

DISSERTATION

BRIDGING LAB AND FIELD: CAPILLARY MICROFLUIDICS FOR RAPID, LOW-
COST, CITIZEN-BASED POLLUTION MONITORING

Submitted by

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ABSTRACT

BRIDGING LAB AND FIELD: CAPILLARY MICROFLUIDICS FOR RAPID, LOW-COST, CITIZEN-BASED POLLUTION MONITORING

There is growing interest in how human activities impact the environment and, in turn, human health. Pollution from point and non-point sources can cause lasting environmental harm, but we lack important information on contaminant distribution. As such, a major gap in addressing this issue is the lack of fast, simple, inexpensive testing solutions. Microfluidic devices can offer a practical solution, enabling fast and affordable testing. Easy-to-operate tools can be used by citizen scientists to test pollution levels, expanding data collection efforts. This reduces the need for frequent visits by professional scientists and can lead to substantial cost savings. This dissertation highlights different capillary-based microfluidic platforms I have developed to overcome the key limitations of traditional sensing platforms for contaminant detection, bridging the “lab-to-field” gap for citizen-based environmental monitoring.

Building on this foundation, a series of capillary flow-driven microfluidic platforms are developed to overcome key limitations of traditional paper-based analytical devices, including slow transport, reliance on precise volume handling, and limited sensitivity. A hollow capillary-driven architecture was first introduced to enable rapid analyte transport and homogeneous signal generation, allowing for the detection of heavy metals such as Ni(II), Cu(II), and Fe(III) within seconds using a simple dip-and-read format. This approach demonstrated that decoupling fluid transport from in-plane paper

wicking can significantly improve assay speed and usability for point-of-need applications.

To address sensitivity limitations inherent to colorimetric detection, a low-cost preconcentration strategy was developed and integrated with the capillary-driven platform. This system enabled multiplexed detection of seven heavy metal ions at low parts-per-billion levels using a simple Fill–Fold–Photo workflow and smartphone-based analysis. The platform achieved detection limits meeting or exceeding regulatory guidelines while maintaining portability and user-friendly operation, demonstrating its suitability for field deployment.

The scalability and real-world applicability of these platforms were further demonstrated through the development of a robust phosphate sensor designed for citizen-based environmental monitoring. By combining fast-flow microfluidic design with improved reagent stabilization, the system enabled accurate and reproducible measurements under ambient conditions. Large-scale deployment of more than one thousand sensors across multiple continents enabled high spatial and temporal resolution mapping of phosphate contamination, highlighting the potential of these technologies for decentralized environmental monitoring.

Finally, the scope of capillary-driven microfluidics was extended to analytes requiring chemical transformation prior to detection. A paper-based microfluidic platform integrating on-chip photochemical nitrosation was developed for the detection of N-nitrosodimethylamine (NDMA), enabling a complete photochemical–colorimetric workflow within a portable format. This approach demonstrates the versatility of

capillary-driven systems and their potential to expand beyond direct detection to more complex analytical challenges.

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I would like to dedicate this dissertation to my late grandmother, Mankumari Aryal. Although I was unable to be by her side and say a final goodbye due to the distance, her love, strength, and quiet resilience have always remained with me. This accomplishment stands as a small tribute to her enduring influence in my life.

PREFACE

This dissertation is presented in a manuscript-based format. Chapters 2–5 are based on peer-reviewed publications and manuscripts that are published or under review. Portions of Chapter 1 are adapted from review articles previously published by the author (*Lab on a Chip* 2024, 24, 1175–1206, and *Frontiers in Lab on a Chip Technologies* 2024, 3, 1467423). The author was a primary contributor to the conception, literature analysis, and writing of these chapters and review articles. Minor edits were made to integrate the adapted material into the overall narrative and objectives of this dissertation. Appendix 1 consists of a first-author publication by the author. Appendices 2 and 3 include additional published works resulting from collaborative efforts with visiting scholars, whom the author had the opportunity to mentor. In these collaborative studies, the author contributed directly to the design and development of the sensors, as well as the execution of experiments and data analysis.

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CHAPTER 1 — INTRODUCTION

1.1 Chapter overview

Parts of this chapter are adapted from previously published review articles by the author, as disclosed in the Preface. This chapter introduces the motivation for developing point-of-need analytical platforms for environmental water monitoring. It outlines the limitations of conventional laboratory-based techniques, including high cost, infrastructure requirements, and limited accessibility for frequent or distributed sampling. The chapter establishes capillary-driven microfluidics and paper-based analytical devices as promising alternatives due to their low cost, portability, and ease of use. It further discusses the key challenges faced by existing paper-based systems—such as slow transport, limited sensitivity, and reliance on precise sample handling—and frames the central objective of the dissertation: to design user-friendly, field-deployable sensing platforms that achieve analytically relevant performance without laboratory infrastructure.

1.2 Environmental pollution—a growing global concern

Human activity has fundamentally altered the Earth's natural systems. Rapid industrialization, urban expansion, and sustained population growth have significantly increased the release of pollutants into the air, water, and soil, resulting in widespread environmental contamination. While these developments have supported economic growth and improved standards of living, they have also imposed substantial costs on human health and ecosystem stability.

A broad range of environmental pollutants—including heavy metals, particulate matter (PM), biogenic toxins, and industrial effluents—have been linked to adverse health outcomes.^{1,2} Comprehensive global assessments have shown that environmental pollution is a major contributor to disease burden, accounting for approximately one-fifth of global morbidity and nearly one-quarter of total deaths worldwide.^{3–6} These impacts are particularly pronounced in developing regions, where large populations are exposed to industrial discharges, inadequate waste management infrastructure, poor sanitation, contaminated water sources, and indoor air pollution arising from biomass combustion.^{7–9} Despite ongoing remediation efforts and regulatory initiatives, environmental pollution remains a persistent and disproportionate threat in these settings.^{10,11}

In parallel, continued industrial and technological advancement in developed nations has led to the emergence of new classes of contaminants that can disseminate rapidly and persist in the environment.^{12,13} Many of these pollutants challenge the natural buffering capacity of ecosystems and complicate conventional monitoring and mitigation strategies.^{14–16} Together, these factors underscore the urgent need for effective technological solutions that enable rapid identification and management of environmental contaminants, thereby supporting timely intervention and long-term environmental protection.

Current analytical techniques used for environmental monitoring—including inductively coupled plasma optical emission spectroscopy (ICP-OES),¹⁷ atomic absorption spectroscopy (AAS),¹⁸ fluorescence spectroscopy,¹⁹ ultraviolet–visible (UV–vis) spectroscopy,²⁰ mass spectrometry (MS),²¹ and high-performance liquid chromatography (HPLC)²²—provide high sensitivity and analytical precision, even at

trace analyte concentrations.²³ However, these methods typically require expensive instrumentation, centralized laboratory infrastructure, lengthy sample preparation, and trained personnel.²⁴ As a result, their application is often limited in field settings and resource-constrained environments, where rapid, decentralized testing is most critically needed.²⁵

To address these limitations, significant research efforts have been directed toward the development of fast, low-cost, user-friendly, and environmentally sustainable sensing technologies.²³ Such approaches aim to complement established laboratory methods by enabling point-of-need monitoring, expanding access to environmental data, and supporting informed decision-making in both developed and developing regions.

1.3 Microfluidic Paper-based Analytical Devices (μ PADs)

Paper has been used as an analytical medium for centuries, most commonly as a surface for writing and record-keeping.²⁶ More recently, however, its intrinsic physical and chemical properties have been recognized as highly advantageous for analytical and sensing applications.²⁷ Advances over the past two decades have established paper as a versatile and accessible platform for low-cost scientific detection and analysis.²⁸

One of the defining features of paper is its ability to transport aqueous fluids through capillary action, enabling passive fluid flow without the need for external pumps or power sources.²⁹ This property makes paper particularly attractive for point-of-need and field-deployable analytical systems.^{30–32} In addition, paper is inexpensive, widely available globally, lightweight, and easily disposable, characteristics that are especially important for applications in resource-limited settings. Paper substrates are available in

a wide range of thicknesses and mechanical strengths, from thin filter papers to more robust cardboard-like materials, allowing device designs to be tailored to specific analytical requirements. The stackability, storability, and ease of transport of paper further enhance its suitability for portable analytical platforms.

From a chemical and biological perspective, paper offers additional advantages. Its cellulose-based composition provides high absorbency and inherent compatibility with biological samples, making it well suited for applications involving proteins, nucleic acids, and other biomolecules.²⁵ Moreover, the surface chemistry of cellulose can be readily modified, enabling functionalization through physical adsorption or covalent attachment of recognition elements such as enzymes, antibodies, nucleic acids, and small molecules.³³ This chemical flexibility significantly broadens the applicability of paper-based analytical devices (PADs) across diagnostics, environmental monitoring, food safety, and related fields.^{34–37}

The modern development of paper-based microfluidic systems was catalyzed by the introduction of the first microfluidic paper-based analytical device (μ PAD) in 2007.³⁸ This initial demonstration highlighted the potential of patterned paper to control fluid flow and perform analytical assays in a low-cost, portable format. Since then, the field has expanded rapidly, with substantial growth in device complexity, analytical performance, and application scope.²⁵ Over the past decade, paper-based analytical systems have gained widespread adoption as platforms for decentralized testing (Figure 1), driven by their simplicity, affordability, and compatibility with point-of-use detection strategies.²⁵

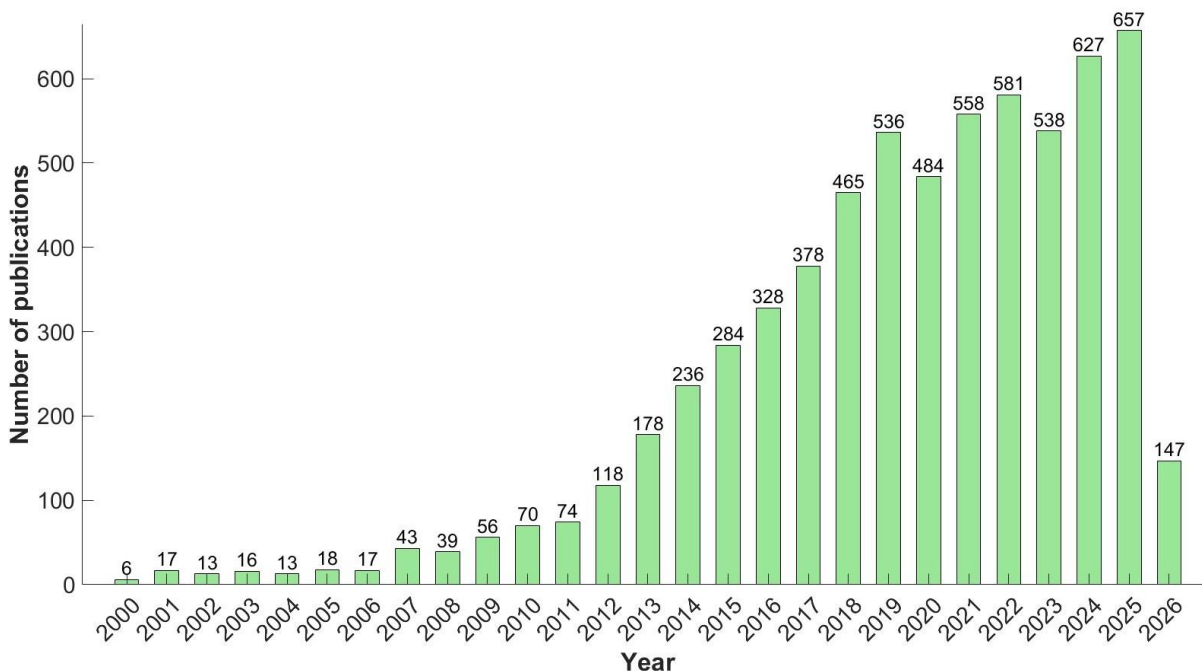


Figure 1. Number of publications involving paper-based microfluidics since 2000, derived from Web of Science search results using search criteria: "paper-based*" OR "patterned paper" OR "paper device*" AND "microfluidic*" (Title), as of 8th April, 2026.

The general principle underlying the design of μ PADs involves the creation of hydrophobic barriers to direct aqueous fluids through predefined hydrophilic pathways within a porous cellulose substrate.²⁵ These barriers confine liquid flow to specific regions, forming capillary-driven microchannels that enable controlled transport without the need for external pumping, as shown in Figure 2.

Fluid transport within μ PADs arises from the intrinsic structure of paper, which consists of an interconnected cellulose fiber network containing abundant hydroxyl (–OH) functional groups.³⁹ These polar groups interact favorably with aqueous and other polar liquids, facilitating spontaneous capillary wicking through the porous matrix.³⁹ Depending on the application, the resulting microchannels may remain open to the environment or be enclosed using transparent films, adhesive tapes, or thin polymer sheets to reduce evaporation, improve reproducibility, or enable multilayer architectures.

The movement of fluid in paper-based microfluidic devices is commonly described by the Washburn equation: (equation 1)⁴⁰

$$l = \sqrt{\frac{\gamma r \cos\theta}{2\eta}} t \dots\dots\dots \text{Eq 1}$$

where l is the distance, γ is the surface tension, θ is the contact angle, η denotes the fluid viscosity, r is the average pore radius, and t is the time.

This relationship indicates that wicking behavior depends on both fluid properties and the physical characteristics of the paper substrate. In practice, the rate of capillary transport is influenced by factors such as paper thickness, pore size distribution, fiber density, and cellulose purity.²⁵

The geometry of microchannels is governed by two primary design parameters. The channel height is determined by the thickness of the paper substrate, while the lateral resolution and uniformity of the hydrophobic barriers depend on the patterning technique employed.³⁹ Common fabrication methods vary widely in cost, accessibility, and achievable feature resolution, requiring careful selection based on the intended application and performance requirements. An optimal patterning approach balances fabrication simplicity, user-friendliness, and the level of fluidic control required.²⁵

Following channel patterning, assay-specific reagents are deposited in designated sensing regions, and any required transducers are incorporated into the device. Upon sample introduction at the inlet, the analyte is transported through the hydrophilic microchannels to the detection zone, where it interacts with immobilized reagents to generate a measurable signal corresponding to the chosen detection modality (Figure 2).

In addition to simple transport and detection, paper-based devices can be engineered to perform sample pretreatment steps, such as filtration, masking of interferents, or pH adjustment, by incorporating functional materials or additional patterned regions within the paper substrate.

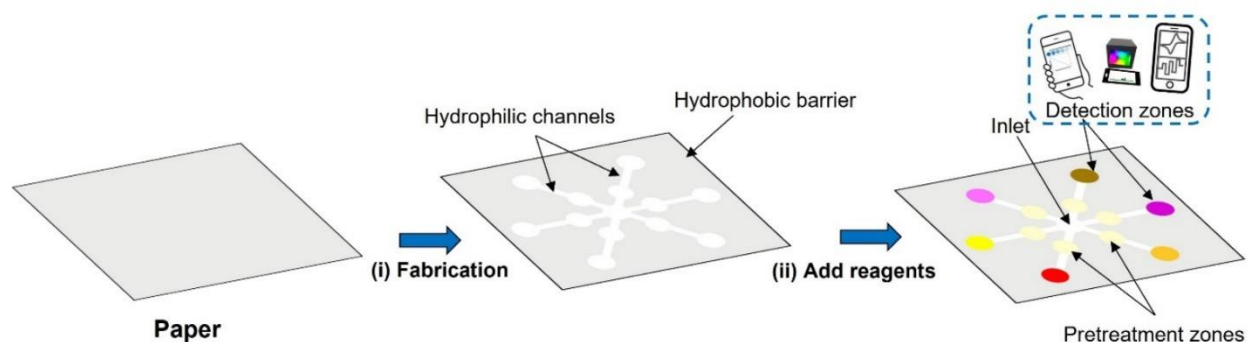


Figure 2. General fabrication technique for μ PADs.

1.4 Limitations of μ PADs

Paper-based analytical devices (μ PADs) have emerged as a promising platform that leverages capillary-driven flow to enable low-cost, disposable, and user-friendly chemical sensing. Their simplicity, compatibility with colorimetric assays, and potential for mass production have driven extensive academic investigation for point-of-care diagnostics and point-of-need environmental testing. Despite this promise, translating μ PADs from laboratory demonstrations to practical field deployment has proven more challenging than initially anticipated. Early field evaluations have revealed recurring limitations related to sample handling, analytical performance, device reproducibility, stability, and usability by non-expert operators.^{41,42}

A central contributor to many of these challenges is the paper-based transport system itself. Fluid flow through cellulose networks is governed by capillary wicking through a porous, heterogeneous, and anisotropic medium.²⁴ As a result, analyte transport is inherently slow, with flow velocities that decrease as the wetted length increases.¹¹ Many μ PADs therefore require extended assay times to achieve complete wetting, reagent rehydration, reaction, and signal development, often ranging from tens of minutes to hours.⁴³ This slow transport delays result acquisition, limits throughput, and reduces practicality for time-sensitive point-of-care and field-based applications.

In addition to slow transport, analyte depletion during wicking further constrains sensitivity. Cellulose fibers present a large internal surface area rich in hydroxyl functional groups, which can promote nonspecific adsorption of analytes, reaction intermediates, or detection products during transport.⁴⁴ For trace-level targets, even modest adsorption losses can significantly reduce the amount of analyte reaching the detection zone, leading to diminished signal intensity and poor quantitative accuracy.⁴⁵

Paper-based transport is also highly sensitive to environmental conditions, particularly temperature and humidity.⁴⁶ Variations in ambient humidity influence evaporation rates and capillary flow behavior, altering effective reagent concentrations and transport dynamics within the paper matrix.^{47,48} Temperature fluctuations can further impact solvent evaporation, reaction kinetics, and color development. These uncontrolled external factors introduce variability that is difficult to mitigate in outdoor or resource-limited settings.⁴⁹

Reproducibility is further affected by the intrinsic anisotropy and microstructural variability of paper.⁵⁰ Cellulose fiber orientation, local density variations, and differences arising from paper manufacturing processes can lead to non-uniform flow paths and spatially heterogeneous wetting.⁵¹ Combined with batch-to-batch variability in paper substrates and non-uniform reagent deposition, these effects contribute to inconsistent signal formation and reduced device-to-device reproducibility, presenting a significant barrier to reliable quantitative analysis and regulatory acceptance.^{50,51}

Another limitation of μ PADs is their limited sample volume capacity, which constrains the achievable detection limits.⁵² Because analytes are distributed throughout the porous matrix during transport, low-concentration targets often fail to generate sufficient signal without amplification or preconcentration.⁵² While various preconcentration strategies have been explored, many require complex substrate modification, multi-step workflows, or external hardware, which undermines the simplicity and accessibility that make μ PADs attractive.⁵³

Finally, many current paper-based and hybrid microfluidic platforms for environmental analysis still rely on pipetting fixed sample volumes into discrete assay zones. This requirement complicates field deployment by increasing operational complexity, dependence on auxiliary tools, and the need for trained users, conditions that are often impractical in real-world monitoring scenarios.

Collectively, these limitations highlight that while μ PADs offer clear advantages in cost and accessibility, the paper-based transport medium imposes fundamental constraints on speed, sensitivity, reproducibility, and robustness. Addressing these

challenges requires alternative capillary-driven architectures that preserve passive, equipment-free operation while enabling faster transport, improved control, and enhanced analytical performance—motivating the capillary-driven microfluidic platforms developed in this dissertation.

1.5 Hollow capillary-flow-driven microfluidics

In 2019, Channon et al. reported a multilayer paper-based device that incorporated a hollow gap channel between two paper sheets to overcome the transport limitations of conventional μ PADs.⁵⁴ By systematically optimizing the gap height and sample loading conditions, they demonstrated that fluid transport could be decoupled from in-plane wicking within the paper substrate. Their approach relied on constructing a sealed slit channel with a gap on the order of several hundred micrometers and employing a loading strategy that maximized Laplace pressure at the inlet. Under these conditions, liquid rapidly traversed the hollow gap and only gradually wicked into the adjacent paper layers, rather than being limited by cellulose-mediated capillary flow. This design achieved flow velocities approximately $145\times$ higher than those of single-layer μ PADs at comparable distances, corresponding to flow rates exceeding 2 mL min^{-1} without the use of external pumps.⁵⁴

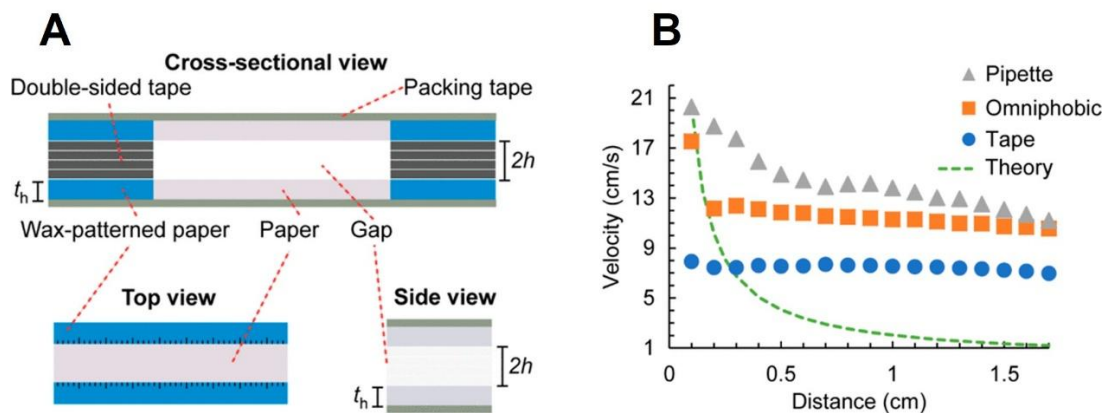


Figure 3. Schematic of the multilayer μ PAD architecture with a central gap channel (A) and decay of flow velocity with distance for different sample loading methods—pipette, super-omniphobic reservoir, and tape well—using a 390 μ m gap and Whatman 3MM paper (B). Copyright © 2019 by American Chemical Society. Reprinted with permission.

Later, in 2021, Jang et al. developed a laminate capillary-driven microfluidic device constructed by stacking transparency films and double-sided adhesive layers to form multilayer hollow capillary channels.⁵⁵ The architecture incorporated shallow inlet channels feeding into a taller main channel, enabling capillary flow that was not only rapid but also precisely controlled in both time and space. By integrating passive push and burst valves, the platform allowed programmable flow initiation and stopping, while geometry-defined Y-junctions enabled tunable laminar, gradient, or fully mixed flow regimes. Importantly, the flow velocity in this system could be set by channel height and inlet length, resulting in nearly constant and predictable flow rates that are not governed by the Washburn-type decay characteristic of paper-based wicking.⁵⁵

The use of hollow capillary channels enabled rapid analyte transport while avoiding many intrinsic limitations of paper substrates. Because fluid transport occurred within enclosed channels rather than porous cellulose networks, analyte loss due to nonspecific adsorption was minimized. In addition, the system was largely insensitive to

environmental factors such as humidity and temperature that strongly affect hygroscopic paper-based devices. The laminate architecture also eliminated dependence on cellulose fiber orientation, which commonly leads to anisotropic and poorly reproducible flow in traditional paper-based microfluidic systems. Together, these features demonstrated that laminate, hollow-channel capillary microfluidics can preserve passive operation while offering substantially improved control, robustness, and analytical reliability compared to conventional μ PADs. This concept was later adopted in the development of several point-of-care biological assays, including laminate and multilayer devices for detecting many clinically relevant biomarkers.^{56–59} It has also been integrated into electrochemical paper- and film-based platforms, where controlled, rapid capillary flow and passive valves enable sequential reagent delivery and more reliable on-chip measurements⁶⁰

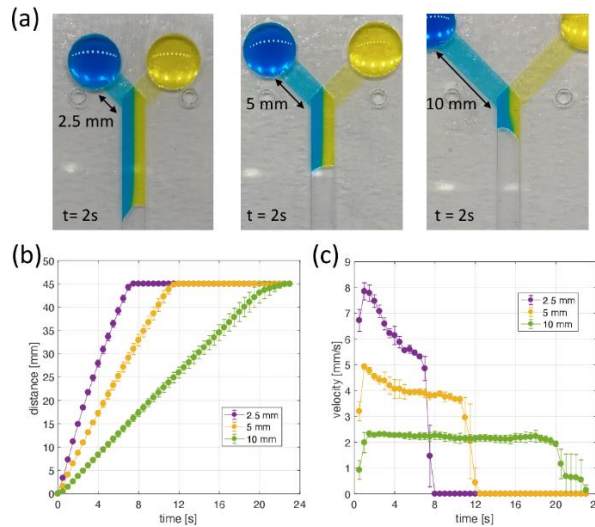


Figure 4. Geometry-controlled capillary flow in laminate hollow-channel microfluidic devices. (a) Y-shaped devices with varying inlet channel lengths showing inlet-dependent fluid delivery 2 s after dye introduction. (b,c) Corresponding flow distance and velocity profiles demonstrate tunable, non-Washburn capillary transport governed by channel

geometry rather than paper wicking. Reproduced from Jang, I. et al., *Analyst* 2021, 146, 2117–2126 with permission from the Royal Society of Chemistry, Copyright © 2021.

While laminate/multilayer capillary-driven platforms have significantly advanced biological point-of-care diagnostics—where defined sample volumes, controlled matrices, and semi-standardized laboratory conditions inherently limit variability, their potential remains largely unrealized in environmental monitoring. In clinical contexts, precise pipetting, buffered samples, and climate control mitigate fluctuations in matrix composition and ambient conditions. In contrast, environmental analysis must contend with heterogeneous and unpredictable samples (e.g., turbid waters, suspended particulates, variable organic loads), undefined volumes, and harsh outdoor environments characterized by temperature shifts, humidity fluctuations, dust, and evaporation. These factors amplify intrinsic μ PAD limitations such as adsorption losses, inconsistent capillary flow rates, and prolonged wicking or drying times, ultimately compromising quantitative reliability outside controlled settings.

This disparity highlights a critical translational gap: although laminate capillary architectures provide deterministic flow paths, passive fluid regulation, and structural enclosure, they have not been systematically adapted for trace environmental contaminant detection. By leveraging multilayer laminate designs to enable self-metered flow, reduced evaporation, improved reaction confinement, and faster assay completion, these systems can overcome many of the constraints that currently limit field deployment. Targets where robustness and operational simplicity are essential represent particularly compelling opportunities for this adaptation.

My dissertation addresses this underexplored niche by developing field-ready sensors for the detection of traditional environmental contaminants, such as heavy metals, nutrients, and pesticides, as well as emerging contaminants like NDMA, using laminate capillary microfluidic platforms. This work bridges the gap between laboratory-scale demonstrations and rugged, field-deployable monitoring technologies.

1.6 My contributions

The work presented in this dissertation advances the development of capillary-driven microfluidic and paper-based analytical platforms for point-of-need environmental sensing. Across the main chapters, I served as the primary conceptual lead, with responsibility for experimental design, device architecture, assay development, data acquisition, data curation, analysis, validation, and manuscript preparation. All main dissertation chapters are based on first-author works, where I led the research from initial concept through execution and dissemination.

Chapter 2 establishes the foundational capillary-driven microfluidic platform combined with paper-based colorimetric detection for rapid heavy metal analysis in water.⁶¹ I fully conceptualized the device architecture, designed the hollow capillary channels and paper integration strategy, developed and optimized the colorimetric assays, and performed all experiments, data analysis, and validation in real water samples. I also led optimization related to flow homogeneity, signal reproducibility, masking strategies, and interference mitigation. This chapter demonstrates that capillary-driven transport can overcome key limitations of conventional μ PADs by enabling rapid, dip-and-read operation without external instrumentation.

Chapter 3 expands this platform through the development of a low-cost preconcentration strategy that enables multiplexed detection of trace-level metal ions at environmentally relevant concentrations.⁶² I conceived the fill–fold–photo workflow, designed the preconcentration device and reagent cards, optimized filtration volume, paper selection, and masking strategies, and established smartphone-based quantitative analysis. I conducted all experimental work, performed method validation against ICP-MS, and led data interpretation. This chapter demonstrates how capillary-driven architecture can be combined with passive preconcentration to achieve regulatory-level sensitivity while maintaining field usability.

Chapter 4 focuses on the development and large-scale deployment of a user-friendly phosphate sensor for surface water monitoring.⁶³ I led the conceptual design of the fast-flow microfluidic architecture, reagent separation strategy, and dip-and-read operation that eliminated the need for pipetting. I developed and optimized the colorimetric assay, performed analytical validation, and assessed stability under ambient conditions. I coordinated large-scale fabrication, quality control, and data analysis for the deployment of more than one thousand sensors across multiple continents, demonstrating the feasibility of citizen-led monitoring and high-spatial-resolution environmental data collection using capillary-driven microfluidic systems.

Chapter 5 extends capillary-driven microfluidics to the detection of N-nitrosodimethylamine (NDMA), a highly toxic and emerging drinking water contaminant that traditionally requires complex laboratory workflows. I conceptualized and developed a paper-based microfluidic platform that integrates on-chip photochemical nitrosation followed by colorimetric detection. I designed the multilayer microfluidic device and UV

irradiation cassette, optimized reagent immobilization and sequential delivery, and performed all analytical performance studies, including selectivity, stability, and validation in environmental water matrices. This work represents the first demonstration of a complete photochemical–colorimetric workflow implemented directly on a capillary-driven paper-based microfluidic platform.

Appendix 1 presents a first-authored study published in *Green Analytical Chemistry* (2024) describing a sustainable, capillary flow-driven microfluidic platform for Fe(III) detection using a natural dye-based sensing strategy.⁶⁴ The work utilizes butterfly pea (*Clitoria ternatea*) anthocyanin as an eco-friendly chromogenic reagent, addressing growing concerns associated with synthetic dyes and hazardous chemical wastes. The sensor integrates a capillary-driven microfluidic architecture with a pH-controlled paper substrate containing dried anthocyanin, enabling direct immersion of the device in water without reagent loss and eliminating the need for external instrumentation or precise volumetric handling. This work represents the first demonstration of natural dye-based sensing implemented within a capillary-driven microfluidic system, highlighting a sustainable approach to point-of-need chemical analysis.

Appendix 2 presents a collaborative study published in *Analytical Chemistry* (2025),⁶⁵ conducted with Dr. Izabela Lewińska, describing a disposable, capillary flow-driven microfluidic device for dissolved oxygen (DO) sensing in water. The device is constructed using a laminate architecture of transparent PET film, double-sided adhesive, and glass microfiber with immobilized reagents, enabling a simple dip-and-read format without external instrumentation. A modified Winkler colorimetric assay is integrated

within the device to convert dissolved oxygen into a quantifiable colorimetric signal. This work establishes a low-cost, field-deployable alternative for DO monitoring suitable for citizen-based water quality assessment. In this study, I contributed to the sensor design and experimental design.

Appendix 3 presents a collaborative study published in *Analytica Chimica Acta* (2025),⁶⁶ conducted with Dr. Pornchanok Punnoy, describing a capillary flow-driven, single-dip dual-sided microfluidic sensor for multiplex detection of heavy metals and nutrients in water. The platform enables simultaneous detection of Ni, Fe, Cu, NO_2^- , and PO_4^{3-} in a single step, with both visual readout and smartphone-based quantitative analysis. This work highlights a scalable approach for rapid, multiplex, and comprehensive water quality monitoring, overcoming key limitations of conventional μPADs . In this study, I conceptualized and designed the dual-sided sensor architecture and led the experimental design and data analysis.

Collectively, the work described in this dissertation has resulted in 10 peer-reviewed publications (6 first-author and 4 co-authored), with four additional manuscripts currently under review. Beyond individual sensors or assays, my contributions establish a coherent design philosophy for capillary-driven microfluidic systems that bridge the gap between laboratory analytical performance and real-world field deployment. The approaches developed here are broadly applicable to environmental monitoring, public health protection, and decentralized chemical analysis.

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CHAPTER 2 — Capillary flow-driven microfluidics combined with a paper device for fast user-friendly detection of heavy metals in water

2.1 Chapter overview

The goal of this chapter is to introduce the first implementation of capillary-driven microfluidic transport combined with paper-based colorimetric detection for rapid, point-of-need monitoring of heavy metals in water. This chapter serves as a foundational demonstration of how hollow, capillary-driven architectures can overcome key limitations of conventional paper-based analytical devices, including slow analyte transport, reliance on pipetting, and long assay times, especially in environmental assays.

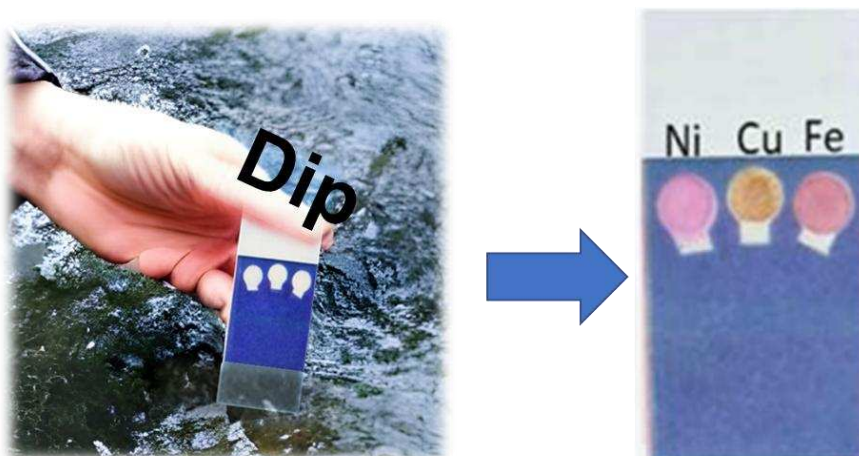
The research described in this chapter was executed under the supervision of Prof. Charles S. Henry, with collaborative input from Eric Brack and Todd Alexander at the U.S. Army DEVCOM Soldier Center. I designed the device architecture, developed and optimized the colorimetric assays, performed all experiments, analyzed the data, and led validation studies using real water samples. This work represents the first demonstration of capillary-driven microfluidic channels used as the primary transport medium for heavy metal detection and was later published as a first-author peer-reviewed article (*Anal. Chem.* 2023, 95, 13, 5820–5827).

In this chapter, a low-cost, laminate capillary-driven microfluidic device is combined with paper substrates to enable rapid, dip-and-read detection of Ni(II), Cu_{total}, and Fe_{total} without the need for external pumps or precise volume handling. By decoupling fluid transport from in-plane paper wicking, the platform achieves homogeneous color development within seconds of sample insertion, drastically reducing assay time relative to conventional μ PADs. Analytical performance, including limits of detection, precision,

selectivity, and recovery in environmental water samples, is evaluated to demonstrate the feasibility of this approach for field-deployable heavy metal monitoring.

While this chapter focuses on a colorimetric environmental application, its broader contribution lies in demonstrating that capillary-driven microfluidic transport can serve as a robust alternative to paper-based wicking. The findings presented here motivate the subsequent development of more advanced capillary-driven platforms, including preconcentration, multiplexed detection, and on-chip chemical transformations explored in later chapters.

Chapter Graphical Summary



2.2 Introduction

Drinking water is an increasingly scarce resource. One in every three individuals worldwide does not have access to safe drinking water.¹ According to the World Health Organization (WHO), 2 billion people use contaminated drinking water sources, causing almost a million deaths annually.² Even today, around 80% of worldwide wastewater is untreated, containing contaminants ranging from organic wastes to highly toxic industrial discharges.³ These contaminants reach different living organisms via several transport modes and can cause havoc within an entire ecosystem. Among the contaminants, heavy metals have drawn much attention from chemists, biologists, environmentalists, and hydrologists over the years due to their hazardous effects on humans, animals, and plants. Environmental factors, including toxic chemical exposure, are responsible for 25% of the total disease burden.⁴

Although most heavy metals are found in nature, the prime cause of heavy metal contamination of water sources is almost entirely anthropogenic. Developments in the industrial sector and urban regions, which also use natural resources and harm water bodies through run-off, are mostly to blame for increased levels of heavy metals. Heavy metals are highly soluble in water, are non-biodegradable, and are readily absorbed by living things.⁵ Hence, in the present context, our exposure to heavy metals is inevitable. Complications of heavy metal toxicity include gastrointestinal and kidney disorders, nervous system disorders, skin lesions, vascular damage, immune system dysfunction, congenital disabilities, and cancer.⁶ Cumulative effects of metal contamination have been extensively studied.⁷⁻⁹ Although drinking water quality is highly regulated with proper treatment plants and quality monitoring in the United States, about 4 million American

households have children under six years of age who live in homes with heavy metals like lead, according to the US Department of Housing and Urban Development.¹⁰ According to a study in 2017, 63 million Americans are exposed to unsafe drinking water containing high levels of bacteria, Arsenic, and other heavy metals.¹¹ This problem of toxic metals contamination in drinking water worsens due to the lack of fast, portable, inexpensive, and user-friendly water testing systems in resource-limited settings.

Inductive Coupled Plasma Mass Spectrometry (ICP-MS), Inductive Coupled Plasma Optical Emission Spectrometry (ICP-OES), and Atomic Absorption Spectrometry (AAS) are traditional heavy metals detection techniques.¹²⁻¹⁴ These methods are advantageous for detecting heavy metals since they are sensitive, specific, and precise even at low concentrations. Molecular spectroscopic techniques, such as UV/Vis molecular absorption and fluorescence spectroscopies, are also indirect techniques for heavy metal detection.¹⁵ Moreover, electrochemical and chromatographic techniques have also been used.¹⁶⁻¹⁸ However, all these methods are either time-consuming, need costly and extensive equipment, or require trained operators, making it almost impossible for point-of-use testing in resource-limited and economically challenged areas. As a result, scientists have been working to create rapid, user-friendly, and environmentally friendly detection technologies. One of the most promising possibilities is the μ PAD. Since the introduction of paper-based colorimetric detection by Whiteside et al. in 2007, paper-based microfluidics has become common.¹⁹ Paper-based devices are easy to fabricate, biodegradable, user-friendly, and economically affordable. μ PADs have been used for heavy metal detection in different fields like wastewater, drinking water, particulate matter, welding fumes, blood, and urine.²⁰⁻²⁶

In paper-based colorimetric chips development, both 2D and 3D approaches have been used depending upon the physical, biological, and chemical properties of the assay. In 2D paper-based chips, hydrophilic channels bordered by hydrophobic barriers guide liquid flow, and the reaction is carried out on the substrate.²⁷⁻²⁸ Whereas in 3D chip development, two-dimensional paper chips are superimposed to achieve the goal of “1+1>2”.²⁹ Photolithography, plasma treatment, and wax printing are the common techniques to create the 2D hydrophobic barrier on paper substrate.³⁰⁻³⁴ These methods result in a slow liquid flow into the detection reservoir via the flow channel. Because most paper-based colorimetric signals are maximized when the substrate is completely dry, quantifying the signal can take several minutes to hours. As a result, incorporating a waste zone beneath the detection reservoir would allow the pad to dry in significantly less time. Origami and lamination methods can be used to superimpose the 2D chips into a 3D.²⁹

Previous research has demonstrated different paper-based devices for detecting toxic heavy metals in water.³⁵⁻³⁷ A highly selective multiplexed system was reported for simultaneous detection of Cu(II), Co(II), Ag(I), Ni(II), and Mn(II) ions.³⁸ An inkjet-printed paper-based device coupled with silver nanoparticles was used with a smartphone for on-site calibration and detection of Hg(II) ions.³⁹ In addition, other approaches like 3D printing, natural cellulose, and doped nanoparticles have been used.⁴⁰⁻⁴² Although these techniques have successfully detected the respective metal ions in water, these current platforms typically rely on pipetting fixed volumes of samples into individual spots on assay cards. This makes it challenging to implement in the field in real-world settings, where fixed volume pipetting adds to the assay complexity and physical

resources required for operation. Moreover, inconsistent lighting conditions while using the smartphone would create less consistent data. Most μ PADs have long analysis and drying times (up to 2 hours), which may not be suitable during in-field testing campaigns and/or when results are needed quickly.^{20, 38} Komatsu et al. created a dip-and-read system for quantification without the need for precise sample insertion.⁴³ Without the use of sophisticated micropipettes, the method allows for sample addition. The transport media in this system, however, are paper-based microfluidics, which takes a longer time for analyte transport. Furthermore, maintaining fluorescent lighting conditions for image analysis would be a challenge in resource-constrained field settings.

Despite their many advantages, μ PADs face a number of significant challenges, including the impact of testing conditions (e.g., humidity), decreased sensitivity and selectivity, sample loss, and so on.⁴⁴⁻⁴⁶ Furthermore, their slow transport velocity frequently results in long assay times (40-120min),^{38, 47-52} making them ineffective for Point of Care assays. It is essential to obtain the signal as soon as possible because some of the complexes formed in colorimetric assays are quasi-stable, and faster flow rates are required for the enhancement of the signal via increased analyte transport rate. Capillary-driven microfluidics is a potential replacement for the transport medium in this scenario. The capillary-driven microfluidics transport system can provide a flow rate increase of up to 145-fold over the conventional 1-layered μ PAD design.⁵³

To address the shortcomings of the previous approaches, we designed a multiplexed system by combining a capillary-driven microfluidic device⁵⁴ with paper for the simultaneous detection of Ni(II), Fe_{total}, and Cu_{total}. The channels in the microfluidic devices were created by laser cutting three layers of transparent films and a double-layer

adhesive. We have previously developed flow control methods for point-of-need diagnostics using capillary-driven microfluidic devices.⁵⁴ We believe that this application can be extended to colorimetric environmental assays. Hence, using paper-based devices in conjunction with the capillary-flow-driven microfluidic device, we created a system that is fast (<1 min),⁵⁵ sensitive, and capable of detecting hazardous heavy metals and other contaminants in water without needing to pipette the sample into the reagent cards. The device developed in this study uses both origami and lamination techniques to superimpose the wax-printed 2D paper substrate and its waste zone. The instrument and the assays were created to detect each element concurrently and even in the presence of potentially interfering metals. Typically, the samples are pipetted into the reagent cards, and it is allowed to dry for several hours before performing the image analysis. Here, we used the capillarity of the microfluidic device so that the device could be run horizontally or directly dipped into the sample solution with color signals appearing in <8sec instead of minutes as compared to the archetypical μ PAD. Also, adding waste pads below the detection zone helped dry the pad quickly. The data were obtained using a smartphone camera and quantified with ImageJ software here to isolate the color intensities of the complex formed in the reagent pads. Moreover, the image analysis was performed as color intensity relative to white control, which counteracts any lighting changes. Using the quantitative study of color intensity, more exact measurements were possible. Interferences from other non-targeted metals and non-metal ions were also extensively studied. Three different metal concentrations were spiked in commercial drinking water and river water samples to analyze the recovery performance of the device. The percent recovery and RSD were found well within the acceptable range.

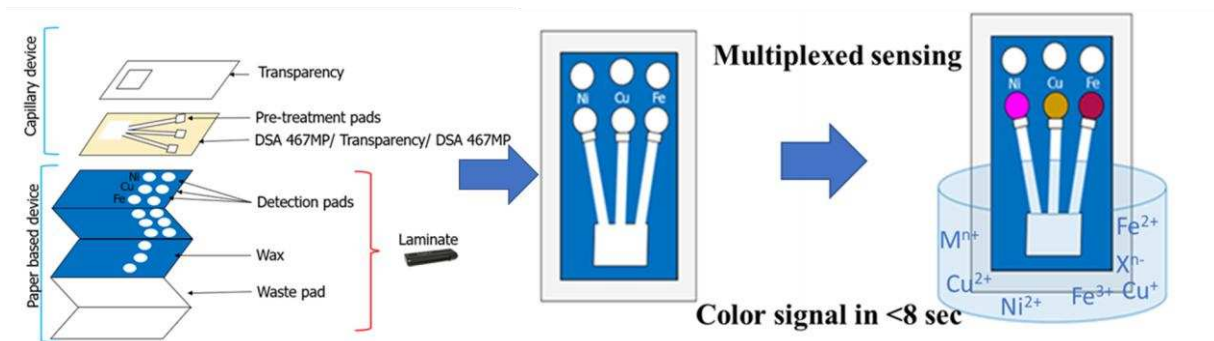


Figure 1. Device Assembly and running

2.3 Experimental section

2.3.1 Materials and chemicals

All metal standards (Nickel(II) Sulfate Hexahydrate, Cupric Sulfate Pentahydrate, and Ferric Chloride Hexahydrate), reagents (Dimethylglyoxime, Bathocuproine, and Bathophenanthroline), and the masking reagents (Sodium Fluoride, Ammonium Acetate) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxylamine hydrochloride and buffer components were purchased from Fisher Scientific. Double-sided adhesive (467MP) and transparency film (9984) were purchased from 3M (St. Paul, Mn, USA), and the Whatman paper was purchased from Fisher Scientific. PVC (0.7mm) foil was purchased from Deecraft Company in Wang Thonglang, Bangkok, Thailand, and the 3mil laminating pouches were purchased from Office Depot in Fort Collins.

2.3.2 Instrumentation

The adhesive tapes and transparency films were cut using an Epilog Engraver WinX64 Zing laser cutter. The Whatman paper was wax printed using Xerox ColorQube-8570. The shape formatting of the paper, ink thickness, and color (CMYK= 100,75,0,60) were

optimized in CorelDRAW to attain optimum hydrophobicity. The paper device was laminated using a Royal Sovereign CL-1223 thermal laminator.

2.3.3 Device design and fabrication

The device consists of two main parts: A microfluidic channel and a paper device. Both parts were designed in OnShape, a computer-aided shape design software, and extracted to CorelDRAW. The microfluidic channel is a 6x3.2cm device that consists of a top 9984 transparency film followed by a flow channel made by laser cutting a thick layer of Double-Sided Adhesive (DSA 467MP) stacked with Transparency film and another layer of DSA 467MP. The flow channel is 1.6cm long, 2.5mm wide, and 0.2mm thick. At the end of each flow channel, a 3mmx3mm glass fiber is placed that acts as a pre-treatment zone. The pre-treatment zones serve three primary purposes: 1. It helps to break the surface tension at the capillary microfluidics and paper interface. 2. The sample is pretreated here to mask the possible interferences from non-targeted ions, and 3. It helps to guide the sample flow into the detection zone. The top transparent layer has a 1cmx1cm sample insertion zone through which the analyte solution enters the flow channel.

The paper device is a 5cm x 2.5cm device that consists of five folds of Whatman grade 1. The top three layers are detection pads, and the bottom two layers are waste pads. The top three layers are wax printed, leaving three 7mm detection zones in each layer, touching each other when folded. The device has control reservoirs so that any colored substances from the sample are not mistaken for the product. The wax-printed paper was then heated by placing it at the center of the hot plate at 160^o °C for 20 s. With this time, the wax flowed entirely into both sides of the paper, creating a complete hydrophobic

barrier, leaving only the detection zones through which the waste solution reaches the waste pad, as shown in Figure 1. With heating, the detection pad size shrunk to 6mm. The microfluidic channel was then attached to the top of the laminated paper-based device so that the pre-treatment pads touched the detection reservoir.

2.3.4 Colorimetric metal detection

Standards

To analyze any interference from non-targeted ions, seven other metallic and non-metallic ion solutions were used. For Ni, Cu, and Fe standards, i.e., Ni(II) Sulfate Hexahydrate, Cu(II) Sulfate Pentahydrate, and Fe(III) Chloride Hexahydrate were used, respectively. Each standard solution was prepared to a 1000ppm concentration in 18.2 MΩcm Millipore water.

Ni-Dimethylglyoxime (DMG) Assay. 1μL of 0.001g/mL glycerol was added to the detection reservoir before adding any reagent. Four aliquots of 0.5μL DMG were pipetted into the detection pad, followed by 0.5μL of Ammonium Hydroxide (pH 9.5). The pad was allowed to dry between each addition of a reagent. In the pre-treatment Zone, two 0.5μL aliquots of ammonium acetate (6.0M), followed by one 1μL NaF (0.5M), were added and dried. Finally, 400μL of the standard sample (NiSO₄·6H₂O) was added to the device through the sample insertion zone. The added reagents did not alter the detection pad's pH condition.

Cu-Bathocuproine assay: 1μL of 0.0007g/mL Polyacrylic acid was added to the detection reservoir before adding any reagent.⁵⁶ The detection pad was then added with two

aliquots of 1 μ L Hydroxylamine (0.1g/mL). 1 μ L NaCl/Acetic acid buffer (pH 4.5) was then added, followed by two 1 μ L aliquots of a solution containing 0.05g/mL bathocuproine with 0.04g/mL PEG-400 prepared in Dichloromethane (DCM). Finally, 400 μ L of the standard sample ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) was added to the device through the sample insertion zone. The detection pad was allowed to dry completely before adding each reagent.

Fe-Bathophenanthroline assay: 1 μ L of 0.0007g/mL Polyacrylic acid was added to the detection reservoir before adding any reagent. Two 1 μ L aliquots of Hydroxylamine (0.1g/mL) prepared in Acetate buffer (4.5pH, 6.3M) were added to the detection pad, followed by 1 μ L Bathophenanthroline (0.025g/mL) prepared in isopropanol (99%). The device was allowed to dry completely with the addition of each reagent. Finally, 400 μ L of the standard sample ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) was added to the device through the sample insertion zone.

2.3.5 Image analysis

After the analyte sample was run, the reaction time was optimized to be 20 min with clearly distinguishable colors for respective metals. Although there was no significant difference between the signal at 2 min and 20 min, the device was dried for 20 min to facilitate better imaging (Figure A6, Appendix A). The top part of the device (capillary microfluidic) is then taken off, and a transparent white 2X2 cm PVC foil is attached to it just above the detection zone. Then, photographs of paper-based devices were taken using a smartphone inside a 15x15x10cm lightbox. The pictures were then extracted in JPEG format into ImageJ, a free image analysis software from the National

Institutes of Health (NIH). The image was split into gray, blue, green, and red channels and inverted. The average grayscale intensity of the complex formed was measured by using a circular tool.

Meanwhile, the average grayscale intensity of white PVC foil was also measured with the exact measurement (Figure A1, Appendix A). Grayscale intensity relative to the white PVC area was calculated. This offset any inconsistency in lighting conditions as both the pad and PVC lighting conditions would change simultaneously. The circular area of interest was kept consistent between the background and the detection zone. Calibration curves were made for each channel (Relative Grayscale Vs Concentration), and the channel with the biggest slope was chosen for further analysis of the respective metal ions. Ni and Fe_{total} were calibrated under the green channel, and Cu_{total} under the blue channel (Figure A4, Appendix A).

2.4 Results and discussion

2.4.1 Device performance

The device performance was analyzed on its user-friendliness, multiplexing ability, flow rate, time for color formation, homogeneity of the complex formed, and drying time. Even though the colorimetric assay for each metal-ligand chemistry was entirely different and selective, it was possible to do a simultaneous analysis of three different metals while validating their performance. The best compromise between the volume and color intensity was made at 400 μ L. Above 400 μ L, the signal started to fade, and it took longer to dry the detection pad (Figure A5, Appendix A). The device can be dipped vertically into the water sources using capillarity to deliver the sample. We calculated the average time

for μ PAD to vertically soak 400 μ L of sample to be 75-80 s (Figure A8, Appendix A). Hence, untrained operators can use the device without needing a specific pipetting system. The pad can hold more than 400 μ L of water; however, limiting it to 400 μ L with dipping time helped the detection pad dry faster, attaining the maximum signal. The device, on the other hand, can be dipped for long enough that the paper pads are completely hydrated, and no more analyte samples can pass through. In this approach, users would not need to control the dipping time, but it may take longer for the detection pad to dry for a better signal.

2.4.2 Edge effect

Because an edge effect on colorimetric assays in paper-based microfluidics is common, it is necessary to modify the surface and adjust the chromogen concentration in PADs so that precipitates can be trapped uniformly in the detection reservoir. Using viscosity and ionic-based trapping by glycerol and polyacrylic acid (PAA), respectively, in the detection pad before inserting the reagents created more homogeneous precipitates. Glycerol prevented DMG from leaching to the waste pad to a significant extent due to its viscosity. Higher viscosity limits the mobility of the complex into the waste pad while producing uniform signals. However, with more volume (>400 μ L), the complex could be washed away. The use of polyacrylic acid was therefore adopted from our previous work on a Fe-Phenanthroline assay.²⁰ PAA was used for ionic trapping of Cu-Bc and Fe-Bph because it has low mobility on paper and can trap positive ions because it is anionic (Figure A10, Appendix A). PAA was not used for the Ni-DMG due to the higher pH requirement for that assay. Due to the application of the waste pad underneath the detection reservoir, the imaging can be done within a few minutes of running the analyte.

The pre-treated glass fibers dried with the masking reagents were easily compressible when stacked with a paper-based device. Stacking double-layered tape with transparency film for the flow channel helped to break the surface tension at the insertion zone easily, rather than using only a single layer of thicker adhesive (Figure A3, Appendix A). This could be because of the stacked transparency film, as its inner side is coated with hydrophilic polymers, allowing better adhesion between water molecules and the channel surface at the sample insertion zone. The best compromise between breaking surface tension at the insertion zone and the flow rate was made at 2.5mm channel width and the stacking mentioned above (Figure A2, Appendix A). The color formation was obtained within seconds of sample insertion, whereas previous paper-based channels would take 10s of minutes for the sample solution to reach the detection zone.¹⁹ All the reagents except Bathophenanthroline were colorless when placed in the detection zone. This made it easier to observe a color change against the white control at the lowest concentration possible. The samples used in the experiments were colorless water samples. However, in a colored analyte sample scenario, reservoirs on the top can be used as the control zones (Eq. 1). The volume of each reagent was optimized to attain the maximum color signal (Figure A7, Appendix A).

$$\text{Relative Intensity} = \frac{I(\text{reaction zone}) - I(\text{control zone})}{I(\text{reference zone})} \dots\dots\dots \text{Eq 1}$$

2.4.3 Colorimetric assays

Ni was detected using Chugaev's method of reacting DMG, a bidentate organic ligand with Nickel (Ni^{2+}) ions in the presence of basic ammonium solution to get pink-colored Nickel Dimethylglyoxime (NiDMG_2) complex.⁵⁷ The pH condition was optimized to be 9.5 at the detection pad. The concentrated ammonium hydroxide at the detection pad helped to maintain a stable pH. Calibration for the Ni concentration is shown in Figure 2. A calibration curve was made for the range of 1ppm to 1000ppm. Above 100ppm, the color intensity started to saturate, showing no or less change in color intensity. A linear range was established between 1-50ppm.

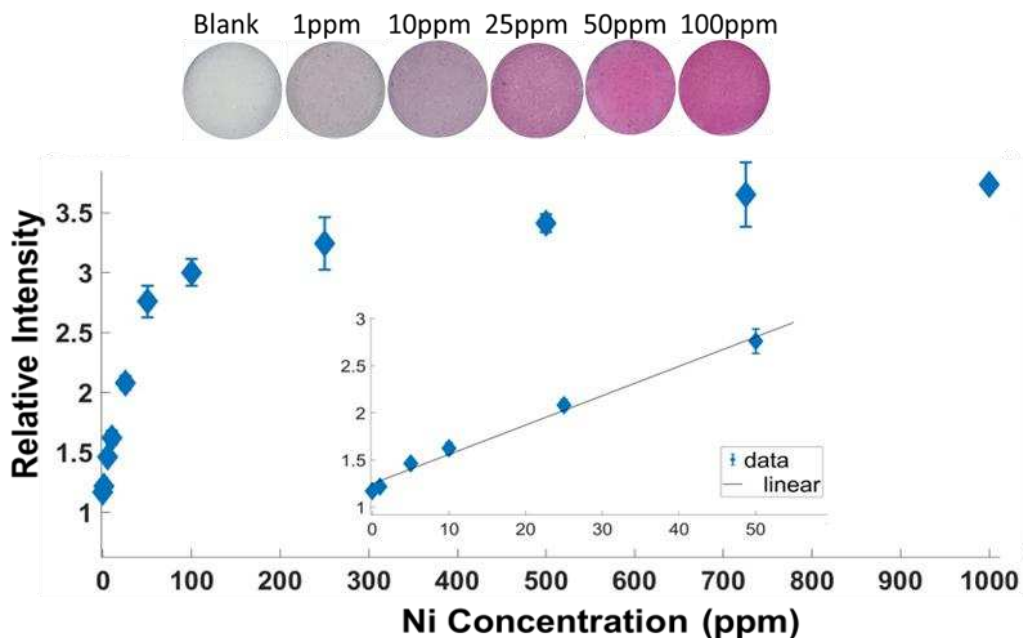


Figure 2. Qualitative Calibration showing representative results (Top), and Quantitative Calibration generated for 1-1000ppm ($n=6$) with a linear range of calibration from 1-50ppm ($R^2=0.99$). The relative grayscale intensity was calculated by dividing the grayscale intensity of the complex by the background control of the PVC sheet.

Cu_{total} was detected using a selective and sensitive Cu-Bathocuproine assay. Cu^+ , when reacted with Bathocuproine under acidic conditions (pH 4.5), gives an orange-colored complex. Bathocuproine does not form any color with Fe(II) , like other cuproine, making it selective for Cu detection.⁵⁸⁻⁵⁹ Hydroxylamine acts as a reducing agent to reduce Cu(II) into Cu(I) . The Cl^- in NaCl /acetic acid buffer stabilizes the cationic Cu-bathocuproine complex.⁶⁰ The bathocuproine made the paper device hydrophobic, so the sample solution could not pass through the waste pad. PEG-400 was mixed with reagent solution to make the surface more hydrophilic to overcome this problem. A quantitative calibration curve for Cu_{total} is shown in Figure 3. A calibration was generated from 1-1000ppm. Since the assay was very sensitive, a linear range was established for 0.1-5ppm. With this assay, Cu_{total} can be quantitatively detected to ppb levels.

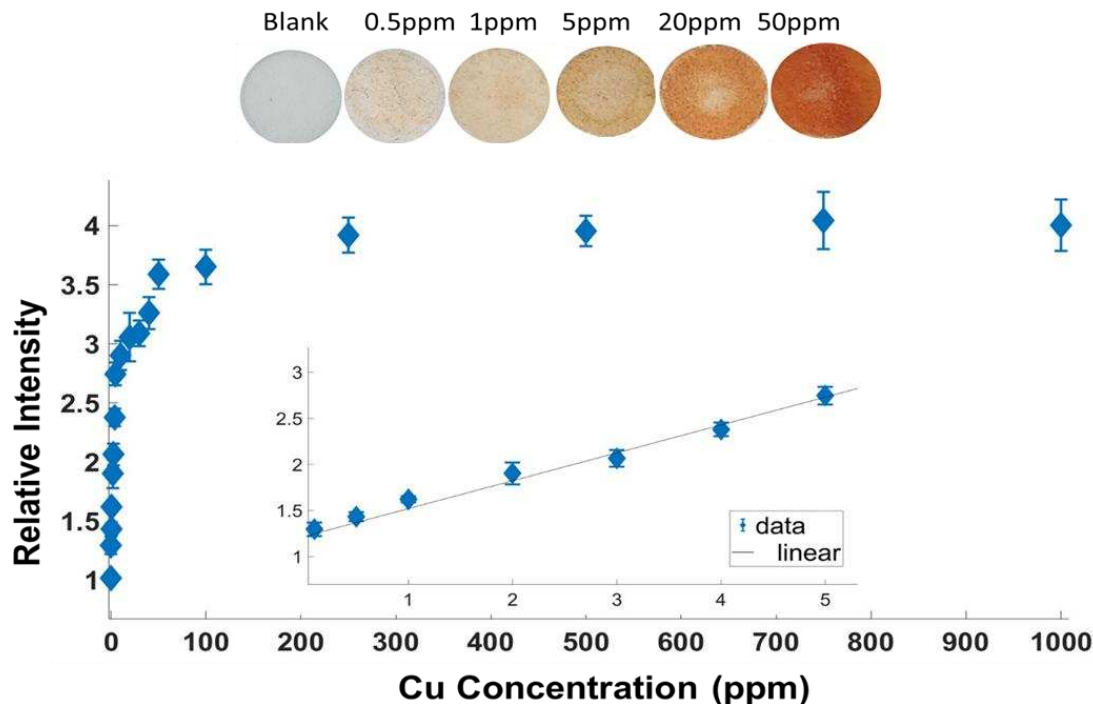


Figure 3. Qualitative Calibration (right) and Quantitative calibration were generated for 1-1000ppm (n=6), with a linear calibration range from 0.1-5ppm ($R^2=0.97$). The relative grayscale intensity was calculated by dividing the grayscale intensity of the complex by the background control of the PVC sheet (bottom).

Fe_{total} was detected using Bathophenanthroline. Bathophenanthroline reacts with Fe^{2+} under acidic conditions (pH 4.5), giving a red-colored complex. Bathophenanthroline was chosen instead of 1,10-Phenanthroline because it is more sensitive than phenanthroline for Fe_{total} detection and has significantly fewer interferences.⁶¹ In addition, the traditional phenanthroline complex is highly soluble and prone to a coffee-ring effect.²⁰ However, no such effect was observed for the bathophenanthroline complex because it is less soluble. A quantitative calibration curve for Fe_{total} is shown in Figure 4. A calibration was generated from 1-500ppm. A linear range was established for 0.5-15ppm.

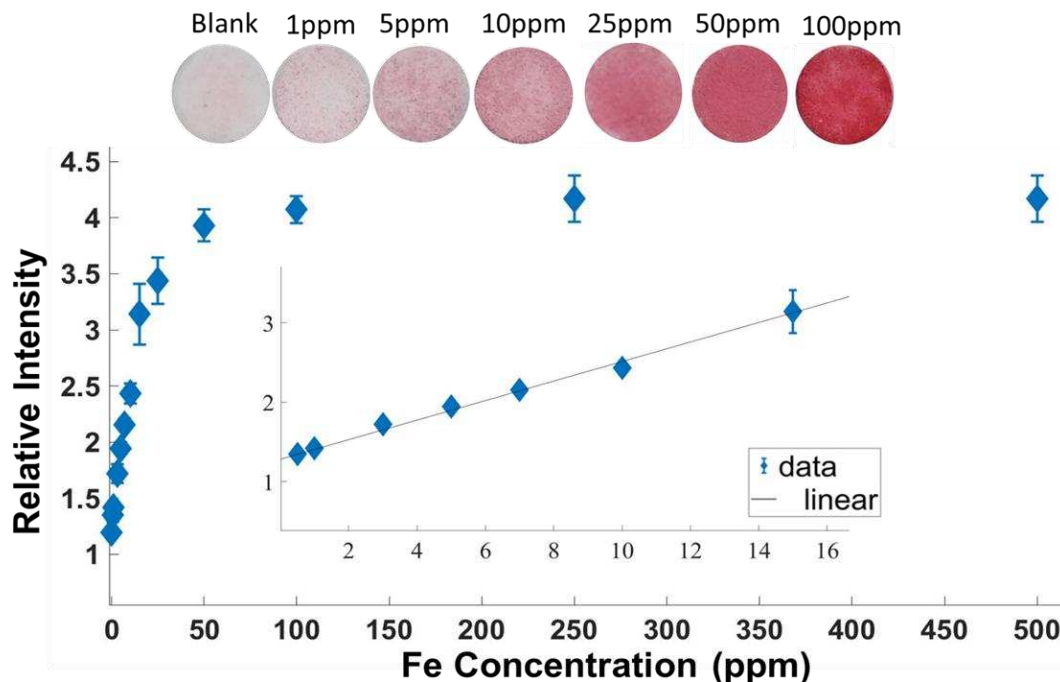


Figure 4. Qualitative Calibration (Top) and Quantitative calibration were generated for 1-500ppm ($n=6$), with a linear calibration range from 0.5-15ppm ($R^2=0.99$). The relative grayscale intensity was calculated by dividing the grayscale intensity of the complex by the background control of the PVC sheet (bottom)

2.4.4 Interference studies

To study the effect of interference of non-targeted ions on different assays. Standard solutions of Cd^{2+} , Zn^{2+} , Co^{2+} , Mg^{2+} , SO_4^{2-} , PO_4^{3-} , and HCO_3^- were prepared in Millipore water. The potential interference was studied at 1:1 and 1:10 ratios (targeted: non-targeted). In addition, interference from Fe(III) was studied for higher levels up to a 1:100 ratio, as most surface water sources are likely to have a higher level of Fe.

