indicate that nanozyme-enhanced colorimetric biosensors have achieved limits of detection (LOD) in the picogram to nanomolar range, with results that can be read using a smartphone²⁴⁻²⁶. The intensity of color correlates with the analyte concentration, as calculated by the Beer-Lambert Law. The chemical reaction is described in the equation below.



Typically, the (chromogenic) substrate used is 3,3',5,5'-Tetramethylbenzidine (TMB), while Horseradish Peroxidase (HRP) serves as the enzyme. Additionally, H₂O₂ is a byproduct of various enzymatic reactions.

Noble metal nanoparticles, such as silver (Ag NPs) and gold (AuNPs), exhibit color changes due to a specific shift in their localized surface plasmon resonance (LSPR). These changes can be easily measured using simple spectrophotometry or even smartphones. According to Mie theory, when AuNPs transition from a dispersed to an aggregated state, there is a red shift in the LSPR peak, typically moving from 520 nm to 600 nm. This shift is visible

as a change in color^{27,28}. These biosensors are simpler and give a visual yes/no output with quantification.

Enzymes or nanozymes cause a color change through the oxidation of chromogens like TMB, which occurs when hydrogen peroxide is present²⁶. Thin-film interference on silica or paper substrates also results in a noticeable change in color²⁵.

1.2.2 Chemiluminescent / Electrochemiluminescent (ECL) Biosensors

When a chemical reaction or the excitation of the electrode surface occurs due to the application of potential, it can produce light, which is useful for biosensing applications. Chemicals such as luminol, peroxyoxalates, dioxetanes, Ru (II) complexes, and nanomaterial emitters are combined with biological receptors for this purpose. These substances have been applied in immunoassays, pathogen detection, pharmaceuticals, and paper-based devices^{29–34}. ECL Biosensors consist of three main components: biorecognition elements, electrochemiluminescent (ECL) luminophores, and conductive substrates. According to the literature these sensors achieve limits of detection (LOD) ranging from femtomolar to picomolar concentrations, with linear measurement ranges spanning 4 to 6 orders of magnitude. A study by Meng et al. reported an ECL biosensor for microRNA that achieved sub-femtomolar LODs by utilizing hybridization cascades and nanostructured electrodes, making it compatible with clinical serum levels³⁵. Novel luminophores integrating porphyrinic-MOF provided LOD of 0.35 fM for circulating tumor DNA³⁶. From 2021–2025, different strategies to work with luminophores have been developed. For example, aggregation-induced electrochemiluminescence (AIECL) was developed, which provided operational stability and tolerance to self-quenching. Additional 2D materials, such as MXenes, are utilized for signal amplification due to their high surface area and conductivity, which also enhances sensitivity and specificity³⁷. These innovations demonstrate that ECL biosensors deliver higher LODs with maintained dynamic ranges and offer shorter assay times^{35,36,38}. ECL and chemiluminescent provides ultra-low backgrounds, high sensitivity, and compatibility with multiplexed and miniaturized formats. However, they require controlled chemical reactions and electrochemical instrumentation, which makes them complex to use at the point of need. Unlike fluorescence biosensors, these sensors do not experience autofluorescence or photobleaching effects. However, their integration with microfluidic systems and low-cost electronics can enhance their application in high-performance clinical diagnostics and environmental monitoring^{35–39}.

1.2.3 Fluorescent Biosensors

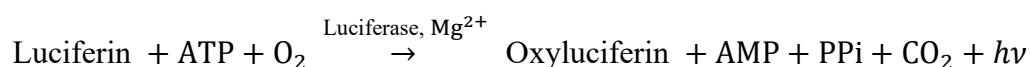
These biosensors detect changes in light intensity, wavelength, Förster resonance energy transfer (FRET) efficiency of fluorophores, or lifetime by absorbing shorter wavelengths and emitting longer wavelengths, leading to a Stokes shift. An external light source is needed to observe the change. Quantum dots, which are nanomaterials with fluorescent properties, as well as dyes, are commonly used. These materials have been applied in sensing ions and metabolites^{40–42}. These measurements are made in steady-state or time-resolved modes, and ratiometric or FRET-based readouts, and these types of sensors provide improved accuracy and extended dynamic ranges. Their LODs are usually picomolar or even subpicomolar ranges (dynamic ranges three or more orders of magnitude), and high temporal resolution enables real-time monitoring of biochemical processes⁴³. Hellweg et al. have engineered the reversible binding interaction between fluorescent proteins (donor) and rhodamine-labeled Halo Tag (acceptor), which made near-perfect FRET efficiency that increased unprecedented dynamic ranges for calcium, ATP, and NAD⁺ sensing, allowing multiplexed imaging⁴⁴. Studies from 2023–2025 concluded that fluorescent nanomaterials—quantum dots, metal nanoclusters, carbon dots, and MOF-based emitters improved quantum yields, narrow emission bands, and excellent photostability, enabling sensitive detection of small molecules, aptamer-based fluorescent biosensors provide very low LODs, and single-molecule fluorescence techniques provide observation of individual biomolecular interactions^{45,46}.

Fluorescent biosensors have several strengths, including high sensitivity, broad dynamic ranges, and excellent spatial and temporal resolutions, which make them ideal for intracellular imaging, high-content screening, and precise quantification of target analytes. However, there are significant challenges, such as photobleaching, background autofluorescence, and limited tissue penetration depth, that need to be addressed^{43,45,46}.

1.2.4 Bioluminescent Biosensors

The bioluminescent reactions involving the enzyme(luciferase) and substrate(luciferin) generate a light-emitting biological system that offers a high signal-to-noise ratio without the need for an external excitation source. The various formats include whole-cell reporters, BRET sensors, caged luciferin (where analyte-triggered deprotection produces light), engineered luciferase mutants, and split luciferase systems^{47–53}. They have been used in vivo imaging,

enzyme or small molecule activity test, neurology tests, and for virus detection. The basic reaction that produces bioluminescence is below



Recently developed CaBLAM Ca^{2+} indicator (from *Oplophorus gracilirostris* luciferase) has shown higher contrast than previous biosensors, with suitable affinity towards Ca^{2+} , with the ability to detect subcellular level activity of a single cell, along with higher frame rates^{54,55}. Nanoluc-based systems provide very high sensitivity and excellent signal-to-background ratios, making them suitable for monitoring protein-protein interactions (PPI) and protein levels. However, their total photon output is generally lower than that of bright fluorescent probes under strong excitation, which makes capturing very fast processes or single-molecule events with high temporal and spatial resolution more challenging. Most bioluminescence (BL) assays report changes in intensity, and truly ratiometric BL designs tend to be more complex than their fluorescent counterparts. BL biosensors should be considered complementary technologies rather than competitors, as they excel in live-animal imaging, low-background high-throughput screening (HTS), and applications where minimal disturbance to the system being studied is crucial^{54–56}.

Colorimetric biosensors offer low-cost, easy-to-use features that require minimal instrumentation. However, subtle changes in intensity, visual thresholds, or matrix effects can lead to variability in output, compromising accuracy. Bioluminescence-based biosensors provide ultra-high sensitivity and low background signals, but they require the addition of luciferin and may face brightness issues. Fluorescence sensors have been tested for multiplexing and spatial imaging; however, they rely on external light sources and suffer from problems such as photo-bleaching and auto-fluorescence. Chemiluminescence and electrogenerated chemiluminescence (ECL) have limits of detection (LOD) in the attomole to picomole range, but their instability is a significant drawback.

1.3 Living, Cell-Free, and Hybrid Biosensing Systems

Synthetic biology has advanced biosensing technology by evolving living, cell-free, and hybrid systems from intact cells to non-living systems with fully intact cellular machinery. This development has expanded the capabilities of biosensing, simplified its operation, and enhanced safety for environmental and medical monitoring.

1.3.1 Living Cell-Based Biosensors

In this approach, whole cells (bacteria, yeast, plants, mammalian) act as biorecognition elements, and in the presence of the analyte of interest, the stimuli are converted into measurable electrical, optical, or mechanical signals. By exploiting metabolic pathways and using synthetic gene circuits, they can monitor toxins, bioavailability, heavy metals, pathogens, drugs, quorum-sensing molecules, or diseases. However, viability and GMO/biosafety concerns limit their usage^{57–61}. Intracellular regulatory networks theoretically function as programmable input-output systems, allowing them to be rewired to sense specific molecular or physical stimuli with increasing thresholds, gains, and dynamic ranges. Using simple or multi-layer gene circuits, genetically coupled sensing elements and reporting elements (e.g., fluorescence, pigments) are utilized for environmental monitoring. Furthermore, amplification, logic, delay, and memory modules can be incorporated into cells that enables multi-analyte detection and temporal integration^{62,63}. Two-component systems (TCSs) can be repurposed using new engineering techniques, such as genetic refactoring, DNA-binding domain swapping, detection threshold tuning, and insulation of phosphorylation crosstalk, to make them sensitive to analytes of interest, including light, metals, nutrients, and host-associated signals.⁶⁴ Their detection time ranges from 1–6 h, and minimum sample preparation is required to measure the buffering capacity of the intracellular environment⁶².

1.3.2 Cell-Free Biosensing Systems

In order to minimize the limitations of cell-based biosensors, such as the requirement of nutrient media, increased response time (due to the cell wall), biosafety-related issues, and long-term storage, the cell-free protein synthesis system has emerged as a potential alternative. Extraction of biological machinery (ribosomes, RNA polymerase, tRNAs, and enzymes) using platforms (*Escherichia coli* extract, *Saccharomyces cerevisiae* extract, Insect cell extract (ICE), Wheat germ extract (WEG), Rabbit reticulocyte extract (RRE), etc.) and utilizing it out of the cell is a major process in cell-free biosensing platforms^{65–71}. They are abiotic analogues of whole-cell sensors; the open architecture permits direct addition of nucleic acid logic circuits, including toehold switches, strand-displacement cascades, and amplification chemistries that are difficult to implement *in vivo*^{72–74}. They even allow logic circuit implementation. Jung et al. built hybrid RNA–DNA logic circuits (AND, OR, NAND, etc.) by interfacing a small-molecule sensing platform (ROSALIND) with toehold-mediated DNA strand-displacement, enabling discrete digital outputs⁷³. Cell-free biosensors can be freeze-

dried, thus removing cold chain requirements, making them deployable by rehydration at the point of need. Wang and Lu (2022) similarly highlight freeze-drying, optimized extract preparation, and microfluidic paper analytical devices (μ PADs) as key enablers for point-of-care and environmental monitoring⁶³. Li et al. have provided a strategy for improving sensitivity by introducing polymerase strand recycling (PSR), and it yields up to 3.6–4.6-fold decreases in EC50 for transcription factor-based biosensors⁷⁴. Key advantages include operational efficiency in matrices, easy integration with the substrate, and improved biosafety. However, concerns exist regarding batch-to-batch variability, sample interferences due to the lack of a membrane barrier, and the high cost of reagents.^{63,75,76}

1.3.3 Hybrid and Artificial-Cell Platforms

Advancements in biosensors involve integrating synthetic materials, such as carbon nanotubes, graphene, and metal-organic frameworks (MOFs), to enhance signal transduction and stability. These improvements enable upstream metabolic transducers to feed into transcription factor (TF)-based circuits, thereby extending the range of detectable chemistries in complex media like urine and beverages through the introduction of hybrid systems.⁷⁷. Another approach involves utilizing cell-free machinery encapsulated in lipid vesicles, which serve as membrane gates. This setup mimics the response of living cells, creating artificial cells that act as micro-compartments and providing greater reliability and longevity for biosensors.^{78–80}. Additionally, there are cell-based hybrid systems that incorporate multiple modes of transduction. These systems integrate cell-based strategies with hybrid nanomaterials and microelectronics to enhance functionality.^{81,82}. A study by Boyd et al. shared the proof-of-concept of a hybrid biosensing platform by encapsulating a fluoride-responsive cell-free riboswitch in lipid vesicles. The membrane was optimized for permeability and kinetics while providing protection against nucleases and inhibitors in a complex matrix. The biosensor produced either colorimetric or fluorescent outputs, making the system compatible with both simple and advanced systems.⁸³. Mathur et al. used hybrid nucleic acid–quantum dot assemblies as modular multiplex reporter in a cell-free system, turning enzyme activities into fluorescent outputs (FRET-based QD–DNA–dye constructs)⁷².

Collectively, these hybrid systems aim to resolve a central trade-off: whole-cell sensors offer protection and long-term function but suffer from viability constraints and regulatory barriers, whereas open cell-free systems are programmable and stable when lyophilized but vulnerable to interference and lack compartmental control. Artificial cells attempt to

combine abiotic programmability and safety with membrane-mediated robustness and tunable transport, enabling operation in complex environmental and clinical samples^{63,78,83}.

1.4 Applications in Environmental and Waste Management

Biosensors can be transformative for environmental monitoring and waste management, while aligning with circular economic frameworks and sustainable development goals (SDG 6, 12). They provide the benefits, not limited to:

1.4.1 Rapid, Real-Time, and Onsite Monitoring

Real and continuous monitoring quality and pollutants (BOD, COD, heavy metals, pathogens, pesticides) allows laboratory-based methods (Culturing, HPLC, etc.) to use confirmatory analysis only. Portable monitoring in situ at treatment plants, rivers, or landfills allows immediate transfer of information and reduces sample transportation time and costs^{84–91}.

1.4.2 High Sensitivity, Selectivity, and Early Warning

As biosensors have highly specific biorecognition elements, they are highly specific towards metals, emerging contaminants, pharmaceuticals, pathogens, and microplastics at trace levels. Toxicity monitoring using cell-based biosensors provides early warning systems and acts as a filtering instrument for toxic discharges^{84–86,91–102}.

1.4.3 Cost-Effectiveness and Sustainability

Conventional analytical systems, such as chromatography and mass spectrometry, require sample preparations, reagents, storage conditions, space, and disposability, the issues that biosensors can overcome. Smaller carbon footprints are due to miniaturize nature of biosensors support white, green, and blue chemistry principles. Modern approaches based on biodegradable platforms, such as paper sensors, are eco-friendly, producing low waste, and are good options for field deployments.^{84,85,87,90,93,96,103–106}

1.4.4 Process Control, Smart Management, and Role in Waste Valorization / Circular Economy

Realtime biosensing operations allow online control of wastewater treatment, limit regulatory breaches, and optimize processes. If integrated with IoT and AI, the smart bioremediation for solid-waste leachates and industrial effluents, remote monitoring, and

predictive maintenance is possible^{85,88,90,91,93,100,106}. Monitoring bio-transformation processes turning waste into resources is central to the circular economy, and monitoring can be done through biosensors. For example, a volatile fatty acid measurement system (bio-electrolytic VFA sensor) was developed that improved the process of anaerobic digestion and stable biogas production. Further monitoring of heavy metals and toxic organics in wastewater and agricultural effluents is used to measure the safety threshold of byproducts of sludge and agro-industrial waste. This maintains quality and safety during valorization and supports the recirculation of harmful contaminants^{86,93,95,105,107–110}.

1.5 Paper as a Sustainable Analytical Substrate

Paper(cellulose) acts as a green platform for biosensors because of its materialistic and life cycle properties and is a leading green platform. Cellulose paper is biodegradable and produced from renewable sources, making its production and disposal more sustainable than ceramics, polymers, or glass. And disposal of paper is natural, with the generation of low volume for proper waste management^{111–116}. Paper substrates offer several advantages, including cost-effectiveness, availability, and compatibility with various scalable printing methods such as wax, screen, inkjet, or laser printing. This facilitates the mass production of biosensors in decentralized locations. Additionally, the internal structure of paper—characterized by intrinsic porosity and capillary action—allows for passive sample transport without the need for external instrumentation. Moreover, the low volume requirements for reagents and samples (measured in microliters) minimize chemical use and ultimately reduce waste^{114–122}. In terms of functionalization, paper provides a fibrous surface with a high surface-to-volume structure for immobilization of enzymes, antibodies, nucleic acids, aptamers, and cells. Many strategies are available to chemically modify the paper with aldehydes, grafted polymers, and amides to make a bioactive paper. These functionalizations and modifications allow an increase in the sensitivity and selectivity of the biosensor. The assays that require multiple steps, paper provides flexibility and foldability, allowing origami and 3d paper fabrication. These eco-designed biosensors perfectly align with green and white analytical chemistry concepts^{112,115–117,119,120,123,124}.

1.5.1 Paper Microfluidics and Origami-Based Designs

As previously discussed, the 3D paper-based biosensor concept, known as paper microfluidics (μ PADs), enables capillary-driven flow in cellulose. The design incorporates patterns with hydrophobic regions created through methods like wax printing, inkjet printing,

laser printing, or stamping confinement. These patterns allow for the routing of liquids in multiple dimensions. The 3D devices feature both vertical and lateral channels, which provide precise control over the flow of liquids. These configurations can be achieved by either stacking multiple sheets or by using origami techniques to fold a single sheet of paper^{125–130}. Origami design utilizes predefined fold lines and trap geometries to regulate the sequence and timing of contact between samples and immobilized reagents. This approach includes fold-to-start assays, sequential incubation, washing, and detection processes. It allows for the separation of incompatible reagents into distinct layers of paper until the step where they are needed. Additionally, plasma separation, washing, multiplexing, and reaction zones can be incorporated into a compact single piece of paper^{125,126,128,129,131–133}.

1.5.2 Advances in Paper Microfluidics and Origami Biosensors

A study has described the 3D capillary-driven sequential devices that combine oPAD (origami PAD) and rPAD (movable reagent pad) in two configurations to deliver reagents automatically through either flow-through or stopped-flow. These strategies eliminated the manual multistep and allowed self-calibration plots, electrode modification steps, and label-free immunosensing¹³³. The origami system overcomes the Lucas–Washburn regime (lateral-flow length limitation), with vertical transport and stacked layers. Further, it enhances mass transport and longer effective path length without sacrificing footprint¹²⁶. Several paper microfluidic-based sensors have been developed to improve performance for different analytes along with different transduction mechanisms (Electrochemical, Electrochemiluminescence, Chemiluminescence, etc.) those are summarized in Table 1.1.

Target/application	Origami design & transduction	Key performance	Citations
Tumor markers in blood	3D origami CL immunodevice	Multiplex CL, good agreement with clinical tests	134

Glucose (serum)	Cu@CuS@N-C origami μ PAD	Range 2–7500 μ M; LOD 0.16 μ M; no pretreatment	135
Sweat biomarkers	Wearable origami paper microfluidics	Multiplex colorimetric + electrochemical sensing on-the body	136
Dopamine & NADH (neurometabolic biomarkers)	3D origami μ PAD on filter paper with graphene/Pt Nano catalysts; smartphone colorimetric readout	18 $\times$ faster reactions; 2.48 $\times$ higher color intensity; LOD 0.56 mM (dopamine), 0.99 mM (NADH)	127
Glucose, lactate, uric acid, Mg²⁺, pH, and cortisol in sweat	Wearable 3D origami paper microfluidic chips with separated colorimetric and electrochemical zones	On-body multiplex sweat sensing; thread-based channels control flow and reaction sequence; validated in human tests	132
H₂O₂ and glucose in saliva	Origami microfluidic PAD integrating SPE modification and detection; RGO/Au–Pt nanocomposite; Amperometric	29.2-fold sensitivity gain vs bare SPE; H ₂ O ₂ LOD 0.15 μ M; glucose LOD 1.5 μ M; ranges cover salivary levels	137
Glucose in serum	Origami μ PAD with core–shell Cu@Cu ₂ S@N-doped	Range 2–7500 μ M (R = 0.996); LOD 0.16	130

	carbon hollow nano cubes; nonenzymatic amperometric	μM ; good selectivity, stability; direct serum analysis without pretreatment	
Zearalenone (mycotoxin) in cereals/wastewater	Origami microfluidic paper immunosensor; carbon-nano horn-decorated nanoporous Au electrode; Amperometric	Linear 10–1000 $\mu\text{g kg}^{-1}$ ($R^2 = 0.998$); LOD 4.40 $\mu\text{g kg}^{-1}$; intra/inter-day RSD <5%; good selectivity vs other mycotoxins	138
E. coli (model microorganism)	Colorimetric origami μPAD on PVDF/cellulose; folding-driven immunoassay with HRP–TMB	LOD 2 CFU mL^{-1} ; dynamic range 10^2 – 10^6 CFU mL^{-1} ; simple POC format with image-based quantification	139
Phycocyanin protein (environmental/food)	3D paper@QDs@MIP origami chip; fluorescence quenching of QDs by QDs@MIP–phycocyanin complex	Dynamic range 10–50 mg L^{-1} ; LOD 2 mg L^{-1} ; reusable imprinted layer; simple fluorescence readout	140
Mg²⁺ (animal health)	Flexible hydrophobic paper microfluidic FET biosensor; DNAzyme-based DNA nanostructure (DNA-origami-like) on SWNTs	Linear 1–1000 μM ; LOD 0.57 μM ; high stability and anti-interference for Mg ²⁺ monitoring	141

Cortisol in sweat	3D origami microfluidic chip with MoS ₂ -FRET aptasensor; smartphone fluorescence readout	Range 10–1000 ng mL ⁻¹ ; LOD 6.76 ng mL ⁻¹ ; 25 min assay; good agreement with ELISA (R = 0.988)	142
Rabbit IgG; HIV-p24 in serum	Origami μ PAD with ZnO nanowires; label-free EIS immunosensor	LOD 60 fg mL ⁻¹ (IgG) in buffer; 300 fg mL ⁻¹ (HIV-p24) in serum; assay <25 min	143,144
HSA and human IgG	Origami electrical biosensor array; five paper FET/chemiresistor channels with SWNT networks	LOD 1.5 pM for HSA/HIgG; μ L-volume samples split by paper microfluidics; cost-effective multiplex platform	145
Enzyme activity & organophosphate pesticides	3D origami potentiometric μ PAD; solid-contact ISE + all-solid-state reference with enzyme/inhibitor chemistry	Versatile potentiometric biosensing of BChE activity and OP pesticides using fold-controlled reagent contact	128
Cu²⁺, Cd²⁺, glucose (ions + metabolite)	3D origami ePAD with solid-contact ion-selective electrodes and MnO ₂ -CPE glucose biosensor	10 μ L sample; high sensitivity to Cu ²⁺ /Cd ²⁺ ; ultra-sensitive potentiometric	146

		glucose detection in small volumes	
Four tumor markers in whole blood	3D origami microfluidic paper immunodevice; chemiluminescence readout with luminol–H ₂ O ₂ –AgNP system	Multiplex CL assay with built-in plasma separation and automated rinsing; good agreement with clinical tests	129

Table 1. 1 Overview of Microfluidics-Based and origami paper Biosensors: Target Applications, Origami Design Strategies, Transduction Mechanisms, and Key Performance Metrics.

1.5.3 Alignment with ASSURED and Sustainability Principles

Paper-based biosensors, μ PADs, and origami designs are compliant to WHO'S ASSURED criteria, including affordability, sensitivity, specificity, user-friendly, Rapid/robust, minimal equipment requirement, and deliverable to users¹⁴⁷. These architectures allow automating multi-step protocols, integrating sample pretreatment, storing reagents in dry form in different layers, ultra-sensitive detection ranges, and Broad analyte coverage, making them complementary to conventional microfluidic chips and bench-top assay equipment.

1.6 Immobilization of Chemical Reagents and Biological Elements on Paper

The hydrophilic and porous nature of cellulose (paper) allows reagents and biomolecules to be stored within fibers and later rehydrated, making them all-in-one or reagent-free μ PADs. The hydroxyl group of cellulose can be chemically modifiable, and hydrogen bonding, electrostatic, and hydrophobic interaction allows retention of bioreceptors. Proper immobilization controls the orientation, activity, and stability of biomolecules, resultantly controlling sensitivity and reproducibility of biosensors^{126,148–152}. Studies have classified immobilization into physical, bioaffinity, and chemical approaches (Figure 1.3).

1.6.1 Physical Adsorption / Entrapment

This type of immobilization is based on non-covalent forces (electrostatic, van der Waals, hydrogen bonding, hydrophobic) between biomolecules and cellulose. Directly spotting or drop-casting the biomolecules or dropping after entrapping them inside bioactive ink (e.g., agarose, pullulan, chitosan, alginate, dextran, sol-gels) over paper is a common method. This methodology is simple, good for disposable biosensors, and compatible with large-area printing. However, leaching during flow, random orientation, and reduced reproducibility in microfluidic processes are major drawbacks^{149,150,153–155}.

1.6.2 Bioaffinity-Based Immobilization

Bioaffinity is highly specific, relying on interactions such as streptavidin/biotin, Ni²⁺/His-tag, and protein G/IgG. It enables precise control over the orientation of antibodies, aptamers, or tagged enzymes on surfaces like paper. This method allows for site-specific attachment, preserves the activity of the biomolecules, and enhances accessibility to their recognition sites. However, it requires engineering of biomolecules and the use of additional reagents, which can make the process costly and complex^{150,153,156–158}.

1.6.3 Chemical Immobilization (Covalent Bonding to Cellulose or Polymer Coatings)

This methodology begins with the chemical functionalization of cellulose, which can involve several techniques such as periodate oxidation to create aldehydes, salinization followed by glutaraldehyde treatment, carbodiimide coupling, or the use of epoxides. These

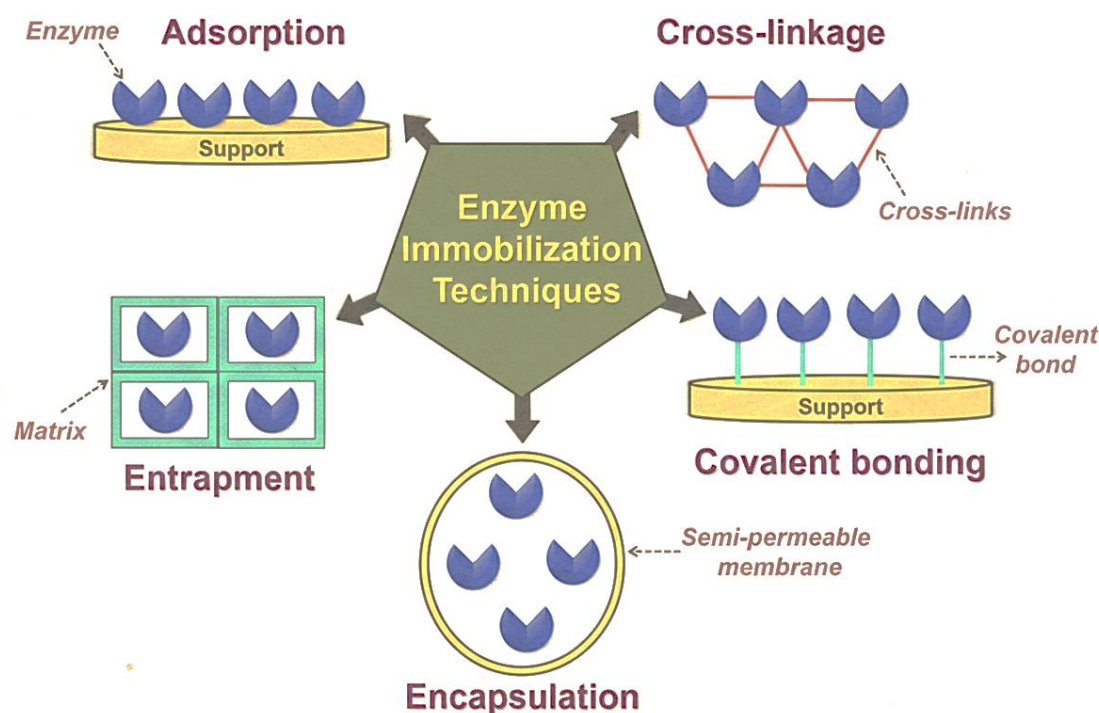


Figure 1.3 Illustration of Major Enzyme Immobilization Strategies Including Adsorption, Covalent Binding, Entrapment, and Cross-Linking¹⁹. Reproduced from Naresh & Lee, *Sensors*, Vol. 21(4), 2021, under CC BY 4.0.

processes allow for the formation of Schiff base or amide linkages with the amino groups of enzymes and proteins. Another approach involves using hydrophilic polymers that have reactive groups, such as esters, which are then attached to the paper through photochemical crosslinking. After this, enzymes are chemically bonded to these polymers, enhancing their resistance to washing steps. These strategies lead to chemically and thermally stable attachments, ensuring that the biosensor remains stable and reusable. A P-ELISA study has shown that using silane-glutaraldehyde chemistry on paper enhances both the sensitivity and reproducibility of the biosensor. But partly damage to biomolecules is a common issue^{150,153,159}.

1.6.4 Immobilization of Different Recognition Elements

Enzymes can be immobilized using various methods, including adsorption, entrapment in polymers or sol-gels, and covalent coupling^{150,152–154,160,161}. Antibodies and ELISA formats are typically attached using covalent methods involving aldehyde or silane-glutaraldehyde, or through protein A/G or biotin/streptavidin interactions^{149,150,159}. Nucleic acids and aptamers are immobilized by adsorbing them to cationic surfaces, creating covalent linkages to modified cellulose, or utilizing bioaffinity tags^{122,149,150,156,157}. There is no universal method for immobilization; instead, physical, affinity, and other approaches are often combined to enhance sensitivity, storage stability, and compatibility with 3D origami structures.

1.7 Current Limitations and Gaps for Field Deployment

Most of the studies concluded that despite the impressive sensitivity, quantitative accuracy, lab-scale performance, and efficiency reproducibility, paper-based biosensors are still inefficient for regulated applications. Limited clinical validations on diverse and large patient cohorts are major limitations, as most of the paper-based sensors are tested against spiked samples or at small pilot studies. Variability in paper substrates, fabrication issues, and immobilization chemistry errors are affecting consistency issues in different lots, and shelf-life in different environments is also an issue. Also, transportation requires robust biosensors towards humidity, temperature fluctuations, and mechanical damage, along with storage at field. Most of the paper-based biosensors are in prototype or in translation stages and requires figure out issues such as standardization and clinical validation before use in low-resource systems^{117,147,162–169}.

1.8 Smartphones as Analytical Detectors

The most appealing choice for an analytical detector is smartphones. They are equipped with sensing hardware, a computational system, and network connectivity, making them low-cost devices. With over 6 billion subscriptions worldwide, smartphones are ideal for democratizing diagnostics, especially when paired with affordable biosensors^{55,170–173}(list of smartphone-based sensors along with smart-phone's component role, and the different analytes detection is provided in Table 1.2, and Table 1.3 respectively) They possess several hardware and software capabilities that qualify them as a choice for an analytical detector.

1.8.1 Hardware Capabilities Relevant to Sensing

For colorimetric, fluorescence, chemiluminescence, and label-free sensing, high-resolution CMOS sensors of smartphones act as array detectors. Exploiting cameras (typically ≥ 12 MP) with simple optical functionalities, such as lenses, filters, and gratings make them sensitive to tabletop instruments^{170,174,175}. A white flashlight of a mobile phone camera and display backlit have been used to serve as an excitation or illumination source. For absorbance and fluorescence-based sensors, the ambient light sensor serves the functions¹⁷⁶. Computation and storage capabilities of smartphones (Processors, RAM, and GPUs) enable real-time image processing, multiple processing, and quantifications directly on devices^{177,178}. Furthermore, the electrical interfaces (such as USB-C, audio jack, Bluetooth, NFC, Wi-Fi) allow display and control of potentiostats, and custom circuitry for electrochemical sensing. Many reviews have highlighted the USB/Bluetooth/NFC of smartphones as an important tool for electrochemical-based biosensors^{55,176,179–181}.

1.8.2 Software Capabilities Relevant to Sensing

Smartphones enable the use of native and custom-built applications to control camera settings such as ISO, exposure time, and focus. They can extract color values (RGB or CIE, etc.), perform calibrations, and output concentration levels. AI-based applications help overcome the limitations of limits of detection (LOD) for optical detection, allowing measurements in the low micromolar (μM) or sub-micromolar range¹⁷⁶. Image processing steps, such as denoising, flat-field correction, and statistical analysis, such as curve fittings can be performed using applications. Advanced applications provide further tools, such as multiple fitting options that reduce human intervention and error¹⁷⁷. For remote monitoring or diagnostics, cloud storage, AI/ML pattern recognition, calibration transfer, and drift correction, smartphones' network capabilities can be integrated into applications^{55,175,181,182}. Furthermore, touchscreen UI can be used for step-by-step guidance to end-users and immediate visualization. Additionally, results can be geo-tagged and shared for surveillance and telemedicine purposes^{170,175,181,183}. Overall, smartphones are upgradable, modular devices that provide a hardware and software ecosystem that can enhance analytical capabilities.

