Published in final edited form as:

Environ Toxicol Chem. 2015 May ; 34(5): 983–992. doi:10.1002/etc.2893.

The Gellyfish: An In-Situ Equilibrium-Based Sampler for Determining Multiple Free Metal Ion Concentrations in Marine Ecosystems

Zhao Dong^{*,†}, Christopher G. Lewis[†], Robert M. Burgess[‡], and James P. Shine[†]

[†]Exposure, Epidemiology and Risk Program, Department of Environmental Health, Harvard T. H. Chan School of Public Health. 665 Huntington Avenue, Building 1, B27 Boston, MA 02115, U.S.A.

[‡]U.S. Environmental Protection Agency, ORD/NHEERL - Atlantic Ecology Division. Narragansett, RI, U.S.A.

Abstract

Free metal ions are usually the most bioavailable and toxic metal species to aquatic organisms, but they are difficult to measure due to their extremely low concentrations in the marine environment. Many of the current methods for determining free metal ions are complicated, time-consuming, and can only measure one metal at a time. We developed a new version of the ‘Gellyfish’, an in-situ equilibrium-based sampler, with significantly reduced equilibration time and the capability of measuring multiple free metal ions simultaneously. By calibrating the Gellyfish to account for its uptake of cationic metal complexes and validating them in multi-metal competition experiments, we were able to determine free metal ion concentrations previously collected over ten months at five locations in Boston Harbor for Cu, Zn, Pb, Ni, and Cd. This work generated one of the largest free metal ion datasets and demonstrated the applicability of the Gellyfish as an easy-to-use and inexpensive tool for monitoring free ion concentrations of metal mixtures in marine ecosystems.

Keywords

Metal; Free ion; Passive sampler; Speciation; Mixture

INTRODUCTION

In natural waters, metals exist in a variety of chemical forms, or ‘species’, each of which exhibits potentially different biogeochemical behavior. Among metal species, the most chemically reactive and bioavailable form in aquatic ecosystems is usually the free metal ion [1-3]. For this reason, concentration of free metal ions, rather than total dissolved metal, often determines metal bioaccumulation and toxicity in aquatic ecosystems [2, 4]. As a result, ambient water quality criteria [5] for metals, which have long been based on total

*Address correspondence to: Tel: 617-432-5554, Fax: 617-432-3349, zdong@hsph.harvard.edu.

Conflict of Interest: The authors declare they have no actual or potential competing financial interests.

dissolved concentrations, have in recent years shifted towards a new framework focusing on free metal ions [6].

A focus of environmental research on metals has been the development of an easy and accurate analytical method for measuring free metal ions in aqueous systems, which occur at extremely low concentrations (usually far below nM) and represent only a small fraction of the total dissolved phase. Widely used and accepted laboratory-based methods for measuring metal speciation include competitive ligand exchange (CLE, usually coupled with other methods), anodic [7, 8] (ASV) and cathodic stripping voltammetry [9] (CSV). However, they are ex-situ, indirect, labor-intensive, operationally complex, time-consuming, may be subject to chemical interferences, and measure the labile fraction of metals rather than free ions. In addition, these methods measure speciation for only one metal at a time, while metals almost always exist in mixtures in the environment.

Several in-situ methods have been developed and refined over recent years [3], such as ion selective electrodes [10] (ISE), diffusive gradients in thin films (DGT) [11-13], the Donnan membrane technique (DMT) [14, 15], supported liquid membrane extraction (SLM) [16-18], and sonic-assisted free ion recorder (SAFIR) [19]. Due to their vastly different mechanisms and characteristics, these methods differ in strengths and limitations, and are not always comparable to each other or to the ex-situ methods. For example, ISE is the simplest to use, but is largely restrained by its relatively high detection limit, although it has been used to measure Cu as low as 10^{-11} to 10^{-12} M in some studies [20-22]; DGT is a diffusion-based sampler widely used in both water and sediment, but it measures a 'DGT-labile' fraction of the metals instead of the true free metal ions, since the kinetics of metal uptake into the DGT may also lead to the accumulation of metals associated with colloidal organic matter or other complexes [23, 24]; SAFIR reaches equilibrium quickly with the assistance of a sonic device, but has less flexibility in design (using a commercially made Petri dish) [19]. Furthermore, although many of these methods have the potential to measure multiple metals simultaneously, this aspect has largely been underexplored, and the majority of data generated still focus on a single metal (usually Cu). A method is still needed to create large datasets of multi-metal free ions for the purpose of environmental monitoring or routine sampling of metal mixtures.

To address the need in monitoring free metal ion concentrations of metal mixtures, we developed the 'Gellyfish' [25, 26], an equilibrium-based sampler consisting of a metal-binding resin (beads coated with iminodiacetic acid, or IDA) impregnated within a polyacrylamide gel matrix. Although using the same binding ligand (IDA) as in Chelex or DGT, the Gellyfish is not a sampler of flux; instead, it aims to measure free ion concentrations at equilibrium. It contains a very small amount of IDA (1.05×10^{-7} mol in a sampler with a hydrated gel volume of 0.375 mL, about 5,900 times less than a typical DGT sampler with 10 mL of resin gel [11]) and it can be assumed that the surrounding solution is infinite with respect to metal concentrations during a field deployment. Conceptually, this is similar to polyethylene samplers commonly used to measure the freely-dissolved activity of organic contaminants in water [27]. Both devices contain a substrate, and the contaminant (freely dissolved organic contaminant vs. free metal ion) has a known partition coefficient between the substrate (polyethylene vs. IDA embedded in polyacrylamide gel) and the

surrounding solution. Both are also designed to provide enough sorptive capacity to amplify the signal and achieve desirable detection limits. In addition, samplers with blank beads (without the IDA coating) are used alongside samplers with IDA to correct for background diffusion of non-binding species into the gel. Our previous work on Cu has shown that Gellyfish measurements respond to changes in Cu^{2+} but not total Cu [25]. Another study in Boston Harbor found significant correlation between Gellyfish measurements of Cu and Pb and biomonitoring results using mussels [28], suggesting that it is a valid tool for studying metal speciation and bioavailability.