As previously reported, Fe, Cu, and Co can interfere with the Ni-DMG complex formation.²⁰⁻²¹ The pre-treatment zone was modified with sodium fluoride and ammonium acetate to address this issue.²⁰ To avoid the possible interferences on Cu-bathocuproine assay from Zn(II), Co(II), and Cd(II), the pre-treatment zone was added

with 0.5g/mL of hydroxylamine.³⁸ Cu(II) lowered the Fe(Bphe)₂ signal when mixed with Fe in a 1:10 ratio. To overcome this, the pre-treatment zone for Fe was treated with 0.5g/mL of hydroxylamine.⁶² For the Cu-bathocuproine and Fe-bathophenanthroline assays, none of the non-targeted ions showed any significant interference, as all the changes in the intensity were within $\pm 10\%$ of the original signal, as shown in Figure 5. For Ni, Cu, and higher Fe mixing (1:100) changed the signal by slightly more than 10%.

2.4.5 Detection limits and stability

The limits of detection (LoD) and quantification (LoQ) were calculated to be 2 ppm and 7 ppm for Ni, 0.3 ppm and 1 ppm for Cu_{total}, and 1 ppm and 4 ppm for Fe_{total}, respectively. LoD was calculated using 3.3 times the standard deviation (SD) of the blank divided by the slope of the calibration curve. The LoQ was quantified using ten times the SD of the blank divided by the slope of the calibration curve (n=9 for both). A clear visual signal was attained at 0.5 ppm for Fe_{total}, 0.1 ppm for Cu_{total}, and 1 ppm for Ni(II). The stability of devices was also assessed over time. The effects of storing the devices in cold storage (5°C) versus room temperature (22°C) were compared. All the detection and sample insertion zones were sealed before storing in the respective conditions to avoid cross-contamination. Ni-DMG and Cu(Bc)₂ device assays showed a stable signal for four weeks of study (n=3). In comparison, Fe-Bph was more stable under cold storage conditions (Figure A9, Appendix A). The stability findings suggest that the device is stable for a reasonable timeframe for batch manufacturing, sealed packaging, and delivery to end users. The edge effect was not observed even after 4 weeks of storage under both conditions.

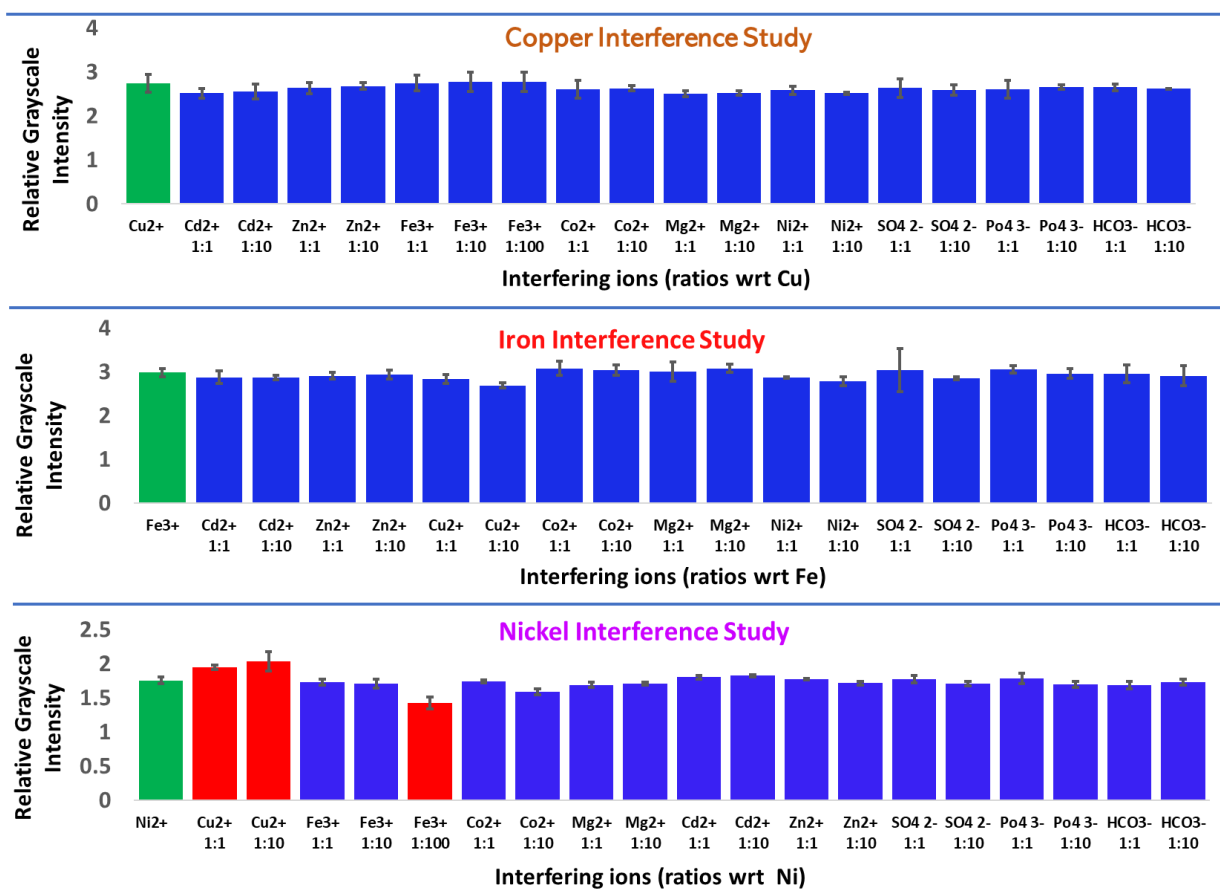


Figure 5. Interference study for all three assays for 1:1 and 1:10 molar ratios (targeted: non-targeted). Blue bars indicate a less than 10% change from the original intensity(green). Red bars indicate more than 10% signal change.

2.4.6 Application in real water samples

To validate the device reproducibility, three different concentrations of each analyte were spiked in a commercial drinking water sample (King Soopers) and a water sample from the Poudre River (GPS coordinates 40.5704685, -105.0821559). For commercial drinking water samples, recovery was determined to be 80-90% for Fe_{total}, 92-109% for Ni, and 85-106% for Cu_{total}. For the collected river sample, the recovery was 62-83% for Fe_{total}, 83-102% for Ni, and 80-124% for Cu_{total} (Table 1).

Table 1. Analyte recovery performance analysis by spiking with real samples (n=3).

	Metal	Spiked (ppm)	Recovered (ppm)	Recovery %	RSD %
<i>Drinking Water</i> 	Ni(II)	—	ND	—	—
		30	28.58±1.92	95.29	6.73
		20	18.50±1.61	92.51	8.69
	Cu_{total}	10	10.91±1.85	109.12	5.93
		—	ND	—	—
		5	5.30±0.54	106.04	10.28
	Fe_{total}	3	2.57±0.06	85.74	2.66
		1	0.91±0.07	90.54	10.40
		—	ND	—	—
		12	10.34±0.86	86.20	8.29
		8	6.46±0.77	80.69	11.92
		4	3.60±0.03	90.12	0.96
<i>River Water</i> 	Ni(II)	—	ND	—	—
		30	29.61±4.33	98.71	14.61
		20	16.70±2.20	83.48	13.20
	Cu_{total}	10	10.21±0.91	102.06	8.92
		—	ND	—	—
		5	4.34±0.64	86.72	14.82
	Fe_{total}	3	2.40±0.09	80.11	3.75
		1	1.25±0.05	124.77	3.93
		—	ND	—	—
		12	9.72±0.91	81.03	9.38
		8	6.64±0.65	83.07	9.76
		4	2.49±0.12	62.17	4.83

2.5 Conclusion

The capillary-driven microfluidic system, combined with a paper-based device developed in this study, provides a fast, user-friendly, and economical way to assess our exposure to heavy metals in water. Using capillary-driven microfluidics as a transport medium reduced the flow rate significantly, hence producing the color signal in a dramatically reduced time. As most of the current paper-based platforms take longer run times and multiple pipetting steps, using this device, the heavy metal contaminants in water can be detected almost immediately without needing to pipette the analyte onto assay cards. Running an adequate volume of analyte samples allowed the metal detection to reach levels near or below the maximum contaminant limits in drinking water established by

governing bodies like the EPA and the WHO.⁶³⁻⁶⁴ In addition, the Cu-bathocuproine assay on this device can give the signal well below the allowed limit. This device can be a promising, cheap alternative to be used by untrained users across countries with high heavy metal exposure, with limited available resources. Assay conditions can be optimized depending on the analyte of interest by eliminating the other possible interferences. Although simultaneous detection of only Ni, Cu_{total}, and Fe_{total} is studied here, the device can be utilized in water quality monitoring for detecting other heavy metals and other organic contaminants as well.

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CHAPTER 3 — Fill, Fold, Photo: Preconcentration and Multiplex Detection of Trace Level Heavy Metals in Water

3. 1 Chapter overview

The goal of this chapter is to address the sensitivity limitations of rapid, point-of-need colorimetric sensing platforms by integrating a low-cost preconcentration strategy with capillary-driven microfluidic transport. While Chapter 2 established that capillary-driven architectures can dramatically reduce assay time and eliminate the need for precise volume handling, detection limits remained constrained by the mass of analyte delivered to the sensing zone. This chapter builds upon that foundation by introducing a scalable, field-deployable preconcentration system that enables multiplexed detection of trace-level heavy metals at environmentally relevant concentrations.

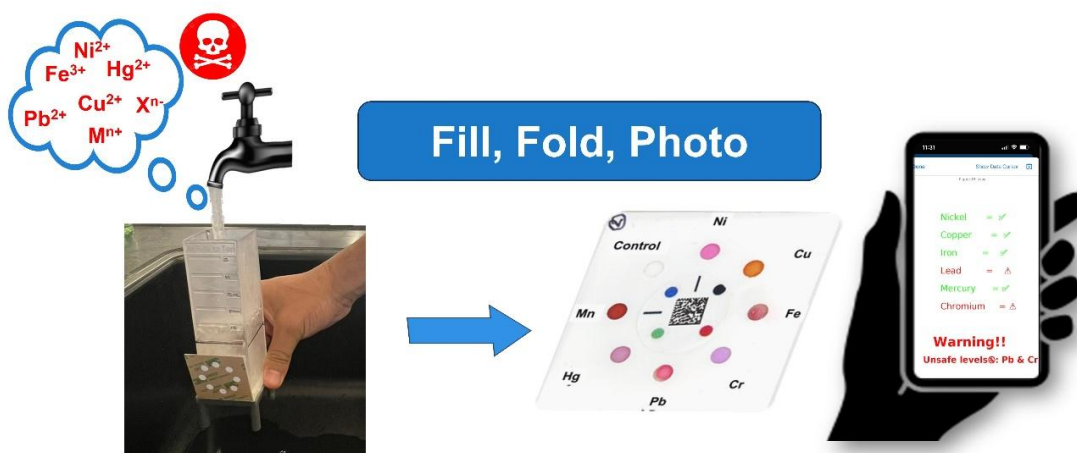
The work presented in this chapter was conceptualized and executed by the author under the supervision of Prof. Charles S. Henry, with collaboration from Eric Brack and Todd Alexander at the U.S. Army DEVCOM Soldier Center. Jason Boes contributed to the initial mechanical design of the preconcentration tank; following this contribution, I led all subsequent aspects of the research, including assay development, experimental execution, data curation, analytical validation, and manuscript preparation. This work was published as a first-author peer-reviewed article (*ACS Sens.* 2024, 9, 10, 5479–5488).

In this chapter, a gravity-driven preconcentration system is coupled with a paper-based colorimetric reagent card to enable simultaneous detection of seven metal ions—Ni(II), Cu_{total}, Fe_{total}, Cr_{total}, Pb(II), Hg(II), and Mn(II)—at low parts-per-billion levels. The

platform processes large sample volumes without pumps or external power and employs a Fill, Fold, Photo workflow that eliminates complex pretreatment steps and minimizes user intervention. Selectivity is achieved using targeted masking strategies, while smartphone-based image analysis enables rapid, portable quantification under field conditions.

Beyond demonstrating improved analytical sensitivity, this chapter highlights how capillary-driven microfluidics and passive preconcentration can be combined without sacrificing simplicity or field usability. Validation against ICP-MS and testing with environmental water samples confirm that the platform achieves regulatory-relevant performance with acceptable accuracy and precision. Collectively, the results presented here demonstrate a practical pathway for extending capillary-driven microfluidic systems to trace-level, multiplex environmental monitoring and motivate their broader application to other contaminant classes explored in subsequent chapters.

Chapter Graphical Summary



3. 2 Introduction

Water pollution is an increasing global concern that poses significant threats to both human health and the environment. For the past decade, the World Economic Forum has consistently ranked the degradation of water resources among the top ten most critical global risks.¹ Worldwide, 80% of the wastewater remains untreated, carrying a mixture of household waste and harmful industrial pollutants.² These contaminants disrupt the delicate ecosystem balances and cause widespread environmental damage.³ Exposure to these environmental toxins contributes to a significant portion (almost 1/4th) of the global disease burden with 50% of global child death attributed to poor water quality.^{4, 5}

Among different classes of water contaminants, heavy metals have garnered significant attention across various sectors due to their detrimental effects on human health and ecological balance.³ While heavy metals can naturally occur in the environment, anthropogenic activities are the predominant source of water contamination.^{3, 6} The inherent properties of heavy metals, including their water solubility, resistance to biological degradation, and high bioavailability, contribute to their persistent presence in the environment.⁵ Heavy metal toxicity can result in various health issues, including gastrointestinal and renal disorders, neurological disorders, dermatological issues, vascular harm, impaired immune system function, congenital disabilities, and malignancy.⁷⁻⁹ Despite existing regulations, many households and public water systems across the United States continue to have elevated levels of heavy metals present in their water.¹⁰⁻¹³ Numerous studies have shown that drinking water in the United States can contain toxic metals such as Pb(II), Cu(II), and As(III&V), posing significant health risks.¹⁴⁻¹⁷ The situation is even more dire in underdeveloped countries,

where regulatory frameworks and resources for water quality management are further limited. The lack of accessible, affordable, and user-friendly water testing systems in these resource-limited settings further compounds this problem.^{13, 18-20} Traditional techniques for quantifying heavy metals include Inductive Coupled Plasma Mass Spectrometry (ICP-MS) and Optical Emission Spectrometry (ICP-OES), as well as Atomic Absorption Spectrometry (AAS).²¹⁻²³ These techniques offer notable advantages in sensitivity, specificity, and precision, especially when measuring low concentrations. Molecular spectroscopy methods, such as UV/Vis molecular absorption and fluorescence spectroscopy, provide indirect approaches for detecting heavy metals.²⁴ Other techniques include electrochemical and chromatographic methods, which have proven valuable in the realm of heavy metal detection.²⁵⁻³⁰ Despite the wide range of techniques available for heavy metal analysis, these methods come with limitations, including time-consuming processes, the need for costly equipment, and skilled operators. As a result, the practical considerations of conducting point-of-need testing in resource-limited and economically disadvantaged areas become challenging, if not impossible.

The emergence of paper-based analytical devices (PADs) has played a significant role in bridging the gap between contaminant testing and end-users. These platforms enable point-of-use analysis with their affordability, diverse designs, and compact size, making them highly portable.³¹⁻³⁴ Common chromogenic agents such as Dimethylglyoxime, Bathocuproine, Bathophenanthroline, Phenanthroline, Diphenyl Carbazone, Rhodizonic acid, Dithizone, 4-(2-pyridylazo) resorcinol (par), Ferrozine, Xylenol Orange, Chrome azurol S, natural dyes etc. are employed for the colorimetric detection of different heavy metals in water.³⁵⁻³⁹

While various paper-based analytical systems have utilized these reagents to detect heavy metals in water, most face challenges in achieving regulated detection limits.³³ Developing a sensor capable of detecting metals at levels compliant with relevant legislative guidelines, typically in parts per billion (ppb), is necessary to execute a system suitable for monitoring water contamination. Therefore, developing a simple, cost-effective, and user-friendly technique for heavy metal monitoring is crucial, particularly for resource-limited locations. The technique should prioritize ease of use and widespread accessibility while ensuring it adheres to established legislative guidelines for accuracy and reliability.

A preconcentration technique can be used to detect the analytes at very low concentrations.^{40, 41} In a preconcentration system, the capture and concentration of target analyte onto a preconcentration medium results in accumulating these analytes in a confined area. Concentrating the analytes increases the overall available mass of the target elements, leading to an increased color signal from the targeted analyte. Some methods have been proposed for preconcentration systems for colorimetric heavy metals detection. Satarpai et al. devised a cost-effective and straightforward method utilizing a PAD modified with Zirconium Silicate for the on-site sampling, preconcentration, and analysis of Pb(II) in water samples.⁴² Ninwong et al. developed a sensitive distance-based method for quantifying mercury ions using fluorescent distance-based paper device coupled with an evaporating preconcentration system.⁴³ Suarez et al. developed a preconcentration system that uses a chitosan and superabsorbent pads to preconcentrate and detect Co(II) in water.⁴⁴ It is crucial to highlight that non-substrate-modified paper-based multiplexed systems for heavy metal detection often lack the sensitivity to attain environmentally relevant Limits of Detection (LoD). In contrast, preconcentration

systems achieving the necessary LoD are focused on single heavy metal detection or require extensive capture substrate modification. Therefore, the challenge lies in developing a method that overcomes the sensitivity limitations while also allowing for multiplexed detection, ensuring a more comprehensive and practical solution for environmental monitoring.

To overcome the aforementioned shortcomings, we developed a 3D-printed preconcentration system combined with colorimetric paper-based detection, enabling the simultaneous detection of seven different metal ions at low ppb levels in water. The proposed system can process up to 250 mL of water to concentrate heavy metals on Ahlstrom Blotting Grade 319 (AB319) paper. Out of the seven metals analyzed, the system achieved a LoD well below the legislative guideline values for six metal ions without any paper substrate modification. Our smartphone-enabled system allows for on-site quantification of the color signals. Various masking strategies were implemented to attain a higher level of selectivity for the targeted metals with minimal interference. The sensor demonstrates stability suitable for batch manufacturing and timely delivery to end-users. Real water samples were tested to validate the sensor's effectiveness, and the results were benchmarked against industry-standard inductively coupled plasma mass spectrometry (ICP-MS) data. Spike recovery analysis was also used to further confirm the sensor's accuracy.

3.3 Experimental section

3.3.1 Materials and chemicals

All metal standards, reagents, and masking agents were obtained from Sigma-Aldrich (St. Louis, MO, USA), while hydroxylamine hydrochloride and buffer components were sourced from Fisher Scientific (Waltham, MA, USA). Double-sided adhesive (DSA, 468MP) and transparency film (9984) were purchased from 3M (St. Paul, MN, USA), and Whatman paper was obtained from Fisher Scientific (Waltham, MA, USA). Additionally, PVC foil (0.7 mm) was purchased from Deecraft company in Wang Thonglang-Bangkok, Thailand. The resin for stereolithography was purchased from Formlabs.

3.3.2 Instrumentation

The adhesive tapes, PVC sheets, paper circles, glass fiber (GF) circles, and transparency films were cut using an Epilog Engraver WinX64 Zing laser cutter. The preconcentration tank was printed using a Formlabs Form 3 3D Printer. Method validation was performed using Perkin Elmer NexION 350D ICP-MS.

3.3.3 Device design and fabrication

The reagent card was fabricated by bonding a white PVC sheet and DSA. The dimensions of the reagent card are 10 cm x 5 cm. Using the laser cutter, eight 4 mm (diameter) circular holes are cut on the left side of PVC and DSA. Between these holes, eight 6 mm AB 319 grade paper circles were inserted within the layers. On the right side of the DSA, eight 6 mm circular holes were laser cut. Eight 8 mm circular glass fibers were placed between the layers in this region, as illustrated in Figure 1A. To ensure controlled water flow and filtration exclusively through the detection circles, the left side of the reagent card is attached with a 6 mm wide inner DSA lining. This design element acts as a barrier,

preventing lateral water leakage and guaranteeing that the water sample flows vertically through the intended detection areas. The reagent card features a laser-cut perforated fold line running precisely through its center. This fold line allows the left and right sides of the card to be folded together, enabling the delivery of the colorimetric substrates for analysis. The preconcentration tank measures 4.2 cm × 4.2 cm × 14.2 cm. The tank is connected to the card station using a hinge. Please see Figure B1 (Appendix B) for detailed schematic on 3D printed preconcentration tank. To operate, the top DSA lining is first peeled off, and the preconcentration zone inserted into the card station, followed by closing the tank. Next, the tank was filled with the sample, and vertical filtration was allowed until the water reached the 150 mL mark. Once the water reached the 150 mL mark, the excess water was drained, the card was removed, the back DSA layer was peeled off, and the card was folded along the perforation line to get the colorimetric signal (Figure 1B).

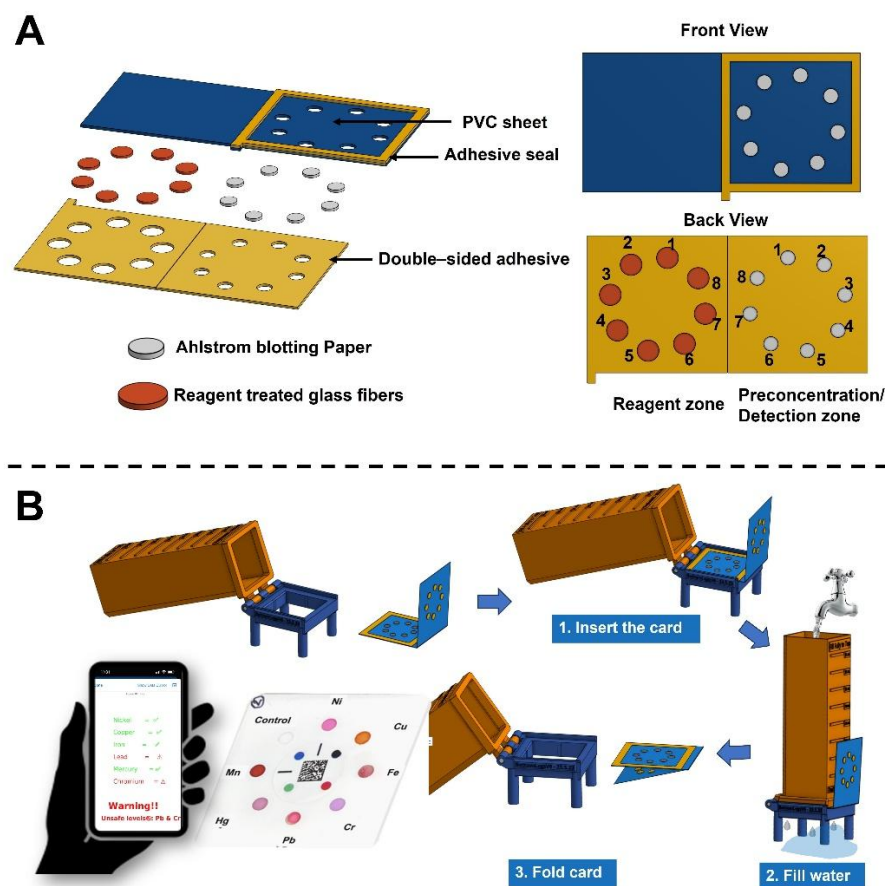


Figure 1. Device schematic and running. (A) Lateral view schematic of reagent card (left), front and back view of the reagent card (right). The detection paper (AB 319) and glass fibers were attached to the left and right side of the reagent card respectively by laser cutting holes into the PVC sheet and Double-Sided Adhesive (DSA). (B) Device running. The reagent card is inserted into the preconcentration tank using adhesive lining. Subsequently, water is added to the tank until it reaches full capacity. The tank is then allowed to filter until it reaches the 150 mL mark. Following this, the adhesive on the backside is peeled off, and the card is folded to observe the color signal.

3.3.4 Colorimetric metal detection

Standards. Metal standards, including Nickel(II) sulfate hexahydrate, Copper(II) sulfate pentahydrate, Iron(III) chloride hexahydrate, Potassium Dichromate (VI), Mercury(II) Chloride, Lead(II) nitrate, and Manganese(II) Sulfate, were prepared at a concentration of 1000 ppm in $18.2 \text{ M}\Omega \text{ cm}^{-1}$ Millipore water. Additionally, 15 solutions containing other

non-targeted metallic and non-metallic ions were prepared to analyze potential interferences.

Assay Card Preparation. To prepare the assay card, reagent and pretreatment solutions were added to the paper preconcentration pads & the glass fiber reagent pads. All the respective concentrations for each assay is outlined in Table B1, Appendix B. After each reagent addition, the card was left to dry completely at room temperature. Post-fabrication, cards were stored as indicated in packaging protocols, below.

Image analysis. After the optimized reaction time, the MATLAB Phone app captured images under controlled lighting. The code automates analysis: it identifies the circular region of interest (ROI), creates a mask to isolate it, calculates its average gray value, and normalizes it using a background ROI. The normalization step in the code accounts for variations in lighting conditions during image capture, enhancing the method's applicability in field settings. Additionally, it analyzes grayscale intensity in specific color channels for optimal sensitivity to different analytes. This process runs in parallel for all ROIs. Ni(II), Fe_{total}, Cr_{total}, Pb(II), Hg(II), and Mn(II) were calibrated in the green channel, and Cu_{total} in the blue channel (see Appendix B, Figure B2). Since the sensor is primarily designed for drinking water systems, the samples used in this study are colorless water samples. However, if the water already exhibits some color, the 8th circle can be utilized as the control zone (similar to the approach in chapter 2). As shown in equation 1, this approach will address both lighting inconsistencies and variations in analyte color.

$$\text{Relative Intensity} = \frac{I(\text{reaction zone}) - I(\text{control zone})}{I(\text{reference zone})} \dots \text{Eq 145}$$

3.4 Results and discussion

3.4.1 Optimization and device performance

Paper substrate optimization. To maximize ionic retention, it is crucial to use an optimal paper substrate that effectively captures the metal ions and exhibits a sensitive color intensity when the paper is folded. We examined various paper types (Whatman 1, 3, and 4, Ahlstrom Blotting grade 319), revealing that the highest color intensity was observed with AB 319 grade paper (see Appendix B, Figure B3). Although we also tested other Whatman papers (Grade 5 and Grade 6) with smaller pore sizes, the filtration time was excessively long. As a result, the AB 319 grade paper emerged as the best compromise between color intensity and filtration time.

We hypothesize that the metal ion trapping ability of Ahlstrom AB 319 paper can be attributed to its unique combination of thickness, structure, surface area, tortuosity, and composition. The increased thickness of AB 319 (483 μm) compared to W1 (180 μm) and W4 (210 μm) provides a longer flow path, increasing the likelihood of metal ions colliding with the paper fibers, promoting adsorption. Additionally, the denser structure and greater thickness offer a larger surface area for adsorption and introduce more tortuous paths that metal ions must navigate. The increased tortuosity hinders the rapid movement of metal ions, thereby enhancing their interactions with the fibers and facilitating their retention. Furthermore, the proprietary nature of blotting paper manufacturing suggests the possible presence of undisclosed functional groups that may exhibit a specific affinity for metal ions, forming complexes and contributing significantly to the observed improvement in metal ion trapping during filtration.

Metal ion volume optimization. The sample volume was optimized to process the maximum volume while maintaining a consistent flow rate. The preconcentration tank is

designed to hold 250 mL of water. Filtration is facilitated solely by the force of gravity, eliminating the need for an external pump. We observed that the first 150 mL of water flows within a duration of 11.77 ± 1.23 minutes ($n=5$). However, filtering the remaining volume would take nearly an hour due to a decrease in water pressure for filtration as the liquid height decreases. Consequently, we halted the process after filtering 150 mL of sample. The excess water is discarded, and the reagent card is removed from the preconcentration tank and folded. In instances where the treated preconcentration pads exhibited hydrophobic properties (in the case of Cu-Bc and Hg-Dtz assay), the reagents were mixed with PEG-400 to make these regions hydrophilic. This modification was implemented to guarantee that the flow rate/filtration rate remained consistent across all detection pads (see Appendix B, Figure B4).

Signal Homogeneity. Because most reagents were immobilized on the glass fibers, establishing a tight seal between the detection pads and fibers was crucial. In this configuration, the thicker paper fibers and active evaporation upstream resulted in convective mass transport, causing the color formation to migrate up toward the surface of the detection pad. Glass fibers were chosen over paper for reagent immobilization due to their higher surface energy. Their higher surface energy facilitates improved wetting, adhesion, and allows for the easy release of reagents into solution.

Reagent optimization. The concentration of reagents for each assay was optimized to ensure reaction was not limited by the reagent concentration. Reagent optimization figures can be found in Figures B5 through B11 in Appendix B. The selected optimum concentrations for the reagents are as follows: 60 mM for Ni-DMG, 8.5 mg/mL for Cu-Bc, 1.25 mg/mL for Fe-Bph, 15 mg/mL for Cr-DPC, 0.625 mg/mL for Pb-Rh, 12 μ g/mL for Hg-Dtz, and 5 mM for Mn-PAR assay.

Reaction time. Multiplexed detection involving seven metal ions necessitates consideration of varying reaction kinetics for each complexation chemistry. While most reactions reached peak intensity within 3 minutes after folding, Mn-PAR and Cr-DPC complexations required an 8-minute reaction period. To ensure uniform performance across all assays and facilitate better imaging, 8 minutes post-folding was chosen as the optimal reaction time. Figure B12 in Appendix B demonstrates this optimization, where signal intensities stabilize after 8 minutes for each assay.

Metals retention. To assess the system's ability to capture a specific amount of metal at various concentrations consistently, tests were conducted at low, medium, and high concentrations for each metal under neutral pH (with $n=5$ devices for each concentration). Significant differences in color signals were found among the tested gradients. Moreover, the RSD for all examined points remained below 10%, as shown in Appendix B, Figure B13. This indicates a reliable and consistent metal retention across the concentrations. The analyte retention rate was assessed by comparing the signal obtained from the sensor with the signal obtained from a direct application of the same amount of mass onto the paper substrate (spot test). The analyte retention rate was calculated to be $25.8 \pm 3.2\%$.

To study the effect of pH on metal retention, Ni(II) ion solutions were processed through the sensor at various pH levels. After filtration, pH was adjusted directly on the paper. No significant effect on metal retention was observed between pH 1 and 7. However, at pH 9, the metal retention decreased by approximately 5% (see Appendix B, Figure B14). While most natural waters containing metals are either acidic or neutral, the presence of non-dissolved metal species at higher pH levels may occasionally affect metal retention. Our study focused exclusively on ionic species due to the detection chemistries

employed. In real-world applications, where we anticipate the system's use after acid digestion, the presence of non-ionic species should not be a concern.

3.4.2 Calibration

Ni-DMG assay. Ni(II) was detected by Chugaev's method, which involves reacting dimethylglyoxime (DMG) with nickel (Ni^{2+}) ions under basic conditions, resulting in the formation of a pink-colored nickel dimethylglyoxime (NiDMG_2) complex.⁴⁶ The pH was adjusted using an ammonium hydroxide/ammonium chloride buffer at the glass fiber. The calibration for Ni concentration is illustrated in Figure 2A, with a calibration curve established for the range of 20–400 ppb. Beyond 400 ppb, the color intensity exhibited non-linearity due to surface saturation. The surface saturation is common in colorimetric assays.⁴⁷⁻⁴⁹ The LoD and LoQ were determined to be 15 ppb and 50 ppb, respectively. These values were calculated using the equations $(3s)/b$ and $10s/b$, where "s" is the standard deviation of the blank ($n = 8$), and "b" is the slope of the calibration curve.⁵⁰ Importantly, the LoD is well below the guideline limit for Ni(II) in water.⁵¹

Cu-Bc Assay. Cu_{total} was detected using a Cu–bathocuproine assay known for its selectivity and sensitivity.⁵² In this method, Cu^+ reacts with Bathocuproine under acidic conditions, forming an orange-colored complex. Bathocuproine exhibits selectivity for Cu detection as it does not produce any color with Fe(II), unlike other cuproine compounds.^{52, 53} The reduction of Cu(II) to Cu(I) is done by using hydroxylamine as a reducing agent. The presence of Cl^- in the NaCl/acetic acid buffer helps stabilize the cationic Cu–Bc complex.⁵⁴ The hydrophobic nature of the Cu-Bc complex hindered its movement upstream for color formation. Therefore, the reagent was treated directly within the detection pads. However, this adjustment led to an extended filter time

exceeding 30 minutes. To enhance hydrophobicity and maintain an efficient flow rate, an optimized solution containing 0.12g/mL PEG-400 was mixed with the reagent. Consequently, the flow and filtering rates matched those achieved with other detection pads. The quantitative calibration curve for Cu_{total} is depicted in Figure 2B, showcasing a linear calibration range from 12.5 to 200 ppb. LoD and LoQ were determined to be 7 ppb and 24 ppb, respectively. The LoD falls significantly below the guideline value for Cu_{total} in water.⁵¹

Fe-Bph Assay. Fe_{total} detection was performed using bathophenanthroline, a compound that reacts with Fe^{2+} under acidic conditions, forming a red-colored complex. Hydroxylamine served as the chosen reducing agent to convert Fe(III) to Fe(II) in an acetate buffer. A quantitative calibration curve for Fe_{total} is shown in Figure 2C. A log-linear range was established specifically for 5 to 80 ppb. The LoD and LoQ were determined to be 2 ppb and 9 ppb, respectively. These values significantly fall below the established guideline limits for Fe_{total} in water.⁵¹

Cr-DPC Assay. Cr_{total} was detected through the complexation reaction of Cr(VI) with diphenylcarbazide (DPC), forming a purple-colored complex on paper.⁵⁵ A phthalic anhydride, polydiallyldimethylammonium chloride (PDDA), and DPC mixture was prepared in acetone. PDDA and phthalic anhydride were added to stabilize the reaction product between Cr and 1,5-DPC and prevent the edge effect, while phthalic anhydride stabilized 1,5-DPC on the glass fibers.^{55, 56} Figure 2D illustrates the calibration of the Cr-DPC assay on the proposed sensor. A calibration curve was established within the linear range of 50 to 500 ppb. The LoD and LoQ were calculated to be 20 ppb and 67 ppb, respectively. These values are well below the legislative guideline for Cr_{total} in water, which is set at 100 ppb.⁵¹

Lead-Rh assay. Lead metal detection was achieved utilizing sodium rhodizonate as a complexation agent, where the chemical reaction induces a color transition from colorless to red in acidic conditions.^{42, 57} A 10 mM tartrate buffer solution was used to enhance the stability of the complexation reagent.⁵⁸ Figure 2E includes a linear curve established within 25 to 150 ppb range for the Lead-Rh assay. The calculated LoD and LoQ were determined to be 13 ppb and 43 ppb, respectively. Importantly, the LoD falls below the EPA action limit for lead in drinking water.⁵⁹

Mercury-Dtz assay. Dithizone (DTZ) and Hg(II) readily form a stable complex with a high formation constant ($\log K_f$, $\text{Hg}-(\text{DTZ})_2 = 40.3$),⁶⁰ evident in the dramatic color shift from yellow to reddish-purple.^{61, 62} Similar to challenges encountered in the Cu-Bc assay with glass fibers, treating the reagents directly on paper resulted in color formation in glass fibers rather than the paper itself. Like Cu-Bc assay, all the reagents were added in the detection zone itself for this assay to overcome this issue. Similarly, the paper's hydrophilicity was fine-tuned by mixing 0.08 g/mL PEG-400 with the reagent, optimizing both flow rate and filtering efficiency. Figure 2F illustrates the calibration results for this assay. A linear calibration range was established within the range of 50 ppb to 300 ppb. LoD and LoQ were calculated to be 26 ppb and 89 ppb, respectively. While the LoD in this assay is lower than that of many multiplexed platforms, it still exceeds the legislative guidelines, which are established at 2 ppb.⁵¹ Ongoing and future studies include exploring additional strategies for paper modification as part of efforts to enhance the sensor's sensitivity and bring it within the desired cutoff range.

Mn-PAR assay. 4-(2-Pyridylazo) resorcinol (PAR) is a commonly employed analytical reagent for heavy metal analysis.^{35, 38} PAR exhibits the ability to form red-colored complexes with various metal ions. This versatility makes PAR a highly used colorimetric

reagent, particularly for Mn(II), Ni(II), Zn(II), and Pb(II) detection under basic conditions.⁶³ In prior work, we have successfully developed a strategy for masking interference against the Mn-PAR assay, resulting in a high degree of selectivity.³⁸ In this colorimetric reaction, the observed color change progresses from yellow to red. Borate buffer was used to stabilize the pH, and Polydiallyldimethylammonium Chloride (PDADMAC) was added to the reagent to ensure the stability of the Mn-PAR complex. Figure 2G presents the calibration results for the Mn-PAR assay. A linear calibration range was defined from 25 ppb to 150 ppb. The LoD and LoQ were calculated to be 15 ppb and 49 ppb, respectively. Importantly, these values fall well below the legislative limit for Mn(II) in water.⁵¹

Table 1 presents a comparative analysis of our Fill, Fold, Photo system with previously documented μ PADs in the literature, focusing on their capability for the multiplexed colorimetric determination of metal ions. Our system stands out in two key aspects: first, it can detect up to seven metal ions simultaneously without substrate modification, surpassing the capabilities of previously reported designs. Second, our limits of detection fall within the lower ppb range, adhering to legislative requirements.

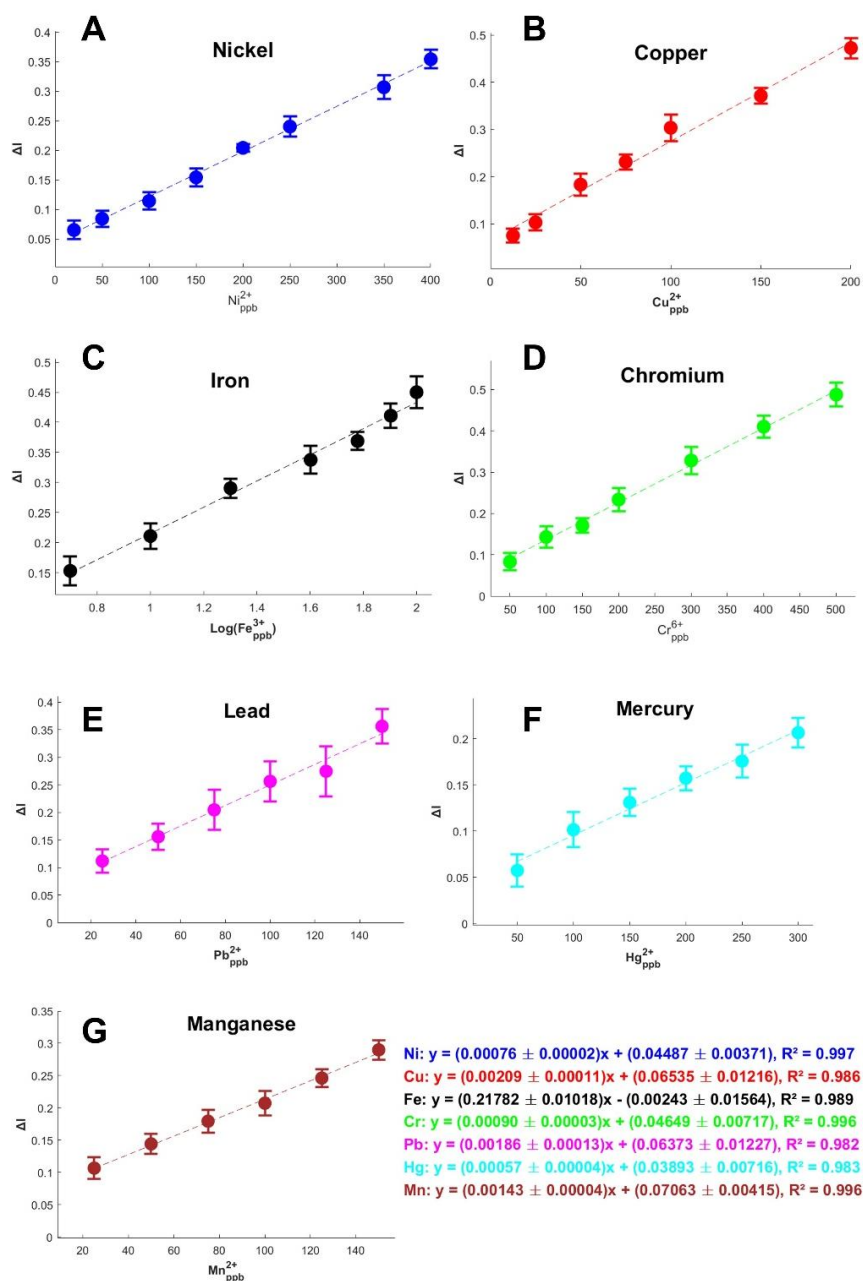


Figure 2. Calibration for Ni-DMG (A), Cu-Bc (B), Fe-Bph (C), Cr-DPC (D), Pb-Rh (E), Hg-Dtz (F), and Mn-PAR (G) assays on the developed sensor (n=8).

3.4.3 Interference Study

We implemented multiple masking approaches to achieve selectivity across various assays on the device. This involved strategically applying masking reagents directly on the

detection pads themselves or adding them to the glass fibers. The masking process was optimized to establish the highest possible tolerance limit without adversely impacting the vertical flow time and cross-reactivity among the reagents. To determine this tolerance limit, we conducted testing at analyte: interferent ratios of 1:1, 1:5, and 1:10. In this context, the tolerance ratio is defined as the specific ratio between targeted and non-targeted ions that results in a change exceeding 5% in the control signal, which originates from the targeted ion. All masking strategies are comprehensively explained in masking protocol S1 in the supplementary Information. In summary, most tested ions exhibited no interference at a 1:1 ratio, and we achieved over 80% selectivity even at a higher mixing ratio of 1:10, as shown in Figure 3.

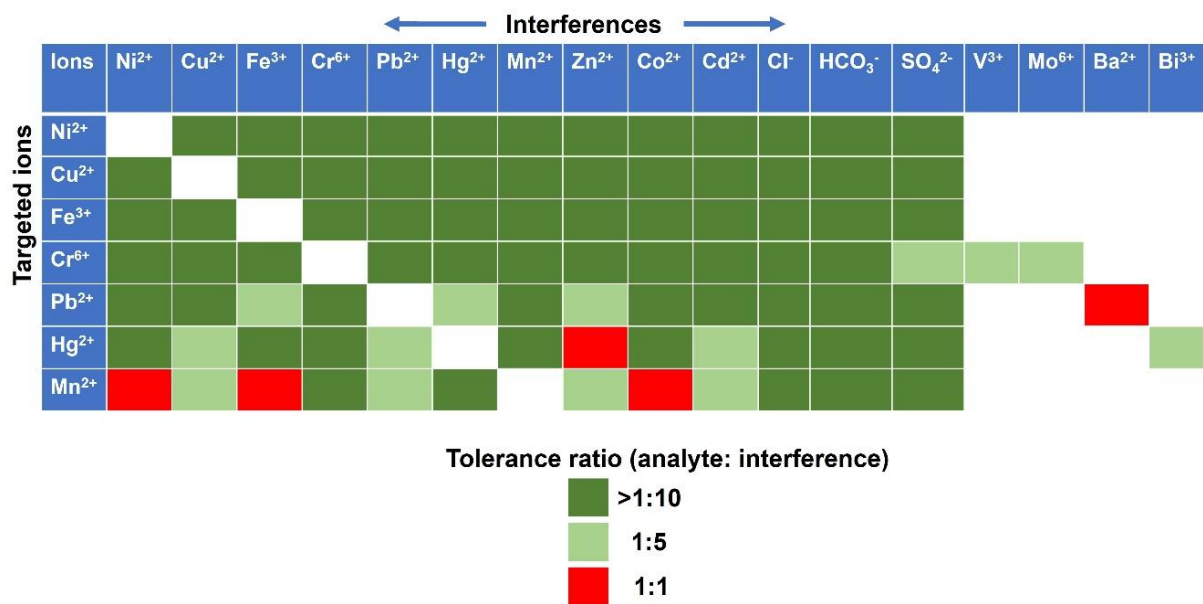


Figure 3. Interference study on the developed sensor. The targeted ions are down the rows, and interfering ions are across the columns. Dark green boxes indicate the interference at >1:10 (targeted: non-targeted), light green at 1:5, and red boxes at 1:1.

Table 1. Comparison of analytical performance of the developed system with other multiplexed colorimetric paper-based systems.

Ref	M ⁿ⁺	Reagent	Linear range (ppm)	LoD (ppm)
[64]	Ni(II)	Dimethylglyoxime	15-60	4.8
	Cr(VI)	PAR	0.5-10	0.18
	Cu(II)	Bathocuproine	5.0-80	1.6
[65]	Fe(III)	Bathophenanthroline	20-1300	20
	Ni(II)	Dimethylglyoxime	100-1100	100
	Cu(II)	Bathocuproine	100-1300	100
[37]	Fe(III)	1,10-Phenanthroline	0.3-18	0.2
	Ni(II)	Dimethylglyoxime	0.4-23	0.3
	Cr(VI)	1.5-DPC	0.2-17	0.1
	Cu(II)	Bathocuproine	0.05-24	0.03
	Al(III)	Chromeazurol S	0.1-10	0.08
	Zn(II)	Dithizone	0.05-24	0.04
[35]	Cu(II)	Bathocuproine	0.32-63.55	0.32
	Co(II)	PAR	0.59-4.71	0.59
	Ni(II)	Dimethylglyoxime	5.87-352.16	5.87
	Hg(II)	Dithizone	0.20-12.04	0.2
	Mn(II)	PAR	0.11-0.55	0.11
[66]	Fe(III)	1,10-Phenanthroline	30-300	15
	Ni(II)	Dimethylglyoxime	30-300	15
	Cr(VI)	1,5-DPC	7.6-120	2.4
	Cu(II)	Bathocuproine	60-300	15
This Work	Ni(II)	Dimethylglyoxime	0.02-0.4	0.015
	Cu _{total}	Bathocuproine	0.0125-0.2	0.007
	Fe _{total}	Bathophenanthroline	0.005-0.1	0.002
	Cr _{total}	Diphenylcarbazide	0.05-0.5	0.020
	Pb(II)	Rhodizoinic Acid	0.025-0.15	0.013
	Hg(II)	Dithizone	0.05-0.3	0.026
	Mn(II)	PAR	0.025-0.15	0.015

3.4.4 Stability and packaging protocols

The stability of the developed sensor was evaluated by storing them under various conditions: room temperature (23°C), fridge (5°C), and freezer (-20°C). Across all these conditions, consistent signals were obtained for Ni(II), Cu_{total}, Fe_{total}, Cr_{total}, and Mn(II) throughout the 28-day study period. However, for Pb(II) and Hg(II), better stability was observed under fridge and freezer conditions, as depicted in Appendix B, Figure B16. In

order to improve the stability of reagents for Pb(II) and Hg(II) at room temperature, the sensors were stored in nitrogen-purged bags with impulse heat sealing. This packaging protocol resulted in retaining over 90% of the signal for up to 5 days at room temperature as shown in Appendix B, Figure B16. This indicates that with our packaging protocols, the sensors can be shipped to end users, refrigerated upon arrival, and subsequently utilized for monitoring purposes.

3.4.5 Method Validation

Device reproducibility was validated by spiking drinking water samples with varying concentrations of each metal ion (Table 2). All RSD values remained below 15%, indicating consistent device performance across varying concentrations. These results suggest the sensor's potential for reliable and precise heavy metal detection in real drinking water. Figure 4 shows the representative images of the sensor for three different levels of spike recovery analysis.

Moreover, to evaluate the sensor's applicability for real water samples, we conducted tests using various samples: drinking water, laboratory water samples, and water samples from a river in Colorado. Details regarding the locations and sample collection protocols are provided in Sampling protocols S1. Additionally, the sensor's performance was validated utilizing results obtained from ICP-MS analysis following EPA method 200.8 guidelines.⁶⁷ The evaluation of the proposed sensor for metal ion detection in various water sources yielded promising results. Notably, Fe_{total} levels in drinking water remained within regulatory limits, with a recovery of 77%. In both Lab Water 1 and 2, the sensor successfully detected Cu_{total} and Fe_{total}, with recoveries ranging from 81% to 94%

and relative standard deviations (RSD) between 6.4% and 10.8%, demonstrating good accuracy and precision.

Furthermore, the sensor detected Ni(II), Cu_{total}, and Fe_{total} in river water in Colorado, achieving recoveries exceeding 79% and RSDs below 15%. Most of the detected levels were below the guideline limits. The on-card color response for each water sample is shown in Appendix B, Figure B17. The proposed sensor demonstrated a performance comparable to the established industry standard (ICP-MS) as shown in Table 3. The on-card responses for the real samples and full results are included in the Appendix B, Figure B17 and Table B2 respectively. These results highlight its potential for widespread adoption in environmental monitoring applications, offering valuable insights for future implementation.

The sensor has certain limitations. In natural water, heavy metal ions can exist in various forms, including precipitates, colloidal particles, and those bound to organic matter. However, the system's colorimetric chemistry is designed to detect only dissolved ion species. Therefore, metal digestion is necessary to ensure that all forms of metal ions are detected. Our ongoing work includes the development of a portable and user-friendly acid digestion system. Additionally, the system's metal retention capacity requires a careful balance between filtration rate and tank height. Lastly, although the system is more user-friendly compared to existing multiplexed systems, the reliance on a smartphone for on-site quantification may be challenging in resource-limited settings. However, the color change is sufficiently pronounced to allow for semi-quantitative analysis.

Table 2. Spike recovery analysis for the developed sensor in real drinking water samples (ND = not detected, NT = not tested).

Metal	ICP-MS (ppb) (n=3)	Sesor (ppb) (n=3)	Spiked (ppb)	Recovered (ppb)	Rec %	RSD %
Ni (II)	0.11 ± 0.02	ND	75	70.97 ± 5.21	95	7.3
			150	139.85 ± 10.62	93	7.6
			300	281.68 ± 18.96	94	6.7
Cu_{total}	6.00 ± 0.04	ND	30	26.27 ± 2.68	88	10.2
			60	56.16 ± 8.20	94	14.6
			100	91.25 ± 12.71	91	13.9
Fe_{total}	13.20 ± 0.37	10.16 ± 1.84	10	8.46 ± 1.11	85	13.1
			20	17.89 ± 2.63	89	14.7
			50	44.98 ± 5.83	90	13.0
Cr_{total}	ND	ND	75	63.01 ± 7.9	84	12.5
			150	130.21 ± 15.83	87	12.2
			300	264.18 ± 36.05	88	13.6
Pb (II)	0.28 ± 0.03	ND	50	42.18 ± 6.02	84	14.3
			100	87.94 ± 11.94	88	13.6
			150	127.46 ± 11.78	85	9.2
Hg(II)	NT	ND	100	75.75 ± 11.09	76	14.6
			200	154.89 ± 14.65	77	9.5
			300	231.47 ± 30.86	77	13.3
Mn(II)	3.91 ± 0.02	ND	50	57.55 ± 8.11	115	14.1
			100	118.11 ± 17.14	118	14.5
			150	178.23 ± 19.33	119	10.8

Table 3. Summary of metal ion detection results in various water sources using the developed sensor.

Sample Type	Metal Ion	ICP-MS (ppb) (n=3)	Sensor (ppb) (n=3)	Rec (%)	RSD (%)
Drinking water	Fe _{total}	13.20 ± 0.37	10.16 ± 1.84	77	18.1
Lab water 1	Cu _{total}	627.67 ± 8.18	540.11 ± 34.67	86	6.4
	Fe _{total}	15.86 ± 0.45	12.83 ± 1.39	81	10.8
Lab water 2	Cu _{total}	690.00 ± 5.72	645.37 ± 41.10	94	6.4
	Fe _{total}	7.71 ± 0.10	5.32 ± 0.97	69	18.3
River water	Ni(II)	50.77 ± 2.01	40.39 ± 5.85	80	14.5
	Cu _{total}	689.00 ± 24.59	621.13 ± 43.14	90	7.0
	Fe _{total}	721.33 ± 20.81	662.72 ± 71.15	92	10.7

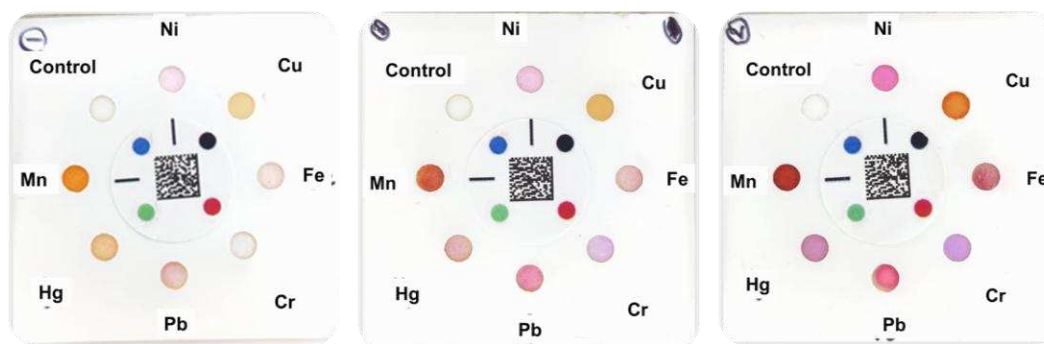


Figure 4. Representative images for spike recovery analysis showing on-card responses for lower to higher spiking images (left to right).

3.5 Conclusions

In this study, we developed a novel preconcentration system coupled with colorimetric detection to simultaneously detect seven heavy metal ions at low ppb levels in water. The system achieved guideline-relevant LoDs of 15 ppb (Ni(II)), 7 ppb (Cu_{total}), 2 ppb (Fe_{total}), 20 ppb (Cr_{total}), 13 ppb (Pb(II)), 26 ppb (Hg(II)), and 15 ppb (Mn(II)). The simple preconcentration method allows for ease of use and eliminates the need for complex pre-treatment steps. Furthermore, the Fill, Fold, Photo detection method offers a portable, user-friendly quantification platform. Implementing various masking strategies ensured high selectivity for the targeted metals, minimizing interference from other potentially present elements. The system maintained stable signal performance with the proper packaging and storage protocols for over four weeks, highlighting its long-term usability and suitability for mass production and deployment. Different real-world water samples were analyzed, demonstrating good recovery compared to the standard ICP-MS method. Spike recovery analysis further confirmed the sensor's accuracy and precision, achieving less than 15% RSD. This work presents a significant advancement in the field of low-cost on-site environmental monitoring. We envision this technology evolving into a convenient and portable solution for in-field monitoring of various water contaminants, including pesticides, PFAS, fertilizers, and beyond.

3.6 Acknowledgements

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CHAPTER 4 — Citizen-Based Water Quality Monitoring: Field Testing a User-Friendly Sensor for Phosphate Detection in Global Surface Waters

4.1 Chapter overview

The goal of this chapter is to demonstrate the translation of capillary-driven microfluidic sensing from laboratory validation to large-scale, real-world field deployment through the development of a user-friendly phosphate sensor for citizen-based water quality monitoring. While earlier chapters focus on improving transport, sensitivity, and multiplexing under controlled experimental conditions, this chapter addresses the critical challenges of operational simplicity, robustness, and long-term stability required for global, in-field use by non-expert users.

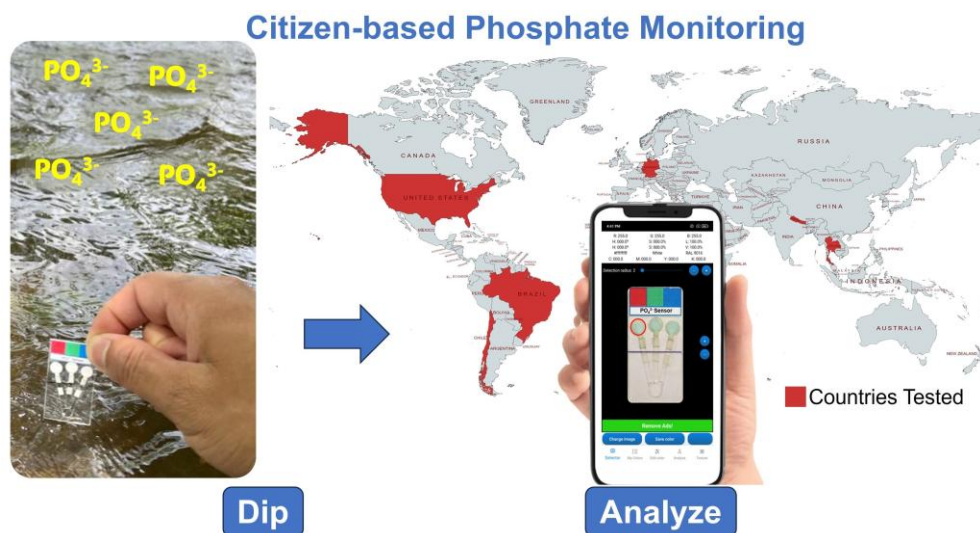
The work presented in this chapter was conceptualized, designed, and led by me, under the supervision of Prof. Charles S. Henry, with collaboration from Eric Brack at the U.S. Army DEVCOM Soldier Center. I developed the microfluidic device architecture, optimized the phosphomolybdenum blue colorimetric assay, established reagent stabilization and packaging strategies, and performed data curation, analysis, and interpretation. Data acquisition during the global sampling campaign was assisted in part by Claire E. Hefner. This work was published as a first-author peer-reviewed article (*Anal. Chem.* 2024, 96, 46, 18369–18376) and represents one of the largest demonstrations of citizen-led deployment of a microfluidic water quality sensor.

In this chapter, a fast-flow, dip-and-read microfluidic device is introduced for phosphate detection in surface waters. The device ensures accurate and consistent sample delivery without pipetting, enabling reliable operation in resource-limited and field

settings. The sensor achieves an environmentally relevant limit of detection near regulatory thresholds, maintains high selectivity, and exhibits extended stability under ambient storage conditions. Analytical performance is validated against the industry-standard UV–Vis method, confirming suitability for real-world deployment.

Beyond analytical performance, this chapter emphasizes the scalability and societal impact of capillary-driven microfluidic systems. More than one thousand sensors were deployed across multiple continents, enabling high spatial resolution mapping of phosphate concentrations in diverse surface water sources. By integrating citizen participation with robust sensor design, this work demonstrates how microfluidic technologies can support decentralized environmental monitoring and provide actionable insights into eutrophication risks at a global scale.

Chapter graphical summary



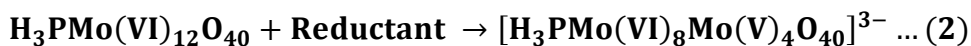
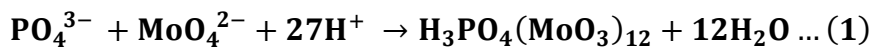
4.2 Introduction

Phosphorus is an essential macronutrient for the direct and indirect sustenance of living organisms.¹ It occurs in various forms, with phosphate (PO_4^{3-}) being the most naturally abundant and bioavailable form in water.²⁻⁴ While essential at low concentrations, high levels (>0.1 ppm) of phosphates pose a serious environmental threat.^{5, 6} Human activities are often the cause of elevated phosphate, with common sources being fertilizers, feeds, detergents, and foods.^{5, 6} When excessive nutrients, like phosphates, accumulate in bodies of water, they promote the growth of algae and aquatic plants. As these organisms decompose, they not only consume oxygen but also create zones within the water that are depleted of oxygen.^{7, 8} Additionally, cyanobacteria can rapidly grow in nutrient-rich environments, forming dense and potentially toxic blooms. The toxins produced by some cyanobacteria species can harm aquatic ecosystems and wildlife and even pose risks to human health when contaminated water is consumed.⁹ Studies have shown that 78% of the US coastal waters and 65% of Europe's Atlantic coastal waters exhibit symptoms of eutrophication, while an alarming 30-40% of the world's surface water systems are already facing this environmental threat.^{10, 11} Therefore, continuously monitoring the active phosphate species in all surface water systems is crucial.

Currently, most regulatory bodies like the US EPA employ sophisticated instrumentation like UV-Vis spectroscopy and high-performance liquid chromatography (HPLC) for phosphate detection and quantification in water.¹²⁻¹⁵ Methods using ion chromatography,¹⁶ fluorescence spectroscopy,¹⁷ or electrochemical detection,¹⁸ also exist; however, their lack of standardization has limited their widespread adoption for routine analysis.¹⁹ Moreover, these techniques are impractical for frequent on-site testing due to

the need for more sophisticated infrastructure, operative complexity, and higher training and operational costs. As a result, there is a pressing need for alternative, cost-effective, easy-to-use methods to enable frequent monitoring of phosphate levels in surface water sources.

Paper-based microfluidics presents a promising solution for on-site detection as they offer straightforward sampling techniques that non-experts can efficiently perform.²⁰⁻²² A typical paper-based microfluidic sensor is comprised of flow channels defined by hydrophobic barriers in the paper.²³ Capillary flow transports liquid samples through the flow channels to carry the target analyte to a reaction zone. Here, the analyte reacts with a colorimetric reagent stored in the paper to generate a colorimetric signal that is directly or indirectly correlated to the analyte concentration.^{24, 25} Most colorimetric paper-based phosphate sensors use the phosphomolybdenum blue (PMB) reaction (Reactions 1 and 2). The PMB reaction occurs through a two-stage process.²⁶⁻²⁹ The first stage involves the interaction of the analyte anion with molybdate, leading to the formation of 12-molybdophosphoric acid (12-MPA). This process occurs under strong acidic conditions, facilitating the reaction and subsequent complexation. Second, the heteropoly acid is reduced, producing a dark blue product.²⁹ Notably, the PMB only reacts with phosphate and other labile phosphorus species, collectively identified as the molybdate reactive phosphorus (MRP) fraction. The MRP fraction is a measure of the total amount of phosphorus that is available for biological uptake which includes the labile forms of phosphorus other than phosphate.⁴



Jayawardane et al. developed the first paper-based device to measure MRP levels in lakes and soil water samples.³⁰ Similarly, Racicot et al. developed another paper-based device capable of colorimetric phosphate detection in seawater.³¹ Both methods used the PMB reaction as their colorimetric mechanism. These paper-based analytical sensors provide a range of benefits, including ease of operation, good analytical performance, compact size, minimal reagent consumption, and low cost (<\$1). However, it is crucial to acknowledge that these techniques involve using highly corrosive 6.6 M sulfuric acid on the paper, necessitating meticulous handling and optimization to prevent both damage to the cellulose through hydrolysis and potential harm to users.³² Additionally, sample introduction to the device is performed using a pipette, which may limit the usability of these techniques for non-trained users. Richardson et al. addressed this challenge by introducing dry p-toluenesulfonic acid (PTSA) as a substitute for sulfuric acid. Unlike sulfuric acid, PTSA is a non-oxidizing organic acid that does not hydrolyze cellulose, thereby preserving the structural integrity of the paper-based device.³³ This innovation allowed the researchers to develop a sensor that facilitated citizen-led sampling. However, the folding design of the device and the hygroscopic nature of PTSA posed limitations in achieving optimal stability at room temperature. The device also lacked sensitivity with a limit of detection at 3 mg/L (3 ppm). The US Environmental Protection Agency (US EPA)

recommends a maximum total phosphate concentration of 0.150 ppm (150 ppb) for streams flowing into lakes or reservoirs.³⁴

It has been established that sensors employing sulfuric acid exhibit high sensitivity but are not stable under ambient conditions for end-user deployment. Conversely, systems utilizing stable acid platforms struggle with higher limits of detection. Existing sensor design has also led to the potential for cross-contamination between individual reagents, rendering them unstable for more than a week under ambient conditions. Therefore, the research presented here aims to develop a portable, field-stable sensor that can be used in diverse locations without cross-contamination of reagents. The ideal sensor should also maintain prolonged stability for months and achieve a limit of detection comparable to sulfuric acid-based systems on sensitive platforms.