Smartphone element/role	Analytical function	Typical performance/examples	Citations
CMOS camera + optics	Array detector for colorimetric, fluorescence, CL, imaging, microscopy, spectrometry	Optical biosensors with LODs from fg mL ⁻¹ –μM; sensitivity comparable to microplate readers in optimized systems	170,174,184–186
LED flash, screen, ambient sensor	Illumination and controlled excitation; absorbance/fluorescence readout	Salinity LOD 0.04 ppt; Hg ²⁺ , Pb ²⁺ , Cu ²⁺ , F ⁻ , antibiotics down to nM–μM	176
CPU/GPU, memory	On-device image/signal processing, curve fitting, chemometrics	Multisignal fluorescence platform with 99.48% localization hit rate: MSE ≈ 3.16	175,177
USB, audio, Bluetooth, NFC	Interface to electrochemical/ECL circuits and readers	Open-source potentiostats and dual-mode EC–colorimetric glucose readers with LOD down to 0.467 μM	55,176,179,187
OS, apps, connectivity	Workflow control, data logging, cloud/ML, telemedicine	Remote diagnostics, ML-enhanced ECL sensing, multiplexed POC biosensing	55,170,175,181–183

Table 1. 2 Roles of Smartphone Components in Analytical Systems: Functional Integration and Representative Performance Metrics

Detection Mode/Example	Analytical Figures of Merit	Smartphone Role	Citations
Dual- λ fluorescence, 17- β -estradiol	LOD 1 pg mL ⁻¹ ; AUC 0.922 vs. ELISA 0.956	Imaging, quantification	188
CL-LFIA, salivary cortisol	LOD 0.3 ng mL ⁻¹ ; range 0.3–60 ng mL ⁻¹	CL imaging, app analysis	189
Colorimetric aflatoxin B ₁ on paper	Range 0.01–1000 ng mL ⁻¹ ; LOD 3.9 pg mL ⁻¹	Image acquisition & quantitation	24
Lactate in oral fluid (paper)	LOD 0.1 mM; 1 min assay	Reflectance reader, dark box	190
Lactate in sweat (thread CL)	LOD 0.25 mM; 3 min; 2 μ L sample	CL detection & processing	191
TRI-analyzer (multi-mode)	LODs <0.5nm	Spectrometer, multi-mode reader	192
ECL glucose/lactate platform	LOD 0.04/0.10 mM; linear to 1–4 mM	ECL imaging, app-based analysis	193
cpb2 DNA electrochemical sensor	Linear 10 ⁻¹⁵ –10 ⁻⁸ M; LOD 1.5 fM	Interface, visualization	194
Iron colorimetric sensor	CV 5.13% across phones; 8.8% accuracy gain	Robust imaging under varying light	173

Table 1. 3 Detection Modes in Smartphone-Assisted Analytical Platforms: Performance Characteristics and System Roles

1.9 Image-Based Quantitative Analysis

Smartphones based biosensors assay system provides data as digital images, and the pixel values of these data provide the concentrations of analytes. The major steps include acquisition of images, preprocessing, feature extraction, and finally calibration.

Image acquisition is typically performed under dark boxes (in cases of bioluminescence, chemiluminescence, or fluorescence systems), with controlled LEDs, and fixed geometries to reduce signal variations and phone-to-phone differences. Smartphones also provide higher limits of detection (LOD)¹⁹⁵. There are several approaches to feature extraction, including using the mean pixel intensity in a region of interest (ROI), the RGB ratio, or converting signals to HSV, CIE Lab, and CIE xyY color spaces. These methods aim to improve robustness against varying ambient lighting conditions and to better align with human perception. Research has shown that using HSV saturation is more effective than the ROI and RGB ratios method, based on an analysis of over 10,000 images. This improvement leads to better limits of detection (LOD) and precision, even under different field conditions^{196–199}. One study reported a quantitative accuracy of 94% for lateral-flow assays (LFIA) utilizing a custom algorithm that involved denoising, binarization, pixel accumulation, and convolution techniques to accurately locate control lines¹⁹⁸. Additionally, smartphone imaging for microplate and strip assays has achieved a limit of detection for melamine at 0.07 mg/L, and for proteins and antibodies at the nanogram per milliliter (ng/mL) level^{195,199}. This suggests that quantitative analysis using smartphone-based imaging is a promising tool for real-world applications.

1.10 Role of AI in Smartphone-Based Biosensors

Artificial Intelligence (AI) is transforming the field of biosensing by replacing manual image processing with data-driven feature learning and decision-making. Various approaches can be utilized for biosensing purposes, including supervised learning, a method that relies on labeled data and is optimized for loss functions to minimize classification errors. It employs validation strategies to reduce overfitting and enhance generalizability. In contrast, unsupervised methods cluster unlabeled data to identify patterns, which aids in exploratory

analysis^{200–202}. Classical machine learning techniques can be applied to regression or classification tasks. For example, U-net region of interest (ROI) segmentation combined with XGBoost regression achieved a Mean Absolute Percentage Error (MAPE) of approximately 17%, which improved low-concentration quantification compared to human readings. Additionally, machine vision has demonstrated over 97.2% accuracy in sample-type classification and over 97.3% accuracy for concentration estimates using edge detection trained on 180 images from electrophoresis-titration distance assays²⁰³. Deep learning techniques have been employed for complex imaging and microfluidics-based imaging tasks. A smartphone-microfluidic mobile health (mHealth) application utilized convolutional neural networks (CNNs) to recognize cells, parasites, and viruses, as well as for image segmentation tasks, achieving 98% accuracy with images captured via smartphone microscopes²⁰⁴. Another multilingual fluorescence smartphone platform, based on a preprocessing algorithm, produced a hit rate of 9.48%, a mean squared error (MSE) of 3.16, and a mean absolute error (MAE) of 1.36 (in grayscale units) for quantitative biosensing¹⁷⁷. Furthermore, a nanozyme colorimetric sensor array was developed, with image segmentation and feature extraction conducted using deep learning (specifically MobileNetV3, trained on 38,868 images). The R^2 values from this system reached up to 0.997 for fatty acids identified with just one click²⁰⁵. A colorimetric pathogen detection biosensor was created that utilized YOLOv5 for the detection of bacteria at a concentration of 10 CFU/mL within 60 minutes, successfully distinguishing between Gram-positive and Gram-negative bacteria. This biosensor achieved 100% sensitivity in clinical samples²⁰⁶. Overall, AI-enhanced smartphone-based biosensors have shown a systematic increase in detection limits (LOD) by 1 to 2 orders of magnitude, demonstrated robustness against varying external lighting conditions, and accounted for user variability and heterogeneity.^{207–209}.

1.10.1 Challenges and Design Considerations

Many AI-based platforms rely on synthetic augmentation, transfer learning, or mid-range datasets (100-200 images) curated for model training. This results in data scarcity and an increase in labeling costs that limit the AI models. Furthermore, constraints of light-weight models (e.g., MobileNet, quantized CNNs) or cloud implementation raise privacy and latency issues over implementation on smartphones. Studies have suggested that the use of interpretable and uncertainty-aware AI, along with SHAP/LIME or attention mechanisms are needed to meet regulatory requirements and build trust.^{197,203,205,208–211}

AI implementation in smartphone-based sensing has increased from RGB intensity measurements to more complex pipelines that combine image processing, optimization of color spaces, and finally interpretations. From classical AI implementation to increase LOD and accuracy to improved sensitivity, compensate imaging conditions, automated, high-throughput, multimodal diagnostics over smartphones is the new norm in research.

1.11 Monitoring-to-Valorization Framework for Agri-Food and Agro-Industrial Waste

The heterogeneous nature in composition, moisture, and toxicity of Agri-food and agro-industrial wastes directly determines the valorization options in terms of technicality, economics, and environmental feasibility. The physicochemical and biological parameters (organic load, nutrient content, water activity, and toxic compounds) decide the specific valorization technology (bioactive, bioenergy, biopolymers, animal feed, adsorbents, packaging, etc.). And, without robust monitoring of these parameters, the outcome may lead to process inhibition, unsafe products, or secondary pollution, undermining circular-economy goals. So, monitoring (including water and toxicity monitoring) becomes not an add-on but a first decision gate in any valorization strategy^{212–217}.

1.11.1 Stepwise Framework: From Monitored Waste Stream to Valorization Route

The first stage involves monitoring, which includes characterization and risk screening. Low-cost sensors, biosensors, and analytical methods are used to map indicators such as moisture levels, Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), and nutrients (nitrogen, phosphorus, potassium), as well as organic micropollutants, heavy metals, and the ecotoxicity of process waters and slurries. Waste is further classified into categories such as pre-consumption vs. post-consumption, wet vs. dry, and oily vs. lignocellulosic. This classification helps in predicting possible methods for waste valorization.^{218–221} At the same time, data for pollutant amount and potential for greenhouse emissions are monitored, making management options available^{222,223}.

Secondly, data from monitoring devices is utilized to make routing decisions among major pathways. For streams that are high in moisture, high in organic matter, and low in toxicity, biorefineries for energy recovery (such as biogas, bioethanol, bio-oils, and hydrochar) are suitable options^{214,216,217}. Extraction of bioactive compounds applies to streams rich in polyphenols, carotenoids, fibers, and similar substances; however, it requires careful management of contaminants and solvent usage^{212,215,224–226}. Additionally, these streams can also be processed into animal feed and feed additives, though this must be done with rigorous

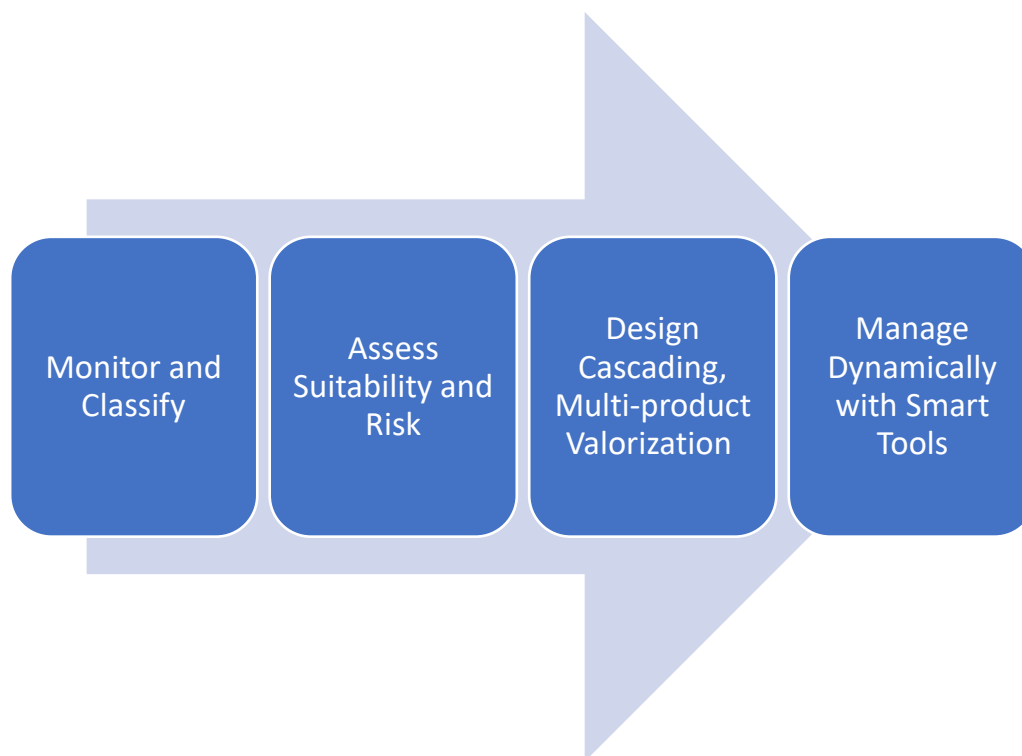


Figure 1. 4 Conceptual Framework Illustrating the Monitoring-to-Valorization Logic in Practical Applications

monitoring and adherence to safety regulations²²⁷. Lignocellulosic, carbon-rich residues can be transformed into bio-based packaging, while hydrochars can be used for water treatment as materials and adsorbents, provided that toxicity is monitored closely^{214,228–230}.

Typically, single-product schemes are not profitable, whereas multi-product, cascading schemes (multi-valorization) are more effective. This approach fosters a zero-waste method: first, bioactives are extracted from the waste stream; the remaining solid can then be converted into bioenergy or other materials, and any residual process water should be re-treated and reused. Monitoring all stages of this process ensures safety and efficiency concerning toxicity and quality^{214,216,217,228,229}.

Finally, Real-time decisions about optimizing bioprocesses, predicting inhibition or toxicity events, and recommending routing of waste streams can be transformed from data provided by IoT-enabled biosensors and AI-based continuous monitoring. Multicriteria decision frameworks combine technical indicators (yield, toxicity), economic metrics, and policy constraints to prioritize smart solutions such as connectivity, real-time sensing, and data sharing along the supply chain (Figure 1.4)^{231,232}.

1.12 Importance of Monitoring Waste Management in Agri-Food and Agro-Industrial Systems

The only possibility to obtain effective valorization is by continuously assessing waste and wastewater quality with respect to water use, pollution loads, and toxicity. Agri-food and agro-industrial activities can degrade surface and groundwater if not managed properly, as they generate nutrient- and organic-rich effluents. Notable issues include salts, metals, pathogens, drugs, emerging contaminants, and high COD/BOD. Agro-industrial vinasses and food processing effluent-related literature have highlighted the exceedance of discharge limits of waste streams. Thus, require treatment before release or reuse, especially when regulations require color and toxicity parameters. Monitoring provides data for compliance (see Table 1.4 for discharge standards, assessing risks of eutrophication, soil degradation, and ecotoxicity, and deciding whether a stream can be reused, treated, or valorized^{94,222,233,234}.

A comprehensive scheme about indicators that should be monitored provides coupling of physicochemical and toxicity-based parameters, includes Core water-quality parameters (COD/BOD, nutrients (N, P), EC/salinity, pH, turbidity, TDS, temperature)^{222,234–237} Priority pollutants (metals, pesticides, antibiotics, endocrine disruptors, and other emerging contaminants) and Ecotoxicological tests (micro biotests with algae and crustaceans). Biosensors and portable devices have been proposed to enable in situ and real-time monitoring of indicators in complex agro-industrial effluents. They provide multiplexing, low-cost screening, and are suitable for decentralized facilities^{87,94,97,104,105,235,238}.

Monitoring target	Role in management/valorization	Citations
COD/BOD, N, P, EC, pH, turbidity	Decide on discharge, reuse, or need/type of treatment	222,234–237
Metals, antibiotics, pesticides, ECs	Identify toxicity risks; constrain reuse, feed, or soil application	94,97,104,235,238
Whole-effluent toxicity (bioassays)	Integrate mixture effects; classify hazard of lagoons/outfalls	235,239
Continuous online/portable sensing	Enable early warning, process control, and dynamic routing	87,90,93,97,104,105,240

Table 1. 4 Key Monitoring Parameters Supporting Waste Management and Circular Valorization Approaches

Smart-phone-based Biosensors/PADs have shifted the paradigm towards decentralized waste monitoring, delivering results rapidly at the point of need, reducing technicalities and cost associated with conventional laboratory-based assays. This has democratized environmental surveillance even in non-expert or low-resource settings^{241–244}. The major strengths of this approach are high sensitivity/selectivity through advanced biorecognition elements, Real-time data processing/visualization via custom apps, and finally Seamless data transfer/sharing through wireless/cloud connectivity^{245–247}. However, there are still challenges, like standardization across smartphones, long-term stability of biosensors, adoption barriers and training issues, data security issues, and finally scaling up from proof-of-concept to commercial deployment^{104,244,248}.

1.13 References

- 1 G. Markou, L. Wang, J. Ye and A. Unc, *Biotechnol. Adv.*, 2018, **36**, 1238–1254.
- 2 H. Al-Hazmi, A. Mohammadi, A. Hejna, J. Majtacz, A. Esmaeili, S. Habibzadeh, M. Saeb, M. Badawi, E. Lima and J. Małkinia, *Environ. Res.*, 2023, 116711.
- 3 V. Trotta, O. Baaloudj and M. Brienza, *Front. Environ. Sci.*, 2024, **12**, 1358842.
- 4 B. J. Singh, A. Chakraborty and R. Sehgal, *J. Environ. Manage.*, 2023, **348**, 119230.
- 5 M. Mainardis, D. Cecconet, A. Moretti, A. Callegari, D. Goi, S. Freguia and A. Capodaglio, *Environmental pollution*, 2021, 118755.
- 6 J. Fito, S. W. H. Van Hulle, S. V. Be, J. Fito and S. W. H. Van Hulle, *Environment, Development and Sustainability 2020 23:3*, 2020, **23**, 2949–2972.
- 7 A. Moretti, H. L. Ivan and J. Skvaril, *Journal of Water Process Engineering*, 2024.
- 8 S. N. Zainurin, W. W. Ismail, S. N. I. Mahamud, I. Ismail, J. Jamaludin, K. N. Z. Ariffin and W. W. A. Kamil, *Int. J. Environ. Res. Public Health*, 2022, **19**.
- 9 R. Sharma, N. Verma, Y. Lugani, S. Kumar and M. Asadnia, 2020, 1–48.
- 10 Md. J. H. Mridha, Md. M. Hossain, S. Md. Imran, S. A. Al Wahid, A. Tahsin, Md. G. R. Alam, A. Talukder and A. W. Reza, *IEEE Access*, 2025, **13**, 98729–98746.
- 11 R. La Cognata, S. Piazza and G. Freni, *Water (Basel)*, 2025.
- 12 M. Sharma, P. Mahajan, A. Alsubaie, V. Khanna, S. Chahal, A. Thakur, A. Yadav, A. Arya, A. Singh and G. Singh, *Materials Today Sustainability*, 2024.
- 13 C. W. Huang, C. Lin, M. K. Nguyen, A. Hussain, X. T. Bui and H. H. Ngo, *Bioengineered*, 2023, **14**, 58–80.
- 14 A. Bharti, U. Jain and N. Chauhan, *Plant Nano Biology*, 2024.
- 15 Y. Wang, J. Liao, D. Zhu, W. Ma and Y. R. Danesh, *Sensors 2025, Vol. 25, Page 6631*, 2025, **25**, 6631.

- 16 M. Thakur, B. Wang and M. Verma, *Environ. Technol. Innov.*, 2022.
- 17 X. Huang, Y. Zhu and E. Kianfar, *Journal of Materials Research and Technology*, 2021, **12**, 1649–1672.
- 18 A. N. Kozitsina, T. S. Svalova, N. N. Malysheva, A. V. Okhokhonin, M. B. Vidrevich and K. Z. Brainina, *MDPI*, 2018, preprint, DOI: 10.3390/bios8020035.
- 19 V. Naresh and N. Lee, *Sensors 2021, Vol. 21, Page 1109*, 2021, **21**, 1109.
- 20 X. Huang, Y. Zhu and E. Kianfar, *Journal of Materials Research and Technology*, 2021, **12**, 1649–1672.
- 21 R. Karunakaran and M. Keskin, *Analytical Techniques in Biosciences: from Basics to Applications*, 2022, 179–190.
- 22 C. Yudin Kharismasari, Irkham, M. I. H. L. Zein, A. Hardianto, S. Nur Zakiyyah, A. Umar Ibrahim, M. Ozsoz and Y. Wahyuni Hartati, *Bioelectrochemistry*, 2024, **155**, 108600.
- 23 M. S. Sumitha and T. S. Xavier, *Hybrid Advances*, 2023, **2**, 100023.
- 24 S. S. Moshirian-Farahi, H. Rahmanian, J. Wu, Q. Huang, Y. Sun, T. Ma, H. Wu, Y. Fu, K. Cheng and J. Pan, *Biosens. Bioelectron.*, 2025, **282**, 117500.
- 25 A. Piriya V.S, P. Joseph, K. Daniel S.C.G., S. Lakshmanan, T. Kinoshita and S. Muthusamy, *Materials Science and Engineering: C*, 2017, **78**, 1231–1245.
- 26 V. G. Panferov and J. Liu, *Adv. Opt. Mater.*, 2024, **12**, 2401318.
- 27 P. Proposito, L. Burratti and I. Venditti, *Chemosensors 2020, Vol. 8, Page 26*, 2020, **8**, 26.
- 28 H. Aldewachi, T. Chalati, M. N. Woodroffe, N. Bricklebank, B. Sharrack and P. Gardiner, *Nanoscale*, 2017, **10**, 18–33.
- 29 B. Babamiri, D. Bahari and A. Salimi, *Biosens. Bioelectron.*, 2019, **142**, 111530.
- 30 A. Roda, M. Mirasoli, E. Michelini, M. Di Fusco, M. Zangheri, L. Cevenini, B. Roda and P. Simoni, *Biosens. Bioelectron.*, 2016, **76**, 164–179.

- 31 R. Abbasi, X. Hu, A. Zhang, I. Dummer and S. Wachsmann-Hogiu, *Bioengineering* 2024, Vol. 11, Page 912, 2024, **11**, 912.
- 32 A. Barhoum, Z. Altintas, K. S. S. Devi and R. J. Forster, *Nano Today*, 2023, **50**, 101874.
- 33 E. Martínez-Periñán, C. Gutiérrez-Sánchez, T. García-Mendiola and E. Lorenzo, *MDPI*, 2020, preprint, DOI: 10.3390/bios10090118.
- 34 E. M. Gross, S. S. Maddipati and S. M. Snyder, *Future Science Ltd*, 2016, preprint, DOI: 10.4155/bio-2016-0178.
- 35 X. Meng, X. Pang, J. Yang, X. Zhang and H. Dong, *Small*, 2024, **20**, 2307701.
- 36 M. Qing, L. Fu, X. Hu, Z. Mu, Y. Wu and L. Bai, *Talanta*, 2025, **288**, 127734.
- 37 K. Zhang, *MedComm – Biomaterials and Applications*, 2025, **4**, e70027.
- 38 M. Cox, J. January, K. V. Mokwebo, S. T. Yussuf, N. A. Sanga, Z. D. Leve, S. F. Douman and E. I. Iwuoha, *ACS Sens.*, DOI:10.1021/acssensors.4c03517.
- 39 L. Zhou, J. Fei, S. Zhang and T. Shan, *Biosensors* 2025, Vol. 15, Page 471, 2025, **15**, 471.
- 40 A. Camarca, A. Varriale, A. Capo, A. Pennacchio, A. Calabrese, C. Giannattasio, C. M. Almuzara, S. D’auria and M. Staiano, *MDPI AG*, 2021, preprint, DOI: 10.3390/s21030906.
- 41 S. Ghanbarlou, D. Kahforoushan, H. Abdollahi, P. Zarrintaj, A. Alomar, C. Villanueva and S. M. Davachi, *Talanta*, 2025, **294**, 128176.
- 42 S. Alharthi, S. Alharthi, M. Madani, H. Alkhaldi, N. Aldaleeli, A. Almarri, F. Alnass, D. Alzahrani, S. A. Mahmoud, M. A. Haque, M. S. Attia, A. Siddiq, S. S. Aly and M. M. Ghobashy, *ChemBioEng Reviews*, 2025, **12**, e70006.
- 43 A. Kidanemariam and S. Cho, *Sensors* 2025, Vol. 25, Page 5081, 2025, **25**, 5081.
- 44 L. Hellweg, A. Edenhofer, L. Barck, M. C. Huppertz, M. S. Frei, M. Tarnawski, A. Bergner, B. Koch, K. Johnsson and J. Hiblot, *Nat. Chem. Biol.*, 2023, **19**, 1147–1157.

- 45 X. Sun, T. Xiang, L. Xie, Q. Ren, J. Chang, W. Jiang, Z. Jin, X. Yang, W. Ren and Y. Yu, *Mater. Today Bio*, 2025, **32**.
- 46 J. Yan, L. Cheng, Y. Li, R. Wang and J. Wang, *Biosensors 2025, Vol. 15, Page 283*, 2025, **15**, 283.
- 47 T. Azad, H. J. Janse Van Rensburg, J. Morgan, R. Rezaei, M. J. F. Crupi, R. Chen, M. Ghahremani, M. Jamalkhah, N. Forbes, C. Ilkow and J. C. Bell, *ACS Nanoscience Au*, 2021, **1**, 15–37.
- 48 T. A. Su, K. J. Bruemmer and C. J. Chang, *Curr. Opin. Biotechnol.*, 2019, **60**, 198–204.
- 49 M. M. Calabretta, D. Gregucci, H. Martínez-Pérez-Cejuela and E. Michelini, *Biosensors (Basel)*, DOI:10.3390/bios12090742.
- 50 A. C. Love and J. A. Prescher, *Elsevier Ltd*, 2020, preprint, DOI: 10.1016/j.chembiol.2020.07.022.
- 51 K. Niwa, H. Kubota, T. Enomoto, Y. Ichino and Y. Ohmiya, *MDPI*, 2023, preprint, DOI: 10.3390/bios13020223.
- 52 K. M. Townsend and J. A. Prescher, *Neurophotronics*, DOI:10.1117/1.nph.11.2.024204.
- 53 C. Reporters, B. Hsien, W. Yeh and H.-W. Ai, DOI:10.1146/annurev-anchem-061318.
- 54 G. G. Lambert, E. L. Crespo, J. Murphy, K. L. Turner, E. Gershowitz, M. Cunningham, D. Boassa, S. Luong, D. Celinskis, J. J. Allen, S. Venn, Y. Zhu, M. Karadas, J. Chen, R. Marisca, H. Gelnaw, D. K. Nguyen, J. Hu, B. N. Sprecher, M. O. Tree, R. Orcutt, D. Heydari, A. B. Bell, A. Torreblanca-Zanca, A. Hakimi, T. Czopka, S. Shoham, K. I. Nagel, D. Schoppik, A. Andrade, D. Lipscombe, C. I. Moore, U. Hochgeschwender and N. C. Shaner, *Nature Methods* 2025 23:1, 2025, **23**, 205–215.
- 55 M. Zhang, X. Cui and N. Li, *Mater. Today Bio*, 2022, **14**, 100254.

- 56 U. Anand, A. K. S. Chandel, P. Oleksak, A. Mishra, O. Krejcar, I. H. Raval, A. Dey and K. Kuca, *Applied Microbiology and Biotechnology* 2022 106:8, 2022, **106**, 2827–2853.
- 57 F. Liu, F. Li, A. N. Nordin and I. Voiculescu, *Sensors (Switzerland)*, 2013, **13**, 3039–3055.
- 58 N. Gupta, V. Renugopalakrishnan, D. Liepmann, R. Paulmurugan and B. D. Malhotra, *Biosens. Bioelectron.*, 2019, **141**, 111435.
- 59 J. Narang, N. Rana, A. Chauhan, A. Kumari and V. Minhas, *Applied Biochemistry and Biotechnology* 2025 197:7, 2025, **197**, 4224–4262.
- 60 G. Mihaela and G. Mihaela, *Journal of Biomedical Research*, 2021, Vol. 35, Issue 4, Pages: 255-263, 2021, **35**, 255–263.
- 61 S. Gaba, N. Chauhan, R. Chandra and U. Jain, *Talanta Open*, 2024, **9**, 100301.
- 62 S. Chen, X. Chen, H. Su, M. Guo and H. Liu, *International Journal of Molecular Sciences* 2023, Vol. 24, Page 7989, 2023, **24**, 7989.
- 63 T. Wang and Y. Lu, *Biosensors* 2022, Vol. 12, Page 318, 2022, **12**, 318.
- 64 J. Lazar and J. Tabor, *Curr. Opin. Syst. Biol.*, 2021, **28**.
- 65 K. H. Lee and D. M. Kim, *Frontiers Media S.A.*, 2019, preprint, DOI: 10.3389/fphar.2019.01166.
- 66 X. Lin, Y. Li, Z. Li, R. Hua, Y. Xing and Y. Lu, *RSC Adv.*, 2020, **10**, 39261–39265.
- 67 Y. Chen, W. Xia, F. Lu, Z. Chen, Y. Liu, M. Cao and N. He, *Microbiol. Res.*, 2025, **293**, 128079.
- 68 Y. Lu, *SpringerBriefs in Applied Sciences and Technology*, 2020, 21–25.
- 69 A. Tinagar, Y. Zhou, F. Hong, K. L. Swingle, A. A. Tang, A. A. Green and K. Pardee, *Handbook of Cell Biosensors*, 2020, 1–39.
- 70 D. K. Karig, A. Reno, L. E. Franklin and A. C. Timm, *Handbook of Cell Biosensors*, 2020, 1–20.

- 71 P. L. Voyvodic and J. Bonnet, *Curr. Opin. Biomed. Eng.*, 2020, **13**, 9–15.
- 72 D. Mathur, M. Thakur, S. A. Díaz, K. Susumu, M. H. Stewart, E. Oh, S. A. Walper and I. L. Medintz, *ACS Synth. Biol.*, 2022, **11**, 4089–4102.
- 73 J. K. Jung, C. M. Archuleta, K. K. Alam and J. B. Lucks, *Nature Chemical Biology* 2022 18:4, 2022, **18**, 385–393.
- 74 Y. Li, T. Lucci, M. Villarruel Dujovne, J. K. Jung, D. A. Capdevila and J. B. Lucks, *Nature Chemical Biology* 2025 21:6, 2025, **21**, 949–958.
- 75 T. P. Green, J. P. Talley and B. C. Bundy, *Biosensors* 2025, Vol. 15, Page 499, 2025, **15**, 499.
- 76 S. H. N. Joshi, C. Jenkins, D. Ulaeto and T. E. Gorochoowski, *BioDesign Research*, 2024, **6**, 0037.
- 77 P. L. Voyvodic, A. Pandi, M. Koch, I. Conejero, E. Valjent, P. Courtet, E. Renard, J. L. Faulon and J. Bonnet, *Nat. Commun.*, DOI:10.1038/s41467-019-09722-9.
- 78 S. Gaba, N. Chauhan, R. Chandra and U. Jain, *Talanta Open*, 2024, **9**, 100301.
- 79 M. A. Boyd and N. P. Kamat, *Trends Biotechnol.*, 2021, **39**, 927–939.
- 80 P. L. Voyvodic and J. Bonnet, *Curr. Opin. Biomed. Eng.*, 2020, **13**, 9–15.
- 81 T. H. Kim, D. Lee and J. W. Choi, *Biosens. Bioelectron.*, 2017, **94**, 485–499.
- 82 F. Liu, F. Li, A. N. Nordin and I. Voiculescu, *Sensors (Switzerland)*, 2013, **13**, 3039–3055.
- 83 M. A. Boyd, W. Thavarajah, J. B. Lucks and N. P. Kamat, *Sci. Adv.*, DOI:10.1126/sciadv.add6605.
- 84 A. Fdez-Sanromán, N. Bernárdez-Rodas, E. Rosales, M. Pazos, E. González-Romero and M. Á. Sanromán, *Multidisciplinary Digital Publishing Institute (MDPI)*, 2025, preprint, DOI: 10.3390/bios15030189.

- 85 Y. Hui, Z. Huang, M. E. E. Alahi, A. Nag, S. Feng and S. C. Mukhopadhyay, *MDPI*, 2022, preprint, DOI: 10.3390/bios12070551.
- 86 C. W. Huang, C. Lin, M. K. Nguyen, A. Hussain, X. T. Bui and H. H. Ngo, *Taylor and Francis Ltd.*, 2023, preprint, DOI: 10.1080/21655979.2022.2095089.
- 87 C. I. L. Justino, A. C. Duarte and T. A. P. Rocha-Santos, *MDPI AG*, 2017, preprint, DOI: 10.3390/s17122918.
- 88 S. Sinha and G. Kaur, *Tools, Techniques and Protocols for Monitoring Environmental Contaminants*, 2019, 75–94.
- 89 M. H. Do, H. H. Ngo, W. Guo, S. W. Chang, D. D. Nguyen, Y. Liu, S. Varjani and M. Kumar, *Science of The Total Environment*, 2020, **712**, 135612.
- 90 F. Ejeian, P. Etedali, H. A. Mansouri-Tehrani, A. Soozanipour, Z. X. Low, M. Asadnia, A. Taheri-Kafrani and A. Razmjou, *Biosens. Bioelectron.*, 2018, **118**, 66–79.
- 91 A. Bindu, S. Bhadra, S. Nayak, R. Khan, A. A. Prabhu and S. Sevda, *Open Life Sci.*, DOI:10.1515/BIOL-2022-0933/XML.
- 92 F. Ejeian, P. Etedali, H. A. Mansouri-Tehrani, A. Soozanipour, Z. X. Low, M. Asadnia, A. Taheri-Kafrani and A. Razmjou, *Biosens. Bioelectron.*, 2018, **118**, 66–79.
- 93 S. Gavrilăș, C. Ștefan Ursachi, S. Perța-Crișan and F. D. Munteanu, *MDPI*, 2022, preprint, DOI: 10.3390/s22041513.
- 94 Y. Xiao, Z. Du, Y. Li, L. Cao, B. Zhu, T. Kitaguchi and C. Huang, *Sensors* 2025, Vol. 25, Page 4945, 2025, **25**, 4945.
- 95 A. Popena, E. Wiśniowska and C. Manuel, *Desalination Water Treat.*, 2024, **319**, 100456.
- 96 G. Hernandez-Vargas, J. E. Sosa-Hernández, S. Saldarriaga-Hernandez, A. M. Villalba-Rodríguez, R. Parra-Saldivar and H. M. N. Iqbal, *MDPI*, 2018, preprint, DOI: 10.3390/bios8020029.
- 97 E. M. McConnell, J. Nguyen and Y. Li, *Frontiers Media S.A.*, 2020, preprint, DOI: 10.3389/fchem.2020.00434.

