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A Systematic Analysis of Metal and Metalloid Concentrations in Eight Zebrafish Recirculating Water Systems

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Abstract

Metals and metalloids are integral to biological processes and play key roles in physiology and metabolism. Nonetheless, overexposure to some metals or lack of others can lead to serious health consequences. In this study, eight zebrafish facilities collaborated to generate a multielement analysis of their centralized recirculating water systems. We report a first set of average concentrations for 46 elements detected in zebrafish facilities. Our results help to establish an initial baseline for trouble-shooting purposes, and in general for safe ranges of metal concentrations in recirculating water systems, supporting reproducible scientific research outcomes with zebrafish.

Keywords: aquatic models, animal welfare, environment, metal requirement, metal toxicity, fish nutrition, reproducibility, standards, water quality, zebrafish husbandry

Introduction

The earth's crust is composed of ~92 elements,[1] 16 of which are essential for living organisms, 9 of which are metals or metalloids: boron (B), calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), potassium (K), sodium (Na), and zinc (Zn).[2] With respect to nutrition, availability and required concentrations for physiological and biological processes are used to distinguish and define these elements as macronutrients (Ca, Mg, Cl, K, and Na) or micronutrients (cobalt [Co], Cu, Fe, iodine [I], Mn, molybdenum [Mo], selenium [Se], and Zn).[3,4] The remaining elements have either no known biological function or are classified as nonessential because of their well-known toxicity or lack of evidence for roles in biological functions.[2,5]

Metal uptake in fish is more diversified than in other species, and various elements are absorbed and passed into the bloodstream through gills (waterborne),[4,6,7] intestines (foodborne),[4,7] and skin (passive diffusion).[4,8,9] Furthermore, specific cell-membrane transport mechanisms transfer these elements into cells to make them available as essential components for biological processes or simply to accumulate them in specific organs such as liver, gills, or kidney.[10–12] Portions of these metals are excreted into the water, mainly through the gills and via the liver (bile) and kidney (urine).[4]

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Biological problems arise when the concentrations of micro- and macronutrients are insufficient or exceed their required minimal or maximal physiological range. Elemental deficiencies usually lead to the slowing or the breakdown of physiological processes, causing various disease symptoms.[13,14] Conversely, high concentrations can also lead to severe symptoms and disorders in reproduction, growth and development,[13,15–17] metabolism,[10,18,19] and behavior.[20–22] The toxicity of particular metals is typically defined by their concentration of free ions in water, which is highly dependent on environmental parameters such as water hardness, conductivity, temperature,[23–26] and pH.[27,28]

Environmental agencies such as the Environmental Protection Agency (EPA, USA) and the Australia, New Zealand Environment, and Conservation Council (ANZECC) developed guidelines for freshwater and saltwater levels for various metals (Table 1). However, innumerable organic and inorganic pollutants still lack reported toxicity thresholds for different aquatic organisms. In addition, many countries have not established their own water criteria guidelines for aquatic life[29] and rely on EPA or ANZECC standards.

Zebrafish (*Danio rerio*), a well-established model organism in biomedical sciences, is an example for which there is a significant lack of information about acceptable concentrations and ranges of metals for animal well-being, health, husbandry, and breeding performance.[13,15,30–34] Centralized recirculating water systems (CRWS) are widely used for zebrafish husbandry and colony maintenance, because they offer control of water chemistry parameters and concentrations of elements in the water. CRWS typically have three key components: the water source, the recirculating water filtration system (RWFS), and the aquatic housing system.

There are typically three main sources of water: groundwater, surface water, and municipal water. These sources can differ seasonally in their quality by carrying varying levels of risks for water contamination. However, the proximity to the source, the availability of sufficient water, and economics of the institution also play key roles.[35–37] A majority of zebrafish facilities rely on municipal water because they are located in urban areas. However, municipal water may also carry different types of pollutants, depending on the economic and geographic area. Thus, there exists intra- and interfacility variability for the quality of source water that needs to be controlled.[35–37]

To this end, facilities eliminate pollutants in the municipal water by reverse osmosis water filtration systems (ROWS), which can significantly reduce, but not completely eliminate, the relative amount of total dissolved solids and pollutants. Freshwater conditions are reconstituted by adding commercially available synthetic sea salts, or laboratory-formulated salts and buffering agents such as sodium bicarbonate or calcium carbonate. While ROWS provide source water with reproducible quality, their main disadvantages are the high cost of maintenance and the amount of discharge water they generate.