In this study, we developed and field-tested a user-friendly dip sensor for phosphate detection in global surface water sources. The sensor uses a lateral flow channel design with reagents, including stable organic acid, Mo(VI), and reducing agents, separated within hollow capillary channels using glass fibers. The hollow capillary channels were created by stacking alternate double-sided adhesive (DSA) layers and PET films. The sensor achieved a detection limit of 190 ppb with method validation by UV-Vis (EPA 365.1).³⁵ This is more than a magnitude lower than the previous PTSA technique and is near the EPA-recommended MCL for phosphate.³⁴ The sensor showed excellent selectivity and stability when tested against non-targeted analytes in water for 2 months under ambient conditions, demonstrating its potential for field applicability. The dip-and-read design eliminates the need for manual pipetting steps while ensuring controlled volume delivery of samples and makes it easy to operate for untrained users. Over 1,000

sensors were distributed to volunteers in different countries across multiple continents and the results were used to map phosphate levels for different surface waters in these locations. The sensor is a promising new tool for phosphate monitoring in surface water environments, offering a cost-effective and user-friendly way to collect data more frequently.

4.3 Experimental section

4.3.1 Material and instrumentation

The materials and chemicals used in this research were purchased from the following companies: potassium phosphate monobasic, ammonium molybdate, interfering ions, and surfactants (The Sigma-Aldrich, St. Louis, MO, USA); P-toluenesulfonic acid (The Santa Cruz Biotechnology, CA, USA); antimony potassium tartrate trihydrate (The Alfa Aesar, Ward Hill, MA, USA); double-sided adhesive (468MP), glass fibers, and PET transparency film (9984) (The 3M, St. Paul, MN, USA); Ahlstrom blotting paper (The VWR International, Radnor, PA, USA). The adhesive tapes, PET films, glass fibers, and Ahlstrom Blotting paper were cut using an Epilog Engraver WinX64 Zing laser cutter (The Epilog Laser, Inc., Golden, CO, USA). The color for references (RGB values) was optimized in CorelDRAW (The Corel Corporation, Ottawa, ON, Canada) to attain constant color print. The device was pressed using a Royal Sovereign CL-1223 laminator (The Royal Sovereign Manufacturing, Inc., Oak Creek, WI, USA). UV-Vis analysis was performed using a GENESYS 150 UV-Vis Spectrophotometer (The Thermo Fisher Scientific, Waltham, MA, USA).

4.3.2 Sensor design and fabrication

A 2.5 cm x 2.5 mm flow channel was laser-cut into a double-sided adhesive. The device was assembled by stacking alternating layers of PET film (9984 on the bottom and the pre-cut DSA (468MP) in the middle), followed by another layer of PET film on top.³⁶⁻³⁸ The hydrophilic side of the PET film faced into the flow channel to facilitate rapid analyte transport through strong capillary action. A 6 mm circle was cut out of the middle DSA and top PET layer at the endpoint of the flow channel to place the paper circles. A 6 mm diameter Ahlstrom blotting paper was cut with a 3 mm x 2.5 mm tail at the end. The tail helps guide the sample flow to the paper circle while reducing the surface tension at the paper-capillary flow interface. Each sensor has three flow channels that connect three paper endpoints to a common dip zone. Above the detection zone of each sensor, printed color references (red, green, and blue) were placed to mitigate the inconsistencies due to lighting conditions. Two 3 mm x 2.5 mm glass fibers are placed inside each flow channel between the dip zone and the paper endpoints. The first glass fiber is pre-treated with PTSA, while the second is pre-treated with Mo(VI) and its catalyst. Figure 1 illustrates the device's design and working mechanism of the sensor. Overall, the described fabrication process and working mechanism of the device enable the sequential chemical reactions of the sensor in a single dip.

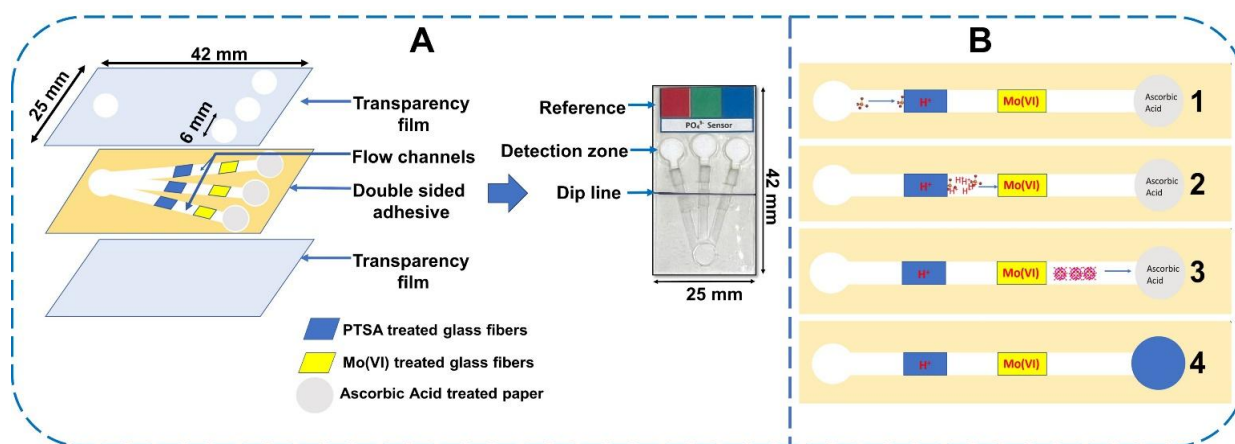


Figure 1. Detailed assembly and final appearance of the device (A). The top layer consists of 9984 PET film with its hydrophilic side facing inwards towards the flow channel. A thicker flow channel is created by alternating layers of 468MP double-sided adhesive on the top, PET film in the middle, and another adhesive at the bottom. Finally, the bottom layer is another PET film with its hydrophilic side facing upwards. The device's working mechanism on a single flow channel (B). When the device is dipped into the sample (1), the solution gets acidified (2), the acidified solution reacts with Mo(VI) to get 12-MPA (3), and 12-MPA gets reduced on paper when it reacts with ascorbic acid to give the blue colored product (4).

4.3.3 Colorimetric Assay

The first glass microfiber pad was pre-treated with a 1 M PTSA solution, and the second glass microfiber pad was treated with a 19 mM Mo(VI) solution mixed with antimony potassium tartrate (0.01 g/mL). The paper endpoint was pre-treated with a 10 mM ascorbic acid solution. All reagents were dried at room temperature before using the sensor. A 1000 ppm phosphate stock solution was prepared in Milli-Q water and serially diluted for calibration. A calibration curve was constructed for the concentration range of 0.625 to 40 parts per million (ppm). The color intensity of the solution began to saturate at concentrations above 20 ppm, indicating that there was little or no change in color intensity.

4.3.4 Data Analysis

After the reaction, the sensors' photographs were taken using a smartphone. The captured images were then processed in ImageJ software by converting them to grayscale and separating them into blue, green, and red channels. The images were then inverted. The average grayscale intensity of the resulting complex was measured using a circular tool. The average grayscale intensity of the blue reference was also measured as a precise reference for measurement. The grayscale intensity was calculated relative to the area of the blue reference to mitigate any inconsistencies in lighting conditions. The reference zone will normalize the intensity when the lighting conditions of both the pad and the reference zone will change. The circular area of interest remained consistent between the reference and the detection zone. Calibration curves were constructed for each channel (Relative Grayscale vs. Concentration). The red channel with the sharpest slope was selected for further analysis for its maximum sensitivity (See Figure C1, Appendix C). The RGB Color Detector, a readily available color quantification application, was also used to calibrate the sensor. The sensor's image was captured within the app, and the red intensity of the color was subsequently calculated using a circular region of interest (See Figure C2 and C3, Appendix C). Moreover, the system can be adjusted to be compatible with other applications such as ColorPicker, ColorMeter, MATLAB Mobile, and more.

4.4 Results

4.4.1 Sensor performance

The sensor's performance was evaluated based on physical and chemical performances. The flow rate was optimized by adjusting channel geometry and choosing the best substrate (See Figure C4 and C5, Appendix C). The signal to noise ratio and homogeneity of the colored product was optimized by optimizing reagent concentrations and reaction time (See Figure C6 through C9, Appendix C). Once the paper endpoints are fully hydrated and channels are filled, the liquid flow within the channel stops. Since the flow is controlled within a dip, the system allows for controlled delivery of a fixed sample volume in each sensor, removing any need for pipetting water samples. A linear calibration was performed between 0.625 and 10 ppm, as shown in Figure 2. The limit of detection (LoD) was calculated to be 190 ppb by $3.3\sigma/m$ method, and the limit of quantification was calculated to be 570 ppb by $10\sigma/m$, where σ is the standard deviation of blank ($n=9$) and m is the slope of the linear range of calibration curve. This LoD is more than an order of magnitude lower than the previously developed sensor that utilized PTSA as a stable, non-oxidizing acid source. The LoD is significant for actively monitoring water sources contaminated with phosphate, particularly in light of the United Nations Environment Programme's conclusion that approximately 30-40% of global water sources have already experienced eutrophication.³⁹

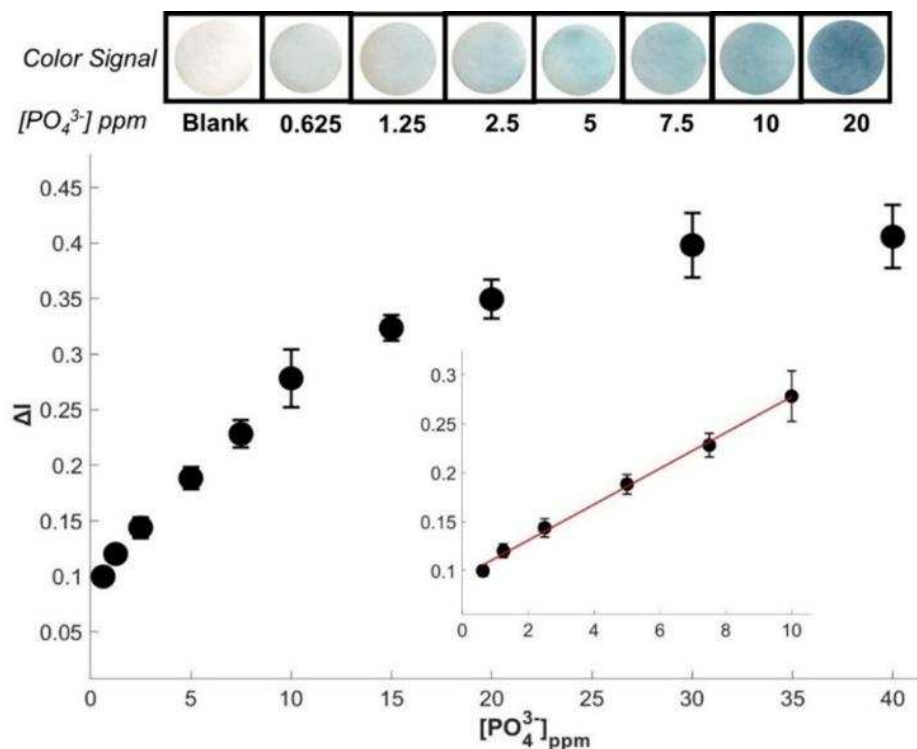


Figure 2. Qualitative (top) and quantitative calibration for determining PO_4^{3-} in solution. A quantitative calibration curve was generated for concentrations between 0.625 and 40 ppm, exhibiting a linear range of 0.625 to 10 ppm ($R^2 = 0.997$). The corresponding linear fit equation was determined to be $y = (0.019 \pm 0.001)x + (0.094 \pm 0.003)$. Error bars indicate the standard deviation of relative grayscale intensity between 6 replicates. Relative grayscale intensity was calculated by dividing the grayscale intensity of the complex by the grayscale intensity of a reference blue color.

4.4.2 Field applicability assessment

Interferences. For the field deployment of the sensor, the device must provide precise and accurate measurements by mitigating potential interferences arising from diverse substances present in real-world water samples. The interference was evaluated by mixing the known interfering ions (AsO_4^{3-} , SiO_4^{4-}) that also undergo the MB reaction, and common water ions/surfactants (Cl^- , Cd^{2+} , Cu^{2+} , Fe^{3+} , SO_4^{2-} , NO_3^- , Ca^{2+} , Zn^{2+} , Co^{2+} , Ni^{2+} , HCO_3^- , Mg^{2+} , sodium dodecyl sulfate, tetramethyl(tetradecyl) ammonium chloride, and Tween 20) at different concentration levels to investigate the potential effect of non-

targeted ions on the assay. No significant difference was observed between the signal from the control and any of the environmentally relevant mixtures. Please refer to Figure C10, Appendix C for a detailed elucidation of the interference study.

Stability and Environmental Resilience. To facilitate user-friendly sampling campaigns, the sensor needs to maintain stability over an extended period in typical environmental conditions. Prolonged stability under ambient conditions is necessary to distribute and utilize devices in resource-limited areas where special storage conditions are not readily available. The sensor's stability was assessed across four storage conditions: room temperature (RT), room temperature with desiccant in mylar bags, 5°C fridge, and -20 °C freezer (Figure 3). The results demonstrate consistent and stable signals for over 60 days under fridge/freezer conditions and room temperature conditions when sealed in mylar bags with desiccant. This extended stability, alongside effective sealing and packaging protocols, ensures the device's viability for real-world testing and deployment in resource-limited areas without requiring specialized handling. Dunnett's multiple comparison test was employed to assess the outcomes for each condition, designating day 0 as the control group. The results of the analysis affirm that no statistically significant differences ($p > 0.05$) exist between the control group and the alternative storage conditions except RT without desiccant. In addition, impulse heat sealing using Mylar bags ensures a moisture-free environment for the sensor, which is important because PTSA is hygroscopic and can absorb moisture. Importantly, we achieved consistent signals for the blanks for the entire storage period. The consistency in the blanks is indicative that the molybdenum complex is not undergoing the auto-reduction process to form isopolymolybdates. The result allowed us to carry out more accurate measurements

in real-field settings. Compared to the previous study, our sensor's design and sealing/packaging protocols significantly improved the lifetime of the reagents under ambient conditions while preventing the auto-reduction of the molybdenum complex for a reasonable timeframe. In a real-world testing scenario, when batch manufacturing and shipping the sensor, it can be taken out of the bag at the time of testing. The stability and interference results suggest that the device can be batch-manufactured and implemented in real-world water environments in a realistic timeframe without special handling.

To assess the sensor's applicability to field deployment, we evaluated its performance across water temperatures and humidity levels (See Figure C11, Appendix C). For the temperature tests, we tested the sensor to water temperatures from 3°C to 45°C. The sensor performed consistently across this range, with no significant differences in the color signals obtained. We then conducted humidity tests by subjecting the sensor to varying humidity levels within a controlled chamber, ranging from 25% relative humidity (RH) to 96% RH ($\pm 1\%$). Again, the sensor performed consistently under all these humidity conditions without significant performance differences. Furthermore, higher humidity levels did not affect the sensor's flow rate. These results suggest the sensor is highly adaptable to field deployment and can function effectively across a wide spectrum of temperature and humidity conditions, provided the packaging protocols are properly followed.

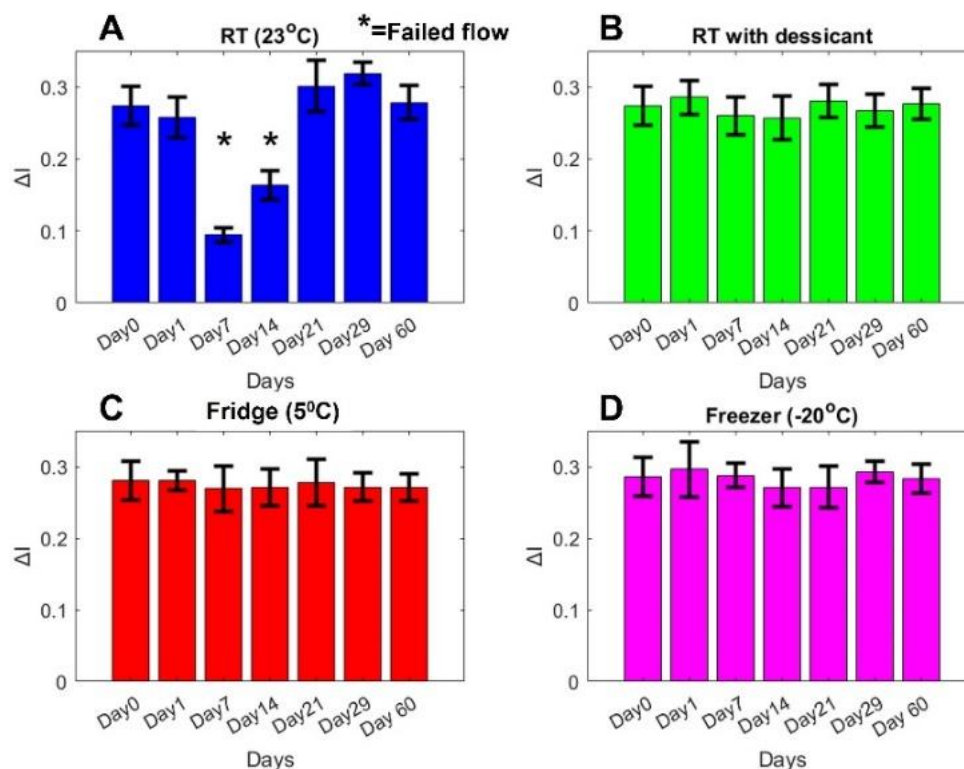


Figure 3. Prolonged stability testing under four storage conditions: room temperature (A), room temperature with dessicant in mylar bags (B), 5°C fridge (C), and -20 °C freezer (D).

Smartphone Compatibility and Environmental Sustainability. In addition to the environmental conditions, variations in lighting conditions across smartphone platforms can lead to discrepancies in grayscale intensity when capturing the same color signal. To address this challenge, we conducted compatibility tests to evaluate the sensor's performance across iOS and Android devices. Identical images were captured using a Xiaomi 9T and an iPhone 13 Pro Max, with the filter disabled on both devices. Subsequently, intensity normalization with respect to a reference zone and calibration were performed. As illustrated in Figure C12, Appendix C, the results demonstrate that our sensor achieves consistent sensitivity across different smartphone platforms. These findings underscore the sensor's adaptability, confirming its effectiveness for use with any

smartphone, if photographs are taken under reflection-free conditions and with the camera angle perpendicular to the sensor. Moreover, we employed the Analytical Greenness (AGREE) metric to assess the environmental sustainability of our sensor, considering 12 principles of green analytical chemistry.⁴⁰ As depicted in Figure C13, Appendix C, our sensor demonstrates a greenness value of 0.85, positioning it in the greener zone.

4.4.3 Method validation

The performance of the sensor was validated by comparing its results to the standard UV-Vis method (EPA Method 365.1).³⁵ We conducted tests on water sources near Fort Collins, CO, USA. The sensor exhibited a signal 90% consistent with the UV-Vis across various concentrations, as shown in Figure 4A. The agreement with the gold standard technique indicates that the sensor is capable of reliable performance in complex matrices encountered in field conditions. To further evaluate the device's user-friendliness, we deployed it for testing by local high school students. The students followed an instructional video and an instruction sheet and utilized the sensor to analyze water samples from stationary sources near their school. The students collected the sampling sites' images, GPS coordinates, and water samples. The sensor's performance was again compared to the UV-Vis method, and the results were similar (See Figure 4B). These findings demonstrate the successful validation of the developed sensor's performance compared with a well-established method. Additionally, the involvement of high school students in the testing process highlights the user-friendly nature of the device and its potential for broader applications. The sample collecting and storage protocols are extensively discussed in Sampling Protocols C1, Appendix C.

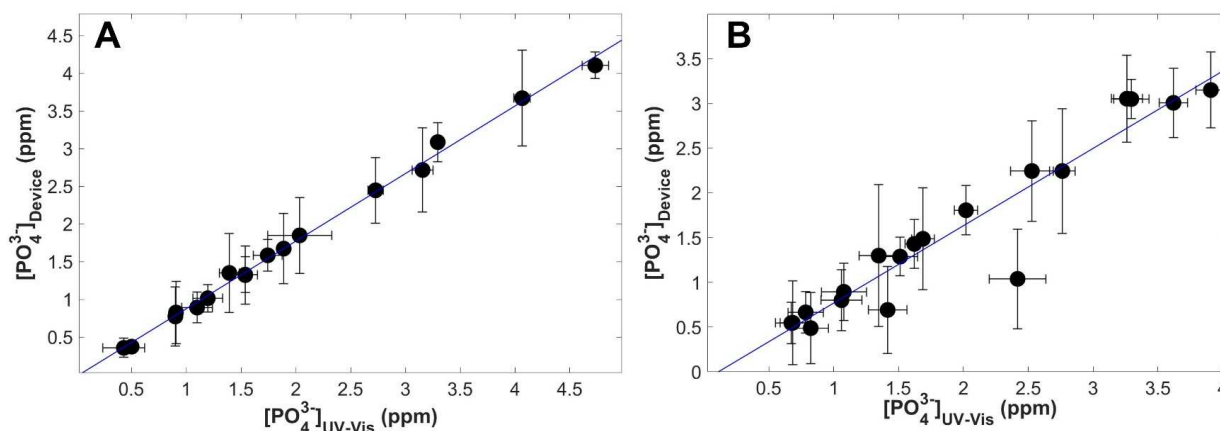


Figure 4. Method comparison between the developed sensor and the gold standard UV-Vis technique by authors (A) and high school students (B) using real samples from water sources near Fort Collins. The linear fit function is $y = 0.900x - 0.021$ with $R^2 = 0.996$ for samples collected by authors. The linear fit function is $y = 0.870x - 0.096$ with $R^2 = 0.987$ for samples collected by high school students in water sources near Thompson Valley High School. The error bars indicate the standard deviation of the concentrations calculated by respective methods among three to six replicates.

4.4.4 Citizen-led Global Monitoring

For volunteered-led phosphate monitoring in global water sources, more than 1000 sensors were manufactured and sent to collaborators in Bangkok, Thailand, Kathmandu, Nepal, Sao Paulo, Campinas, and Goiania, Brazil, Santiago, Chile, Massachusetts and Colorado, USA, and Frankfurt, Germany. The volunteers came from diverse backgrounds, including graduate students, engineers, plumbers, stay-at-home parents, electricians, mechanics, and high school students. Based on the results, the mapping was categorized into eutrophicated regions, with phosphate levels reaching up to 4.5 ppm (Figure 5), and excessively eutrophicated regions (Figure 6), where phosphate levels reached up to 10 ppm or higher. These classifications were determined using lab-generated calibration curves to calculate phosphate concentrations.

Eutrophicated regions:

Using the sensor, we detected the phosphate levels along the Cache la Poudre River, starting from the Gateway Natural Area in the river's headwaters and extending downstream to the urbanized Fort Collins area (Figure 5A). The results revealed a contrast in phosphate concentrations between the pristine and urbanized river sections. In the upstream mountainous region, phosphate levels were consistently low, indicating minimal anthropogenic impact. However, a significant increase in phosphate levels was observed as sampling progressed downstream towards the urbanized areas. Phosphate concentrations reached levels as high as 4.5 ppm in several eutrophicated regions. Based on these eutrophicated locations, agricultural runoff, wastewater discharge, and lawn fertilizers were identified as potential contributing factors for elevated phosphate levels. In comparison, we observed higher levels of phosphate concentrations than the previous monitoring performed in the same river by Son et al. in 2015.⁴¹

Another group of citizen scientists investigated phosphate levels in surface water systems in the vicinity of Santiago, Chile (Figure 5B). They collected samples from eight locations, including the Maipo River and San Antonio, as well as Baños Morales, San Gabriel, El Manzano, El Canelo, and its mouth in Llole, covering the entire stretch of the river from its source to the heart of the city. The results indicated elevated phosphate concentrations in at least one area of each sampled location, with the highest levels observed at the mouth of the Maipo River in Llole near Baños Morales. Volunteer contributions highlighted that the river runoff exhibited strong eutrophication colors in some regions of the Maipo River, and some communities were found to discharge laundry wastewater directly into the river. Many laundry detergents contain phosphates, which are added for their ability to break down dirt and stains. The volunteers also identified

industrial activities around the mega port, contributing to increased runoff from non-point sources and anthropogenic activities. These multiple potential sources of eutrophication were identified, emphasizing the need for further investigation and environmental management in the region.

To investigate phosphate levels in surface water systems near Bangkok, Thailand, a team of citizen scientists sampled >20 locations, traversing from the northern region of Bangkok near Chinat to the heart of Bangkok city (Figure 5C). The sampling spanned several locations, including Lop Buri, Pathum Thani, Ban Pong, and Bangkok near Chulalongkorn University. The results revealed elevated phosphate concentrations in at least one area of each sampled region, with Bangkok exhibiting the highest levels. Volunteer input suggested that agricultural runoff was the potential culprit for eutrophication in Chinat, Khlong Luang, Pathum Thani, and the Ban Pong River. In contrast, Bangkok's elevated phosphate levels were attributed to increased drainage runoff and a higher level of anthropogenic activities, leading to multiple non-point sources of phosphate.

Additionally, volunteers employed the sensor to evaluate phosphate levels in the water sources of São Paulo and Campinas, Brazil (Figure 5D and 5E). Our testing included the Raia Olímpica River in São Paulo and various stationary water bodies near Campinas and *Barão Geraldo*. While we identified instances of eutrophication in Campinas, most of the tested areas exhibited phosphate concentrations below eutrophication thresholds. However, due to the limited sample size, we refrain from making specific speculations regarding the sources and causes of high phosphate levels in the tested areas of São Paulo and Campinas.

Excessively Eutrophicated regions:

All the water sources tested in Goiânia were located close to areas of high public traffic, significant runoff from public spaces, and a notable lack of vegetation. This configuration strongly suggests a link to an inadequate drainage system, where runoff from public areas, potentially carrying pollutants, could directly influence water quality.⁴² Insufficient vegetation exacerbates the situation by limiting natural filtration processes. This observation highlights the need for an improved and well-managed drainage infrastructure to mitigate the impact of urban runoff on water sources in Goiânia (Figure 6A). On the other hand, the high eutrophication levels of water tested in the Kathmandu and Lalitpur valleys can be intricately linked to the city's inadequate waste disposal and sewage systems, compounded by elevated agricultural runoff. Particularly concerning is the scenario in the small canals of the Lalitpur region, where agricultural runoff is exceptionally high. Upon contacting locals, it was discovered that there was no clear guidance regarding the appropriate amount of fertilizer to be used. This unregulated and intensive farming practice leads to the runoff of phosphate-rich water into these canals, speeding up eutrophication ($\geq 10\text{ppm}$) (Figure 6B).

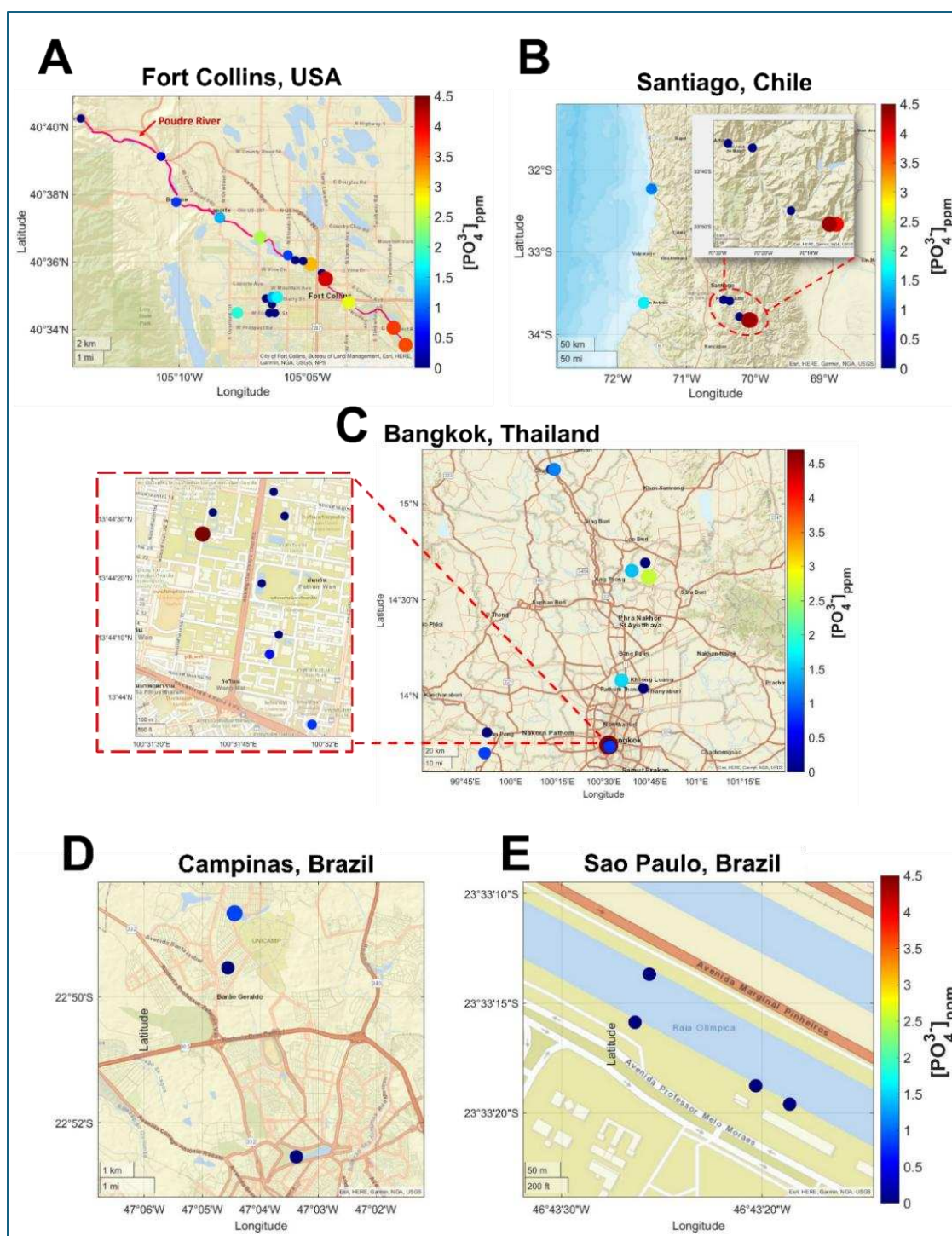


Figure 5. Phosphate mapping results from (A) Fort Collins, USA; (B) Santiago, Chile; (C) Bangkok, Thailand; (D) Campinas, Brazil; and (E) São Paulo, Brazil. Phosphate concentrations are represented using a jet color scale (0–4.5 ppm), with increasing circle size corresponding to higher phosphate levels, demonstrating spatial variability across river systems and urban water sources.

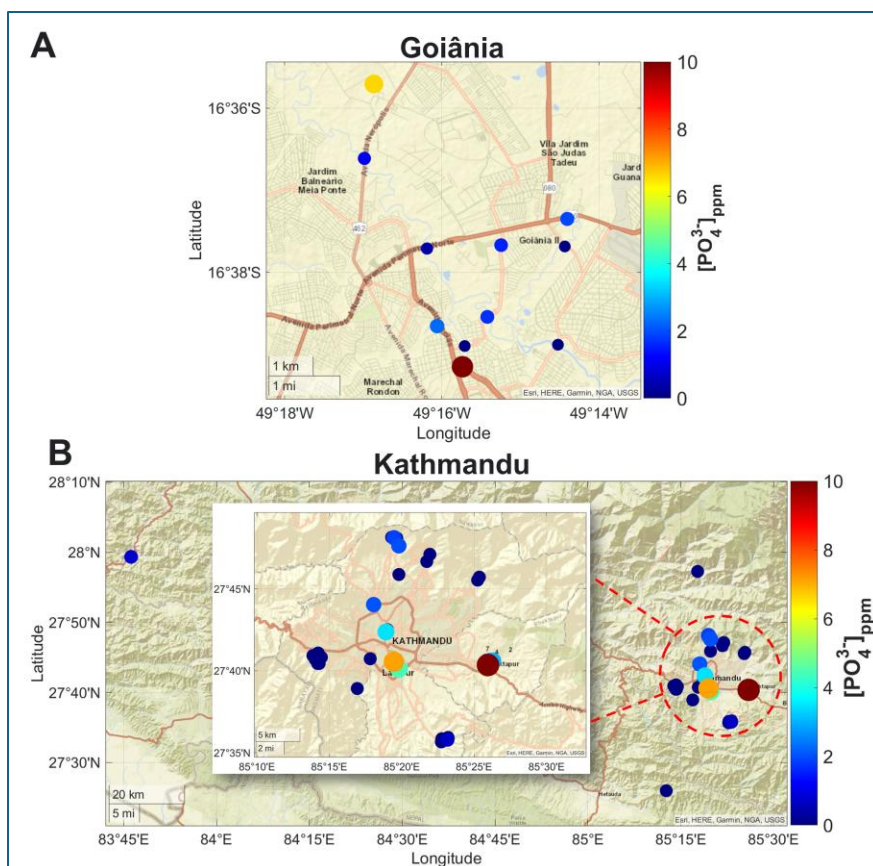


Figure 6. Phosphate mapping results from (A) Goiânia, Brazil, and (B) Kathmandu, Nepal. The 'jet' color gradient visually represents the rising phosphate concentrations, transitioning from deep blue (0 ppm) to dark red (≥ 10 ppm). Concurrently, the size of the circles expands in proportion to the increase in phosphate levels in respective images. The findings from these mapping efforts, along with insights from local volunteers, highlight the prevalence of excessive eutrophication in surface water sources across these regions.

4.5 Discussion

In this study, we developed a novel, inexpensive sensor for fast and user-friendly detection of phosphate concentrations in global water sources via volunteer-led sampling. The sensor requires only a single user step (dip) to obtain qualitative results within ten minutes. The sensor successfully employs a stable acid and additional reagents in the lateral flow channels, ensuring the prolonged stability of the sensor under ambient conditions without compromising the limit of detection. The sensor's performance was

validated using the gold standard UV-vis method, and results showed a very strong correlation between the methods. The sensors were then batch-manufactured and sent to citizen scientists worldwide. We were able to map the phosphate distribution in many locations, including Thailand, the USA, Chile, Brazil, and Nepal. Every tested country showed multiple locations where the water sources had already undergone eutrophication. Some areas, such as Kathmandu and Goiânia, seemed to have an excessive level of phosphate in the water sources that needed immediate attention. The overall results of testing phosphate levels in different surface water matrices worldwide present an alarming truth of increasing eutrophication. The sensor does have a limitation: highly turbid water can obstruct microfluidic channels, making the sensor ineffective and necessitating filtration beforehand. However, despite this limitation, our global campaigns demonstrate promising potential for the system's usability even among a wide range of inexperienced users.

4.6 Conclusion

The sensor developed in this study and its application in global-level phosphate mapping is a testament to citizen science's power in monitoring global water contamination. With further adoption, the developed system can be utilized to detect further contaminants like heavy metals, pesticides, PFAS, and others. The widespread phosphate contamination revealed by our citizen-led global phosphate mapping initiative highlights the alarming reality that eutrophication incidents extend beyond economically developing nations and afflict economically developed countries as well. While developing and deploying such monitoring systems are crucial, addressing the remediation of excess phosphate levels in water sources is equally imperative. If not regulated in time, we may lose many surface

water locations as dead zones. Despite excessive phosphate levels in many stagnant lake waters, no international framework currently exists for classifying in situ lake water remediation techniques.⁴³ To address the issue, a concerted focus must be on developing more robust and sustainable in situ technologies. Strategies such as biological nutrient removal, constructed wetlands, and permeable reactive barriers hold promise in effectively mitigating phosphate pollution and restoring the ecological balance of water bodies. By integrating these environmentally friendly techniques with sustainable monitoring platforms like the one described in this study, we can make significant contributions to the remediation of phosphate contamination, fostering a more holistic and sustainable approach to preserving global water resources.

4.7 Acknowledgments

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CHAPTER 5 — Capillary Flow-Driven Paper-Based Microfluidic Sensor for NDMA Detection in Water

5.1 Chapter overview

The goal of this chapter is to extend capillary flow-driven paper-based microfluidic systems to the detection of N-nitrosodimethylamine (NDMA), a highly toxic and emerging drinking water contaminant that typically requires complex laboratory-based workflows. While previous chapters focused on improving transport, sensitivity, multiplexing, and field deployment for inorganic analytes, this chapter addresses the challenge of integrating chemical transformation and detection directly on a paper-based microfluidic platform, enabling rapid and decentralized NDMA monitoring.

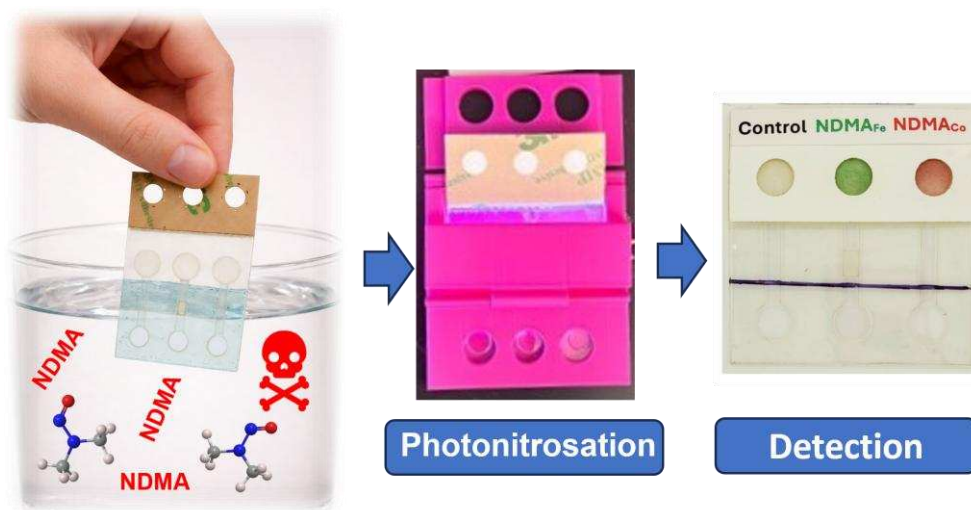
The work presented in this chapter was conceptualized, designed, and led by me, under the supervision of Prof. Charles S. Henry, with collaboration from Eric Brack at the U.S. Army DEVCOM Soldier Center. I developed the overall sensing strategy, designed the capillary-driven device architecture, optimized the on-chip photochemical nitrosation reaction, and performed assay development, data curation, analysis, and validation. Device fabrication was assisted by Jade Manna-Rubenstein, and portions of data collection were supported by Tessa Whitaker. This chapter is the first demonstration of a complete photochemical-colorimetric workflow implemented on a paper-based microfluidic device. The manuscript is under review for publication.

In this chapter, a dip-and-fold paper-based microfluidic sensor is introduced that integrates UV-driven nitrosation with dual-channel colorimetric detection using Fe^{2+} - and Co^{2+} -based complexation. The device employs hollow PET capillary channels to

enable rapid analyte transport while minimizing nonspecific adsorption and environmental sensitivity. Complementary green and red chromogenic responses provide enhanced selectivity and internal validation across two detection channels. Analytical performance, including linear range, limits of detection, selectivity, stability, and spike recovery in real water matrices, is evaluated to demonstrate robustness and suitability for field use.

Beyond NDMA detection, this chapter demonstrates the broader potential of on-chip photochemistry within capillary-driven microfluidic platforms. By combining passive fluid transport, photochemical transformation, and colorimetric detection in a low-cost and user-friendly format, this work establishes a framework for expanding paper-based microfluidic systems to a wider class of contaminants that require chemical derivatization prior to detection.

Chapter Graphical Summary



5.2 Introduction

N-Nitrosodimethylamine (NDMA) is a volatile, water-soluble N-nitrosamine that forms readily from common precursors and acts as a potent genotoxic environmental contaminant.¹ NDMA was first produced on an industrial scale as a byproduct in the manufacturing of unsymmetrical dimethylhydrazine (a liquid rocket propellant). Initially, NDMA was used as an antioxidant, lubricant additive, and polymer softener; but over subsequent decades, NDMA appeared as an unintended contaminant in various manufacturing processes. Most notably, NDMA was found as a nitrosamine impurity in certain pharmaceuticals, prompting major recalls of contaminated Valsartan and other drugs.^{2–6} Although NDMA has arisen from a variety of historical and incidental sources, its presence in contemporary drinking water primarily stems from formation as a disinfection byproduct.⁷ In the early 2000s, U.S. water treatment systems attempted to improve microbial control and reduce other byproducts such as trihalomethanes by switching disinfectants from free chlorine to chloramines.^{8,9} During chloramination, monochloramine and dichloramine react with dimethylamine-containing precursors from quaternary amine polymers, pharmaceuticals, and wastewater-derived organic nitrogen through chloramine substitution, oxidation, and nitrosation steps to form NDMA, which can persist into finished drinking water.^{10–13}

Despite being a semi-volatile liquid with limited sorption to organic carbon, NDMA possesses sufficient environmental stability to migrate through different water sources as a persistent contaminant plume.^{14,15} Consequently, concern about NDMA exists due to its high mobility in aquatic systems, multiple pathways into water supplies, and severe health effects at extremely low doses.^{16,17} NDMA can leach or be transported into surface and groundwater, enabling contamination of drinking water sources far from

the original point of release. Toxicologically, NDMA is classified by the U.S. Environmental Protection Agency (EPA) as a probable human carcinogen (Group B2) and by the International Agency for Research on Cancer (IARC) as “probably carcinogenic to humans” (Group 2A).^{16,18} Acute human exposures to high NDMA doses have caused fulminant hepatic failure.¹⁸ Recent epidemiological investigations related to NDMA-contaminated pharmaceuticals examined potential associations with gastric and colorectal cancers, and highlighted concern about long-term systemic exposure even when environmental concentrations are low.^{19–21} Chronic low-level exposure is linked to progressive liver injury, hematological abnormalities (e.g., thrombocytopenia), and elevated risks of liver and other cancers. Acute high-dose exposure to NDMA results in nausea, vomiting, headache, jaundice, and fulminant hepatic failure.^{18,22,23} Despite these risks, the U.S. EPA has not established a federal Maximum Contaminant Level (MCL) for NDMA in drinking water. Instead, it has derived a non-regulatory health reference level of 0.7 ng/L (ppt),¹⁶ but this value serves as a risk-assessment guideline rather than an enforceable standard. Even so, the risks of NDMA have driven several state-level actions, including California’s establishment of a 3 ng/L public health goal for NDMA in drinking water.^{24,25}

Regular monitoring and testing of NDMA levels in water systems as a preventative public health measure is critical to identify and mitigate contamination before it leads to widespread health crises, much like the decades-long oversight of per- and polyfluoroalkyl substances (PFAS), which were initially dismissed as inert but later revealed as persistent toxins linked to cancers, immune dysfunction, and massive remediation costs exceeding billions.^{26–28} Frequent testing enables regulatory bodies and

industries to track pollution sources, enforce safety standards, and implement timely remediation efforts. The absence of accessible, affordable, and rapid tools leaves communities vulnerable to undetected chronic exposure, progressive environmental degradation, and increased long-term health and economic burdens.²⁹

The gold standard for NDMA quantification in drinking water, EPA Method 521, relies on solid-phase extraction (SPE) of large-volume samples followed by gas chromatography–tandem mass spectrometry (GC-MS/MS).^{30–32} This workflow requires long (up to 14-day) sample holding times, specialized instrumentation, extensive sample cleanup, and highly trained operators.³³ Other established techniques, including high-performance liquid chromatography with chemiluminescence detection after UV photolysis,³⁴ GC-MS/MS variants using triple-quadrupole detectors,³⁵ and liquid chromatography–high-resolution mass spectrometry (LC-HRMS),³⁶ also require centralized laboratories, incur high analytical costs (~\$500–1000 per sample), and involve turnaround times of days to weeks, limiting their suitability for timely field-based decision-making.³⁷ Emerging alternatives, such as molecularly imprinted polymer (MIP)–based electrochemical sensors,³⁸ can achieve high sensitivity but depend on costly portable potentiostats, face challenges related to incomplete template removal, and require complex electrode fabrication steps that hinder practical field deployment.^{39,40}

Recently, Beard et al. introduced a promising colorimetric assay for aqueous NDMA detection based on photochemical nitrosation of a commercial naphtholsulfonate indicator.⁴¹ In their method, NDMA functions as a photodonor under UV irradiation, transferring a nitroso group to the naphtholsulfonate to generate a nitrosated product that subsequently forms a green Fe(II)–complex and red Co(II)-complex, hence

establishing a new analytical pathway.⁴¹ However, the quantitative analysis requires a benchtop UV–Vis spectroscopy, which limits suitability for point-of-need applications due to the associated instrumentation cost, power requirements, and operational complexity. To the best of our knowledge, no studies have reported a paper-based microfluidic platform for NDMA detection, nor has on-chip photochemistry been demonstrated for microfluidic systems in this context. These limitations underscore the need to bridge the gap with lab-on-a-chip platforms capable of fast, low-cost, and portable NDMA detection suitable for decentralized monitoring.

To address this need, we developed a capillary flow–driven, paper-based microfluidic system coupled with a 3D-printed UV irradiation chamber for NDMA detection in water samples. This platform provides high portability, rapid analysis times, and automated operation. Unlike conventional paper-based devices, our system transports samples through hollow capillary channels within seconds, minimizing sample adsorption to channel walls and ensuring efficient reagent delivery. To the best of our knowledge, this study is the first to demonstrate an NDMA assay implemented on a paper-based microfluidic platform and is the first study to integrate the photochemical nitrosation reaction directly on-chip, which enables a complete photochemical–colorimetric workflow. By leveraging microfluidic technology, this approach broadens detection capabilities and paves the way for rapid, portable NDMA screening on fast-flow paper-based systems suitable for field deployment by non-experts.

5.3 Experimental section

5.3.1 Chemicals and materials

NDMA was purchased from MilliporeSigma (Burlington, MA, USA). Sodium 1-naphthol-4-sulfonate, iron(II) chloride, and cobalt(II) sulfate heptahydrate were obtained from Fisher Scientific (Pittsburgh, PA, USA). Materials used for fabrication of the capillary flow-driven device included 468MP double-sided adhesive (DSA) and 9984 PET transparency film, both supplied by 3M Company (St. Paul, MN, USA). All aqueous solutions were prepared using Milli-Q water (18.2 M Ω ·cm). UV LED strips were purchased from Amazon (Seattle, WA, USA). Device components were patterned and cut using a CO₂ laser cutter (Epilog Zing; Golden, CO, USA). The UV irradiation chambers were fabricated using a fused deposition modeling (FDM) 3D printer (Bambu Lab P1S; Bambu Lab USA Inc., Austin, TX, USA).

5.3.2 Device fabrication

The capillary-driven microfluidic device consists of three inlets, three microfluidic channels, and three outlets, as illustrated in Figure 1. The device is constructed as a three-layer laminate composed of alternating sheets of 9984 PET transparency film and double-sided adhesive (Figure 1A). Device layers were patterned using a CO₂ laser to define the microchannels, inlets, outlets, and reagent zones.^{42–46} The detection zone is located at the bottom layer of the device and is folded over the outlet region during operation. During assembly, circular glass fiber pads (6 mm diameter) and Whatman Grade 4 filter paper discs (6 mm diameter) were cut and placed into designated regions of the device for reagent pretreatment, as shown in Figure 1A. Sodium 1-naphthol-4-sulfonate (20 μ L) was deposited and dried onto each glass fiber pad. Fe²⁺; 5 μ L and Co²⁺; 5 μ L solutions were deposited and dried onto the paper discs in channels 2 and 3, respectively. Additionally, after drying 1-naphthol-4-sulfonate on the third glass fiber pad, 10 μ L of Co²⁺ solution

was added and dried on the same pad. A glass fiber (2.5mm X 5mm) dried with 0.1M NaF was placed in the middle of the second flow channel as a masking zone. A polyvinyl chloride (PVC) backing was added beneath the detection zone to facilitate image acquisition by providing a uniform, non-reflective background. The fully assembled device measures 6 cm × 4 cm and is reduced to 4 cm × 4 cm after folding along the indicated line. Each microfluidic channel measures 18 mm × 2.5 mm (Figure 1B). Channel 1 serves as a control, while channels 2 and 3 are designated for NDMA detection using Fe^{2+} and Co^{2+} , respectively.

A 3D-printed on-chip holder was developed to facilitate the photonitrosation reaction between NDMA and sodium 1-naphthol-4-sulfonate. The device was fabricated using polylactic acid (PLA) filament via fused deposition modeling (FDM) 3D printing. The design consists of three integrated components: (1) a microfluidic sensor-insertion module, (2) a UV irradiation hinge, and (3) a folding panel (Figure 1C). The sensor-insertion module is designed to securely hold the disposable capillary-driven microfluidic sensor in a fixed position. At the distal end of this module, three inlet ports are aligned to correspond precisely with the three inlets of the microfluidic device, ensuring consistent sample introduction. The UV irradiation hinge incorporates a UV LED strip connected to an external power source. The hinge is positioned such that, when closed, the UV LEDs are aligned directly above the glass fiber reaction zones of the microfluidic sensor, enabling uniform and reproducible UV exposure during the photonitrosation reaction. The folding panel contains three circular apertures (9 mm diameter) that, upon folding, apply constant and uniform pressure across the reaction zones. This controlled folding ensures consistent contact between the metal-ion-impregnated paper pads and the

photonitrosation complex formed on the glass fiber, thereby improving the reproducibility of color signal generation.

5.3.2 Image analysis

Images of the paper pads on the microfluidic device were captured using a smartphone camera under controlled lighting conditions. Image analysis was performed using ImageJ software (NIH, USA). Each image was first separated into its red, green, and blue (RGB) color channels.⁴³ The blue channel was used for NDMA-Fe²⁺ analysis while the green channel was selected for NDMA-Co²⁺ analysis, as these channels exhibited the highest sensitivity for the respective assays (see Appendix D, Figure D1). To minimize background interference, the selected color channel image was inverted before analysis. A circular region of interest (ROI) was defined over each paper pad, and the mean grayscale intensity was calculated. For each device, intensity values were averaged across three ROIs corresponding to the three flow channels. Measurements obtained for the same NDMA concentration were further averaged. Unless otherwise stated, all experiments were performed in triplicate using three parallel channels within a single capillary flow-driven device, and method validation was conducted using three independent devices, each incorporating a control channel along with one Fe²⁺-based detection channel, and one Co²⁺-based detection channel. This standardized image acquisition and processing workflow was effective during assay development and laboratory validation. For on-site or field-based applications, a simplified analysis protocol may be employed, such as the use of a color reference chart or a uniform white background to normalize lighting variations.^{47–49} In addition, the compatibility of the rapid-flow sensor with the RGB Color Detector—a readily available mobile image-analysis smartphone application—was

demonstrated, enabling portable and on-site quantitative analysis when required (Appendix D Figure D2).

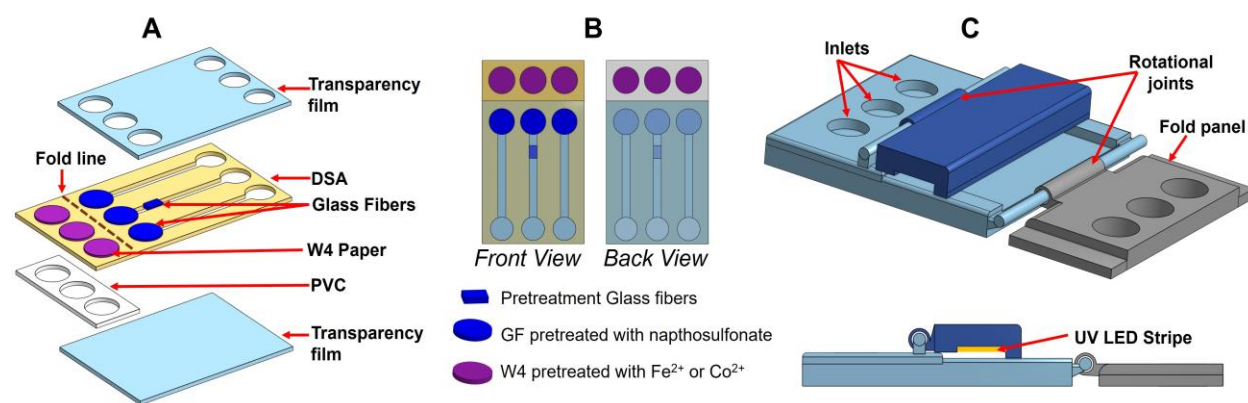


Figure 1. Design and assembly of the capillary-driven microfluidic NDMA sensor and UV irradiation cassette. (A) Exploded view of the three-layer laminated microfluidic device showing patterned PET and double-sided adhesive layers, reagent-loaded glass fiber and paper pads, masking zone, and fold-over detection region. (B) Top and rear views of the assembled sensor illustrating inlet/detection zone alignment and channel layout. (C) Isometric (top) and side views of the 3D-printed UV cassette, highlighting the sensor-insertion module, UV LED irradiation hinge, and folding panel.

5.3.4 Assay principle

The proposed mechanism for the on-chip colorimetric reactions illustrated in Figure 2 is adapted from a previously reported study.⁴¹ Under UV irradiation, NDMA undergoes photolysis to generate nitrosating species that react with the photoacidic sodium 1,4-naphtholsulfonate, resulting in a photonitrosation that converts the naphthol into an ortho-naphthoquinone-oxime via a naphthol-as-acid excited-state proton transfer

mechanism analogous to Chow's naphthol nitrosation chemistry.⁵⁰ The quinone-oxime product on its own is only weakly colored, but its oxime N/O donors and adjacent quinone O atoms form a strong chelating ligand for Fe(II) and Co(II); mixing the ortho-naphthoquinone-oxime with Fe(II) or Co(II) in near-neutral aqueous solution produces a tris-chelate Fe(II) or Co(II) complex, respectively (Figure 2).⁴¹ The tris-chelate Fe(II) complex is green, whereas the tris-chelate Co(II) complex is red.

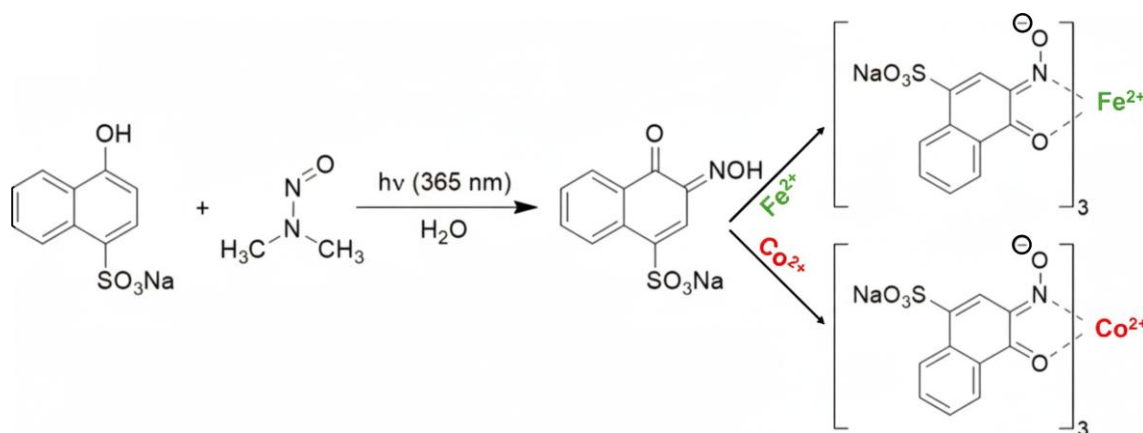


Figure 2. Proposed chromogenic reaction mechanism of NDMA with 1-naphthol-4-sulfonate and Fe²⁺ or Co²⁺. Adapted from ref. 41.

5.4 Results and discussion

5.4.1 Sensor performance

The performance of the capillary-driven microfluidic sensor was evaluated in terms of user-friendliness, multiplexing capability, flow rate, reaction time, time required for color formation, and homogeneity of the colorimetric complex. A key challenge in NDMA detection using the photochemical method is performing multiple chemical reactions on a single chip while preventing reagents from reacting prematurely and ensuring they are available when needed. This was addressed through an on-chip design that uses lateral flow, allowing reagents to be separated within hollow channels and stored without interacting until the test is performed. Moreover, the device is designed so that capillary flow transports the analyte sample upon dipping, enabling rapid flow while minimizing analyte loss due to adsorption, a common limitation of conventional paper-based microfluidic systems.^{29,51} Once the channels are filled, the flow stops automatically, allowing controlled volume delivery without precise pipetting. Another challenge was enabling UV irradiation on-chip, as most miniaturized systems focus on colorimetry, electrochemistry, or qualitative readouts. In this system, the photochemical reaction is followed by colorimetric detection, so incorporating a UV source directly into the hollow channels was not feasible. To address this, a reusable 3D-printed cassette was designed to perform photonitrosation easily. The cassette is inexpensive, user-friendly, portable, and can be reused, allowing the UV step to be carried out on-site.

The microfluidic device's channel geometry was optimized for fast and consistent flow, with a width of 2.5 mm, a detection zone diameter of 6 mm, a sample inlet diameter

of 6 mm, and a channel height of 350 μm (see Appendix D, Figure D3 and D4). Whatman Grade 1, Whatman Grade 3, Whatman Grade 4, and Ahlstrom blotting paper 319 were evaluated for signal intensity and response homogeneity (see Appendix D, Figure D5). Whatman Grade 4 provided the most uniform and reproducible signal and was selected for all subsequent experiments.

Naphthosulfonate pads were primarily composed of glass microfiber to ensure rapid release of immobilized reagents due to their high surface chemistry. Technical specifications for different substrates tested in this study are provided in Table D1 (Appendix D). The concentration of sodium 1-naphthol-4-sulfonate was optimized by evaluating concentrations of 0.25, 0.5, 1.0, 1.5, and 2.0 mg mL^{-1} (Figure 3A). Among these, 0.5 mg mL^{-1} produced the highest color intensity at the lowest reagent concentration and was therefore selected for subsequent experiments. Reaction time was optimized for both Fe^{2+} - and Co^{2+} -based detection by monitoring signal development from 1 to 15 min (Figure 3B). The color intensity increased rapidly within the first 5 min and showed no statistically significant enhancement thereafter; thus, 5 min was chosen as the optimal imaging time. UV irradiation time was optimized by testing exposure durations of 5, 10, 15, 20, 25, and 30 min (Figure 3C). Signal intensity increased up to 20 min and then reached a plateau, indicating completion of the photonitrosation reaction; therefore, 20 min was selected. Finally, the concentrations of metal salts immobilized on the paper pads were optimized by evaluating Fe^{2+} concentrations of 10.25, 20.5, 41, 82, and 164 mM and Co^{2+} concentrations of 8.5, 17, 34, 68, and 136 mM (Figure 3D and 3E). Optimal signal intensity with minimal variation was obtained at 41 mM Fe^{2+} and 17 mM

Co^{2+} , which were used for all subsequent measurements. No further improvements were observed at higher concentrations.

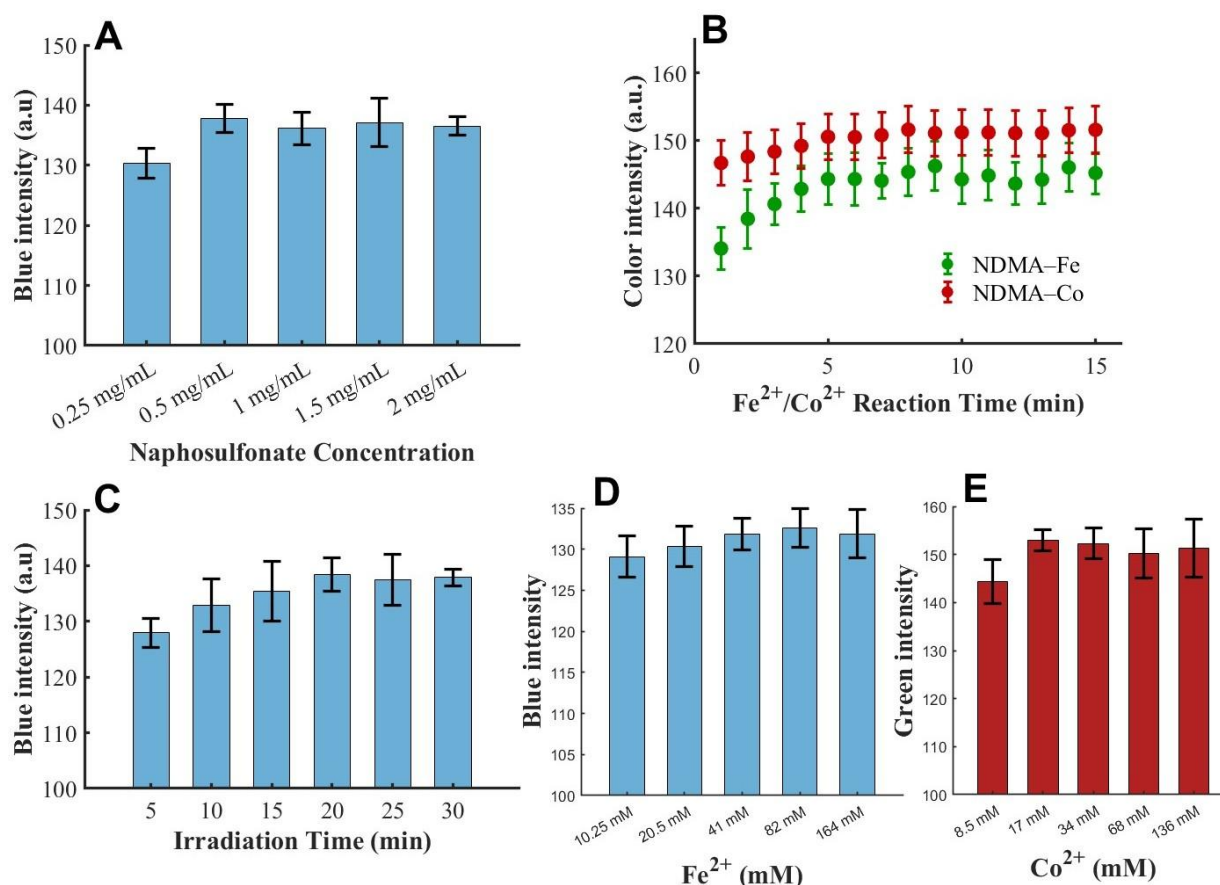


Figure 3. Optimization of assay parameters for paper-based NDMA detection. (A) Effect of sodium 1-naphthol-4-sulfonate concentration on color intensity, with 0.5 mg/mL selected as the optimal concentration. (B) Optimization of reaction time for Fe^{2+} - and Co^{2+} -based assays, showing signal saturation at 5 min. (C) Effect of UV irradiation time on signal development, with a plateau observed at 20 min, indicating completion of the photonitrosation reaction. (D) Optimization of Fe^{2+} concentration and (E) optimization of Co^{2+} concentration on the paper pads to maximize signal intensity while minimizing variability.