The present study represents a significant advance in the development and application of the Gellyfish sampler. We created a new design with substantially reduced equilibration times compared to the original version [25, 26]. To calibrate the Gellyfish for measuring free metal ions in metal mixtures, we estimated stability constants for the binding of cationic metal complexes to the IDA. The predominant cationic metal complexes were either hydroxide or chloride complexes, for Cu, Zn, Pb, Ni and Cd individually. These five metals were chosen because they are among the most studied and commonly found metals in marine ecosystems, and have the highest affinities for IDA. We performed laboratory tests to validate the ability of the Gellyfish to accurately account for competition among metals for complexation with IDA, so that it is able to simultaneously determine multiple free metal ions at environmentally-relevant concentrations. We then used the measured stability constants to calculate multiple free ion concentrations from a previously collected multi-metal dataset, where Gellyfish samplers were deployed biweekly in Boston Harbor from March to December 2005. This work demonstrates the potential of the Gellyfish as an easy-to-use and inexpensive tool for routine monitoring of multi-metal free ion concentrations in marine systems.

MATERIALS AND METHODS

General procedures

Trace metal clean techniques were applied to all experimental steps. Plasticware (made of low density polyethylene, acrylic, or Teflon) and all other materials were soaked in 10% HCl for 24 hours, rinsed with ultrapure de-ionized (DI) water (Barnstead Nanopure Diamond, Dubuque, IA) three times, then soaked in 10% HNO_3 for 24 hours, and rinsed again with DI water three times. Sample preparation and handling were conducted in a Class-100 clean bench whenever possible to minimize potential contamination.

With the exception of the salinity experiment, all experiments in the present study were conducted at an estuarine salinity of 18 ppt for simplicity. Artificial seawater (Aquil, containing $4.20\text{E-}1$ M of NaCl, $2.88\text{E-}2$ M of Na_2SO_4 , $9.39\text{E-}3$ M of KCl, $2.38\text{E-}3$ M of NaHCO_3 , $8.40\text{E-}4$ M of KBr, $4.85\text{E-}4$ M of H_3BO_3 , $5.46\text{E-}2$ M of MgCl_2 and $1.05\text{E-}2$ M of CaCl_2 , with a salinity of 36 ppt) was used for all laboratory experiments, prepared according to Price et al. [29] (excluding fluoride, nutrients and trace metals), and filtered through a Chelex-100 (Sigma-Aldrich) column to remove any trace metal contaminants. The Chelex-100 resins were pre-conditioned as described in Price et al. [29] The prepared Aquil was diluted two-fold with DI water to approximate an estuarine salinity of 18 ppt before use. Metals were spiked into the purified Aquil as dissolved trace metal grade chloride salts

(Fisher Scientific; Sigma-Aldrich; EMD), and free metal ion concentrations were controlled by trisodium-nitrilotriacetic acid (Na_3NTA ; Aldrich Chemical Company). Metal and NTA speciation were calculated in MINEQL+ (version 4.07, Environmental Research Software) to model free ion concentrations at equilibrium. To ensure full mixing, all spiked solutions were contained in 2.6 L polypropylene canisters (Rubbermaid, Atlanta, GA) and placed on a rotary shaker table (VWR) at 75 rpm for at least 24 hours prior to deployment of the Gellyfish and for the entire duration of all laboratory experiments. Based on the design criteria of the Gellyfish, the amount of spiked metals in 2 liters represented an infinite solution (less than 5% depletion of total dissolved metals at maximal number of inserted samplers). To monitor possible metal contamination in Aquil, an Aquil solution without metal or NTA addition and one with NTA only were always used alongside the spiked containers. pH was measured for each solution at the end of all experiments, and used to account for the competitive effect of hydrogen ions.

After exposure, Gellyfish samplers were retrieved from the canisters, rinsed thoroughly with DI water, and extracted in polyethylene Petri dishes containing 20 mL of 5% high purity HNO_3 (Mallinckrodt Baker) for 48 hours. Preliminary leaching tests showed the extracted concentrations remained constant after 24 hours, and this result was unaffected by varying free metal concentration in the surrounding solution (Supplemental Data, Figure S1).

Extracts were measured on an inductively-coupled plasma mass spectrometer (ICP-MS; Perkin Elmer Elan 6100 DRC) calibrated with external standard, with indium as the internal standard to correct for potential instrument drift. NIST 1643e (trace elements in water) was used as an independent standard reference material and measured after every 10 to 15 samples. The average NIST recoveries and detection limits of the metals are summarized in Supplemental Data, Table S1. The detection limits were calculated as three times the standard deviation of the method blanks, and at a typical estuarine pH of 8.0 and salinity of 18 ppt, this translates into an overall detection limit by the Gellyfish of $4.8\text{E-}15\text{ M}$ for Cu^{2+} , $3.6\text{E-}12\text{ M}$ for Zn^{2+} , $1.2\text{E-}13\text{ M}$ for Pb^{2+} , $2.4\text{E-}13\text{ M}$ for Ni^{2+} , and $2.5\text{E-}14\text{ M}$ for Cd^{2+} , estimated by our model (described in the “multi-metal concentration modeling” section below).

Construction of Gellyfish samplers

The Gellyfish samplers consisted of either IDA-coated beads or blank beads (Toyopearl AF-Chelate-650M and HW 65F; TOSOH Bioscience LLC) embedded in a polyacrylamide gel matrix. However, the surface area-to-thickness ratio was altered substantially the original design described in Senn et al. [25]. To reduce equilibration time, the new design was thinner, with a larger surface area to volume ratio and a polyacrylamide backbone to provide physical support (Gelbond, a $< 0.1\text{ mm}$ film of polyacrylamide on a 0.7 mm thick polyester sheet, cut to a mean diameter of 57 mm ; Lonza). The beads were shipped suspended in 20% methanol, with a reported mean binding site concentration of $28\text{ }\mu\text{mol/mL}$ (suction-dried beads) for the IDA-coated beads. To prepare the Gellyfish, the beads were first rinsed with DI and suction-dried three times in a polycarbonate bottle top filter (Nalgene). An equivalent volume of DI was added to make a 1:1 slurry, yielding an ion-exchange capacity of $14\text{ }\mu\text{mol/mL}$. To make a 5 mL gel batch, 0.375 mL of IDA-coated or blank bead slurry, 2