Table 1. Aquatic Water Criteria for Freshwater Species from the Environmental Protection Agency (USA) and the Australia and New Zealand Environment and Conservation Council

	EPA		*Australia/New Zealand*
Elements	*Freshwater CMC (μg L^{-1})*	*Freshwater CCC (μg L^{-1})*	*Concentration/level of protection (80%–99% species) (μg L^{-1})*
Aluminum (pH >6.5)	—	—	27–150
Arsenic	340	150	1–360 (AsIII); 0.8–140 (AsV)
Barium	—	—	—
Beryllium	—	—	—
Boron	—	—	90–1300
Cadmium	1.80	0.72	0.06–0.8
Cesium	—	—	—
Chromium	570 (CrIII); 16 (CrVI)	74 (CrIII); 11 (CrVI)	0.01–40 (CrVI)
Cobalt	—	—	—
Copper	—	—	1–2.5
Gallium	—	—	—
Iron	—	1000	—
Lead	82	3.20	1–9.4
Manganese	—	—	1200–3600
Mercury	1.4	0.77	0.06–5.4
Molybdenum	—	—	—
Nickel	470	52	8–17
Rubidium	—	—	—
Selenium	—	—	5–34
Silver	3.2		—
Strontium	—	—	—
Thallium	—	—	—
Thorium	—	—	—
Vanadium	—	—	—
Zinc	120	120	2.4–31

Maximal water concentrations for metals and metalloids according to the aquatic water criteria for freshwater species of EPA and ANZECC agencies. Hyphen: lack of information. The trigger values from EPA and ANZECC come from multiple-species toxicity tests and are not specific to zebrafish. *Sources:* Refs.[76,77]

ANZECC, Australia and New Zealand Environment and Conservation Council; CCC, Criterion Continuous Concentration; CMC, Criterion Maximum Concentration; EPA, Environmental Protection Agency.

RWFS are composed of a mechanical filter that removes coarse detritus and debris from food residue and feces that are generated in aquaria. A biological filter (containing sand, ceramic, or plastic beads as filter material) harbors nitrifying bacteria that oxidize ammonia and convert it into less toxic nitrites and nitrates. An aeration system or a trickle column provides gas exchange and establishes atmospheric levels of oxygen in water. Depending on the filter materials used in the mechanical and biological filters, fine sediment filters (FSFs) (containing zeolite or similar minerals) can be used to trap flocculants that would otherwise reduce the efficacy of UV water sterilizer units by shielding microorganisms from UV exposure. UV sterilizers are typically last in line before water flows back to the aquatic housing systems. These differ by manufacturers but are typically made of glass or polycarbonate. Pumps are required to move water between the filter components and the fish tanks. Various probes are placed in the water to monitor flow or quality, and water-heating systems are needed to establish species-specific water temperatures. Water recirculates constantly and a portion of it, typically 10%–20% every day, is exchanged with fresh, reconstituted source water.[36–40]

With this study, we aim to determine the ranges of metal and metalloid concentrations for zebrafish in seven institutions: (1) the Champalimaud Center for the Unknown (CCU, Lisbon, Portugal), (2) the Centro Nacional de Investigaciones Cardiovasculares (CNIC, Madrid, Spain), (3) the European Zebrafish Resource Center (EZRC, KIT, Eggenstein-Leopoldshafen, Germany), (4) two CRWS in the Institute of Anatomy (IA) at the University of Bern (IA.1 and IA.2, Bern, Switzerland), (5) the Karolinska Institutet (KI, Stockholm, Sweden), (6) the Norwegian University of Life Sciences (NULS, Oslo, Norway), and (7) the Zebrafish International Resource Center (ZIRC, Eugene, USA). We analyzed 45 elements using inductively coupled plasma mass spectrometry (ICP-MS) and Ca using atomic absorbance spectrometry (AAS). Our evaluation of trace metals in different systems is unprecedented for zebrafish facilities and sheds light on some sources of elements in the water. Our findings help to establish a baseline and ranges for zebrafish water composition, provide strategies to troubleshoot problems with water quality, and consequently support reproducible animal welfare and husbandry conditions for zebrafish and other aquatic models.

Materials and Methods

Elemental analysis

Mercury analysis. Triplicates of fish water samples from the IA.1 and IA.2 were collected for mercury (Hg) analysis in 10 mL glass tubes. As diluents, concentrated acids were added for a final concentration of 1% nitric acid (HNO_3, 70%; #438073; Sigma) and 0.5% hydrochloric acid (HCl, 37%; #320331-500ML; Sigma). The samples were treated with HNO_3 and HCl as a diluent to keep the elements in