5.4.2 Calibration and analytical figures of merit

Calibration curves for the NDMA-Fe²⁺ and NDMA-Co²⁺ assays were constructed by plotting color intensity as a function of NDMA concentration (Figure 4). For the NDMA-Fe²⁺-based detection system, calibration was performed over a concentration range of 0–150 ppm, with a linear response observed from 0–100 ppm (Figure 4A). Similarly, the NDMA-Co²⁺ system was calibrated over a range of 0–100 ppm but exhibited a lower linear dynamic range of 0–30 ppm (Figure 4B). Beyond the respective linear ranges, little to no additional increase in color intensity was observed, as the paper-based detection zones reached maximum color saturation and the signal plateaued. The limits of detection (LoD) and quantification (LoQ) were determined to be 4 ppm and 14 ppm for the NDMA-Fe²⁺ system, and 1.5 ppm and 5 ppm for the NDMA-Co²⁺ system, respectively. The LoD was calculated as three times the standard deviation (SD) of the blank divided by the slope of the calibration curve, while the LoQ was calculated as ten times the SD of the blank divided by the slope ($n = 9$).⁵² Although the LoD values reported here are higher than NDMA concentrations typically found in drinking water, most regulatory detection methods, such as GC-MS or LC-HRMS incur their major costs from the need for sophisticated instrumentation, specialized columns, and highly trained personnel. Therefore, when existing preconcentration approaches are integrated with this portable paper-based sensor, the platform offers a cost-effective and field-deployable alternative that maintains analytical reliability for high-frequency, on-site NDMA monitoring.

Moreover, unlike other reported sensing systems, the capillary flow-driven devices presented here are inherently disposable, simple to operate, and require minimal external hardware. The reagents are strategically separated within the detection zones to prevent cross-reactivity, enhancing the device's long-term stability.⁴⁸ Each sensor is

designed for single use, and the 3D printed cassette is reusable without any maintenance, therefore there is no need for regular maintenance or calibration. Furthermore, unlike electrochemical sensors, the developed platform operates reliably without the need for sample stirring. To further improve its user-friendliness, the colorimetric signal could be processed using a custom-developed or commercially available smartphone application instead of ImageJ. Previous studies support the effectiveness of quantifying colorimetric signals in fast-flow microfluidic devices.^{47,48}

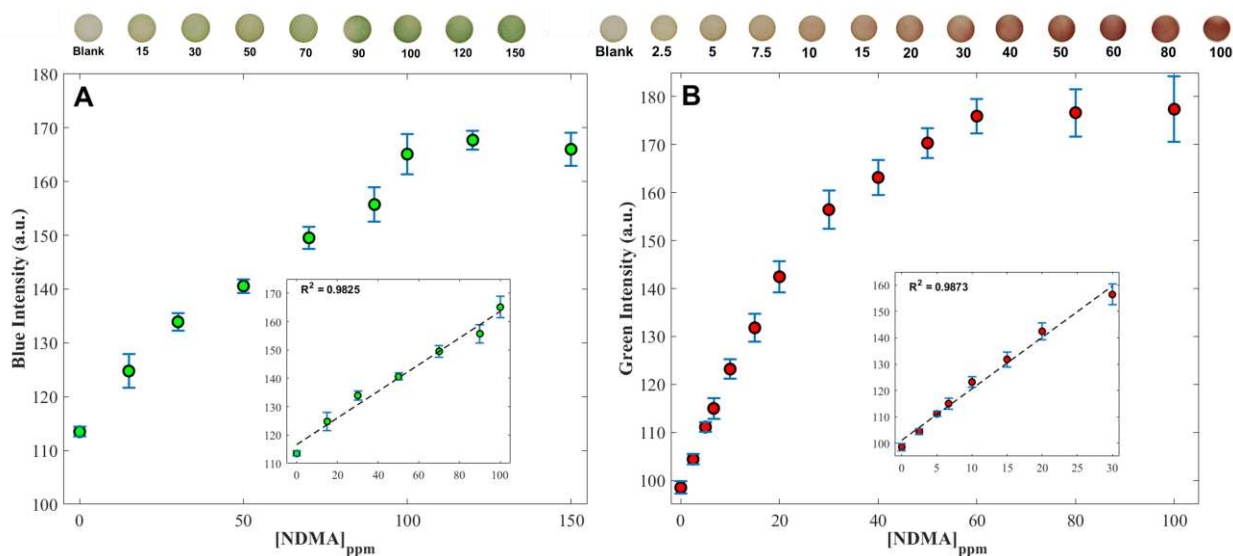


Figure 4. Calibration curves for NDMA detection using (A) NDMA-Fe²⁺ and (B) NDMA-Co²⁺ paper-based assays. Color intensity is plotted as a function of NDMA concentration. The NDMA-Fe²⁺ system was calibrated over 0–150 ppm with a linear response from 0–100 ppm, while the NDMA-Co²⁺ system was calibrated over 0–100 ppm with a linear dynamic range of 0–30 ppm. Error bars represent $\pm$ SD ($n = 3$). The linear equations for the systems were determined as $y = (0.47 \pm 0.03)x + (116.60 \pm 1.73)$ for the NDMA-Fe²⁺ system and $y = (1.95 \pm 0.09)x + (101.10 \pm 1.32)$ for the NDMA-Co²⁺ system.

5.4.3 Interference study

To evaluate potential interference in the NDMA assays, interference studies were conducted for both the NDMA–Fe²⁺ and NDMA–Co²⁺ systems at a 1:1 ratio of NDMA to potential interferents (Figure 5A and 5B, respectively). The following ions and species were examined: Fe³⁺, Li⁺, Na⁺, Pb⁺, Ag⁺, Mg²⁺, Ca²⁺, Ba²⁺, Zn²⁺, Cd²⁺, SO₄²⁻, PO₄³⁻, HCO₃⁻, Co²⁺, Ni²⁺, and Cu²⁺, along with two representative surfactants (Surf 1: sodium dodecyl sulfate, Surf 2: tetramethyl(tetradecyl) ammonium chloride). In the bar plots, green bars represent the control (NDMA only) for the respective reactions, blue bars represent responses from NDMA mixed with potential interferents showing no significant interference, and red bars indicate significant interference. For the NDMA–Fe–based system, significant interference was observed from Cu²⁺ and Co²⁺. Interference from Cu²⁺ was successfully mitigated by pretreating the glass fiber in the second channel with NaF.^{43,53} In contrast, interference from Co²⁺ could not be effectively masked using reported methods in the literature without compromising assay sensitivity. In the presence of Co²⁺, the color response changed from green to red. However, this does not pose a practical limitation because in water samples containing substantial Co²⁺ levels, detection can instead rely on the NDMA–Co²⁺ system, for which no significant interference was observed (Figure 5B). Notably, the color signal produced by the NDMA–Co²⁺ system was sufficiently strong to overshadow any potential interference from Cu²⁺ or Fe²⁺. The use of two detection systems with distinct dynamic ranges, therefore, provides a complementary, hybrid sensing strategy. Although a previous solution-based study reported faint color development from Ni²⁺ in the NDMA–naphthosulfonate assay, no significant interference from Ni²⁺ was observed in either of the paper-based assays.⁴¹ This is likely due to suppression of the weak Ni²⁺-related signal by the much stronger

color responses associated with Fe^{2+} or Co^{2+} on the paper platform, consistent with the very faint color formation reported in solution.⁴¹

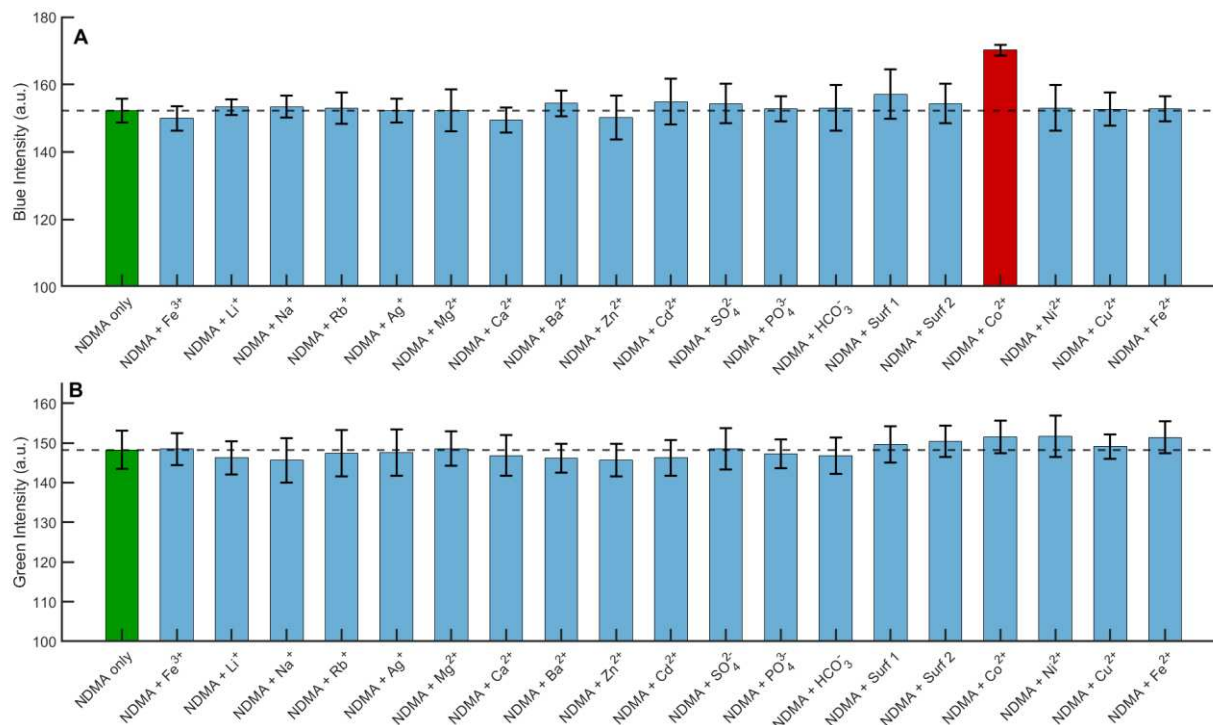


Figure 5. Interference studies for NDMA detection using (A) NDMA- Fe^{2+} and (B) NDMA- Co^{2+} paper-based assays. Assays were performed at a 1:1 ratio of NDMA to potential interferents. Green bars represent the NDMA-only control, blue bars indicate NDMA in the presence of potential interferents showing no significant interference, and red bars denote significant interference. Significances were tested using Dunnett's multiple comparison test ($\alpha=0.05$).

5.4.4 Stability and sustainability

The stability of Fe^{2+} -based (Figure 6A) and Co^{2+} -based (Figure 6B) NDMA sensors was assessed under three storage conditions: room temperature (RT), refrigeration ($5\text{ }^{\circ}\text{C}$), and freezing ($-20\text{ }^{\circ}\text{C}$). Devices were stored in black Mylar bags containing activated molecular sieves, purged with nitrogen, and vacuum sealed. Sensor performance was evaluated over four weeks. Under refrigerated ($5\text{ }^{\circ}\text{C}$) and frozen ($-20\text{ }^{\circ}\text{C}$) storage, both sensor types maintained signal intensities comparable to day-0 values throughout the study period. In

contrast, sensors stored at room temperature lost ~10% of their initial signal over four weeks, with measurable signal degradation observed after the first week. These results indicate that the devices can be batch-manufactured, shipped, and stored for practical field deployment without significant loss of performance. Additionally, the environmental sustainability of the sensor was assessed using the Analytical GREEnness (AGREE) metric, which evaluates adherence to 12 principles of green analytical chemistry, including minimization of reagent consumption and waste, reduction of toxic reagent usage, and ensuring operator safety.⁵⁴ As shown in Appendix D Figure D6, the sensor achieved a greenness score of 0.83, placing it firmly within the “greener” zone and demonstrating its environmentally friendly design.

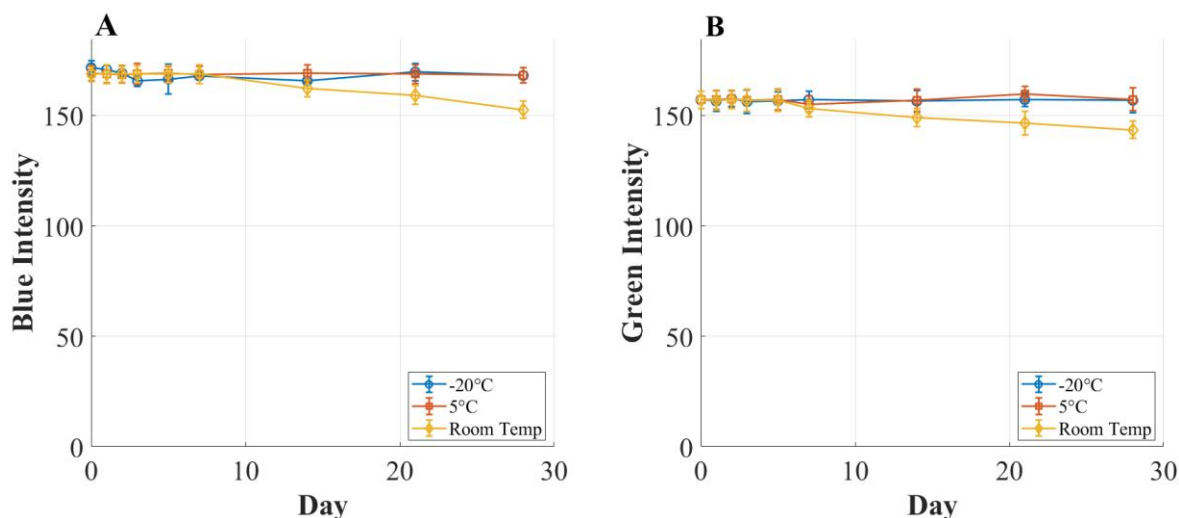


Figure 6. Stability of NDMA sensors under different storage conditions. Stability of (A) Co^{2+} -based and (B) Fe^{2+} -based NDMA sensors stored at room temperature, 5 °C, and -20 °C, with signal intensity monitored over four weeks.

5.4.5 Analysis of real water samples

To evaluate the sensor’s performance in complex matrices, surface water samples were collected from campus tap water, the Cache La Poudre River, and Sheldon Lake (GPS coordinates: 40°35'42.2"N 105°04'32.5"W, and 40°34'56.7"N 105°06'09.9"W,

respectively). Since the natural concentrations of the target analyte were below the sensor's detection limits, spike recovery experiments were performed as an analytical validation method.⁵⁵ Three concentrations of NDMA were spiked into each sample, selected to exceed the sensor's limit of detection (LoD) while remaining below saturation levels. For the NDMA–Fe²⁺–based system, NDMA was spiked at concentrations of 25, 50, and 75 ppm. In tap water, recoveries ranged from 93–95 % with relative standard deviations (RSDs) of 7.8–9.5 %. River water samples showed recoveries of 81–83 % with RSDs between 6.6 and 9.9 %, while lake water samples exhibited recoveries of 76–81 % with RSDs of 5.7–9.0 %. The analytical performance of the NDMA–Co²⁺–based sensor was similarly assessed by spiking NDMA at 5, 15, and 25 ppm to evaluate sensor performance at lower concentration levels. In tap water, recoveries ranged from 95–98 % with RSDs of 6.1–7.4 %. River water samples yielded recoveries of 81–86 % with RSDs between 5.9 and 7.3 %, while lake water samples showed recoveries of 76–80 % with RSDs of 7.5–9.4 %. The recovery values within each specific water type were highly consistent across the tested concentration levels, typically varying within a narrow range ($\approx 5\%$), with RSD <10 %, highlighting the robustness of both detection systems for real-water analysis.

Table 1. Spike recovery results for NDMA in real-water samples using the NDMA–Fe²⁺–based detection system.

Water Type	Spiked (ppm)	Recovered (ppm)	%Recovery	%RSD
	0	ND	-	-
<i>Tap water</i>	25	23.3 ± 2.2	93	9.5
	50	47.2 ± 3.7	94	7.8

	75	71.3 ± 6.5	95	9.2
	0	ND	-	-
	25	20.4 ± 2.0	81	9.9
<i>River water</i>	50	41.3 ± 3.6	83	8.8
	75	62.6 ± 4.2	83	6.6
	0	ND	-	-
	25	19.6 ± 1.8	78	9.0
<i>Lake water</i>	50	40.7 ± 2.3	81	5.7
	75	56.9 ± 4.5	76	7.9

Table 2. Spike recovery results for NDMA in real-water samples using the NDMA–Co²⁺–based detection system.

Water type	Spiked (ppm)	Recovered (ppm)	%Recovery	%RSD
	0	ND	-	-
	5	4.9 ± 0.3	98	6.1
<i>Tap water</i>	15	14.2 ± 1.1	95	7.4
	25	23.8 ± 1.6	95	6.9
	0	ND	-	-
	5	4.3 ± 0.3	86	6.4
<i>River water</i>	15	12.3 ± 0.7	82	5.9
	25	20.4 ± 1.5	81	7.3
	0	ND	-	-
	5	3.8 ± 0.3	76	8.0
<i>Lake water</i>	15	11.6 ± 0.9	77	7.5
	25	20.0 ± 1.9	80	9.4

5.5 Conclusion

In this study, we report the development of the first capillary flow-driven paper-based microfluidic platform for rapid NDMA detection, featuring integrated on-chip photochemical nitrosation. A key challenge in NDMA detection using photochemical methods is performing multiple reactions on a single chip without premature reagent mixing. This was addressed using lateral-flow channels that store reagents separately and

allow controlled analyte transport upon dipping, minimizing loss and eliminating the need for pipetting. Another challenge was enabling UV irradiation for the photochemical reaction, which cannot be integrated directly into the channels. This was solved with a reusable, portable 3D-printed cassette that performs photonitrosation easily and cost-effectively, followed by colorimetric detection on the chip. The sensor operates in two simple steps: dip and fold—without the need for pipetting or specialized instruments for readout. The device achieves detection limits of 1.5 ppm and 4 ppm for Co^{2+} - and Fe^{2+} -based sensors, respectively, with complementary linear ranges extending to 30 ppm and 100 ppm. Dual-channel multiplexing combined with a proper masking strategy provides higher selectivity in real sample matrices. The assay is completed in under 25 minutes, exhibits excellent storage stability, and demonstrates high analytical reliability with RSD <10% in real water samples. With a high greenness score (0.83, AGREE metrics), low device cost (<\$0.20), and straightforward smartphone-based readout, this platform offers a portable, user-friendly, and sustainable alternative to conventional lab-based methods such as GC-MS/MS, enabling decentralized, high-frequency NDMA monitoring. Furthermore, the approach is adaptable for other on-chip photochemical reactions in paper-based and other microfluidic systems that can be used to detect a wide variety of further contaminants.

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CHAPTER 6 – Conclusions and Future Directions

6.1 Conclusion

There remains a clear need for environmental sensing platforms that enable rapid, reliable, and accessible chemical measurements at the point of need, particularly in resource-limited and field settings. Conventional laboratory-based techniques provide excellent sensitivity and selectivity but rely on expensive instrumentation, centralized facilities, and trained personnel, limiting their suitability for high-frequency and spatially distributed monitoring. Meanwhile, many point-of-use paper-based analytical devices suffer from slow transport, limited sensitivity, poor reproducibility, and operational complexity that hinder real-world deployment. The work presented in this dissertation seeks to address these gaps by developing capillary flow-driven microfluidic platforms that preserve passive operation while overcoming key limitations of traditional paper-based systems.

Initial work in this dissertation demonstrated that decoupling fluid transport from in-plane paper wicking through the use of hollow capillary-driven microfluidic architectures enables rapid and homogeneous analyte delivery without external pumps or precise volume handling.¹ This concept was validated through the development of a fast, dip-and-read colorimetric sensor for heavy metal detection, establishing capillary-driven microfluidics as a viable transport medium for environmental analysis. While this approach significantly reduced assay time and improved usability, sensitivity remained constrained by the mass of analyte delivered to the detection zones.

Subsequent chapters addressed this limitation through the integration of passive preconcentration and multiplexing strategies. By combining gravity-driven sample processing with paper-based colorimetric detection, the work demonstrated simultaneous detection of multiple heavy metal ions at low parts-per-billion levels using a simple Fill–Fold–Photo workflow.² This platform achieved regulatory-relevant performance while maintaining low cost, portability, and compatibility with smartphone-based readout, highlighting a practical pathway for extending capillary-driven systems to trace-level environmental monitoring.

The dissertation further demonstrated the translation of these platforms from laboratory validation to large-scale, real-world deployment through the development of a user-friendly phosphate sensor for citizen-based water quality monitoring. Emphasis was placed on operational simplicity, reagent stability under ambient conditions, and robustness to environmental variability.³ The successful deployment of more than one thousand sensors across multiple continents illustrated the scalability of capillary-driven microfluidic systems and their ability to generate high spatial and temporal resolution environmental data through citizen-led initiatives.

Finally, this work expanded the scope of capillary-driven paper-based microfluidics beyond direct detection by integrating on-chip chemical transformation for the analysis of N-nitrosodimethylamine (NDMA), a highly toxic and emerging water contaminant. By implementing photochemical nitrosation directly on-chip and coupling it with dual-channel colorimetric detection, this chapter demonstrated the first complete photochemical–colorimetric workflow on a paper-based microfluidic platform. This achievement establishes a foundation for extending capillary-driven microfluidic systems

to a broader class of contaminants that require derivatization or chemical activation prior to detection.

Taken together, the work presented in this dissertation establishes capillary flow–driven microfluidics as a unifying design framework for point-of-need environmental sensing. By combining passive transport, thoughtful device engineering, reagent stabilization, and simplified user operation, these platforms bridge the gap between laboratory analytical performance and real-world usability. Beyond the specific analytes studied here, the approaches developed are broadly adaptable and provide a foundation for future environmental, public health, and decentralized chemical monitoring technologies.

6.2 Future directions

6.2.1 Laminate capillary-driven microfluidics for 6PPD detection in water

N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (6PPD) is an emerging environmental contaminant originating from vehicle tires and other rubber products.^{4,5} Mechanical abrasion during road use releases 6PPD-containing particles into the environment, where subsequent reaction with ozone forms 6PPD-quinone, a highly toxic transformation product implicated in aquatic toxicity, particularly in salmon species.^{6,7} Given the ecological importance of aquatic systems and the role of fish in global food security, there is a growing need for simple, field-deployable methods to monitor 6PPD and related contaminants in water.

To address this need, the laminate capillary-driven microfluidic framework developed in this dissertation was extended to the detection of 6PPD. The device consists of layered polyethylene terephthalate (PET) film and double-sided adhesive (DSA), with laser-defined inlet and flow channels terminating in absorbent paper detection zones. The paper substrates are pretreated with potassium persulfate ($K_2S_2O_8$), enabling a colorimetric response upon interaction with the analyte.⁸ During operation, the device is simply dipped into the sample, and capillary action drives fluid through the channels to the detection zones, producing a visible color change.

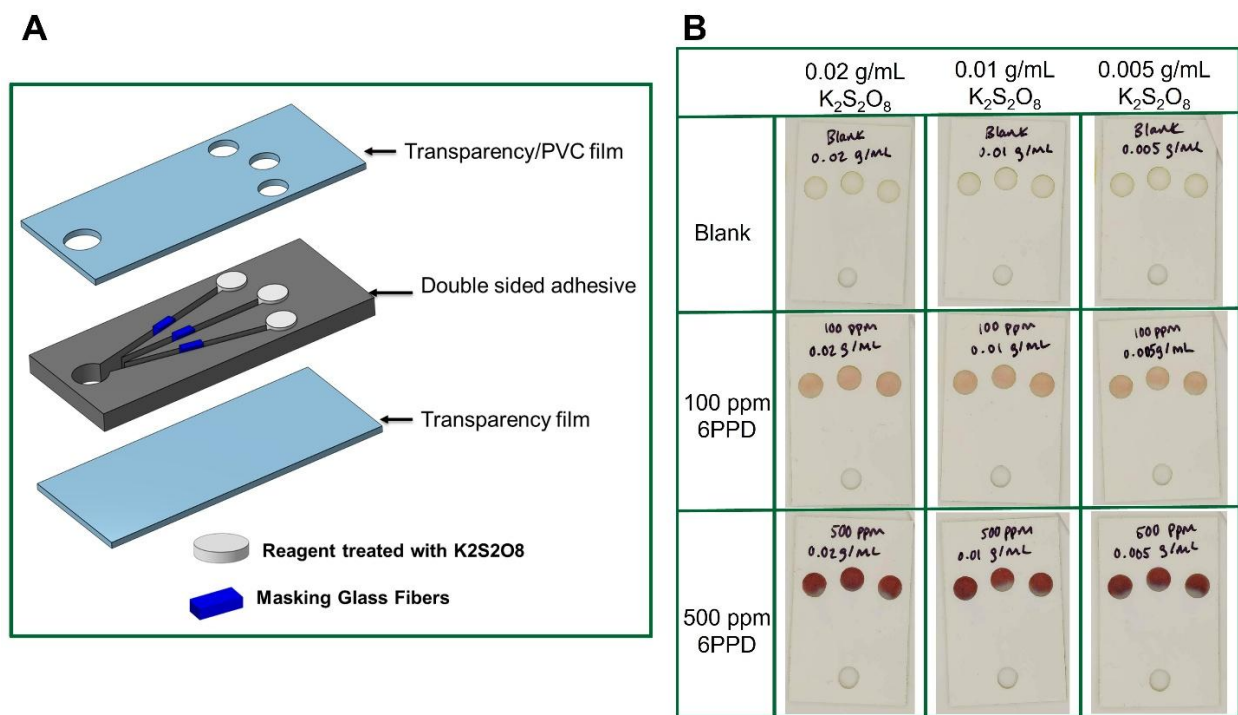


Figure 1. (A) Assembly of the laminate capillary microfluidic device with PET and DSA layers and paper detection zones. (B) Preliminary colorimetric detection of 6PPD showing concentration-dependent signal gradients.

Preliminary results demonstrate that 6PPD can be detected using this approach, with observable color gradients across concentrations (Figure 1B). However, further optimization is required to improve selectivity and enhance quantitative performance. Future work will focus on refining channel geometry, reagent loading, and substrate selection strategies. Integration with the preconcentration strategies developed in Chapter 3 may be necessary to achieve detection limits relevant to environmental guidelines.

6.2.2 Advancing sample pretreatment on-site

While the sensing platforms developed in this dissertation enable rapid and reliable detection of a wide range of contaminants in water, it is important to recognize that the measurements primarily target labile species—those present in readily detectable ionic or reactive forms. In natural waters, however, a significant fraction of heavy metals, nutrients, and other contaminants exists in non-labile forms, including species complexed with natural organic matter, adsorbed to particulate or mineral surfaces, incorporated into colloids, or bound within stable inorganic or organometallic complexes. As a result, direct analysis without prior sample processing can underestimate total contaminant concentrations and, in some cases, lead to false negatives in field measurements.

In conventional laboratory workflows, conversion of non-labile species into detectable forms is typically achieved through acid digestion, often assisted by microwave or high-temperature reactors.^{9–11} While effective, these methods rely on expensive instrumentation, hazardous liquid acids, and trained personnel, making them impractical

for on-site or resource-limited applications. Consequently, most portable sensing technologies assume that analytes are already present in labile forms, representing a critical bottleneck in translating laboratory-grade analytical accuracy to field-deployable systems.

To address this limitation, ongoing work has focused on the development of a fully integrated, portable sample pretreatment platform designed to enable safe, on-site conversion of non-labile metal species into sensor-compatible ionic forms. The central innovation involves coupling a battery-powered thermal digestion module with encapsulated solid acid reagents, eliminating the need to transport or handle corrosive liquid acids in the field (Figure 2). In this approach, premeasured p-toluenesulfonic acid capsules are activated under controlled thermal conditions to liberate metals bound to organic and inorganic matrices, providing sufficient proton donation for effective digestion while maintaining a closed and user-safe system.

Following digestion, on-device pH adjustment is achieved through encapsulated buffering agents, such as sodium bicarbonate, producing solutions compatible with downstream colorimetric or electrochemical platforms. The encapsulated chemistry approach ensures reproducible reagent dosing, long-term storage stability, and minimal user intervention. Importantly, the closed-loop design reduces exposure to acidic vapors and enhances operational safety during field use. As shown in Figure 2B, 2C, 2D, and 2E, effective on-site digestion enabled the conversion of non-labile Fe (B), Cu (C), Ni (D), and Pb (E) into labile, sensor-detectable species.

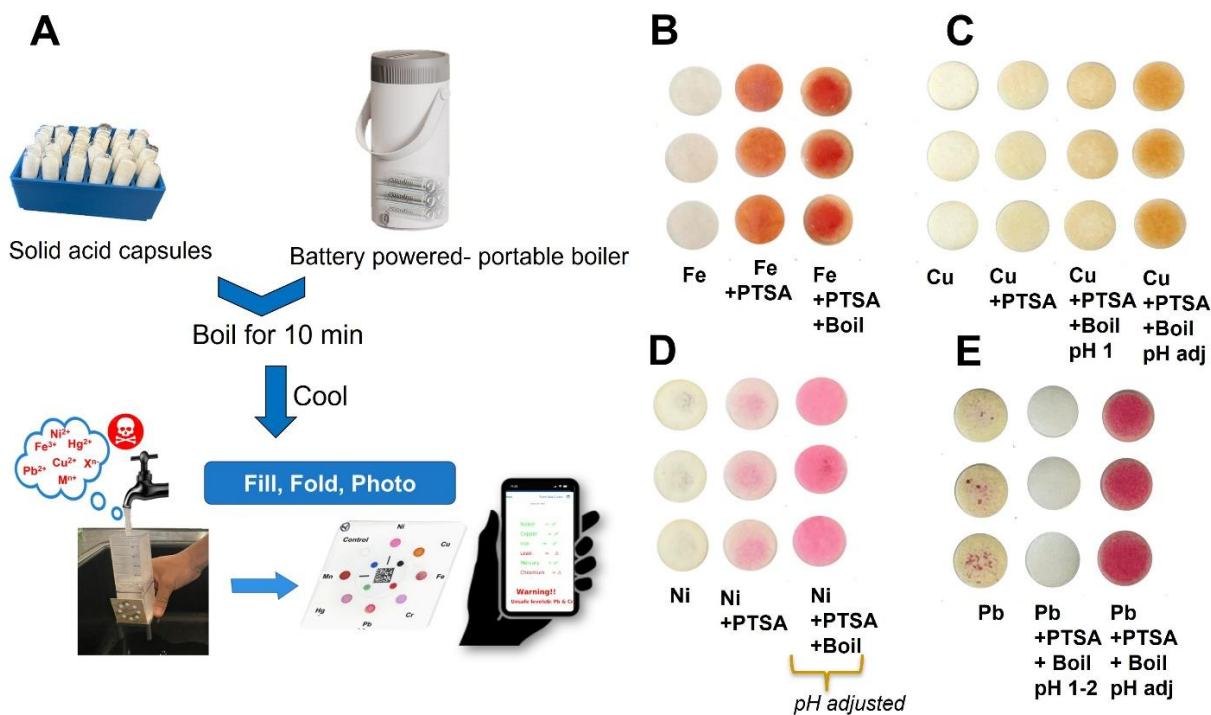


Figure 2. (A) Schematic workflow of the portable, on-site acid digestion system integrating thermal digestion, encapsulated acid delivery, for sensor-compatible analysis. (B–E) Demonstration of successful digestion and conversion of non-labile (B) Fe, (C) Cu, (D) Ni, and (E) Pb into labile, detectable forms.

By integrating sample digestion, pH modulation, and sensor-ready output into a single portable system, this approach addresses one of the most significant unmet needs in field-deployable environmental analysis. Advancing on-site sample pretreatment has the potential to transform point-of-need sensing by enabling regulatory-relevant total metal measurements, rather than limiting analysis to labile fractions alone. Future integration of this pretreatment platform with the capillary-driven microfluidic sensors developed in this dissertation represents a critical step toward achieving comprehensive, laboratory-grade environmental analysis entirely in the field.

6.3 Toward field-deployable environmental monitoring systems

A key remaining challenge for truly field-deployable environmental monitoring systems is achieving sufficient sensitivity for analytes with extremely low regulatory limits. Contaminants such as PFAS, 6PPDQ, NDMA, and toxic metals including Pb and Hg often have maximum contaminant levels (MCLs) in the low ppb or even ppt range, which are difficult to detect using direct, on-site colorimetric or portable sensing approaches alone. As a result, future development of capillary flow-driven and paper-based microfluidic platforms must incorporate integrated preconcentration strategies to bridge this sensitivity gap. Coupling these portable devices with on-site preconcentration methods such as sorbent-based extraction, electrostatic enrichment, or membrane-assisted accumulation can enable sufficient analyte enrichment prior to detection while maintaining the simplicity and accessibility required for field use. The integration of such preconcentration modules with rapid, user-friendly sensing platforms is therefore essential to enable real-time, in-field quantification of ultra-trace contaminants and to fully realize the potential of decentralized environmental monitoring systems.

A persistent challenge in the development of point-of-need (PoN) sensing technologies lies not in the lack of innovation, but in the disconnect between academic method development and real-world implementation. Within academia, there is often an implicit assumption that the primary role of researchers is to develop new analytical methods or demonstrate proof-of-concept devices, while the responsibility for translation, validation, and deployment is left to industry. While this division of labor has merit, it has also resulted in many promising technologies remaining confined to academic publications, producing limited tangible impact beyond scholarly recognition.¹²

This challenge is particularly evident for μ PADs and other portable sensing platforms. Although such systems are frequently designed with field use in mind, early field evaluations have revealed recurring barriers, including inadequate sample preparation, limited quantitative accuracy, batch-to-batch variability, short reagent shelf life, reliance on controlled readout conditions, usability challenges for non-expert users, high production costs, and the complexity of regulatory approval. Importantly, addressing these challenges often requires expertise that extends beyond traditional academic training in chemistry or biology. This imbalance reflects a broader structural issue: the skillsets required for successful field deployment—logistics, user training, data validation under uncontrolled conditions, and stakeholder engagement—are rarely emphasized in academic research programs. In contrast, public health professionals, environmental scientists, and practitioners experienced in fieldwork often possess complementary expertise that is critical for successful implementation.

Despite these challenges, the work presented in this dissertation demonstrates that meaningful progress toward field translation is possible within an academic framework. The successful deployment of more than one thousand phosphate sensors across multiple continents, enabled through collaboration with citizen scientists and local partners, illustrates that academic research can move beyond laboratory validation when field considerations are incorporated early in the design process. While this effort does not fully resolve the broader translational gap, it represents a constructive step toward demonstrating feasibility and impact.

Moving forward, bridging the gap between academic innovation and real-world adoption will require deliberate, collaborative efforts. Increased partnerships between

academic researchers, public health experts, environmental agencies, and community stakeholders are essential to ensure that sensing technologies address real operational constraints and data needs. Equally important is the integration of field evaluation, deployment logistics, and user-centered design training into academic curricula for students developing PoC and PoN technologies. By fostering interdisciplinary collaboration and redefining success beyond laboratory performance metrics alone, future sensing platforms can achieve broader adoption and deliver meaningful societal and environmental impact.

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Appendix 1—Smartphone-Enabled Green Anthocyanin Sensor for Fe(III) Sensing on Paper Using Capillary-Driven Microfluidics

Abstract

Chemical wastes, such as synthetic dyes and coloring agents, pose significant concerns due to their detrimental impact on human health and ecosystems. As an eco-friendly alternative, the study of natural dyes and coloring agents has gained momentum over the last decade, owing to their biodegradability and reduced environmental and health risks. Here, we report a novel approach employing a butterfly pea anthocyanin paper-based sensor to quantify Fe(III) ions in water. Compared to previous green sensing techniques reliant on heavy instrumentation, this approach offers enhanced accessibility, cost-effectiveness, and user-friendliness. Notably, this study is the first example of natural dye sensing on a capillary microfluidics system. The capillary-driven microfluidic system ensures user-friendly operation and allows the sensing platform to be directly immersed in water without reagent loss. The device's design involves drying the butterfly pea-derived anthocyanin reagent within a pH-controlled paper substrate embedded in the capillary-driven microfluidic system. Smartphone-based colorimetric signal generation and quantification were achieved within seconds for a working range of 50-800 mg/L, with the device's detection limit of 43 mg/L using ImageJ and 55 mg/L using the smartphone app. This green sensing methodology has the potential to be used for groundwater sources with elevated Fe(III) levels. Additionally, this platform can be used in chemistry educational settings as an effective means to understand fundamental concepts such as acid-base reactions, base strength, ionic equilibrium, and metal

complexation in a user-friendly microfluidic system. This work has been published in *Green Analytical Chemistry* 2024, 8, 100091.

Introduction

Iron is a ubiquitous heavy metal widely present in the natural environment and living organisms. In the human body, iron is vital in facilitating hemoglobin synthesis, cellular metabolic transformations, enzymatic activities, and the production and repair of DNA and RNA.^{1,2} However, maintaining optimal iron distribution within the human body is paramount due to the potential deleterious outcomes of iron imbalance. Inadequate iron levels lead to iron-deficiency anemia, characterized by fatigue, compromised immune response, and impaired oxygen delivery to cells.^{3–5} Conversely, excessive iron accumulation is implicated in the onset of severe diseases like hepatitis.⁶ Oxidative damage inflicted by these surplus iron ions upon nucleic acids, lipids, and proteins results in long-term organ impairment, coma, hemorrhagic disorders, hepatic insufficiency, and fatality.⁷

In water, iron generally exists as ferrous Iron (Fe^{2+}) and oxidizes to ferric Iron (Fe^{3+}) under environmental conditions such as pH or air.⁸ Given the rising utilization of iron-containing materials in hydrometallurgical and various industrial applications, such as steel production, mining, wastewater treatment, chemical processing, foundries, etc., the need to develop effective strategies for detecting trace iron levels, especially in water samples, becomes even more pressing.^{9–11} To achieve high-frequency monitoring, it is essential to devise user-friendly, cost-effective, selective, and environmentally benign techniques for assessing and monitoring iron concentrations in water.

Among different detection techniques for iron,^{12–16} the colorimetric approach has several distinct advantages, including lower costs, simple instrumentation (or even the absence of equipment for direct detection), and the ability to perform qualitative or semi-quantitative assessments through unaided observation.¹⁶ Detection of iron using colorimetry has been performed using several chemical reagents, such as thiocyanate, 2,2-bipyridyl, and phenanthroline and its derivatives.^{17–21} These straightforward processes require fewer chemical reagents, are simple to produce, and can be used in many applications to detect environmental pollutants. But these synthetic reagents can also become a source of pollution as they can bioaccumulate in the environment through different mechanisms and disrupt entire food chains and ecosystems.^{22,23} As a result, there is a pressing need to replace the synthetic dyes used for different tests with dyes derived from natural sources to minimize further harm to ecosystems and the environment.²⁴ These eco-friendly alternatives can be sourced from plants, minerals, or other renewable materials, ensuring minimal adverse effects on the ecosystem and human health.

Anthocyanins are the natural color pigments found in different fruits and vegetables. These pigments are members of the flavonoid family and are characterized by a positively charged oxygen atom on the C-ring of their basic structure. The stability of anthocyanins is intricately tied to various factors, including pH, light exposure, temperature, and their molecular structure.²⁵ Anthocyanins have recently been studied a lot as metal ion sensors because of their potential use as green and sustainable colorimetric sensors.^{26–28} As one of the subclasses of phenolic phytochemicals, Anthocyanin can respond to environmental changes and can cause complexation with metal ions, making it an excellent research subject in green analytical sensor research.^{29,30}

The *Clitoria ternatea* flower, also known as the butterfly pea flower, is a particular type of plant that contains natural blue pigments. In Asian countries, *Clitoria ternatea* is frequently used as a natural colorant in foods, beverages, and snacks.^{30,31} It is hypothesized that ternatin, an anthocyanin component in *Clitoria ternatea*, is the source of the blue Anthocyanin in the flower extract.³² Ternatin consists of malonylated delphinidin 1, 3O, and 5O-triglucosides with side chains of D-glucose and p-coumaric units, and its high degree of glycosylation makes the stability of ternatin higher.³³ The conjugated bonds in Anthocyanin are responsible for the change in their pigments under different conditions.³⁴

Anthocyanin-based paper sensors have emerged as versatile analytical tools for a wide range of applications in environmental monitoring, diagnosis, food quality control, process intensification, and more. These sensors have been successfully used to detect ammonia and heavy metals in environmental samples,^{35,36} to monitor bacteria for diagnostic purposes,³⁷ assess liquid food quality by measuring pH changes,^{38,39} as well as temperature,⁴⁰ and other applications.^{41,42} These works collectively showcase the significance of anthocyanins in paper-based analytical systems, offering green and innovative solutions for a variety of analyte detection challenges. However, a noteworthy limitation across many of these systems is their reliance on either manual pipetting steps or the use of resource-intensive laboratory instrumentation for signal quantification. This approach may not align seamlessly with the in-situ measurement principle advocated in green analytical chemistry. Furthermore, the necessity of user-driven pipetting and the demand for energy-intensive tools for detection could pose significant challenges in resource-constrained settings where such resources are not readily accessible for on-site operations.

El-Nagger et al. developed a rapid colorimetric sensor using polyvinyl alcohol microfibers and anthocyanin extract from red cabbage to detect ferrous ions in aqueous media.²⁷ Khattab et al. also developed a cellulose dipstick using the same extract, utilizing chitosan nanoparticles as a hosting biopolymer.²⁸ Both methods provide a fast, responsive, and environmentally friendly sensor for detecting Iron levels in water and have great potential to replace the synthetic dyes for Iron sensing in aqueous media while using spectrophotometry to quantify the ions. Spectrophotometry is a widely used technique for quantitative and qualitative analysis of various compounds. However, its application in field settings is limited due to the need for specialized equipment and trained personnel. The use of absorbance-based anthocyanin sensors, relying on the absorbance spectra, further complicates the analysis, making it inaccessible for untrained users in resource-limited settings.

To address the constraints observed in previous green sensing techniques, we propose an alternative strategy employing a colorimetric quantification method. This approach can be conveniently executed on paper pads and analyzed using readily accessible image processing software, enabling a simplified and efficient "click and quantify" procedure. This approach eliminates the need for specialized equipment and trained personnel, making it more accessible, cost-effective, and user-friendly for on-site quantification. The ImageJ app and other smartphone applications, such as RGB color detectors and MATLAB smartphone apps, can further simplify and enhance the analysis. When compared to expensive instruments and laborious operating procedures, such as inductively coupled plasma mass spectrometry (ICP-MS), atomic absorption spectrophotometer (AAS), inductively coupled plasma optical emission spectroscopy

(ICP-OES), and UV-vis spectrometry, our method is more user-friendly, environmentally sustainable, and economically affordable (<50 ¢).^{43–45}

In addition to these advantages, the fast capillary-driven flow system allows up to 145 times faster flow than traditional paper-based flow channels,⁴⁶ drastically reducing the assay time from 10+ minutes to seconds. The capillary-driven microfluidic system allows the detection pad to remain separate from the water source, minimizing the reagent loss and interference and allowing the use of stable buffers for optimal pH. Additionally, running quadruplets in a single device eliminates the need for multiple dipsticks, reducing waste and simplifying the analysis process. Overall, our approach offers a novel and innovative solution to the limitations of current anthocyanin sensors, providing a faster, cost-effective, user-friendly, and environmentally safe alternative for the detection and on-site quantification of Fe(III) ions.

This study aimed to use capillary-driven microfluidics containing a paper impregnated with Anthocyanin from *Clitoria ternatea* flower extract to create a green, sustainable, and user-friendly colorimetric sensor for detecting Fe (III) in aqueous samples. Here, we report the first use of *Clitoria ternatea* flower extract on hollow capillary-driven microfluidic devices to detect Fe(III) ions in an aqueous medium, in which the device produced a consistent color signal within seconds of sample addition. Validation of the sensor's performance was conducted through spike recovery analysis in various surface water matrices, affirming its reliability. Notably, the sensor achieved a greenness score of 0.88 out of 1 and demonstrated excellent stability over a 3-week study period under ambient conditions. Moreover, our smartphone-enabled on-site quantification system produced consistent results, comparable to traditional ImageJ

software. This innovative capillary flow green sensor has the potential to advance the field of water contaminant detection while being in line with the principles of green chemistry.

Experimental

Materials and chemicals

Metal standard (Ferric Chloride Hexahydrate), all interfering ions, and buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Whatman paper was purchased from Fisher Scientific, and the double-sided adhesive (468MP) and transparency film (9984) were purchased from 3M (St. Paul, MN, USA). The *Clitoria ternatea* flower was obtained from Yogyakarta, Indonesia.

Instrumentation

Fourier transform infrared spectroscopy analysis (FTIR, Vertex 80, Bruker, Germany) was performed to evaluate the functional groups in the *C. ternatea* extract at a wavelength of 4000-600 cm^{-1} . The GENESYS 150 UV-Vis Spectrophotometer was used for metal complexation analysis. An Epilog Engraver WinX64 Zing laser cutter was used to cut the double-sided adhesives, transparency films, and Whatman paper detection pads. The images for ImageJ were scanned using the Xerox DocuMate 3220 Scanner.

The extraction of Anthocyanin

Flowers of *Clitoria ternatea* were collected from Sleman Regency, Yogyakarta Province, Indonesia. The flower was dried, powdered, and macerated with ethanol 70% (1:10 w/w) at room temperature for 24 hours. The mixture was filtered and evaporated, and a crude

extract of *Clitoria ternatea* flower containing Anthocyanin was obtained. The anthocyanin content in the *Clitoria ternatea* flower extract was measured using a UV-visible spectrometer using the following equation: ⁴⁷

$$A = (A_{520} - A_{700})_{pH_{1.0}} - (A_{520} - A_{700})_{pH_{4.5}} \dots \dots \dots \text{Eq 1}$$

$$\text{Total anthocyanins (mg/L)} = (A * M_w * DF * 1000) / (\epsilon * l) \dots \dots \dots \text{Eq 2}$$

where A = absorbance; M_w = molecular weight of cyanidin-3-glycoside; DF is the dilution factor; ϵ is the molar absorptivity; and l is the optical path length.

The monomeric anthocyanin content in the *Clitoria ternatea* extract was 569.99 ± 7.71 mg/L.

Characterization of the extract

A flavonoid-specific test based on Shinoda's approach was conducted to characterize the anthocyanin extract (Figure S8, Supplementary information). The test involved adding four magnesium fillings (in ribbon form) to the ethanolic extract and a few drops of concentrated hydrochloric acid. The reddish color indicated the presence of anthocyanins in the extract.^{48–50} FTIR (Fourier Transform Infrared Spectroscopy) and UV-vis (Ultraviolet-Visible) spectroscopy were also performed for further characterization and confirmation of complexation.

Device development

The device consists of four 20 mm long and 2.5 mm wide flow channels made of stacking transparency film (9984 polyester film) and double-sided adhesive (468MP). At the end of each flow channel, 6 mm detection (diameter) pads are placed. Each channel has a sample inlet (4 mm diameter) through which the analyte solution is added. The center of the device has a 6 mm diameter color reference zone that can be used in case the sample solution is already colored. This design allows quantification in quadruplicates within the

same device, facilitating assay simplicity. The device's shape was designed through Onshape, exported into CorelDRAW, and cut using a laser cutter. Following device assembly, anthocyanins are pipetted into the detection reservoir and allowed to air-dry. This means that the measurement process only requires one simple user action – adding the sample. Side-view assembly, and sensor's operation is shown in Figure 1.

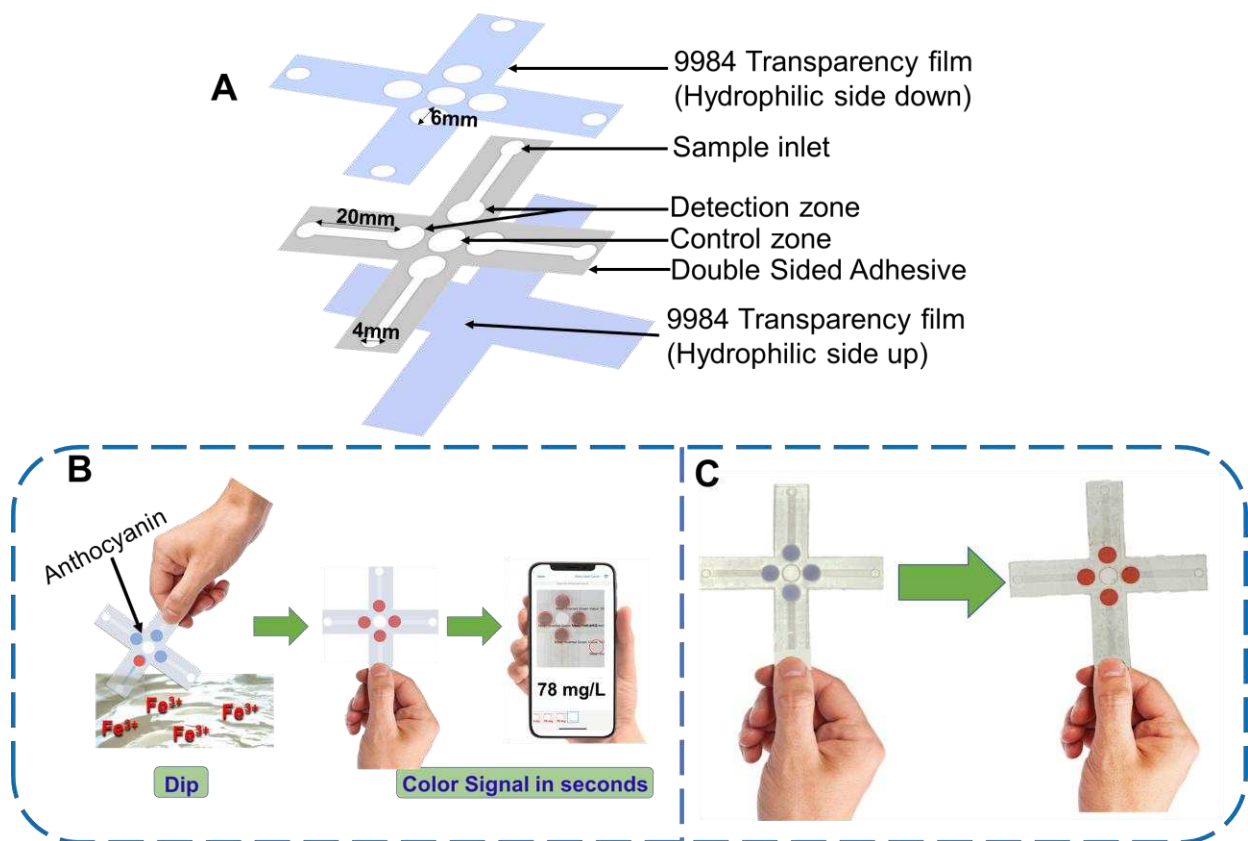


Figure 1. Device Schematic (A), the device running, and quantification top view(B), and real sensor top-view color response (C)

Colorimetric metal detection

Anthocyanins' dihydroxyl group serves as an essential chelating site, where the lone pairs of electrons from the oxygen atoms coordinate with the central Fe^{3+} ion.^{51,52} The extent of color depends on the pH condition and the amount of free Fe(III) available to bind. Fixing the pH condition on the paper by drying a stable buffer on the reaction pad allowed the

colorimetric quantification of Fe(III). The standard Fe(III) solution was prepared to 1000 mg/L in Millipore water. 4 μ L of anthocyanin reagent extract (50 mg/mL) prepared in PBS buffer (0.1 M) was added to the detection reservoir. The device was allowed to dry for 5 minutes. 15 μ L of the sample was then added to each sample inlet. The device changed the color of the pretreated blue extract to red within seconds of sample addition.

Image analysis

For the analysis using ImageJ, once the analyte solution was loaded onto reagent cards, the device was allowed to dry before imaging. After drying, the device was scanned using a flatbed scanner at 600 DPI (no extra lighting adjustments) and exported to ImageJ in JPEG format. The image was divided into blue, green, and red channels and inverted. A circular tool was used to measure the complex's average grayscale intensity. Among the channels, the green channel produced the maximum sensitivity and was chosen for calibration. Conversely, for the calibration using the MATLAB Phone app, the image was taken in a light-controlled box and quantified using our code in the app within seconds.

Results and discussion

FTIR and UV-Vis analysis of *Clitoria ternatea* flower extract

FTIR spectroscopy was used to analyze the functional groups of chemical constituents in the samples. The FTIR of *Clitoria ternatea* flower extract is presented in Fig S10. This study analyzed the *Clitoria ternatea* extract under 600-4000 cm^{-1} . The functional groups are extensively analyzed in the Supplementary Information S10. The overall FTIR results suggest that *Clitoria ternatea* ethanolic extract consisted of phenolic and flavonoid groups.

UV-Vis spectroscopy was used to study the effect of Fe (III) concentrations on anthocyanin extract spectra. The spectra were measured for Anthocyanin alone, Fe(III) alone, and with increasing Fe(III)— Figure 2 displays spectra revealing concentration-dependent absorbance increase, indicating Fe-anthocyanin complex formation. UV-Vis spectra exhibit distinct absorption peaks corresponding to anthocyanin transitions, with notable absorbance rise as Fe(III) concentration increases. Enhanced absorbance suggests interaction-induced complex formation with higher light absorption than individual components.

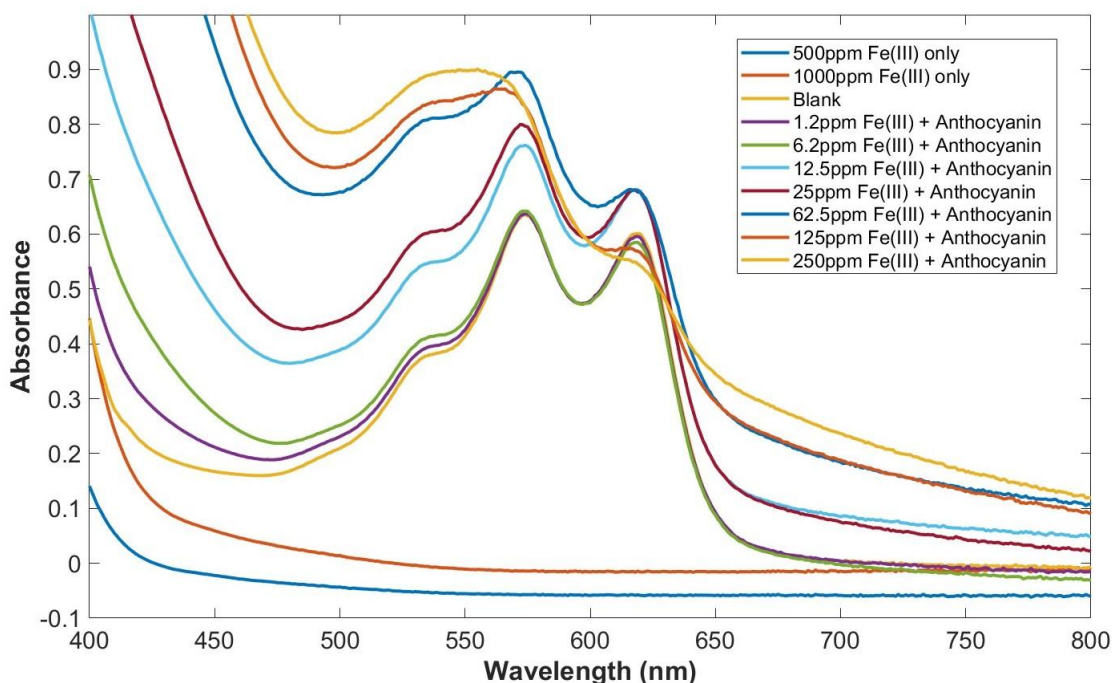


Figure 2. Effect of different concentrations of Fe^{3+} on UV-visible spectra of CT

Device performance

The device's performance was thoroughly evaluated based on two key factors: the homogeneity of the complex and the assay time. The device's flow channel width was

optimized to achieve the maximum sample flow rate, with the best flow rate observed at a channel width of 2.5 mm (Figure S3, Supplementary information). The pH condition was also optimized to ensure optimal differentiation between the blank and the analyte (Figure S5, Supplementary information). Due to the capillary action of the flow channel created, the device can be dipped into the sample solution without needing pipetting. This feature enhances the device's user-friendliness and supports its green design. The flow rate was consistent throughout the flow channels in the device. When tested, all devices passed the flow into the detection reservoir within 4 seconds of sample addition (n=10). The analytes used in this experiment were colorless water samples; however, if the surface water sample has some color, the middle circle can be used as a color reference. The plant extract volume was also optimized to attain the maximum color signal and homogeneity on the detection pads (Figure S4, Supplementary information).

Calibration

ImageJ: A quantitative calibration was done between 50-1000 mg/L. A linear range was established between 50 and 800 mg/L. Above 800 mg/L, the color was saturated, yielding the same grayscale intensity. The limit of detection was calculated to be 43 mg/L. LOD was calculated by dividing 3.3 times the blank's standard deviation (SD) by the calibration curve's slope (n=8).⁵³

Smartphone app: A quantitative calibration was obtained from 50 mg/L to 800 mg/L using a readily available smartphone app called Matlab mobile (Figure 3). The sensor's image was taken in a lightbox, and upon running our code, it identifies the circular region of interest, creates a binary mask, and quantifies the average grey value within that region. Additionally, it calculates the average mean value of the background using a fifth region

of interest (ROI) circle, allowing for the normalization of the mean grey values obtained from the sensor. This normalization accounts for variations in lighting conditions during image capture, enhancing the method's applicability in field settings. The limit of detection was calculated to be 55 mg/L. Although these results are higher than those of the previous studies,^{18,54} they offer a different strategy for metal detection that is expected to be more sustainable and environmentally friendly. The developed green sensor exhibits compatibility with various imaging applications, such as the RGB Color Detector, which is readily accessible in the app stores.

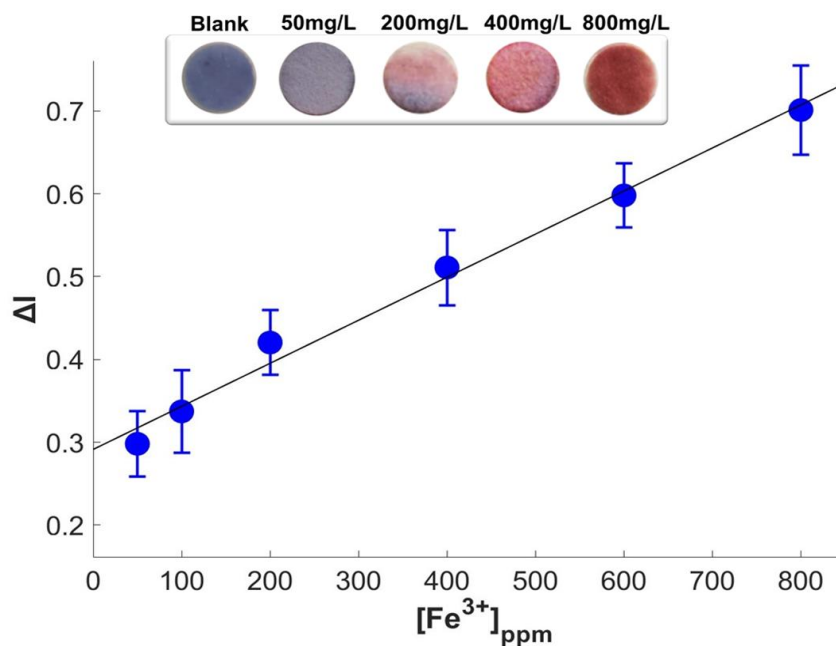


Figure 3. Qualitative calibration (top) and quantitative calibration (bottom) using smartphone application (linear fit- $y = 0.00052 \pm 0.00015 * x + 0.2913 \pm 0.0455$, $R^2 = 0.9897$). Error bars represent the standard deviations of respective intensities ($n=4$). The "lack of fit test" showed that the calibration model adequately represents the data.

Interferences

Anthocyanin reagent is prone to interfering factors like pH and aqueous ions.^{25,53,55,56} To study the effect of non-targeted ions, interferences from nine common ions (Cu^{2+} , Mg^{2+} ,

Co^{2+} , SO_4^{2-} , Ni^{2+} , Zn^{2+} , HCO_3^- , Ca^{2+} , Cr^{6+}) were analyzed by testing the change in color intensity when mixed with Fe(III) (Figure 4). When using a lower buffer concentration (1 mM), metallic ions like Zn^{2+} , Ca^{2+} , and Cr^{6+} altered the pH, changing the color intensity. The buffer concentration was increased (0.1 M) to minimize this problem. HCO_3^- significantly decreased the grayscale intensity of the blank. However, when mixed with Fe(III), the Fe(III) color dominated the signal, yielding the same intensity as Fe(III) alone. This result suggests the device is more suitable for water sources where the Fe(III) concentration is relatively higher than the bicarbonates. This outcome is in line with earlier research, which revealed that *C. ternatea* extract in an aqueous medium has no significant color change in the presence of cations, while in the presence of anions, the visual color change from blue to purple in the presence of HSO_4^- ion, and the color change from blue to green in the presence of HCO_3^- and CO_3^{2-} .⁵⁷ Because anthocyanin pigments are affected by pH, the color change could be due to a change in the solution's pH from added sodium bicarbonate that raised the pH of the anthocyanin to an alkaline level.⁵⁸ Although high concentrations of Zn^{2+} , Cr^{6+} , and HCO_3^- caused some interference, these tested levels are not physiologically abundant in most surface water matrices. In contrast, iron is commonly found in high concentrations in industrial runoffs, mines, and many municipal waters. We tested the interference of these ions at high concentrations to simulate the worst-case scenario.

Following our high-concentration interference study, we performed a second study at more realistic concentrations of 10 mg/L for the ions that showed significant interferences (Zn(II) , HCO_3^- , and Cr(VI)) at higher concentrations. These concentrations were tested against the quantification limit (142 mg/L) of the developed sensor (Figure S6, Supplementary information). Dunnett's tests confirmed that there was no significant

difference between the blank signal and the signal from the interfering ions alone. Additionally, the test also confirmed that there was no statistically significant difference between the signal from Fe(III) alone and the signal from Fe(III) mixed with 10 ppm of the different interfering ions. Hence, the interference tests have successfully validated the sensor's ability to provide selective results, even in the presence of interfering ions at concentrations that are relevant to physiological conditions.

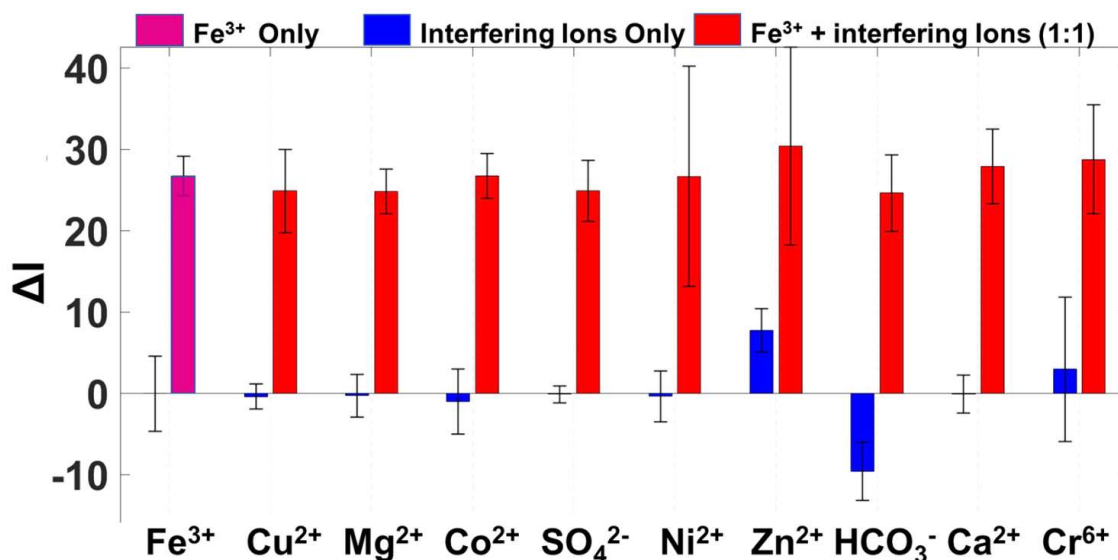


Figure 4. Interference Study for the Fe-Anthocyanin assay in the developed device. Red bars indicate intensity when Fe(III) was mixed with interfering ions in a 1:1 ratio, and blue bars indicate the interfering ions alone. All intensities are subtracted from the blank (n=4). According to Dunnett's multiple comparison test, all the signals from the (1:1) ratios are the same.