- 98 M. Herrera-Domínguez, G. Morales-Luna, J. Mahlkecht, Q. Cheng, I. Aguilar-Hernández and N. Ornelas-Soto, *MDPI*, 2023, preprint, DOI: 10.3390/bios13030370.
- 99 S. Singh, V. Kumar, D. S. Dhanjal, S. Datta, R. Prasad and J. Singh, 2020, pp. 317–335.
- 100 M. H. Do, H. H. Ngo, W. Guo, S. W. Chang, D. D. Nguyen, Y. Liu, S. Varjani and M. Kumar, *Science of The Total Environment*, 2020, **712**, 135612.
- 101 M. Cheliukanov, G. Gurkin, R. Perchikov, A. Medvedeva, T. Lavrova, T. Belousova, A. Titova, Y. Plekhanova, S. Tarasov, A. Kharkova, V. Arlyapov, P. Mandin, H. Nakamura and A. Reshetilov, *Multidisciplinary Digital Publishing Institute (MDPI)*, 2025, preprint, DOI: 10.3390/bios15050290.
- 102 S. Sinha and G. Kaur, *Tools, Techniques and Protocols for Monitoring Environmental Contaminants*, 2019, 75–94.
- 103 P. M. Nowak, R. Wietecha-Posłuszny and J. Pawliszyn, *TrAC Trends in Analytical Chemistry*, 2021, **138**, 116223.
- 104 C. W. Huang, C. Lin, M. K. Nguyen, A. Hussain, X. T. Bui and H. H. Ngo, *Taylor and Francis Ltd.*, 2023, preprint, DOI: 10.1080/21655979.2022.2095089.
- 105 J. Manjunathan, M. Revathi, H. Sowmya, S. S. Meenambiga, R. Sudha, B. Prakash, S. Nangan, L. Gnanasekaran and N. Thirumalaivasan, *Clean Technologies and Environmental Policy* 2023 26:11, 2023, **26**, 3667–3683.
- 106 N. Kuppan, M. Padman, M. Mahadeva, S. Srinivasan and R. Devarajan, *Waste Management Bulletin*, 2024, **2**, 154–171.
- 107 R. B. González-González, E. A. Flores-Contreras, E. González-González, N. E. Torres Castillo, R. Parra-Saldívar and H. M. N. Iqbal, *Ind. Eng. Chem. Res.*, 2022, **62**, 4503–4520.
- 108 G. Elli, S. Hamed, M. Petrelli, P. Ibba, M. Ciocca, P. Lugli and L. Petti, *Sensors*, DOI:10.3390/s22114178.

- 109 H. C. Liu, J. X. Du, J. Wang, J. Liu, L. Yang and Y. C. Yong, *Biosensors (Basel)*., DOI:10.3390/bios15060341.
- 110 X. Jin, X. Li, N. Zhao, I. Angelidaki and Y. Zhang, *Water Res.*, 2017, **111**, 74–80.
- 111 A. Mishra, S. Patra, V. Srivastava, L. Uzun, Y. K. Mishra, M. Syväjärvi and A. Tiwari, *Biosens. Bioelectron. X*, 2022, **11**, 100166.
- 112 N. Seddaoui, N. Colozza, L. Gullo and F. Arduini, *Int. J. Biol. Macromol.*, 2023, **253**, 127409.
- 113 A. Mishra, S. Patra, V. Srivastava, L. Uzun, Y. K. Mishra, M. Syväjärvi and A. Tiwari, *Biosens. Bioelectron. X*, 2022, **11**, 100166.
- 114 S. Kummari, L. R. Panicker, J. Rao Bommi, S. Karingula, V. Sunil Kumar, K. Mahato and K. Y. Goud, *MDPI*, 2023, preprint, DOI: 10.3390/bios13040420.
- 115 S. R. Benjamin, F. de Lima, V. A. do Nascimento, G. M. de Andrade and R. B. Oriá, *Multidisciplinary Digital Publishing Institute (MDPI)*, 2023, preprint, DOI: 10.3390/bios13070689.
- 116 S. Kumar, J. B. Kaushal and H. P. Lee, *Multidisciplinary Digital Publishing Institute (MDPI)*, 2024, preprint, DOI: 10.3390/bios14060300.
- 117 A. T. Singh, D. Lantigua, A. Meka, S. Taing, M. Pandher and G. Camci-Unal, *MDPI AG*, 2018, preprint, DOI: 10.3390/s18092838.
- 118 S. Malik, J. Singh, K. Saini, V. Chaudhary, A. Umar, A. A. Ibrahim, S. Akbar and S. Baskoutas, *Royal Society of Chemistry*, 2024, preprint, DOI: 10.1039/d3ay02258g.
- 119 N. Colozza, V. Caratelli, D. Moscone and F. Arduini, *MDPI*, 2021, preprint, DOI: 10.3390/BIOS11090328.
- 120 A. Kumar and P. Maiti, *Royal Society of Chemistry*, 2024, preprint, DOI: 10.1039/d3ma01019h.
- 121 Y. Liu, S. Lu, Z. Zhang, Z. Yang, X. Cui and G. Liu, *Lab Chip*, 2023, **23**, 3328–3352.

- 122 N. Seddaoui, N. Colozza, L. Gullo and F. Arduini, *Int. J. Biol. Macromol.*, 2023, **253**, 127409.
- 123 M. M. Bordbar, A. Sheini, P. Hashemi, A. Hajian and H. Bagheri, *MDPI*, 2021, preprint, DOI: 10.3390/bios11090316.
- 124 F. Arduini, *Curr. Opin. Electrochem.*, 2022, **35**, 101090.
- 125 R. Kumari, A. Singh, U. P. Azad and P. Chandra, *Multidisciplinary Digital Publishing Institute (MDPI)*, 2023, preprint, DOI: 10.3390/bios13090891.
- 126 N. Colozza, V. Caratelli, D. Moscone and F. Arduini, *MDPI*, 2021, preprint, DOI: 10.3390/BIOS11090328.
- 127 A. F. Putra, A. S. Ningrum, Suyanto, V. M. Pratiwi, M. Y. H. Widiyanto, Irkham, W. T. Wahyuni, I. Rahmawati, F. M. Wang, C. H. Huang and R. A. Wahyuono, *ADMET DMPK*, DOI:10.5599/admet.2833.
- 128 J. Ding, B. Li, L. Chen and W. Qin, *Angewandte Chemie - International Edition*, 2016, **55**, 13033–13037.
- 129 L. Ge, S. Wang, X. Song, S. Ge and J. Yu, *Lab Chip*, 2012, **12**, 3150–3158.
- 130 Y. Li, H. Chen, R. Huang, D. Deng, X. Yan and L. Luo, *Anal. Chim. Acta*, 2024, **1316**, 342828.
- 131 Y. Zhu, X. Tong, Q. Wei, G. Cai, Y. Cao, C. Tong, S. Shi and F. Wang, *Biosens. Bioelectron.*, 2022, **196**, 113691.
- 132 Y. Cheng, S. Feng, Q. Ning, T. Li, H. Xu, Q. Sun, D. Cui and K. Wang, *Microsyst. Nanoeng.*, DOI:10.1038/s41378-023-00514-2.
- 133 A. Yakoh, S. Chaiyo, W. Siangproh and O. Chailapakul, *ACS Sens.*, 2019, **4**, 1211–1221.
- 134 L. Ge, S. Wang, X. Song, S. Ge and J. Yu, *Lab Chip*, 2012, **12**, 3150–3158.
- 135 Y. Li, H. Chen, R. Huang, D. Deng, X. Yan and L. Luo, *Anal. Chim. Acta*, 2024, **1316**, 342828.

- 136 Y. Cheng, S. Feng, Q. Ning, T. Li, H. Xu, Q. Sun, D. Cui and K. Wang, *Microsyst. Nanoeng.*, DOI:10.1038/s41378-023-00514-2.
- 137 C. Chen, B. Ran, B. Liu, Y. Xiao, G. Tang, T. Shen, J. Liang and Z. Liang, *Microchemical Journal*, 2025, **210**, 112966.
- 138 A. Laza, S. V. Pereira, G. A. Messina, M. A. Fernández-Baldo, J. Raba, M. D. Regiart and F. A. Bertolino, *Chemosensors*, DOI:10.3390/chemosensors12010010.
- 139 A. B. Sözmen, A. E. Bayraktar and A. Arslan-Yildiz, *Emergent Materials* 2024 7:6, 2024, **7**, 2985–2993.
- 140 B. Li, Z. Zhang, J. Qi, N. Zhou, S. Qin, J. Choo and L. Chen, *ACS Sens.*, 2017, **2**, 243–250.
- 141 H. Wang, Y. He, Z. Yu, R. Chen, Z. Feng, D. Chen, W. M. E. S. Shakweer, F. Zhang, X. Nan, M. Mijit, B. Xiong, L. Yang and X. Tang, *Biosensors (Basel)*., DOI:10.3390/bios15070405.
- 142 X. Weng, Z. Fu, C. Zhang, W. Jiang and H. Jiang, *Anal. Chem.*, 2022, **94**, 3526–3534.
- 143 X. Li and X. Liu, *Adv. Healthc. Mater.*, 2016, **5**, 1378.
- 144 X. Li and X. Liu, *Adv. Healthc. Mater.*, 2016, **5**, 1326–1335.
- 145 Y. Shen, S. Modha, H. Tsutsui and A. Mulchandani, *Biosens. Bioelectron.*, 2021, **171**, 112721.
- 146 S. Pesaran, E. Rafatmah and B. Hemmateenejad, *Biosensors 2021, Vol. 11, Page 44*, 2021, **11**, 44.
- 147 S. Smith, J. G. Korvink, D. Mager and K. Land, *RSC Adv.*, 2018, **8**, 34012–34034.
- 148 N. Seddaoui, N. Colozza, L. Gullo and F. Arduini, *Int. J. Biol. Macromol.*, 2023, **253**, 127409.
- 149 F. Kong and Y. F. Hu, *Analytical and Bioanalytical Chemistry* 2012 403:1, 2012, **403**, 7–13.

- 150 M. Nemiwal, T. C. Zhang and D. Kumar, *Enzyme Microb. Technol.*, 2022, **156**, 110006.
- 151 A. Sassolas, L. J. Blum and B. D. Leca-Bouvier, *Biotechnol. Adv.*, 2012, **30**, 489–511.
- 152 G. Sun, X. Wei, D. Zhang, L. Huang, H. Liu and H. Fang, *Biosensors 2023, Vol. 13*, Page 886, 2023, **13**, 886.
- 153 A. Böhm, S. Trosien, O. Avrutina, H. Kolmar and M. Biesalski, *Front. Chem.*, 2018, **6**, 373094.
- 154 A. Dabhade, S. Jayaraman and B. Paramasivan, *3 Biotech 2021 11:4*, 2021, **11**, 183-.
- 155 F. Mustafa and S. Andreescu, *ACS Sens.*, 2020, **5**, 4092–4100.
- 156 S. Khan, B. Burciu, C. D. M. Filipe, Y. Li, K. Dellinger and T. F. Didar, *ACS Nano*, 2021, **15**, 13943–13969.
- 157 F. V. Oberhaus, D. Frense and D. Beckmann, *Biosensors 2020, Vol. 10*, Page 45, 2020, **10**, 45.
- 158 X. Tao, X. Wang, B. Liu and J. Liu, *Biosens. Bioelectron.*, 2020, **168**, 112537.
- 159 Y. Chen, K. Danchana and T. Kaneta, *Analytical and Bioanalytical Chemistry 2024 416:28*, 2024, **416**, 6679–6686.
- 160 N. Seddaoui, N. Colozza, L. Gullo and F. Arduini, *Int. J. Biol. Macromol.*, 2023, **253**, 127409.
- 161 D. Zhang, Y. Bai, H. Niu, L. Chen, J. Xiao, Q. Guo and P. Jia, *Biosensors (Basel).*, 2024, **14**, 168.
- 162 M. Sher, R. Zhuang, U. Demirci and W. Asghar, *Expert Rev. Mol. Diagn.*, 2017, **17**, 351–366.
- 163 N. Jiang, N. D. Tansukawat, L. Gonzalez-Macia, H. C. Ates, C. Dincer, F. Güder, S. Tasoglu and A. K. Yetisen, *ACS Sens.*, 2021, **6**, 2108–2124.

- 164 J. Hu, S. Q. Wang, L. Wang, F. Li, B. Pingguan-Murphy, T. J. Lu and F. Xu, *Biosens. Bioelectron.*, 2014, **54**, 585–597.
- 165 A. P. Gayatri, N. R. Bandaru, M. G. Bonthu and D. S. Tandu, *Curr. Biotechnol.*, 2025, **14**, 83–107.
- 166 S. Ahmed, M. P. N. Bui and A. Abbas, *Biosens. Bioelectron.*, 2016, **77**, 249–263.
- 167 K. Mahato, A. Srivastava and P. Chandra, *Biosens. Bioelectron.*, 2017, **96**, 246–259.
- 168 B. Kuswandi, M. A. Hidayat and E. Noviana, *Biosensors 2022, Vol. 12, Page 1088*, 2022, **12**, 1088.
- 169 S. Cinti, D. Moscone and F. Arduini, *Nature Protocols 2019 14:8*, 2019, **14**, 2437–2451.
- 170 X. Huang, D. Xu, J. Chen, J. Liu, Y. Li, J. Song, X. Ma and J. Guo, *Analyst*, 2018, **143**, 5339–5351.
- 171 S. Banik, S. K. Melanthota, Arbaaz, J. M. Vaz, V. M. Kadambalithaya, I. Hussain, S. Dutta and N. Mazumder, *Analytical and Bioanalytical Chemistry 2021 413:9*, 2021, **413**, 2389–2406.
- 172 D. Quesada-González and A. Merkoçi, *Biosens. Bioelectron.*, 2017, **92**, 549–562.
- 173 N. A. Nguyen, A. Hendricks, E. Montoya, A. Mayers, D. Rajmohan, A. Morrin, M. McCaul, N. Dunne, N. O’Connor, A. Spanias, G. Raupp and E. Forzani, *Sensors*, 2025, **25**, 4693.
- 174 Z. Geng, X. Zhang, Z. Fan, X. Lv, Y. Su and H. Chen, *Sensors 2017, Vol. 17, Page 2449*, 2017, **17**, 2449.
- 175 D. V. Baker, J. Bernal-Escalante, C. Traaseth, Y. Wang, M. V. Tran, S. Keenan and W. R. Algar, *Lab Chip*, 2025, **25**, 884–955.
- 176 T. H. Bui, B. Thangavel, M. Sharipov, K. Chen and J. H. Shin, *Chemosensors 2023, Vol. 11, Page 468*, 2023, **11**, 468.

- 177 W. Zhan, Q. Geng, B. Wang, Y. Liu and Z. Geng, *IEEE Sens. J.*, 2025, **25**, 18312–18322.
- 178 D. V. Baker, J. Bernal-Escalante, C. Traaseth, Y. Wang, M. V. Tran, S. Keenan and W. R. Algar, *Lab Chip*, 2025, **25**, 884–955.
- 179 A. Ainla, M. P. S. Mousavi, M. N. Tsaloglou, J. Redston, J. G. Bell, M. T. Fernández-Abedul and G. M. Whitesides, *Anal. Chem.*, 2018, **90**, 6240–6246.
- 180 D. Zhang and Q. Liu, *Biosens. Bioelectron.*, 2016, **75**, 273–284.
- 181 T. Beduk, D. Beduk, M. R. Hasan, E. Guler Celik, J. Kosel, J. Narang, K. N. Salama and S. Timur, *Biosensors 2022, Vol. 12, Page 583*, 2022, **12**, 583.
- 182 M. L. Bhaiyya, S. K. Srivastava, P. K. Pattnaik and S. Goel, *IEEE Trans. Instrum. Meas.*, DOI:10.1109/TIM.2023.3296819.
- 183 W. K. Abdelbasset, S. V. Savina, D. Mavaluru, R. A. Shichiyakh, D. O. Bokov and Y. F. Mustafa, *Talanta*, 2023, **252**, 123769.
- 184 M. Rezazadeh, S. Seidi, M. Lid, S. Pedersen-Bjergaard and Y. Yamini, *TrAC Trends in Analytical Chemistry*, 2019, **118**, 548–555.
- 185 J. Tang, Z. Gao, L. Xu, Q. Zhao, T. Hu, Y. Luo, J. Dou, Y. Bai, L. Xia and K. Du, *Food Chem.*, 2025, **462**, 141026.
- 186 H. Xu, C. Li, R. Mao, X. Wang, Y. Fan, H. Lu, J. Liu and H. Zhou, *Biosens. Bioelectron.*, 2024, **262**, 116569.
- 187 J. Xu, J. Shen, B. Zhang, Y. Zhang, X. Lv and G. Zhu, *Electrochim. Acta*, 2024, **481**, 143952.
- 188 W. Il Lee, S. Shrivastava, L. T. Duy, B. Yeong Kim, Y. M. Son and N. E. Lee, *Biosens. Bioelectron.*, 2017, **94**, 643–650.
- 189 M. Zangheri, L. Cevenini, L. Anfossi, C. Baggiani, P. Simoni, F. Di Nardo and A. Roda, *Biosens. Bioelectron.*, 2015, **64**, 63–68.

- 190 D. Calabria, C. Caliceti, M. Zangheri, M. Mirasoli, P. Simoni and A. Roda, *Biosens. Bioelectron.*, 2017, **94**, 124–130.
- 191 E. Maiorano, M. M. Calabretta, E. Lunedei and E. Michelini, *Biosensors (Basel)*., 2025, **15**, 530.
- 192 K. D. Long, E. V. Woodburn, H. M. Le, U. K. Shah, S. S. Lumetta and B. T. Cunningham, *Lab Chip*, 2017, **17**, 3246–3257.
- 193 A. Kumar, D. Jain, J. Bahuguna, M. Bhaiyya, S. K. Dubey, A. Javed and S. Goel, *Biosens. Bioelectron.*, 2023, **238**, 115582.
- 194 Q. Wu, B. Yang, Y. Wang, Y. Xiong, J. Zhang, Y. Wen and A. Rajkovic, *Microchimica Acta 2025 192:4*, 2025, **192**, 264-.
- 195 M. Mesas Gómez, E. Julián, L. Armengou and M. I. Pividori, *Talanta*, 2024, **275**, 126106.
- 196 Z. Geng, Y. Miao, G. Zhang and X. Liang, *Sens. Actuators A Phys.*, 2023, **349**, 114056.
- 197 T. Karademir, G. Kaleli-Can and B. E. Köktürk-Güzel, *Biosensors 2025, Vol. 15, Page 507*, 2025, **15**, 507.
- 198 S. Zhang, X. Jiang, S. Lu, G. Yang, S. Wu, L. Chen and H. Pan, *Sensors 2023, Vol. 23, Page 6401*, 2023, **23**, 6401.
- 199 Y. C. Chang, K. H. Wu, J. C. Wang and W. C. Huang, *Pure and Applied Chemistry*, 2025, **97**, 235–244.
- 200 P. Garg, M. K. Sharma and P. Kumar, *Arabian Journal for Science and Engineering 2025 50:19*, 2025, **50**, 15751–15767.
- 201 Z. Salahuddin, H. C. Woodruff, A. Chatterjee and P. Lambin, *Comput. Biol. Med.*, 2022, **140**, 105111.
- 202 V. M. Rao, M. Hla, M. Moor, S. Adithan, S. Kwak, E. J. Topol and P. Rajpurkar, *Nature 2025 639:8056*, 2025, **639**, 888–896.

- 203 Z. Guo, Y. Cao, Y. Tian, L. Fan, W. Liu, Y. Ma, Q. Zhang and C. Cao, *Biosens. Bioelectron.*, 2025, **267**, 116832.
- 204 B. Wang, Y. Li, M. Zhou, Y. Han, M. Zhang, Z. Gao, Z. Liu, P. Chen, W. Du, X. Zhang, X. Feng and B. F. Liu, *Nature Communications* 2023 14:1, 2023, **14**, 1341-.
- 205 X. Zhong, Y. Qin, C. Liang, Z. Liang, Y. Nong, S. Luo, Y. Guo, Y. Yang, L. Wei, J. Li, M. Zhang, S. Tang, Y. Liang, J. Wu, Y. M. Lam and Z. Su, *ACS Sens.*, 2024, **9**, 5167–5178.
- 206 R. Cui, H. Tang, Q. Huang, T. Ye, J. Chen, Y. Huang, C. Hou, S. Wang, S. Ramadan, B. Li, Y. Xu, L. Xu and D. Li, *Biosens. Bioelectron.*, 2024, **259**, 116369.
- 207 P. Shende, B. Prabhakar and A. Patil, *Sens. Actuators A Phys.*, 2023, **349**, 114056.
- 208 D. H. Hagos, S. K. Aryal, P. Ymele-Leki and L. L. Burge, *Comput. Struct. Biotechnol. J.*, 2025, **27**, 2219–2232.
- 209 Y. Yang, F. Xu, J. Chen, C. Tao, Y. Li, Q. Chen, S. Tang, H. K. Lee and W. Shen, *Biosens. Bioelectron.*, 2023, **229**, 115233.
- 210 T. Wasilewski, W. Kamysz and J. Gębicki, *Biosensors* 2024, Vol. 14, Page 356, 2024, **14**, 356.
- 211 R. Qureshi, M. Irfan, H. Ali, A. Khan, A. S. Nittala, S. Ali, A. Shah, T. M. Gondal, F. Sadak, Z. Shah, M. U. Hadi, S. Khan, Q. Al-Tashi, J. Wu, A. Bermak and T. Alam, *IEEE Access*, 2023, **11**, 61600–61620.
- 212 R. Kour, S. Singh, H. B. Sharma, T. S. S. K. Naik, N. Shehata, P. N. W. Ali, D. Kapoor, D. S. Dhanjal, J. Singh, A. H. Khan, N. A. Khan, M. Yousefi and P. C. Ramamurthy, *Chemosphere*, 2023, **317**, 137822.
- 213 F. M. Antony, D. Pal and K. Wasewar, *Physical Sciences Reviews*, 2021, **6**, 1–21.
- 214 P. R. Yaashikaa, P. Senthil Kumar and S. Varjani, *Bioresour. Technol.*, 2022, **343**, 126126.

- 215 S. Yadav, K. Malik, J. M. C. Moore, B. R. Kamboj, S. Malik, V. K. Malik, S. Arya, K. Singh, S. Mahanta and D. K. Bishnoi, *Molecules* 2024, Vol. 29, Page 2055, 2024, **29**, 2055.
- 216 J. Painsi, V. Benedetti, S. S. Ail, M. J. Castaldi, M. Baratieri and F. Patuzzi, *Waste and Biomass Valorization* 2021 13:1, 2021, **13**, 31–50.
- 217 I. Prado-Acebo, J. Cubero-Cardoso, T. A. Lu-Chau and G. Eibes, *Waste Management*, 2024, **183**, 42–52.
- 218 J. Painsi, V. Benedetti, S. S. Ail, M. J. Castaldi, M. Baratieri and F. Patuzzi, *Waste and Biomass Valorization* 2021 13:1, 2021, **13**, 31–50.
- 219 P. R. Yaashikaa, P. Senthil Kumar and S. Varjani, *Bioresour. Technol.*, 2022, **343**, 126126.
- 220 F. M. Antony, D. Pal and K. Wasewar, *Physical Sciences Reviews*, 2021, **6**, 1–21.
- 221 R. Kour, S. Singh, H. B. Sharma, T. S. S. K. Naik, N. Shehata, P. N. W. Ali, D. Kapoor, D. S. Dhanjal, J. Singh, A. H. Khan, N. A. Khan, M. Yousefi and P. C. Ramamurthy, *Chemosphere*, 2023, **317**, 137822.
- 222 D. Díaz-Vázquez, D. Carrillo-Nieves, D. A. Orozco-Nunnally, C. Senés-Guerrero and M. S. Gradilla-Hernández, *Front. Environ. Sci.*, 2021, **9**, 682093.
- 223 P. Roy, A. K. Mohanty, P. Dick and M. Misra, *ACS Environmental Au*, 2023, **3**, 58–75.
- 224 E. Shawky, S. Gibbons and D. Selim, *Bioresour. Technol.*, 2025, 132640.
- 225 A. Arias, G. Feijoo and M. T. Moreira, *J. Environ. Manage.*, 2025, **380**, 124935.
- 226 S. de O. Silva, A. K. C. Mafra, F. M. Pelissari, L. Rodrigues de Lemos and G. Molina, *Microorganisms* 2025, Vol. 13, Page 1789, 2025, **13**, 1789.
- 227 D. Malenica, M. Kass and R. Bhat, *Sustainability* 2023, Vol. 15, Page 117, 2022, **15**, 117.

- 228 S. Agro-Food Waste, M. Ragoubi, F. Becquart, L. S. de C Carnaval, A. K. Jaiswal and S. Jaiswal, *Journal of Composites Science* 2024, Vol. 8, Page 41, 2024, **8**, 41.
- 229 I. Akkari, M. M. Kaci and M. Pazos, *Environmental Monitoring and Assessment* 2024 196:11, 2024, **196**, 1035-.
- 230 P. Rao and V. Rathod, *The Chemical Record*, 2019, **19**, 1858–1871.
- 231 Z. Said, P. Sharma, Q. T. B. Nhuong, B. Bora, E. Lichtfouse, H. Khalid, R. Luque, X. P. Nguyen and A. T. Hoang, *Bioresour. Technol.*, 2023, 128952.
- 232 Q. Zhang and H. Zhang, *Sustainability* 2024, Vol. 16, Page 6169, 2024, **16**, 6169.
- 233 H. El-Ramady, E. Brevik, Y. Bayoumi, T. Shalaby, M. El-Mahrouk, N. Taha, H. Elbasiouny, F. Elbehiry, M. Amer, N. Abdalla, J. Prokisch, S. Solberg and W. Ling, *Sustainability*, 2022.
- 234 B. Koul, M. Yakoob and M. Shah, *Environ. Res.*, 2021, 112285.
- 235 S. Šariri, Ž. Cvetković, T. M. Pavin, Z. Kljaković-Gašpić, D. Valić, T. Kralj, A. Brkić, Z. Redžović and V. F. Marijić, *J. Contam. Hydrol.*, 2025, **274**, 104667.
- 236 W. Czekala and J. Pulka, *Water* 2024, Vol. 16, Page 1817, 2024, **16**, 1817.
- 237 R. K. Nishan, S. Akter, R. I. Sony, Md. M. Hoque, M. J. Anee and A. Hossain, *Discover Water* 2024 4:1, 2024, **4**, 43-.
- 238 J. Burkholder, B. Libra, P. Weyer, S. Heathcote, D. Kolpin, P. Thorne and M. Wichman, *Environ. Health Perspect.*, 2006, **115**, 308–312.
- 239 A. G. Yeshiwas, G. M. Bayeh, T. D. Tsega, S. S. Tsega, A. A. Gebeyehu, Z. Alamrie Asmare, R. M. Anteneh, A. G. Ejigu, A. F. Ahmed, Z. A. Yigzaw, A. Temesgen, A. S. Enawgaw, G. Yirdaw, H. Mekonen and C. Yenew, *Environ. Health Insights*, DOI:10.1177/11786302251329795.
- 240 T. N. D. Trinh, N. K. S. Tran, H. A. Nguyen, N. M. Chon, K. T. L. Trinh and N. Y. Lee, *Biomicrofluidics*, DOI:10.1063/5.0224217.

- 241 S. Mansouri, S. Boulares, S. Chabchoub, Y. Alharbi and A. Alqahtani, *Microchemical Journal*, 2025, **209**, 112670.
- 242 Q. ul ain Zahra, S. A. H. Mohsan, F. Shahzad, M. Qamar, B. Qiu, Z. Luo and S. A. Zaidi, *Biosens. Bioelectron.*, 2022, **215**, 114509.
- 243 A. R. Sharifi, V. Mazzaracchio, L. Duranti, L. Gullo, S. Brannetti, M. Peyravian, M. A. Kiani and F. Arduini, *ACS Sens.*, 2024, **9**, 6542–6552.
- 244 S. Upadhyay, A. Kumar, M. Srivastava, A. Srivastava, A. Dwivedi, R. K. Singh and S. K. Srivastava, *Talanta*, 2024, **275**, 126080.
- 245 C. Li, Z. Zhu, J. Yao, Z. Chen and Y. Huang, *Molecules* 2024, *Vol. 29*, Page 4891, 2024, **29**, 4891.
- 246 A. R. Sharifi, V. Mazzaracchio, L. Duranti, L. Gullo, S. Brannetti, M. Peyravian, M. A. Kiani and F. Arduini, *ACS Sens.*, 2024.
- 247 K. Mao, H. Zhang, Y. Pan and Z. Yang, *Water Res.*, 2020, **191**, 116787.
- 248 R. E. Madrid, A. Ramallo, F. ; Barraza, D. E. ; Chaile, R. E. Madrid, F. A. Ramallo, D. E. Barraza and R. E. Chaile, *Bioengineering* 2022, *Vol. 9*, Page 101, 2022, **9**, 101.

Chapter 2

An All-in-One Sustainable Smartphone Paper Biosensor for Water Toxicity Monitoring Combining Bioluminescence Detection with Artificial Intelligence

This chapter is based on the following published article:

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Author contributions are detailed in the separate section of this thesis.

2.1 Introduction

Water monitoring is crucial for ecosystems and human health. Unsustainable practices in industry, agriculture, and urban-ization contribute to surface water pollution, releasing harmful chemicals like heavy metals (lead, mercury, cadmium) as well as organic compounds such as polychlorinated biphenyls (PCBs) and toxins derived from harmful algal blooms, posing continuous challenges to both analytical chemists as well as regulators^{1,2}. In addition to agricultural and industrial activities, urban wastewater discharges containing chemicals such as pharmaceuticals and microplastics significantly contribute to water contamination^{3,4}. All of these pollutants pose significant threats to aquatic ecosystems and human health. Therefore, it is of outmost importance to treat these polluted water sources before they are discharged to surface water bodies, especially in the case of water reuse that is becoming vital⁵. Different water treatment technologies can be implemented, but to evaluate the degree of performance needed and the quality of the final effluent, precise and easy to use measuring instruments are required, possibly at the point of need^{6,7}.

Highly sensitive laboratory-based techniques, including liquid chromatography high-resolution mass spectrometry (LC-HRMS) and gas chromatography mass (GC-MS), are available to detect several analytes, including polar and nonpolar contaminants of emerging concern (CEC).^{8,9} Nevertheless, not all CECs are detected by these techniques, and the implementation and harmonization of protocols for nontarget screening is required to support regulatory bodies. In addition to conventional screening, biosensors represent a viable solution especially for screening purposes and on-site analysis^{10–12}. The implementation of portable devices integrating biosensors was reported with applications spanning from toxicity monitoring to detection of heavy metals. The implementation of portable devices integrating biosensors was reported with applications spanning from toxicity monitoring to detection of heavy metals^{13–15}. More promising devices rely on the use of a smartphone as a detector, avoiding the need for hand-held optical detectors. Despite the development of several prototypes, their uptake in the market remains limited^{16,17}. Among the main reasons there are regulatory issues connected to i) the on-site use of genetically modified (GM) organisms, ii) the sustainability and cost of these analytical devices, iii) the need to perform pipetting or dispensing steps and data elaboration to obtain quantitative results, and iv) the scarce robustness of smartphone-based devices, i.e., the quality of results is highly dependent on the smartphone camera.