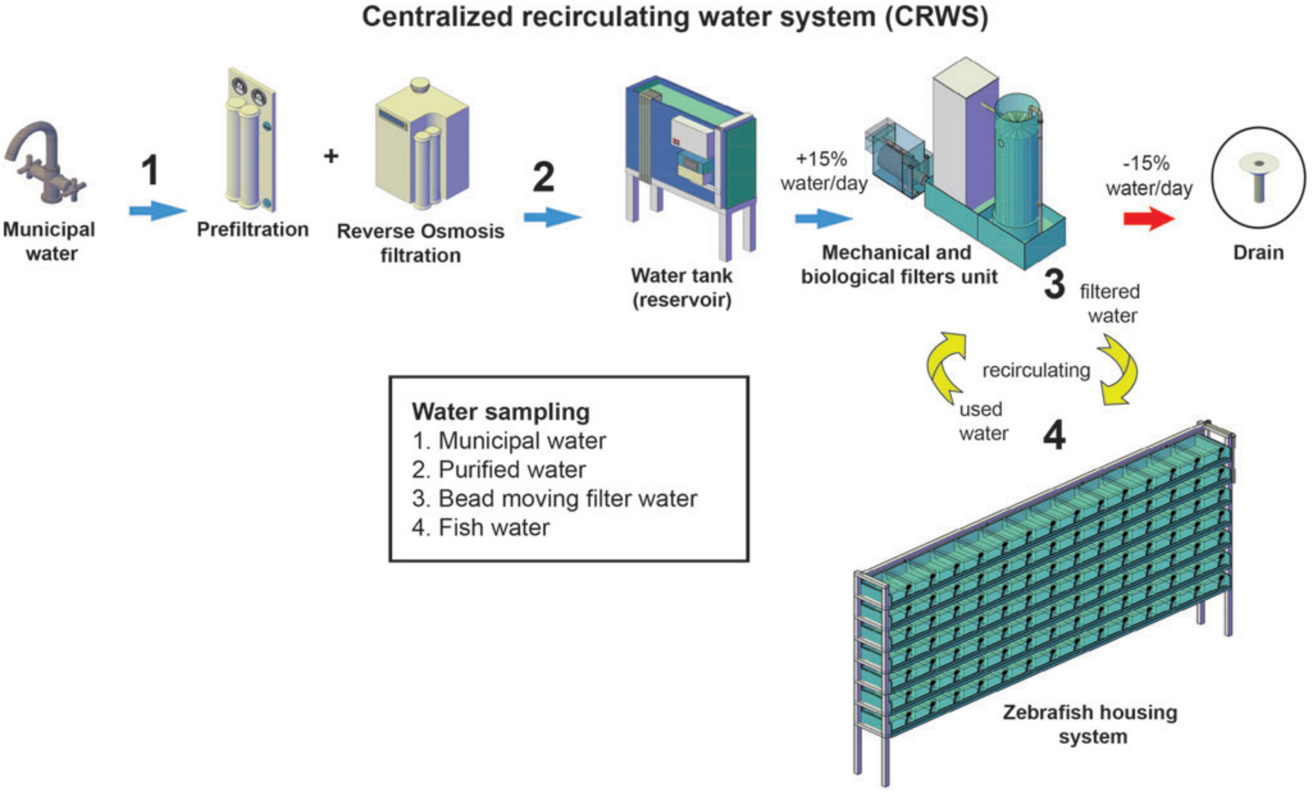


FIG. 1. Schematic of a centralized, recirculating water system. Shown is a scheme of the CRWS, at the IA from Zebcare (IA.1 according to the nomenclature used in this article). *Blue arrows* represent direction of water flow from the municipal water source (1), through a prefilter unit and ROWS, to the reservoir or water tank (2). *Yellow arrows* indicate the recirculating, filtered system water between RWFS (3) and fish housing system (4). The *red arrow* indicates daily water removal to drain (15% of the total facility water volume). CRWS, centralized recirculating water systems; IA, Institute of Anatomy; ROWS, reverse osmosis water filtration systems; RWFS, recirculating water filtration system. Color images are available online.

Table 2. Water Quality Parameters, Added Compounds, and Technical Specifications from Eight Fish Facilities