mL of DI water, 0.75 mL of a patented 2% agarose-derived cross-linker (DGT Research Ltd.) and 1.875 mL of 40% acrylamide gel solution were mixed together, then divided in half into two 2.5 mL aliquots. To make the Gellyfish sampler, 20 μ L ammonium persulphate (10%, newly prepared) and 8 μ L of TEMED (N,N,N',N'-tetramethylethylenediamine, 99%) were added to a 2.5mL gel aliquot. Quickly mixed several times with pipettes, 200 μ L of this mixture was pipetted into each of 10 Gellyfish press molds made of acrylic discs (McMaster-Carr, Santa Fe Springs, CA). A circle-shaped acid-clean Gelbond sheet was placed in each mold in advance, with a polyacetate shim (thickness = 0.1 mm, inner diameter = 57.2 mm, outer diameter = 76.2 mm; McMaster-Carr) on top, and the mixture was pressed in the mold to form a thin layer of gel matrix on the Gelbond. The Gellyfish samplers and blanks produced this way had an unhydrated thickness of approximately 100 μ m and a diameter of about 50 mm (Supplemental Data, Figure S2). They were allowed to polymerize in the molds overnight and hydrate in DI for another 24 hours, during which the DI water was changed three times. At this point, the Gellyfish samplers were ready for use, with an average hydrated volume of 375 μ L. This volume was used to calculate metal concentrations accumulated in the Gellyfish samplers (M-Id). Blank samplers were produced in the same way, except that blank beads without IDA-coating were used.

During each experiment, one binding sampler (with IDA-coated beads) and one blank sampler (with blank beads) were placed back-to-back, sandwiched between two polyethersulfone membrane filters (0.45 μ m pore size, 90 mm diameter; Pall Corporation) to prevent biofouling on the surface of the gel when used in the field, then housed in a specially designed filter holder [26]. The samplers were placed vertically in the solution and on a shaker table at 75rpm for 48 hours, to facilitate diffusion and equilibration.

Equilibration time experiment

Procedures for equilibration time experiments were similar to what was described in Senn et al. [25], but multiple metals were used instead of Cu only. Twelve of the 2.6 L canisters, each containing 2 L of 18 ppt Aquil, were spiked with metal chlorides (M-Cl₂) and Na₃NTA to create a metal mixture containing 1.02E-10 M of Cu²⁺, 1.39E-8 M of Zn²⁺, 8.48E-9 M of Pb²⁺, 4.63E-10 M of Ni²⁺ and 1.51E-8 M of Cd²⁺. MINEQL+ was used to calculate the proper amount of total M-Cl₂ and Na₃NTA required, while taking into account of extra Cl⁻ and Na⁺ concentrations introduced to the Aquil solution.

After the initial mixing of the solutions on the shaker table, triplicates of assembled Gellyfish were inserted into each canister. Samplers were retrieved from the containers at 12 fixed times during an 88-hour experimental period (t=0h, 1h, 3h, 7h, 12h, 18h, 25h, 32h, 40h, 52h, 64h, 88h). Blank-corrected data were then fitted to an empirical curve [25] to determine time needed to achieve 90% equilibrium (t₉₀):

$$C_t = C_{eq} \times (1 - e^{-bt}) \quad (1)$$

where C_t is the average metal concentration in Gellyfish at time t , C_{eq} is the concentration at equilibrium, and b is the variable to be estimated; $t_{90} = -\ln(1-0.9)/b$. This equation was

derived assuming first-order kinetics during the equilibration and assuming a homogenous distribution of IDA ligands within the gel layer.

Calibration of Gellyfish for cationic metal complexes

For modeling purposes, literature values for the equilibrium reaction between free metal ions and IDA were adopted [30, 31]. The uptake of metals in Gellyfish was initially thought to be dominated by the binding of free ions only to IDA [25], but preliminary experiments showed an underestimation of Gellyfish uptake relative to model expectations using only the stability constants of M-IDA in literature [30], indicating that some small, positively-charged metal complexes have indeed bound with IDA and accumulated within the Gellyfish. Because there were no literature values for binding of these cationic complexes to IDA, a series of experiments were undertaken for their estimation. To calibrate the Gellyfish for measuring free metal ions, we estimated the binding stability constant for the predominant cationic metal complex (ML^+) for each metal based on equilibrium thermodynamics.

The metal concentration measured in the Gellyfish, $[M-Gf]$, can be described by the following equation:

$$[M-Gf] = [M-Id] + [M-Id_2] + [ML-Id] \quad (2)$$

in which $M-Id$ and $M-Id_2$ are complexes formed by the binding of free metal ion to IDA, $ML-Id$ is the binding product of small metal complex ML^+ to IDA, in which ML^+ acts like a cation. Here we assumed that under seawater pH and salinity, the predominant ML^+ is MOH^+ for Cu, and MCl^+ for Zn, Pb, Ni, and Cd [31].

At equilibrium, Equation (2) can be written as:

$$[M-Gf] = K_{M-Id} [M^{2+}] [Id_{free}] + K_{M-Id_2} [M^{2+}] [Id_{free}]^2 + K_{ML} [M^{2+}] [L^-] * K_{ML-Id} [Id_{free}] \quad (3)$$

where $[M^{2+}]$ is the free ion concentration in the surrounding solution, $[Id_{free}]$ is the concentration of unbound IDA groups, $[L^-]$ is the total concentration of inorganic ligand L (either OH^- or Cl^- ; here $[L^-]$ was assumed to be approximately equal to $[L_{free}]$, or the change in pH and $[Cl^-]$ was negligible before and after the deployment of Gellyfish), K_{M-Id} , K_{M-Id_2} , K_{ML} , K_{ML-Id} are the apparent stability constants of $M-Id$, $M-Id_2$, ML^+ and $ML-Id$, respectively. Here K_{M-Id} , K_{M-Id_2} and K_{ML} were corrected for ionic strength (Supplemental Data, Appendix) based on literature reported conditional stability constants at $I = 0$ [31], while K_{ML-Id} was to be estimated.

On the other hand, the speciation of IDA is represented as follows:

$$\begin{aligned} [Id_{total}] &= [Id_{free}] + [M-Id] + 2[M-Id_2] + [ML-Id] \\ &= [Id_{free}] + K_{M-Id} [M^{2+}] [Id_{free}] + 2 * K_{M-Id_2} [M^{2+}] [Id_{free}]^2 + K_{ML} [M^{2+}] [L^-] * K_{ML-Id} [Id_{free}] + \sum K_{i-Id} [M_i^{n+}] [Id] \end{aligned}$$

where $[Id_{total}]$ is the total concentration of IDA in the Gellyfish, which is a fixed number of 280 μM based on the Gellyfish recipe; $\sum K_{i-Id} [M_i^{n+}]$ represents the sum of the products of

stability constants and concentrations for the major ions (Mg^{2+} , Na^+ , K^+ , Ca^{2+}) as well as H^+ that can also bind with IDA.