To address these issues, we developed a bioluminescence (BL) sustainable paper sensor relying on *Aliivibrio fischeri* bacteria that provides a novel approach for on-site water analysis. Although several attempts have been reported to implement bacterial bioreporters on portable devices for water monitoring^{18,19} no complete integration of the entire assay procedure has been achieved. *A. fischeri* strain has the capability of emitting visible light due to the presence of the lux operon encoding both the luciferase (luxAB) and proteins needed for synthesis of the aldehyde substrate (luxCDE)²⁰. *A. fischeri* bacteria are widely used for toxicological assessment due to their sensitivity to a broad range of toxic compounds, including metals, organic pollutants, and pesticides. Their luminescence response is highly reproducible, making them ideal for consistent toxicity evaluation. The International Organization for Standardization (ISO) has defined the ISO 11348 method (<https://www.iso.org/standard/40518.html>), based on *A. fischeri*, for water quality monitoring. This test requires cell culture facilities, benchtop instrumentations and skilled personnel, increasing the cost and time of the analysis.

To turn this method into a low-cost and user-friendly toxicity biosensor, a bioluminescent paper sensor obtained by entrapping *A. fischeri* has been developed with the goal of implementing both analytical steps (one-step assay) and post-analytical steps (data analysis performed by the Android app) for providing real-time quantitative user-friendly results, in

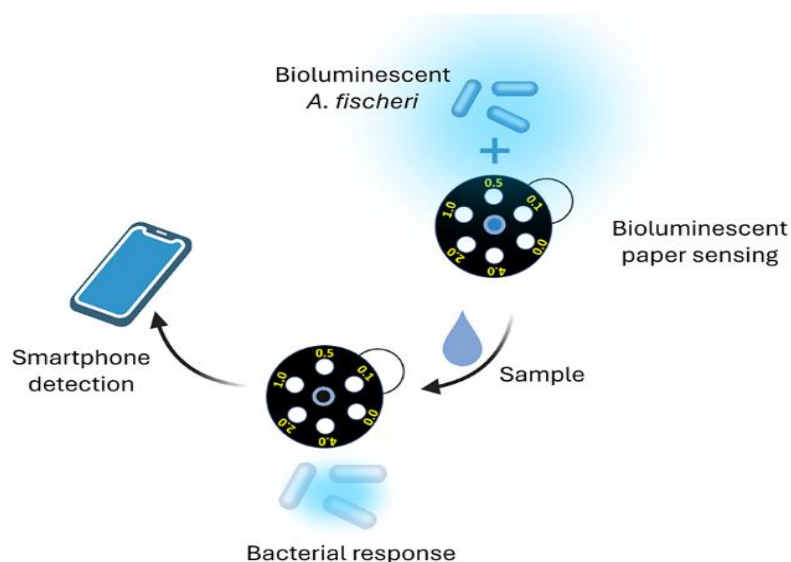


Figure 2. 1 Schematic representation of the paper biosensor principle.

terms of toxicity equivalents, within 15 min. The paper biosensor also integrates a calibration curve, which allows, combined with an AI algorithm, to obtain information on the toxicity

directly from a smartphone-captured photo of the sample (Figure 2. 1). This algorithm was implemented into an Android application, and the assay was validated with tap and wastewater samples spiked with different contaminants to simulate different conditions such as the presence of disinfectant agent residues, toxins deriving from algae bloom and pesticide residues. The app combined with the paper sensor was also tested with different smartphone models, showing promising results both in terms of analytical performance and ease of use with potential applicability for use by the general population, also in perspective of citizen science.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents.

Naturally bioluminescent *A. fischeri* bacteria were kindly gifted from Prof. Stefano Girotti²⁰. NaClO was from commercially available bleach with a declared NaClO percentage of 3.5%_{v/v}. Microcystin-LR, 3,5-dichlorophenol, lead nitrate, lysogeny broth (LB) medium and all reagents for cell culture were from Merck (St. Louis, MO). Whatman 1 CHR cellulose chromatography paper was from GE Healthcare (Chicago, IL, USA) and was used as support for the design of the bioluminescent sensing paper. OnePlus 6T (OnePlus, Shenzhen, China), Motorola edge 40 neo (Motorola Mobility LLC, Chicago, IL, USA), Huawei P20 (Huawei Technologies Co. Ltd, Shenzhen, GD, China), Samsung Galaxy S20 (Samsung Electronics, Suwon-si, South Korea), iPhone 12 mini and iPhone 13 mini (Apple Inc., Cupertino, CA, USA) were used for bioluminescent signal acquisitions. Wax printer Phaser 8400 office (Xerox, Norwalk, CT, USA) was used for wax printing.

2.2.2 Immobilization of *A. fischeri* Bacteria.

A. fischeri strain was cultured in LB medium with high salinity (10 g/L peptone, 30 g/L NaCl, 5 g/L yeast extract) at 19 °C and with orbital shaking at 140 rpm. Different experimental parameters were evaluated for cell immobilization on paper including volumes and cell number. An array of 3x6 circular hydrophilic wells (7 mm diameter) was designed using PowerPoint software (Microsoft, Redmond, WA, USA) and printed onto W1 paper as host platform to immobilize *A. fischeri*. The wax printed paper sensor was then heated at 150 °C for 1 min to allow wax penetration through the paper thickness effectively forming well-defined

hydrophobic boundaries. To prevent leakage during sample addition, the back side of the sensor was sealed with adhesive tape.

Different bacterial concentrations ranging from 1.2×10^7 to 2.0×10^7 cells/well and supplements (trehalose and glycerol) were tested for *A. fischeri* entrapment in agarose-based hydrogels (See Supporting Information)

In optimized conditions, the BL paper sensor was obtained by entrapping *A. fischeri* (cell suspension with OD600 = 5.0 in LB medium) in 0.5%_{w/v} agarose hydrogel matrix. A 3%_{w/v} agarose hydrogel was first prepared in sterile milliQ water by heating, then, when the agarose hydrogel reached a temperature of around 60°C, an 80 µL-volume was added to 420 µL of *A. fischeri* suspension in LB medium (final temperature of about 30°C) (see Heat Conduction Calculation in Supporting Information). A volume of 20 µL of the bacterial suspension - agarose was deposited immediately into each well. Then, the wells were equilibrated at room temperature (25°C) for 30 minutes before performing the analysis.

2.2.3 Real Samples Analysis.

The suitability of the BL sensing paper was tested with real water samples, including six tap water and six industrial wastewater samples (provided by a carwash activity) spiked with different concentrations of the model analytes (NaClO from 0.1 to 4.0 ppm, microcystin-LR from 1.5 to 40 ppb, 3,5-dichlorophenol from 1.0 to 6.0 ppm and lead nitrate from 5.0 to 100 ppb) and analyzed following the assay procedure described above. Since their viability is affected by toxic agents, the decrease in BL was used to assess the toxicity of samples. BL signals were normalized with respect to the control. Each sample was tested in triplicate and experiments were repeated with three different paper sensors.

2.2.4 Design of Toxicity Sensing Paper and NaOCl Toxicity Assay.

To obtain ready-to-use portable sensing paper for toxicity assessment of water samples, a circular flower-like paper (30.0 mm diameter) has been created by wax printing technology using a Phaser 8400 office wax printer (Xerox, Norwalk, CT, USA). The toxicity sensing paper was designed to contain seven hydrophilic wells (diameter of 5 mm each) to immobilize the BL bacteria: six external wells for the calibration curve (S0, S1, S2, S3, S4, S5) and a central well for the sample. The optimized procedure requires i) dispensing of 30 µL-volume of standard solutions and sample, ii) incubation from 1 to 15 min at room temperature, iii)

placement of the paper sensor in the cardboard dark box (8.5 x 11.5 x 10.0 cm) to avoid external light interference, iv) acquisition with the OnePlus 6 smartphone camera (30-s integration time, ISO1600). Images were analyzed in parallel with ImageJ software and the Android-based application.

2.2.5 Android Application Development.

An Android app, called *Sc Sentinel*, was developed in Python using the Kivy framework library to perform image analysis, within an integrated development environment (IDE), focusing on code validation, statistical analysis through curve fitting, and adjustments of AI parameters. Then, the program was converted into an Android app by finetuning the probability parameter and non-maximum suppression (NMS) for the AI model to 0.67 and 0.01 respectively to increase confidence level for the prediction of BL signal and filtering low-level of signals.

Figure 2. 2 illustrates the user interface of the *Sc Sentinel* application and its functionality. Initially, the image of the paper sensor was uploaded to the *Sc Sentinel* app and transferred to the Microsoft Azure server. On this server, image processing tasks such as instance segmentation and feature extraction were performed. The processed and labeled image along with the extracted features were sent back to the *Sc Sentinel* app (Figure 2. 2a). The OpenCV library was used to label each region of interest (ROI) and extract the corresponding BL signal value. All details are provided in the Supporting Information section.

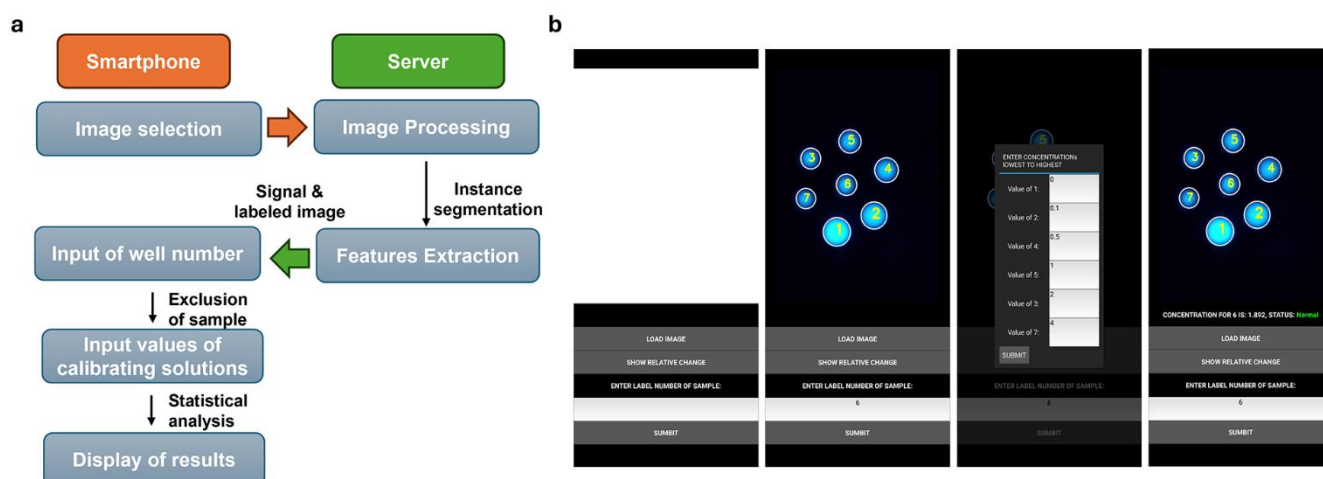


Figure 2. 2 (a) Representation of services handled by smartphone and server. (b) Screenshots of application graphical user interphase (GUI) representing buttons, input prompts, image and concentration display.

2.2.6 Smartphone Acquisitions and Statistical Analysis.

For optimization, a OnePlus 6 smartphone secondary sensor (20 MP Sony Exmor RS IMX 376 K, BSI CMOS color sensor with 1.0 μm pixels, $f/1.7$ aperture) was used with ISO 1600 and 30 s integration time. All pictures were analyzed with ImageJ (v. 1.53k, National Institutes of Health, Bethesda, MD, USA), and the BL signal intensities, expressed as relative light units (RLUs), were quantified over the ROI defined in correspondence of the sensor wells. GraphPad Prism v.8 software (GraphPad Software, LaJolla, CA, USA) was used for data elaboration. The limit of detection (LOD) was calculated as the mean value of the blank minus three times the standard deviation. The limit of quantification (LOQ) was calculated as the mean value of the blank minus ten times the standard deviation. All measurements were performed in triplicate and repeated at least three times.

2.2.7 Analytical Performance of Different Smartphones and Data Elaboration with *Sc Sentinel* Application.

To evaluate the analytical performance of different smartphone-integrated CMOS sensors and to compare data elaboration obtained with ImageJ software and *Sc Sentinel* application, BL images of toxicity sensing papers (3x6 wells, 7mm diameter) incubated with 50 μL of NaOCl (0.0-4.0 ppm) for 1 min at room temperature ($25\pm 2^\circ\text{C}$), were acquired using five different smartphones: Motorola edge 40 neo (16 s acquisition time, ISO 1600), Samsung Galaxy S20 (30 s acquisition time, ISO 1600), Huawei P10 (8 s acquisition time, ISO 1600), iPhone 12 mini (10 s acquisition time, night mode), and iPhone 13 mini (20 s acquisition time, night mode).

2.2.8 Stability and Reproducibility Studies.

Biosensor stability and reproducibility were evaluated over a 14-day period by storing the biosensors at 4°C in petri dishes sealed with Parafilm tape. BL intensities were normalized with respect to BL signal obtained from freshly immobilized *A. fischeri* at day 0. At the same time reproducibility studies were performed in triplicate by incubating the paper-toxicity biosensor with 1 ppm NaOCl for 1-min at room temperature. BL intensities obtained in the sample wells were normalized to BL data in control wells. Images were acquired with OnePlus 6 smartphone (30 sec, ISO 1600) and analyzed with ImageJ. All measurements were performed in triplicate and repeated at least three times with different toxicity paper-based biosensor.

2.3 Results and Discussion

Several biosensors for monitoring water toxicity have been reported; however, none fully incorporate all the essential analytical and post-analytical steps typically performed in a standard analytical laboratory before delivering results. We addressed all the needs required to obtain a robust all-in-one biosensor which only requires sample addition and a photo taken with the smartphone. As proof of concept, we applied this workflow to the standard toxicity bioassay based on bioluminescent *A. fischeri* bacteria. Our approach tackles key challenges in transforming an analytical method, the ISO 11348 method “Water quality - Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* -Luminescent bacteria test”, into a sustainable, all-in-one toxicity biosensor. Since the biosensor is not selective we first investigated its suitability for detecting target key contaminants with the required sensitivity. In particular we tested toxins produced by cyanobacteria, residues of disinfectants, pesticide residues and heavy metals. The frequency of extreme weather events is causing concerns about harmful cyanobacterial algal blooms producing highly toxic metabolites, including microcystin-LR²¹. Another target analyte is 3,5-dichlorophenol, a metabolite of polychlorinated phenols (i.e., Pestanal®) and benzene hexachloride pesticides²². As a model heavy metal, lead (Pb) was chosen due to frequent exposures from lead-containing plumbing and severe health effects including high neurotoxicity, especially in children²³.

2.3.1 Optimization of *A. Fischeri* Immobilization.

Preliminary experiments were performed to optimize *A. fischeri* immobilization on paper. Agarose-based hydrogels were selected using different cell concentrations as previously reported²⁴ with slight modifications. *A. fischeri* cells with OD₆₀₀ 5.0 were entrapped in 0.25% w/v agarose + 10% w/v trehalose hydrogel and deposited on paper. Different bacterial concentrations were tested (range 1.2×10^7 - 2.0×10^7 cells/well); a 5.2 % decreased bioluminescence signal was obtained using the lowest concentration while the highest number of cells resulted in a 26.1 % signal increase compared to the same cell suspension in liquid culture. BL intensities of entrapped *A. fischeri* cells (OD₆₀₀ 5.0) were evaluated using different hydrogel compositions with 0.5% w/v agarose, 0.25% w/v agarose + 15% w/v glycerol, and 0.25% w/v agarose + 10% w/v trehalose (Figure S2. 1), showing increased BL intensities (+ 28.2 %) for both 0.5% w/v agarose and 0.25% w/v agarose + 10% w/v trehalose, and a 4.1 % increase for the hydrogel composed by 0.25% w/v agarose and 15% w/v glycerol.

2.3.2 Toxicity Test optimization with Model Analyte.

After these preliminary evaluations, the hydrogel composition, incubation times, and volume of samples were investigated to identify the suitable combination providing active bacterial cells on paper and the highest sensitivity of the toxicity paper sensor. The analytical performance of the toxicity paper biosensor was assessed by using four environmental contaminants as model analytes: NaClO, microcystin-LR, 3,5-dichlorophenol, and lead nitrate. For each analyte, the incubation time (1, 15, and 30 min) and the volume of the sample (30 and 50 μL) were evaluated with cell suspension (OD_{600} 5.0) in 0.5% w/v agarose and 0.25% w/v agarose + 10% w/v trehalose. As expected, BL signals obtained in the presence of an analyte that causes immediate toxicity, such as NaClO, did not significantly differ using the two hydrogel compositions (Figure S2. 2). This confirms that for NaClO, both entrapment methods represent a viable solution. Incubation times exceeding 15 min result in a complete loss of bioluminescence, even at the lowest tested NaClO concentration (Figure S2. 3), confirming that for chemicals causing an immediate cell death, images must be acquired 1 min after sample addition.

When bacteria entrapped in the trehalose-containing hydrogel were incubated with 3,5-DCP (0.0, 2.0, 3.5, and 6.0 ppm), bioluminescence signals acquired at min 1 showed a negligible inhibitory effect (0.7% decrease in the presence of 2.0 ppm 3,5-DCP) with no concentration-dependent effect (Figure S2. 4). After 30 min of incubation, the decrease in the BL signal was not proportional to 3,5-DCP concentration increase with a nonsignificant decrease in BL (i.e., 99.0% of the signal with 6.0 ppm 3,5-DCP). This effect could be ascribed to the presence of trehalose, which is known to protect bacteria under stress conditions, as previously reported for plants and bacteria²⁵. Bacteria entrapped in 0.5% w/v agarose (without trehalose) showed a significant decrease in the BL signal when incubated with increasing concentrations of 3,5-DCP with a 11.0% decrease in the BL signal after 15 min incubation with 6.0 ppm 3,5-DCP (Figure 2.4a). A similar behavior was observed with MC-LR, which did not produce an immediate toxicity effect on bacteria. In the presence of 40.0 ppb MC-LR, a 16% decrease of the BL signal was observed with agarose-entrapped bacteria, while only 4% decrease was observed with bacteria entrapped with the 0.25% w/v + trehalose 10% w/v hydrogel (Figure S2. 5a,b). We evaluated the hydrogel composition effect in the presence of a heavy metal, i.e., lead, which can leach into water from pipes. No concentration-dependent effect was observed in both the two hydrogel matrices, with only a 7, 8, and 9% signal decrease obtained

with bacteria entrapped in the 0.25% w/v + trehalose 10% w/v hydrogel after 30 min incubation time with 5, 15, and 75 ppb of lead nitrate, respectively (Figure S2. 6). For the other incubation times, however, the same behavior observed with MC-LR was observed. Consequently, the initial hypothesis that trehalose could interfere with the biosensor response due to its nutrient and protective action was confirmed. In optimized conditions, the BL paper sensing was obtained with cell suspension (OD₆₀₀ 5.0) entrapped in the 3.0% w/v agarose hydrogel (final agarose concentration 0.5%_{w/v}). Different sample volumes were tested (30 and 50 μ L). BL signals were acquired after 1 min for NaClO (Figure S2. 7) and after 15 min for microcystin-LR (Figure S2. 8), 3,5 dichlorophenol (Figure S2. 9), and lead nitrate (Figure S2. 10). The 50 μ L sample volume was selected because it allowed better discrimination between the blank and analyte-containing samples. This volume offered the best compromise to enable BL signal detection via the smartphone camera with the required sensitivity.

2.3.3 Analytical Performance of the Toxicity Sensing Paper.

In optimized conditions, the toxicity sensing paper is prepared by adding a 20 μ L volume of the bacterial suspension–agarose to each well; then, the paper sensor is equilibrated at room temperature (25 °C) for 10 min. To perform the toxicity test, a volume of 50 μ L of either the sample or standard analyte is added to the paper sensor and a picture is taken at 1 or 15 min, depending on the analyte toxicity. Because no substrates are required, this poses no inconvenience to the end-user, who can simply capture images at both time points. With the optimized method, toxicity dose–response curves were obtained by using the sensing paper for the analysis of NaClO (from 0.1 to 4.0 ppm), microcystin-LR (from 1.5 to 40 ppb), 3,5-dichlorophenol (from 1.0 to 6.0 ppm), and lead nitrate (from 5.0 to 100 ppb) (Figure 2. 3). Calibration curves were obtained with ImageJ software and with the *Scntinel* app, and a coefficient of determination of 0.990 or higher was obtained for all cases. The LODs and LOQs for NaClO were 0.11 and 0.15 ppm, respectively, for ImageJ, and 0.17 and 0.58 ppm for the *Scntinel* app (Figure 2. 3a), respectively. These results confirm the suitability of the toxicity paper sensor to measure the maximum allowed concentration of free residual chlorine in drinking water of 4 and 5 ppm fixed by the U.S. Environmental Protection Agency (EPA) and the World Health Organization (WHO), respectively. Concerning microcystin-LR, for which the maximum allowed concentration in waters for human consumption is fixed at 1.0 ppb by the WHO (WHO/HEP/ECH/WSH/2020.6), LODs of 0.65 and 0.23 ppb were obtained with ImageJ analysis and *Scntinel* app, respectively; LOQs were 8.55 and 26.5 ppb,

respectively (Dose–response curves using BL signals obtained with the OnePlus6 smartphone camera (30 s integration time, ISO 1600) and quantified by ImageJ software (colored) and AI-assisted application (black) for (a) NaClO in distilled H₂O (1 min incubation time), (b) microcystin-LR in EtOH 5% v/v (15 min incubation time), (c) lead nitrate distilled H₂O (15 min incubation time), (d) 3,5-dichlorophenol EtOH 5% v/v (15 min incubation time). Figure 2. 3 b). For the 3,5-dichlorophenol, LODs of 4.50 and 4.93 ppm and LOQs of 6.76 and 5.12 ppm were obtained with ImageJ analysis and the *Scintinel* app, respectively. In this case, due to a lower toxicity of 3,5-DCP, the sensor analytical performance does not fulfill the required regulatory limits (e.g., the WHO limit for 3,5-DCP in drinking water is 0.5 ppm); however, the toxicity sensor is not specific and is intended for the first screening assay (Figure 2. 3c). When a sample contains multiple contaminants, potentially having additive or synergistic effects on total toxicity, the sensor would provide the total effect of the sample; therefore, we believe this is not a shortcoming of the method. LODs for lead nitrate were 74.5 and 5.0 ppb, and the LOQs were 71.4 and 26.2 ppb, obtained with ImageJ analysis and the *Scintinel* app, respectively (Figure 2. 3 d), supporting the potential use of the application for the analysis of lead in drinking water (maximum allowed concentration according to the WHO is 10 ppb). According to a recent study several commercial kits for lead analysis were not able to detect such levels of lead since showed detection limits in the order of 10-20 mg/L²⁶. We compared these results to a recently reported biosensor in which a *A. fischeri* were immobilized on a 96-wells plate with two strategies, either agar or graphene oxide, and the bioluminescence signal was read with a smartphone²⁷. Despite the promising results obtained by Bergua et al., full integration of the *A. fischeri* in a portable device was not reported, and the limits of detection for the tested pesticides (from milligrams per liter to µg per liter) were still not adequate to those requested by the regulatory agencies (ng/L range). Our smartphone-based biosensor offers several advantages over traditional *A. fischeri*-based toxicity assays such as Microtox and DeltaTox systems, which are either laboratory-based assays or transportable systems requiring power supply and pipetting steps with skilled personnel. In contrast to the Microtox assay, which requires a temperature of 15 °C to rehydrate freeze-dried bacteria and conduct the analysis, our biosensor operates effectively at 25 °C while maintaining satisfactory analytical performance. Moreover, in terms of cost-effectiveness, these conventional approaches can be prohibitively expensive because of the instrument costs and maintenance, whereas our method is based on a low-cost paper sensor and a smartphone, making it accessible to a wider range of customers²⁸. Traditional systems often require trained personnel and laboratory facilities, increasing the cost and the time of the analysis. Thanks to an analysis via image capture and AI-based processing,

our system can perform real-time analysis and provides instant feedback. Besides, AI integration ensures automated and consistent data interpretation,²⁹ reducing human error and enhancing reproducibility.

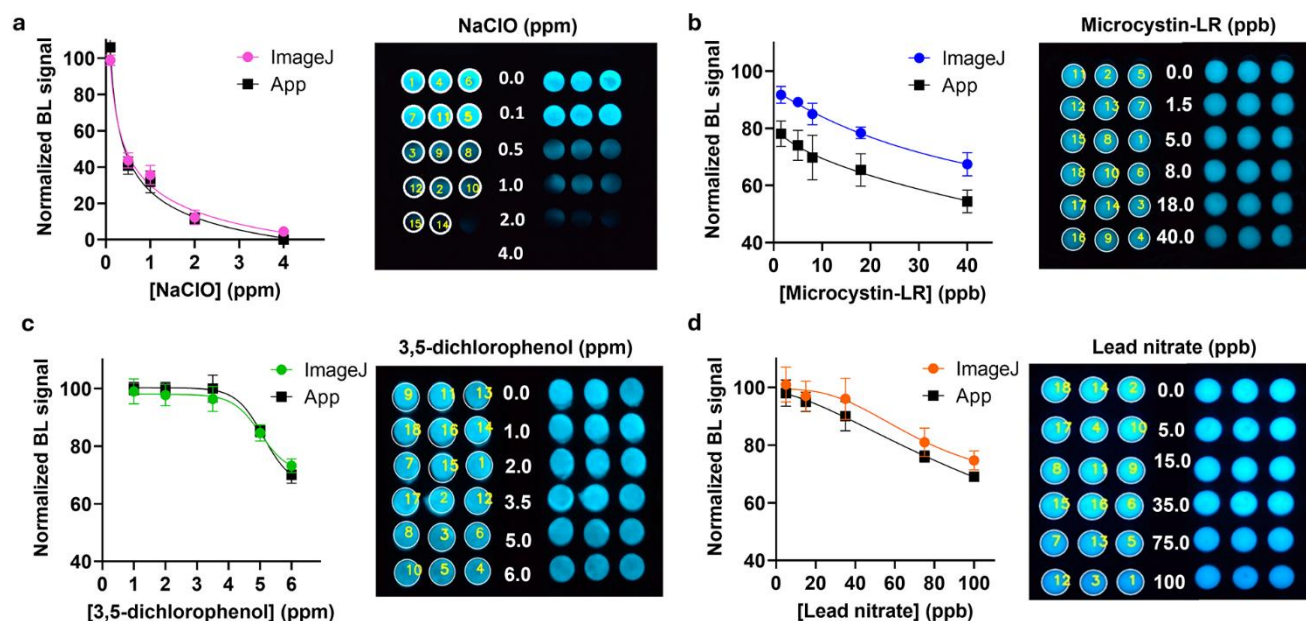


Figure 2. 3 Dose–response curves using BL signals obtained with the OnePlus6 smartphone camera (30 s integration time, ISO 1600) and quantified by ImageJ software (colored) and AI-assisted application (black) for (a) NaClO in distilled H₂O (1 min incubation time), (b) microcystin-LR in EtOH 5% v/v (15 min incubation time), (c) lead nitrate distilled H₂O (15 min incubation time), (d) 3,5-dichlorophenol EtOH 5% v/v (15 min incubation time).

2.3.4 Real Samples and Recovery Studies.

To evaluate the analytical performance of the paper biosensor and investigate the potential matrix effects, real water samples of tap water and wastewaters were spiked with NaClO (Figure S2. 11) microcystin-LR, 3,5-dichlorophenol, and lead nitrate at different concentrations. Toxicity tests were performed with the optimized assay procedure, and data extractions were obtained from the analysis of the bioluminescent signals with both ImageJ software and the *Scintinel* App (Figure 2. 4) The toxicity observed in the presence of the four analytes in tap and wastewaters reflects that obtained in dH₂O both for ImageJ and the App analysis. Differences in the inhibition profile of bacteria are due to the complexity of the matrices. Recovery values for microcystin-LR, 3,5-dichlorophenol and lead nitrate were in the range 91-120% for drinking waters (Table S2. 1) and 92-128% for wastewaters (Table S2. 2) while, as expected, the acute toxicity of NaClO yielded lower recoveries.

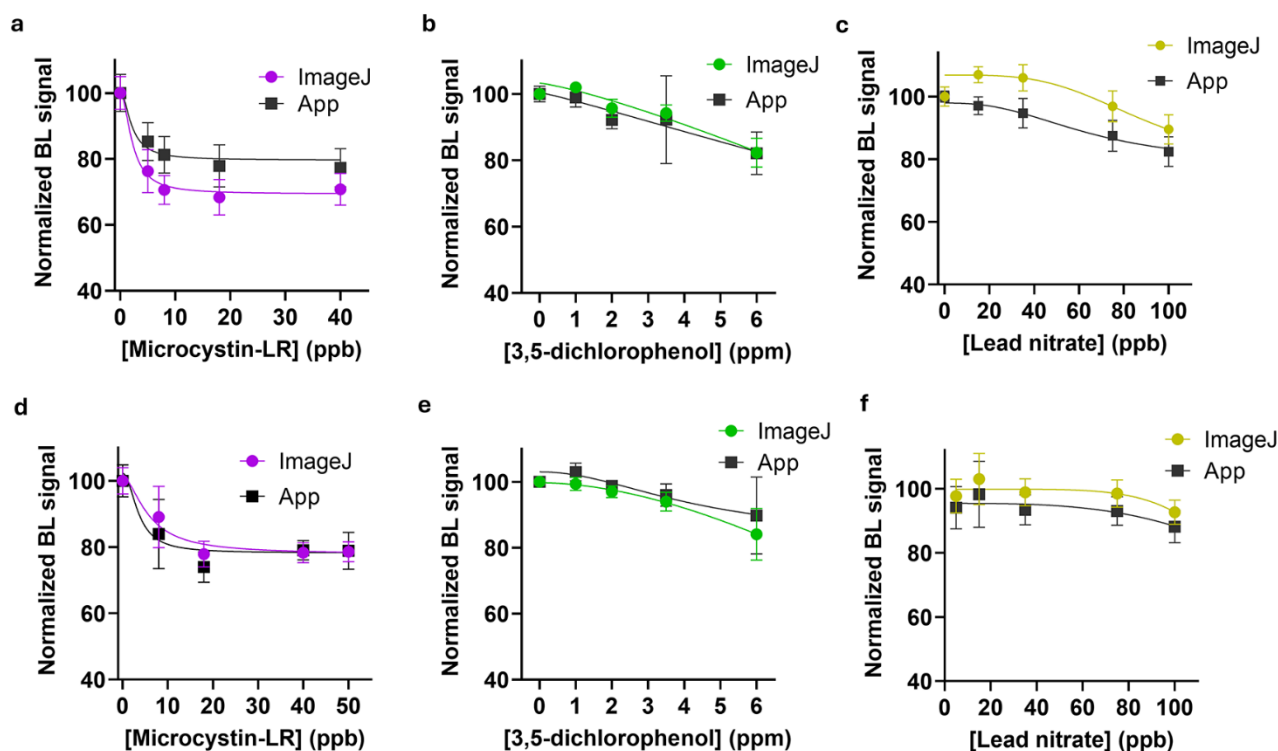


Figure 2. 4 Dose–response curves in (a–c) drinking water and (d–f) in wastewaters obtained with the paper-based biosensor. BL signals were acquired with a OnePlus6 smartphone camera (30 s integration time, ISO 1600) and quantified by ImageJ software (colored) and the AI-assisted application (black) for (a,d) microcystin-LR (15 min incubation time), (b,e) 3,5-dichlorophenol (15 min incubation time), (c,f) lead nitrate (15 min incubation time).

