During this time of vigilance against terrorist activity, in situ detection and testing of seawater for trace levels of explosives is a high priority of the Navy and the DoD, both for protection of U.S. personnel and assets and for environmental monitoring of Navy-patrolled littoral waters. NRL has immersed itself in this project and surfaced with a solution: the use of Hydroid REMUS100 autonomous underwater vehicles (AUVs) equipped with high throughput microfluidic immunosensors (HTMIs) and high aspect ratio microstructures (HARM) containing immobilized antibodies.

As the AUV glides through the marine environment, its high throughput microfluidic sensors collect, detect, and quantify trace levels of trinitrotoluene (TNT) at a flow rate of 2 ml/min; this yields a 30 s lag time, but at the 9 ml/min possible flow rate, nearly instantaneous detection is eventually expected. In initial tests, the REMUS100 operated at 6 to 9 ft depth for 2-h deployments, successfully analyzing seawater contaminated with ~20 to 200 ppb of TNT without sample pretreatment and without HTMI failures due to clogging from biomass infiltration or device leaks.

This technology represents a significant time savings over offsite chemical analysis and brings laboratory-level sensitivity to a field-deployable system that can be used to assess the environmental safety of marine environments for dispatched medical personnel and other first responders and to monitor levels of contaminants leached by unidentified unexploded ordnance (UXO) in defunct munitions testing grounds and military installations.

This is just another demonstration of NRL's willingness to go deep to defuse potentially explosive situations.
REMUS100 AUV with an Integrated Microfluidic System for Explosives Detection

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Detecting explosive materials at trace concentrations in the marine environment is critical to protecting U.S. citizens, waterways, and military personnel during this era of potential domestic terroristic activity. Presented here are results from recent field trials that demonstrate detection and quantitation of nitroaromatic materials using novel high-throughput microfluidic immunosensors (HTMIs) to perform displacement-based immunoassays onboard a Hydroid® REMUS100® autonomous underwater vehicle (AUV). Antibodies were immobilized on high aspect ratio microstructures (HARM) incorporated into high-throughput microfluidic devices, thereby creating larger surface-area-to-volume ratios that facilitated more efficient analyte extraction. Missions were conducted 6 to 9 ft below sea level, and no HTMI failures were observed due to clogging from biomass infiltration or device leaks. HTMIs maintained immunoassay functionality during 2-h deployments while continuously sampling seawater at a flow rate of 2 ml/min, which resulted in reduced lag time (101). Contaminated seawater with ~20 to 200 ppb trinitrotoluene (TNT) was successfully analyzed in situ without sample pretreatment.

INTRODUCTION

Monitoring the composition of waterways about the continental United States, its territories, and its allies is an ongoing concern for the Navy and the Department of Defense. Highly energetic small molecules classified as explosives and their associated degradation products pose major security and environmental concerns during this era of imminent domestic terroristic activity.1 In addition to being a major cause for concern relative to security, the leaching of explosives in the vicinity of various operational/defunct military installations and munitions testing grounds throughout the U.S. indicates the need for autonomous environmental monitoring of unidentified unexploded ordnance (UXO) and the associated degradation products of these materials. Finally, forensic investigations, as they pertain to domestic terroristic activities, also dictate that field-deployable assessment tools be readily available for untrained field technicians in order to rapidly determine the safety of dispatched emergency medical personnel and other first responders.

Traditionally, offsite chemical analysis has been performed using conventional analytical instrumentation including high performance liquid chromatography, gas chromatography coupled to mass spectrometers, surface-enhanced Raman spectroscopy, ion mobility spectrometry, cyclic voltammetry, and energy dispersive X-ray diffraction in the analysis of explosives. These methods have successfully delivered high sensitivity and selectivity for the analytes, yet these methods offer limited amenability to portability, which detracts from their potential field readiness. Size notwithstanding, these instruments require significant expertise to employ. Real-time reporting from remote analysis of trace levels of explosives has been an elusive target.

The most prolific field-deployable detection system has been the extensively trained canine. These animals are expensive to train, require continuous maintenance, and are subject to fatigue and injury. Further, the canine’s strong sense of smell is rendered ineffective when the analyte or target exists in a submerged plume within a body of seawater such as those that exist in the U.S. coastal waters.