At a given $[\text{M}^{2+}]$, we know $[\text{M-Gf}]$ from measurement, and by combining Equation (3) and (4) we can solve for $K_{\text{ML-Id}}$. Therefore, by fitting a curve at varying $[\text{M}^{2+}]$ and $[\text{M-Gf}]$, we would be able to estimate $K_{\text{ML-Id}}$. For this purpose, a series of increasing free metal ion concentrations was achieved by adding the same amount of Na_3NTA and increasing the amount of total concentration for an individual metal (i.e. Cu, Zn, Pb, Ni or Cd). Modeled free ion concentrations in solution ranged between four to six orders of magnitude, while the majority (> 90%) of the total metals added was controlled by NTA. The curves were fitted using the nls function in RStudio 0.98.978 (R version 3.1.1).

Multi-metal concentration modeling

When multiple metals are present, because the IDA binding sites within the Gellyfish are not metal-specific, there could be significant competition among metals for binding with IDA, which may affect uptake of individual metals into the Gellyfish. To address this issue, we developed an R-based model with the name “GELLYMOD”, which corrects for ionic strength and competition among metals within the Gellyfish itself. In this model, all five metals (i.e. Cu, Zn, Ni, Pb, Cd) were included in equation (4), and stability constants for ML-Id estimated from the calibration experiment above were used (Supplemental Data, Appendix). Given basic Gellyfish characteristics (hydrated volume, bead slurry volume per sampler, ion exchange capacity of beads), salinity and pH at the site, and measured $[\text{M-Gf}]$, the model calculates $[\text{M}^{2+}]$ in the surrounding solution. Inversely, the model can also predict $[\text{M-Gf}]$ based on an input of free ion concentrations: a feature used in our laboratory validation experiments.

pH is a key factor affecting the performance of Gellyfish, since hydrogen ion is also a cation, binding to IDA with a similar or even stronger affinity compared with the metals of interest [30]. At low pHs, the detection limit of Gellyfish may become significantly higher. However, typical marine water pHs (above 7) should not impact Gellyfish measurements, with competition between hydrogen ions and metal ions accounted for by the GELLYMOD.

Multi-metal competition experiments

To test the accuracy of metal competition modeled by GELLYMOD, a series of validation experiments were designed and conducted with two or three metals present in the same solution. For each validation experiment, an array of free ion combinations was generated by keeping free ion concentration of one metal constant while varying that of the competing metal(s), which ranged between two to four orders of magnitude. Total metal and NTA spikes to achieve these combinations were calculated in MINEQL+.

Salinity experiment

Without consideration for the binding of MCl^+ , previous experimental work has shown that the Gellyfish accumulation for Cu was unaffected by a change in salinity from 5 to 35 ppt in the surrounding water [25]. However, this could differ for other metals, especially Cd, which has a higher affinity for chloride. Therefore, we tested the accuracy of our Gellyfish

calibration over salinity for multiple metals, by comparing the measured and modeled [M-Gf] in a multi-metal solution over a range of salinities. In this experiment, the total concentrations of Cu, Zn, Pb, Ni, Cd and NTA were kept constant, while the salinity varied from 5 to 35 ppt. The measured-to-modeled ratio of [M-Gf] was calculated to describe the effect of salinity on Gellyfish recovery.

Reusability of Gellyfish samplers

In previous work, Gellyfish samplers were only used once [25, 26], while their appearance seemed unaltered after acid extraction. To explore the potential reusability of Gellyfish, previously deployed and extracted samplers were “cleaned” and re-deployed as duplicates alongside newly made samplers during equilibration experiments, and the Gellyfish measurements were compared using paired t-tests. The “cleaning” step involved soaking the samplers in 1 M NaCl for at least 24 hours to replace the H⁺-loaded binding sites with Na⁺, and rinsing thoroughly with DI water.

Field deployment

Gellyfish samplers were deployed at two-week intervals from March to December 2005, at five inshore locations (Mystic River (MR), Inner Harbor (IH), Fort Point Channel (FPC), Savin Hill Cove (SHC), and Marina Bay (MB)) in Boston Harbor (Figure 1), an urban estuary with a history of organic and metal contamination primarily sourced from industrial effluents and urban sewage [32]. These sites were chosen based on the distance between them, their proximity to potential contaminant sources, and ease of access.

Triplicates of assembled Gellyfish samplers were mounted within a plastic basket for further protection and deployed at all five sites on the same day, hung by nylon lines in the water to a depth of approximately 1.5 m from the surface. Upon deployment, temperature and salinity were measured in the field using a portable YSI 30 salinometer (YSI Incorporated). All samplers were retrieved after six days and brought back to the laboratory for extraction and chemical analysis. Simultaneously, one seawater grab sample was collected at each site in a 1 L low density polyethylene (LDPE) bottle for subsequent determination of total dissolved (operationally defined as species passing through a 0.45 µm filter) metal concentrations.

Upon retrieval, Gellyfish samplers were transported in sealed plastic bags back to the Trace Metals Laboratory at Harvard School of Public Health. Within one hour, the samplers were removed from their protective housings and transferred to 15 mL centrifuge tubes containing 5 mL of 10% high purity HNO₃. After 24 hours of extraction at minimum, metal concentrations in the extracts were analyzed on an ICP-MS (Elan 6100 DRC; Perkin Elmer) as described earlier. Immediately after the seawater samples were brought back to the laboratory, pH was measured with a pH meter (Corning Inc.). Water samples were then filtered through a 0.45 µm polycarbonate membrane filter using an acid-cleaned polysulfone filtration apparatus (Nunc Nalgene) under less than 5 psi of pressure from ultrapure nitrogen gas. Filtered water samples were acidified using 0.1% concentrated high purity HCl. One blank and one replicate were created for every five samples filtered.

Dissolved trace metals were extracted from all filtered seawater samples using a modified version of EPA method 200.10 [33]. One method blank, one replicate, and one standard reference estuarine water (SLEW-3; National Research Council, Canada) were generated for every five estuarine samples extracted. The SLEW-3 recoveries, detection limits and method blanks are summarized in Supplemental Data, Table S1.