2.3.5 Data Elaboration with AI *Sc Sentinel* App Using Different Smartphones.

One of the main limitations of smartphone-biosensors is the high variability of the response due to the different sensitivity of smartphone-integrated CMOS^{30,31}. Image acquisition from different mobile phones is affected by different sensitivity of sensors and processing algorithms limiting the accuracy of analytical procedures and applicability of Machine Learning (ML) algorithms^{30,31}. To address this issue, we designed a BL toxicity paper sensing which includes an on-board calibration curve enabling the analysis of standard solutions as well as the unknown sample; this strategy avoids artifacts due to signal variations deriving from non-controlled environmental conditions (e.g., temperature, humidity). We evaluated our approach by capturing images of toxicity paper biosensors (three biosensors for each smartphone analysis) incubated with different concentrations of NaClO with various mobile phones, from

flagship to low-tech models. For iOS smartphones, the picture was first taken with the iOS smartphones and then transferred to an Android smartphone with the *Scntinel* app installed. Pictures of the paper toxicity biosensor incubated with different concentrations of NaClO (range 0.0 - 4.0 ppm) were taken with five smartphones (Motorola edge 40 neo, Samsung Galaxy S20, Huawei P10, iPhone 12 mini, and iPhone 13 mini) and compared with those obtained with the OnePlus6. All the tested mobile phone cameras successfully allowed us to

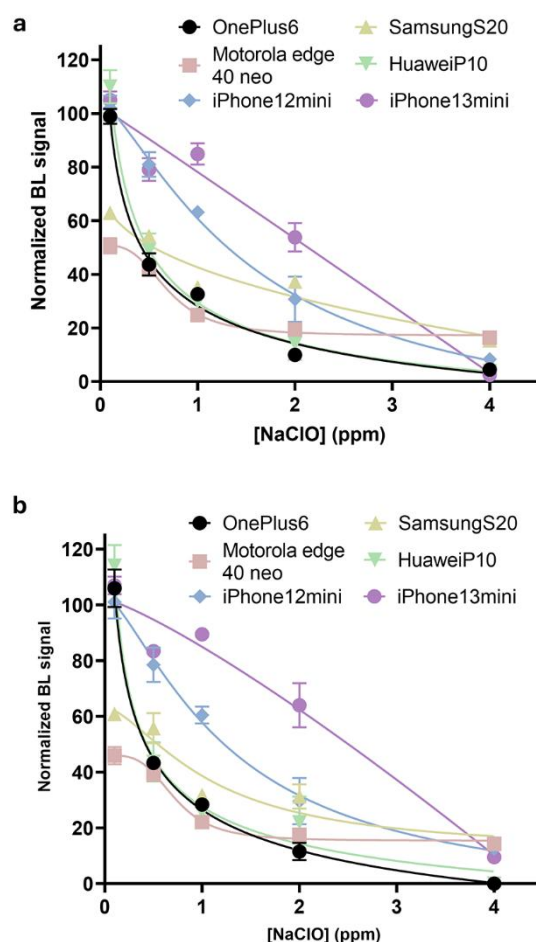


Figure 2. 5 NaClO toxicity curves obtained in ddH₂O, by incubating for 1 min the paper toxicity biosensor with NaClO (from 0.0 to 4.0 ppm) and acquiring BL signals with different smartphones (OnePlus 6, Motorola edge 40 neo, Samsung S20, HuaweiP10, iphone 12 mini and iPhone 13 mini). BL intensities were quantified by using a) ImageJ software and the b) Scntinel app.

retrieve quantitative information with decent dose-response curves obtained with ImageJ analysis (Figure 2. 5a) or the *Scntinel* App (Figure 2. 5 b). Motorola edge 40 neo provided the

lowest LOD was the with 0.06 ppm for NaClO obtained both with imageJ analysis and the *Scentinel* app (Table S2. 3 shows LODs obtained with different smartphones).

Furthermore, our application is compatible with all Android-based mobiles, enabling quantification even with lower ISO ranges and acquisition times. To the best of our knowledge this is the first bioluminescence paper biosensor in which an AI algorithm enables to obtain quantitative results by interpolating the bioluminescent signals from an on-board calibration curve. Since the biosensor is not selective for specific analytes, the read-out provides both

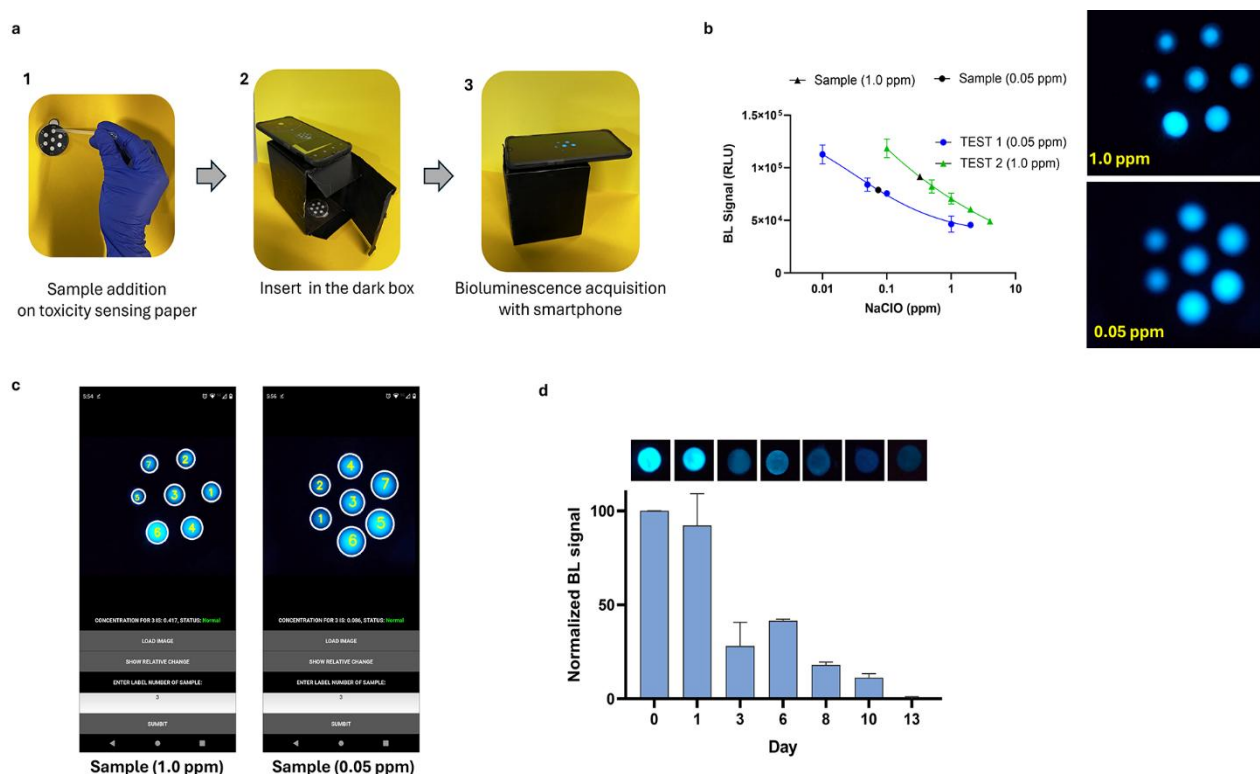


Figure 2. 6 Representation of the procedure for toxicity assessment with the sensing paper containing six external wells (diameter 0.5 mm) used for calibration curve and one well for sample testing. (1) Deposition of 30 μ L-volume of standard solutions and sample into the paper-based sensor. (2) Setup of the signal acquisition using a dark box and a smartphone. 3) Picture of the sensor in the dark box. b) Images of the toxicity sensing paper incubated with 0.05 and 1.0 ppm of NaClO for 1 min and acquired with OnePlus 6T smartphone and calibration curves. c) Read-out of the Scentinel app providing the “status” of the sample (normal) and interpolated concentration. d) Stability of sensing paper stored at +4 °C for 13 days. BL signals are acquired with Oneplus 6 (30 s at ISO 1600) and analysed with ImageJ. BL images obtained on different days are normalized with data on day 0.

quantitative and qualitative information, the latter in terms of general warning, for example “Toxic”, “Safe”, these parameters have been preliminary set according to a pre-defined level of toxicity caused by the sample and can be modified according to the needs. This application could have more general use for all optical biosensors, including those with colorimetric and fluorescence output, for turning a qualitative response into quantitative information, without the need for separate data elaboration.

2.3.6 Design of Flower-Like Paper Toxicity Biosensor.

After characterization of the biosensor analytical performance, we designed a circular flower-like paper sensor containing five external wells for the calibration curve with standard solutions of analyte and a central well for the sample test (Figure 2. 6 a). For easy handling, a 1.5 cm paper grip has been added to the sensor (Figure S2. 12).

NaClO was used as model analyte to test drinking water samples, which were spiked with three different concentrations (0.05, 1.0, and 4.0 ppm) and analyzed using the toxicity sensing paper after 1 min of incubation time at 25°C. As shown in Figure 2. 6 b, the intensity of light emitted by the central well containing the sample is compared to the control (0 ppm) and the calibration solutions (0.1 - 4.0 ppm). For the sample with 0.05 ppm NaClO, the calibrators range from 0.05 to 2.0 ppm. For the samples with 1.0 ppm and 4.0 ppm NaClO, the calibrators range from 0.5 to 4.0 ppm. Even a preliminary comparison achieved by the naked eye confirmed that all three samples showed intensities similar to the corresponding wells on the calibration curve. Figure 2. 6 c shows the corresponding values obtained with the *Scntinel* app and the graphs obtained with the standard ImageJ analysis and the values were interpolated using the NaClO calibration curve (Figure S2. 13 and Figure S2. 14, Table S2. 4).

2.3.7 Stability and Reproducibility Studies.

The stability and responsiveness of *A. fischeri* bacteria entrapped on paper with the agarose hydrogel and stored at 4 °C was evaluated for a two-week period. As shown in Figure 2. 6 d, although we observed a proportional decrease in BL intensities during the days, the toxicity sensing paper response was maintained within 13 days (about 48% of the initial response on day 6). At the same time, triplicate wells were incubated with 1 ppm NaClO for 1 min and BL intensities acquired with the OnePlus 6 camera.

2.3.8 Sustainability Assessment.

Ensuring the sustainability of a biosensor is essential for reducing environmental impact in healthcare and environmental monitoring³². The sustainability of the toxicity sensing paper was evaluated using the RGB 12 algorithm which incorporates 12 principles of white analytical chemistry (WAC)³³. The developed toxicity sensor was compared to three previously reported laboratory-based standard methods, which utilized freshly prepared, liquid-dried, and freeze-dried *A. fischeri* bacteria for assessing the toxicity of water samples. Data shown in Supporting Information report the RGB scores of these methods as well as the score assessment criteria. In terms of sustainability (Green score), the smartphone-based toxicity sensor ranked first with a score of 89.2%, compared to 59.6% for the analogous assay performed with standard methods (ISO 11348). Standard methods and test kits involve the use of chemical reagents that require careful disposal to avoid secondary contaminations, plastic cuvettes and microtiter plates. To reduce the environmental impact, our toxicity sensing paper was designed to be environmentally friendly thanks to the green properties of the paper, low sample volumes and no additional steps of rehydration. Considering the feasibility, our method achieved a blue score of 100%, as it does not require benchtop or sophisticated instrumentation such as luminometers or strict cold chain (Table S2.5, S2.6, and S2.7). Therefore, unlike the standard laboratory methods, the developed sensing paper allows for easy and rapid monitoring of water toxicity. It is highly suitable for integration with portable detectors for point-of-need analysis. Additionally, it offers the advantages of low cost and a low carbon footprint. The blue score was notably increased due to the reduced time and cost efficiencies of the developed system. The AI *Scentinel* App, compared to the ImageJ tool analysis, allowed to reduce the total time of the analysis from about 1 h to 5 min avoiding the use of a laptop for data elaboration and software for graphical elaboration and statistical analysis.

2.4 Conclusion

This work presents a sustainable, paper-based biosensor using bioluminescent marine bacteria for rapid water toxicity assessment. This is the first report in which bioluminescence detection has been combined with AI algorithms to avoid manual signal elaboration and provide a truly user-friendly interface. A flower-like paper sensor configuration was designed to integrate an onboard calibration curve with a model toxic analyte, thus enabling the evaluation of toxicity of a water sample in terms of toxicity equivalents.

The analytical performance of the biosensor was assessed with harmful substances like disinfectants, toxins, and heavy metals, showing the required sensitivity in tap and wastewater samples. While traditional lab methods remain standard, this portable system is ideal for quick screenings in diverse settings, especially in low-resource areas. The integrated Android app with AI-driven image analysis provides instant results, minimizing the need for technical skills and supporting citizen science. Future developments aim to enhance performance with broader data sets and expand testing to polluted sources like agricultural runoff. With its affordability (about €0.20 per cartridge), ease of use, and remote accessibility, the sensor supports better water quality monitoring and environmental health.

2.5 Supporting Information

Experimental Section

Heat Conduction Calculation

Heat conduction calculation of the bacterial suspension - agarose was investigated to avoid the effect of high temperature of agarose hydrogel on the *Aliivibrio fischeri* vitality. By applying Richmann's law³⁴ to solutions of agarose mixed with LB containing bacteria the temperature change was approximated using equation:

$$T_f = \frac{V_1 \cdot T_1 + V_2 \cdot T_2}{V_1 + V_2}$$

where T_f is final temperature of solution, C_1 and C_2 are specific heat capacities of solutions, V_1 and V_2 are volumes of solutions and T_1 and T_2 are their respective temperatures at the time of mixing. The agarose's specific heat capacity³⁵ is 2.85 J/g°C and volume and temperature are 80 μ L and 60°C respectively, while the specific heat capacity of LB is considered 4.2 J/g°C, higher near to water. The volume of LB with bacteria is 420 μ L, with the temperature of 19°C. The calculated rise in temperature was of about 30°C, not enough to destroy bacterial cells.

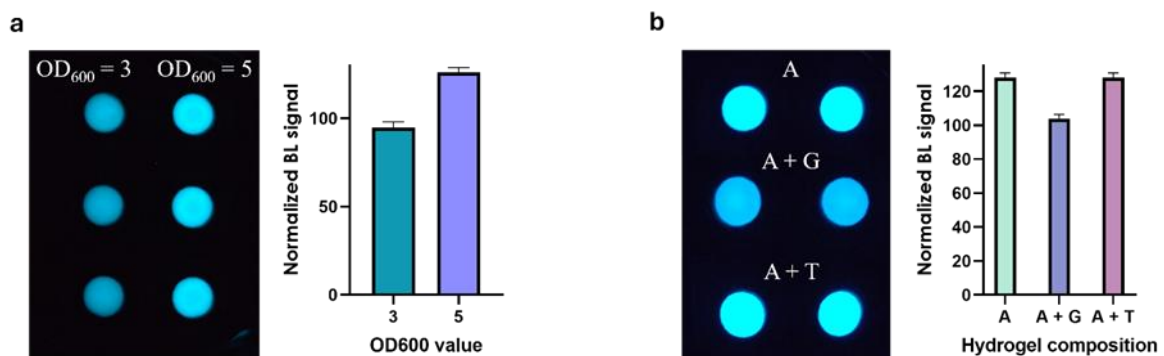


Figure S2. 1 Bioluminescent signal of *A. fischeri* cells (a) with an OD600 value of 3.0 and 5.0, and (b) with OD600 of 5.0 entrapped in agarose 0.5%w/v, agarose 0.25%w/v + glycerol 15%w/v, and agarose 0.25%w/v + trehalose 10%w/v.

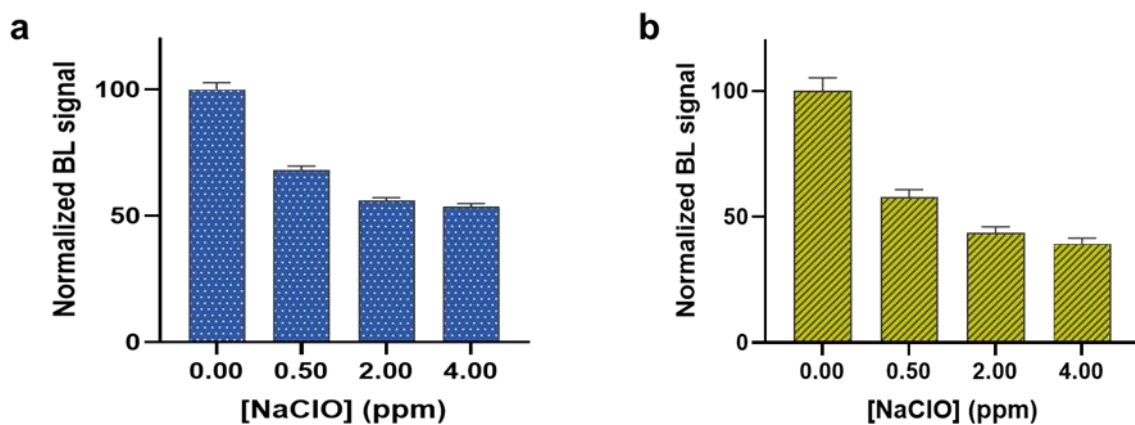


Figure S2. 2 Normalized bioluminescent signal of *A. fischeri* cells entrapped in a) agarose 0.5%w/v and in b) agarose 0.25%w/v + trehalose 10%w/v hydrogel incubated with NaClO (concentration range 0-4 ppm) for 1 min at room temperature.

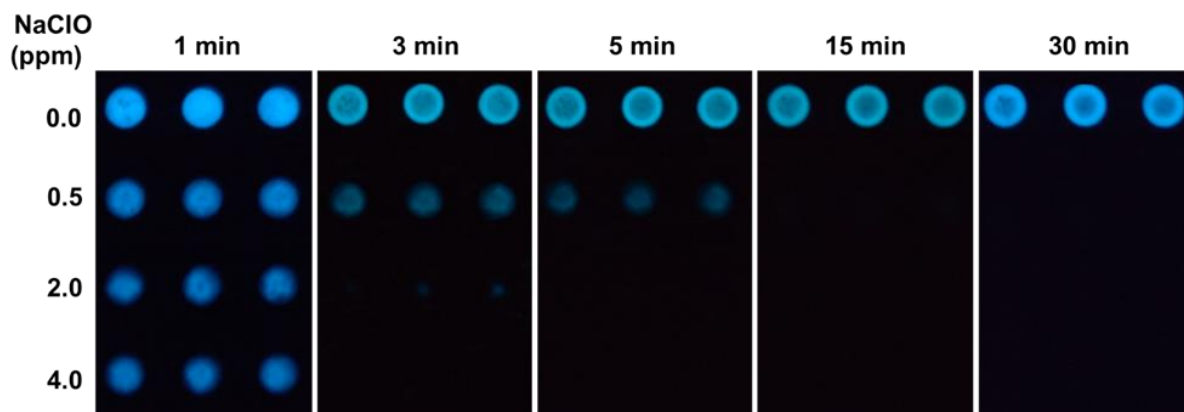


Figure S2. 3 Bioluminescence emissions of *A. fischeri* entrapped in agarose 0.5%w/v incubated at room temperature for 1, 3, 5, 15, and 30 minutes with NaClO 0.0, 0.5, 2.0, and 4.0 ppm. Images were acquired with OnePlus 6T smartphone camera with ISO 1600 and 30 s of acquisition time.

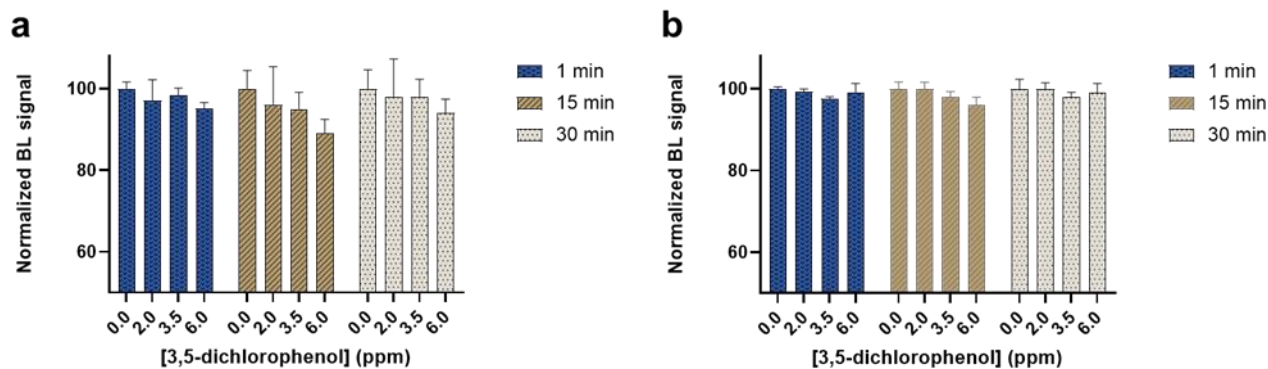


Figure S2. 4 Normalized bioluminescent signal of *A. fischeri* cells entrapped in a) agarose 0.5%w/v and in b) agarose 0.25%w/v + trehalose 10%w/v hydrogel incubated with increasing concentrations of 3,5-dichlorophenol for 1 min, 15 and 30 min.

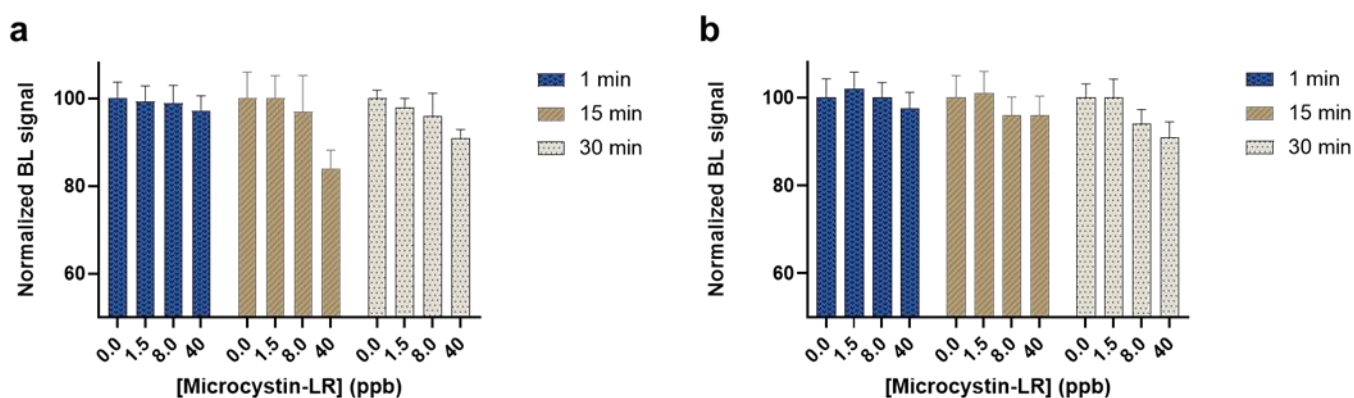


Figure S2. 5 Normalized bioluminescent signal of *A. fischeri* cells entrapped in a) agarose 0.5%w/v and in b) agarose 0.25%w/v + trehalose 10%w/v hydrogel incubated with increasing concentrations of Microcystin-LR for 1 min, 15 and 30 min.

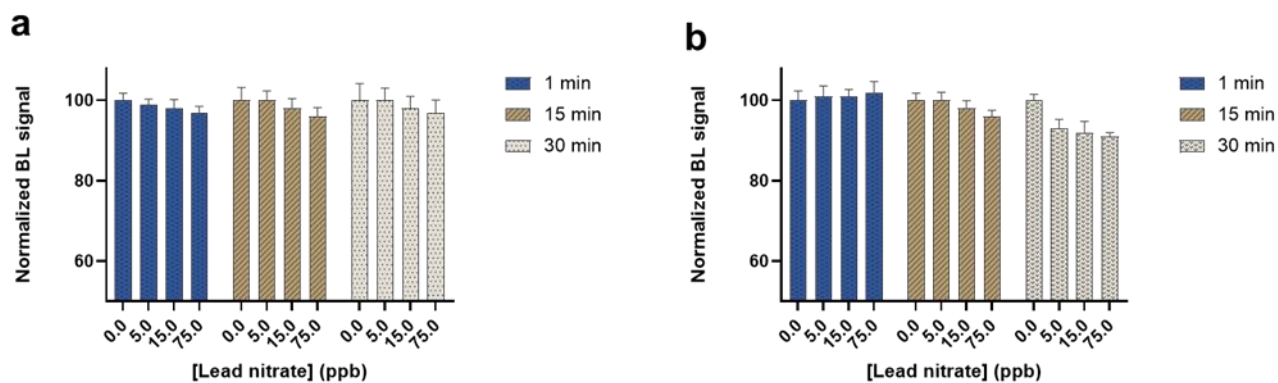


Figure S2. 6 Normalized bioluminescent signal of *A. fischeri* cells entrapped in a) agarose 0.5%w/v and in b) agarose 0.25%w/v + trehalose 10%w/v hydrogel incubated with increasing concentrations of lead nitrate for 1 min, 15 and 30 min.

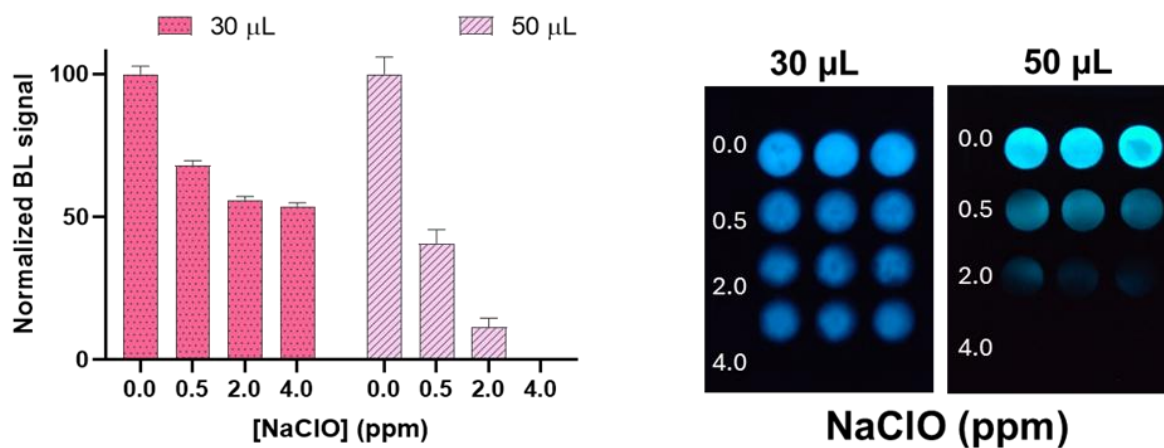


Figure S2. 7 Normalized bioluminescent signal of *A. fischeri* after addition of 30 and 50 μ L of NaClO solutions and incubation time of 1 min, and images acquired with the OnePlus 6T smartphone camera (ISO 1600, integration time of 30 sec).

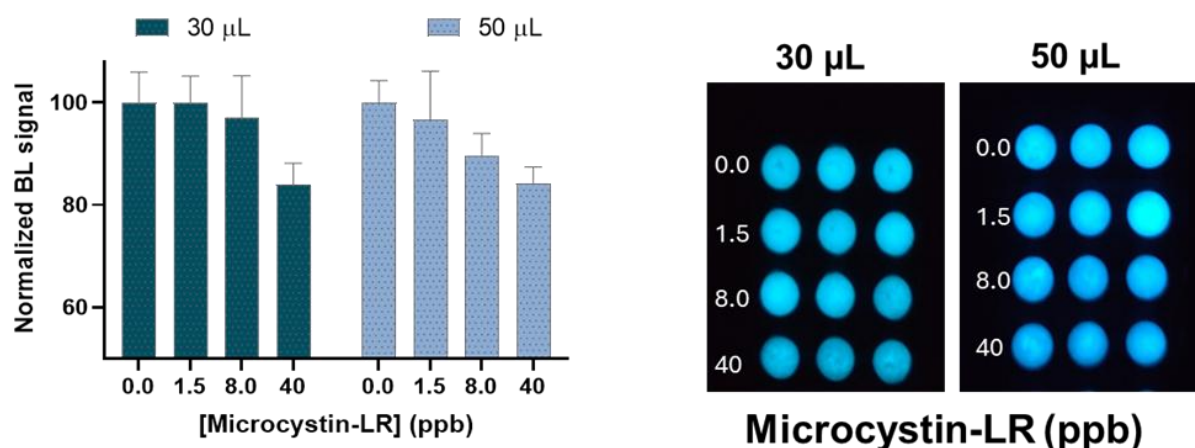


Figure S2. 8 Normalized bioluminescent signal of *A. fischeri* after addition of 30 and 50 µL of Microcystin-LR solutions and incubation time of 15 min, and images acquired with the OnePlus 6Tsmartphone camera (ISO 1600, integration time of 30 sec).

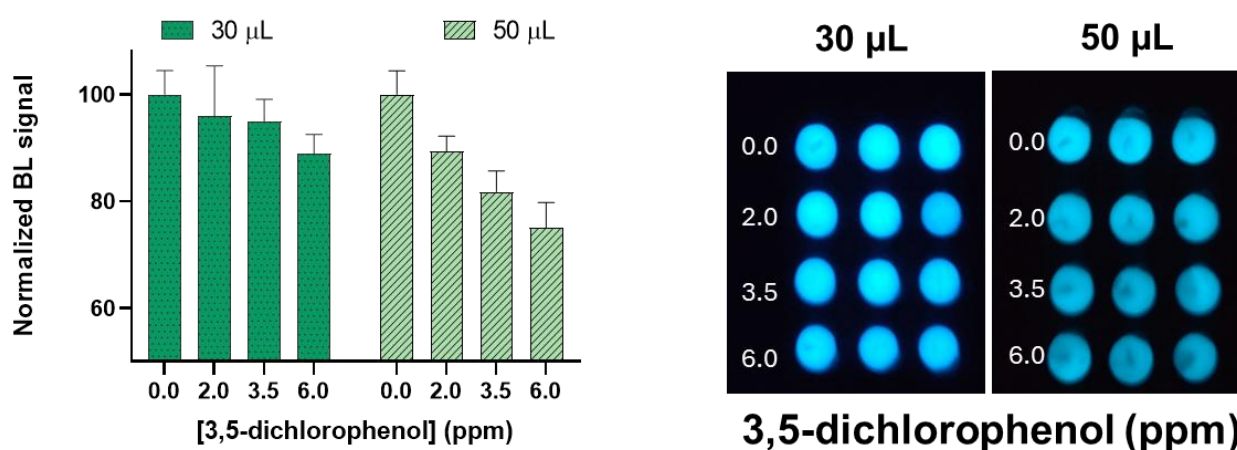


Figure S2. 9 Normalized bioluminescent signal of *A. fischeri* after addition of 30 and 50 µL of 3,5-dichlorophenol solutions and incubation time of 15 min, and images acquired with the OnePlus 6Tsmartphone camera (ISO 1600, integration time of 30 sec).

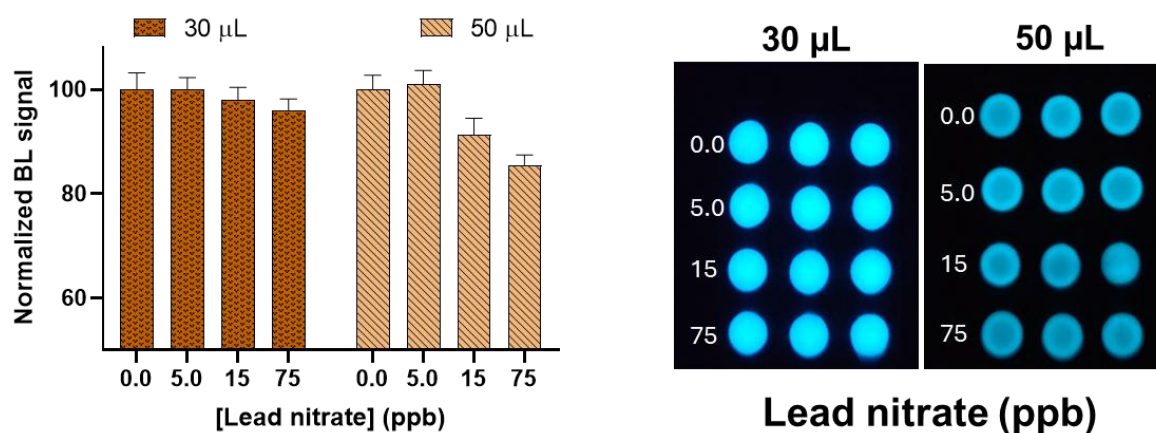


Figure S2. 10 Normalized bioluminescent signal of *A. fischeri* after addition of 30 and 50 μ L of 3,5-lead nitrate solutions and incubation time of 15 min, and images acquired with the OnePlus 6Tsmartphone camera (ISO 1600, integration time of 30 sec).

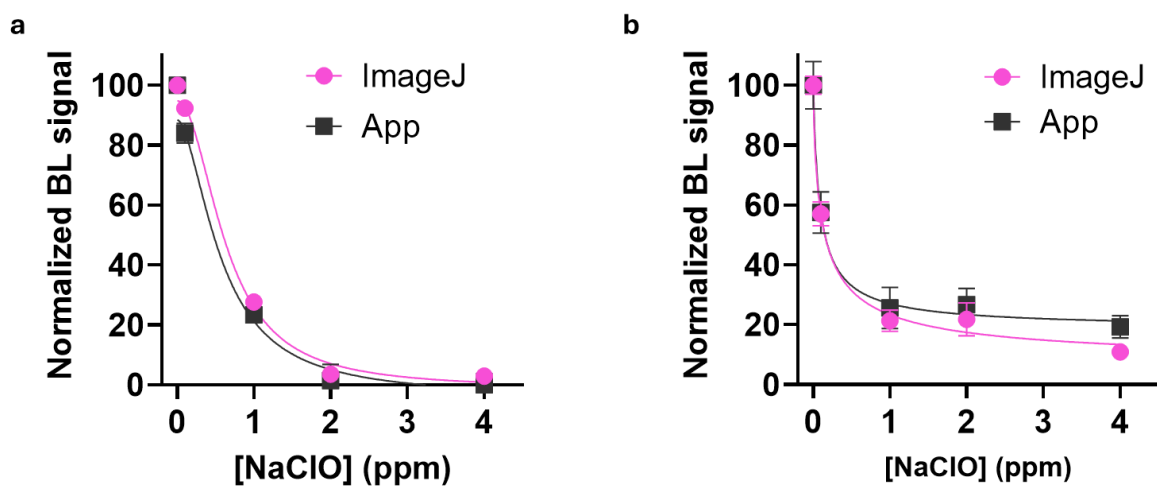


Figure S2. 11 NaClO dose-response curves in (a) drinking water and (b) in wastewaters obtained with the paper-based biosensor (1-min incubation time). BL signals were acquired with OnePlus6smartphone camera (30-sec integration time, ISO 1600) and quantified by ImageJ software (colored) and the AI assisted application (black).