The nature of explosives and concern for the safety of the analyst and first responders demonstrate the need for sensing these dangerous and potentially toxic (≥ 0.002 µg/mL as reported by the Environmental Protection Agency) environmental contaminants remotely.2 For underwater operations, there is a significant threat posed by UXO, both to maritime traffic and to
A new breed of highly sensitive immunosensors embedded into autonomous underwater vehicles (AUVs) such as the commercially available Hydroid® REMUS100® offers distinct advantages such as (1) enabling on-site in situ assessment that eliminates the low bias due to sample storage, degradation, and transport; (2) remote sensing capability that minimizes putting people in harm’s way; and (3) programmable vehicles that can survey the area of interest for the targets. Sensors for explosive nitroaromatic compounds that offer better approaches to the rapid, highly sensitive and selective remote detection of trace levels of various explosives are undeniably warranted. Presented herein is a novel approach with application of high-throughput microstructures designed to meet three primary requirements: (1) reducing false positive and false negative responses in complex marine matrices; (2) providing for rapid analysis; (3) increasing sensitivity by more efficient extraction.

**AUTONOMOUS UNDERWATER VEHICLE, IMMUNOSENSOR, AND PLUME GENERATOR**

The vehicle used in these field trials was the Hydroid REMUS100 AUV, which has been widely employed for port and harbor surveillance by the U.S. Navy as well as by NATO countries. The standard REMUS100 is 7.5 in. in diameter, 63 in. long, and weighs approximately 80 lb (see Fig. 1). The expandability of the REMUS100 is provided by an interface connector on the front of the REMUS100 vehicle supplying operational commands, communications, and power to the sensor payloads. The first part of the REMUS100 augmentation systems is the adapter collar. The collar provides access to the electrical connector on the front of the REMUS100 and provides a mechanical attachment for the additional sensors. This collar has access ports for the programming connector; conductivity, temperature, and depth (CTD) sensor; the acoustic transponder; and EcoPuck Triplet for optical measurements. The external CPU section (ECPU) of REMUS100 was custom designed by SubChem Systems (Narragansett, RI) to provide an external controller for adaptive missions with the REMUS100. The section consists of a single-board computer, power conditioning, and an acoustic modem (Benthos ATM-885 board set). For each mission, once transponders (underwater navigational aids) are positioned within the operational area, the REMUS100 can be programmed to navigate a specified path while continuously assaying the contents of its environment and reporting the results over the acoustic modem to the main controller. Results can also be broadcast to a secure IP address for visualization by offsite individuals over the Internet.

The immunosensor was fitted with a pump capable of delivering flow rates of 9 ml/min that can be controlled independent of the rest of the system via acoustic modem commands. Once directed, the pump turned on at the requested flow rate and pushed the trinitrotoluene (TNT) sample through the sample injector shown in Fig. 2. The REMUS’s forward velocity moved fluid through the sample injector and TNT sample was mixed into that flow, producing the final concentration that was assessed by the immunosensor. The flow rate through the manifold was determined by the vehicle’s forward velocity. The REMUS velocity for this trial was programmed to be 1 m/s throughout the mission. The corresponding flow rate through the manifold was approximately 1.9 L/min through the 0.25-in.-diameter flow path. As configured, with the high flow pump set to 1 ml/min, the corresponding TNT concentration of the plume was 20 ppb. Plume concentration was linearly controlled with the high flow pump flow rate, and a 9 ml/min setting yielded a TNT concentration of 175 ppb. The immunosensor’s filtered intake was positioned at the aft end of the manifold flow path (see Fig. 2).

![FIGURE 1](image1.png)

**FIGURE 1**

Hydroid® REMUS100® autonomous underwater vehicle.

![FIGURE 2](image2.png)

**FIGURE 2**

Nose of REMUS100 with plume generator and the filter capped inlet for the immunosensor.

The immunosensor is shown in Fig. 3. The unit was optimized for TNT detection in seawater matrices during bench scale experiments. The system was designed to be submersible in order to be used in chemical sensing for submerged UXO. In the REMUS100
framework, the immunosensor was poised immediately aft of the nose. The free flooded REMUS100 nose housed the sample intake and buffer solution. The sample intake filter was located bottom dead-center just outside the nose within the REMUS100’s free flow boundary layer. On the forward bulkhead of the immunosensor was the high-throughput microfluidic immunosensor (HTMI) or coupon interface. In the face sealing design, as coupons were exhausted, they were exchanged by removing the nose and replacing the spent coupon with a charged coupon. This was accomplished by removing the mounting bracket and sliding the coupon off the alignment pegs between the ~2-h REMUS100 deployments. The quick coupon removal interface of the immunosensor is illustrated in Fig. 3(a).