Stability

Drying the reagent onto paper eliminates the need for users to add anthocyanins during testing. To maintain consistency and reliability, the anthocyanin extract was stored frozen, and fresh solutions were prepared for each testing session, ensuring a consistent signal in the experiments. The stability of the anthocyanin on the sensor was evaluated over three weeks under different storage conditions. We compared the device's

performance when stored at cold temperature (5°C with a desiccator) and at room temperature (22°C). All detection and sample insertion zones were carefully sealed before storage in their respective conditions to ensure minimal on-chip evaporation of buffer and reagents. Throughout the three-week study, we observed that the device exhibited higher stability when stored under cold, sealed conditions, although no drastic changes were observed for storage at room temperature (Figure S7-A, Supplementary information). Using the Dunnett's multiple comparison test, we found no significant difference in the color signal obtained on day zero compared to subsequent days for cold-sealed conditions. The stability results using our packaging protocols suggest that the device can be batch-manufactured and sent to the end users in a realistic timeframe.

Additionally, we examined the stability of the color signal itself. Following the analyte run, the signal on paper was tested at regular intervals and the next day (Figure S7-B, Supplementary information). We observed no drastic loss or increase in the color signal on the paper during this period, indicating that the assay can be reliably used in the field and later transported to the laboratory for data analysis, especially in situations where color-quantifying resources are not immediately available. No significant edge effects were observed after three weeks under cold and room temperature conditions, further corroborating the sensor's robustness.

Analysis of real water samples

To assess the device's performance under a complex matrix like surface water, three concentrations of Fe(III) were spiked in water samples from the Cache La Poudre River, Stone Creek, and a pond from Spring Creek (GPS coordinates: 40.5950209 - 105.0751458, 40.5658458 - 105.0992506, and 40.5643398 - 105.0834326, respectively). We chose three spiked iron concentrations that exceeded the sensor's limit of detection

(LOD) but stayed below saturation levels. The percent recovery and RSD using ImageJ were found in the range of 114-126% and 13.7-18.9% for Poudre River, 104-110% and 4.5-13.4% for Spring Creek Pond, and 104-117% and 5.2-18.2% for Stone Creek (Table 1). While using the Matlab mobile app, the % recovery and RSD were found in the range of 111-112% and 11.5-18.5% for Poudre River, 111-118%, and 9.5-15.8% for Stone Creek, 94-109%, and 4.1-14.5% for Spring Creek Pond. Another study of paper-based analytical devices (PADs) from the ethanolic extract of *Curcuma longa* showed a comparable recovery range.⁵⁹

Table 1. Recovery performance analysis in three different surface water matrices using ImageJ and a smartphone

Source	Spiked (mg/L)	<i>Image J</i>		<i>Smartphone</i>	
		Recovery %	RSD %	Recovery %	RSD %
<i>Poudre River</i>	—	ND	—	ND	—
	150 (BQL)	126	18.9	112	18.5
	250	119	15.6	111	11.5
	500	114	13.7	112	15.6
<i>Spring Creek</i>	—	ND	—	ND	—
	150 (BQL)	108	13.4	94	14.5
	250	110	2.6	103	4.1
	500	104	4.5	109	9.6
<i>Stone Creek</i>	—	ND	—	ND	—
	150 (BQL)	117	18.2	111	9.5
	250	118	16.8	112	19.4
	500	104	5.2	118	15.8

Table 2 shows the analytical performance of different anthocyanin colorimetric sensors using different plant extracts and different reaction mediums. Compared to other techniques, our approach allows on-site quantification that can be easily used by the least trained users without any complicated analysis. Moreover, the device showed a working range similar to the other cellulose-based techniques without additional hosting bypolymer.

Table 2. Analytical performance of different colorimetric anthocyanin sensors for Fe(III)

Method	Anthocyanin Source	LOD	Quantification technique	Field applicability
This work	Butterfly Pea	44 & 55 mg/L	Image analysis	Enables on-site quantification using free imaging apps and software
Hands-on experiment kit ⁶⁰	Fever root	0.03 mg/L	Image Analysis	Suitable for classroom conditions only
Nanofibrous metallochromic sensor ²⁷	Red Cabbage	1 mg/L	Spectrophotometry	Requires trained personnel to do quantitative analysis
Metallochromic cellulose dipstick ²⁸	Red Cabbage	10 and 400 mg/L	Spectrophotometry	Requires trained personnel to do quantitative analysis
Anthocyanins from bugnaya ⁶¹	Bugnaya fruits	NA	Spectrophotometry	Requires trained personnel to do quantitative analysis
Spectrofluorimetric method based on the grape skin ⁶²	Grape skin	7.5 nM	Spectrofluorometry	Requires trained personnel to do quantitative analysis

Greenness assessment of the sensor

We evaluated the environmental friendliness of our sensor using the Analytical Greenness (AGREE) metric approach, considering 12 principles of green analytical chemistry.⁶³ As shown in Figure 5, our sensor achieved a greenness value of 0.88, placing it among the

dark green side. Explanations for each parameter are included in Figure S9, Supplementary information. Compared to traditional methods and other alternative platforms that employ anthocyanin as a probe, the anthocyanin sensor presents several distinct advantages aligned with the principles of green chemistry. These attributes include the capability for in-field sampling and direct analysis, minimal sample volume requirements of just 0.06 mL, the capacity for in-line measurement, and a streamlined process involving a single user step. The sensor offers a semi-automatic and miniaturized platform, eliminating the need for derivatization and significantly reducing waste generation to a mere 0.6 grams per analysis. Notably, the anthocyanin sensor is an energy-efficient technique with negligible energy consumption (0.015 kWh for >1000 sample analyses using a smartphone). Some of the reagents utilized in this sensor are bio-based, and it refrains from employing toxic reagents, ensuring operator safety and environmental responsibility. These features collectively position the developed sensor as an attractive and sustainable choice for analytical applications.

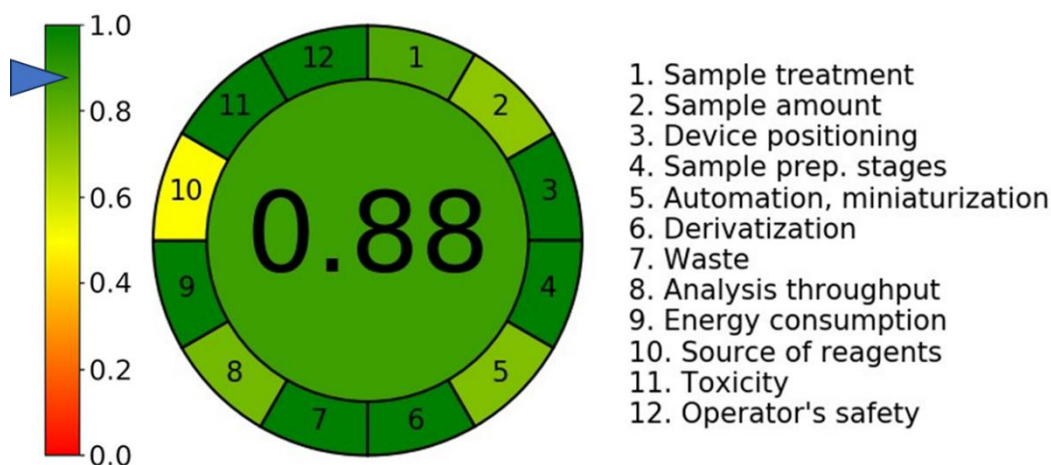


Figure 5. An annotated greenness report of the sensor from the Analytical Greenness Calculator.

Conclusion

The increasing demand for sustainable and environmentally friendly detection of water contaminants, particularly heavy metals, has driven the development of innovative sensing technologies. Our capillary-driven microfluidic sensor helps to bridge the gap between green sensing techniques and end users by moving away from the traditional non-user-friendly and energy-intensive techniques to a more versatile smartphone-enabled approach. Using the natural anthocyanin dye from Butterfly Pea in our novel fast-flow-driven paper-based colorimetric device produced a consistent and rapid signal response. It required only one user step (sample addition), creating a user and environment-friendly platform for detecting and quantifying Fe(III). Consistent results were obtained even when testing for Fe(III) in complex surface water samples with acceptable recovery performance. Additionally, the developed colorimetric device can be used by either dropping the analyte solution into the sample inlet or dipping the device directly into the water source, making the device's operation versatile. Moreover, combining environmentally friendly dyes with our smartphone-enabled sensor makes for a simple, cost-effective (i.e., <50¢ per device) and user-friendly strategy for detecting pollutants within aqueous environments. With further research and adoption, these smartphone-enabled capillary flow green sensors can potentially improve the field of water contaminant detection, promoting environmental stewardship and safeguarding public health while following the principles of green chemistry.

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Supplementary Information for Appendix 1—Smartphone-Enabled Green Anthocyanin Sensor for Fe(III) Sensing on Paper Using Capillary-Driven Microfluidics

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Image Analysis procedure on ImageJ

- After the device was dried, the image was scanned using flatbed scanner at 600dpi.
- The picture was then imported into the free ImageJ program.
- Three channels, blue, green, and red, were separated into the image.
- Optimized green channel was then inverted under Edit menu.
- Using a circle tool, the grayscale of the detection area was measured. The circle's size and area were maintained uniformly across regions.

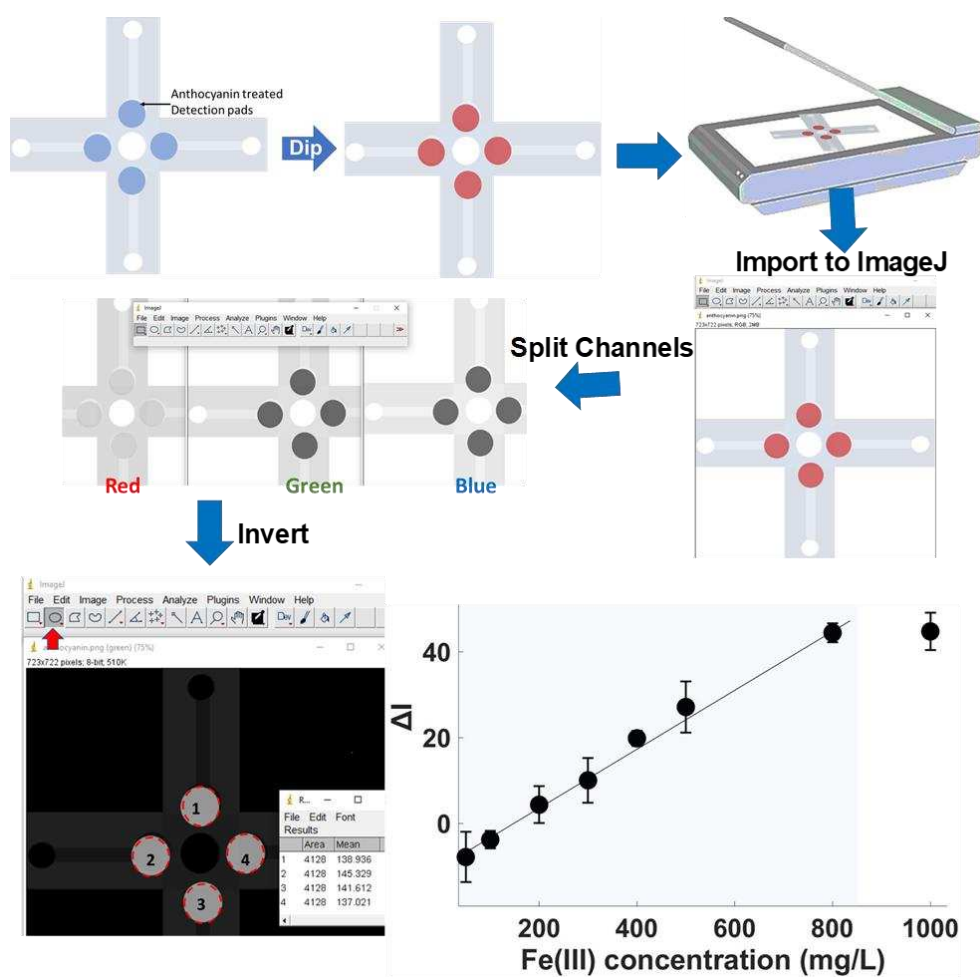


Figure S1. Quantitative processing of image using ImageJ

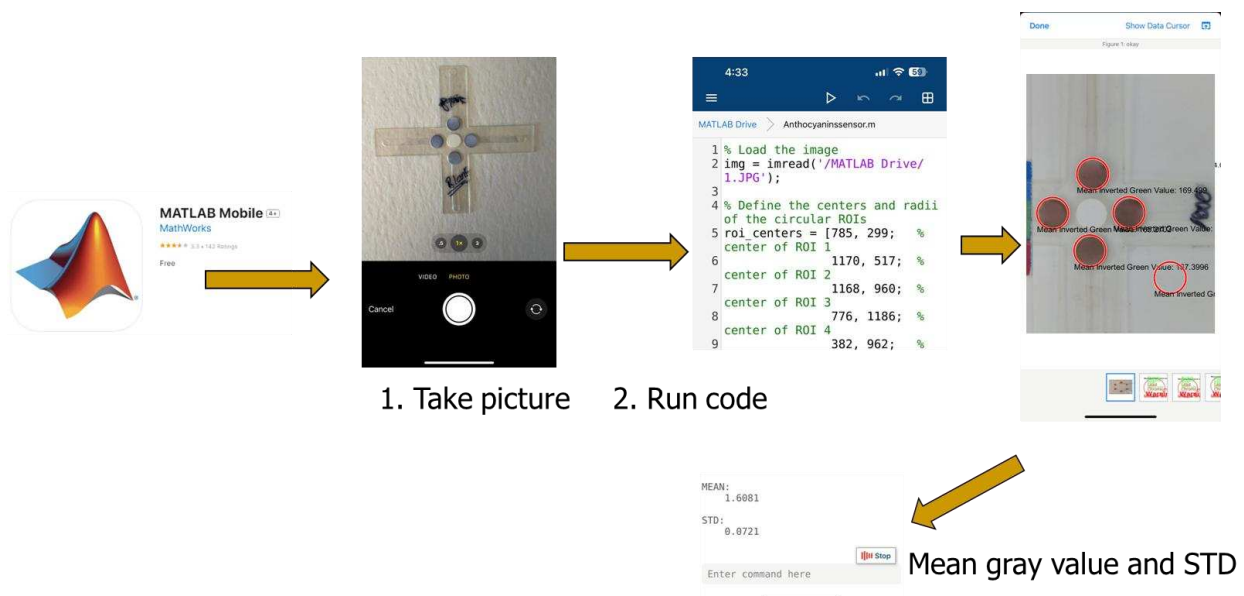


Figure S2. Quantitative image processing using the Matlab phone app.

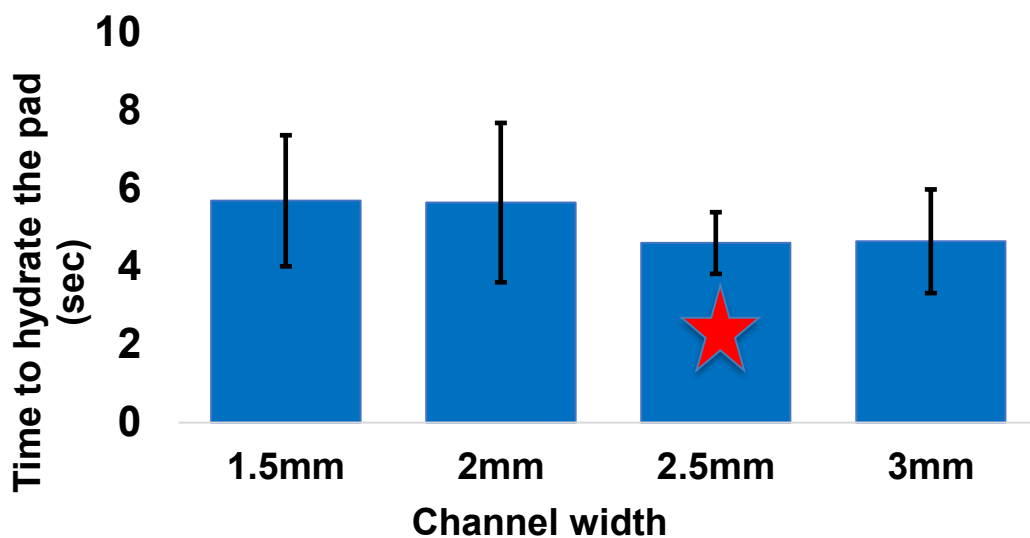


Figure S3. Channel Width Optimization Using 468MP double sided adhesive.

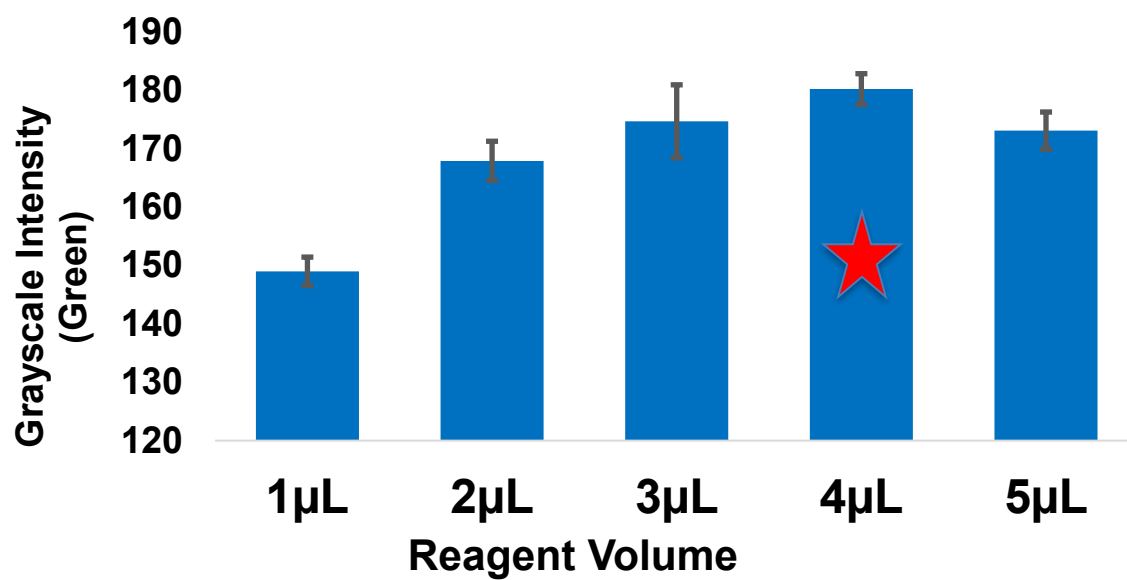


Figure S4. Anthocyanin Volume optimization

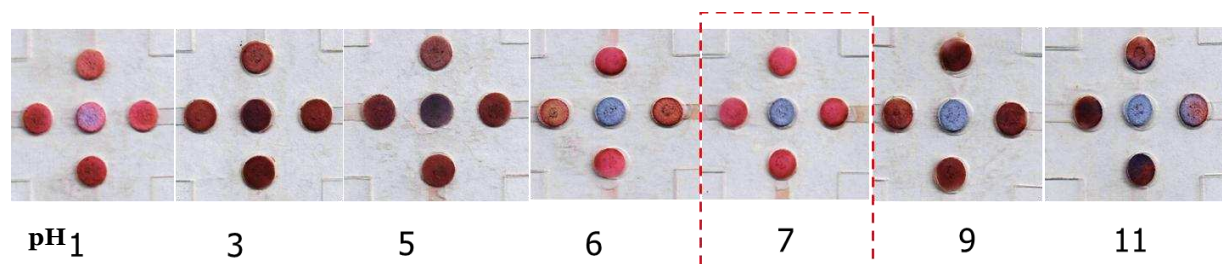


Figure S5. pH optimization for the maximum differentiation between blank (middle circle) and reagent signal (outer 4 circles).

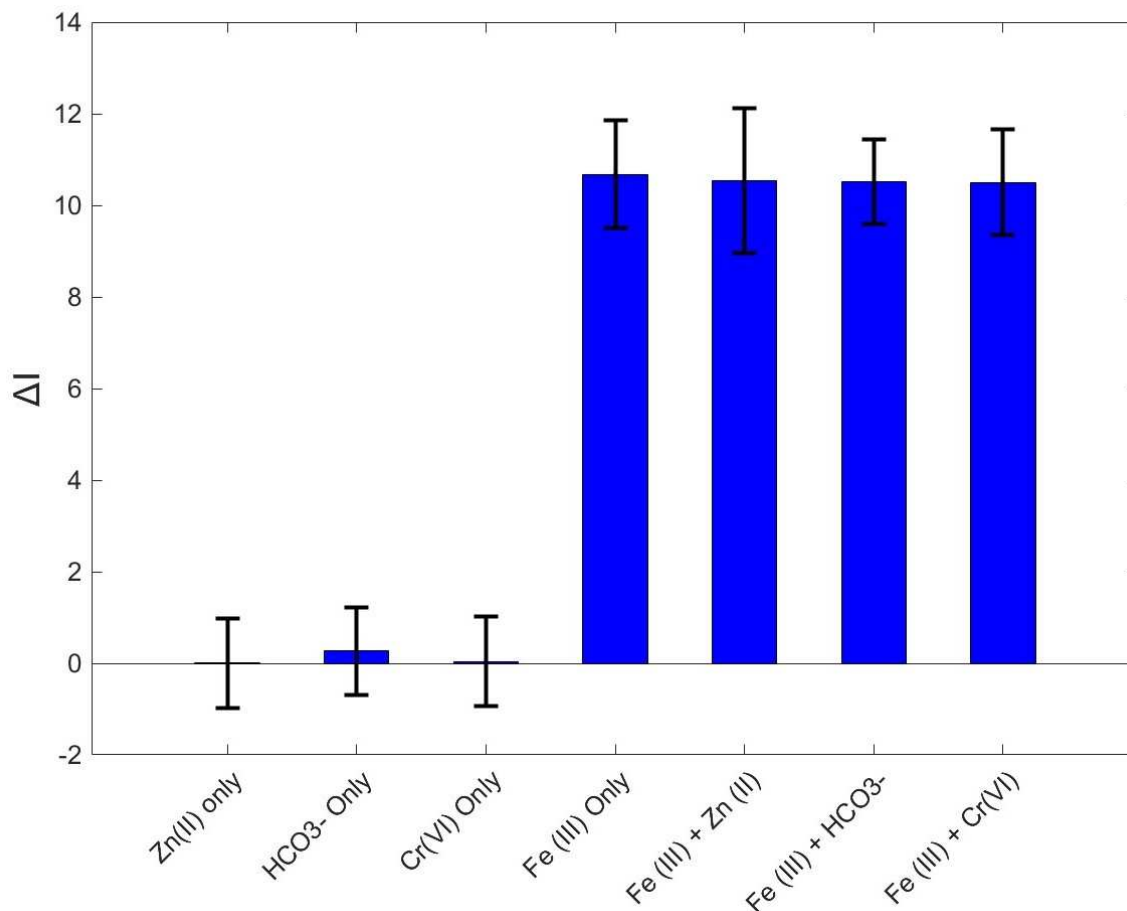


Figure S6. Interference study against Zn(II), HCO₃⁻, and Cr(IV) at more realistic concentration (10 mg/L each).

These concentrations were tested against the LOQ(142 mg/L) of the sensor developed. The dunnets multiple comparison test confirmed that there was no significant difference between blank signal and the signal from interfering ions only. Moreover, the test also confirmed that there was no statistical significant difference between signal from Fe(III) only and when mixed with 10ppm of different interfering ions. All these data were obtained by scanning the image and using ImageJ.

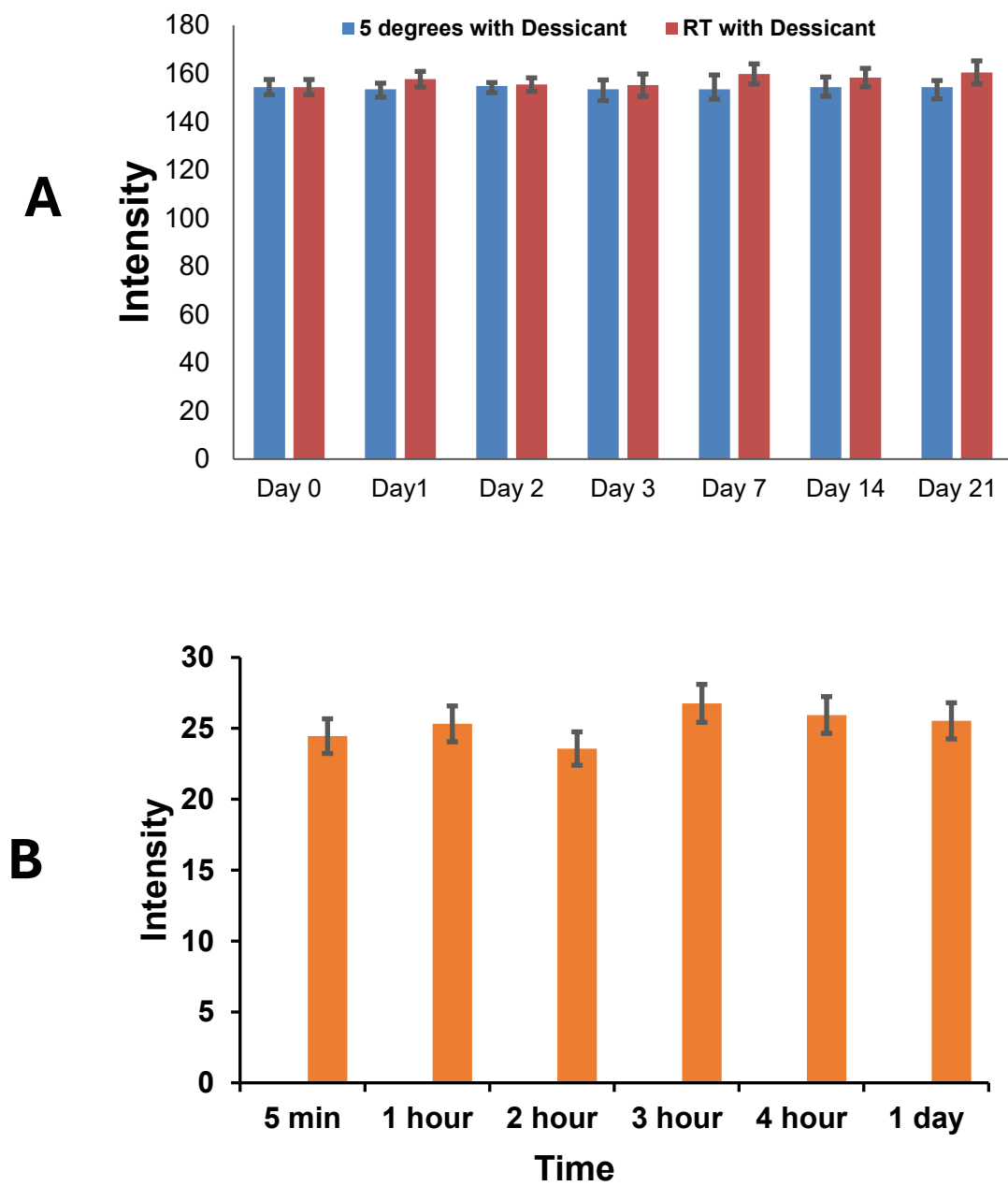


Figure S7. Device's stability test(A) and Complex's color stability test (B)

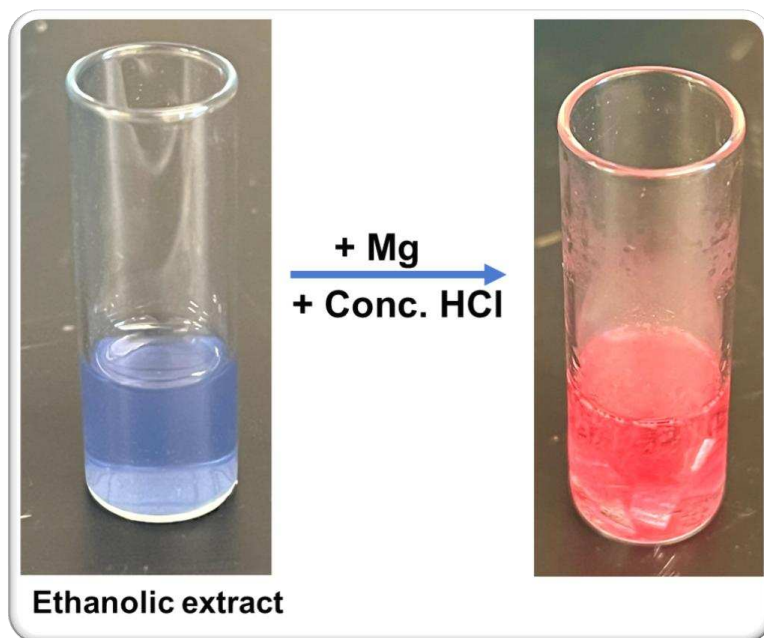


Figure S8. Shinoda's test for anthocyanin

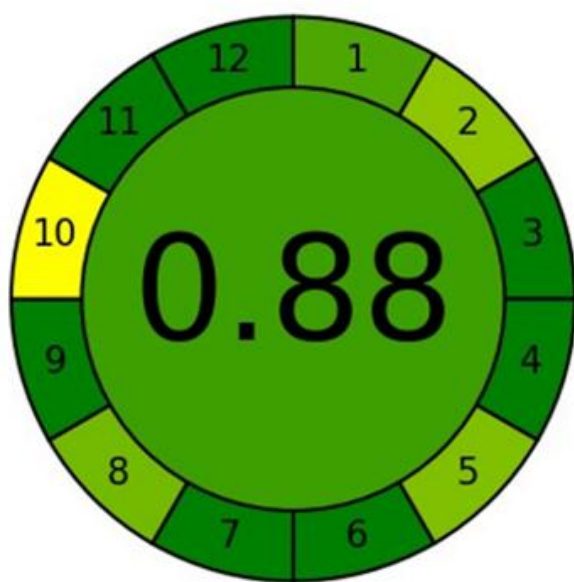


Figure S9. Greenness of the sensor. All parameters are equally weighted

Analytical Greenness Inputs

1. In-field sampling and direct analysis	7. 0.6g of waste per analysis (this is the weight of one sensor when done analyzing)
2. In-Line measurement	8. No. of analytes in single run:1 and sample throughput : 30 samples in an hour or even more
3. Sample volume 0.06mL	9. Non energy intensive technique: Phone takes about 0.015kWh energy to be fully charged (input: 0.015kWh)
4. 3 or fewer steps	10. Some reagents are bio-based
5. Semi-automatic and miniaturized	11. No toxic reagents are used
6. No Derivatization	12. No operator's safety concerns

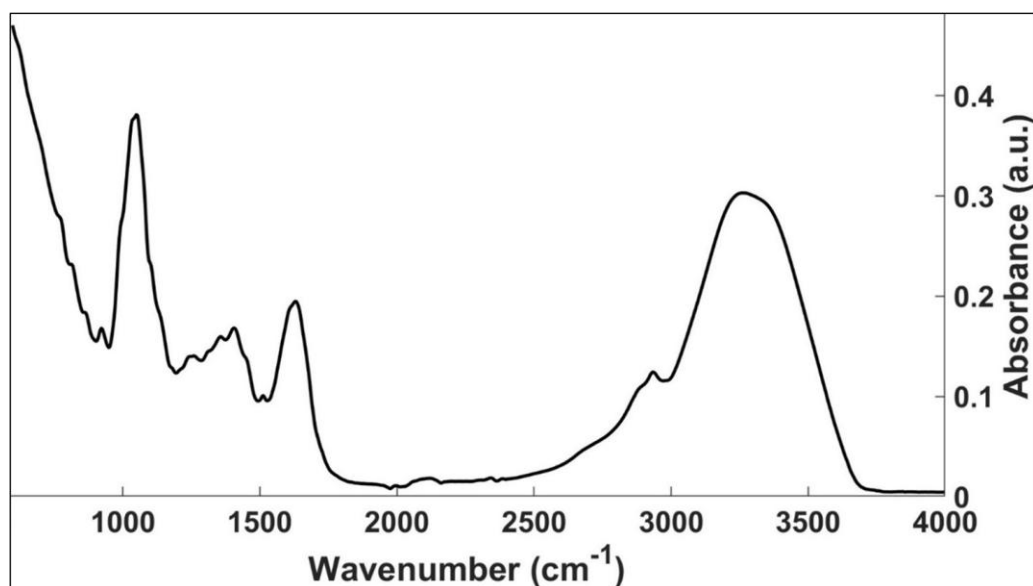


Figure S10. FTIR analysis of *Clitoria ternatea* flower extract

The broad peak around 3155-3405 cm⁻¹ indicated the hydroxyl groups of anthocyanins.

The peak around 2912 cm⁻¹ arises from the C-H stretching of alkane constituents.

Carboxylic acid presence was indicated by peak absorbance around 1616 cm⁻¹. The peak

rise at 1056 cm^{-1} probably indicated the aliphatic amines, while the peak around 1234 cm^{-1} indicated alkyl halides. Furthermore, the peak around 1338 cm^{-1} possibly comes from aromatic amines.

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APPENDIX 2 — Capillary Flow-driven, Disposable Device for Dissolved Oxygen Sensing in Water Samples

Abstract

Fluctuations of dissolved oxygen (DO) levels in many waterbodies have been observed in the last decades. While well-established technology for DO monitoring exists, it is rather expensive and requires user training to use. Here, we propose a simple, user-friendly, and disposable capillary flow-driven microfluidic device for colorimetric DO determination. The device is constructed with transparent PET film, double-sided adhesive, and glass microfiber with immobilized reagents in such a way, that sample is taken by dipping the device in the analyzed water to yield the results. Modified Winkler colorimetric assay has been employed to transform oxygen into colored species. The limit of detection of the developed device was 1.7 ppm. Field applicability assessment was performed to ensure that the devices are not susceptible to interferences and that measurements can be performed in various environmental conditions by untrained users. The developed devices were validated with freshwater samples from various water bodies as well as with aquarium water, demonstrating high accuracy. The findings presented here open possibilities to deploy these sensors for citizen-based water quality monitoring campaigns. This work has been published in *Analytical Chemistry* 2025, 97 (45), 25078–25086.

Introduction

In recent decades, a decline in dissolved oxygen (DO) levels in many water bodies has been observed as a consequence of global warming and nutrient discharges.¹⁻³ DO plays a vital role in maintaining healthy aquatic ecosystems. The term *Dissolved oxygen* refers to gaseous oxygen dissolved in water. Many aquatic organisms, such as fish, rely on adequate DO levels for respiration, and low concentrations can limit their survival and activity.⁴ Consequently, DO concentration is a key parameter to consider when assessing water quality as a habitat for aquatic life. Several thresholds of DO requirements in water can be distinguished for various species of fish. DO levels below 3 ppm do not support fish populations. DO levels greater than 3 ppm, but lower than 5 ppm indicate stressful conditions for most fish species. Finally, DO >6 ppm can support spawning, and in 8-9 ppm of DO in water, the fish population thrives.⁵ DO levels affect not only fish populations but also biodiversity, eukaryotic biomass, and the occurrence of energy-requiring interactions (e.g., predation).² Because of that, regular monitoring of DO levels in multiple locations and time points is of great importance.³ Other applications of DO sensing include, but are not limited to bioprocesses monitoring,⁶ aquarium water quality testing,⁷ wine and beer-making control,^{8,9} and many more.

There are a few routinely used methods for DO determination. For the past 140 years, the iodometric Winkler titration has been a standard method to determine DO concentration. In this method, just after sample (here water) collection, oxygen is immediately fixed by the addition of Mn(II) salt and alkaline I⁻ solution. Mn(II) is oxidized by oxygen to Mn(IV) and a brown precipitate of MnO(OH)₂ appears. This precipitate is then dissolved in acid and liberated Mn(IV) ions oxidize I⁻ to I₂. I₂ is typically quantified using titration with

thiosulfate, using starch as an endpoint indicator,¹⁰ although spectrophotometric variants of this method, measuring the absorbance of I_2 and I_3^- species have also been described in the literature.^{11–13} What is worth noting, DO is a parameter highly sensitive to both sample collection and storage, therefore measuring the DO level *in situ* in the sample is preferred. In this context, electrochemical and optical sensors designed for in-field measurements should be mentioned. Electrochemical O_2 sensors are based on the work of Clark.¹⁴ In such systems, electrodes polarized with external voltage are located in a body with a gas-permeable membrane filled with electrolytes. O_2 diffuses through the membrane and dissolves in the inner electrolyte. The current of O_2 molecule reduction at the cathode is measured.¹⁵ Galvanic DO sensors are also widely used and rely on oxygen reduction at the cathode and anode material oxidation without external polarization voltage.¹⁵ Finally, optical DO sensors have gained attention in the last decades. They operate based on a fluorescence quenching mechanism by O_2 present in the sample. Fluorescent dye (typically a Pt or Ru derivative) is immobilized in a membrane, which is in contact with the analyzed sample. Dye is excited using LED in the body of the sensor and fluorescence intensity is measured using a photodiode.¹⁵

While all of the mentioned technologies enable accurate DO determination, there are some difficulties in implementing them in on-site sensing scenarios by citizen users. Winkler titration is a complex, multistep method with four different reagents addition. Also, taking a sample from waterbody is required. Finally, the reagents and their volume used in Winkler titration do not satisfy demands for green chemistry. During DO determination using electrochemical sensors, oxygen is consumed. As a consequence, to obtain accurate results constant sample stirring is necessary. Also, electrochemical

sensors need periodic maintenance (replacement of membrane, electrolyte). Optical sensors are the easiest to use but also are the most expensive out of the discussed technologies. All of these issues highlight a need for a disposable, user-friendly device development with minimal hardware requirements for on-site DO sensing.

A few lab-on-a-chip devices for optical or electrochemical DO determination have been reported in the literature as an attempt to tackle the mentioned problems. Optical chips for DO determination rely on quenching of luminescence of O_2 -sensitive dye, which is immobilized on a solid support. In the role of solid support custom-made superhydrophobic polymers were employed.^{16,17} Also, O_2 -sensitive dye was immobilized in polystyrene nanoparticles, which were cured on polycarbonate substrate.¹⁸ Electrochemical detection more commonly employed for on-chip DO determination. Kaufman et al. developed a 3D-printed microfluidic device with integrated gold electrodes covered with platinum black for oxygen reduction reaction.¹⁹ DO electrochemical sensing chips were also fabricated on glass and flexible plastic²⁰ solid support²¹. Again, the source of electrochemical signal was oxygen reduction reaction. Finally, Seymour et al. recently proposed an interdigitated electrode arrays fabricated on silicon wafer.²² In their device, O_2 was reduced to H_2O_2 on a gold electrode and H_2O_2 was subsequently oxidized on a platinum electrode to determine DO with low susceptibility to interferences. While all of the described technologies offer great analytical features, they still have rather high hardware and skill requirements, making them unsuitable for untrained users.

Despite the importance of DO determination, to our best knowledge the literature lacks disposable, colorimetric, on-chip solutions with minimal hardware requirements dedicated to this problem. We associate this gap with the difficulty in creating a device

sealed in a way, that atmospheric oxygen would not interfere. Here we propose to use for this purpose a capillary flow-driven device made of inexpensive PET transparency film and double-sided adhesive.^{23,24} Such devices utilize capillary flow to collect and transport samples, so there is no need for external pumping devices.^{25–28} Reagents required for a specific assay are immobilized on paper or glass microfiber pads within the flow channel. They are rehydrated when the sample is introduced, allowing for the reaction between reagents and analyte to occur.²⁹ In the case of colorimetric detection of the target analyte, the devices are simply photographed to obtain information on the color intensity of the detection area.

In this paper, we adapted the Winkler colorimetric method to be applied in a capillary flow-driven device. This method has been used in bulk flow analysis systems in the past.^{30,31} However, owing to difficulties in precipitate transportation with the capillary flow, the chosen method did not show sufficient sensitivity. To resolve this problem, we employed starch to detect I_3^- in the Winkler colorimetric method, which was the first reported use of starch in this context. This allowed us to develop presented here the first disposable, user-friendly colorimetric sensor for DO determination in water samples.

Experimental section

Chemicals and materials

Manganese chloride tetrahydrate, sodium Iodide, sodium hydroxide, sodium azide, hydrogen peroxide (30% wt solution), sodium chloride, sodium nitrite, soluble starch, p-toluenesulfonic acid monohydrate (PTSA), and catalase from bovine liver (3809 U mg⁻¹) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Materials for capillary flow-driven device manufacture, namely 468MP double-sided adhesive (DSA) and 9984 PET transparency film, were from 3M (St. Paul, MN, USA). The thickness of the used DSA and PET transparency was 0.13 mm and 0.1 mm, respectively. Glass microfiber (0.3 mm thick, 0.007 g cm⁻² weight) was purchased from Sigma Aldrich (St. Louis, MO, USA). All solutions were prepared in Milli-Q water (18.2 MΩ cm).

DO standardization/calibration

DO standards were made using Milli-Q water. DO standards were prepared just before each set of measurements. For device optimization purposes, two DO standards were made: 100% DO saturated water and 0% DO saturated water. Water was saturated with oxygen by pumping air into the water using an aquarium pump (Cornwall Aquatics) to obtain DO concentration of around 6.5 ppm. To remove DO from water, N₂ gas was purged through ca. 250 mL of water for 30 min.

To calibrate the devices, DO standards were made by *in situ* oxygen synthesis using H₂O₂ decomposition by catalase. This approach allowed a more precise control of DO concentration in each standard. To prepare DO standards, 200 μL of 15 kU mL⁻¹ catalase solution was added to 200 mL of N₂ purged water. Then, portions of 30% wt hydrogen peroxide solution were added to obtain the desired DO concentration.

DO concentration was controlled with MW600 PRO DO Meter (Milwaukee Instruments, Rocky Mount, NC, USA). The displayed value was corrected for the altitude of Fort Collins

(5000 ft above sea level), according to the instructions provided by the manufacturer. The DO meter was validated against the Winkler titration method, using LaMotte® DO Test Kits as the reference standard.

Device design and fabrication

Components for microfluidic device fabrication (PET transparency, DSA, and glass microfiber pads) were cut using an Epilog Engraver WinX64 Zing CO₂ laser cutter (Epilog Laser, Golden, CO, USA). The design and operating principle of the device is shown in Figure 1. The flow channel was fabricated from sandwiched DSA and PET transparency in the following order: DSA-PET-DSA-DSA-PET-DSA. This allowed us to achieve the desired height of the flow channel (720 μm). The flow channel had dimensions of 23 by 2 mm and began with a sample aspiration zone (3 mm in diameter circle) and it ended with a 4 mm circular detection zone. Each device held three flow channels to allow for triplicate measurements in one take. The cut flow channels layer was glued on PET transparency with the hydrophilic side of the transparency facing the flow channel. Then glass microfiber pads with predried reagents were inserted into the appropriate locations in the flow channel. The device was sealed with another layer of PET transparency, also with a hydrophilic side facing the flow channel. This transparency layer had 3 mm circles cut, which were aligned with the sample aspiration zone, as well as 1 mm in diameter perforations aligned with the detection zone in the flow channel to serve as air outlets (Figure 1A). Such a design promoted capillary flow after dipping the sample aspiration zones in the sample. A scheme of the Winkler reaction happening in a single flow channel is shown in Figure 1B

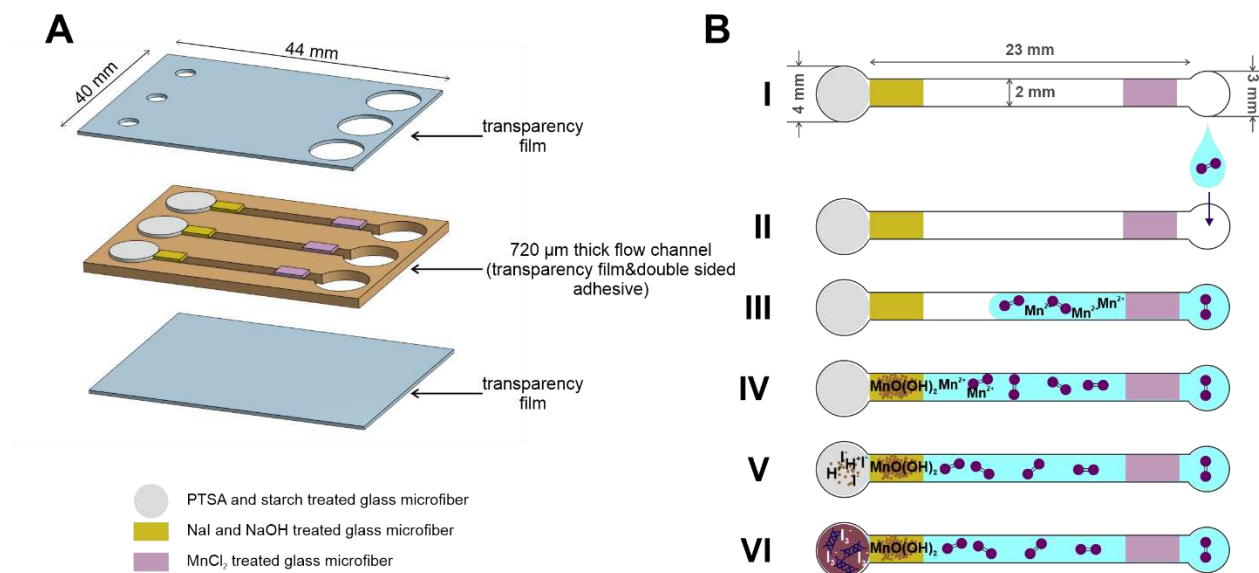


Figure 1. A: Scheme of capillary flow-driven device assembly. The top and bottom parts of the device are made of 9984 PET transparency film, with the hydrophilic side facing the flow channel. A flow channel of 720 μm thickness is formed by alternating layers of double-sided adhesive and transparency sheets. B: A scheme of the Winkler reaction happening in a single flow channel. (I) Dimensions of the flow channel; (II) The Sample is introduced to the device by dipping the device in the sample; (III) The Flowing sample releases Mn^{2+} ions from the glass microfiber pad; (IV) As the sample reaches the second glass microfiber pad, Mn^{2+} ions form with oxygen and OH^- ions stored in the second glass microfiber pad a brownish precipitate of MnO(OH)_2 ; (V) Flowing sample pushes a fraction of the precipitate towards the detection zone, alongside with stored in second glass microfiber pad I^- ions; (VI) MnO(OH)_2 dissolves in PTSA present in the detection zone and released Mn^{4+} ions oxidize I^- to I_2 , which then form together I_3^- , reacting with starch to give a colorful complex.

The method chosen for DO determination was the colorimetric variant of Winkler titration. The required reagents were immobilized on glass microfiber pads and placed in the flow channel in the order shown in Figure 1. Pads located in the flow channel were 4 by 2.5 mm and the circular pad in the detection zone was 4 mm in diameter. The first glass microfiber pad was pretreated with 5 μL of 0.5 mol L^{-1} MnCl_2 . The second one contained 5 μL of 1 mol L^{-1} NaOH, 0.75 mol L^{-1} NaI, and, if real samples were being analyzed, 150 mmol L^{-1} NaN_3 . In the final, circular glass microfiber pad 7 μL of 1.25 mol

L⁻¹ PTSA with 1% w/v starch was dropcasted. All glass microfiber pads were dried for 1 hour in ambient conditions before they were used to construct the microfluidic device.

Data capture and analysis

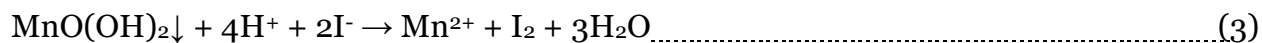
After the reaction between oxygen and reagents had been completed, a photograph of the device was captured with the Motorola Edge 20 Lite smartphone (Motorola, Schaumburg, IL, USA) using the built-in camera application. An exemplary photograph is shown in Supplementary Information Figure S1. To ensure constant lighting conditions, the photographed device was placed in an illumination box. The photographs obtained were then transferred to a PC and analyzed using ImageJ software. A circular region of interest (ROI) was first selected, comprising the entire detection zone. Then, images were converted to 8-bit color, inverted, and mean color intensity was read inside ROI. The obtained readings for the same DO concentration were averaged. Unless stated otherwise, each measurement was conducted in triplicate i.e. using three flow channels in one capillary flow-driven device. This standard data acquisition and processing procedure proved to be effective in the assay development phase. However, for on-site analyses a simplified measurement protocol could be implemented, for, utilizing a color reference chart or a constant white background to normalize variances in lighting and a custom-written application for color intensity readings.²³

RESULTS AND DISCUSSION

Device optimization

The most challenging aspects of capillary flow-driven devices for DO sensing development were firstly, selecting a colorimetric detection method and secondly,

minimizing interference from atmospheric oxygen. In the course of preliminary experiments, the colorimetric Winkler titration variant was chosen to determine DO. This method relies on insoluble $\text{Mn}(\text{OH})_2$ formation (eq. 1), which is oxidized by oxygen to form a brownish precipitate, $\text{MnO}(\text{OH})_2$ (eq. 2). Then, the oxidized precipitate is dissolved in acid, which causes the release of Mn(IV) ions. These ions oxidize I^- ions to form I_2 (eq. 3). Finally, excess I^- ions react with the liberated I_2 to give I_3^- , which is then complexed by starch to form a purple complex (eq. 4). The color intensity of the formed starch-triiodide complex was the source of analytical signal in these microfluidic devices. A scheme of reactions occurring in flow channels is shown in Figure 1B.



To adapt the Winkler colorimetric method to be used in a microfluidic device, a few alterations had to be introduced. First of all, typically used sulfuric acid was substituted with PTSA as a stable, crystalline source of H^+ ions.²³ Although other H^+ ions sources were not investigated, any strong acid, which is solid in the ambient conditions, water soluble, and safe to handle for untrained users could be used instead PTSA. Secondly, in all reports on the spectrophotometric Winkler method, the compound being detected is I_2 , which absorbs at a maximum wavelength of around 450 nm.¹¹ In the case of on-chip detection presented here, such an approach did not yield any measurable analytical signals. This is because the precipitate in the glass microfiber pad containing hydroxide does not freely flow towards the pad with PTSA but rather stays where it was

formed. As a result, a very limited amount of I_2 is formed. To boost the sensitivity, soluble starch was introduced in the detection zone. This procedure, reported for the first time for colorimetric Winkler assay, allowed us to obtain linear readings within the relevant DO concentration range. The use of capillary flow-driven devices made of DSA and PET transparency allowed for spatial separation of reagents that cannot be premixed and their sequential addition, as well as for almost completely sealing the device to minimize interference from atmospheric oxygen. Both features highlight the advantages of this type of capillary flow-driven device.

In the following step, the dimensions of the microfluidic device and the concentrations of all reagents were optimized to achieve the most sensitive readings. In all cases signals for 0% and 100% DO were measured, according to *DO standardization/calibration* section. The results obtained are presented in Figure 2. The optimal channel width was chosen to be 2 mm (Figure 2A) and the optimal diameter of the detection zone was 4 mm (Figure 2B) as both conditions ensured the largest signal difference between the measured standards. The sample inlet diameter was fixed at 3 mm and was not subject to optimization. Channel height was selected based on the flow rate in the flow channel and was set to 720 μm . While pads inside the flow channel were by default made of glass microfiber, as it rapidly releases immobilized chemicals, a few materials (glass microfiber and various paper types) were investigated to fabricate the detection zone. The technical parameters of materials used in this study can be found in Table S1 in Supplementary Information. Out of the tested materials, glass microfiber provided the most sensitive and precise readings (Figure 2C), so it was used to fabricate the detection zone in further experiments. In the next steps, the concentrations of all reagents were optimized. The

number of moles of MnCl_2 , NaOH , and PTSA are linked by reactions stoichiometry. Because of that, the molar ratio of MnCl_2 to NaOH was fixed at 1:2 and the number of moles of PTSA should exceed n_{NaOH} . The latter condition was not satisfied in the case of 6 mol L^{-1} NaOH , because of the limited solubility of PTSA. Out of the tested combinations (Figure 2D), the use of 2 mol L^{-1} MnCl_2 , 4 mol L^{-1} NaOH , and 3.5 mol L^{-1} PTSA provided the highest sensitivity. However, NaOH and PTSA, as highly hygroscopic reagents, did not fully dry on glass microfiber pads at these concentrations. This would most likely negatively impact the long-term stability of microfluidic devices therefore 0.5 mol L^{-1} MnCl_2 , 1 mol L^{-1} NaOH , and 1.25 mol L^{-1} PTSA were chosen for further experiments. Finally, 0.75 mol L^{-1} NaI (Figure 2E) and 1% (w/v) starch (Figure 2F) were chosen as optimal concentrations of these reagents. Results presented in Figure 2F prove that the addition of starch in the detection zone is necessary to obtain a measurable signal difference between 0 and 100% oxygen-saturated water.

Using the optimized device, the kinetics of the signal development were registered to establish the time between dipping the devices in the sample and capturing photos of the detection zone. The obtained signal vs time dependencies for 0 and 100% oxygen-saturated water are presented in Figure S2 in Supplementary Information. After 2 min incubation, both dependencies have almost identical slopes, indicating that any incubation time longer than 2 min can be selected without compromising sensitivity. Interestingly, a plateau in the reaction kinetics was not reached in the studied time. This is probably caused by atmospheric oxygen diffusing through the sample inlet causing a constant increase of the measured signal. However, by capturing photos for each standard and sample after the same incubation time, the influence of this effect is negligible.

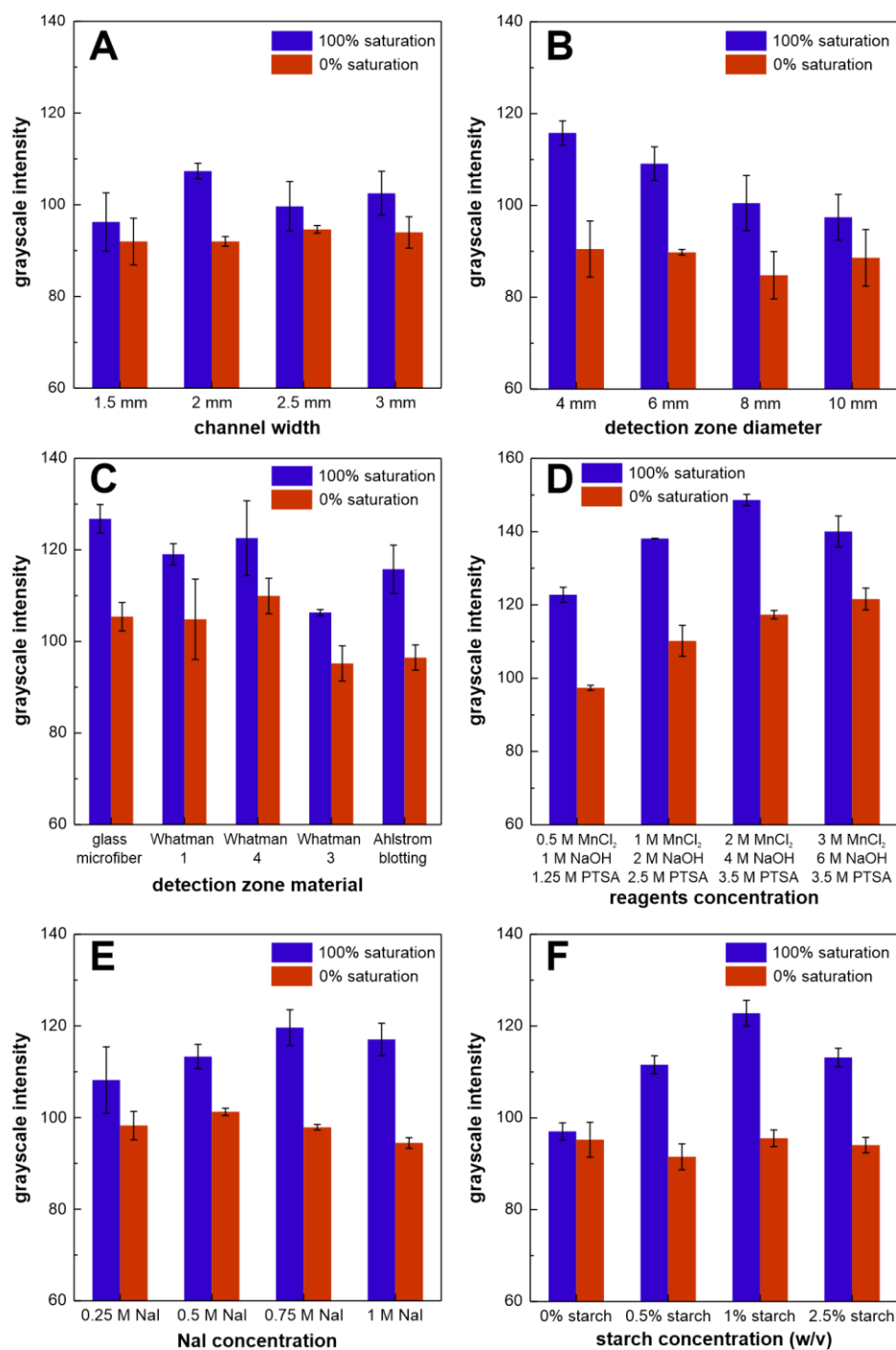


Figure 2. Capillary flow-driven device for DO determination optimization. A: Flow channel width optimization; B: Detection zone diameter optimization, C: Detection zone material optimization; D: MnCl₂, NaOH, and PTSA concentration optimization; E: NaI

concentration optimization; F: starch concentration optimization. Error bars represent the standard deviation of $n=3$ measurements.

To calculate the limit of detection (LOD), usually a signal value for a blank sample (without analyte) is necessary. In the case of DO sensing, it is not feasible to obtain a blank (zero DO) solution by simply flushing nitrogen gas through water.³² Typically, for DO probe calibration a solution of sodium sulfite is used as a blank, as in such a solution oxygen is reduced by sulfite ions. However, in the case of the presented colorimetric sensor, the use of such a blank solution yields unrealistically low detection limits. This is because all atmospheric oxygen diffusing to the sample during the measurement phase would also get reduced, while in reality, LOD should reflect its presence. Consequently, LOD was calculated as $3.3SD_y s^{-1}$, where SD_y is the standard deviation of the intercept and s is the slope of the calibration curve, to give 1.7 ppm DO. The precision of the measurements, expressed as relative standard deviation ($n = 6$, 2 devices, 3 flow channels per device), was 3.0% for 3.7 ppm DO and 3.6% for 10.8 ppm DO. The minimum detectable change in dissolved oxygen (resolution) for this device is 0.91 ± 0.07 ppm at 95% confidence level. This resolution allows the device to resolve ecologically meaningful shifts in DO, such as the boundary between hypoxic, critical, and stressful conditions for aquatic organisms. Moreover, the qualitative color changes visible in the images demonstrate that this sensor can be used as a semi-quantitative field tool, similar to pH test strips.

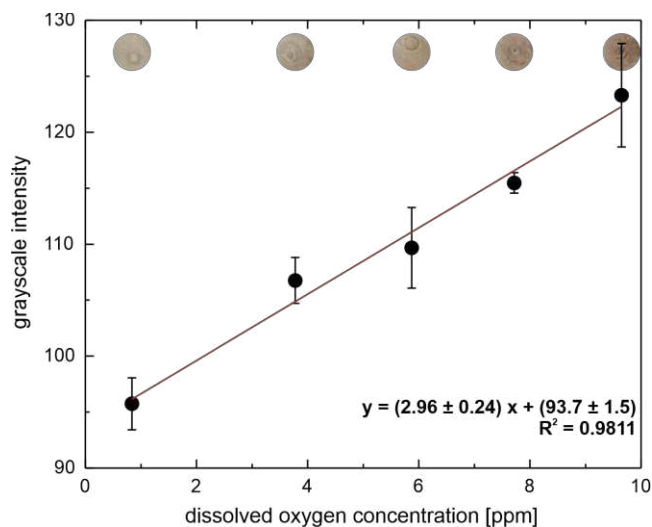


Figure 3. Calibration curve registered using the developed devices. Actual photos of the detection zones are presented for each standard. Error bars represent the standard deviation of $n=3$ measurements.

Since the developed capillary flow-driven device for DO determination is the only sensor of such kind found in the literature, it was not feasible to compare their performance with similar sensing solutions. Because of that, in Table 1, analytical parameters and operational features of the developed devices are compared with different kinds of DO sensors as well as with commercially available DO meters. This comparison reveals that the developed microfluidic devices exhibit higher than other sensors' limit of detection. However, in the case of on-site measurements of DO, this parameter is not crucial when evaluating the sensor's performance, because it is still well below critical level for aquatic life. On the other hand, contrary to other sensing solutions, capillary flow-driven devices presented here combine disposability, user-friendliness, and minimal hardware requirements. The reagents are spatially separated to avoid cross-reactivity, which ensures its long term stability. Owing to the device disposability, there is no need for regular maintenance of the sensor. Additionally, compared to electrochemical sensors, the developed device does not require sample stirring to obtain accurate results. To

further increase user-friendliness of the developed DO sensing platform, a custom-written or commercially available application could be employed for colorimetric signal processing instead of ImageJ. Such applications have already proved their usefulness to quantify colorimetric signal in fast-flow microfluidics in our previous works.^{23,27,33}

Table 1. Comparison of selected analytical parameters and operational features of DO sensors published in the literature with capillary flow-driven device presented here. PtTFPP - platinum(II) meso-tetrakis (pentafluorophenyl) porphyrin, [Ru(dpp)3] [4-Clph4]B]2 - tris(4, 7-diphenyl-1, 10-phenanthroline)ruthenium(II) ditetrakis(4-chlorophenyl)borate, PtOEPK - platinum(II) octaethylporphyrin ketone, nd – no data.

Determination method	LOD [ppm]	Response time	Equipment needed	Disposable	ref.
<i>Oxygen reduction on inkjet-printed Au electrodes</i>	0.23	20 – 30 s	potentiostat	yes	20
<i>Oxygen reduction, followed by H₂O₂ oxidation on micro interdigitated electrode arrays on a silicon wafer</i>	0.36	nd	potentiostat	yes	22
<i>Fluorescence quenching of PtOEPK dye in polystyrene nanoparticles cured on polycarbonate support</i>	nd	depends on DO concentration	home-made hardware platform coupled with spectrometer	yes	18
<i>Fluorescence quenching of Pt-based dye in superhydrophobic polymer membrane on PET support</i>	nd	6 s	home-made flow cell coupled with fluorometer	yes	17
<i>Oxygen reduction on Au or platinum black modified electrode in microfluidic system</i>	0.38 or 0.49	1 min	potentiostat, pumps	no	19

<i>PtTFPP fluorescence quenching, dye immobilized on optical fiber</i>	0.98	130 s (N ₂ to O ₂), 649 s (O ₂ to N ₂)	LED, spectrometer	no	34
<i>[Ru(dpp)₃][4-Clph₄]B]₂ fluorescence quenching, dye immobilized in rubber film</i>	0.01	3 min	home-made meter with LED and photodiode	no	35
<i>Commercial meter for polarographic DO determination</i>	0.01	nd	meter, probe	no	36
<i>Commercial meter for optical (fluorescence quenching) DO determination</i>	0.01	nd	meter, probe	no	37
<i>Winkler colorimetric assay in capillary flow-driven device</i>	1.7	>2 min	smartphone	yes	this work

Field applicability assessment

The most important step in devices for on-site sensing development is validation if they can be used for real sample analysis conducted by untrained users. In the first step of such validation, the influence of potentially interfering compounds on DO determination was studied. The compounds selected for this study are ions and nutrients commonly occurring in water samples or that water can be polluted with. Also, DO sensing was performed in water with NaCl in the range from 10 to 35 parts per thousand (ppt) to establish if the developed devices were to be used for DO measurements in salt water. The results obtained are summarized in Figure 4.