RESULTS AND DISCUSSION

Equilibration time

Equilibration time of the “redesigned” Gellyfish sampler for Cu was significantly shorter than the previous design. According to our experiment, the new design reached 90% equilibrium in one third of time as the original design (Figure 2). By fitting the observed levels of M-Gf over time to Equation (1) (Supplemental Data, Figure S3), 90% equilibration time was calculated for each metal in a multi-metal experiment: 20.9 hours (95% CI 17.7 ~ 25.6 h) for Cu, 3.4 hours (95% CI 2.0 ~ 13.5 h) for Zn, 2.4 hours (95% CI 1.6 ~ 5.5 h) for Pb, 57.6 hours (95% CI 46.1 ~ 76.8 h) for Ni and 17.7 hours (95% CI 15.4 ~ 23.0 h) for Cd. The difference in equilibration time among metals may be explained by binding kinetics. For example, nickel-water dissociation is relatively slow compared to other metals [34, 35] due to its hexacoordinative capability, therefore it takes longer for Ni to achieve equilibrium (Supplemental Data, Figure S3). All subsequent laboratory experiments allowed 48 hours for equilibration, during which Cu, Zn, Pb and Cd equilibrated to greater than 90% of full equilibrium with the Gellyfish, while Ni reached about 85% equilibrium.

Overall, the significantly reduced equilibration time of the Gellyfish is much more useful for both laboratory and in-situ field deployments, making it applicable to the monitoring of free ion concentrations on a daily basis. Moreover, the Gellyfish has the potential to utilize certain physical approaches to further reduce equilibration time, such as sonication proposed by Li et al. [19] for the SAFIR sampler.

Apparent stability constants for cationic complexes

The estimated stability constants for the IDA binding of CuOH^+ , ZnCl^+ , PbCl^+ , NiCl^+ and CdCl^+ , as well as the stability constants for all species used in the model, are shown in Table 1. Supplemental Data, Figure S4 shows the empirically fit curves to determine the Log K values for cationic ML complexes. These estimated logK values ranged from 6.70 (PbCl^+) to 10.98 (CuOH^+), suggesting significant binding of these cationic complexes to IDA. The logK for CuOH^+ can also be derived (around 10.8) from literature reported stability constants (8.5 ± 0.9) [30] for the binding of CuOHIDA to H^+ to form CuIDA . This is slightly lower than our estimate, but still within the calculated 95% confidence interval (95% CI = 10.76 – 11.13). The estimated log K for the other four cationic metal complexes had narrower 95% CIs than Cu, indicating better fits and potentially more accurate estimations (Supplemental Data, Figure S4).

In field measurements, other cationic metal species may also be present, such as ZnOH^+ and PbOH^+ [31], which might lead to uncertainty in our model. However, the effect of these species should be small at a typical seawater pH and salinity. Larger metal complexes may

also enter the gel matrix, such as fulvic or humic acids [24], but it is unlikely that they would bind with IDA without dissociating into free ions, which should not affect the Gellyfish measurement in an infinite solution at equilibrium.

Blank Gellyfish measurements

Our results suggest that blank Gellyfish reflects total metal concentrations in the surrounding solution. Assuming that all metals accumulated in the blank Gellyfish are due to diffusion, and that the gel matrix is not restrictive to dissolved metal species, that is, metals are distributed in the blank Gellyfish just like they are in the same volume of surrounding solution, then the total dissolved metals (M_T) in the solution can be estimated as:

$$M_T \text{ (mol/L)} = \frac{\text{Metals Extracted from Blank Gellyfish (mol/L)} \times \text{Extraction Volume (mL)}}{\text{Hydrated Gellyfish Volume (mL)}} \quad (5)$$

In our calibration experiments, M_T calculated from Equation (5) had a strong correlation with the spiked total metals across tested concentration ranges and the relationship was close to 1:1 for nearly all metals (Supplemental Data, Figure S5). This shows the potential of Gellyfish to determine total metal concentrations and free metal ions at the same time. The detection limit might be too high (~ 100 nM) for some field samples, although the extraction volume may be reduced from 20 mL to 5 mL to lower the current detection limit.

Multiple metal competition experiments

We first considered the effect of Zn ions on Cu binding to the Gellyfish, because both are important and ubiquitous trace metals in the marine environment, and because Cu^{2+} usually exists in very low concentrations due to its strong affinity for natural ligands [36], while Zn is more abundant in aquatic systems and may compete markedly with Cu for uptake into the Gellyfish. In this competition experiment, we kept Cu^{2+} at a constant level of $7.5\text{E-}11$ M, in a series of solutions with increasing Zn^{2+} concentrations. The GELLYMOD estimated [Cu-Gf] matched well with the measured levels (Figure 3A), especially at higher Zn^{2+} concentrations, where our model predicted a 71% decrease in Cu binding from baseline. This indicates that GELLYMOD was able to capture the competition between Cu and Zn accurately, and that our calibration of the Gellyfish was valid.

Secondly, we extended the competition experiment to two other metals, Ni and Pb. Although we observed a slight deviation between measurements and model predictions and larger variations among duplicates, mainly due to lower concentrations of both metals, the decreasing trend in Pb-Id over increasing Ni^{2+} could again be adequately modeled (Figure 3B).

Finally, we tested the Gellyfish samplers in more complex metal mixtures with three metals, Cu, Zn and Pb. This experiment was designed such that six of the containers were spiked with increasing Cu^{2+} concentrations while free Zn and Pb levels were kept constant, and the other six solutions contained the same concentration series of Cu^{2+} and Pb^{2+} , but all with a higher Zn^{2+} level. The results (Figure 4) supported the validity of our calibration to include small metal complexes, that is, GELLYMOD was able to accurately predict the measured

metals in the Gellyfish, demonstrating its ability to account for metal competition for binding with IDA at environmentally relevant concentrations. Therefore, in a field deployment, M^{2+} of multiple metals in aqueous solution can be estimated simultaneously based on the knowledge of measured [M-Gf].