**HIGH-THROUGHPUT MICROFLUIDIC IMMUNOSENSOR (HTMI)**

The high-throughput microfluidic immunosensor was created via a high-precision micromilling process whereby a molding tool was cut from brass feedstock with a positive impression of the desired microarchitectures. The molding tool was used in a
hot embossing process that transferred the positive features of the molding tool into poly(methylmethacrylate) (PMMA) blanks. PMMA was chosen due to its well-characterized surface chemistry, high impact resistance, durability, and resistance to biofouling. Next, the substrate was coupled to PMMA coverslips via a trisolvent-assisted thermal bonding process, resulting in the production of high-throughput microfluidic devices. The microchannels were then treated to provide a scaffold that was later used to covalently immobilize antibodies directed against TNT. After the antibody immobilization was complete, the devices were saturated with a solution containing fluorescently labeled analogues of TNT. The devices were sealed with a plastic film and encased in aluminum foil to prevent leaks and photodegradation of the bound fluorophores. All devices were refrigerated or stored on ice in a cooler until used. A representation of the HTMI (labeled “coupon”) is shown in Fig. 3(a). The HTMI uses 39 parallel 1-in.-long centrally located microchannels. The high aspect ratio microstructures (HARM) increased the surface-area-to-volume ratio of the HTMI, thereby enabling more efficient analyte extraction, subsequently resulting in improving overall assay sensitivity. The highly parallelized microchannels enabled unequaled throughput by reducing the pressure drop across the device to a negligible amount, allowing for operational flow rates of up to 9 ml/min, which is 90× greater than previous reports while maintaining favorable kinetic conditions for the displacement-based immunoassay.

FIELD DEMONSTRATION

All tests were performed in Narragansett Bay, Rhode Island, within the Greenwich Bay. Tests were conducted August 9 and 10, 2010. A topographical representation of the Greenwich Bay is shown in Fig. 4(a). The green diamonds show the position of the transponders and a green plus symbol represents the ship location. The REMUS100 assay path is shown in purple while the location of the REMUS100 at the time of the image is illustrated by a white plus symbol. A prelaunch photo of the REMUS100 is shown in Fig. 4(b) during the ballasting procedure in which the unit’s buoyancy was optimized for the mission.

REMUS100 IMMUNOSensor PERFORMANCE

Prior to the mission, the immunosensor was calibrated against matrix-matched TNT standards as shown in Fig. 5. Triplicate analyses of TNT standards of 0.01, 0.1, 1, and 10 ppb prepared from a TNT stock solution were performed. An analysis of TNT samples at 1 and 10 ppb prepared using nonhazardous explosive for security training and testing (NESTT) material was also done in triplicate to compare the sensor response for other available TNT variants. Peak-to-peak reproducibility was in excellent agreement with %RSD (relative standard deviation) values of less than 8%. Figure 6(a) shows the sensor response over time as a black trace and the plume activation as a blue trace.
In agreement with bench testing, lag times during the field trial of approximately 30 s from plume initiation to signal production were observed during the trials. The operational flow rate for the TNT assay was 2 ml/min, which was 20× higher than previously reported. The inset in Fig. 6(b) shows the path of the REMUS100 during the mission, and the contour plot shows the response at the given position. Although the plume was simulated as detailed earlier, the contour plot is in excellent agreement with the TNT releases as time correlated with Fig. 6(a). The deviation peak symmetry in the field trial was expected. In the bench assays, a finite volume was injected into the column for quantitation; however, in the field, the volume injected was dependent upon crossing currents as well as other environmental effects.

SUMMARY

We have demonstrated the potential for low ppb detection of TNT at significantly higher flow than previously demonstrated. The monoclonal antibodies used here were demonstrated to function in the harsh aquatic environment of the Narragansett Bay. The trial was conducted with an operational flow rate of 2 ml/min, yielding nearly 30 s lag times, but the immunosensor is capable of operating at flow rates as high as 9 ml/min. At that flow rate, lag time would be negligible, providing nearly instantaneous results. The immunosensor as configured for these trials successfully detected TNT from a simulated plume at 20 ppb in seawater while underway. Experiments are continuing to further extend the sensitivity of the assay by increasing the density and bioavailability of the receptors. In addition, small molecules that are toxic to indigenous species are also being evaluated as possible analytes for future versions of the immunosensor.

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PAUL T. CHARLES has been a research chemist in the Center for Bio/Molecular Science and Engineering at the Naval Research Laboratory since 1991. He received his master’s degree from the University of Maryland, University College (health care/business administration) and a bachelor of arts degree from the University of Maryland, Baltimore County (biological sciences). Mr. Charles has utilized his expertise in the immobilization of biomolecules for the design of novel fluorescence-based biomolecular sensors for the detection of small molecular weight molecules such as explosives (TNT, RDX, PETN, and HMX), polychlorinated biphenyls (PCBs) and drugs of abuse (cocaine, morphine, and marijuana). His work has been adapted for field monitoring applications of environmental contaminants in groundwater, soil, and seawater. His current research involves the development of an underwater autonomous vehicle (UAV) prototype biosensor for use in the marine environment.

References
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