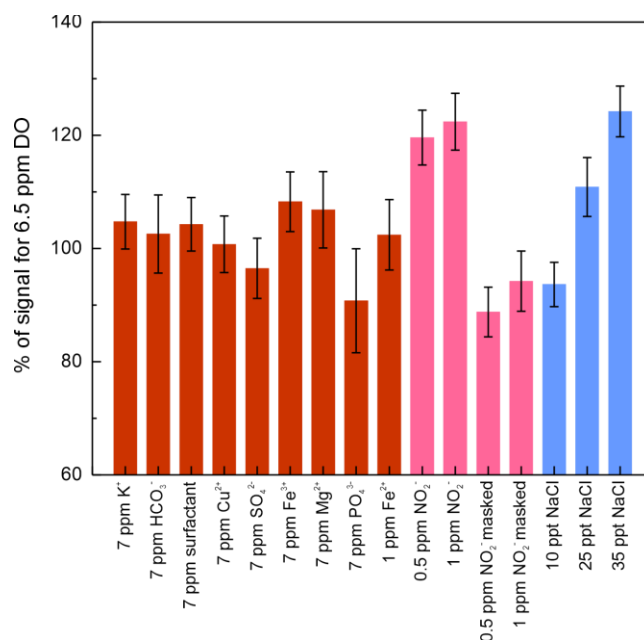


Figure 4. The influence of selected matrix components on DO determination. All signals were normalized by dividing grayscale intensity by signal measured for white background and by the measured with DO probe oxygen concentration. ‘Masked’ refers to masking of nitrite ions with sodium azide. Error bars represent standard deviation of $n=3$ measurements.

Nearly all of the studied interfering compounds do not cause significant differences in the measured signal. The presence of nitrite ions elevates the signal for DO by around 20%. This outcome is not surprising as nitrite ions are known to interfere in both Winkler titration and Winkler colorimetric methods.¹⁰ To minimize their influence, nitrite ions are typically masked with azide ions, which are included in alkaline iodide reagents.¹⁰ The same procedure proved to be effective in the case of the developed capillary-flow-driven device. Sodium azide (150 mmol L⁻¹) was added to reagent containing sodium iodide and sodium hydroxide. This reagent was immobilized on a glass microfiber pad, which was placed in the flow channel, as described in *Device design and fabrication* section. As shown in Figure 4, masking nitrite significantly reduces interference resulting from its

presence in the samples analyzed. Winkler titration method is generally very well studied and validated procedure. According to the literature, other, not tested in this experiment compounds are unlikely to interfere. The only exception are sulfides, which might react with the generated I_2 .³⁸

The influence of sample salinity on DO measurements was tested on three levels of salinity: 10, 25, and 35 ppt, which is the typical salinity range for seas and oceans. The data obtained clearly show an increase in salinity results in an increase in the measured signals for DO (Figure 4). A possible explanation for this outcome is the more effective transfer of manganese oxide hydroxide precipitate from the second glass microfiber pad to the adjacent detection zone with increasing ionic strength of the sample. As a consequence, the developed devices can be used to accurately determine DO in water with salinity levels lower than 10 ppt (for example Baltic Sea³⁹ and freshwater). Interestingly, the dependence of the measured signal (normalized for DO concentration) on salinity is linear (see Figure S3, supplementary information). This opens an opportunity to introduce a mathematical correction in the determined DO concentration if the salinity level is known, which is a standard procedure in commercially available DO meters. Alternatively, a detection area for chloride ions, as an indicator of salinity, could be incorporated in the capillary-flow driven device for distance-based⁴⁰ or colorimetric⁴¹ Cl^- detection. It has been demonstrated previously that capillary-flow-driven devices have great potential to be used for multiplexed sensing of several analytes.³³

Another important factor in the field applicability of the developed devices is their robustness in terms of user-related operations. In other words, the response of the sensors should not drastically depend on end-user adherence to operating procedures as

end-users may lack manual skills and analytical experience. Several factors that might potentially influence DO readings were selected for testing: device dipping time, defined as the time that the device is immersed in the sample, dipping depth – the length of the device which is immersed in the sample, and dipping angle, indicating the position of the device in respect to the surface of the sample during the sampling process. 90° angle means that the device was perpendicular to the sample surface, while for measurements at 0° angle, an excess of the studied sample was pipetted on the sample introduction zone of the device, lying horizontally. Additionally, the influence of sample temperature on DO readings was studied to verify if the developed sensors can be used for on-site DO testing in samples differing in temperature.

The results obtained are shown in Figure 5. If the dipping time is lower than or equal to 40 s, the differences in this parameter do not influence the measured signal. Although dipping the device in the sample for 60 s might provide higher signals and sensitivity, this option was not further investigated as it is easy to execute in the field. Changing the dipping and angle does not significantly affect the measured signals. These results strongly support the thesis that the developed flow devices are sufficiently robust and user-friendly for on-site testing. On the other hand, the difference in sample temperature causes a difference in the measured signal, especially since decreasing the sample temperature to 14°C results in a significant rise of the signal in comparison to the 25°C sample. The influence of temperature on the photometric Winkled method has been described previously by other Authors, who pointed to a shift in I_3^-/I_2 equilibrium as a potential source of temperature error.¹¹ This effect could be mitigated in a few ways: (i) performing calibration using standards that have similar temperature to the studied

samples; (ii) temperature correcting the obtained result, which is a standard operating procedure for commercial DO probes; (iii) collecting the samples and bringing them to a constant temperature.

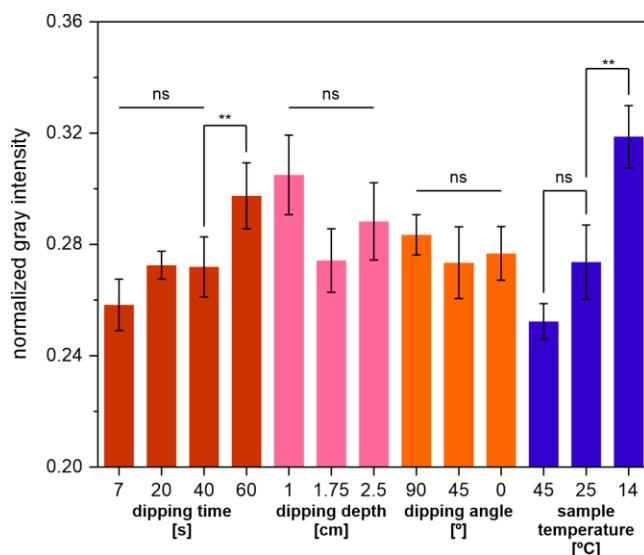


Figure 5. The influence of device dipping time, dipping depth, dipping angle, and sample temperature on DO determination using the developed devices. All signals were normalized by dividing the grayscale intensity by the signal measured for white background and by the measured with DO probe oxygen concentration. Error bars represent standard deviation of $n=3$ measurements.

Finally, the long-term stability of the developed capillary flow-driven devices was studied to ensure that they are susceptible to various storage and shipping conditions. Devices were prepared and stored in the following conditions sealed in plastic bags with moisture absorbers: room temperature, 5°C fridge, and 5°C fridge with additional vacuum sealing in a pouch. A moisture absorber was used to prevent hygroscopic reagents (PTSA, NaOH) from absorbing water from the environment which might compromise the device's stability. To test if the devices are long-term stable, every few days signal for 6.5 ppm DO was measured and compared with the signal registered for freshly prepared devices. The results of this study are shown in Figure 6. Statistical analysis of the obtained data

confirmed that the developed devices can be stored at room temperature or in the fridge in vacuum-sealed pouches for at least 15 days without significant change in the measured signal ($p>0.05$). The small variations in the measured signals are most likely a result of manual assembly of the devices and they should not affect accuracy of DO determination. The stability study results demonstrate that, with appropriate packaging, the sensor can be manufactured in batches and reliably stored and shipped to end users within a practical timeframe. Based on our previous experience,²³ no loss in sensors performance is anticipated during longer than studied periods of storage. This is because all immobilized reagents are long-term stable both as a solid and in solution, reagents which might prematurely react with each other are spatially separated, and finally chemically inert solid support (glass fiber) is used to construct the capillary flow device.

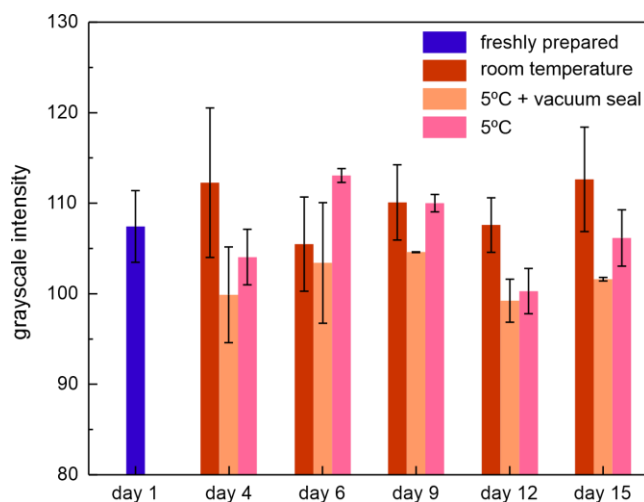


Figure 6. Long-term stability study. Devices were stored in plastic bags at room temperature, in a 5°C fridge sealed in a vacuum, and in a 5°C fridge without additional protection. Error bars represent standard deviation of $n=3$ measurements.

Real samples analysis

In the final step of the presented research, the developed devices were applied for DO determination in real samples. Samples from three water reservoirs in Fort Collins, Colorado, USA (Spring Creek, Sheldon Lake, and Arthur Canal Lagoon) and two aquariums (professional and amateur) were taken into narrow-mouth flasks, allowing for the sample to overflow. They were immediately analyzed using the developed devices as well as with Winkler titration as a reference method without any sample preparation. The results of this experiment are summarized in Table 2.

Table 2. DO determination in real samples of freshwater reservoirs and aquarium water (n = 6).

Sample	On-chip determined DO [ppm]	Relative standard deviation [%]	Winkler titration determined DO [ppm]	Relative error [%]
Spring Creek	6.5	3.2	7.0	-7.1
Sheldon Lake	5.0	1.9	5.5	-9.1
Arthur Canal Lagoon	3.3	6.0	3.4	-2.9
professional aquarium	5.4	5.9	5.4	0.0
amateur aquarium	6.3	4.3	6.5	-3.1

The analysis of the calculated relative errors reveals a small systematic error when comparing DO determination with the developed devices and with standard Winkler titration. The source of this error might be the sample matrix. For example high levels of phosphate ions, according to Figure 4, could contribute to lowering in the measured signal. The magnitude of the relative error does not compromise DO determination with acceptable accuracy for such a simple device. Most importantly, the developed devices are capable of differentiating between lethal levels for fish ($\text{DO} < 3 \text{ ppm}$), stressful levels ($\text{DO} > 3 \text{ ppm}$ and $\text{DO} < 5 \text{ ppm}$), and levels supporting aquatic life ($\text{DO} > 7 \text{ ppm}$).⁴

CONCLUSIONS

In this paper, we have demonstrated the capabilities of capillary flow-driven microfluidic devices made of alternating layers of hydrophilic PET and double-sided adhesive to determine a challenging analyte such as DO. The challenges in DO determination arise from significant interference from atmospheric oxygen and the limited number of available colorimetric methods for this analyte determination. Interference from atmospheric oxygen was limited by almost completely sealing the device and the modified Winkler colorimetric method proved to be sufficiently fast and sensitive for accurate DO determination. We have used starch in the Winkler colorimetric method as a novel addition to this assay to boost its sensitivity. The devices were successfully validated with real samples of fresh water and aquarium water. The main limitation of the proposed DO sensors is their limited applicability to salt water. More research is required to examine the most effective way to compensate for water salinity and expand the collection of samples that can be analyzed using developed devices. A possibility of including an

additional flow channel in the developed capillary flow-driven device for Cl⁻ determination, for salinity and DO multiplexed detection, will be explored in the future. Finally, the developed sensor fulfills requirements for Green Analytical Chemistry, such as minimization of reagents consumption, minimization of waste, safety for the operator or reduction of toxic reagents usage.⁴²

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Supplementary Information for Appendix 2— Capillary Flow-driven, Disposable Device for Dissolved Oxygen Sensing in Water Samples

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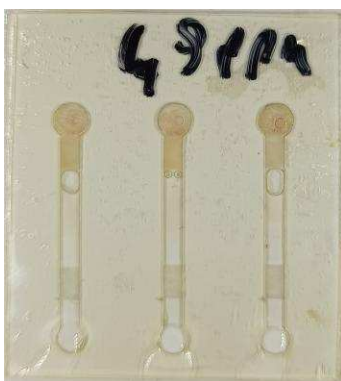


Figure S1. Photograph of capillary flow-driven microfluidic device subjected to a sample containing 4.9 ppm of dissolved oxygen.

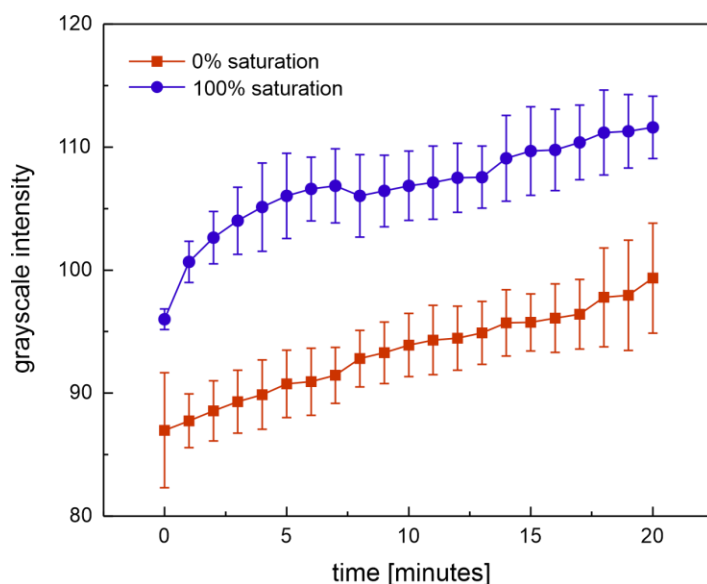


Figure S2. The dependence of the measured grayscale intensity on devices incubation time for 0% and 100% oxygen saturated water. Error bars represent standard deviation of $n=3$ measurements.

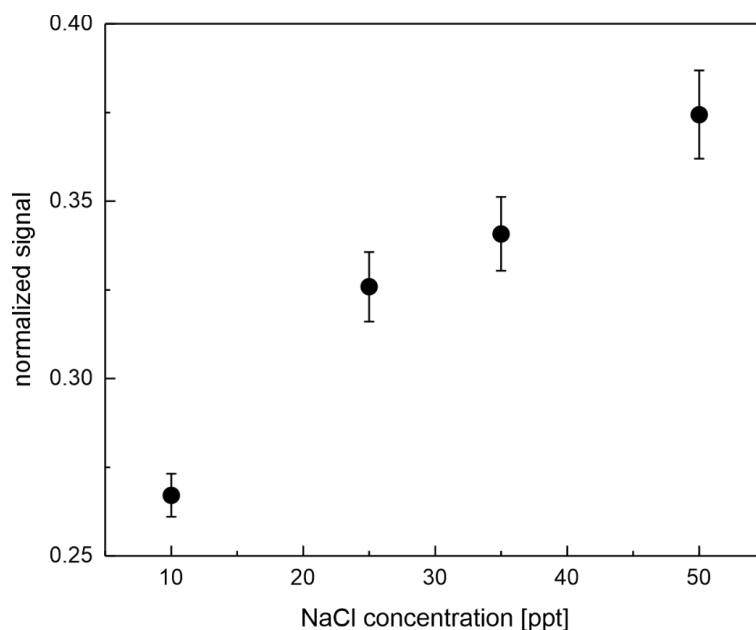


Figure S3. The dependence of the registered grayscale intensity, normalized by dividing by dissolved oxygen concentration and signal measured for white background, on sodium chloride concentration in the sample in parts per thousand (ppt). Error bars represent standard deviation of $n=3$ measurements.

Table S1. Technical parameters of paper types used for detection zone of capillary flow-driven microfluidic device manufacturing. nd – no data

Material	Thickness [mm]	Porosity [μm]	Flow rate	Grammage [g m^{-2}]
Whatman Grade 1 Chromatography Paper	0.180	nd	57 mL/min	87
Whatman Grade 3 Qualitative Filter Paper	0.390	6	28 mL/min	187
Whatman Grade 4 Qualitative Filter Paper	0.205	20-25	247 mL/min	96
Ahlstrom Blotting Paper Grade 319	0.5	19	360 mL/min	179
Glass fiber GFDX203000	0.3	nd	nd	75

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Appendix 3 — Smartphone-Assisted Dual-Sided Capillary Microfluidic Device for Multiplex Detection of Heavy Metals and Nutrients in Drinking Water

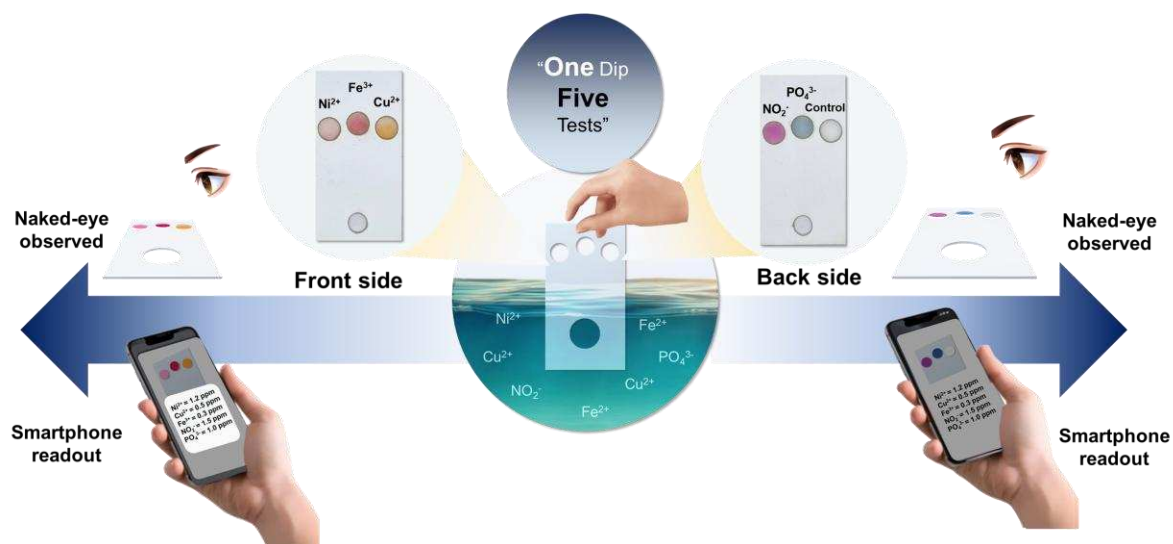
Abstract

Background: Heavy metal and nutrient contamination are growing global issues necessitating the monitoring of water resources. While laboratory-based platforms for detecting these contaminants are sensitive and accurate, they require centralized facilities, trained personnel, and high costs. Microfluidic paper-based analytical devices have emerged as a low-cost alternative for on-site detection of these water contaminants; however, these platforms struggle with slow assay times, loss of analyte, and the need for precise volumetric pipetting. Moreover, these platforms often focus on detecting only one subgroup of contaminants, limiting the potential for comprehensive measurements.

Results: We developed a capillary flow-driven, single-dip dual-sided detection system, enabling rapid, multiplex detection of heavy metals and nutrients in a single user step. The sensor enables both qualitative visual analysis and quantitative analysis via a smartphone app for real-time and on-site detection of Ni, Fe, Cu, NO_2^- and PO_4^{3-} . The limits of detection (LoD) and quantification (LoQ) were calculated as 1.3 and 4.4 ppm for Ni, 0.3 and 0.9 ppm for Fe, 0.2 and 0.6 ppm for Cu, 0.4 and 1.2 ppm for NO_2^- , and 0.5 and 1.6 ppm for PO_4^{3-} . Selectivity was achieved through masking strategies in each detection zone. The sensors were stable for >4 weeks under ambient conditions. Spike-recovery analysis was performed using river, tap, pond, and commercial drinking water, achieving recoveries between 86–112% with accuracy and precision below 15% RSD for all samples.

Significance: This multiplex sensor offers a solution to overcome the current limitations of paper-based devices, allowing for a more comprehensive analysis of multiple contaminant classes. This work has been published in *Analytica Chimica Acta*, Volume 1356, 22 June 2025, 344031.

Graphical abstract



Keywords: Heavy metals, Nutrients, Colorimetric sensor, Microfluidic device, Paper-based device, Smartphone readout

1. Introduction

Every day, 80% of the world's wastewater is released into the environment untreated, carrying a range of pollutants, from highly toxic industrial chemicals to excess agricultural

and urban runoff, putting more than 2 billion people at risk of exposure to unsafe water.^{1,2} Among these contaminants, heavy metals and nutrients have drawn particular attention due to their chronic and long-term impacts on human health and ecosystems.³⁻⁵ Even at low exposure levels, heavy metals such as Fe, Cu, and Ni can exceed the maximum contaminant level (MCL), or health advisory levels set to protect human health. The limits are 0.1 ppm for Ni, 1.3 ppm for Cu, and 0.3 ppm for Fe.⁶⁻⁸ These metals, even at trace concentrations, can pose significant risks to both environmental and public health.⁸ Chronic exposure can result in neurotoxicity, hepatotoxicity, nephrotoxicity, and disruption of cellular homeostasis.^{9,10} These contaminants, often introduced into water sources through industrial effluents, mining activities, and improper waste management, pose a critical public health threat, contributing to an estimated 1.6 million child fatalities annually.^{11,12}

At the same time, nutrient pollution, primarily from excess nitrogen and phosphorus, is another significant issue.^{13,14} Human activities, including agricultural runoff from fertilizers, wastewater discharge, emissions from automobiles, and waste from livestock, introduce these nutrients at concentrations that far exceed the natural processing capacity of the water ecosystem. This overabundance drives a surge in biomass production, depletes oxygen, and promotes harmful algal blooms (eutrophication),^{15,16} a process with extensive ecological consequences. Eutrophication can lead to the death of fish and aquatic species, while excessive algal growth clogs rivers and streams, disrupting water flow in pipelines and turbines. Additionally, toxic algal blooms along coastlines, such as red tides and dinoflagellate outbreaks,¹⁷ pose serious risks to human health by releasing harmful toxins that can cause respiratory issues and other health

complications.^{18,19} NO_2^- is a critical nitrogen-based nutrient in the environment,²⁰ while PO_4^{3-} s are the primary form of phosphorus nutrients.²¹ To protect public health, the USEPA has set maximum allowable concentrations for NO_2^- in drinking water at 1 mg L^{-1} .²² While there is no federally established Maximum Contaminant Level (MCL) for phosphate (PO_4^{3-}) in drinking water by the U.S. EPA, the EPA has set a guideline of 0.10 mg/L for total phosphorus to help prevent cultural eutrophication in flowing water.²³

While advanced analytical techniques like inductively coupled plasma mass spectrometry (ICP-MS),²⁴ inductively coupled plasma optical emission spectrometry (ICP-OES),²⁵ atomic absorption spectrometry (AAS),²⁶ and UV-Vis spectroscopy,²⁷ can detect heavy metals and/or nutrients, they have limitations. Specifically, these methods require complex sample preparation, skilled personnel, and expensive, bulky equipment, making them impractical for portable and on-site use.²⁸ Microfluidic paper-based analytical devices (μ PADs) offer a promising solution, as they are simple, portable, and cost-effective.^{29–32} These devices rely on capillary action through cellulose microchannels for fluid movement, eliminating the need for external pumps and making them ideal for field applications.^{33–35} Many μ PADs target a single analyte or multiplex for one class of analytes at a time, such as metals, nutrients, pesticides, or PFAS.^{36–42} It has been well established in the literature that the presence of one contaminant class can influence the fate and occurrence of another.^{43–45} For instance, iron and copper affect phosphorus bioavailability, disrupting nutrient cycling in ecosystems and water treatment.⁴⁶ In anoxic conditions, reduced iron (Fe^{2+}) drives iron-dependent denitrification, depleting nitrogen levels.⁴⁷ Therefore, there is a need to expand the comprehensiveness of these sensing platforms to multi-class analyte detection in a single test to better understand

contaminant interactions and inform more effective water management strategies which could save significant time, money, and resources, particularly in resource-limited settings.⁴⁸

However, developing a single μ PAD capable of detecting such chemically distinct species presents a significant technical challenge. Conventional μ PADs have advanced field-deployable sensing but face significant limitations due to their reliance on cellulose matrices. These include slow transport rates, leading to long assay times (up to hours), uncontrolled fluid dynamics requiring precise pipetting for volumetric accuracy, substantial analyte loss from adsorption to cellulose fibers, and the inability to separate chemically incompatible reagents within a single device.⁴⁹

In response to these challenges, we developed a single-dip, dual-sided detection system capable of simultaneously detecting both heavy metals and nutrients in a single test. Specifically, this system detects Cu, Fe, Ni, NO_2^- , and PO_4^{3-} —analytes selected due to their environmental and regulatory significance. Fe, Ni, and Cu are common contaminants originating from industrial, mining, and geological sources, often co-occurring and exhibiting synergistic toxic effects.^{50,51} NO_2^- and PO_4^{3-} serve as key indicators of water quality, eutrophication, and ecosystem health.^{52,53} This selection enables a comprehensive water quality assessment while considering interrelated effects, such as Fe influencing NO_2^- detection and PO_4^{3-} bioavailability, and Cu/Ni affecting microbial cycling.^{46,47} These analytes are widely monitored due to their health and environmental risks, making their detection crucial for regulatory compliance.

Unlike conventional μ PAD-based systems, our platform employs a capillary flow-driven microfluidic approach that overcomes key limitations of paper-based devices. The open laminate design achieves flow velocities $\sim 145\times$ faster than μ PADs, eliminating slow transport rates and long assay times.³⁵ The hollow capillary action enables fixed-volume dipping without requiring precise pipetting, while adsorption-resistant polymer surfaces minimize analyte loss, enhancing detection sensitivity. Additionally, the platform's dual-sided fluidic isolation allows simultaneous detection of chemically incompatible species (e.g., Fe^{3+} and PO_4^{3-}) without cross-talk.⁴⁷ These advancements enable rapid, multi-contaminant detection within five minutes, while maintaining smartphone compatibility for real-time field-based water quality analysis.

A key innovation of this platform is its dual-sided sensor design, where one side detects heavy metals and the other detects nutrients, mitigating interference effects and enabling comprehensive water quality assessment. This approach is particularly critical for improving nitrite (NO_2^-) detection, as iron-catalyzed reactions can otherwise lead to its underestimation. Integrated monitoring of Fe^{3+} , PO_4^{3-} , and NO_2^- is essential for understanding contaminant interactions that influence biofouling, harmful algal blooms, and oxygen transport in aquatic systems. By employing digital image colorimetry (DIC) via a smartphone application, the device quantitatively analyzes colorimetric changes across analyte concentrations, correcting for variations in lighting and device inconsistencies. This method achieves detection limits near or below regulatory thresholds without requiring complex substrate modifications, making it a practical and accessible tool for real-time, field-based water quality assessment. By streamlining multi-contaminant detection in a single test, this platform provides an effective solution for

water monitoring in resource-limited settings, facilitating improved environmental management and public health protection.

2. Experimental

2.1 Materials and Chemicals

All metal standards, including nickel (II) sulfate hexahydrate, cupric sulfate pentahydrate, and ferric chloride hexahydrate, reagents such as dimethylglyoxime, bathocuproine, and bathophenanthroline, along with the masking reagents sodium fluoride and ammonium acetate, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxylamine hydrochloride and buffer components were obtained from Fisher Scientific. Double-sided adhesive (467MP) and transparency film (9984) were sourced from 3M (St. Paul, MN, USA), while Whatman paper was also acquired from Fisher Scientific. Additionally, PVC foil (0.7 mm) was purchased from Deecraft Company in Wang Thonglang-Bangkok, Thailand.

2.2 Instrumentation

The developed platform, comprised of adhesive tapes, transparency films, and PVC sheets, was initially designed using OnShape and then cut with an Epilog Engraver WinX64 Zing laser cutter. The device was subsequently laminated using a Royal Sovereign CL-1223 thermal laminator.

2.3 Device Design and Fabrication

The device was created by stacking alternating layers of double-sided adhesive (DSA) and transparency film (9984). The bottom layer (Layer 1) is a transparency film (TF) with dimensions of 4 cm x 2.5 cm, followed by a flow channel layer consisting of DSA/TF/DSA (Layer 2), another transparency film (Layer 3), double-sided adhesive (Layer 4), and a PVC sheet (Layer 5), from bottom to top. A 6 mm circle was cut in each layer above the 1st layer, along with an 8 mm inlet. On the flow channel layer, a 2.5 cm x 2.5 mm flow channel was laser-cut, creating three channels with a common dip zone/inlet of 8 mm (**Figure 1a**). A 6 mm diameter Ahlstrom blotting paper or Whatman 1 were cut with a 3 mm x 2.5 mm tail at the end, which helps guide the flow and mitigate surface tension at the paper and transparency film interface. This entire assembly constitutes one side of the sensor. The same pattern was followed for the opposite side, and the two sides were attached using DSA of the same dimensions (4 cm x 2.5 cm) (**Figure 1b**). Additionally, upon immersion into the analyte-containing water, the sensor undergoes a distinct, observable color change. This change results from the specific interaction between the analyte and the colorimetric reagents embedded within the sensor's detection zone (paper), leading to a shift in color that visually reflects the presence and concentration of the analyte. This color transition is qualitative, indicating the presence of a target compound, and quantitative, allowing for analyte concentration assessment based on the intensity and hue of the color change (**Figure 1c**).

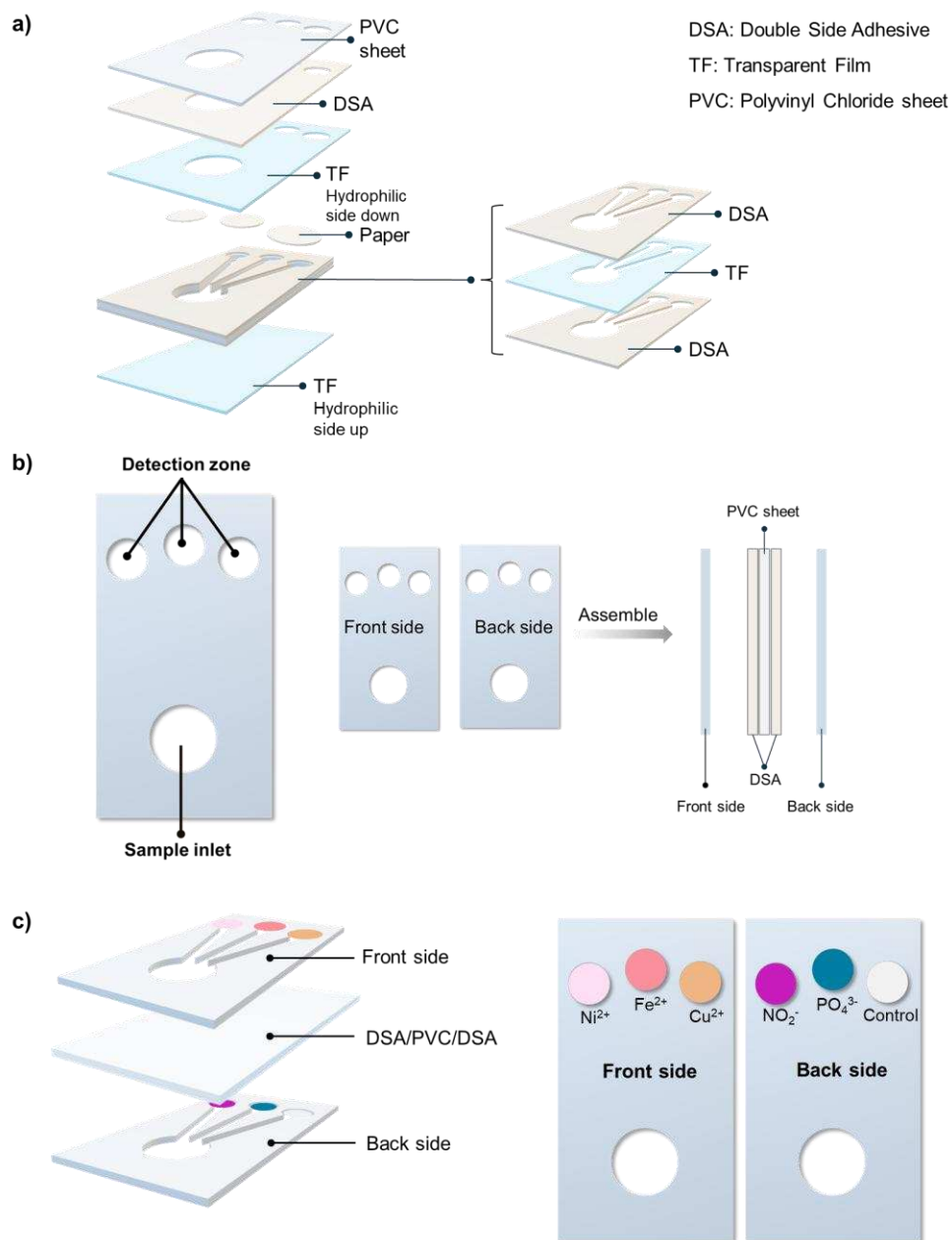


Figure 1. Stacking alternating layers of the Multiplex Colorimetric Sensor (a), the finalized two-sided pattern of the Multiplex Colorimetric Sensor (b), and the visible color change after dipping into the analyte-containing water source (c).

2.4 Colorimetric Metal Detection

2.4.1 Standards: Nickel(II) sulfate hexahydrate, Copper(II) sulfate pentahydrate, Iron(III) chloride hexahydrate, potassium dihydrogen phosphate ($\text{H}_2\text{K}_2\text{P}_2\text{O}_7$), and sodium nitrite (NaNO_2) were used as standards for Ni(II), Cu(II), Fe(III), PO_4^{3-} , and NO_2^- , respectively. Each standard solution was prepared at a concentration of 1000 ppm in 18.2 M Ω ·cm Millipore water, and all assays for detecting heavy metals and nutrients are shown in **Figure 2a-b**. To analyze potential interference from non-target ions, solutions of seven other metallic and non-metallic ions were used.

2.4.2 Ni–Dimethylglyoxime (DMG) Assay: Two aliquots of 4 μL of DMG were precisely pipetted onto the detection pad, followed by the addition of 2 μL of ammonium hydroxide (pH 9.5). Each reagent addition was allowed to dry completely before the subsequent addition. In the pretreatment zone, two aliquots of 5 μL ammonium acetate (6.0 M) were applied, followed by one aliquot of 1 μL NaF (0.5 M), with drying periods between each application. Subsequently, the device was dipped in the standard sample solution through the sample inlet.

2.4.3 Cu–Bathocuproine Assay: Two 8 μL aliquots of hydroxylamine (0.1 g/mL) prepared in acetate buffer (4.5 pH, 6.3 M) were added to the detection pad followed by 4 μL of bathocuproine (0.005 g/mL) prepared in methanol. The detection pad was allowed to dry completely before adding each reagent. Subsequently, the developed sensor was dipped in the standard sample solution through the sample inlet.

2.4.4 Fe–Bathophenanthroline Assay: Two 8 μL aliquots of hydroxylamine (0.1 g/mL) prepared in acetate buffer (4.5 pH, 6.3 M) were added to the detection pad followed by 4 μL of bathophenanthroline (0.025 g/mL) prepared in isopropanol. The device was

allowed to dry completely with the addition of each reagent. Subsequently, the developed sensor was dipped in the standard sample solution through the sample inlet.

2.4.5 PO_4^{3-} – Molybdenum Blue Assay: Initially, 5 μ L of 30.0 mM ascorbic acid was pipetted onto the detection pad. In the pretreatment zone, 2 M p-toluene sulfonic acid was added to the first glass fiber followed by 5 μ L of 30 mM molybdenum (VI) in the second glass fiber, with drying periods allowed between each application. Subsequently, the developed sensor was dipped in the standard sample solution through the sample inlet.

2.4.6 NO_2^- – Griess’s Reagent Assay: Two 7 μ L aliquots of Griess reagent (0.05 g/mL) prepared in Millipore water were added to the detection pad. The device was allowed to dry completely. Finally, the developed sensor was dipped in the standard sample solution through the sample inlet.

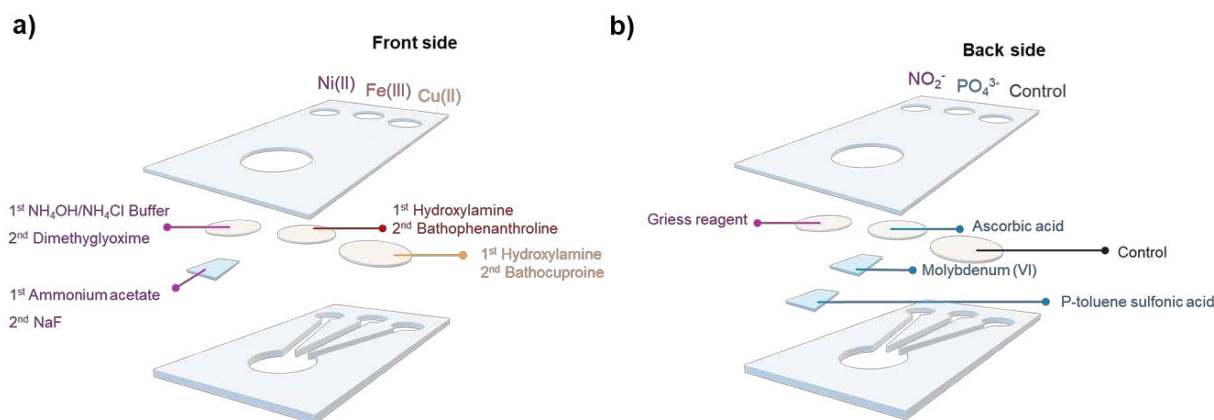


Figure 2. The finalized front and back sides of the developed device for detecting heavy metals (a) and nutrients (b).

2.5 Image Analysis

For image analysis, after the optimized time, the sensor image was captured in a controlled lightbox using the MATLAB Mobile app to quantify the signals. Our code automates the entire imaging process. Upon running the code, it prompts the user to take an image of the sensor. Then, it creates a binary mask and isolates the region of interest (ROI). Next, it inverts the colors on the optimized channel scales. Simultaneously, it normalizes the intensity using the area of the PVC located above the detection zones. This step mitigates inconsistencies in lighting conditions during real-world applications, where lighting may vary. As the color signal is always normalized to a constant white background, the relative grayscale value is always consistent. After normalization, the code displays the normalized intensity on the screen. Once calibrated, the system can display the corresponding concentration based on the response received.

2.6 Selectivity of the Multiplex Device

To assess the selectivity of the proposed multiplex device, various potential interfering species were evaluated. Standard solutions of each interfering compound, including PO_4^{3-} , AsO_4^{3-} (1:100), Cl^- , SiO_4^{4-} (1:10 and 1:100), Cd^{2+} , Cu^{2+} , Fe^{3+} , SO_4^{2-} , NO_3^- , Ca^{2+} , Zn^{2+} , Co^{2+} , Ni^{2+} , HCO_3^- , Mg^{2+} , as well as surfactants (Surf 1: sodium dodecyl sulfate, Surf 2: tetramethyl(tetradecyl) ammonium chloride) were prepared. The potential interference was examined at different ratios (targeted: non-targeted). The color shift on the detection pad was quantitatively analyzed with MATLAB software.

Real Sample Analysis: Spike recovery analysis was performed to validate the sensor's performance in various water samples, including commercial drinking water, tap water, and river water from the Poudre River. Three concentrations of these heavy metals and

nutrients were spiked to these different water samples and recovery and precision were analyzed using the smartphone platform.

3. Results and Discussion

3.1 Device Performance

The device utilizes capillary action to generate flow, allowing it to be immersed directly into the sample solution without the need for pipetting (**Video S1**). This design feature enhances user-friendliness, making the device more accessible and easier to use. We first sought to understand and optimize device performance by studying the impact of each functional element on performance.

Color uniformity is crucial for reliable colorimetric readouts in μ PADs as it enhances analytical performance, with the paper type being a key factor.⁵⁴ To optimize this parameter, several paper types were tested, including Whatman® Grades 1, 3, and 4, as well as Ahlstrom blotting paper. Among these, for detecting Fe, Cu, and NO_2^- , Whatman® Grade 1 paper emerged as the best choice, offering the best average color intensity with the lowest RSD (Figure S1 and Table S1). The superior performance of Whatman® Grade 1 is linked to its optimal thickness and surface properties, as thicker substrates and surface heterogeneity tend to reduce signal intensity and signal-to-noise ratio. For Ni and PO_4^{3-} detection that requires pretreatment zones or glass fibers within the channels, thicker paper substrates were found to work better. Consequently, Ahlstrom blotting paper was selected for Ni and PO_4^{3-} detection in this study due to the higher intensity and less variability (Figure S1, S2, and Table S1). Another advantage of our system is that we can use multiple types of paper in a single device. The reaction times for detecting heavy

metals and NO_2^- were optimized to 5 min (Figure S3). After 5 min, the color signal plateaus, indicating the completion of the reaction. In contrast, PO_4^{3-} detection required a longer reaction time of 8-10 min. Overall, the results of time optimization indicate that measurements should be taken after 8 min to ensure comprehensive and accurate detection.

The smartphone-based imaging was optimized for sensitivity to different analytes by analyzing grayscale intensity in specific color channels. Ni, Fe, Cu, and NO_2^- were calibrated using the green channel, while PO_4^{3-} was measured using the red channel. These channels were chosen based on the best calibration slopes obtained in previous studies.^{39,48,54,55} To ensure consistent and accurate readings, even with colored water samples, a control zone was included to account for background interference, minimizing the impact of lighting or color variations.

3.2 Colorimetric Assays

Metals detection

Dimethylglyoxime (DMG) is a well-known chemical reagent utilized for the analysis of nickel ions (Ni^{2+}) that forms a characteristic pinkish-red precipitate in the presence of Ni^{2+} .^{56,57} The pH was fixed to be 9.5 at the detection pad using a dried concentrated $\text{NH}_4\text{OH}/\text{NH}_4\text{Cl}$ buffer. **Figure 3a** shows the calibration for Ni(II). A linear range was found from 1–20 ppm. The LoD and LoQ were calculated to be 1.3 and 4.4 ppm, respectively. The LoD was calculated using 3.3 times the standard deviation (SD) of the blank divided by the slope of the calibration curve. The LoQ was quantified using

10 times the SD of the blank divided by the slope of the calibration curve ($n = 9$ for both).⁵⁸

Fe was detected using bathophenanthroline, a chelating agent that forms a stable, red-colored complex $[\text{Fe}(\text{bathophenanthroline})_3]^{2+}$.⁵⁹ This agent is more selective for Fe(II) over other metal ions compared to 1,10-phenanthroline. This increased selectivity is attributed to its larger and more complex structure, which fits better with Fe(II) ions,⁶⁰ thereby reducing the likelihood of forming complexes with other metal ions present in the sample. The use of hydroxylamine as a reducing agent converts other forms of iron to Fe(II), enabling the system to detect all labile oxidation states of Fe. A linear calibration curve for Fe(III) was established from 0.1 - 20 ppm (**Figure 3b**). The LoD and LoQ were calculated to be 0.3 ppm and 0.9 ppm, respectively. Furthermore, the LoD for Fe achieved with the proposed PADs meets the EPA's recommended secondary maximum contamination level (SMCL) of 0.3 ppm for Fe content in drinking water, thus aligning with regulatory requirements.⁷

Cu was detected using the Cu(I)–bathocuproine assay, where Cu(I) reacts with bathocuproine under acidic conditions (pH 4.5) to form an orange-colored complex after the reduction of all Cu(II) to Cu(I). Unlike other cuproines, bathocuproine does not produce a color with Fe(II), making it highly selective for Cu(I) detection.^{61,62} The use of hydroxylamine as a reducing agent converts other forms of copper to Cu(I), enabling the system to detect all labile oxidation states of Cu. The Cl^- ions in the NaCl/acetic acid buffer stabilize the cationic Cu–bathocuproine complex.⁶³ A quantitative calibration curve for Cu(II) is shown in **Figure 3c**, with a linear range from 0.5 to 25 ppm. The LoD and LoQ were determined to be 0.2 ppm and 0.6 ppm, respectively. The LoD for Cu

using the proposed sensor complies with the EPA's recommended maximum level of 1.3 ppm for Cu in drinking water, thereby adhering to regulatory standards.²²

Nutrient Detection

The Griess assay uses a diazo-coupling reaction to detect NO_2^- .⁶⁴ Under acidic conditions, NO_2^- diazotizes amino-containing organic molecules, forming a diazonium salt. This salt then reacts with N-(1-naphthyl)ethylenediamine (NED), creating a larger conjugated structure that produces a color change.²⁰ A calibration curve for NO_2^- in the developed sensor is shown in **Figure 3d**. A linear range was established from 0.25 to 10 ppm. The LoD and LoQ were determined to be 0.4 ppm and 1.2 ppm, respectively. These values are comparable to those of previously developed paper-based colorimetric sensors for NO_2^- .^{65–67} Notably, the achieved LoD for NO_2^- concentration using the proposed analytical device adheres to the guideline level of 1 ppm for NO_2^- in drinking water, thereby conforming to regulatory standards.²²

The phosphomolybdenum blue reaction is widely used for PO_4^{3-} determination. In this process, PO_4^{3-} reacts with molybdate under acidic conditions to form a polyoxometallate species, known as a heteropoly acid. This intermediate is then reduced to produce the intensely colored phosphomolybdenum blue (PMB) species.⁶⁸ For this method, we followed our optimized protocol from recently published work.⁶⁹ A quantitative calibration curve for PO_4^{3-} is shown in **Figure 3e**. A linear range was established for 0.5–20 ppm. The LoD and LoQ were determined to be 0.5 ppm and 1.6 ppm, respectively. Significantly, the proposed sensor achieved a LoD for PO_4^{3-}

concentration that is relevant for active monitoring of real water phosphate contaminants, as demonstrated in our previous study.^{68,70}

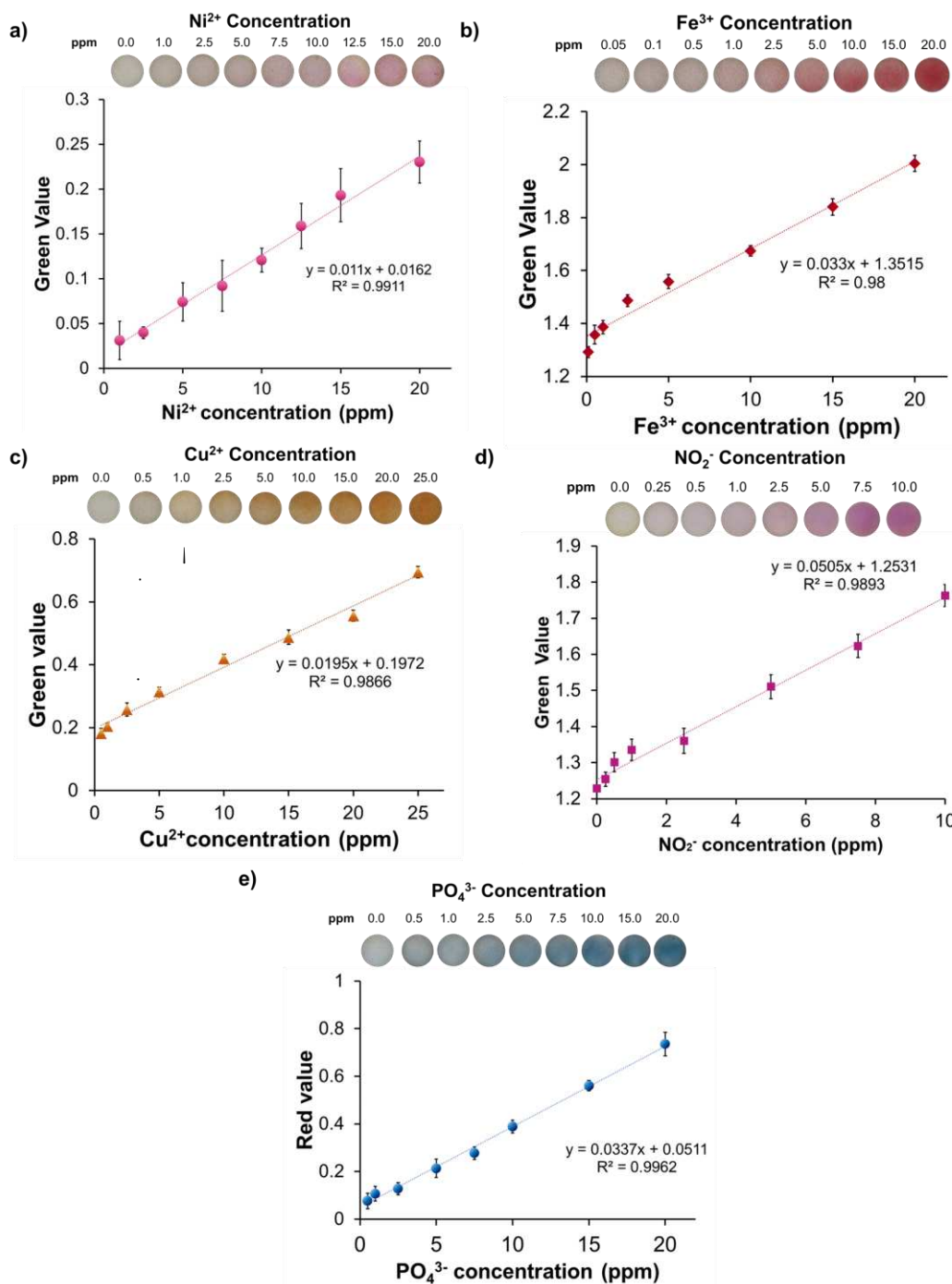


Figure 3. Quantitative calibration and standard color charts for μ PADs -based detection of heavy metals, including Ni(II) (a), Fe(III) (b), Cu(II) (c), and nutrients including NO₂⁻ (d) and PO₄³⁻ (e) on the developed sensor. The linear fits were calculated to be $y = 0.011x$

+ 0.016 for Ni, $y = 0.033x + 1.352$ for Fe, $y = 0.020x + 0.197$ for Cu, $y = 0.051x + 1.253$ for NO_2^- , and $y = 0.034x + 0.051$ for PO_4^{3-} . Error bars indicate the standard deviation across five replicates.

Table 1. Summary of the analytical performance of the multiplex sensor for detecting heavy metals and nutrients, enabling simultaneous monitoring of multiple contaminants.

Analytes	Limits (ppm) (EPA)	Linear range (ppm)	Color	Reagent	LoD (ppm)	LoQ (ppm)
Ni	0.1	1–20	Pinkish red	DMG	1.3	4.4
Fe	0.3	0.1–20	Red	Bathophenanthroline	0.3	0.9
Cu	1.3	0.5–25	Orange	Bathocuproine	0.2	0.6
NO_2^-	1.0	0.25–10	Purple	Griess's reagent	0.4	1.2
PO_4^{3-}	0.1	0.5–20	Blue	Molybdenum (VI)	0.5	1.6

3.3 Interference Studies

As previously reported, Fe(III), Cu(II), and Co(II) can interfere with the formation of the Ni–DMG complex. To mitigate this, the pretreatment zone was modified with sodium fluoride and ammonium acetate.⁷¹ Similarly, to prevent interference in the Cu–bathocuproine assay from Zn(II), Co(II), and Cd(II), 0.5 g/mL of hydroxylamine was added to the detection zone.^{71,72} Cu(II) was found to suppress the Fe(Bphe)₂ signal when mixed with Fe at a 1:10 ratio; this was addressed by treating the Fe detection zone with 0.5 g/mL of hydroxylamine.^{55,71} Interference tests for Ni, Fe, Cu, and NO_2^- were conducted at 1:10 ratios, while certain known interferences for PO_4^{3-} , which can also undergo the molybdenum blue reaction, were examined at 1:100 ratios. Due to the masking strategies combined with the sensor's unique design, these results are more robust than those of previously developed metal and nutrient assays, showcasing higher selectivity levels.^{71,73} This level of selectivity ensures that the system provides sturdy and accurate results, demonstrating resistance to interference from multiple ions (**Figure 4**).

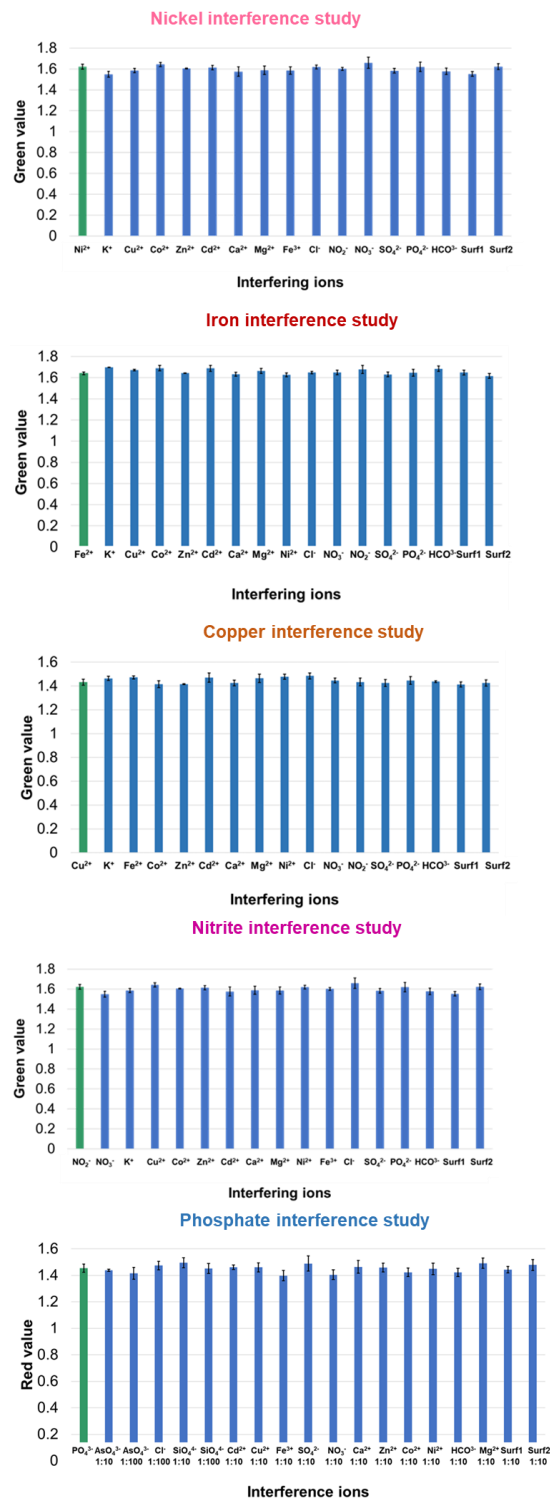


Figure 4. Interference study for all three assays for different ratios (targeted: nontargeted).

3.4 Stability Test

Device stability was evaluated over time under various storage conditions, including freezer (-20 °C), refrigerator (5 °C), and room temperature (22 °C). To minimize the evaporation of chromogenic and masking reagents, devices were sealed in opaque foil bags for storage. The heavy metal assay devices demonstrated stable signals over four weeks (n = 3) at all temperatures. In contrast, the NO₂⁻ assay was more stable when stored at 5°C or -20°C. It is well-documented that the Griess reagent is prone to degradation due to self-oxidation if not stored properly.⁷⁴ These stability findings indicate that the devices are sufficiently stable for batch manufacturing, sealed packaging, and distribution to end users within a reasonable timeframe, as shown in **Figure 5**. The long-term stability of phosphomolybdenum blue assay in the same lateral flow channel has already been established.⁶⁹

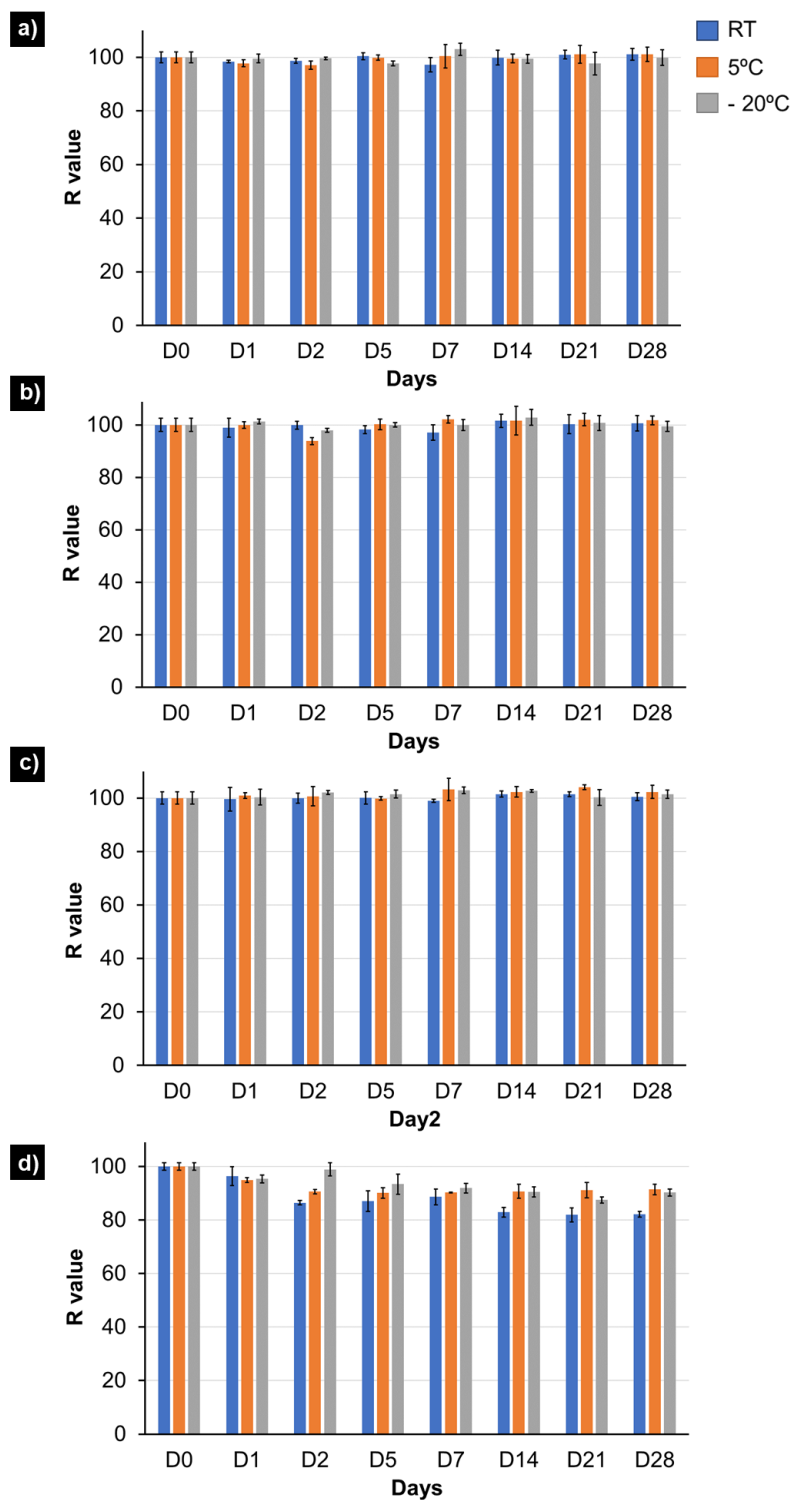


Figure 5. Stability study for Ni (a), Fe (b), Cu (c), and NO₂⁻ (d) assays on the developed device.

3.5 Application in Real Water Sample

To evaluate the sensor's performance with real water samples, water was collected from various sources, including commercial drinking water (King Soopers grocery store), the Poudre River (GPS coordinates 40.5704685, -105.0821559), a pond (40.5950209, -105.0751458), and tap water. Since the natural concentrations of the target analytes in these samples were below the sensor's detection limits, spike recovery analysis was conducted as an analytical method validation protocol. Three different concentrations of each analyte, chosen to fall within the dynamic range and exceed the system's LoD, were spiked into the water samples. Before calculating recovery, a blank test was conducted, and the native concentration of the target analytes in the real samples was quantified. These values were subtracted from the measured concentration after spiking, ensuring that the reported recoveries accurately reflect the method's true analytical performance rather than an overestimation due to pre-existing analytes. The accuracy and reliability of the detection system under conditions resembling real-world scenarios were assessed by calculating percent recovery and standard deviations. For the collected river sample, recovery was determined to be 93–105% for Ni, 100–105% for Fe, 86–107% for Cu, 104–108% for PO_4^{3-} , and 99–105% for NO_2^- . For commercial drinking water samples, the recovery was 98–108% for Ni, 91–104% for Fe, 95–107% for Cu, 93–104% for PO_4^{3-} , and 105–109% for NO_2^- . For the collected pond sample, the recovery was 107–109% for Ni, 95–108% for Fe, 106–112% for Cu, 102–124% for PO_4^{3-} , and 94–104% for NO_2^- . For the tap water, the recovery was 106–114% for Ni, 92–111% for Fe, 86–101% for Cu, 100–110% for PO_4^{3-} , and 104–114% for NO_2^- (**Table 2**).