Recovery % for NaClO 1 ppm

	ImageJ	Scentinel
Tap water	77.7	73.4
Wastewaters	59.6	80.3

Recovery % for MC-LR 8 ppb

	ImageJ	Scentinel
Tap water	83.1	116.5
Wastewaters	91.7	106

Recovery % for DCP 3.5 ppm

	ImageJ	Scentinel
Tap water	97.7	90.9
Wastewaters	97.4	95.7

Recovery % for Pb 35 ppb

	ImageJ	Scentinel
Tap water	110	105
Wastewaters	103	104

Table S2. 1 Recovery studies in water samples (tapwater and wastewater samples) spiked with NaClO 1ppm, MC-LR8 ppb, DCP 3.5 ppm and Pb 35 ppb. Recovery was calculated as follows: Recovery % =[recovered (measured spiked sample-baseline)/ concentration added] x 100

NaClO in tap water

[NaClO] ppm	ImageJ	Scentinel

MC-LR in tap water

[MC-LR] ppb	ImageJ	Scentinel

0	100	100	0	100	100
0.1	93.5	79.6	1.5	94.8	117
0.5	98.4	87.2	5.0	85.6	115
1.0	77.7	73.4	8.0	83.1	117
2.0	29.0	12.8	18	87.3	119
4.0	20.6	n.d.	40	105	142

DCP in tap water

Pb in tap water

[DCP] ppm	ImageJ	Scentinel	[Pb] ppb	ImageJ	Scentinel
0	100	100	0	100	100
1.0	103	98.5	5.00	106	98.3
2.0	98.0	91.7	15.0	111	102
3.5	97.7	90.9	35.0	110	105
5.0	98.7	107	75.0	120	116

6.0	112	117	100	120	120
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Table S2. 2 Recovery studies in tap water samples spiked with different concentrations of NaClO , MC-LR, DCP and Pb. Recovery was calculated as follows:

Recovery % =[recovered (measured spiked sample-baseline)/ concentration added] x 100

Smartphone model	LOD (ppm) obtained with ImageJ-based analysis	LOD (ppm) obtained with the <i>Scntinel app</i>
OnePlus6	0.10	0.21
Motorola edge 40 neo	0.06	0.06
SamsungS20	0.06	0.06
HuaweiP10	0.29	0.31
iPhone12mini	0.40	0.46
iPhone13mini	0.43	0.59

Table S2. 3 Limits of detections for NaClO obtained by analysing the pictures of different smartphone models either with ImageJ and the *Scntinel app*. The procedure is described in Materials and Methods section.

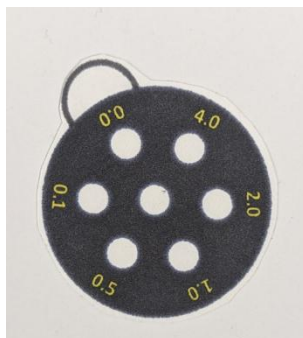


Figure S2. 12 The paper sensor printed with an office wax printer onto chromatography paper. The paper biosensor includes 6 wells for the calibration curve and a central well for the sample.

Scentinel App Development and Guide for Users

The Scentinel application provides a simple graphical user interface (GUI) (basic flow of work is described in Figure S2. 14, written using Kivy³⁶ framework of python, to upload the captured images from the smartphone gallery. The image processing functionality is deployed on Microsoft Azure³⁷, utilizing Flask's API³⁸, a server-side web framework for managing HTTP requests and responses. The quantification of sample concentration is performed on the smartphone.

In the first phase, a Python³⁹ program was developed within an integrated development environment (IDE), focusing on code validation, statistical analysis through curve fitting, and adjustments of AI parameters. In the second phase, a program to application transition was performed. The probability parameter and non-maximum suppression (NMS) for the AI model were fine-tuned to 0.67 and 0.01 respectively to increase confidence level for prediction of bioluminescence signal and filtering low level of signals.

Pre-Processing of Image

In this step, the input image is pre-processed for faster segmentation aiming to utilize fewer computing resources such as bandwidth and storage, while aligning with the AI model. First, the image decimation is performed preserving the aspect ratio, followed by converting to grayscale by applying Eq. (1) to each pixel⁴⁰. Click or tap here to enter text. In the equation

R, G and B are integers representing red (R), green (G) and blue (B) with values from 0 to 255. Finally, the pixel values of the image are normalized before being fed to the AI model.

$$Y = 0.299 R + 0.587 G + 0.114 B \quad (1)$$

Image Segmentation and Extraction of Properties

In the application, StarDist⁴² library was utilized, commonly applied for cell detection and segmentation in microscopy images. StarDist predicts star-convex polygons rather than traditional axis-aligned bounding boxes by regressing radial distances from each pixel to object boundaries. Object probabilities are derived from the Euclidean distance to background pixels, with non-maximum suppression (NMS) refining the final object instances⁴³. The pre-trained model 'Versatile (fluorescent nuclei)' of StarDist was employed to segment each well, representing bioluminescence signal, on the sensor image. Subsequently, measuring properties (area, bounding box) of detected well was performed by scikit-image algorithms⁴⁴. The bioluminescence value of each well was calculated by dividing its total pixel values to the total number of pixels of control. Then OpenCV⁴⁵ library was used to draw circles around segmented wells and to label them with numbers. Coding files are freely available in GITHUB repository (<https://github.com/faisalnazir1213/Scentinel>) Regression curve fitting and interpolation of the sample value

In this phase, based on user input, the bioluminescence signal value assigned to the sample well (unknown) is extracted, separating it from the known wells (calibration solutions). The application then prompts the user to input the concentration values of the calibration solutions. This is necessary to provide actual values to the corresponding captured signal values in order to perform statistical analysis. If the user submits without entering values, the relative toxicity is calculated using the default NaOCl calibration values (0.0 ppm, 0.1 ppm, 0.5 ppm, 1.0 ppm, 2.0 ppm, 4.0 ppm). Alternatively, the user can manually enter the concentration values.

Then a 4-parametric regression model is implemented using SciPy library on calibration solution correlating each concentration to its corresponding signal value using Eq. (2). Then, the app computes the final measurement by interpolating the sample concentration with respect to the calibration curve using Eq. (3). Where x is the independent variable (concentration values of calibration solutions), y is the dependent variable (bioluminescence

values of calibration solutions), A is the minimum value that can be obtained, B is the Hill's slope of the curve, C is the point of inflection, and D is the maximum value that can be obtained.

$$y = \frac{A - D}{1 + \left(\frac{x}{C}\right)^B} + D \quad (2)$$

$$x = C \left(\frac{A - D}{y - D} - 1 \right)^{\frac{1}{B}} \quad (3)$$

Step By Step Guide for Scentinel App Use

Step 1: Open The App and Load Your Image

- A. Start by launching the application on your android device.
- B. Click on the 'LOAD IMAGE' button here you can upload/select an image of biosensor. Choose the image you wish to analyze.
- C. The processed image will be displayed assigning all wells with numbers (the sample well number will be used for further analysis)

Step 2: View Relative Changes (Optional)

- D. Once the image is loaded, locate and click the 'SHOW RELATIVE CHANGE' button. This button is found under the 'LOAD IMAGE' button.
- E. After clicking, the app will display the relative changes/signal values of each well on the new prompt. These values represent the intensities of wells.

Step 3: Enter Sample Well Number

- F. To proceed with the analysis, you need to specify the sample well number. This is done by entering the number into a designated input box under text 'ENTER LABEL NUMBER OF SAMPLE'.

G. Ensure you enter the correct number corresponding to your sample (central well). This step is crucial for the app to associate the correct data with your sample.

H. After entering the sample well number, click the 'SUBMIT' button to finalize this step.

Step 4: Enter Calibration Solution Concentrations

I. In a new prompt of the app, you will be asked to enter the calibration solution concentrations. These concentrations must be numbers (e.g., 0, 1, 2, 3, 4...).

J. Input the calibration solution concentrations in the increasing order (4, 3, 2, 1, 0...)

K. After entering all the required concentrations, press the 'SUBMIT' button. The app will then process this data to estimate the tentative concentration of the sample.

Step 5: Review Tentative Concentration

L. Once the app has processed the calibration solution concentrations, it will display the tentative concentration of the sample on the screen under the labeled image. This value is an estimate based on the data entered and the calibration solutions used.

Comparison Between Manual and AI Based Analysis

Before developing the android based Smartphone application, a comparative analysis was conducted between the results of AI-based analysis procedures (Figure S2. 13a) and with manual ImageJ software (Figure S2. 13b). The paper sensor, printed with 4 x 4 arrays of wells, immobilized with *Aliivibrio fischeri* was prepared and concentrations of NaClO (0, 0.01, 0.05, 0.1, 0.5, 1, 2, 4 ppm) were introduced to it. After the proposed duration images were acquired under dark box and analyzed to obtain values of bioluminescence signal. The curve was fitted (Figure S2. 13c) on the values using GraphPad Prism 8 to determine the limit of detections (LOD) those were 0.039 ppm for manual and 0.044 ppm for AI-based approach, validating the model's suitability for detection and quantification. However, it is noteworthy to mention that due to the absence of zone boundaries in higher concentrations 4ppm (wells below 13 and 14) due to no bioluminescence signal as displayed in the last row of Figure S2. 13a. the AI model could not segment and detect anything.

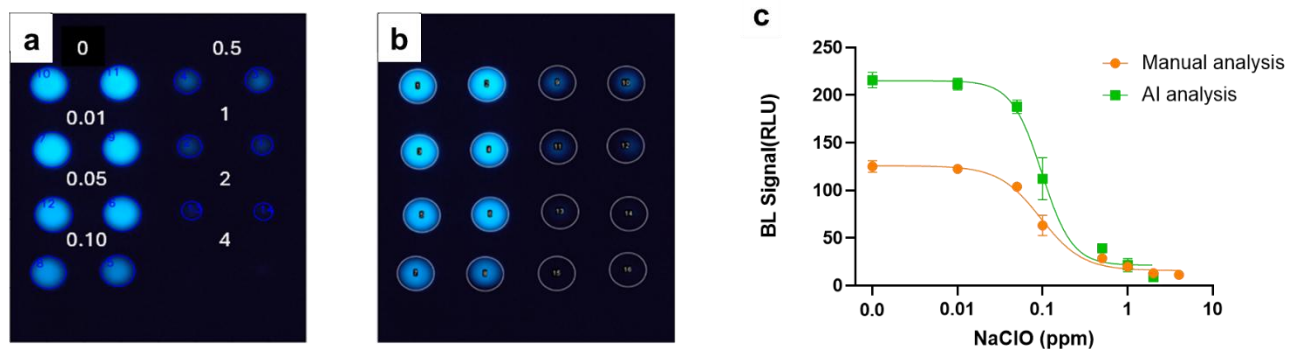


Figure S2. 13 Regions of interest (ROIs) of the toxicity paper sensor with NaClO concentrations in ppm, measured with AI based analysis (a) and with manual segmentation with Image J software (b). The lower panel shows the calibration curves obtained with either Image or the Scentinel App (c).

Disposable Flower-Like Paper Toxicity Biosensor Evaluation

To enable users to quantify samples on-site using the *Scentinel Application*, a disposable flower-like paper toxicity biosensor was developed, with a calibration curve established around the central sample well (Figure 2.6c). Different concentrations of NaClO (0.05, 1.0 ppm, and 4.0 ppm) were tested in the central well, the interpolated values from both methods, ImageJ and AI-based, were compared as shown in Table S2.4. The introduction of toxicity curve provides the solution to quantify relative toxicity according to calibration solutions of known concentrations. Both the *Scentinel* application and manual approach (computer-based opensource software ImageJ) produced similar results (Table S2.4), unlike manual approach *Scentinel* application requires no effort from the user side except image acquisition

Manual Interpolation (ppm)	App-based Interpolation (ppm)	Standard Deviation(σ)
0.079	0.081	0.001
0.35	0.40	0.02
1.47	1.41	0.03

Table S2. 4 Comparison of manually interpolated values and the application interpolated values along with the standard deviation.

Practical Application of Biosensor (stability, reproducibility, technological improvement AI)

Technological Improvement by AI-Based Detection

Manual image processing and quantitative analysis require technical expertise, time, and access to hardware (e.g., personal computers) and software (e.g., image processing, statistical tools, spreadsheets), making participation in citizen science challenging. In contrast, our Android-based *Scintinel* application automates all steps previously performed manually, streamlining image analysis and quantification, and eliminating the need for bulky hardware, software and manual effort. The blue principles of the RGB 12 algorithm⁴⁶ discussed in a later section, further support the effectiveness of this smartphone-based approach, streamlining the process and enhancing accessibility for users without technical expertise.

Red principles

R1: Scope of application. Any given analytical method should be capable of being applied to a multitude of different situations and samples, as well as being able to determine a wide range of different analytes. It is crucial that it can be applied over a vast range of concentrations, and that it be compatible with a diverse range of sample types. Additionally, the method must demonstrate resilience to potential interferences.

R3: Precision. Precision is a fundamental aspect of analytical methods, and it is essential that the precision of these methods be characterized by the best possible repeatability and reproducibility of the results.

Score	R1. Scope of Application	R3. Precision RSD (Reproducibility)
100	$0 < \text{pictograms} \leq 1$	$0 < \% \leq 5$
100 - 75	$1 < \text{pictograms} \leq 2$	$5 < \% \leq 10$
75 - 50	$2 < \text{pictograms} \leq 5$	$10 < \% \leq 20$
50 - 25	$5 < \text{pictograms} < 10$	$20 < \% \leq 40$
25 - 0	$\geq 10 \text{ pictograms}$	≥ 40

Table S2. 5 Criteria assignment of red principles scores according to Nowak et al. for the efficiency assessment of bioluminescent cell-based methods relying on paper-based or standard approaches. For comparison we are using standard ISO methods, and we compared only the precision of the methods. Other analytical parameters are not provided by ISO.

Green principles

G1: Toxicity of reagents. It is of utmost importance that reagents exhibit minimal toxicity. When feasible, it is advisable to select biodegradable, renewable, or naturally occurring reagents and materials. In order to assess the safety of the reagents, the safety data sheets were evaluated, taking into account the quantity of pictograms for each reagent utilized in the cell-based method.

G2: Amount of reagents and waste. It is recommended that the use of reduced volumes be employed in order to minimize the waste.

G3. Energy and other media. In the development of an analytical method, it is essential to consider the lowest possible electricity consumption. Therefore, our assessment focused on the energy consumption required for the maintenance of cell cultures (incubators, safety cabinets, optical density meters, refrigerators, centrifuges, etc.) and for light detection (the use of benchtop luminometers, spectrophotometers, smartphones, and other portable detectors) without considering the storage conditions.

G4. Direct impacts. It is important to make sure that the deployment of an analytical method does not affect humans or animals. In this case, we focused on the impact on humans and the environment related to the reagents used for the cell-based assays.

Score	G1. Toxicity of reagents	G2. Amount of reagents and waste	G3. Energy consumption	G4. Direct impact
100	$0 < \text{pictograms} \leq 1$	$0 < \text{ml} \leq 0.5$	Exceptionally low or non-consuming	No hazardous activity
100 - 75	$1 < \text{pictograms} \leq 2$	$0.5 < \text{ml} \leq 1$	< 0.1 kWh	Exceptionally low hazardous activity
75 - 50	$2 < \text{pictograms} \leq 5$	$1 < \text{ml} \leq 2.5$	0.1 - 1.5 kWh	low hazardous activities

50 - 25	5 < pictograms < 10	2.5 < ml ≤ 5	> 1.5 kWh	medium hazardous activities
25 - 0	≥ 10 pictograms	> 5 ml	+ tools > 1.5 kWh	Dangerous activities

Table S2. 6 Criteria assignment of green principles scores according to Nowak et al. for the sustainability assessment of cell-based methods. Pictograms of the 'Globally Harmonized System of Classification and Labelling of Chemicals', for energy consumption kWh are considered.

Blue principles

B1. Cost-efficiency. It is important to minimize the total cost as much as possible. In this context, the cost of all necessary reagents, materials, equipment, and personnel was taken into consideration in order to calculate the total analysis cost.

B2. Time-efficiency. A reduction in analysis time is a desirable outcome, encompassing method development and all steps of the workflow. In this context, the time required for preparation of the experimental set-up and incubation time with the sample were considered.

B3. Requirements. It is recommended to minimize the use of equipment, sample quantity, and skilled personnel. In this context, the necessity for incubators, luminometers, spectrophotometers, image analysis software, and statistical analysis software for the assay was considered.

B4. Operational simplicity. In the optimal scenario, an analytical method should be fully automated, employ artificial intelligence techniques, and should be miniaturized and portable, thus enabling on-site measurements.

Score	B1. Cost Efficiency	B2. Time efficiency	B3. Requirements		B4. Operational Simplicity		
			B3.1 Sample consumption	B3.2 Other needs*	B4.1 Portability	B4.2 Integration automation	B4.3 Miniaturization
100	Very low-cost	$1 < \text{min} \leq 30$	$0 < \text{ml} \leq 0.1$	Simple Accessories & tools (mobile, pipette, dark box, etc.)	Online analysis	High automation or simple, online data	Handheld
100 - 75	Low-cost	$30 < \text{min} \leq 60$	$0.1 < \text{ml} \leq 1$	Simple Equipment (glassware, dark box, basic equipment for manual preparation, mobile/camera/ccd, image processing, statistical software)	At line analysis, camp laboratory	High automation or simple	Portable
75 - 50	Medium cost	$1 < \text{hrs.} \leq 2$	$1 < \text{ml} \leq 2.5$	Medium complexity instrumentation (spectrophotometer), statistical analysis, equipment for basic preparation	In lab analysis	Partially automation	Transportable
50 - 25	Expensive	$2 < \text{hrs.} \leq 4$	$2.5 < \text{ml} \leq 5$	Complex instrumentation, equipment for preparation (Luminometer, statistical analysis, Incubator, Biological safety cabinet, Refrigerator)	In lab analysis with sample storing, batch working, results obtained the same day of analysis	No automation	Bench
25 - 0	Extremely expensive	$\geq 1 \text{ Day}$	$> 5 \text{ ml}$	High complexity instrumentation (luminometer, cell culturing equipment (centrifugation, storage, etc.), optical density meter, Statistical analysis software, other complex equipment	In lab analysis with sample storing, planned batch working, results obtain, days after the analysis	No automation	Complex facilities

Table S2. 7 Criteria assignment of blue principles scores according to Nowak et al. for the economic assessment of bioluminescent cell-based methods relying on paper-based or standard approaches.

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Conceptualization: F.N. and E.M.; methodology and design: D.G. M.M.C., C.C.; formal analysis and investigation: F.N., D.G., P.V., S.L., A. T. and M.M.C.; Data curation and visualization: F.N., D.G. and M.M.C; writing-original draft: F.N. and D.G; writing-review and editing: M.M.C, E.M., S.L., A.T.; supervision: M.M.C and E.M.; funding acquisition and resources: E.M., A.T.

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2.8 References

- 1 S. D. Richardson and T. Manasfi, *Anal. Chem.*, 2024, **96**, 8184–8219.
- 2 M. Puri, K. Gandhi and M. S. Kumar, *J. Environ. Manage.*, 2023, **332**, 117344.
- 3 M. Patel, R. Kumar, K. Kishor, T. Mlsna, C. U. Pittman and D. Mohan, *Chem. Rev.*, 2019, **119**, 3510–3673.
- 4 J. Li, H. Liu and J. Paul Chen, *Water Res.*, 2018, **137**, 362–374.
- 5 H. E. Al-Hazmi, A. Mohammadi, A. Hejna, J. Majtacz, A. Esmaeili, S. Habibzadeh, M. R. Saeb, M. Badawi, E. C. Lima and J. Mąkinia, *Environ. Res.*, 2023, **236**, 116711.
- 6 X. Nan, S. Lavrnić, G. Mancuso and A. Toscano, *Water Air Soil Pollut.*, 2023, **234**, 434.
- 7 D. Barceló, *Anal. Bioanal. Chem.*, 2024, **416**, 3479–3485.
- 8 R. Capilla-Flores, R. López-Ruíz, R. Romero-González and A. Garrido Frenich, *J. Hazard. Mater.*, 2025, **489**, 137623.
- 9 L. D. Brunelle, A. L. Batt, A. Chao, S. T. Glassmeyer, N. Quinete, D. A. Alvarez, D. W. Kolpin, E. T. Furlong, M. A. Mills and D. S. Aga, *Environ. Sci. Technol.*, 2024, **58**, 2468–2478.
- 10 M. Sharma, P. Mahajan, A. S. Alsubaie, V. Khanna, S. Chahal, A. Thakur, A. Yadav, A. Arya, A. Singh and G. Singh, *Materials Today Sustainability*, 2025, **29**, 101068.
- 11 R. Nehru, C.-W. Chen and C.-D. Dong, *Science of The Total Environment*, 2024, **935**, 173360.
- 12 H. Sohrabi, A. Hemmati, M. R. Majidi, S. Eyvazi, A. Jahanban-Esfahlan, B. Baradaran, R. Adlpour-Azar, A. Mokhtarzadeh and M. de la Guardia, *TrAC Trends in Analytical Chemistry*, 2021, **143**, 116344.
- 13 H.-F. Tsai, Y.-C. Tsai, S. Yagur-Kroll, N. Palevsky, S. Belkin and J.-Y. Cheng, *Lab Chip*, 2015, **15**, 1472–1480.

- 14 L. Cevenini, M. M. Calabretta, A. Lopreside, G. Tarantino, A. Tassoni, M. Ferri, A. Roda and E. Michelini, *Anal. Bioanal. Chem.*, 2016, **408**, 8859–8868.
- 15 S. Belkin and J.-Y. Cheng, *Curr. Opin. Biotechnol.*, 2023, **82**, 102952.
- 16 L. Shen, Y. Chen, L. Hu, C. Zhang, L. Liu, L. Bao, J. Ma, H. Wang, X. Xiao, L. Wu and S. Chen, *ACS Sens.*, 2024, **9**, 654–661.
- 17 A. Horta-Velázquez, G. Ramos-Ortiz and E. Morales-Narváez, *Biosens. Bioelectron.*, 2025, **273**, 117089.
- 18 J. Stocker, D. Balluch, M. Gsell, H. Harms, J. Feliciano, S. Daunert, K. A. Malik and J. R. van der Meer, *Environ. Sci. Technol.*, 2003, **37**, 4743–4750.
- 19 J. H. Lee and M. B. Gu, *Biosens. Bioelectron.*, 2005, **20**, 1744–1749.
- 20 L. Camanzi, L. Bolelli, E. Maiolini, S. Girotti and D. Matteuzzi, *Environ. Toxicol. Chem.*, 2011, **30**, 801–805.
- 21 W. Ji, J. Ma, Z. Zheng, A. Z. Al-Herrawy, B. Xie and D. Wu, *Science of The Total Environment*, 2024, **940**, 173528.
- 22 E. Jerschow, A. P. McGinn, G. de Vos, N. Vernon, S. Jariwala, G. Hudes and D. Rosenstreich, *Annals of Allergy, Asthma & Immunology*, 2012, **109**, 420–425.
- 23 P. Levallois, P. Barn, M. Valcke, D. Gauvin and T. Kosatsky, *Curr. Environ. Health Rep.*, 2018, **5**, 255–262.
- 24 A. Lopreside, L. Montali, B. Wang, A. Tassoni, M. Ferri, M. M. Calabretta and E. Michelini, *Biosens. Bioelectron.*, 2021, **194**, 113569.
- 25 A. Elkelish, A. M. Alhudhaibi, A. S. Hossain, F. Haouala, B. M. Alharbi, M. F. El-Banna, A. Rizk, A. Badji, N. I. AlJwaizea and A. A. S. Sayed, *BMC Plant Biol.*, 2024, **24**, 460.
- 26 R. Kriss, K. J. Pieper, J. Parks and M. A. Edwards, *Environ. Sci. Technol.*, 2021, **55**, 1964–1972.

- 27 J. F. Bergua, R. Álvarez-Diduk, L. Hu, A. H. A. Hassan and A. Merkoçi, *J. Hazard. Mater.*, 2021, **406**, 124434.
- 28 K. Kalinowska, W. Wojnowski and M. Tobiszewski, *Trends Food Sci. Technol.*, 2021, **111**, 271–279.
- 29 F. Cui, Y. Yue, Y. Zhang, Z. Zhang and H. S. Zhou, *ACS Sens.*, 2020, **5**, 3346–3364.
- 30 S. Soares, G. M. Fernandes and F. R. P. Rocha, *TrAC Trends in Analytical Chemistry*, 2023, **168**, 117284.
- 31 M. Wienhold, A. Kirkpatrick, T. Xu, S. Ripp, G. Sayler and D. Close, *Front. Bioeng. Biotechnol.*, DOI:10.3389/fbioe.2021.767313.
- 32 A. Williams, M. R. Aguilar, K. G. G. Pattiya Arachchillage, S. Chandra, S. Rangan, S. Ghosal Gupta and J. M. Artes Vivancos, *ACS Sustain. Chem. Eng.*, 2024, **12**, 10296–10312.
- 33 P. M. Nowak, R. Wietecha-Posłuszny and J. Pawliszyn, *TrAC Trends in Analytical Chemistry*, 2021, **138**, 116223.
- 34 J. L. Heilbron, *Elements of early modern physics*, University of California Press, 1982.
- 35 E. Myers, M. Piazza, M. Owkes and R. K. June, *J. Biomech.*, 2024, **176**, 112307.
- 36 D. Phillips, *Creating apps in Kivy: mobile with Python*, ‘O’Reilly Media, Inc.’, 2014.
- 37 J. C. Andersson, *Learning microsoft azure*, ‘O’Reilly Media, Inc.’, 2023.
- 38 M. Grinberg, *Flask web development*, ‘O’Reilly Media, Inc.’, 2018.
- 39 G. Van Rossum and F. L. Drake, *CreateSpace*, 2009, preprint.
- 40 A. F. Villán, *Mastering OpenCV 4 with Python: a practical guide covering topics from image processing, augmented reality to deep learning with OpenCV 4 and Python 3.7*, Packt Publishing Ltd, 2019.

- 41 A. F. Villán, *Mastering OpenCV 4 with Python: a practical guide covering topics from image processing, augmented reality to deep learning with OpenCV 4 and Python 3.7*, Packt Publishing Ltd, 2019.
- 42 M. Weigert, U. Schmidt, R. Haase, K. Sugawara and G. Myers, in *Proceedings of the IEEE/CVF winter conference on applications of computer vision*, 2020, pp. 3666–3673.
- 43 U. Schmidt, M. Weigert, C. Broaddus and G. Myers, in *Medical image computing and computer assisted intervention–MICCAI 2018: 21st international conference, Granada, Spain, September 16-20, 2018, proceedings, part II 11*, Springer, 2018, pp. 265–273.
- 44 S. Van der Walt, J. L. Schönberger, J. Nunez-Iglesias, F. Boulogne, J. D. Warner, N. Yager, E. Gouillart and T. Yu, *PeerJ*, 2014, **2**, e453.
- 45 G. Bradski and A. Kaehler, *Learning OpenCV: Computer vision with the OpenCV library*, ‘O’Reilly Media, Inc.’, 2008.
- 46 P. M. Nowak, R. Wietecha-Posłuszny and J. Pawliszyn, *TrAC Trends in Analytical Chemistry*, 2021, **138**, 116223.

Chapter 3

An Origami Colorimetric Paper-Based Sensor for Sustainable On-Site and Instrument-Free Analysis of Nitrite

This chapter is based on the following published article:

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Author contributions are detailed in a separate section of this thesis.

3.1 Introduction

Water quality is of paramount importance, and since the primary strategy for water quality management is contamination prevention, it is essential to monitor the presence of contaminants.

Nitrite (NO_2^-) and nitrate (NO_3^-) represent the most common nitrogen-containing compounds, posing a significant environmental and health challenge due to increased natural and anthropogenic activities. Nitrite, well known inorganic contaminant, naturally occurs in plants, food and water. However, it is also widely present in foodstuffs because of the use of nitrogen-based compounds as food preservatives, especially in meat and meat products. The use of nitrogen-based fertilizers contributes significantly to increasing nitrite levels in water bodies^{1,2} by converting urea or ammonia through the microbial nitrification process. Similarly, animal and human waste, known to be rich in organic nitrogen, releases nitrite and nitrate into the surrounding environment after their decomposition, leaching beyond the soil-plant system and reaching water bodies³. High levels of nitrite can be detected in drinking water sources, posing serious health risks, including methemoglobinemia in infants. The World Health Organization (WHO) set the maximum allowable concentration of nitrite in drinking water at 3 mg/L, based on assessments of health risks associated with agricultural activities^{4,5}. A limit of 1 mg/L for nitrite in drinking water has been established by the Environmental Protection Agency (EPA) <https://www.epa.gov/ground-water-and-drinking-water/national-primary-drinking-water-regulations>.

Nitrite ions are monitored in drinking waters, surface and underground waters, as well as in industrial and municipal water wastes and different analytical methods have been developed,⁴ including spectrophotometric and electrochemical techniques, chromatography⁶ and capillary electrophoresis analysis^{7,8}. The most used method is the International Organization for Standardization (ISO)15923-1:2013 method, which relies on spectrophotometric and turbidimetric analysis of different ions, including nitrite, for water quality. The method is based on the colorimetric Griess reaction,⁹⁻¹² that follows an azo coupling mechanism, where an aromatic amine (e.g. sulfanilamide - SulfAA), in the presence of nitrite and an acidic catalyst, undergoes diazotization to form a reactive diazonium salt, through an electrophilic aromatic substitution. When this diazonium salt is combined with an electron-rich coupling agent (e.g. N-(1-naphthyl)ethylenediamine - NED), it reacts to produce a highly-colored azo dye. The diazo dye absorbs visible light at a wavelength of 540 nm,

resulting in a pink/purple color, with an absorption intensity that is directly proportional to the concentration of nitrites.

Commercially available assays based on the Griess reaction and methods based on redox reactions in acidic condition¹³ rely heavily on either complex sample pretreatment or laboratory instrumentation for absorbance measurements (i.e. benchtop spectrophotometers), making on-site and real-time measurements challenging¹⁴.

Sensors, enabling on-site and low-cost analysis, appear as highly appealing alternatives. The use of sensors in routine agricultural practices would allow a more sustainable management, implementing real-time decision-making, reducing the need for complex laboratory procedures and trained personnel. As an alternative, sensing approaches have been proposed for the detection of nitrite in different matrices, including microfluidic paper-based systems based on the Griess reaction,^{15–19} exploiting reflectance detection¹⁹. Reflectance detection, i.e., the measure of the fraction of light reflected by a material, is widely used in user-friendly portable paper sensors with colorimetric detection. Paper-based sensors provide the potential to create simple, low-cost, and disposable analytical devices, making them particularly suitable for environmental monitoring. A few paper-based devices for the detection of nitrite and nitrate have been reported²⁰. Recently a hydrogel chemosensor was developed by Tai et al.,²¹ whereas Li et al. used a paper strip and a triple-mode sensing method to detect nitrites ions²². Another study presented a paper-based device and a microanalysis cassette for detecting nitrite levels in food samples using LED lights and a smartphone for images acquisition²³. Smartphone-based sensors are receiving significant attention since they replace optical detectors avoiding the use of further instrumentation^{24–32}.

However, most of these devices still require expensive instrumentation for fabrication (i.e., patterning, cutting), pre-concentration of the sample and, most importantly, they are not sustainable considering the full life cycle of a disposable sensing device.