Salinity experiment

With regard to salinity, the Gellyfish calibration accounting for cationic metal complexes worked to a different extent among the five metals of interest (Figure 5). Consistent with our previous findings [25], even without considering the effect of $CuCl^+$, the measured-to-modeled ratio of [Cu-Gf] stayed close to 1 in the entire salinity range of 5-35 ppt. A similar result was found for Cd, suggesting that our calibration for the binding of $CdCl^+$ was successful. For Pb, Zn, and Ni, the calibration on chloride species worked well at a salinity of 15 ppt or above, while the ratio increased significantly below 10 ppt. At a salinity of 5 ppt, the GELLYMOD underestimated the uptake of Pb by a factor of 2.5 and that of Zn and Ni by a factor of 5. This might be due to negative charges introduced by the polymerization products within the polyacrylamide gel, which may cause additional uptake of cations at lower ionic strengths [37]. Nevertheless, the calibration appeared to be valid within the salinity range (>10 ppt) of typical environmental samples in seawater for all five metals.

Reusability

Our results indicate that the Gellyfish samplers could be reused at least twice. After the first use, the metal concentration in the Gellyfish was 3.9% higher on average, but the difference was not statistically significant ($p=0.42$, Supplemental Data, Figure S6). The equilibration time for the reused Gellyfish was 25% shorter than the newly made ones, perhaps because it was much easier for metal ions to displace Na^+ ($\log K_{IDA}=0.36$ at $20^\circ C$) than H^+ ($\log K_{IDA}=9.34$ at $25^\circ C$) [30]. A similar comparison between second-time use and third-time use in another experiment yielded a p -value of 0.69. Beyond three usages, they might still be able to produce valid data, although potential wear on the gel matrix could alter the Gellyfish volume and cause inaccuracies in model predictions. Compared with other in-situ samplers, the Gellyfish is inexpensive (with a consumable material cost of about 10 USD for each data point, including the Gelbond, membrane filters, gel matrix and beads), and its reusability further reduces the cost of both material and labor, extending its applicability to the developing world as well.

Field data

Gellyfish samplers were able to capture interesting temporal and spatial patterns in free metal ion concentrations in Boston Harbor (Figure 6), generating a dataset with unprecedented size and dimension. All five metals showed a small peak around late September, corresponding to the time of increasing sediment ventilation and bioturbation in the harbor [38], when dissolved metals were released from the contaminated sediment through diffusion and resuspension, although this peak was less discernible at MR. In addition, strong correlations may exist among metals, for example, Cu^{2+} and Zn^{2+} appeared to fluctuate in a similar temporal trend, especially at MB, where both metals were elevated from June to July. This could be due to increased boating activities at this site in the summer: of the Zn- or Cu-based anti-fouling paints widely-used on boat hulls, a substantial

percentage of metals may leach into the water as free ions [39]. Our ongoing work involves statistical analysis of this dataset, including additional biogeochemical parameters such as total metals, pH, salinity, temperature and DOC (Supplemental Data, Figure S7 & S8.), to further explain the temporal and spatial variability observed in free ion concentrations.

Estimated total metals from the blanks were comparable to the measured total dissolved metals on average (for example, average estimated total Cu from blanks was 27.0 nM, while the measured total Cu had a average of 22.4 nM), although no significant correlation was found between them. This is probably because metals in blank Gellyfish were mostly below detection limits (around 100 nM for Cu).

While free ion data for Zn, Pb, Ni and Cd in estuarine waters were rarely reported before, our free Cu results were comparable to values measured by CLE-ACSV (pCu ranged from 9.55 to 11.46) in Cape Cod, MA [40]. In addition, our free Cu data were close to the SAFIR measurement, another equilibrium-based free Cu sampler developed by Li et al. [19], and both were slightly higher than CLE-CSV results (measured along with SAFIR), while the dissolved Cu concentrations were comparable. Since our measurements were conducted in a different year (2005) from Li et al. [19] (2009-2010), yearly variation in biogeochemical factors such as total dissolved Cu levels, salinity (Table 2), pH and DOC may all contribute to the observed difference between our data and SAFIR. For example, the DOC concentration at this site in late September of 2005 was 1.5 mg/L (samples collected by us and measured by the department of Environmental, Coastal and Ocean Sciences at the University of Massachusetts, Boston, using the high temperature combustion method 5310 B), while the level was 1.9 mg/L around the same time in 2009 (unpublished data from the same lab at UMass Boston).

CONCLUSIONS

The new Gellyfish sampler, with significantly reduced equilibration time and the capacity to account for competition among metals for binding sites via the use of GELLYMOD, was shown to be a valid tool for simultaneous determination of multiple free metal ion concentrations, both in the lab and in the field. Compared to other commonly-used free ion sampling techniques, it is an easy-to-use and inexpensive equilibrium-based passive sampler that can be useful for large-scale monitoring of metal speciation in marine systems. Its capacity to generate multi-metal free ion datasets may for the first time provide opportunities for complex statistical analysis of metal speciation over space and time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work was funded by Grant P42 ES05947 and ES000002 from the National Institute of Environmental Health Sciences (NIEHS). The contents of the present paper are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS. Thanks to D. Senn and S. Griscom for technical guidance and assistance in general, to N. Lupoli for technical assistance on the ICP-MS analyses, and to L. Schaidler and D. Vasudevan for proofreading the manuscript. We also thank R. Chen and his students for collecting and measuring the DOC data.