Table 2. Analyte Recovery Performance Analysis by Spiking with Real Sample (n=3)

Metal	Spiked	Recovered	Recovery	RSD

	(ppm)	(ppm)	(%)	(%)
Tap water				
Ni	15	16.15±0.35	108	2.2
	10	11.43±0.20	114	1.7
	5	5.33±0.50	107	9.3
Fe	15	14.62±0.51	97	3.5
	10	9.23±0.90	92	9.7
	5	5.56±0.15	111	2.7
Cu	15	12.97±0.26	86	2.0
	10	10.11±0.36	101	3.6
	5	4.55±0.19	91	4.1
PO₄³⁻	15	15.08±0.04	101	0.3
	10	10.02±0.04	100	0.4
	5	5.55±0.17	111	3.1
NO₂⁻	10	10.45±0.16	105	1.5
	5	5.72±0.20	114	3.5
	1	1.09±0.10	109	9.6

Pond water				
Ni	15	16.19±0.99	108	6.2
	10	10.91±0.87	109	8.0
	5	5.47±0.27	109	5.0
Fe	15	15.02±0.89	100	6.0
	10	10.82±0.49	108	4.5
	5	4.78±0.22	96	4.5
Cu	15	15.92±1.72	106	10.8
	10	11.26±0.73	113	6.5
	5	5.36±0.37	107	6.8
PO₄³⁻	15	15.37±0.33	102	2.2
	10	10.55±0.17	105	1.6

NO₂⁻	5	6.24±0.35	125	5.7
	10	10.00±0.39	100	4.0
	5	5.23±0.15	105	3.0
	1	0.95±0.11	95	12
River water	Ni			
	15	15.80±0.90	105	5.7
	10	9.30±0.13	93	1.4
	5	4.96±0.25	99	5.1
	Fe			
	15	15.05±0.67	100	4.4
	10	10.52±0.24	105	2.3
	5	5.27±0.41	105	7.8
	Cu			
	15	16.08±0.52	107	3.2
	10	9.71±0.24	97	2.5
	5	4.34±0.35	87	8.0
	PO₄³⁻			
	15	15.66±0.30	104	1.9
	10	10.60±0.26	106	2.5
	5	5.42±0.25	108	4.6
	NO₂⁻			
	10	10.55±0.09	105	0.9
	5	5.25±0.18	105	3.4
	1	0.99±0.08	99	7.9

**Drinking
water**

Ni

15	16.21±1.20	108	7.4
10	9.14±0.37	91	4.1
5	4.94±0.43	99	8.6

Fe

15	13.68±0.41	91	3.0
10	10.46±0.21	105	2.0
5	4.61±0.27	92	5.9

Cu

15	14.32±0.88	95	6.2

PO_4^{3-}	10	12.11 ± 0.42	107	4.0
	5	5.08 ± 0.15	102	3.0
NO_2^-	15	13.98 ± 0.50	93	3.6
	10	10.23 ± 0.96	102	9.4
	5	5.24 ± 0.19	105	3.5
	10	10.67 ± 0.27	107	2.5
	5	5.48 ± 0.37	110	6.8
	1	0.89 ± 0.09	105	8.7

Conclusion

This study presents a novel approach to water quality monitoring with the development of a single-dip, dual-sided detection system designed to simultaneously detect heavy metals (Cu, Fe, Ni) and nutrients (NO_2^- , PO_4^{3-}) in a single test. The sensor design overcomes the limitations of existing paper-based microfluidic devices by controlled volume delivery, streamlining the testing process, and enabling the rapid, multiplex detection different class of contaminants in a single step. The sensor achieved LoD and LoQ that meet or exceed regulatory guidelines. Additionally, the sensor features digital image colorimetry via a smartphone application, enabling real-time, quantitative analysis. The system exhibited a high level of selectivity and stability for over four weeks under ambient storage conditions and achieved recoveries ranging from 86–112% with precision below 15% RSD in spike-recovery analysis. Moreover, this novel approach offers a powerful tool for researchers to study interactions, occurrence, and correlations among the different classes of contaminants in water sources, while smartphone integration enhances accessibility and real-time capabilities, aligning with the growing demand for decentralized, reliable monitoring tools in resource-limited settings. Future work will

expand the functionalities of the sensor and the smartphone application to support large-scale deployment and broader water quality monitoring networks. Additionally, the sensor could be adapted to detect other classes of contaminants, such as pesticides, pharmaceutical products, and PFAS.

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Supplementary Information for Appendix 3 — Smartphone-Assisted Dual-Sided Capillary Microfluidic Device for Multiplex Detection of Heavy Metals and Nutrients in Drinking Water

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Table S1. Paper substrate optimization

Analyte	Signal (AVG)	SD	RSD (%)	
Phosphate (W1)	105.0537	4.2966	4.0899	a)
Phosphate (W3)	129.7740	6.9242	5.3356	
Phosphate (W4)	112.2437	1.8984	1.6913	
Phosphate (AB1)	130.1853	1.401786	1.076762	
Nitrite (W1)	109.4043	0.4332	0.3960	b)
Nitrite (W3)	92.0293	0.5923	0.6436	
Nitrite (W4)	95.8690	0.6506	0.6786	
Nitrite (AB1)	93.8757	0.6280	0.6690	
Nickel (W1)	94.7623	1.2917	1.3631	c)
Nickel (W3)	104.2397	1.2917	1.2391	
Nickel (W4)	104.2007	0.3932	0.3774	
Nickel (AB1)	111.1577	1.0232	0.9205	
Iron (W1)	111.3613	0.2254	0.2024	d)
Iron (W3)	105.0827	2.1458	2.0420	
Iron (W4)	105.1457	1.4111	1.3420	
Iron (AB1)	114.4663	2.9314	2.5609	
Copper (W1)	95.1780	0.8286	0.8706	e)
Copper (W3)	88.8767	1.0298	1.1587	
Copper (W4)	87.3790	2.3445	2.6831	
Copper (AB1)	90.2333	1.9966	2.2127	



Figure S1. Photograph showing color changes for different paper substrates, including Whatman 1, Whatman 3, Whatman 4, and Ahlstrom blotting paper, used in the detection of (a) Ni, (b) Fe, (c) Cu, (d) NO₂⁻, and (e) PO₄³⁻.

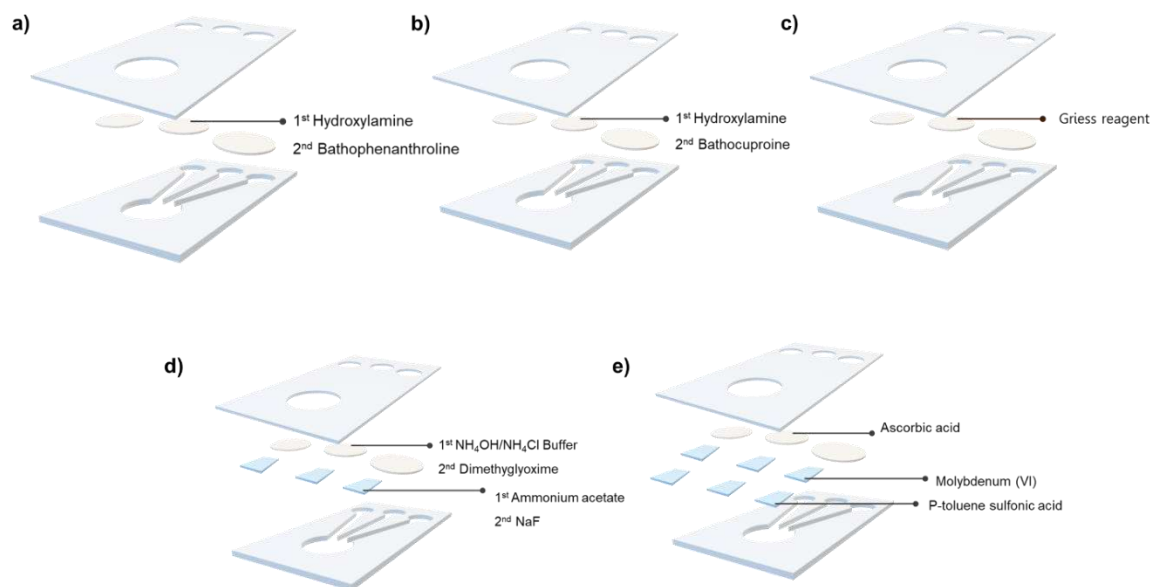


Figure S2. Stacking alternating layers of the Multiplex Colorimetric Sensor without (a-c) and with (d,e) pretreatment zone for detecting Fe, Cu, NO_2^- , Ni, and PO_4^{3-} respectively

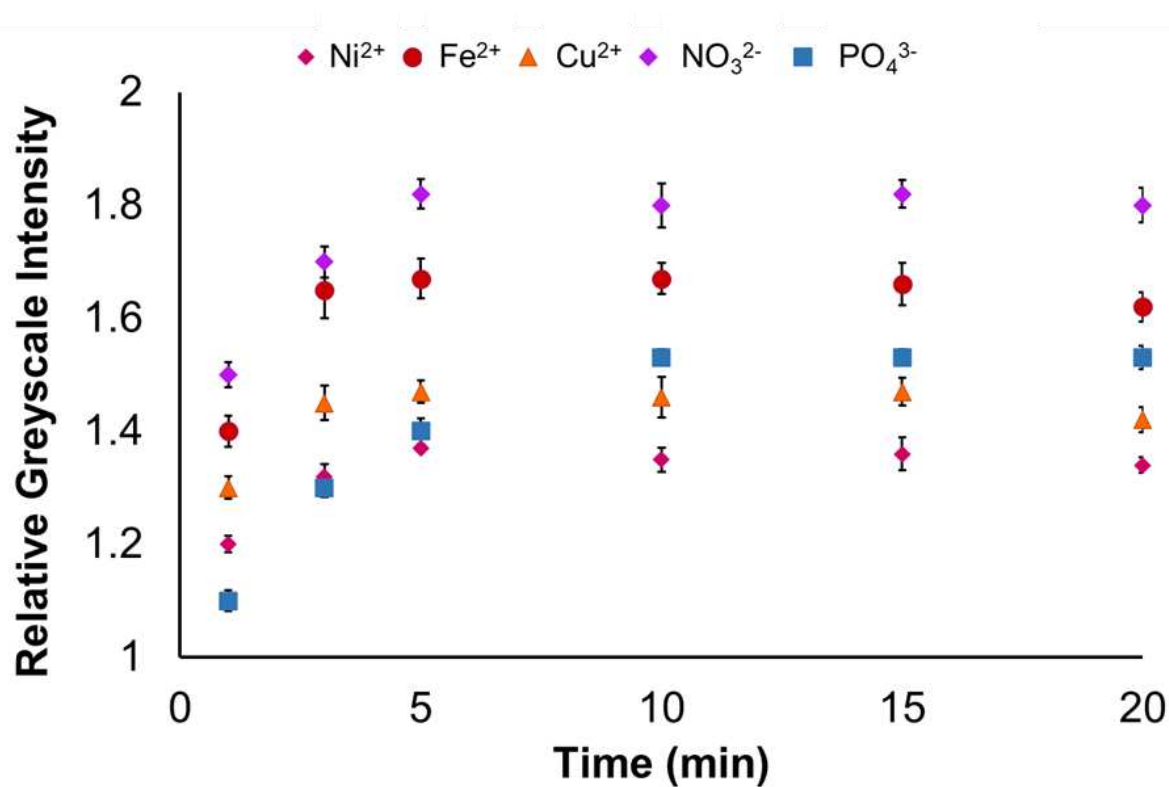


Figure S3. Reaction time optimization (n=3). For the multiplexed system, 8-10 minutes was chosen for reaction time for maximum color intensity

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APPENDIX A – Supporting Information for Chapter 2: flow-driven microfluidics combined with a paper device for fast user-friendly detection of heavy metals in water

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Table A1. Reagents used

Reagents	Concentration	Solvent
Dimethylglyoxime	60 mM	Methanol
Bathocuproine	0.05 g/mL	Dichloromethane
Bathophenanthroline	0.025 g/mL	Isopropanol
Hydroxylamine (for Fe)	0.1 g/mL	Acetate Buffer
Hydroxylamine (for Cu)	0.1 g/mL	Millipore water
Sodium Fluoride	1 M	Millipore water
Ammonium Acetate	6 M	Millipore water

Image Analysis

- After the optimized reaction time, the image was captured using a smartphone (no filter adjustments) inside a light-controlled black box.
- The image was then imported to the open access ImageJ software
- The image was split into three channels: blue, green, and red.
- The respective channels (blue = Copper, green = Nickel) were then inverted using the edit tool.
- The grayscale of the detection area and reference white PVC sheet was measured using a circle tool. The size/area of the circle was kept consistent between the PVC background and the detection region.
- The relative grayscale intensity was calculated by dividing the detection region's intensity by the grayscale on the PVC background.

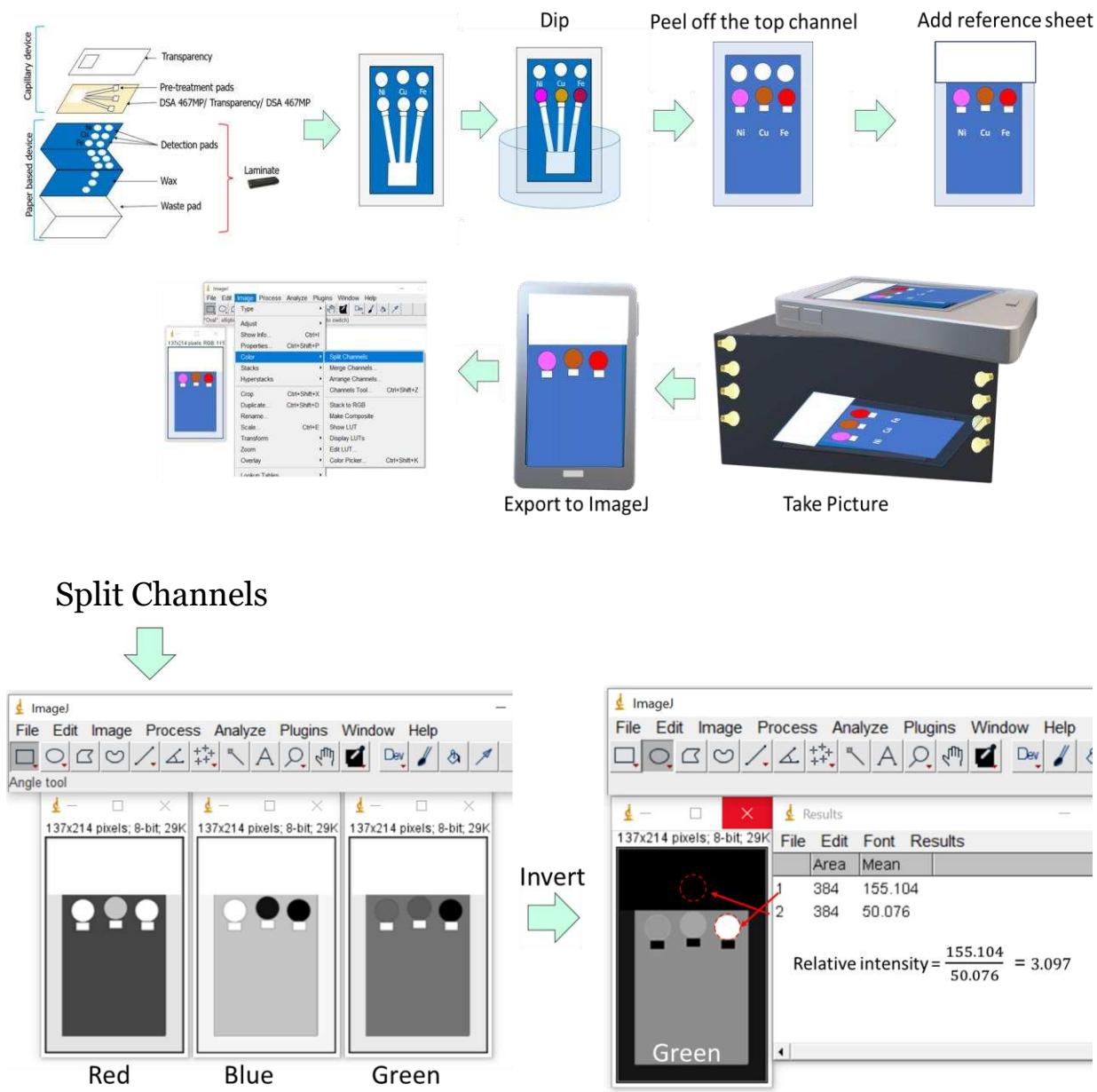


Figure A1. Device Running and Quantitative Image Processing

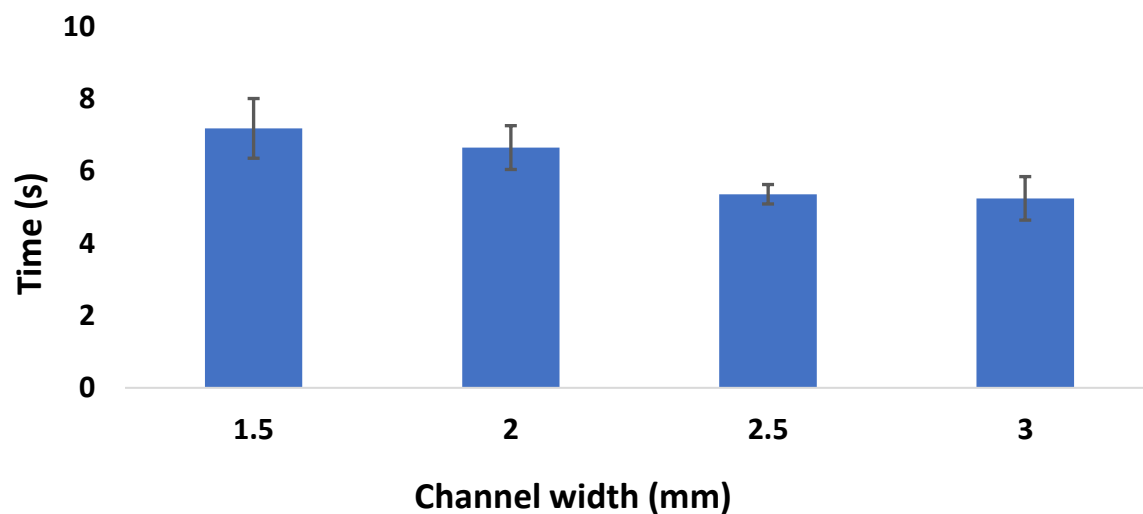


Figure A2. Channel width Optimization with 300 μ L and 467MP adhesive tape (n=4 devices). The time to reach the detection pads were calculated for each channel width.

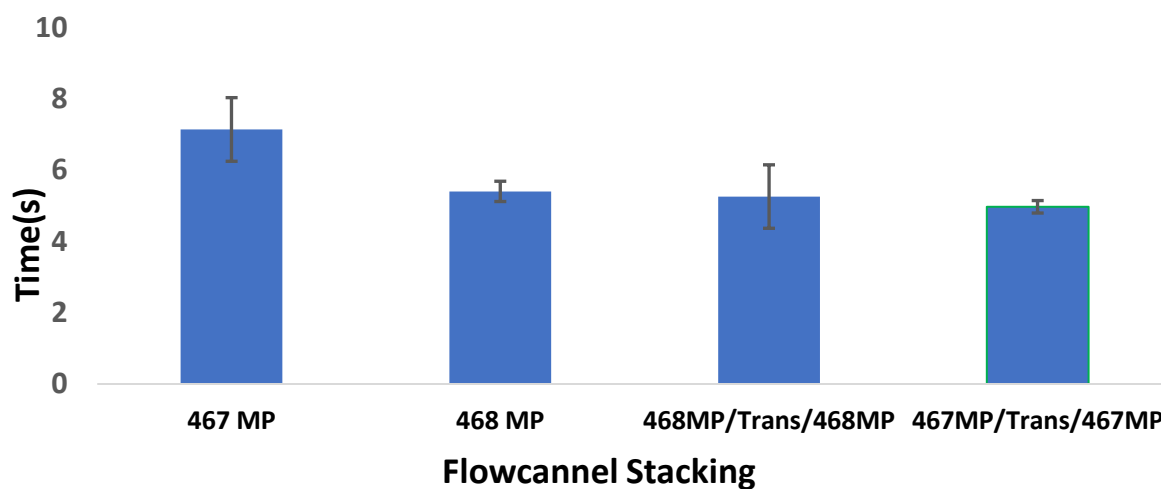


Figure A3. Channel height Optimization with 300 μ L and 2.5mm wide channel (n=4 devices). The flow channel thickness was varied using different stacking patterns of adhesive tapes (467MP and 268MP double-sided adhesive) and transparency film. The time to reach the detection pads were calculated for each channel height.

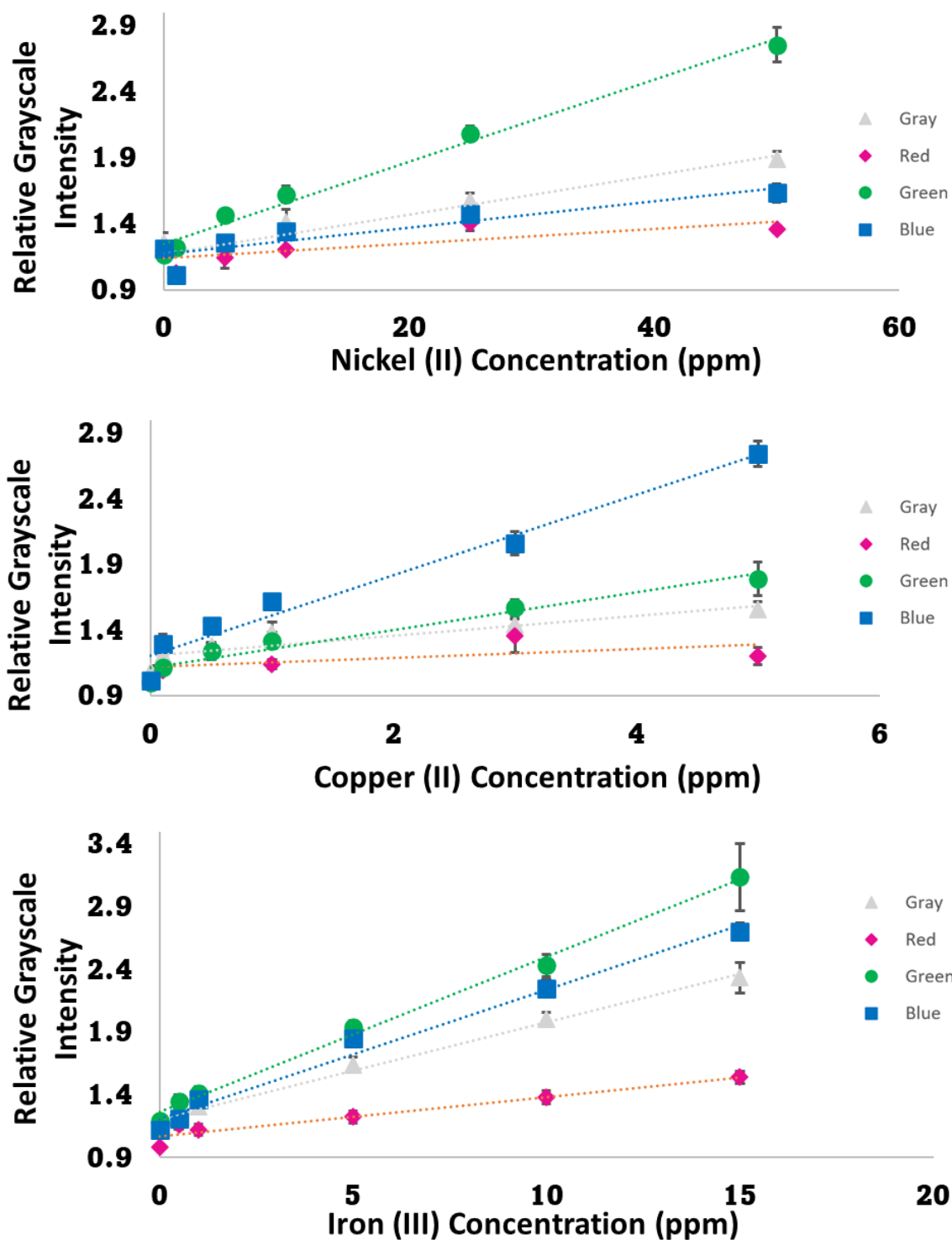


Figure A4. Optimizing the ImageJ channels for maximum slope and color signal. It was found that.

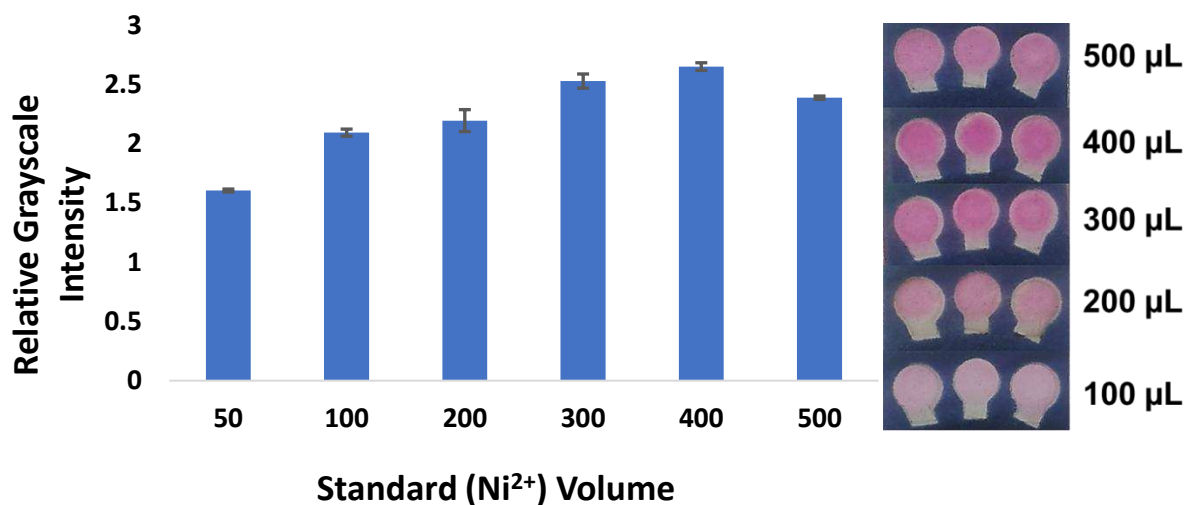


Figure A5. Standard Volume optimization for Nickel. Above 400 μL , the complex started to leach away, causing a decrease in signal. Also, not using the volume to the total capacity of the pad helped the detection zone to dry faster. Copper and Iron complexes were relatively stable and did not leach away. The signal was retained for Cu and Fe even above 500 μL . However, the analyte volume was fixed to 400 μL for each assay for faster drying. The device was allowed to dry before imaging.

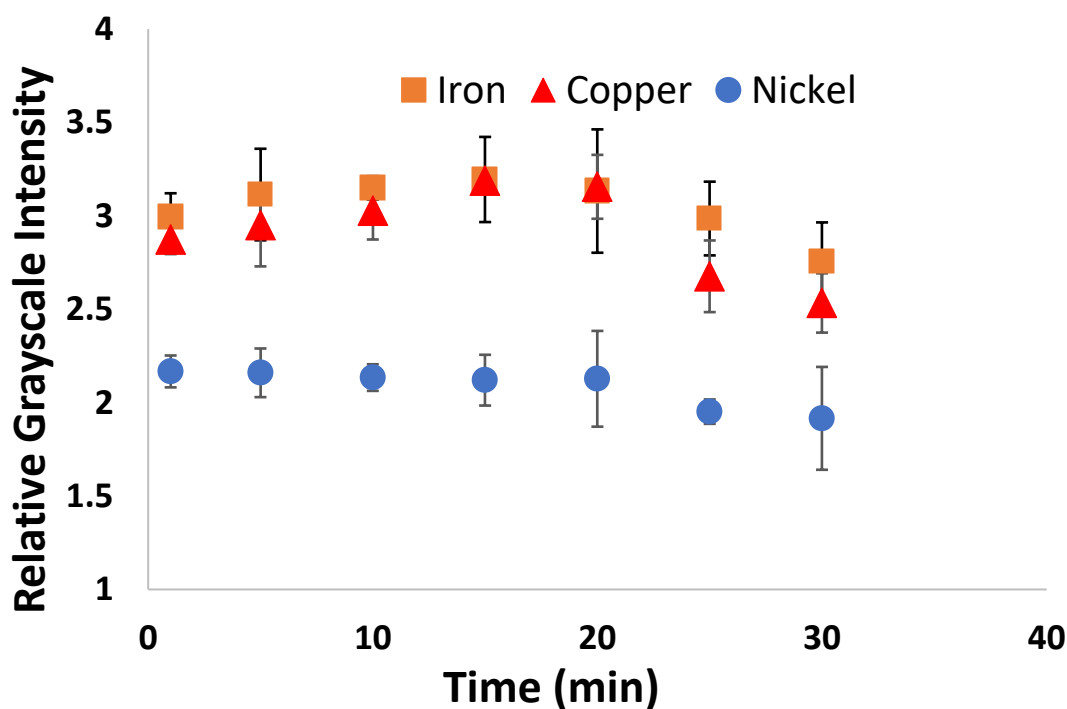


Figure A6. Reaction time optimization (n=3). For a multiplexed system, 20 minutes was chosen for reaction time for maximum color intensity.

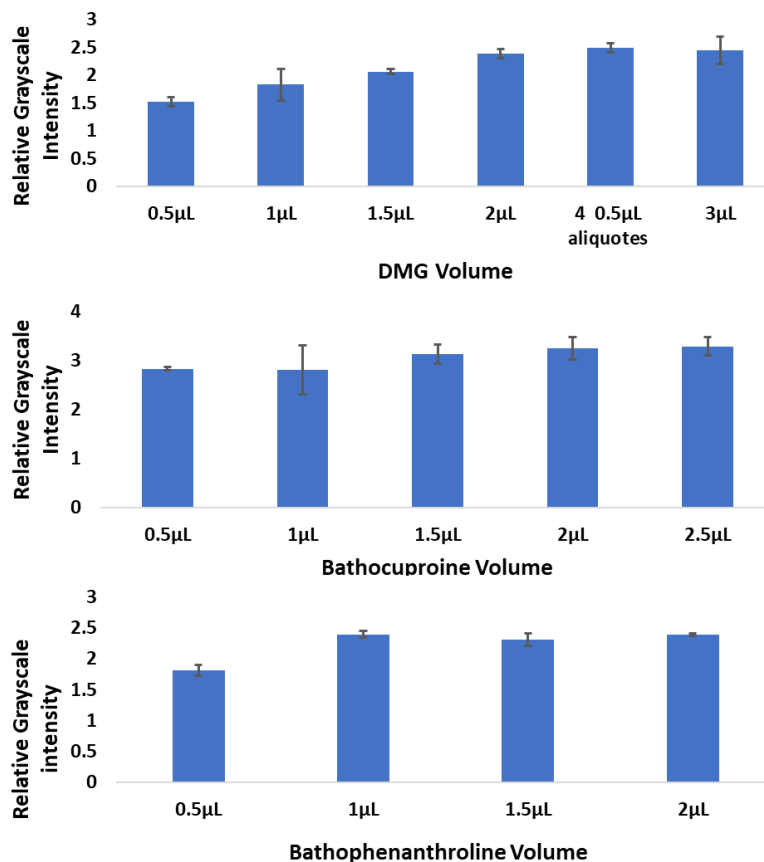


Figure A7. Reagents Volume optimization for complexing agents(n=3).

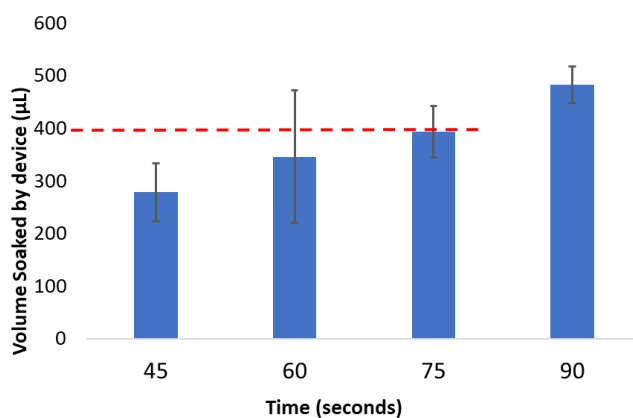


Figure A8. Vertical flowtime study (n=5 devices) for calibrated volume(400 μ L). The volume was calculated by measuring the mass of water-soaked by the device at different times when dipped vertically. The device will take 75 seconds to soak the 400 μ L of the sample when dipped vertically.

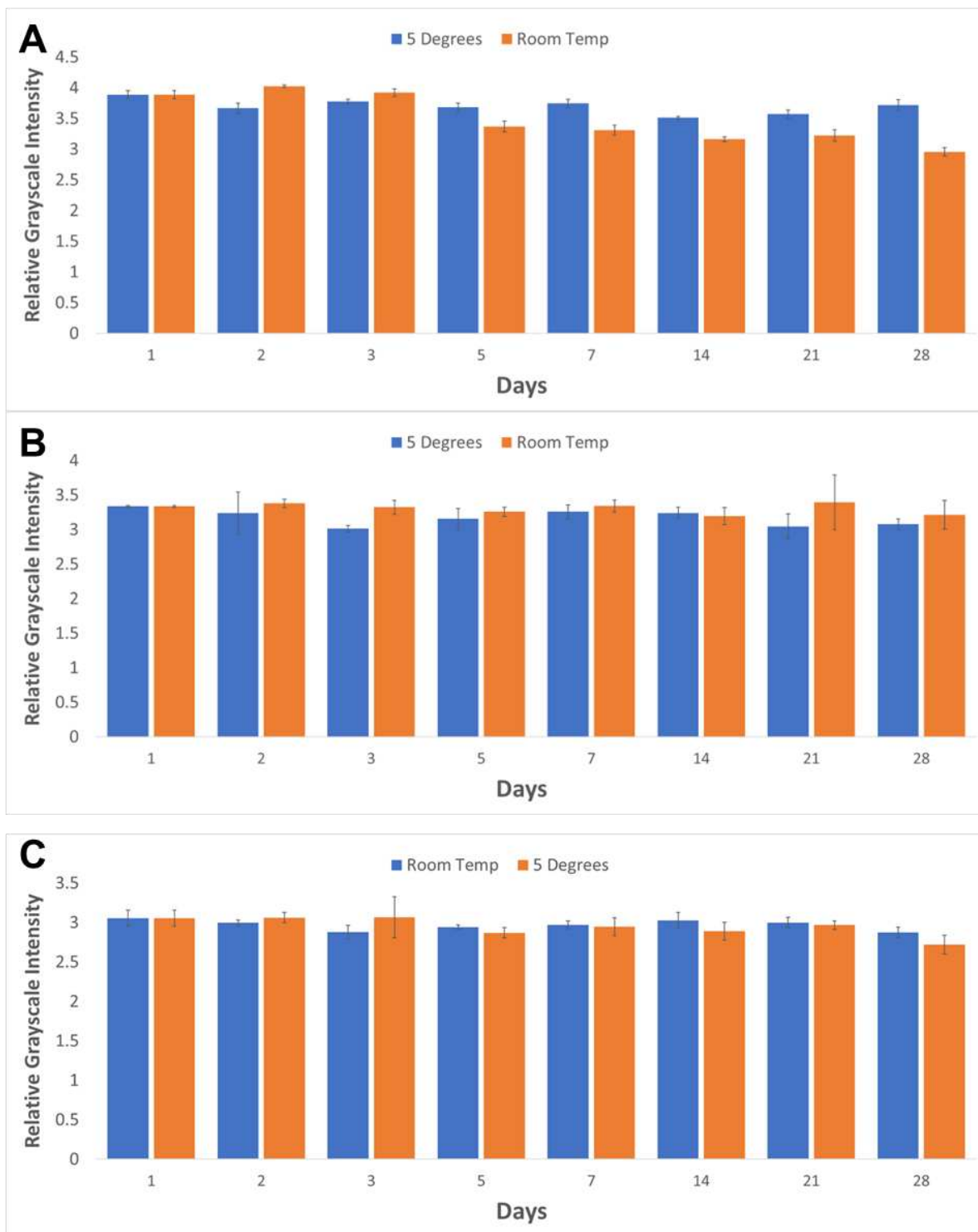


Figure A9. Stability study for Iron (A), Copper (B), and Nickel (C) assays on the developed device.



Pads untreated with PAA



Pads treated with 0.7mg/mL PAA

Figure A10. Poly Acrylic Acid (PAA) was used as an ion-pairing agent to reduce the edge effect and enhance the uniformity of this test.

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APPENDIX B — Fill, Fold, Photo: Preconcentration and Multiplex Detection of Trace Level Heavy Metals in Water

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Table B1. List of reagents (red), masking agents, and hydrophilicity treatment in each zone. DZ = Detection Zone, GF = Glass Fiber.

Assay	DZ	Preconcentration/Detection Zone (DZ) paper Reagents	GF	Reagent Zone /Glass Fiber (GF) Reagents	Hydrophilicity Treatment
Ni-DMG Assay	1	➤ 5 μL 6.0 M ammonium acetate ➤ 5 μL 0.5 M NaF	1	➤ 7.5 μL $\text{NH}_4\text{OH}/\text{NH}_4\text{Cl}$ buffer (pH 9) ➤ Two 10 μL aliquots of 60 mM dimethylglyoxime in methanol	NA
Cu-Bc Assay	2	➤ 5 μL hydroxylamine (0.2 g/mL, Acetate/NaCl buffer, pH 4.5) ➤ 10 μL solution of 8.5 mg/mL Bathocuproine, 0.12 g/mL PEG 400	2	NA	0.12 g/mL PEG added to the reagent mixture
Fe-Bph Assay	3	➤ 5 μL hydroxylamine (0.2 g/mL, acetate buffer, pH 4.5)	3	➤ 20 μL solution of 1.25 mg/mL bathophenanthroline in isopropanol	NA
Cr-DPC Assay	4	➤ 5 μL 0.1 M phosphoric acid	4	➤ 20 μL solution of 15 mg/mL Diphenyl Carbazide, 0.04 g/mL phthalic anhydride, 1 mM ceric(IV) ammonium nitrate, 5% w/v PDDA in acetone	NA
Pb-Rz Assay	5	NA	5	➤ 20 μL Rhodizate (0.625 mg/mL	NA

					in Tartrate buffer, pH 3)
Hg-Dtz Assay	6	➤ 5 µL Ethylenediamine (3 M)	6		0.08 g/mL PEG added to the reagent mixture
		➤ 5 µL trans-1,2- diaminocyclohexa ne-N,N,N',N'- tetraacetic acid (DCTA) (0.1 M)		NA	
		➤ 10 µL diluted stock solution of dithizone (12 µg/mL in dichloromethane) + PEG 400 (0.08 g/mL)			
Mn- PAR Assay	7	➤ Combination of dimercaptosuccini c acid and triethylenetetrami ne in pH 9.3 borate buffer	7	➤ 20 µL solution of 2.5 mM 4- (2-pyridylazo) resorcinol mixed with 5% w/w PDADMAC in borate buffer (pH 9.3, 0.1 M)	NA

Assays B1

Ni-Dimethylglyoxime (Ni-DMG) Assay. 5 µL of 6.0 M ammonium acetate, followed by 5 µL of 0.5 M NaF, was added to the detection zone 1. Subsequently, 7.5 µL of NH₄OH/NH₄Cl buffer (pH 9) was added to glass fiber 1, followed by the addition of two 10 µL aliquots of 60 mM dimethylglyoxime prepared in methanol. After each reagent addition, the detection zone and reagent zone were left to dry at room temperature. Ultimately, 250mL of the standard solution was added to the preconcentration tank.

Cu-Bathocuproine (Cu-Bc) Assay. 5 µL of hydroxylamine, prepared in Acetate/NaCl buffer at a concentration of 0.2 g/mL (pH 4.5), was added to detection zone 2 and dried.

A solution containing 8.5 mg/mL Bathocuproine was prepared in dichloromethane. A 10 μ L of this solution was added to detection zone 2. After each reagent addition, the detection zone was dried completely. Ultimately, 250mL of the standard solution was added to the preconcentration tank. In this assay, all the reagents were added to the detection zone itself.

Iron-Bathophenanthroline (Fe-Bph) Assay. In detection zone 3, 5 μ L of 0.2 g/mL of hydroxylamine, prepared in a 6 M acetate buffer with a pH of 4.5, was initially added. Then, 20 μ L of 1.25 mg/mL of bathophenanthroline, prepared in isopropanol, was added to glass fiber 3. After each reagent addition, the detection zone and reagent zone were dried completely. Ultimately, 250mL of the standard solution was added to the preconcentration tank.

Chromium-Diphenylcarbazide (Cr-DPC) Assay. 5 μ L of 0.1 M phosphoric acid was added to detection zone 4. A solution was prepared by mixing 15 mg/mL of Diphenyl Carbazide (DPC), 0.04 g/mL of phthalic anhydride, along with 1 mM ceric(IV) ammonium nitrate and polydiallyldimethylammonium chloride (PDDA) (5% w/v) in acetone. 20 μ L of this solution was added to glass fiber 4. After each reagent addition, the detection zone and reagent zone were dried completely. Ultimately, 250mL of the standard solution was added to the preconcentration tank.

Lead- Rhodizoate (Pb-Rh) Assay. 20 μ L of Rhodizoate at a concentration of 0.625 mg/mL in Tartrate buffer (pH 3) was added to the glass fibers 5 and exposed to drying. Following this, 250mL of the standard solution was added to the preconcentration tank.

Mercury- Dithizone (Hg-Dtz) Assay. In the detection zone 6, 5 μ L of Ethylenediamine (3 M) was added, followed by the addition of 5 μ L of *trans*-1,2-diaminocyclohexane-N,N,N', N'-tetraacetic acid (DCTA) (0.1 M). Subsequently, a stock solution of dithizone at a concentration of 12 μ g/mL in dichloromethane was prepared. This stock solution was then diluted 20 times in 5 mM NaOH, mixed with 0.08 g/mL of PEG-400, and 10 μ L of the final diluted solution was added to detection zone 6. After each reagent addition, the detection zone was dried completely. Ultimately, 250mL of the standard solution was added to the preconcentration tank. Similar to copper, all the reagents were added in the detection zone itself for this assay.

Manganese- 4-(2-pyridylazo) resorcinol (Mn-Par) Assay. In detection zone 7, a combination of dimercaptosuccinic acid and triethylenetetramine in pH 9.3 borate buffer was added and dried. Subsequently, a solution containing 2.5 mM of 4-(2-pyridylazo) resorcinol (PAR), mixed with 5% w/w PDADMAC, was prepared in borate buffer (pH 9.3, 0.1 M). A 20 μ L aliquot of this solution was applied to the glass fiber 7. After each reagent addition, the detection and reagent zones were completely dried. Ultimately, 250mL of the standard solution was added to the preconcentration tank.

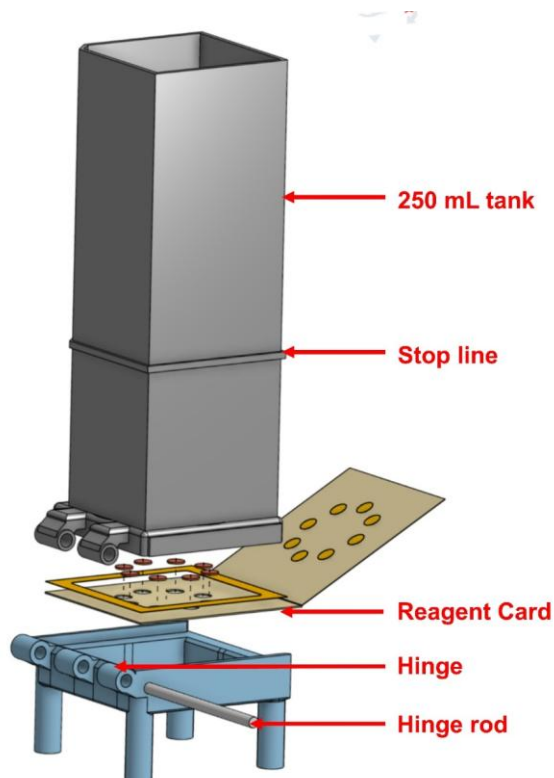


Figure B1. Schematic of 3D printed preconcentration tank

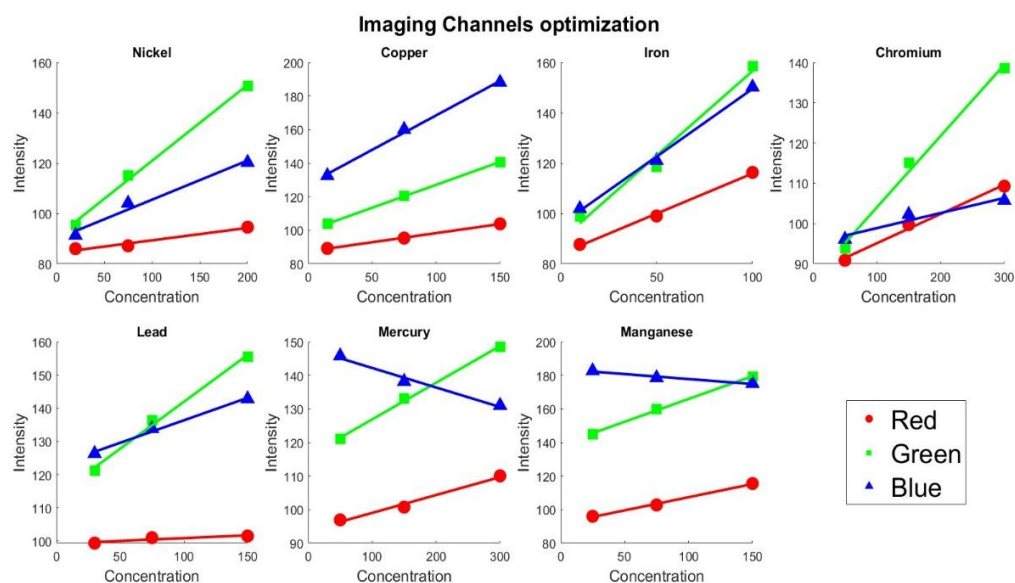


Figure B2. Imaging Channels optimization. The image channels were optimized for green, red, and blue channels. ImageJ established A linear plot for each channel using three points (low, medium, and high concentrations). Subsequently, the plot with the

steepest slope was chosen for calibration. All metals were calibrated under the green channel except for copper, which was calibrated under the blue channel.

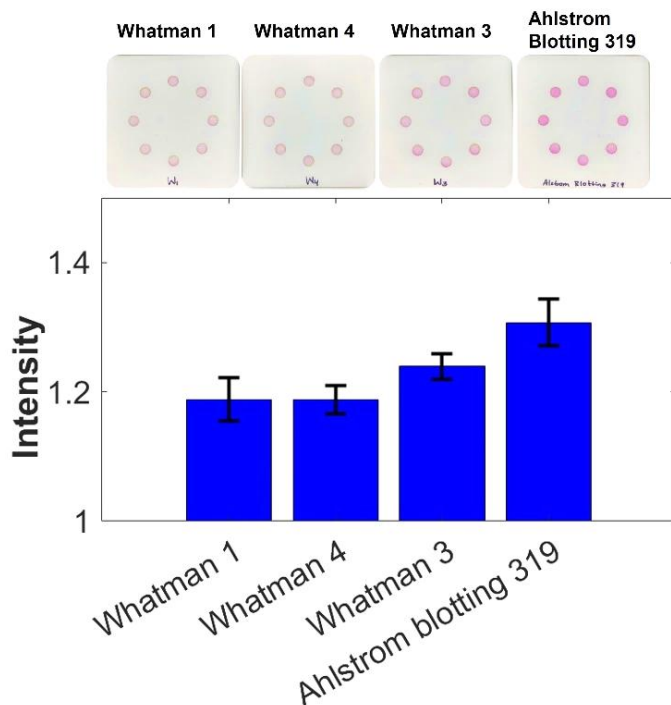


Figure B3. Substrate optimization.

The increased thickness of Ahlstrom AB 319 (Thicker Paper (483 μ m for AB 319 vs. 180 μ m for W1 and 210 μ m for W4) provides an extended diffusion pathway, enhancing the chances of metal ions colliding with the paper fibers. Moreover, AB 319's higher thickness and denser structure likely provide a greater surface area for adsorption. The thicker nature of Ahlstrom AB 319 introduces more tortuous paths for metal ions to navigate. This tortuosity, coupled with potential functional groups, hinders the swift movement of metal ions, increasing the likelihood of interactions with the paper fibers and subsequent retention. In addition, considering the proprietary nature of AB 319, it's reasonable to speculate that the paper may contain undisclosed functional groups that could have an affinity for metal ions, forming complexes that contribute significantly to the enhanced metal ion trapping observed during filtration.

In summary, while the specific chemical composition of Ahlstrom AB 319 remains proprietary, its superior metal ion trapping capabilities can be attributed to a combination of increased thickness, enhanced surface area for adsorption, and tortuosity. The presence of functional groups may play a crucial role in complexing with metal ions, further enhancing the paper's effectiveness in retaining these ions during the experimental filtration process.

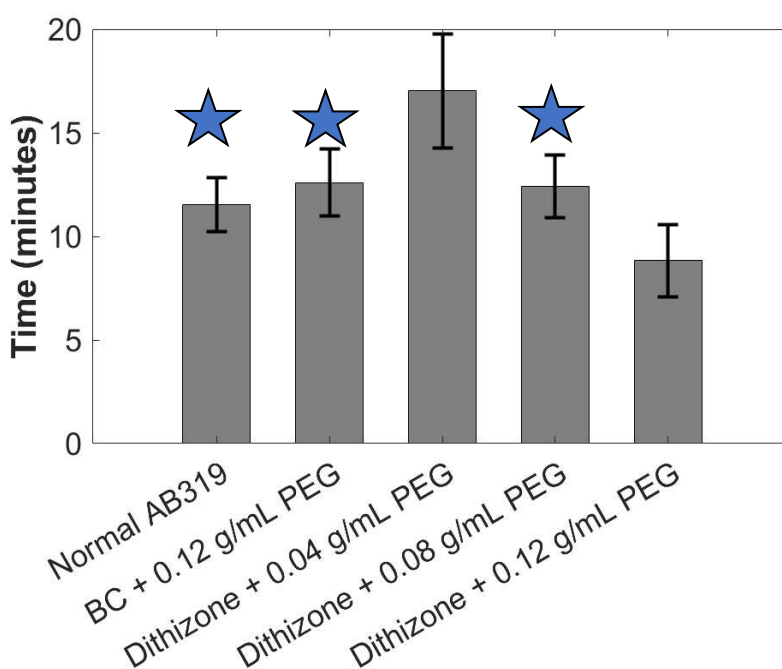


Figure B4. Substrate modification with PEG for Cu-Bc and Hg-Dtz assay to increase the hydrophobicity of the detection zone. The optimization for PEG was to ensure that we had the same filtration time for the reagent-treated paper as compared to those not treated in the case of the other five metal ions. Therefore, the reagents for Cu(II) and Hg(II) were mixed with optimized PEG concentrations.

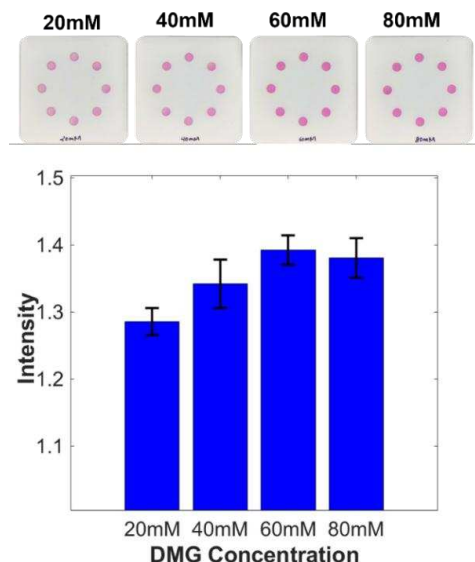


Figure B5. Dimethylglyoxime (DMG) concentration optimization. The best concentration for the optimal color response was determined to be 60mM. This concentration gave the highest average signal with lower standard deviation. Although the average signal difference was not statistically significant compared to the neighboring concentration in the T-test ($p > 0.05$), this concentration was chosen for better consistency.

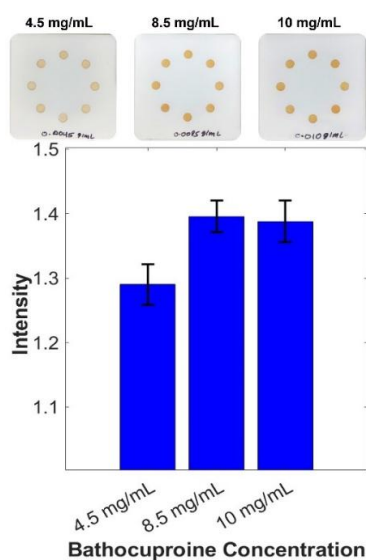


Figure B6. Bathocuproine concentration optimization. The best concentration for the optimal color response was determined to be 8.5 mg/ μ L. This concentration gave the highest average signal with lower standard deviation. Although the average signal difference was not statistically significant compared to 10mg/mL in the T-test ($p > 0.05$), this concentration was chosen for better consistency.

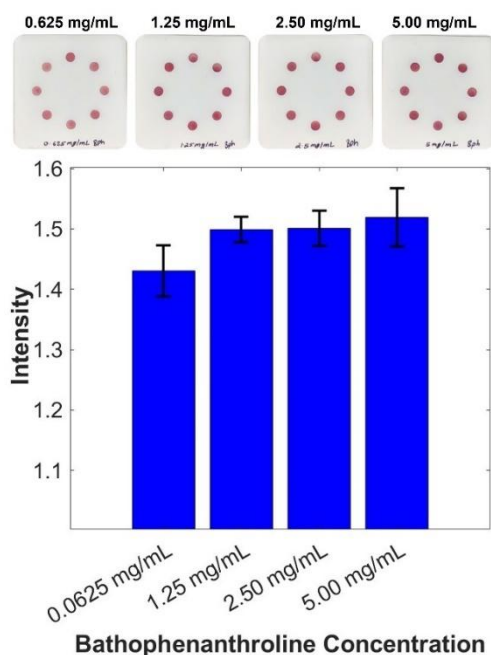


Figure B7. Bathophenanthroline concentration optimization. The best concentration for the optimal color response was determined to be 1.25mg/mL. Although 1.25, 2.5, and 5 mg/mL produced statistically similar signals ($p>0.05$), 1.25 mg/mL was chosen because it had the least standard deviation.

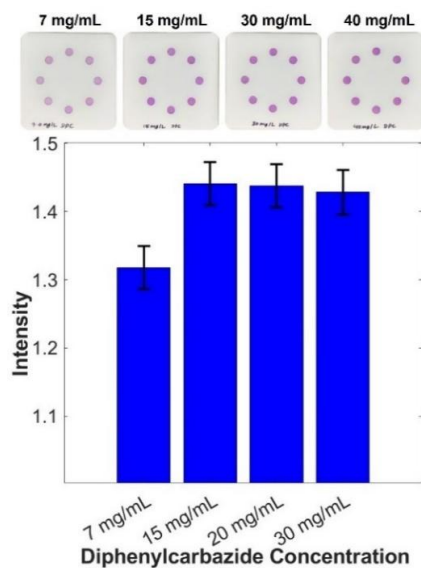


Figure B8. Diphenylcarbazine concentration optimization. The best concentration for the optimal color response was determined to be 15 mg/mL. Although 15 mg/mL, 20 mg/mL, and 30 mg/mL produced statistically similar signals ($p>0.05$), 15 mg/mL was chosen to minimize reagent use. This concentration was sufficient even at higher analyte concentrations.

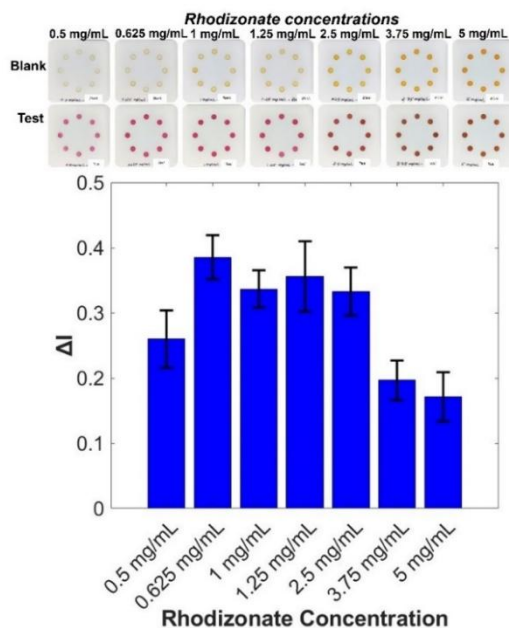


Figure B9. Rhodizonate concentration optimization. The best concentration for the optimal color response was determined to be 0.625 mg/mL. This concentration provided the highest average differentiation between the test and blank, with a lower standard deviation than the next concentration (1.25 mg/mL), which was statistically similar to 0.625 mg/mL ($p > 0.05$).

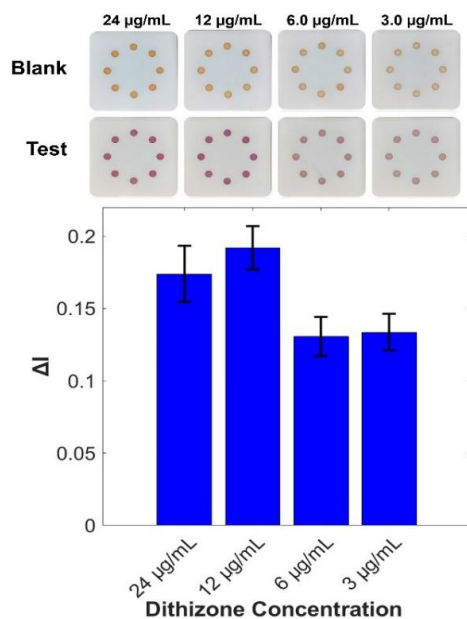


Figure B10. Dithizone reagent optimization. The optimal color response was achieved at 12 µg/mL. While this concentration was statistically similar ($p > 0.05$) to 24 µg/mL, 12 µg/mL had a lower standard deviation. We selected this as the minimal reagent concentration that was not reagent-limited, even at higher analyte concentrations and had better consistency.

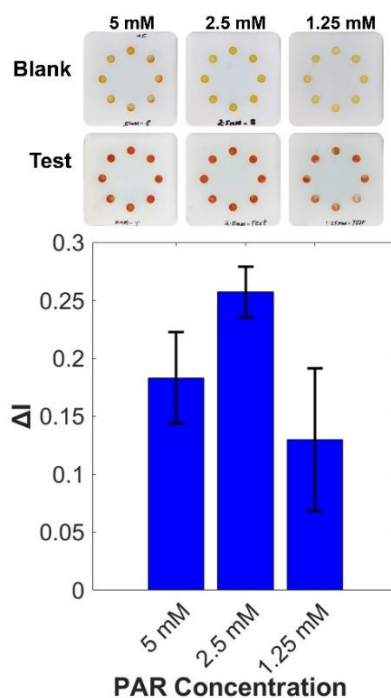


Figure B11. PAR concentration optimization. The best concentration for the optimal color response was determined to be 5 mM.

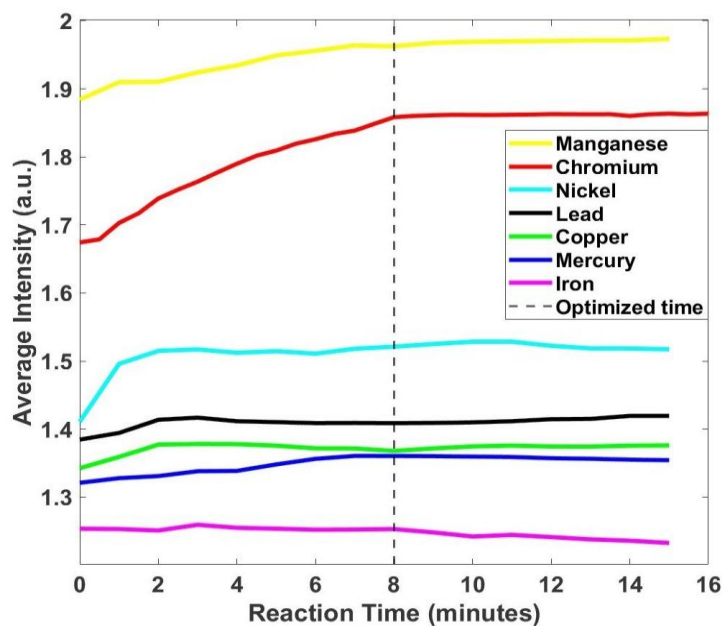


Figure B12. Reaction time optimization for developed sensor (n=8 for each reaction). The optimized time was determined to be 8 minutes after folding the chip.

Masking Protocols B1.

Building on our previous findings, we identified Fe, Cu, and Co as potential culprits interfering with the formation of the Ni-DMG complex in detection zone 1.¹ We modified the detection zone 1 by adding sodium fluoride and ammonium acetate to tackle this challenge.¹ In the Cu-bathocuproine assay, increasing the reducing agent to 0.2 g/mL effectively mitigated interferences from Zn(II), Co(II), and Cd(II).² The same strategy was applied for Cu(II) interference in Fe-bathophenanthroline assay by reducing it to Cu(I). In the Cr-DPC assay, significant interference from Fe(III) was addressed by adding phosphoric acid at the detection zone 4.³ With the implementation of these masking strategies, tolerance ratios for Ni(II), Cu_{total}, Fe_{total}, and Cr_{total} were extended beyond 1:10 (analyte: interference).

Only Barium showed a significant interference at a 1:1 ratio for the Pb-Rh assay. Zn(II), Hg(II), and Fe(III) showed interference at a tolerance ratio of 1:5. In the Hg-dithizone assay, Ethylenediamine (3 M) was employed to mask Cu(II), Ni(II), Zn(II), Cd(II), and Pb(II) in detection zone 6.⁴ Additionally, DCTA (0.1 M) was added to mask Cu(II), Co(II), Ni(II), and Pb(II) at zone 6.⁵ Only Zn(II) exhibited significant interference at a 1:1 ratio among the non-targeted ions. The remaining non-targeted ions demonstrated higher tolerance limits in the Hg-Dtz assay. In the Mn-PAR assay, we employed masking strategies from our previous study by Meredith et al. to achieve higher selectivity for manganese detection.⁶ A combination of dimercaptosuccinic acid and triethylenetetramine in pH 10 borate buffer was used at detection zone 7 to enhance the assay's selectivity. Significant interferences were observed from Fe(III), Co(II), and Ni(II) (<±10% change) at a 1:1 ratio.

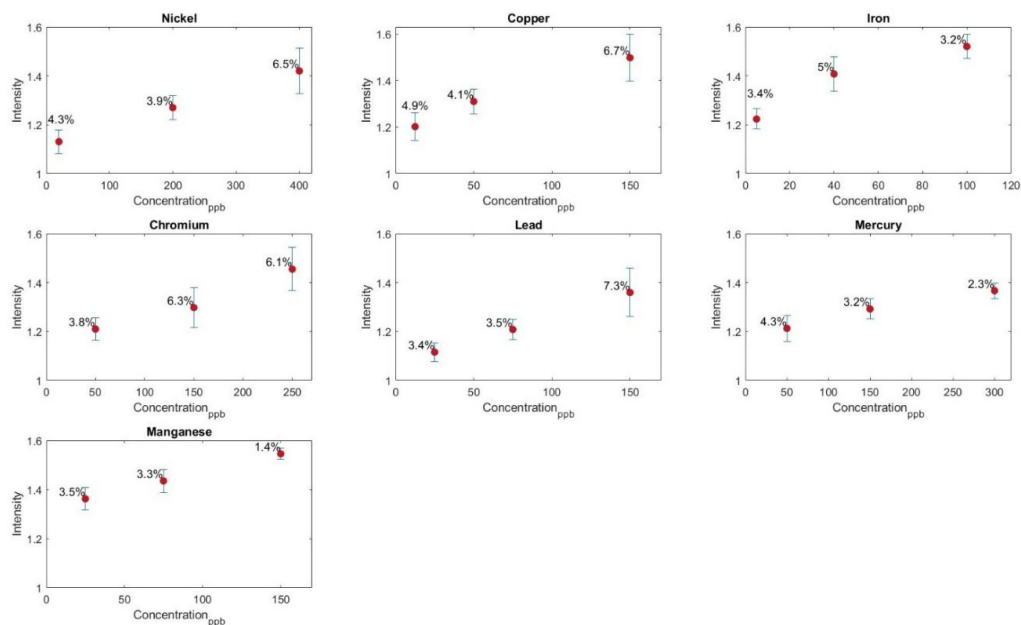


Figure. B13. Filtration consistency test (n=5 devices for each concentration). Tests were conducted at low, medium, and high concentrations for each metal. The RSD for all examined points remained below 10%.