This work aims to develop a ready-to-use reflectance origami paper sensor based on the Griess reaction, to detect low concentrations of nitrite without the need for manual handling liquid reagents. To ensure the portability and the simplicity of the system, without loss of sensitivity, smartphone-based detection was introduced. This approach emphasizes the use of eco-friendly materials, allowing the monitoring of environmental samples, like water sources, in a sustainable way. By exploiting these principles, our sensor addresses the need for effective

environmental monitoring, playing a role in promoting the reuse of resources and contributing to more sustainable and responsible water management.

3.2 Experimental

3.2.1 Chemicals, Reagents and Instrumentation

Sodium nitrite, NaCl, MgSO₄, K₂HPO₄, K₂CO₃, KNO₃, MgCl₂, CaCl₂, FeCl₃, FeCl₂, urea, glucose and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO). Sulfanilamide (SulfAA), and N-(1-Naphthyl)-Ethylenediamine (NED) were from the “Nitrite/Nitrate Colorimetric Test” (Roche) kit from Sigma-Aldrich (St. Louis, MO). Whatman 1 CHR cellulose chromatography paper was from GE Healthcare (Chicago, IL, USA) and was used as support for the design of the colorimetric sensing paper. A Phaser 8400 office wax printer (Xerox, Norwalk, CT, USA) was used for wax printing. OnePlus 6T (OnePlus, Shenzhen, China) was used for colorimetric signal acquisitions. A Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for absorbance measurements.

3.2.2 Design and Method Optimization of Nitripad Paper Sensor

A preliminary design of the NitriPad, comprising 18 circular areas (a 6 x 3 array of 7 mm-diameter wells) was created using PowerPoint (Microsoft, Redmond, WA, USA), and printed on Whatman 1 CHR chromatography paper using an office wax printer. The hydrophobic areas were created by heating the NitriPad for 2 min at 100°C to allow wax to spread through the cellulose fibers. This configuration was used to create a calibration curve for NaNO₂ (concentration range 0.05 – 5.0 mg/L in ddH₂O) using the Griess reagents. Thus, maintaining the same ratio suggested for the standard 96-well assay, 10 mL of SulfAA + NED solution and 15 mL of NaNO₂ standard solutions and a control (0.0 mg/L of NaNO₂ in ddH₂O) were dispensed into each well. Images were acquired every 5 minutes up to 15 minutes, and then every 15 minutes up to 2 hours of incubation at room temperature (RT ~ 22 °C) using a smartphone CMOS sensor and a dark box to avoid light interferences during the incubation of the analyte with SulfAA and NED. SulfAA and NED are light-sensitive reagents due to their aromatic amine and conjugated structures, which can degrade or oxidize under light exposure, causing background interference in the colorimetric measurement.

To create a ready-to-use paper sensor, the adsorption of the Griess reagents on paper was evaluated and the origami configuration was explored. In particular, the volumes of Griess reactant and analyte, their ratio, and the paper folding process were investigated. For each experiment, the incubation time for the colorimetric reaction was monitored by acquiring images of the PAD with a smartphone camera. The final configuration of the NitriPad was designed to include a 3x2 wells array on one side (bottom) and a 3x2 well array on the opposite side (top) (Figure S3. 1). Three configurations (A, B, and C) were tested by adsorbing NED and SulfAA reagents on different sides of the PAD and changing the order in the procedure of sample addition and paper folding. For each PAD, 3 wells were used for the control (ddH₂O) and 3 wells for a standard solution of NaNO₂ 5.0 mg/L). Volumes were optimized by increasing the reagents and analyte total volume (25 and 50 mL) onto the PAD, and changing the Griess reagents/analyte ratio, testing 20 and 30 mL of analyte volume. The adsorption of both NED and SulfAA in the same well was also evaluated, confirming the need to keep the two Griess reagents separate to avoid their competitive reaction during the drying process.

The optimized configuration involves the adsorption of 10 mL of SulfAA to the top side of the NitriPad, and 10 mL of NED to the bottom one for 3 h at room temperature (22°C) in the dark box to avoid the degradation (oxidation) of SulfAA and NED, due to the light-sensitive nature. Then, the NED part of the PAD is sealed with adhesive tape to prevent sample leakage, the SulfAA part is folded over the NED part and 30 mL of samples are added to the reverse SulfAA part. In this way, sample addition causes wetting of the two layers of the PAD, thus triggering the chemical reaction (Figure S3. 1).

3.2.3 Nitripad Assay Optimization and Data Analysis

Once the procedure has been optimized, a calibration curve was obtained for NaNO₂ using the configuration of the NitriPad with an array of 7 x 3 wells for each part of the PAD and the optimized volume for NaNO₂ (0.05 - 5.0 mg/L), standard solutions and control. Images were acquired after 0, 5, 10, 15, 20, and 30 minutes of incubation in a dark box at room temperature with a OnePlus 6T smartphone, equipped with a dual integrated camera, composed of a primary sensor (16 MP Sony Exmor RS IMX 519, BSI CMOS ½.600 color sensor with 1.22 µm pixels, *f*/1.7 aperture) and a secondary sensor (20 MP Sony Exmor RS IMX 376 K, BSI CMOS ½.800 color sensor with 1.0 µm pixels, *f*/1.7 aperture). The native camera app of the smartphone was used to acquire the images with the automatic exposure mode, without any zoom, filter or other tools. The smartphone was placed at a distance (20 cm) from the plane of

the sensing paper to ensure reproducibility of the measurements. To minimize reflectance variations and reduce user input, no other light source apart from the standard room lighting was used uniformly across all sessions, avoiding shadows. Reflectance measurements were analyzed with ImageJ software (v. 1.53t), National Institutes of Health, Bethesda, MD, USA) in the form of colorimetric signals. A circular region of interest (ROI) was defined corresponding to the circular wells. The ROI had a diameter of 70 pixels, corresponding to an area of 3852 pixels. The field of view (FoV) of the image was 418.91 x 192.00 mm, corresponding to the full resolution of 4608 x 2112 pixels, allowing for accurate spatial reference of the selected ROI. The pixel size was calibrated based on the known scale of 7 mm = 77 pixels, resulting in a pixel size of 11.0 pixels/mm. Pixel brightness was calculated as the mean value within the defined ROI using the grayscale, ranging from 0 (black) to 255 (white). The intensity values were evaluated to assess the colorimetric signals associated with the reflectance of the sample. Data were fitted with the second-order polynomial (quadratic) non-linear regression on GraphPad Prism v.8 software (GraphPad Software, LaJolla, CA, USA). The limit of detection (LOD) was calculated as the mean value of the blank (ddH₂O) minus three times the standard deviation of the control (ddH₂O). The limit of quantification (LOQ) was calculated as the mean value of the blank (ddH₂O) minus ten times the standard deviation of the control (ddH₂O). All measurements were performed in triplicate and repeated at least three times.

3.2.4 Real Sample Analysis and Recovery Studies

The applicability of the origami colorimetric paper-based sensor to the analysis of complex samples was also evaluated. Bottled water samples (Vera San Giorgio in Bosco, Vera S.p.a., Milano, Italy) with a declared nitrite concentration < 0.002 mg/L were spiked with NaNO₂ by adding known concentration (0.5, 1.0 and 2.5 mg/L) of NaNO₂ in the samples. The assay procedure was the same as described previously using 30 µL of water sample in triplicate. The % recovery was then calculated using the equation:

$$\%R = \frac{\text{measured concentration}}{\text{spiked concentration}} \times 100$$

3.2.5 Selectivity, pH Effect and Storage Stability Studies

Selectivity studies were performed to evaluate the specificity of the NitrI-Pad sensor in presence of potential interferences. NaCl, MgSO₄, K₂HPO₄, K₂CO₃, KNO₃, MgCl₂, CaCl₂, FeCl₃, FeCl₂, urea, glucose and BSA solutions (2.0 mM) were prepared in ddH₂O and evaluated individually using the optimized procedure described in section “3.2.3 NitrI-Pad assay optimization and data analysis”. Results were compared to that obtained with the NitrI-Pad tested with NaNO₂ 2.5 mg/mL (CTR+) and with ddH₂O (CTR-) solutions. In addition, pH effect on the NitrI-Pad was also evaluated testing NaNO₂ sample (1.75 mg/L) solutions at pH 5.0, 7.0 and 9.0.

Sensor stability was investigated by measuring the colorimetric signals of the NO₂⁻ (or NaNO₂) sensing papers stored at +4 °C in Petri dishes and sealed with Parafilm tape for 28 days. The sensor response was assessed by the addition of 30 µL of NaNO₂ (1.0 mg/L) (or NO₂⁻ 0.667 mg/L) with an incubation period of 15 min at room temperature (~ 22 °C). Colorimetric signals were acquired with a smartphone camera (OnePlus 6T) and analyzed with ImageJ software. The colorimetric signals obtained at day 0 were normalized as 100% of the signal.

3.3 Results and Discussion

In this work, a foldable colorimetric smartphone paper sensor for monitoring nitrite ions was developed. Exploiting the modified Griess reaction, an azo coupling mechanism, in the

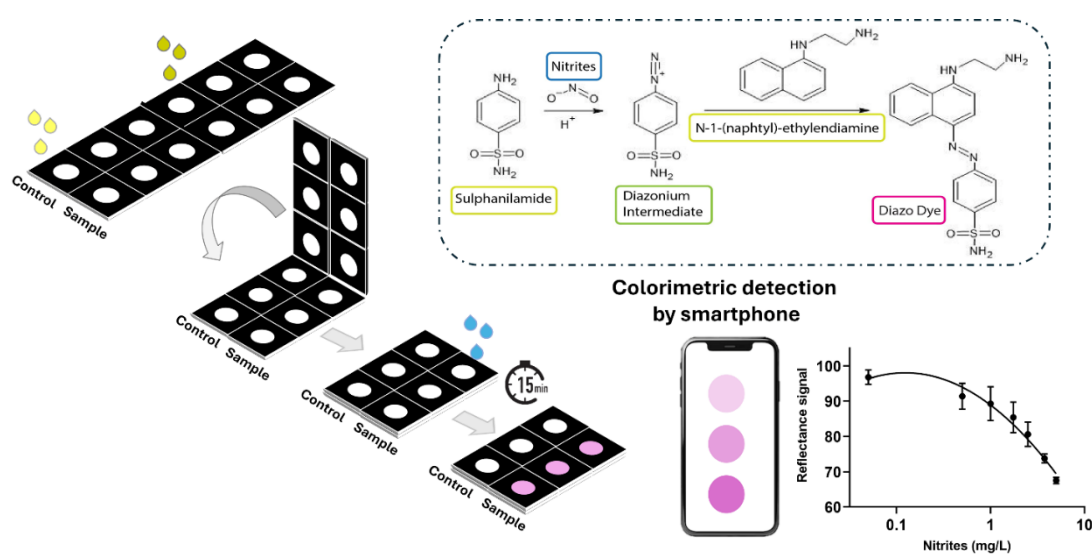


Figure 3. 1 Schematic mechanism of Griess reaction and the NitrI-Pad assay principle with the optimized configuration.

presence of nitrite and an acidic catalyst, an aromatic amine (e.g. sulfanilamide - SA), undergoes in diazotization to form a reactive diazonium salt through an electrophilic aromatic substitution that, combined with an electron-rich coupling agent (e.g. N-(1-naphthyl)ethylenediamine - NED), produces a highly-colored azo dye with a pink color (Figure 3. 1). Sulfanilic acid was replaced by sulfanilamide in hydrochloric acid since it yields higher color stability and faster reaction kinetics using a commercially available reagent³³.

3.3.1 Experimental Design and Method Optimization on Paper

Different optimizations were performed to implement the Griess reaction on a user-friendly paper sensor for NO_2^- detection. Firstly, the method was scaled for the paper format by sequentially adding in each well of the paper sensor a 10 mL-volume of SulfAA/NED solution in a ratio 1:1 and 15 mL of NaNO_2 (concentration range from 0.05 to 5.0 mg/L), and 15 mL of ddH₂O for the control. The colorimetric reaction was monitored by incubating the analyte with the colorimetric reagents, SulfAA and NED, in a dark box at room temperature and by acquiring reflectance images with a OnePlus6T smartphone camera every 5 min for the first 30 min, and every 15 min up to 2 h. The smartphone was used for reflectance measurements to implement the Griess reaction onto a paper-based sensing platform. As shown in Figure S3. 2, the presence of NaNO_2 produced a color change from colorless (white on paper) to pink visible by the naked eye, even immediately after the addition of the standard analyte solutions to the Griess reagents on paper. Images were analyzed in terms of brightness intensity with ImageJ software using the grayscale as a combination of the R (red), G (green), and B (blue) channels of the RGB system. To avoid differences in the signals due to background light interferences, a control sample was tested for each experiment and data were used to normalize the signal of each analyte concentration tested. The normalized brightness intensity was used as an analytical signal, which decreases as the concentration of the analyte increases, as the reflectance signals are calculated in terms of brightness in the grayscale. This signal normalization allowed to improve the robustness of the reflectance data which reproducibility and precision are generally affected by light sources having different spectral characteristics. A first calibration curve for NaNO_2 on paper was obtained after 15 min of incubation time with a LOD of 0.16 mg/L (corresponding to 0.11 mg/L of NO_2^-).

3.3.2 Optimization of The Origami Design and Experimental Procedure

To obtain a ready-to-use sensor, physical adsorption was evaluated to immobilize the Griess reagents on paper and the origami technique for the design of the device. Firstly, we immobilized the Griess reagents, SulfAA and NED, into the same well by creating a solution of SulfAA and NED with a 1:1 ratio and dispensing 10 mL of mix in each well of the PAD. After 1 h incubation at room temperature and in the dark, a color change was observed also by the naked eye in all the wells. Data analysis was performed by comparing the reflectance signals obtained in the wells in which the Griess reactants were immobilized together and the control wells with significant differences (from 8 to 22%) in decrease of reflectance signal (Figure 3. 3). This is in accordance with previous studies, in which one of the main limitations of the Griess reagents is the poor stability when single components are mixed^{34–36}. To minimize the Griess reagents degradation upon storage SulfAA and NED were immobilized on separate wells. To avoid pipetting and handling toxic solutions and simplify the user procedure, we implemented the origami approach, in which folding the paper sensor allows reagents' mixing and triggering of chemical reactions³⁷. By implementing the origami approach, the user needs only to add the sample, also with a disposable dropper to simplify the procedure. PADs were designed to contain a bottom and a top part, each consisting of an array of 3 x 2 wells for the immobilization of SulfAA and for NED. Three configurations A, B, and C, in which a 5 mL-volume of SulfAA and 5 mL of NED, previously absorbed on paper for 1 h at room temperature in the dark, were first explored and tested for nitrite detection. Configuration A was designed for the PAD A to include the SulfAA in the bottom wells and the NED in the top ones. The optimized procedure involved sample addition (15 μ L NaNO₂ 5.0 mg/L) to the SulfAA wells, then the folding of the NED part and the acquisition of the reflectance signals after 0, 5, 10, 15, 20, and 30 min of incubation time at room temperature and in the dark with the OnePlus 6 smartphone. The same configuration was chosen for PAD B, except that the NED part was first folded over the SulfAA one, and then the sample was added to the reverse NED wells. No color changes were observed up to a period of 4 hours (Figure S3. 4). PAD C followed the same procedure used for PAD B, but with SulfAA immobilized onto the top wells, and NED onto the bottom ones. A change in the color of the sample wells was visible immediately after sample addition (15 μ L NaNO₂ 5.0 mg/L), and pictures were taken after 0, 5, 10, 15, 20, and 30 minutes of incubation at room temperature (22°C) and in the dark.

According to the reaction mechanism, the sequential reaction of SulfAA with nitrites ions led to the formation of the diazonium intermediate, which spontaneously reacts with the NED coupling reagent to form a diazo colored compound^{34,36}. Therefore, the configuration C was chosen since it provided the highest color variation for the wells containing 5.0 mg/L NaNO₂ (about 38% decrease in reflectance signal vs a 24% decrease obtained with the configuration A) and the experimental procedure was very easy, making it an accessible tool for any user (Figure S3. 5). Since the sample volume is a critical aspect of the origami technique, acting not only as analyte but also as a carrier fluid with the task of wetting all the paper layers and allowing mixing of the reagents, the total volume and the ratio between Griess reagents and analyte were also studied. We evaluated different reagents:analyte ratios (i.e., 2:3 and 1:1 ratios).

We tested the ratio 2:3 using 5 mL of SulfAA, 5 mL of NED and 15 mL of analyte, and 10 mL of SulfAA, 10 mL of NED and 30 mL of analyte. We also evaluated the 1:1 ratio by adding 10 mL of SulfAA, 10 mL of NED and 20 mL of analyte. Quantitative elaboration of the obtained data (Figure S3.6 and Figure S3.7) confirmed that the optimal ratio Griess reactants/sample to ensure wettability of the paper layers and provide higher sensibility was 2:3 and the total volume was 50 mL.

3.3.3 Analytical Performance of The Nitripad

After optimizing the design and the optimal conditions for the colorimetric reaction on paper, the sensitivity of the method was assessed by creating a calibration curve for NaNO₂ using the NitriPad composed of a 7 x 3 wells array for the NED part and the SulfAA part. After the adsorption of the Griess reactants, adhesive tape was applied to the back side of the NED part, then the SulfAA part was folded over the NED part. 30 μ L of NaNO₂ standard solutions (0.05, 0.50, 1.00, 1.75, 2.50, 3.75, 5.00 mg/L) and control were added to the wells and incubated in the dark at room temperature (22°C). After 0, 5, 10, 15, 20, and 30 minutes, images were acquired with the smartphone, and reflectance signals were extracted and analyzed as described in the Materials and Methods section. The colorimetric reaction started immediately after the addition of the standard solutions, and no significant difference was observed after 10, 15, 20, and 30 minutes of incubation (Figure 3. 2). Therefore 15 minutes of incubation time was chosen as the best compromise to achieve the best sensitivity with a rapid procedure (Table S 3.1).

A LOD of 0.40 mg/L of NaNO_2 (corresponding to 0.27 mg/L of NO_2^-) and a LOQ of 1.67 of NaNO_2 (corresponding to 1.11 mg/L of NO_2^-) were obtained using the optimized conditions.

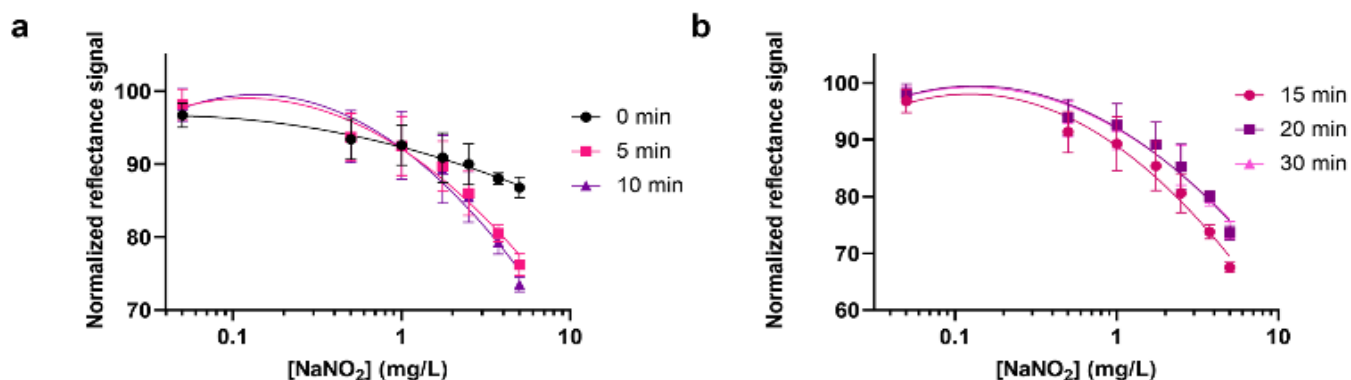


Figure 3. 2 Calibration curve for NaNO_2 obtained at 0, 5 and 10 min of incubation time in the dark, at RT and b) at 15, 20 and 30 min acquiring the reflectance signals with the OnePlus 6 smartphone.

Compared to other paper-based methods,^{32,34,35,38,39} the NitriPad is simple and cost-effective, the preparation of the device does not require any complex apparatus, and no instrumentation is needed for signal detection. This is particularly beneficial for field applications where advanced equipment may not be available. This is a crucial feature for early detection and prevention of nitrite-related issues in environmental monitoring and clinical diagnostics. Klasner et al.³⁹ developed a mPAD for detecting nitrite in saliva samples with a LOD of 5 mM, which is comparable to our sensor. Whereas the mPAD presented by Jayawardane et al. lowered the LOD for nitrites to 1.0 mM²⁰. However, their method includes the manual addition of fresh Griess reagents to the paper PAD, therefore the end-user should handle chemicals, thus complicating the workflow for non-skilled personnel. In addition, most of reported sensors⁴⁰ do not include a calibration curve, therefore, despite having promising analytical performance, separate sensors should be used to construct the calibration curve for achieving quantitative results. Conversely, the proposed paper sensor can be intended for widespread applications in the environmental and agro-food fields, as well as for the analysis of biological fluids.

3.3.4 Real Samples and Recovery Studies

The performance of the NitriPad was evaluated with real samples of drinking water including commercial bottled water in which the declared nitrite ions content was <0.002 (Figure 3. 3).

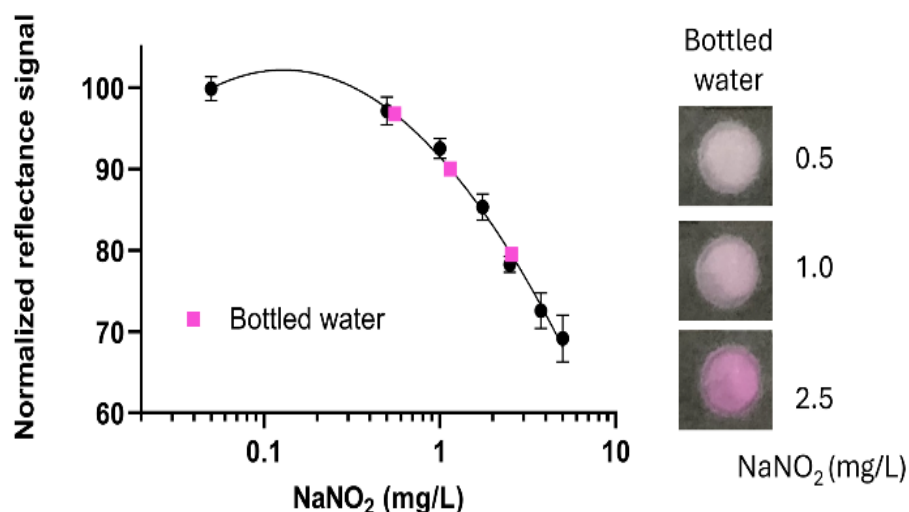


Figure 3. 3 Calibration curves for NaNO₂ obtained with the NitriPad at 15 min and bottled water samples spiked with different NaNO₂ (0.5, 1, 2.5 mg/L)

Samples were spiked by adding three different concentrations (0.5, 1.0, and 2.5 mg/L) of NaNO₂ and recoveries, calculated as described in the Materials and Methods Section, were 110, 87 and 102% respectively, thus demonstrating the applicability of the sensor for drinking water (Table 3. 1).

Added (mg/L)	Found (mg/L)	Recovery (%)	RSD (%)
0.50	0.60	110	5
1.00	0.87	87	9

2.50	2.60	102	7
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Table 3. 1 Recovery of NaNO₂ in commercial bottled water (n=3)

3.3.5 Selectivity and Stability Studies

Selectivity studies were performed to evaluate the specificity of the NitriPad sensor. As shown in Figure 3. 4a. the effect of potential interferents including ions (NaCl, MgSO₄, K₂HPO₄, K₂CO₃, KNO₃, MgCl₂, CaCl₂, FeCl₃, FeCl₂) and compounds present in

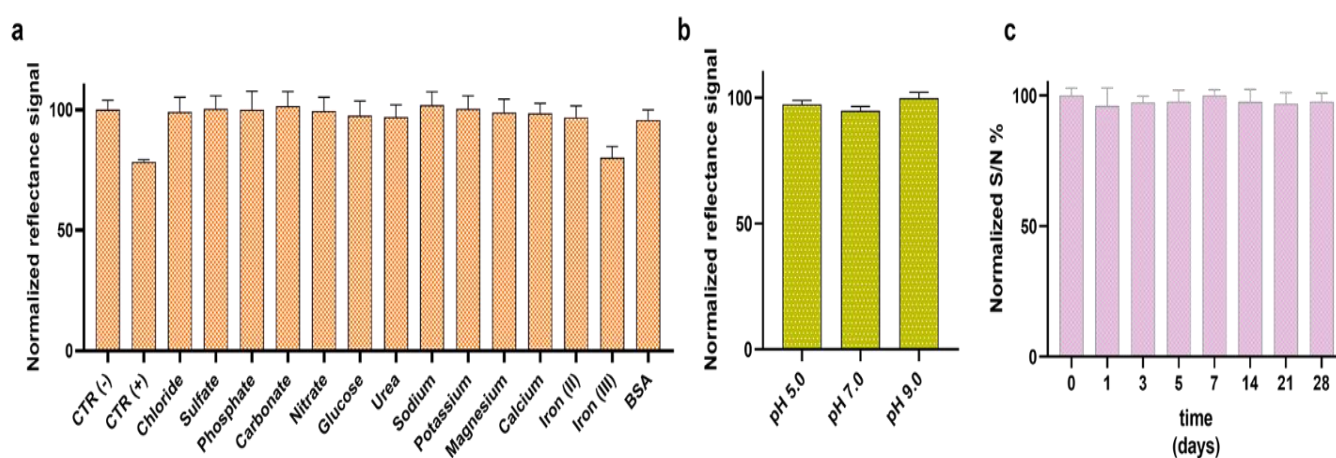


Figure 3. 4 a) Selectivity studies of the NitriPad with different interferents (2mM) compared to the NaNO₂ 2.50 mg/L (CTR+). Reflectance signals are normalized to the CTR (-); b) pH effects on the NitriPad and c) Stability of the NitriPad stored at +4°C. Reflectance signals are normalized to day 0.

environmental or clinical matrices (urea, glucose and BSA) was compared to NaNO₂ (2.5 mg/L) used as CTR(+) and results normalized respect to the ddH₂O solution (CTR-). Only Fe³⁺ was shown to be a potential interferent with a decreased reflectance signal of about 20 ± 4%. This result is in line with tolerance ions studies⁴¹. The effect of pH was also evaluated with NaNO₂ solutions (1.75 mg/L) at pH 5.0, 7.0 and 9.0 (Figure 3. 4b). Decreased reflectance signals of about 5.7 ± 1.3 % and of 2.7 ± 1.5 % were observed at pH 7.0 and 5.0, respectively, confirming that nitrous acid is the predominant species at pH 5.0⁴¹.

NitriPad storage stability was evaluated during storage at +4°C. The analytical performance of the NitriPad method for the detection of NaNO₂ was assessed by performing

the assay after 1, 3, 5, 7, 14, 21, and 28 days of storage, acquiring the colorimetric images, and normalizing the reflectance signals to the values obtained for the freshly prepared sensing PADs (day 0). As shown in Figure 3. 4, the assay provided excellent results also after 28 days of storage, with a responsiveness of $98 \pm 3.3\%$ compared to the test performed on day 0. This level of stability is notably superior to other paper-based sensors, which often exhibit a significant decrease in sensitivity over shorter time frames²⁰. This result supports the future applicability of the sensor even when shipping to remote locations is required.

Additionally, the NitriPad demonstrated excellent reproducibility with a relative standard deviation (RSD%) of about 1.6%, which has been investigated using paper PADs produced on the same day and kept at +4°C using an intermediate concentration of NaNO₂ (1.0 mg/L). The repeatability was evaluated using PADs fabricated the same day, obtaining an impressive RSD% of about 0.2%.

The results of the paper sensor were compared with those obtained with a commercial kit (Nitrite/Nitrate Colorimetric Test, Roche) performed with 96 well-plates and benchtop spectrophotometer and, according to the F-Test, the variances were not significantly different at a 95% confidence level (Table S3 .2). We also performed a paired t-test showing an agreement between the results at a 95% confidence level.

3.3.6 Sustainability Assessment

A fundamental aspect to consider in the development of a new analytical method is sustainability since it ensures that a new analytical method is not only effective but also environmentally friendly, economically feasible, and aligned with broader societal goals. We evaluated the sustainability of the NitriPad through the 12 principles of white analytical chemistry (WAC) using the RGB 12 algorithm⁴². The algorithm analyzes the analytical efficiency, the compliance with the green analytical chemistry, and the productivity, together with the practical/economic efficiency. These parameters are represented as the primary colors red, green, and blue components, respectively, in the algorithm, and they assess the overall whiteness of the method. The more saturation in each primary color, the higher the whiteness of the methods, which means an optimal and consistent analytical method. A darker final color reflects the lower sustainability of the method. The NitriPad method was compared to 5 previously reported microfluidic PADs, all relying on colorimetric detection of nitrites using the Griess reactants using a scanner for the acquisition of the colorimetric signals. Data (Table S3, S4, S5) and explanation of the RGB score assessment criteria are presented in the

Supplementary Materials. The sustainability of the NitriPad showed a total Whiteness score of 71.1% (Table S3.5). The highest score is reached during the blue principles analysis, which considers the feasibility of the method. This method only requires an office wax printer for the preparation of the NitriPad and a smartphone for the detection of the colorimetric signal and achieved a blue score of 93.8%. Additionally, the NitriPads are stable at +4°C and are safe for the user since all the reagents are immobilized on paper and only the sample is added by the end-user.

3.4 Conclusions

In this work, an origami paper-based sensor for the quantitative detection of nitrite has been developed using a modified version of the Griess reaction. It is based on a colorimetric reaction, which produces a colored compound visible to the naked eye, and the intensity of the color is proportional to the presence of nitrite ions. The origami technique was chosen to separate the two Griess reagents before performing the test. This technique enhances the portability of the sensor, the sensitivity of the system, and the efficiency of the interaction between the reactant species and the target analyte. The use of a smartphone camera for the acquisition of reflectance signals creates a portable and easy-to-use system that can be used by non-skilled personnel and on-site analysis.

An important key strength of this work is the stability. The performance of our sensor is maintained at the 98% for at least 1 month when stored at +4°C. To the best of our knowledge, this is the first prototype of a paper-based sensor interfaced with a smartphone camera for the detection of nitrite with a limit of detection suitable for analysis of drinking water and storage stability exceeding 1 month. These findings underscore the NitriPad's potential for long-term storage without significant loss of sensitivity, making it a promising tool for practical nitrite detection applications.

The NitriPad represents a valuable tool for the rapid and reliable monitoring of nitrites across a range of applications, including water quality assessment in agricultural runoff, wastewater treatment, surface and drinking water. Additionally, it represents a versatile nitrite detection tool, since its applicability can be extended to the analysis of biological fluids, such as urine, saliva, and sweat for point-of-care diagnostic, highlighting its potential impact in both environmental and health-related contexts.

3.5 Supporting Information

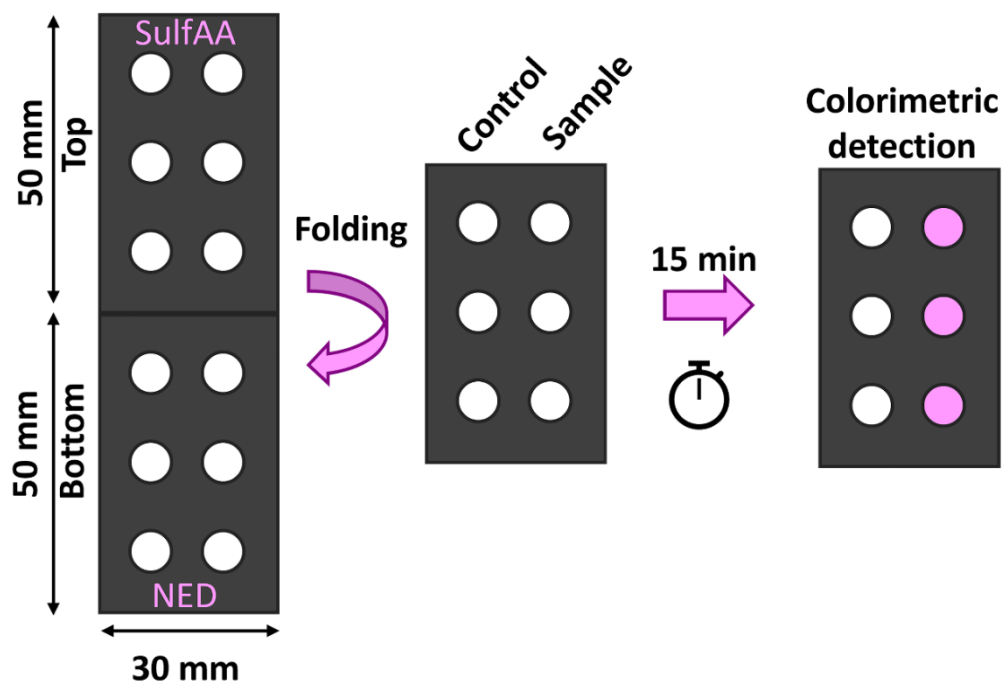


Figure S3. 1 General design of the NitriPad and schematic procedure for nitrites colorimetric detection on paper.