REFERENCES

1. Chapman PM, Wang FY, Janssen C, Persoone G, Allen HE. Ecotoxicology of metals in aquatic sediments: binding and release, bioavailability, risk assessment, and remediation. *Can J Fish Aquat Sci.* 1998; 55:2221–2243.
2. Florence TM. The speciation of trace-elements in waters. *Talanta.* 1982; 29:345–364. [PubMed: 18963140]
3. Batley GE, Apte SC, Stauber JL. Speciation and bioavailability of trace metals in water: Progress since 1982. *Australian Journal of Chemistry.* 2004; 57:903–919.
4. Sunda WG, Huntsman SA. Processes regulating cellular metal accumulation and physiological effects: phytoplankton as model systems. *Science of the Total Environment.* 1998; 219:165–181.
5. U.S.EPA. Aquatic life ambient freshwater quality criteria - copper. EPA 822-R-07-001. U.S. Environmental Protection Agency; Washington D.C.: 2007.
6. Niyogi S, Wood CM. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. *Environmental Science & Technology.* 2004; 38:6177–6192. [PubMed: 15597870]
7. Batley GE, Florence TM. Evaluation and comparison of some techniques of anodic-stripping voltammetry. *Journal of Electroanalytical Chemistry.* 1974; 55:23–43.
8. Skoog, DA.; Holler, FJ.; Nieman, TA. Principles of Instrumental Analysis. 5th ed. Saunders College Pub.; Philadelphia: 1998.
9. Vandenberg CMG. Potentials and potentialities of cathodic stripping voltammetry of trace-elements in natural-waters. *Analytica Chimica Acta.* 1991; 250:265–276.
10. Bakker E, Buhlmann P, Pretsch E. Carrier-based ion-selective electrodes and bulk optodes. 1. General characteristics. *Chemical Reviews.* 1997; 97:3083–3132. [PubMed: 11851486]
11. Zhang H, Davison W. Performance-characteristics of diffusion gradients in thin-films for the in-situ measurement of trace-metals in aqueous-solution. *Analytical Chemistry.* 1995; 67:3391–3400.
12. Zhang H, Davison W, Miller S, Tych W. In-situ high-resolution measurements of fluxes of Ni, Cu, Fe, and Mn and concentrations of Zn and Cd in porewaters by DGT. *Geochimica Et Cosmochimica Acta.* 1995; 59:4181–4192.
13. Warnken KW, Davison W, Zhang H. Interpretation of in situ speciation measurements of inorganic and organically complexed trace metals in freshwater by DGT. *Environmental Science & Technology.* 2008; 42:6903–6909. [PubMed: 18853807]
14. Fitch A, Helmke PA. Donnan equilibrium graphite-furnace atomic-absorption estimates of soil extract complexation capacities. *Analytical Chemistry.* 1989; 61:1295–1298.
15. Kalis EJJ, Weng LP, Dousma F, Temminghoff EJM, Van Riemsdijk WH. Measuring free metal ion concentrations in situ in natural waters using the Donnan Membrane Technique. *Environmental Science & Technology.* 2006; 40:955–961. [PubMed: 16509342]
16. Romero R, Jonsson JA. Determination of free copper concentrations in natural waters by using supported liquid membrane extraction under equilibrium conditions. *Analytical and Bioanalytical Chemistry.* 2005; 381:1452–1459. [PubMed: 15759138]
17. Romero R, Liu JF, Mayer P, Jonsson JA. Equilibrium sampling through membranes of freely dissolved copper concentrations with selective hollow fiber membranes and the spectrophotometric detection of a metal stripping agent. *Analytical Chemistry.* 2005; 77:7605–7611. [PubMed: 16316167]
18. Ndungu K, Hurst MP, Bruland KW. Comparison of copper speciation in estuarine water measured using analytical voltammetry and supported liquid membrane techniques. *Environmental Science & Technology.* 2005; 39:3166–3175. [PubMed: 15926567]
19. Li L, Pala F, Haskins J, Sukola K, Wallace GT. Measurement of free Cu ion activity in seawater using a passive-equilibrium sonic-assisted free ion recorder (SAFIR). *Environmental Science & Technology.* 2011; 45:5660–5667. [PubMed: 21630694]
20. Rivera-Duarte I, Zirino A. Response of the Cu(II) ion selective electrode to Cu titration in artificial and natural shore seawater and in the measurement of the Cu complexation capacity. *Environmental Science & Technology.* 2004; 38:3139–3147. [PubMed: 15224747]

21. Zirino A, VanderWeele DA, Belli SL, DeMarco R, Mackey DJ. Direct measurement of Cu(II)(aq) in seawater at pH 8 with the jalpaite ion-selective electrode. *Mar Chem.* 1998; 61:173–184.
22. Cooper CA, Tait T, Gray H, Cimprich G, Santore RC, McGeer JC, Wood CM, Smith DS. Influence of Salinity and Dissolved Organic Carbon on Acute Cu Toxicity to the Rotifer *Brachionus plicatilis*. *Environmental Science & Technology.* 2014; 48:1213–1221. [PubMed: 24380586]
23. Van Der Veen PLR, Pinheiro JP, Van Leeuwen HP. Metal speciation by DGT/DET in colloidal complex systems. *Environmental Science & Technology.* 2008; 42:8835–8840. [PubMed: 19192806]
24. Zhang H, Davison W. Diffusional characteristics of hydrogels used in DGT and DET techniques. *Analytica Chimica Acta.* 1999; 398:329–340.
25. Senn DB, Griscom SB, Lewis CG, Galvin JP, Chang MW, Shine JP. Equilibrium-based sampler for determining Cu²⁺ concentrations in aquatic ecosystems. *Environmental Science & Technology.* 2004; 38:3381–3386. [PubMed: 15260338]
26. Lewis, CG. Sc.D. Thesis. Harvard School of Public Health; Boston: 2006. Field deployable equilibrium-based sampler for the measurement of free ion copper in aquatic ecosystems.
27. Lohmann R. Critical review of low-density polyethylene's partitioning and diffusion coefficients for trace organic contaminants and implications for its use as a passive sampler. *Environmental Science & Technology.* 2012; 46:606–618. [PubMed: 22122117]
28. Galvin, JP. Sc.D. Thesis. Harvard School of Public Health; Boston: 2005. Use of sensors and indicator approaches to assess the health of the marine environment.
29. Price NM, Harrison GI, Hudson RJ, Nirel PM, Palenik B, Morel FMM. Preparation and chemistry of the artificial algal culture medium Aquil. *Biological Oceanography.* 1989; 6:443–461.
30. Martell, AE.; Smith, RN. Critical Stability Constants. Plenum Press; New York: 1974.
31. Morel, FMM.; Hering, JG. Principles and Applications of Aquatic Chemistry. Wiley-Interscience; New York: 1993.
32. Bothner MH, ten Brink MB, Manheim FT. Metal concentrations in surface sediments of Boston Harbor - Changes with time. *Mar Environ Res.* 1998; 45:127–155.
33. Long, SE.; Martin, TD.; Creed, JT. EPA 200.10. Cincinnati, Ohio: 1997. Determination of Trace Elements in Marine Waters by On-Line Chelation Preconcentration and Inductively Coupled Plasma - Mass Spectrometry.
34. Xue HB, Jansen S, Prasch A, Sigg L. Nickel speciation and complexation kinetics in freshwater by ligand exchange and DPCSV. *Environmental Science & Technology.* 2001; 35:539–546. [PubMed: 11351726]
35. Stumm, W.; Morgan, JJ. Aquatic Chemistry: Chemical Equilibria and Rates in Natural Waters. 3rd ed. John Wiley & Sons, Inc.; New York: 1996.
36. Donat JR, Lao KA, Bruland KW. Speciation of dissolved copper and nickel in south San-Francisco Bay - a multi-method approach. *Analytica Chimica Acta.* 1994; 284:547–571.
37. Warnken KW, Zhang H, Davison W. Trace metal measurements in low ionic strength synthetic solutions by diffusive gradients in thin films. *Analytical Chemistry.* 2005; 77:5440–5446. [PubMed: 16131050]
38. Morford JL, Martin WR, Kalnejais LH, Francois R, Bothner M, Karle IM. Insights on geochemical cycling of U, Re and Mo from seasonal sampling in Boston Harbor, Massachusetts, USA. *Geochimica Et Cosmochimica Acta.* 2007; 71:895–917.
39. Sandberg J, Wallinder IO, Leygraf C, Virta M. Release and chemical speciation of copper from anti-fouling paints with different active copper compounds in artificial seawater. *Mater Corros.* 2007; 58:165–172.
40. Twiss MR, Moffett JW. Comparison of copper speciation in coastal marine waters measured using analytical voltammetry and diffusion gradient in thin-film techniques. *Environmental Science & Technology.* 2002; 36:1061–1068. [PubMed: 11917992]