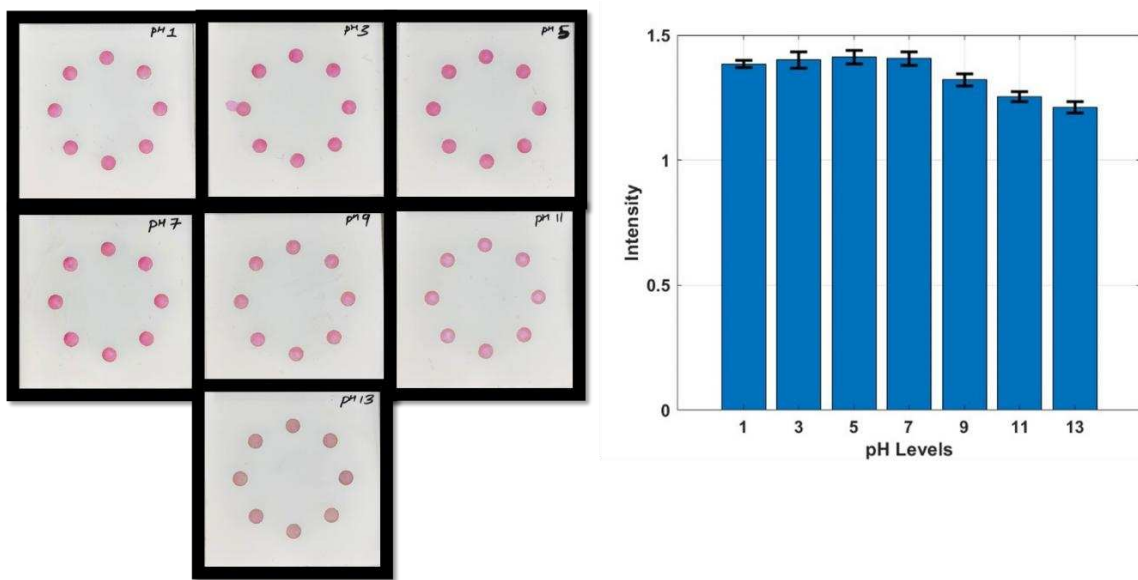


Figure B14. Effect of pH on Metal Retention

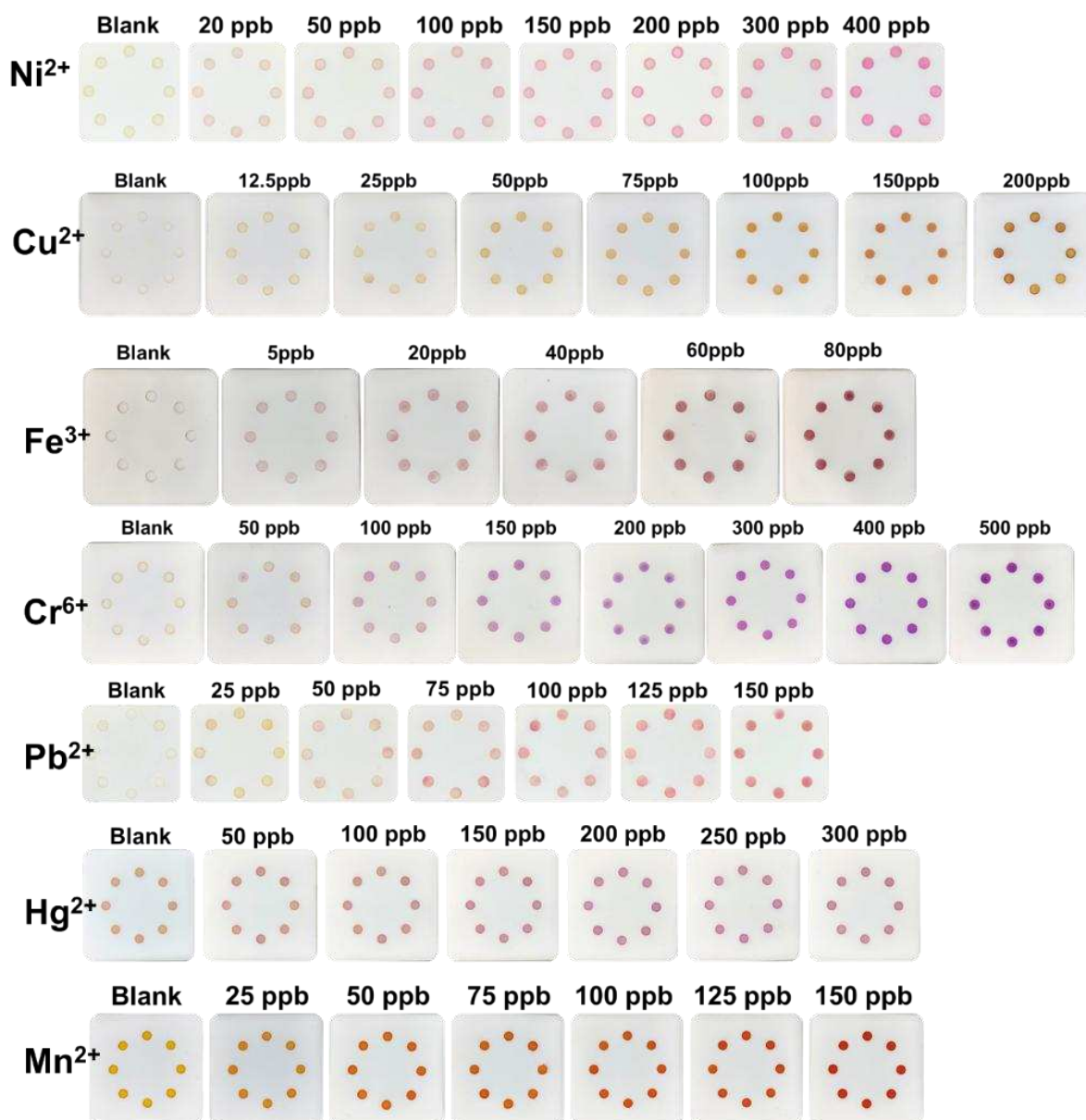


Figure B15. Color gradients for each assay for the calibration on the developed system. The oxidations states are from the standards used to detect. The system is capable of detecting Cu_{total}, Fe_{total}, Cr_{total}.

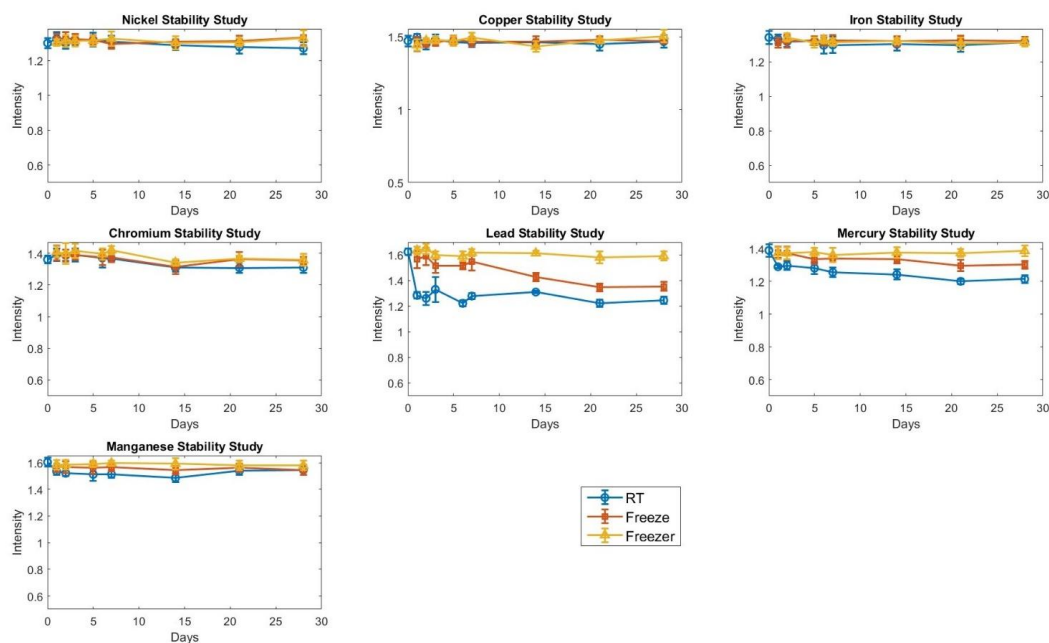


Figure B16.a. Stability study for all the assays under three different conditions for four weeks. To evaluate the stability of the developed sensor, it underwent storage in three distinct conditions: room temperature (23°C), fridge (5°C), and freezer (-20 °C). Over four weeks, the color signal on the chip was systematically tested for Ni(II), Cu_{total}, Fe_{total}, Cr_{total}, and Mn(II). The color signals remained stable across all storage conditions throughout the four-week testing period. However, it was observed that the freeze and freezer conditions proved to be more conducive for the Pb(II) and Hg(II) assay.

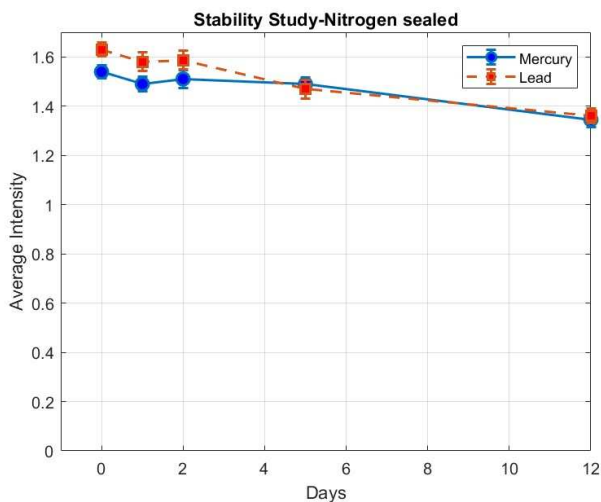


Figure B16.b. In order to improve the stability of Pb(II) and Hg(II) at room temperature, the sensors were stored in nitrogen-purged bags. This packaging protocol resulted in retaining over 90% of the signal for up to 5 days.

Sample protocols B1. Drinking water samples were collected in triplicate using 50 mL Falcon tubes and spiked with 1% HNO₃. In the case of water from the river, the samples were filtered and spiked in triplicate. The tubes were fully submerged, and the lids were closed inside the water. We ensured that water samples were collected from areas where the water was clear. The samples were refrigerated overnight and sent for ICP-MS analysis the following day. Metal testing was conducted using the sensor on the same day.

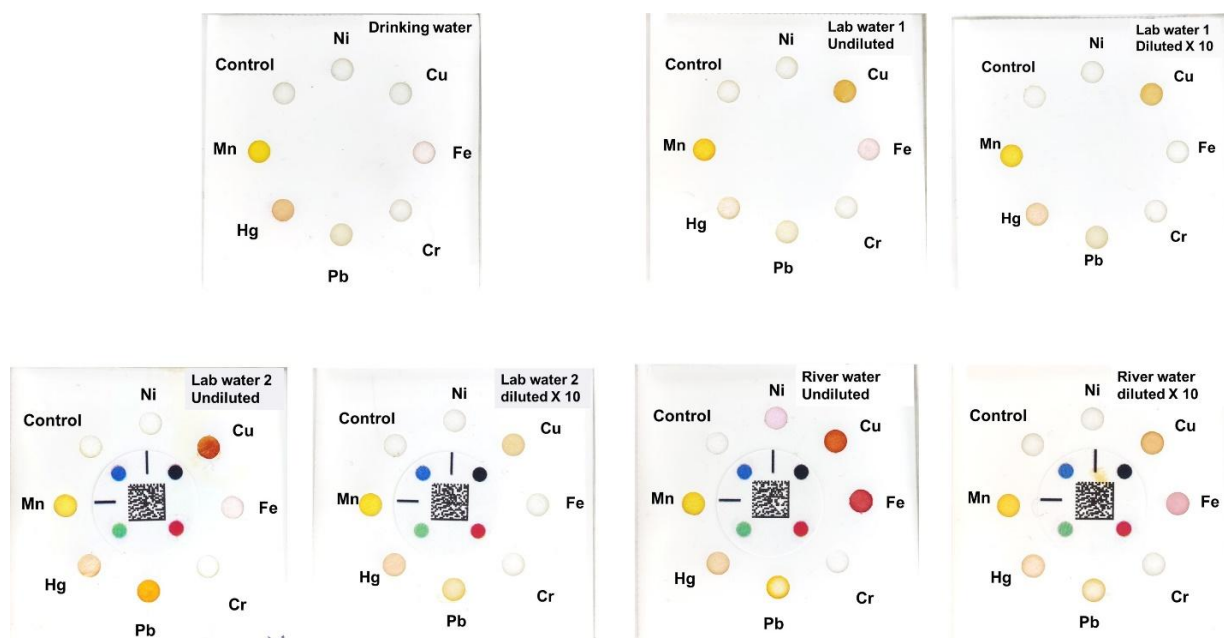


Figure B17. Representative images for Real water samples analysis on the chip response. The test was done immediately for the samples that provided the signal within the signal's dynamic range. However, in case the color was already saturated, the quantification was done after a 10-fold dilution.

Table B2. Summary of metal ion detection results in various water sources using the developed sensor. The results are compared with the industry-standard ICP-MS method (ND = not detected).

Sample Type	Metals	ICP-MS (ppb) (n=3)	Sensor (ppb) (n=3)	% Rec	% RSD
Drinking water	Ni(II)	0.11 ± 0.02	ND	x	x
	Cu _{total}	6.00 ± 0.04	ND	x	x
	Fe _{total}	13.20 ± 0.37	10.16 ± 1.84	77	18.1
	Cr _{total}	ND	ND	x	x
	Pb(II)	0.28 ± 0.03	ND	x	x
	Mn(II)	3.91 ± 0.02	ND	x	x
Lab water 1	Ni(II)	15.15 ± 0.14	<LoD	x	x
	Cu _{total}	627.67 ± 8.18	540.11 ± 34.67	86	6.4
	Fe _{total}	15.86 ± 0.45	12.83 ± 1.39	81	10.8
	Cr _{total}	0.08 ± 0.01	ND	x	x
	Pb(II)	0.41 ± 0.01	ND	x	x
	Mn(II)	4.78 ± 0.02	ND	x	x
Lab water 2	Ni(II)	7.78 ± 0.38	ND	x	x
	Cu _{total}	690.00 ± 5.72	645.37 ± 41.10	94	6.4
	Fe _{total}	7.71 ± 0.10	5.32 ± 0.97	69	18.3
	Cr _{total}	0.07 ± 0.01	ND	x	x
	Pb(II)	0.19 ± 0.01	ND	x	x
	Mn(II)	4.56 ± 0.03	ND	x	x
River water	Ni(II)	50.77 ± 2.01	40.39 ± 5.85	80	14.5
	Cu _{total}	689.00 ± 24.59	621.13 ± 43.14	90	7
	Fe _{total}	721.33 ± 20.81	662.72 ± 71.15	92	10.7
	Cr _{total}	0.34 ± 0.01	ND	x	x
	Pb(II)	3.05 ± 0.04	ND	x	x
	Mn(II)	4.13 ± 0.00	ND	x	x

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APPENDIX C — Citizen-Based Water Quality Monitoring: Field Testing a User-Friendly Sensor for Phosphate Detection in Global Surface Waters

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Protocol C1. Quantitative Image Processing

Quantitative Image processing using ImageJ

- Following the optimized reaction time, an image was captured using a smartphone inside a light-controlled black box without making any filter adjustments.
- Subsequently, the captured image was imported into the open-access ImageJ software.
- Within ImageJ, the image was split into three channels: blue, green, and red.
- The red channel was then inverted using the edit tool.
- The grayscale values of the detection area and the blue reference were measured using a circle tool to quantify the findings. The size/area of the circle remained consistent between the reference and the detection region.
- Finally, the relative grayscale intensity was calculated by dividing the detection region's intensity value by the reference region's grayscale value.

Quantitative Image processing using the RGB Color detector smartphone app

- The image was taken using the RGB color detector application.
- The red intensity of detection zones and the blue reference were calculated using the circular ROI.
- The red intensity was then normalized using the reference intensity.

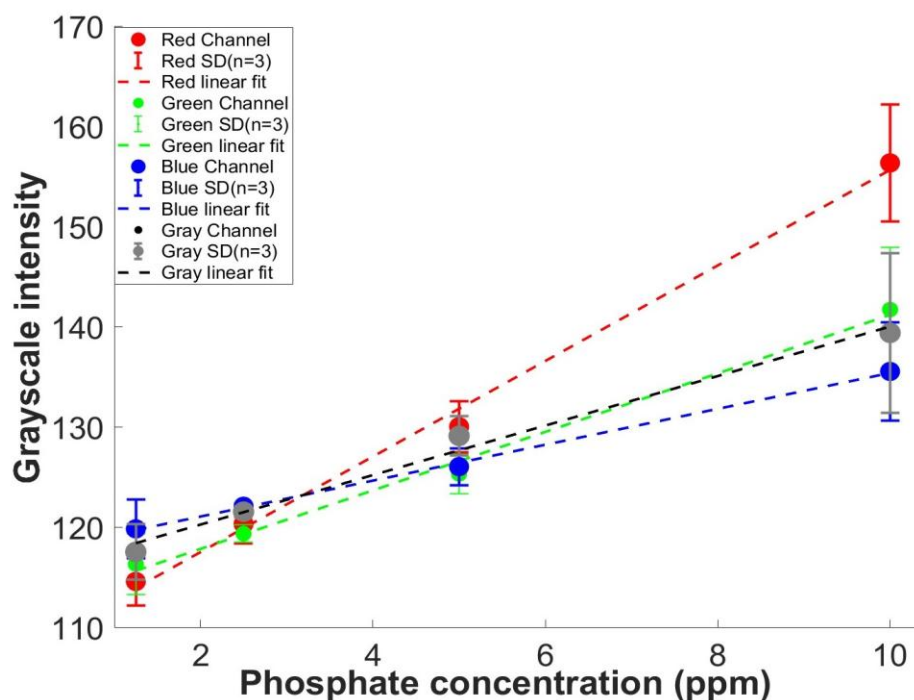


Figure C1. Channel optimization for image analysis.

Once the reaction was complete, a photograph was taken using a smartphone. The images were then processed in ImageJ software by converting them to grayscale and separating them into red, green, and blue channels. The images were then inverted, and the average grayscale intensity of the resulting complex was measured using a circular tool. The average grayscale intensity of the blue reference was also measured as a precise reference for measurement. To mitigate any inconsistencies in lighting conditions, the grayscale intensity was calculated relative to the area of the blue reference. This is because the lighting conditions of both the pad and reference zone would change simultaneously, but the circular area of interest remained consistent between the reference and the detection zone. Calibration curves were constructed for each channel (Relative Grayscale vs. Concentration), and the red channel with the sharpest slope was selected for further analysis due to its maximum sensitivity. For the smartphone app as well, red intensity was chosen.

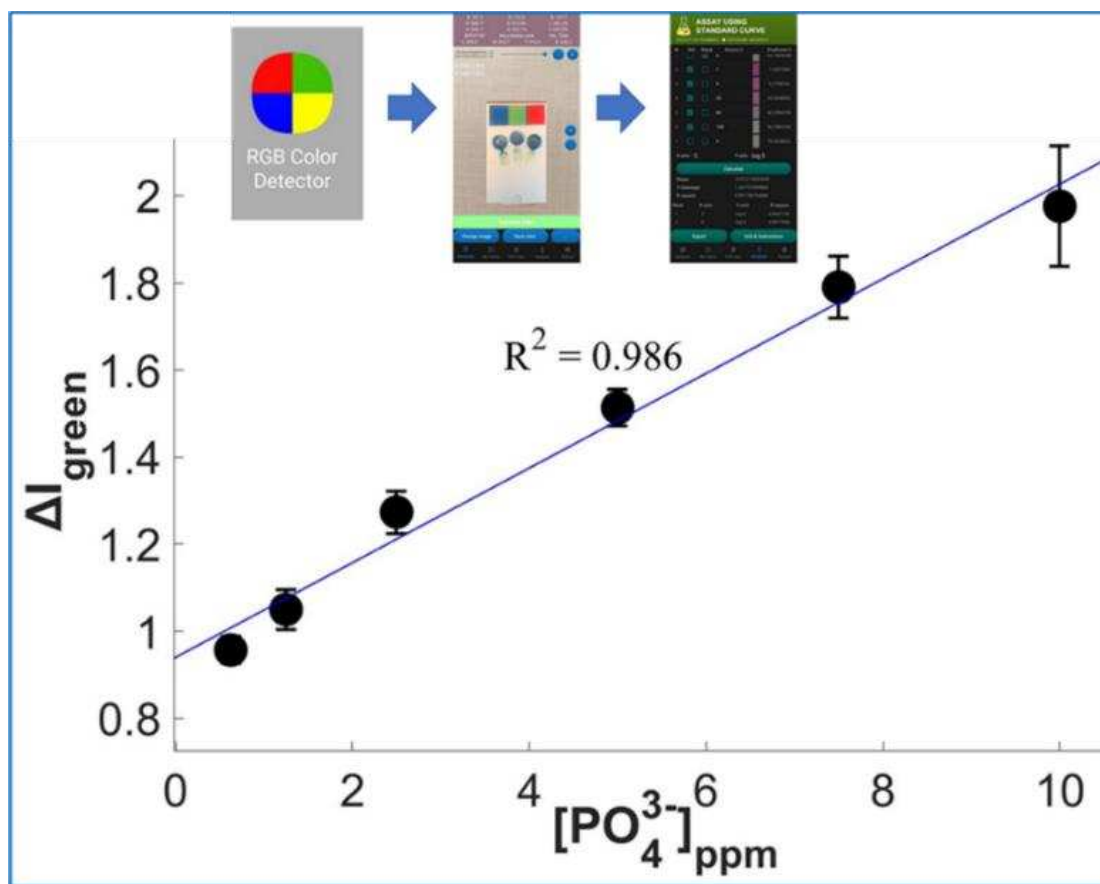


Figure C2. Quantitative calibration using the RGB Color Detector app (n=3).

The limit of detection of the method was calculated to be 0.32ppm. The RGB color detector app is readily available in app stores in both Android and iOS systems. The system allows for on-site calibration followed by the quantification, hence eliminating the need for sample storage and carrying to the laboratory. Moreover, the application-based system allows for choosing the best regression model within the app itself. Some volunteer-led sampling used the app-based detection, while others analyzed using ImageJ.

Location	GPS	Area Photo	River Flow (m/s)	UV/Vis	Device
Site 1	40.5640033, -105.0837675		0.2	1.74±0.13	1.58±0.21
Site 2	40.5639223, -105.0838336		0.2	1.10±0.14	0.89±0.20
Site 3	40.5643477, -105.0834904		0	0.31±0.09	<(LOD)
Site 4	40.5644032, -105.0817600		0.1	ND	ND
Site 5	40.5704685, -105.0821559		0.3	0.43±0.19	0.37±0.13
Site 6	40.5950209, -105.0751458,		0.1	1.19±0.14	1.01±0.18
Site 7	40.5658458, -105.0992506	N/A	0.2	1.53±0.11	1.33±0.24

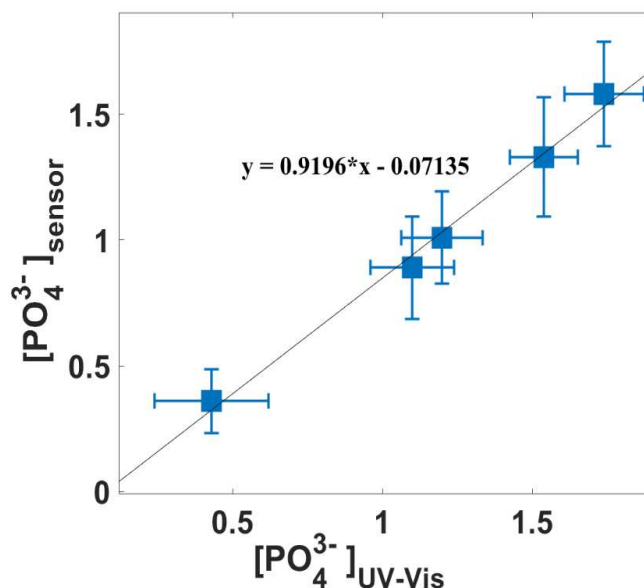


Figure C3. Method validation using the RGB application.

The application of the sensor using an RGB color detector was validated using the Spring Creek Canal near the campus in Fort Collins. Seven sites were sampled, and six of these locations had relevant phosphate concentrations. One of the data points collected was below the limit of detection (LOD) of the sensor using the RGB model. Therefore, the remaining five results were compared to the gold standard method. The results show that the recovery performance was similar to that obtained when validating with ImageJ software, as shown in the calibration image of the manuscript (the slopes are statistically the same).

Optimization

The best flow rate on the capillary-driven channels was obtained using a 2.5 mm wide channel with 220 μm height (Figure S3). The reaction time was also optimized to attain the maximum color response on the chip (Figure S5). The reaction time was optimized to be between 10 and 15 minutes. When tested, all devices passed the flow into the detection reservoir within 10 seconds of sample addition ($n=10$). We tested different papers (Whatman 1, 3, 4, and Ahlstrom blotting paper grade 319) to find the optimum color response (Figure S4). The best compromise between color response and complex homogeneity was obtained with Ahlstrom blotting 319-grade paper. This paper allows for higher water absorption while facilitating strong water pumping within the flow channels. The sensor forms a color signal within seconds of dipping, but it takes a few minutes for the color to become homogeneous. Within the optimized time, the color response was homogeneous at all concentrations. The analytes used in this experiment were colorless water samples. However, if the surface water sample has some color, one of the flow channels can be used as a control zone. The use of glass fibers with larger pore sizes facilitates the release of reagents due to their high surface energy, allowing maximum reagent transport to the detection zone.

Optimizing the PMB reaction on a paper-based system requires a balance between maximizing the formation and reduction of 12-MPA and minimizing the formation of isopolymolybdates. The color intensity due to the analyte's response can be maximized by optimizing the pH, Mo(VI), and reducing conditions. At the same time, the blank's coloration can be minimized by limiting the auto-reduction of molybdate species, which requires a delicate balance between the concentrations of molybdenum (VI), $[\text{Mo(VI)}]$, and protons, $[\text{H}^+]$. As reviewed by Nagul et al., the degree of protonation is crucial in optimizing the colorimetric PMB reaction.¹ However, translating the PMB system with an

alternate acid requires further optimization. The Mo(VI) concentration was optimized first, then the PTSA concentration, and finally the ascorbic acid concentration. A 19 mM Mo(VI) concentration was optimal for the best color response (Figure S6). For the PTSA concentration, the maximum difference between the blank and analyte response was observed at 1 M and 0.13 M PTSA. However, using a lower (0.13 M) concentration of PTSA caused significant interference from silicate species. The Molybdenum Blue reaction between silicate and molybdenum occurs at higher pH, so it was necessary to use a high (1 M) concentration of PTSA to mitigate any interferences (Figure S7). After optimizing the PTSA concentration, the reducing agent (ascorbic acid) concentration was also optimized to be 10mM (Figure C8`). After these thorough optimizations, we achieved a clean blank signal with maximized analytes' colorimetric response.

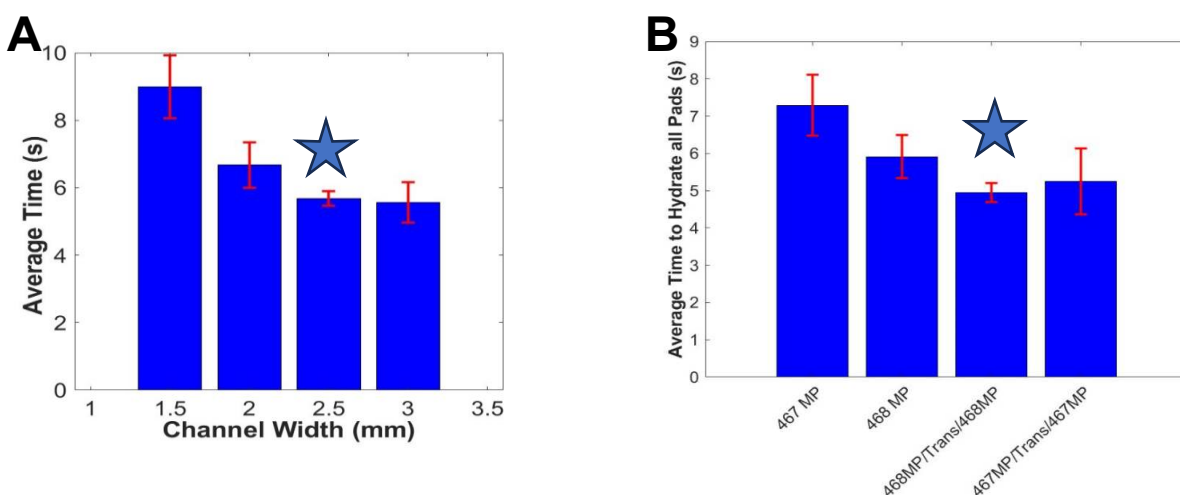


Figure C4. Flow channel width optimization (A) and Flow channel height optimization (B).

Both width and height were optimized based on the flow rate. Time to hydrate all three paper detection zones was recorded. Based on the results, a 2.5mm width was best for the faster flow rate with less standard deviation (A). For the height, the stacking of the flow channel was altered using different adhesives (467 and 468MP). The thicker

adhesive(468MP), when stacked with transparency film in the middle, gave a faster flow rate with consistent timing (B).

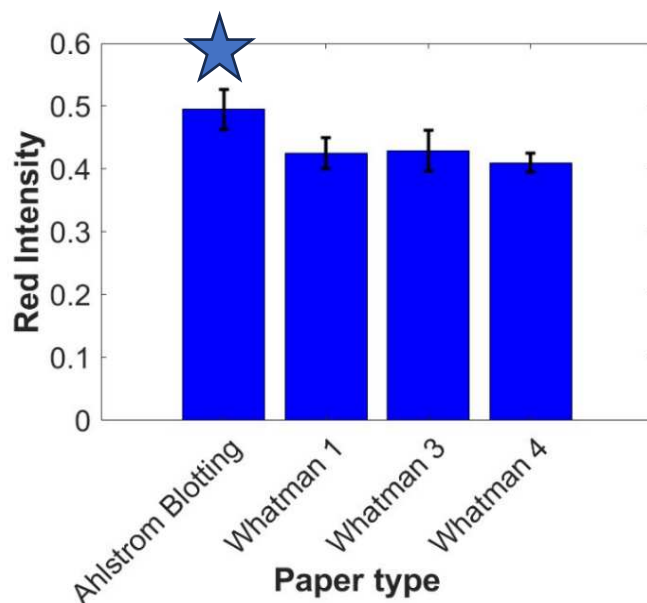


Figure C5. Paper substrate optimization.

We first focused on optimizing the paper substrate to optimize signal intensity in paper-based microfluidic devices. We tested four primary paper types: Whatman 1, Whatman 3, Whatman 4, and Ahlstrom 319 grade blotting paper (AB319). At the same concentration, AB319 demonstrated the highest color intensity. This may be due to AB319's higher water absorption capacity. When the channels are filled, and Whatman-grade papers are fully hydrated, analyte mass transport is facilitated by diffusion alone. In contrast, AB319 grade paper's higher water absorbance allows for increased convective mass transport to the detection zone, resulting in a stronger color signal.

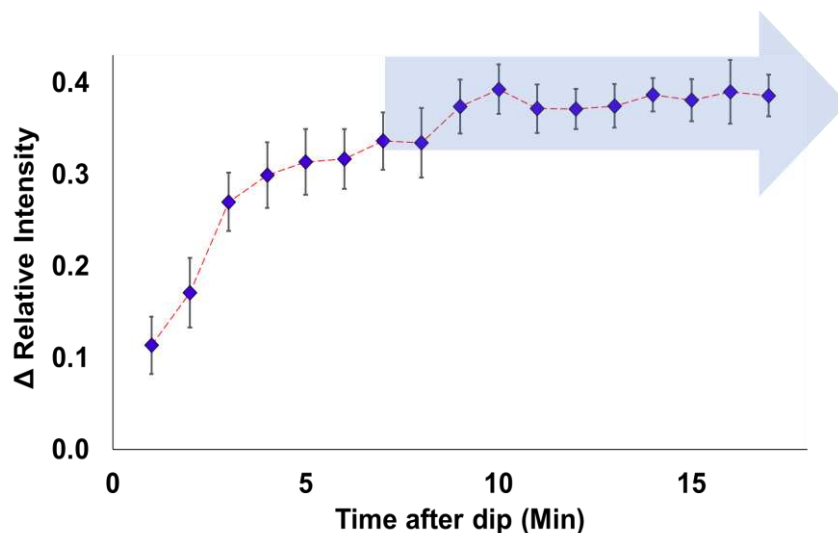


Figure C6. Reaction time optimization. After optimizing all the physical parameters, we tested the color signal on the developed sensor by taking pictures every minute. The highest intensity was obtained after 9 minutes, with no significant difference in signal till 15 minutes. Hence, 10-15 minutes was taken as the optimal time to better facilitate the imaging.

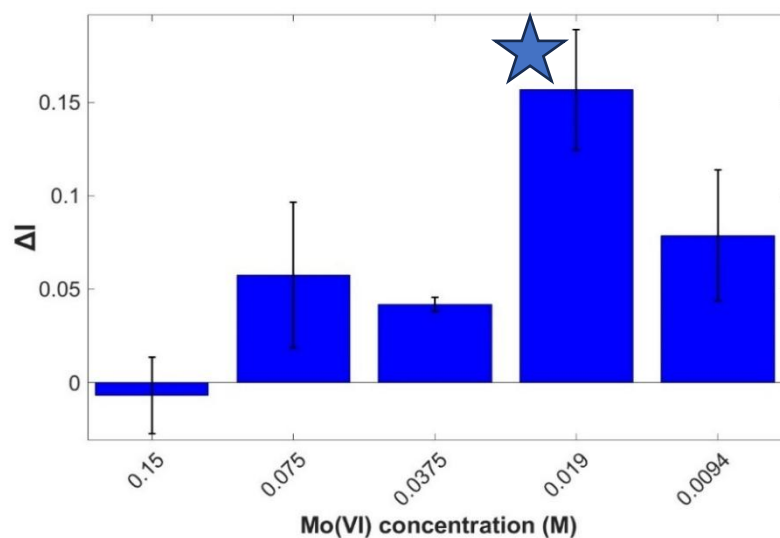


Figure C7. Mo(VI) concentration optimization on the developed sensor.

The optimal concentration of Mo(VI) for the developed sensor was found to be 19mM, at which the greatest color difference between the blank and the test sample was observed. Mo(VI) was mixed with antimony potassium tartrate (0.01 g/mL).

When the concentration of Mo(VI) was increased, the signal difference disappeared, and when the concentration of Mo(VI) was decreased below 19mM, the signal for the blank sample began to reappear. All these testing was done using 1M p-Toulenesulfonic acid.

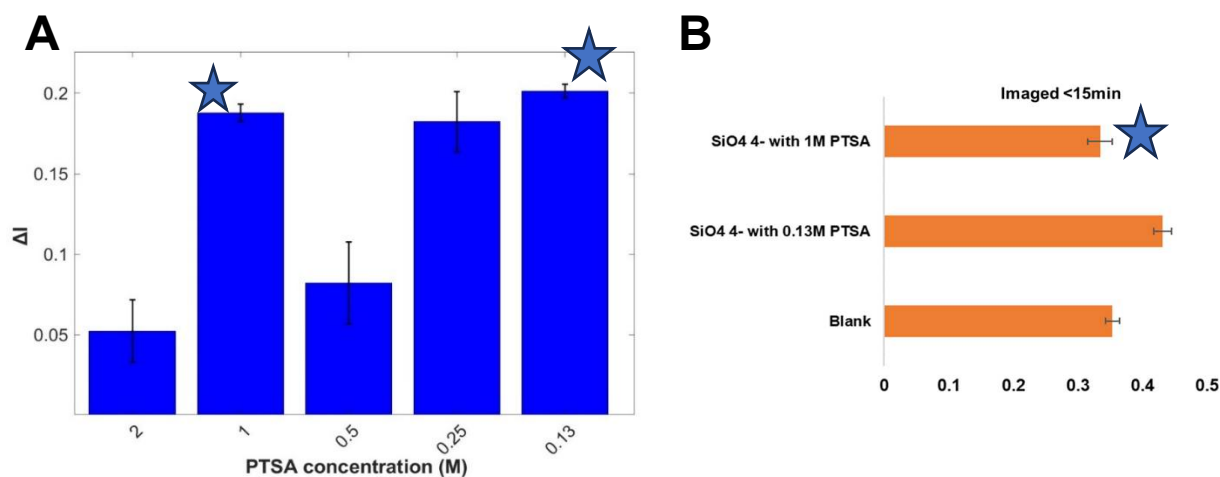


Figure C8. (A) p-Toulenesulfonic acid concentration optimization on the developed sensor. (B) Interference testing from silicates for optimal acidic conditions (1M vs 0.13 M) was obtained from part A.

After the optimization of Mo(VI), we optimized p-Toulenesulfonic acid concentration at different concentrations ranging from 0.13M to 2M. We observed a very random pattern when it comes to the color differentiation between the blank and the sample. This can be described through a Plot of acidity against the maximum tolerable Mo(VI) concentration (C) before the direct reduction of Mo(VI) to isopoly MB begins, as described by m ElShamy and Iskander ². The addition of molybdenum(VI) to the reaction promotes the formation of 12-MPA, but it is important to find the optimal concentration to avoid direct reduction of molybdenum(VI), which is the cause of the reagent blank in MB methods. The concentration of molybdenum(VI) needed to maintain a constant degree of protonation (Z) is a non-linear function of acidity, and the acidity itself strongly affects the stability of 12-MPA. At a given acid concentration, the amount of 12-MPA

formation and reduction increases sharply with increasing molybdenum(VI) concentration, until a certain critical concentration is reached. At this point, the further formation of 12-MPA tapers off, and the direct reduction of molybdenum(VI) to form isopolymolybdenum blues begins. The optimal concentration for the highest signal differentiation was observed at a PTSA concentration of 1M. At a lower concentration of 0.13M, although the signal differentiation was higher, this acidic condition was not enough to suppress the interferences from silicates, as silicate interferences are more prevalent at lower acidic conditions. Therefore, 1M was chosen as the optimal acid condition (B).

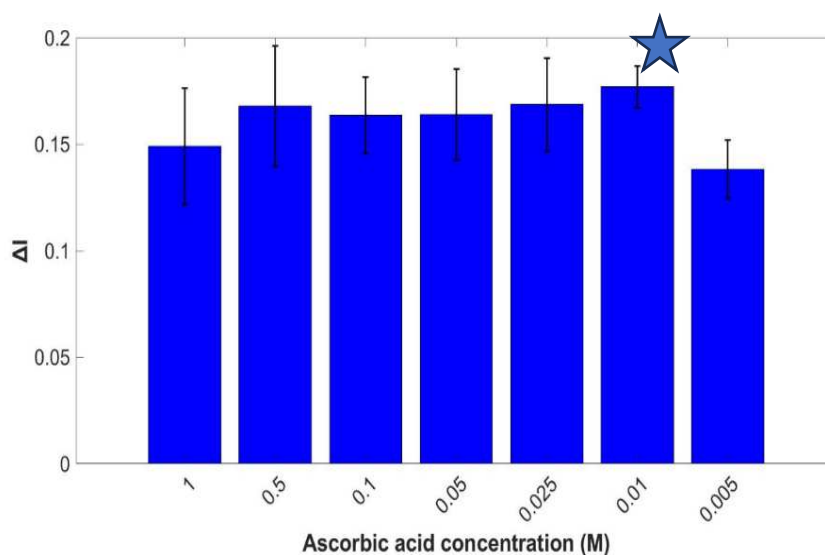


Figure C9. Ascorbic acid concentration optimization on the developed sensor (A).

After optimizing the reagent and organic acid, the signal was enhanced to a maximum, such that there was a very faint to no signal from the blank. Finally, the reducing agent was optimized for maximum signal differentiation such that the blank signal is colorless and only the analyte signal is blue. We tested the range of

concentrations from 5mM to 1M ascorbic acid. The best color differentiation with less variation was obtained at 10mM ascorbic acid concentration.

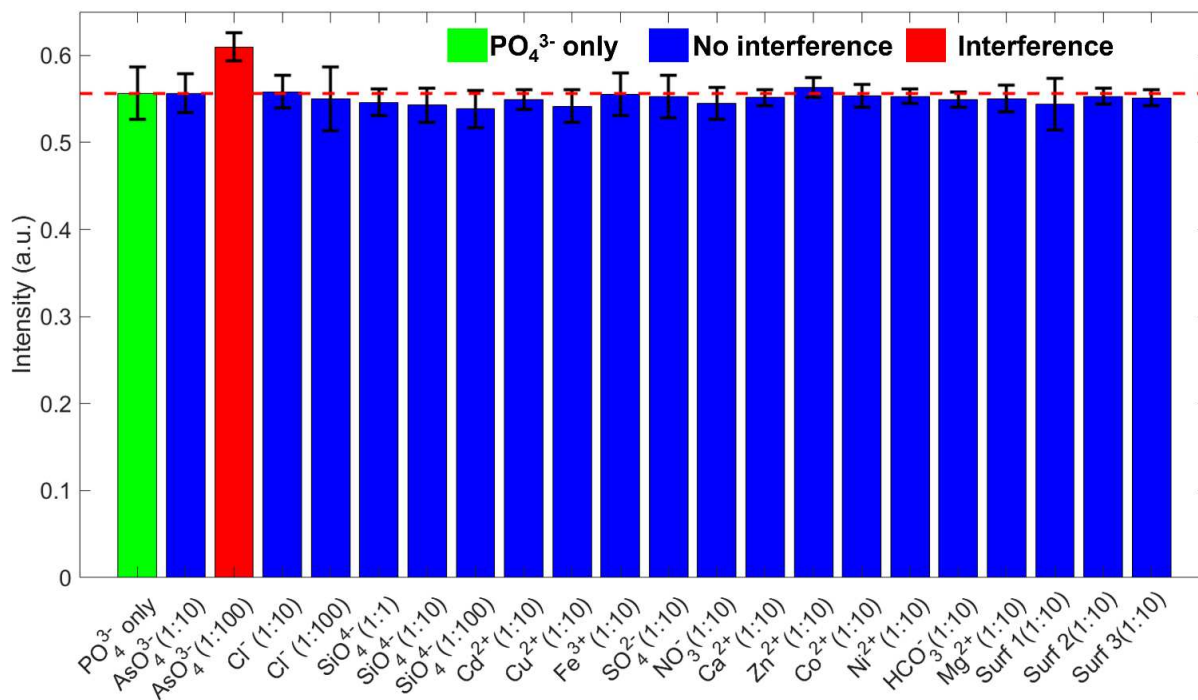


Figure C10. Interference study for PMB assay on the developed sensor. The sensor was tested with different ratios of targeted and non-targeted molecules (n=3). The green bars represent the control signal, the blue bars represent no interferences, while the red bar indicates a significant interference. The horizontal dotted line is the average signal from the control (PO₃³⁻ only). Dunnett's multiple comparison test was conducted to compare the results, with PO₄³⁻ alone serving as the control group at a 95% confidence level. The test outcomes confirmed that there was no statistically significant difference between the control group and the other interfering datasets (p>0.05) except for arsenate at a higher ratio (1:100).

Silicate (SiO₄⁴⁻) has long been considered one of the main interferences in the molybdenum blue (PMB) method, as it also forms heteropolyacids that can be reduced to molybdenum blues.³ Silicate interference becomes problematic when the reaction acidity is too low.¹ We observed this problem during PTSA optimization. In this study, using an optimized high concentration (1 M) of PTSA was sufficient to suppress the formation of molybdosilicate species. When the sensor is dipped in 1000 ppm SiO₄⁴⁻ and left for a

longer time (>15 min), some color formation is observed. However, no interference from silicate was observed at any concentration ratios within our optimized timeframe (10-15 min).

Similar to phosphate (PO_4^{3-}), arsenate (AsO_4^{3-}) also produces a reducible heteropoly acid when combined with molybdenum.⁴ These arsenomolybdenum blue species also display similar spectral features, molar absorptivities, and formation conditions⁵. Many cases of a "synergistic effect" in solution between arsenic and phosphorus have been reported in the literature, wherein their simultaneous presence accelerates the formation of molybdenum blue compared to the presence of either element alone.^{6, 7} While we detected no interference with a 1:10 ratio of phosphate to arsenate, a significant additive effect emerged at a 1:100 ratio. However, it is important to note that the higher mixing ratio was employed to simulate a worst-case scenario. In natural environments, encountering such high arsenate concentrations is very rare.

At elevated ionic strengths, the signal is expected to decrease by approximately 1-4%.^{8, 9} This effect is more pronounced when utilizing stannous chloride (SnCl_2) as the reducing agent, especially in water samples from marine and estuarine environments.¹ No notable subtractive interference arising from ionic strength was observed under the optimized reaction time. Meanwhile, chlorides can also form complexes with molybdate cations.¹⁰ Ascorbic acid, a less sensitive reducing agent, is, therefore, less likely to be influenced by variations in ionic strength. Moreover, the formation constants of these complexes are much weaker (0.29 and 0.036) than that of 12-MP.^{1, 11} Therefore, we observed no significant difference in the color signal while using either of the mixing ratios with Cl^- .

The sensor was also tested against a range of other common aqueous ions and three surfactants (Surf 1: sodium dodecyl sulfate, Surf 2: tetramethyl(tetradecyl) ammonium chloride, and Surf 3: Tween 20) in a 1:10 ratio of phosphate to interferences. Surfactants can be present at high concentrations in some surface water sites.^{12, 13} No significant difference was observed between the signal from the control and any of the mixtures.

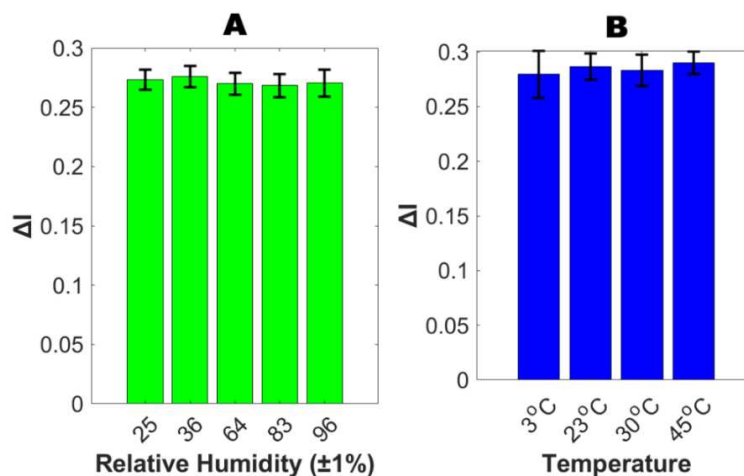


Figure. C11. Environmental Resilience Assessment. (A) Humidity Test: The sensor's performance remains consistent across varying humidity levels within a controlled chamber, ranging from 25% RH to 96% RH. (B) Temperature Test: The sensor exhibits stability and consistency when subjected to a wide range of water temperatures, from 3°C to 45°C.

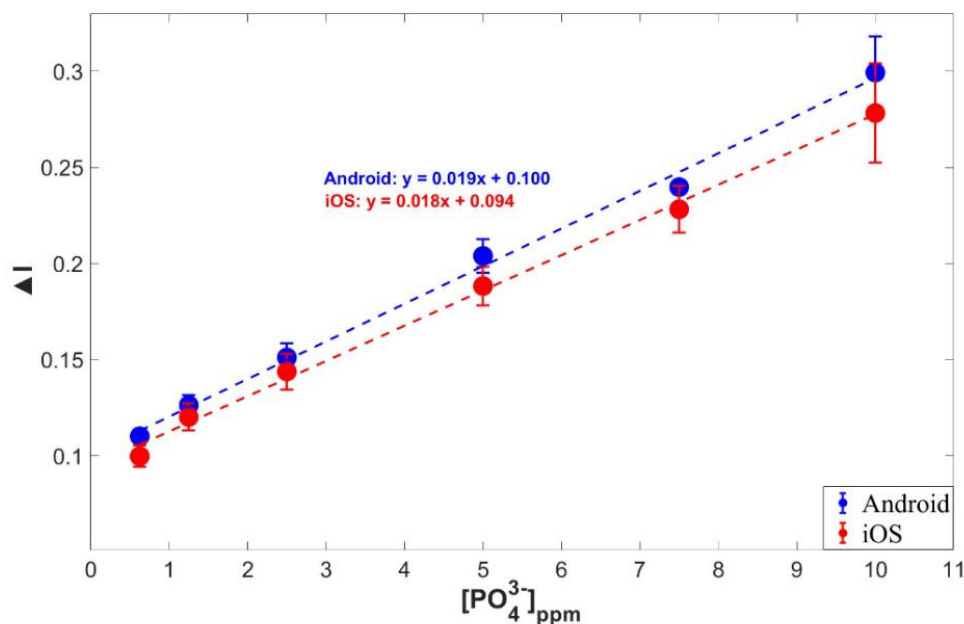


Figure C12. Testing the sensor's compatibility with different smartphone systems.

It is important to note that the lighting conditions on different smartphone systems can vary, leading to differences in grayscale intensity when capturing the same color signal. To address this, we conducted tests to assess the sensor's compatibility across different smartphone platforms, specifically comparing iOS and Android. In our experiments, we captured identical images using a Xiaomi 9T and an iPhone 13 Pro Max, with both the beauty filter turned off. Subsequently, we normalized the intensities with respect to a reference zone and performed calibration. As presented in Figure S9, the results clearly demonstrate that our sensor can achieve consistent sensitivity (slope = 0.018 and 0.019) across different smartphone systems. These findings validate the sensor's adaptability, emphasizing that it can be effectively utilized with any type of smartphone, provided that photographs are taken under conditions free from reflections, and with the camera angle perpendicular to the sensor.

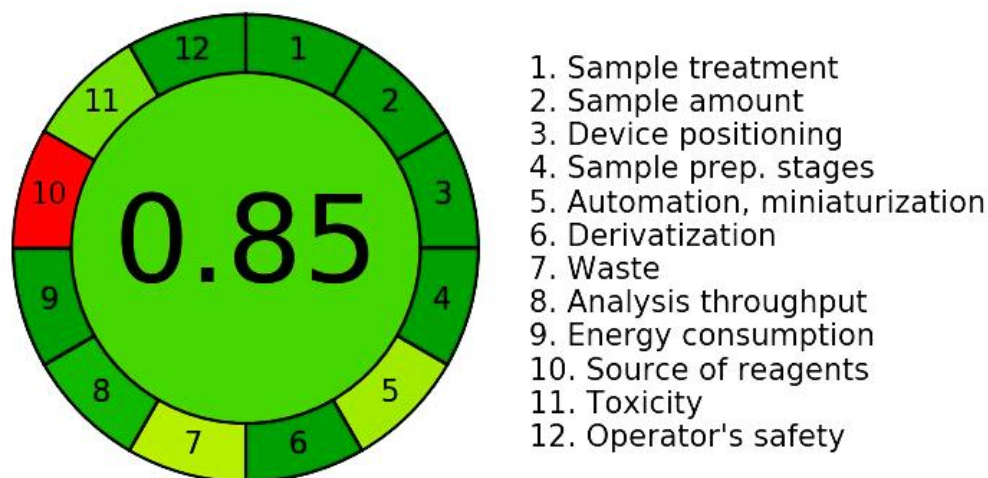


Figure C13. Analytical Greenness (AGREE) sustainability assessment report. Each parameter was assigned an equal weight.

Compared to traditional methods and alternative colorimetric platforms, our sensor displays various advantages aligned with green chemistry principles. These include on-site sampling and analysis capabilities, minimal sample volume requirements (just 0.08 mL), the ability for in-line measurements, and a streamlined process involving a single user step. The sensor is semi-automatic, miniaturized, eliminating the need for derivatization while substantially reducing waste generation to a mere 0.8 grams per analysis. Notably, the sensor is an energy-efficient technique with negligible energy consumption. The sensor is designed with a strong emphasis on user-friendliness, prioritizing operator safety and environmental suitability. These features position the developed sensor as an appealing and sustainable choice for phosphate-sensing applications.

Sampling protocols C1

The highly variable nature of river water characteristics in both spatial and temporal dimensions precludes the establishment of a single standardized methodology for sampling, storage, and analysis of phosphorus (P) in rivers.¹⁴⁻¹⁸ In this study, we adhere to the Precautionary Practice guidelines outlined by Jarvie et al. for protocols governing the sampling, storage, and analysis of collected river water.¹⁴

For the global level volunteered-led sampling, all the volunteers were provided with a sampling video, and an instruction sheet for sampling. The volunteers simply had to dip the sensor in water sources and upload the images with GPS coordinates and site information to the Dropbox cloud storage.

For on-site sampling by high school students to validate the developed sensor, the same process was followed as for sampling by the authors. However, 20 mL glass containers were cleaned with 1 M HCl for UV-Vis analysis and rinsed with Millipore water. To collect the samples, the containers were fully submerged in the water until full, and the lids were closed while still underwater. All sampling was done approximately 2 cm below the water's surface to ensure samples were collected from the same depth as the sensor was dipped. For method validation by the authors in the laboratory, samples were analyzed using UV-Vis within an hour of collection. For samples collected by high school students, samples were stored in a freezer and analyzed within 24 hours. UV-Vis analysis was conducted following the well-established protocol from EPA method 365.1.¹⁹

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APPENDIX D — Capillary Flow-Driven Paper-Based Microfluidic Sensor for NDMA Detection in Water

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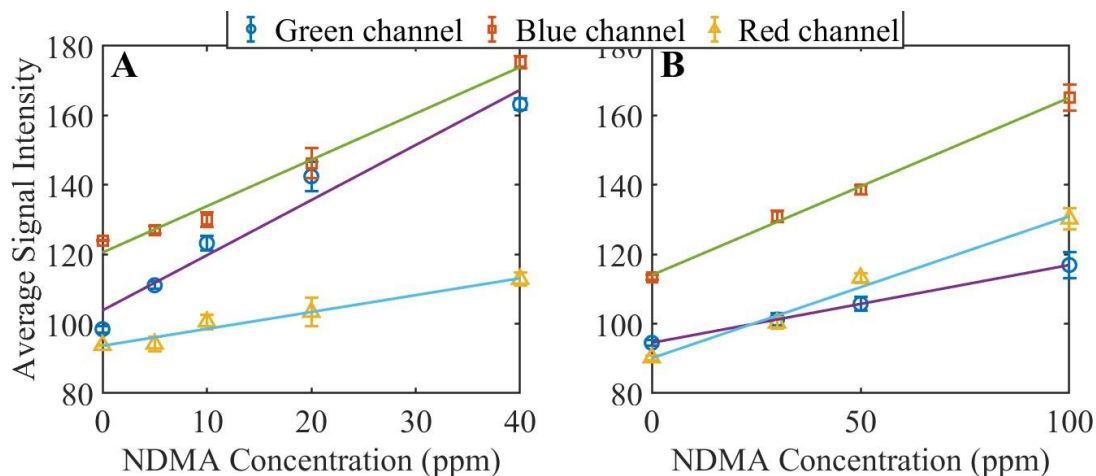


Figure D1. Image analysis color-channel selection for NDMA detection. Images were analyzed using ImageJ and separated into red, green, and blue (RGB) color channels.¹ For NDMA–Co assays (A), the green channel was selected, while for NDMA–Fe assays (B), the blue channel was used, as these channels exhibited the highest sensitivity for their respective reactions.

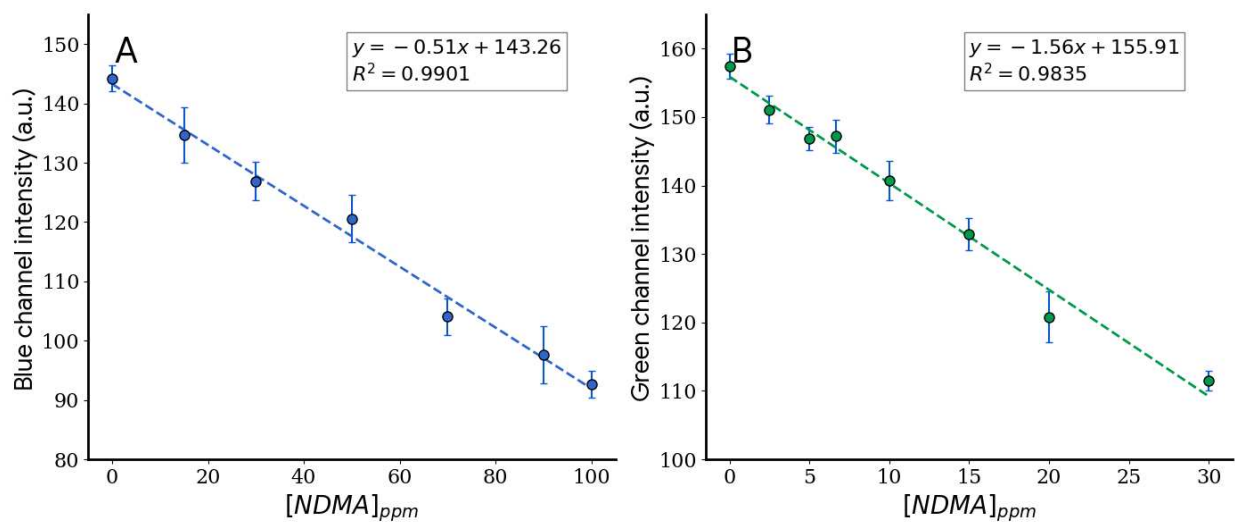


Figure D2. Sensor's compatibility with smartphone-based system (RGB color detector smartphone app). A = Fe²⁺ based system, B = Co²⁺ based system.

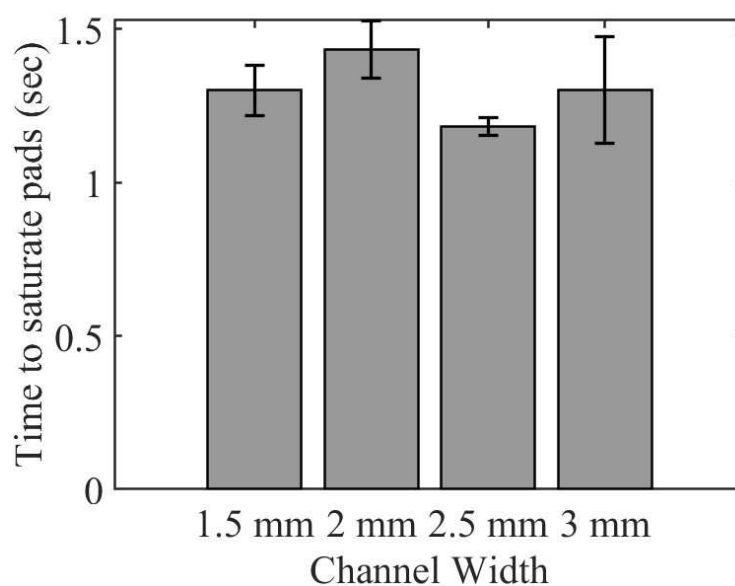


Figure D3. Channel width optimization for the capillary flow–driven microfluidic device. Average flow performance metrics (mean $\pm$ SD) are shown for channel widths of 1.5, 2.0, 2.5, and 3.0 mm, demonstrating that a channel width of 2.5 mm provides the most consistent and reliable flow behavior and was therefore selected for subsequent device fabrication and testing. The replicate measurement seconds were rounded to the nearest 0.5.

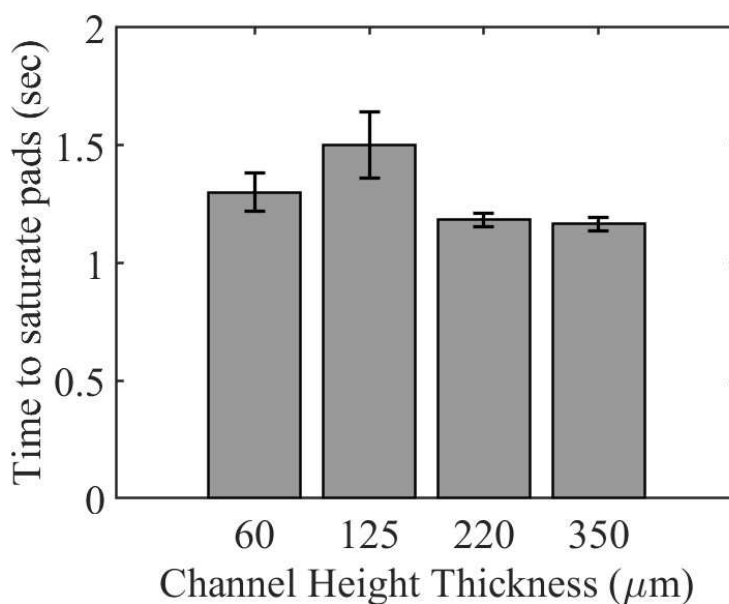


Figure D4. Channel height optimization for the developed sensor. The replicate measurement seconds were rounded to the nearest 0.5.

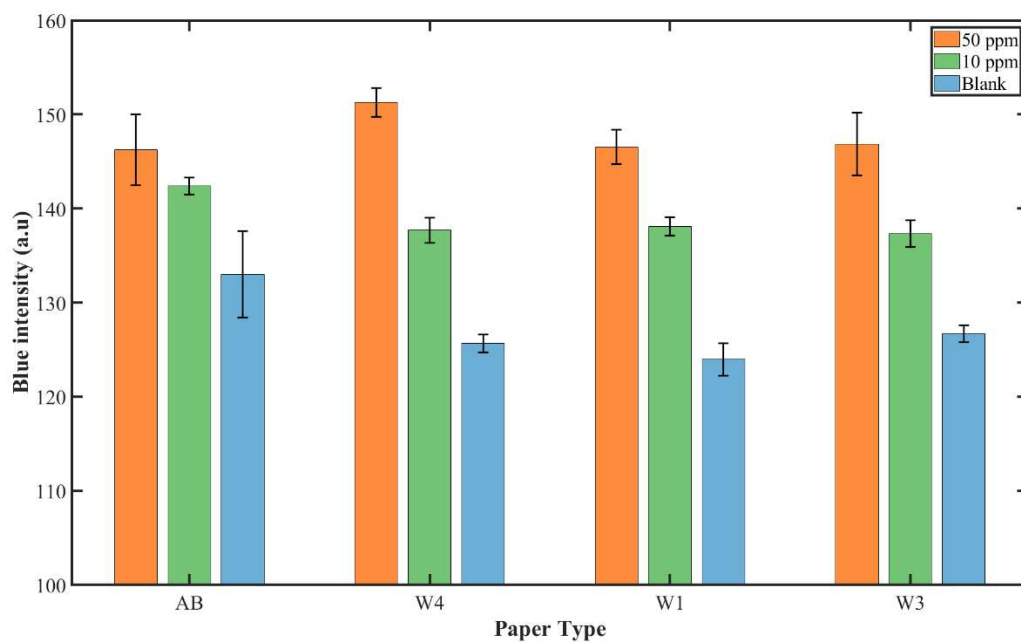


Figure D5. Substrate optimization on the developed sensor. W4 was chosen for its best differentiation of signal with lower SD.

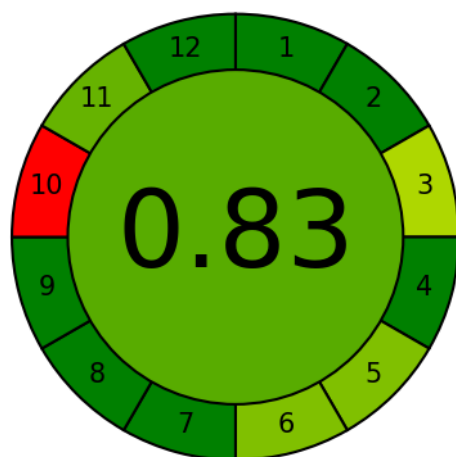


Figure D6. AGREE Greenness score of the developed sensor²⁻⁵

Table D1. Physical and fluidic characteristics of paper substrates evaluated for capillary flow-driven microfluidic device detection zones.⁶ nd – no data

Material	Thickness [mm]	Porosity [μm]	Flow rate	Grammage [g m⁻²]
Glass fiber	0.3	nd	nd	~60
Whatman Grade 1 Chromatography Paper	0.180	nd	57 mL/min	87
Whatman Grade 4 Qualitative Filter Paper	0.205	20–25	247 mL/min	96
Whatman Grade 3 Qualitative Filter Paper	0.390	6	28 mL/min	187
Ahlstrom Blotting Paper Grade	0.5	19	360 mL/min	179

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