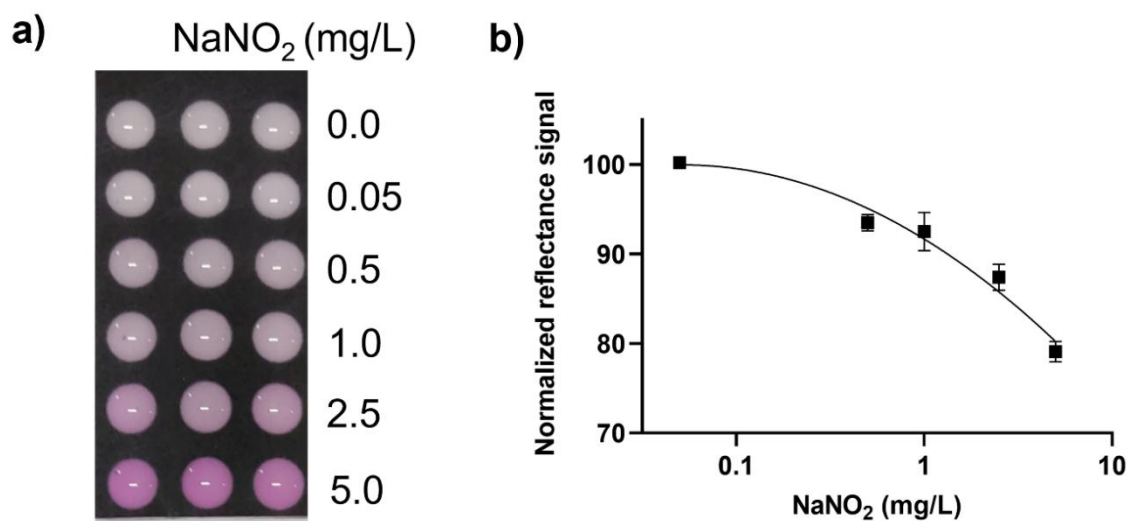


Figure S3. 2 a) Image of NaNO_2 calibration curve on paper after 15 minutes of incubation in the dark, at RT and b) graphical elaboration of the colorimetric signals acquired with the OnePlus 6 smartphone

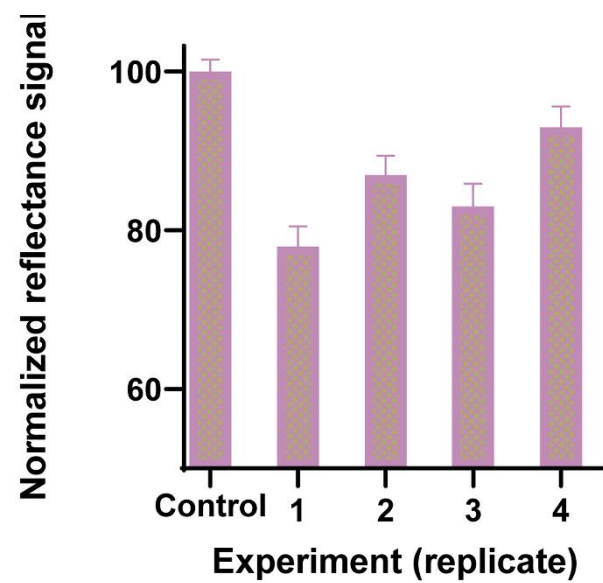
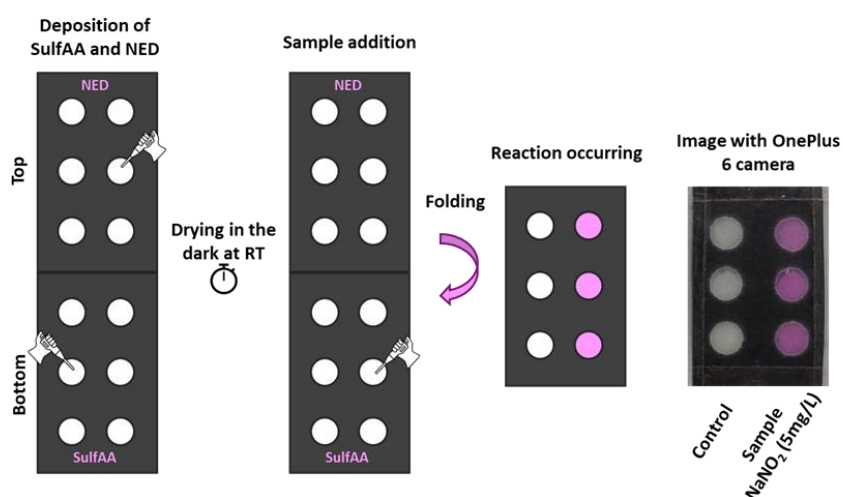
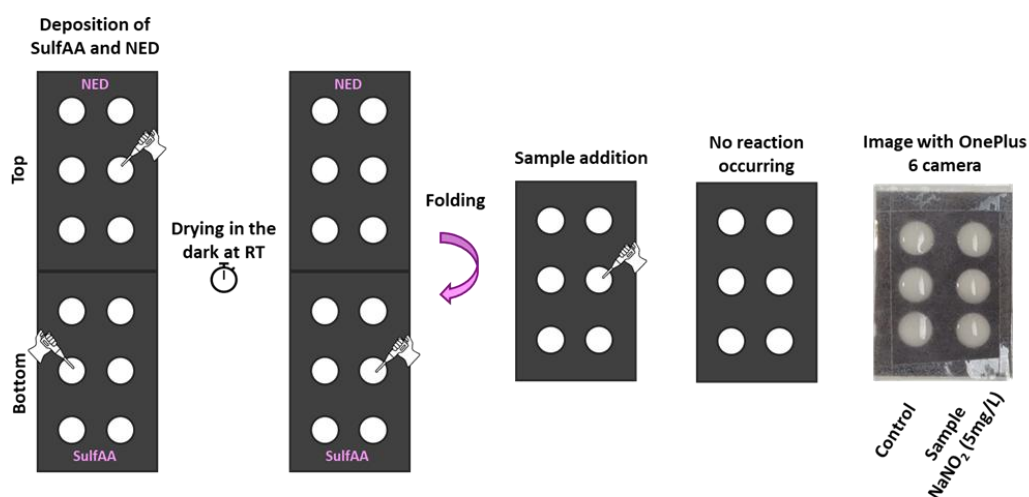


Figure S3. 3 Reflectance signal of four paper sensors in which the mixed solution of SulfAA and NED is adsorbed into each well.

Configuration A



Configuration B



Configuration C

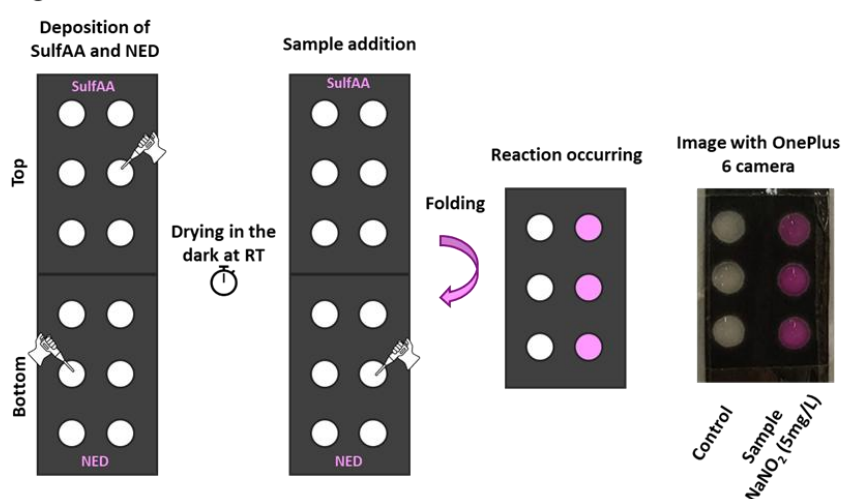


Figure S3. 4 Schematic procedure for the creation of the three configurations A, B, and C, and relative images of the image of PADs acquired with a OnePlus6T smartphone camera after 15 minutes of incubation in the dark, at RT with 5.0 mg/L of NaNO_2

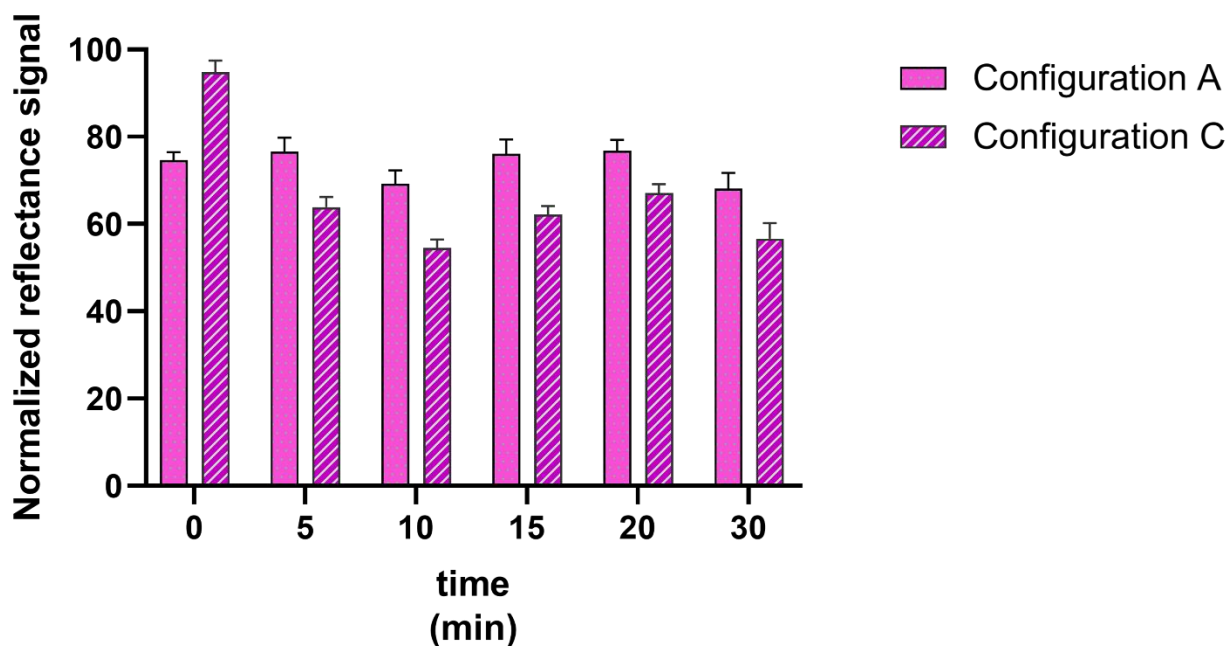


Figure S3. 5 Reflectance signals obtained using the Configuration A and C of the origami colorimetric paper-based sensor and incubation with 5.0 mg/L of NaNO_2 for 0, 5, 10, 15, 20 and 30 min.

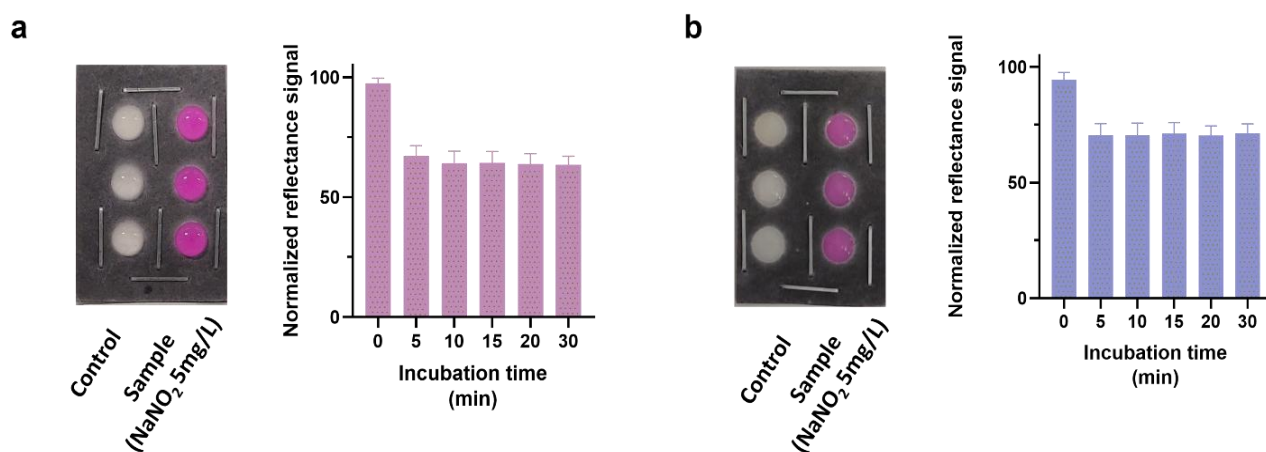


Figure S3. 6 Optimization studies of the volumes of the Griess reagents and analyte (ratio 2:3) and the image of the NitriPad obtained after 15 minutes of incubation with NaNO_2 0.0

and 5.0 mg/L and the normalized reflectance signal obtained after 0, 5, 10, 15, 20, and 30 minutes of incubation time.

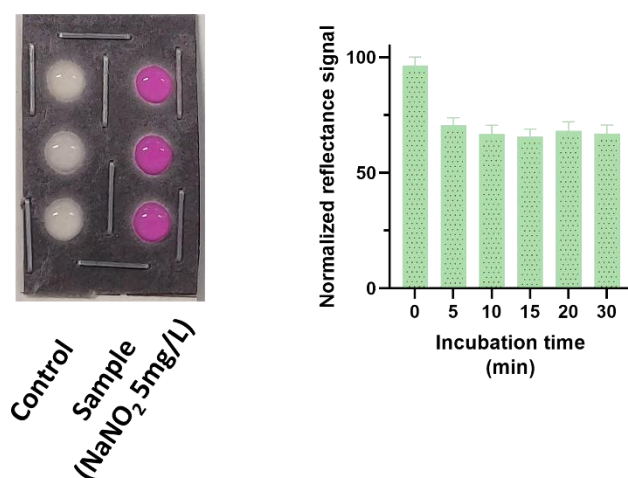


Figure S3. 7 Optimization study of the ratio 1:1 of the Griess reagents:analyte and the image of the NitriPad after 15 minutes of incubation with NaNO₂ 0.0 and 5.0 mg/L and the normalized reflectance signal obtained after 0, 5, 10, 15, 20, and 30 minutes of incubation time.

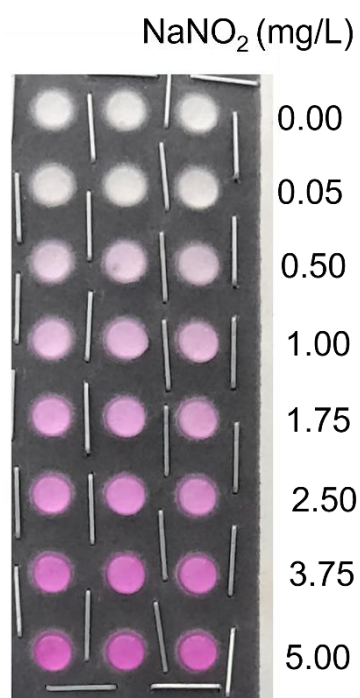


Figure S3. 8 Image of the NaNO₂ calibration curve obtained with the NitriPad sensor after 15 min of incubation time

Incubation time	LOD	LOQ
(min)	NaNO₂ mg/L	NaNO₂ mg/L
0	/	0.73
5	0.09	0.43
10	0.53	1.82
15	0.40	1.67
20	0.79	3.76
30	0.39	1.22

Table S3. 1 Limit of detection (LOD) and limit of quantification (LOQ) obtained with the NitriPad sensor at different incubation times

Sample	Nitrite (mg/L)	
	NitriPad	Nitrite/Nitrate Colorimetric Test kit
Mean	0.60	0.58
Standard deviation	0.03	0.02
Number of measurements (<i>n</i>)	10	8

Table S3. 2 Nitrite concentrations in water sample obtained with the NitriPad and the commercial Nitrite/Nitrate Colorimetric Test (Roche) kit performed in 96-well microtiter plates and benchtop spectrophotometer.

SUSTAINABILITY ASSESSMENT

Red principles

R1: Scope of application

An analytical method should be versatile, adaptable to various applications, and able to detect different types of analytes, and/or the same analyte in different samples. It needs to be robust against potential interferences, and it should be applied over a broad concentration spectrum.

R2: Limit Of Detection (LOD)

A suitable analytical method should have a high sensitivity, enabling the detection of trace amounts of a substance. The limit of detection represents the smallest quantity of analyte that can be reliably distinguished from the background noise. Thus, ensuring a low LOD is essential for the identification of analytes at minimal concentrations, and enhancing the overall reliability and applicability of the method across various sample types.

Score	R1. Scope of Application	R2. LOD
100	sample type ≥ 7	mM ≤ 1
100 -75	$7 < \text{sample type} \leq 5$	$1 < \text{mM} \leq 5$

75 - 50	$5 < \text{sample type} \leq 3$	$5 < \text{mM} \leq 10$
50 - 25	$3 < \text{sample type} < 1$	$10 < \text{mM} \leq 20$
25 - 0	$\leq 1 \text{ sample type}$	$\text{mM} \geq 20$

Table S3. 3 Criteria for the assignment of the Red Principles scores according to Novak et al. for the efficiency of the NitriPad sensor for detecting nitrite ions. We compared our assay to previously published methods for the detection of nitrites, relying on paper-based devices. We reported here only the common parameters reported in all the compared methods.

Green Principles

G1: Toxicity of reagents

A crucial aspect of the development of an analytical method is the toxicity of reagents. They must exhibit minimal toxicity, and it is advisable to select biodegradable, renewable, or natural reagents and materials. In this work, the safety data sheets of the reagents were evaluated to assess the safety of the materials that have been used.

G2: Amount of reagents and waste

In terms of sustainability, it is of utmost importance to reduce waste. Thus, the use of reduced volumes is preferable for the preparation of the devices and the use of samples.

G4: Direct impacts

The direct impacts parameter is aimed at studying the impact of the reagents used for the analysis on humans, animals, and the environment.

Score	G1. Toxicity of reagents	G2. Amount of reagents and waste	G4. Direct impact
100	0 < pictograms ≤ 1	0 < ml ≤ 0.5	No hazardous activity
100 - 75	1 < pictograms ≤ 2	0.5 < ml ≤ 1	Exceptionally low hazardous activity
75 - 50	2 < pictograms ≤ 5	1 < ml ≤ 2.5	low hazardous activities
50 - 25	5 < pictograms < 10	2.5 < ml ≤ 5	medium hazardous activities
25 - 0	≥ 10 pictograms	> 5 ml	Dangerous activities

Table S3. 4 Criteria for the assignment of green principle scores according to Novak et al.⁴³ for the assessment of sustainability of the NiriPad system. We used pictograms of the “*Globally Harmonized System of Classification and Labelling of Chemicals*”, and mL as a unit of quantification of the volumes used for the analysis.

Blue principles

B1: Cost-efficiency

The total analysis cost is an important parameter for the economic assessment of an analytical method. The aim is to minimize the cost of the analysis as much as possible by evaluating the cost of all materials, equipment, and reagents. Also, the qualification of the personnel is to be taken into account for these considerations.

B2: Time-efficiency

A desirable result is a short-time analysis, together with a reduction in the time of preparation of the experimental set-up and the reaction time. These parameters were taken into account for the evaluation of the time efficiency of the methods.

B3: Requirements

Requirements for the economic assessment of the method include the reduction of sample volumes, equipment, and trained personnel. We evaluated the detection systems, image and statistical analysis software used for each assay, and the need for any laboratory equipment.

B4: Operational simplicity

Ideally, an analytical method should be fully automated, miniaturized, and portable, enabling on-site usage for any user.

Score	B1 Cost Efficiency	B2 Time efficiency	B3. Requirements		B4. Operational Simplicity		
			B3.1 Sample consumption	B3.2 Other needs	B4.1 Portability	B4.2 Integration automation	B4.3 Miniaturization
100	Very low-cost	$1 < \text{min} \leq 5$	$0 < \text{ml} \leq 0.05$	Simple Accessories & tools (mobile, pipette, dark box, etc.)	Online analysis	High automation or simple, online data	Handheld

100-75	Low-cost	$5 < \text{min} \leq 15$	$0.05 < \text{ml} \leq 0.1$	Simple Equipment (glassware, dark box, basic equipment for manual preparation, mobile/camera /ccd, image processing, statistical software)	At line analysis, camp laboratory	High automation or simple	Portable
75-50	Medium cost	$15 < \text{min} \leq 30$	$0.1 < \text{ml} \leq 1$	Medium complexity instrumentation (scanner), statistical analysis, equipment for basic preparation	In lab analysis	Partially automation	Transportable
50-25	Expensive	$30 < \text{min} \leq 60$	$1 < \text{ml} \leq 5$	Complex instrumentation, equipment for preparation (spectrophotometer, statistical analysis, Refrigerator)	In lab analysis with sample storing, batch working, results obtained	No automation	Bench

					the same day of analysis		
25 - 0	Extremely expensive	≥ 60 min	> 5 ml	High complexity instrumentation (luminometer, Statistical analysis software, other complex equipment	In lab analysis with sample storing, planned batch working , results obtain, days after the analysis	No automation	Complex facilities

Table S3. 5 Criteria for assigning the blue principles scores according to Nowak et al. for the economic assessment of the NitriPad system.

Method number	R (%)	G (%)	B (%)	Whiteness (%)	Reference
1	47.5	73.8	93.8	71.7	This work
2	33.8	54.2	56.9	48.3	2
3	37.5	66.7	78.8	61.0	3
4	36.3	65.4	68.8	56.8	4

5	43.8	25.0	54.7	41.1	5
6	50.0	65.4	85.0	66.8	6

Table S3. 6 Results from whiteness evaluation for our method and five NitriPad sensors for the detection of nitrites. The average values in percentage for the Red, Green, and Blue parameters of each method are reported.

3.6 Author Contributions

Conceptualization, D.G. and E. M.; methodology, D. G., R. J. R. A., F. B., R. D.; validation, M. M. C., D. G.; formal analysis, D. G., F. N., M. M. C.; investigation D. G., M. M. C and E. M.; resources, E. M.; data curation, M. M. C., D.G; writing – original draft preparation, D.G.; writing – review and editing, M. M. C. and E. M.; visualization, D.G, M. M. C.; supervision, M.M.C and E. M.; funding acquisition, E. M. All authors have given approval to the final version of the manuscript.

1. Conflicts of Interest

There are no conflicts to declare.

2. Data Availability

The article presents reflectance measurements obtained with a OnePlus 6 smartphone and elaborated with ImageJ and Prism v.8 software. All raw data and corresponding images are available at the authors' laboratory at University of Bologna.

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3.8 References

- 1 S. Singh, A. G. Anil, V. Kumar, D. Kapoor, S. Subramanian, J. Singh and P. C. Ramamurthy, *Chemosphere*, 2022, **287**, 131996.
- 2 F. Serio, P. P. Miglietta, L. Lamastra, S. Ficocelli, F. Intini, F. De Leo and A. De Donno, *Science of The Total Environment*, 2018, **645**, 1425–1431.
- 3 Bijay-Singh and E. Craswell, *SN Appl. Sci.*, 2021, **3**, 518.
- 4 M. Moorcroft, *Talanta*, 2001, **54**, 785–803.
- 5 World Health Organization, *Guidelines for drinking-water quality: fourth edition incorporating the first and second addenda. Geneva: World Health Organization; 2022. Licence: CC BY-NC-SA 3.0 IGO.*, Geneva, 2022.
- 6 H. Li, C. J. Meininger and G. Wu, *J. Chromatogr. B Biomed. Sci. Appl.*, 2000, **746**, 199–207.
- 7 J. E. Melanson and C. A. Lucy, *J. Chromatogr. A*, 2000, **884**, 311–316.
- 8 R. Michalski and I. Kurzyca, *Pol. J. Environ. Stud.*, 2006, **15**, 5–18.
- 9 D. Tsikas, *Journal of Chromatography B*, 2007, **851**, 51–70.
- 10 M. Shariati-Rad, M. Irandoust and M. Haghighi, *J. Chemom.*, 2014, **28**, 93–99.
- 11 J. B. Fox, *Anal. Chem.*, 1979, **51**, 1493–1502.
- 12 L. Váradi, M. Breedon, F. F. Chen, A. Trinchi, I. S. Cole and G. Wei, *RSC Adv.*, 2019, **9**, 3994–4000.
- 13 Y. Thipwimonmas, J. Jaidam, K. Samoson, V. Khunseeraksa, A. Phonchai, A. Thiangchanya, K. H. Chang, A. F. L. Abdullah and W. Limbut, *Chemosensors*, 2021, **9**, 161.
- 14 J. Sun, X. Zhang, M. Broderick and H. Fein, *Sensors*, 2003, **3**, 276–284.
- 15 M. Arvand, N. Arjmandi, M. Shakibaie, S. Jafarinejad, R. Shahghadami and P. Sasanpour, *J. Phys. D Appl. Phys.*, 2020, **53**, 355403.

- 16 S. A. Klasner, A. K. Price, K. W. Hoeman, R. S. Wilson, K. J. Bell and C. T. Culbertson, *Anal. Bioanal. Chem.*, 2010, **397**, 1821–1829.
- 17 T. M. G. Cardoso, P. T. Garcia and W. K. T. Coltro, *Analytical Methods*, 2015, **7**, 7311–7317.
- 18 B. M. Jayawardane, S. Wei, I. D. McKelvie and S. D. Kolev, *Anal. Chem.*, 2014, **86**, 7274–7279.
- 19 S. A. Bhakta, R. Borba, M. Taba, C. D. Garcia and E. Carrilho, *Anal. Chim. Acta*, 2014, **809**, 117–122.
- 20 B. M. Jayawardane, S. Wei, I. D. McKelvie and S. D. Kolev, *Anal. Chem.*, 2014, **86**, 7274–7279.
- 21 Y.-T. Tai, C.-Y. Cheng, Y.-S. Chen and F.-H. Ko, *Sens. Actuators B Chem.*, 2022, **369**, 132298.
- 22 D. Li, Y. Ma, H. Duan, W. Deng and D. Li, *Biosens. Bioelectron.*, 2018, **99**, 389–398.
- 23 C.-Y. Hou, L.-M. Fu, W.-J. Ju and P.-Y. Wu, *Chemical Engineering Journal*, 2020, **398**, 125573.
- 24 Z. Geng, Y. Miao, G. Zhang and X. Liang, *Sens. Actuators A Phys.*, 2023, **349**, 114056.
- 25 L. Cevenini, M. M. Calabretta, A. Lopreside, G. Tarantino, A. Tassoni, M. Ferri, A. Roda and E. Michelini, *Anal. Bioanal. Chem.*, 2016, **408**, 8859–8868.
- 26 J. T. Heggestad, D. S. Kinnamon, J. Liu, D. Y. Joh, C. M. Fontes, Q. Wei, A. Ozcan, A. M. Hucknall and A. Chilkoti, 2022, pp. 343–365.
- 27 S. Soares and F. R. P. Rocha, *Microchemical Journal*, 2021, **162**, 105862.
- 28 V. B. dos Santos, L. B. Ayres, H. S. de Sousa, C. D. Garcia and W. Toito Suarez, *Sensors & Diagnostics*, 2024, **3**, 1467–1475.
- 29 I. S. Resque, V. B. dos Santos and W. T. Suarez, *Chemical Papers*, 2019, **73**, 1659–1668.

- 30 F. T. S. M. Ferreira, R. B. R. Mesquita and A. O. S. S. Rangel, *Talanta*, 2020, **219**, 121183.
- 31 L. P. dos S. Benedetti, V. B. dos Santos, T. A. Silva, E. B. Filho, V. L. Martins and O. Fatibello-Filho, *Analytical Methods*, 2015, **7**, 4138–4144.
- 32 P. Rajasulochana, Y. Ganesan, P. S. Kumar, S. Mahalaxmi, F. Tasneem, M. Ponnuchamy and A. Kapoor, *Environ. Res.*, 2022, **208**, 112745.
- 33 M. I. Karayannis, E. A. Piperaki and M. M. Maniadaki, *Anal. Lett.*, 1986, **19**, 13–23.
- 34 S. A. Bhakta, R. Borba, M. Taba, C. D. Garcia and E. Carrilho, *Anal. Chim. Acta*, 2014, **809**, 117–122.
- 35 T. M. G. Cardoso, P. T. Garcia and W. K. T. Coltro, *Analytical Methods*, 2015, **7**, 7311–7317.
- 36 S. C. Pai, Y. T. Su, M. C. Lu, Y. Chou and T. Y. Ho, *ACS ES and T Water*, 2021, **1**, 1524–1532.
- 37 N. Colozza, V. Caratelli, D. Moscone and F. Arduini, *Biosensors (Basel)*, 2021, **11**, 328.
- 38 M. Arvand, N. Arjmandi, M. Shakibaie, S. Jafarinejad, R. Shahghadami and P. Sasanpour, *J. Phys. D Appl. Phys.*, DOI:10.1088/1361-6463/ab8e7a.
- 39 S. A. Klasner, A. K. Price, K. W. Hoeman, R. S. Wilson, K. J. Bell and C. T. Culbertson, *Anal. Bioanal. Chem.*, 2010, **397**, 1821–1829.
- 40 K. Khachornsakkul, R. Del-Rio-Ruiz, H. Creasey, G. Widmer and S. R. Sonkusale, *ACS Sens.*, 2023, **8**, 4364–4373.
- 41 M. Irandoust, M. Shariati-Rad and M. Haghighi, *Analytical Methods*, 2013, **5**, 5977.
- 42 P. M. Nowak, R. Wietecha-Posłuszny and J. Pawliszyn, *TrAC Trends in Analytical Chemistry*, 2021, **138**, 116223.

Conclusion and Future Perspectives

This doctoral research addressed the critical need for sustainable, field-deployable analytical tools for water quality monitoring, in direct support of United Nations Sustainable Development Goal 6 and within the framework of the Italian PNRR AgriTech National Research Center and the EU Horizon FARMWISE project.

Two paper-based biosensors have been designed and validated for use in real-world situations. The first is a bioluminescent biosensor that utilizes the organism *Aliivibrio fischeri*, which was immobilized in agarose hydrogels and adhered to wax printed cellulose paper. This biosensor provides portable, single-use cartridges capable of replacing ISO 11348 toxicity assays and can be used in the absence of laboratory infrastructure. The dual sensor was also developed using the Griess reaction to measure nitrites using paper as a substrate. The sensor achieved a limit of detection of 0.27 mg L⁻¹ and produced recovery percentages between 87 and 110% for real-world drinking water samples. Additionally, the nitrite paper biosensor has an instrument-free operation and demonstrated no loss in analytical response over a 28-day period. The work described herein demonstrates the potential of both of these systems to provide rapid and accurate analysis of chemical contaminants in real-world situations. Major limitation was the inability to assign compound specificity due to the non-specific nature of bioluminescence inhibition.

Future work will build directly on complementary research conducted at the College of Chemical and Biological Engineering, Zhejiang University. The biosensors recently developed were to detect copper, lead, and cadmium in both live (whole-cell) and in vitro (cell-free) systems utilize a combination of plasmid construction, cell-based assays, and cell-free fluorescence detection platforms. These systems will be integrated into multiplexed test cartridges which will convert multiple toxicity signals to quantitative concentrations of contaminants. Furthermore, the cell-free biosensor systems provide a regulatory compliant, genetically modified organism (GMO) free alternative for jurisdictions that have restrictions on deploying microbes in the environment.

The new *ASSESSOR* Android application will be a continuation of the *Scoutinel* ability to embed geolocation into data storage, allow for data retrieval and historical results, and provide data sharing, will naturally evolve into a networked environmental monitoring system; future development will integrate the *ASSESSOR* and *Scoutinel* into a single, multi-modal

platform for processing bioluminescent and colorimetric signals acquired during the same field session. Continuing expansion to include agro-industrial analytes (e.g., pesticide metabolites, mycotoxins, and antibiotic residues). Together, these advances establish a coherent, field-ready analytical ecosystem for sustainable water quality monitoring in direct service of environmental stewardship and global health security.

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