Figure 1.

Map of the five sampling locations in Boston Harbor: 1. Mystic River (MR); 2. Inner Harbor (IH); 3. Fort Point Channel (FPC); 4. Savin Hill Cove (SHC); 5. Marina Bay (MB).

Figure 2.

Equilibration curves of the Gellyfish with Cu for the new and original design, in percentages of the equilibrium concentration (i.e. C_{eq} in the empirical curve $C_t=C_{eq} \times (1-e^{-bt})$). Solid diamonds indicate average Cu-Id concentrations over time measured by the new design, open squares indicate those measured by the original design, based on Lewis [26]. Lines are empirical curves fit to the data, following the shown equations. Error bars represent standard deviations of duplicates. Both experiments were performed under a salinity of 18 ppt, at a Cu^{2+} concentration of $1.0\text{E}-10$ M.

Figure 3.

(A) The effect of Zn on the binding of Cu with Gellyfish. Dots represent Cu accumulated in Gellyfish, with increasing Zn²⁺ concentrations and constant Cu²⁺ concentration at 7.5E-11 M; (B) The effect of Ni on the binding of Pb with Gellyfish. Dots are Pb accumulated in Gellyfish, following increasing Ni²⁺ concentrations and constant Pb²⁺ concentration at 1.0E-8 M. Lines indicate levels predicted by GELLYMOD. Error bars represent standard deviations of duplicates.

Figure 4.

Measured Cu, Zn and Pb concentrations within the Gellyfish compared to M-Gf levels predicted by the GELLYMOD, plotted against a 1:1 line. Pb^{2+} was kept constant at $2.4\text{E-}8$ M throughout the experiment with six different Cu^{2+} concentrations rising from 10^{-12} M to 10^{-8} M, at two Zn^{2+} concentrations of $4.1\text{E-}10$ M and $4.6\text{E-}8$ M controlled by Na_3NTA .

Figure 5.

Ratio of measured-to-modeled metal uptake by Gellyfish over salinity. All solutions were spiked with the same metal and NTA levels: $2.6\text{E-}7$ M of total Cu, $3.0\text{E-}6$ M of total Zn, $6.0\text{E-}7$ M of total Pb, $5.0\text{E-}7$ M of total Ni, $2.0\text{E-}8$ M of total Cd, and $1.0\text{E-}4$ M of total NTA. Error bars represent standard deviation of triplicates.

Figure 6.

Temporal profiles of free metal ions (in pM^{2+} , or negative log of molar concentration) at the five sampling locations in Boston Harbor for Cu, Zn, Pb, Ni and Cd. Error bars represent standard deviations of triplicate Gellyfish measurements.

Table 1

Literature [31] reported conditional stability constants (logK) for the species considered in the GELLYMOD (I = 0 M, T=25°C), and our estimated conditional stability constants (estimated at I = 0.1 M) for the binding predominant cationic metal complexes to iminodiacetic acid (IDA), with 95% confidence intervals (CI) shown in parentheses.

Cation	Species	logK (95% CI)
H ⁺	HIDA	9.73
	H ₂ IDA	12.63
	H ₃ IDA	14.51
Na ⁺	NaIDA	0.8
Mg ²⁺	MgIDA	3.8
Ca ²⁺	CaIDA	3.5
Cu ²⁺	CuIDA	11.5
	CuIDA ₂	17.6
	CuOH	6.3
	CuOHIDA	10.98 (10.76, 11.13)
Zn ²⁺	ZnIDA	8.2
	ZnIDA ₂	13.5
	ZnCl	0.4
	ZnClIDA	9.13 (8.96, 9.25)
Pb ²⁺	PbIDA	8.3
	PbCl	1.6
	PbClIDA	6.70 (6.60, 6.78)
Ni ²⁺	NiIDA	9.1
	NiIDA ₂	15.7
	NiCl	0.6
	NiClIDA	9.94 (9.90, 9.98)
Cd ²⁺	CdIDA	6.6
	CdIDA ₂	11.1
	CdCl	2.0
	CdClIDA	7.24 (7.22, 7.25)

Table 2

A comparison of free Cu (mean $\pm$ sd) measured by Gellyfish, SAFIR and CLE-CSV [19], at the same location (Savin Hill Cove) and months. Salinities and total dissolved Cu (Cu_{diss}) concentrations are also included.

Month ^a	Gellyfish			SAFIR		CLE-CSV	
	Salinity (ppt)	pCu ²⁺ (M)	Cudiss (nM)	Salinity (ppt)	pCu ²⁺ (M)	Cudiss (nM)	pCu ²⁺ (M)
Apr	13.4	12.1 $\pm$ 0.4	12.0	29.3	12.3 $\pm$ 0.2	25.3	12.8
Aug	29.2	12.0 $\pm$ 0.04	25.8	28.0	12.4 $\pm$ 0.2	18.3	12.8
Sep	30.4	12.0 $\pm$ 0.01	12.6	30.3	11.7 $\pm$ 0.2	12.1	12.5
Nov	24.4	12.0 $\pm$ 0.03	11.8	30.0	12.3 $\pm$ 0.1	16.3	12.7

^a All Gellyfish measurements were performed in 2005; SAFIR and CLE-CSV measurements were performed in Aug – Nov, 2009 and April, 2010.