Centralized recirculating water systems

Institution	*CCU*	*CNIC*	*EZRC*	*IA.1*	*IA.2*	*KI*	*NULS*	*ZIRC*
Water quality								
Temperature	28–28.5	28–28.5	27–27.5	28–28.5	28–28.5	28–28.5	28–28.5	28–28.5
Conductivity (μS/cm)	1200–1250	450–500	220	600–700	500–600	750–800	400–500	450–550
pH	7.4–7.6	7.4–7.6	7–7.2	7.4–7.6	7.4–7.6	6.8–7.2	7.5–7.7	7.5–7.6
External compounds added in the CRWS								
Main diet	Sparos Zebrafeed 400–600 μm	Gemma Micron 500	Caviar and TetraMin flakes	Gemma Micron 500	Gemma Micron 300	Sparos Zebrafeed 400–600 μm	SDS400	Master mix
Live food	A and R	A	A	A	A	R	A	A and R
Synthetic sea salt	Sal basic plus	Sera marin	—	Reef crystals, aquatic systems	Instant ocean, aquatic systems	Tetra marine	Instant ocean, aquatic systems	Instant ocean, aquatic systems
Others	SB	SB	—	SB	SB	SB	SB	Ar and Zeo
Technical specifications								
Brand CRWS	Tecniplast	Aquaneering	Aquaschwartz	Zebcare	Tecniplast	Tecniplast	Aquatic habitats	Aquaneering
Year of construction	2011	2010	2003	2015	2010	2018	2008	1999
Percentage of water change per day	10–15	10–15	10–15	15–20	10–15	10–15	10–15	10–15
Water source	MW	MW	GW (1/3) +MW (2/3)	MW	MW	MW	MW	MW
Water treatment; brand; model	ROWS; Merck Millipore; RiOS200	ROWS; Cuno; HP-WM-900	—	ROWS; Merck Millipore; RiOS16	ROWS; Merck Millipore; RiOS16	ROWS; E2 GE Osmonics	ROWS; E2 GE Osmonics	ROWS; Cuno; HP-WM-900
Type of filtration of water	Solids removal, chemical and biological	Solids removal, chemical and biological	Solids removal and biological	Mechanical and biological	Solids removal, chemical and biological	Solids removal, chemical and biological	Solids removal and biological	Solids removal, chemical and biological

Institutions: CCU, Champalimaud Center for the Unknown (Lisbon, Portugal); CNIC, Centro Nacional de Investigaciones Cardiovasculares (Madrid, Spain); EZRC, European Zebrafish Resource Center (Eggenstein-Leopoldshafen, Germany); IA.1 and IA.2, Institute of Anatomy (Bern, Switzerland); KI, Karolinska Institutet (Stockholm, Sweden); NULS, Norwegian University of Life Sciences (Oslo, Norway); ZIRC, Zebrafish International Resource Center (Eugene, USA). *Main diet:* Master mix (Zeigler, O.S.I and Golden Pearl). *Live food:* A, artemia; R, rotifer. *Others:* Ar, aragonite; SB, sodium bicarbonate; Zeo, zeolite. *Water source and water treatment:* GW, groundwater; MW, municipal water; ROWS, reverse osmotic water filtration system. *Water filtration:* solids removal (filter pads, fine sediment filter, drumfilter, or cartridge filters); chemical (activated carbon); biological (bead moving filter or propeller wash bead filter). CRWS, centralized recirculating water system.

suspension until they were analyzed in the ICP-MS. Then, Hg samples were filtered with 450 nm filters (#721-1345; Thermo Fisher). The instrument was calibrated with known Hg concentrations (0.05–2 μg L^{-1}; #28941-100ML-F; Sigma). Hg analysis was performed with a 7700x ICP-MS (Agilent Technologies, Santa Clara, USA). The ICP-MS was rinsed with three different chemical mixtures between each sample to prevent any carryover of Hg between samples.[41]

Ca analysis. Triplicates of fish water samples from three participating facilities (CNIC, IA.1, and ZIRC) were collected for Ca analysis in 50 mL Falcon tubes, concentrated HNO_3 (70%; #438073; Sigma) was added for a final concentration of 1% HNO_3 as diluent, and 50 μL of 2 g/L cesium chloride solution (#51869-250 mL; Sigma) was added as ionization buffer in all samples and standards. The instrument was calibrated with a standard solution of known Ca concentrations (0–10 mg L^{-1}; #1.19778.0500; Merck). Ca analysis was performed with a ZEEnit 700 P AAS (Analytic Jena AG, Jena, Germany). After every tenth sample, two of the standards (2.5 and 8 mg L^{-1}) were measured again to assess instrument stability and avoid drift.

Multielement analysis. Triplicates of municipal water, purified water, and fish water samples from all the participating facilities (CCU, CNIC, EZRC, IA.1, IA.2, KI, NULS, and ZIRC) were collected for multielement analysis of aluminum (Al), arsenic (As), barium (Ba), beryllium (Be), B, cadmium (Cd), cerium (Ce), cesium (Cs), chromium (Cr), Co, Cu, dysprosium (Dy), erbium (Er), europium (Eu), gadolinium (Gd), gallium (Ga), holmium (Ho), Fe, lanthanum (La), lead (Pb), lutetium (Lu), Mg, Mn, Mo, neodymium (Nd), nickel (Ni), K, praseodymium (Pr), rubidium (Rb), samarium (Sm), Se, silicon (Si), silver (Ag), Na, strontium (Sr), thallium (Tl), thorium (Th), thulium (Tm), titanium (Ti), uranium (U), ytterbium (Yb), vanadium (V), and Zn. K, Mg, Mo, Na, Si, and Ti were in 50 mL Falcon tubes. Concentrated nitric acid (HNO_3, 70%; #438073; Sigma) was added for a final concentration of 1% HNO_3. The samples were treated with HNO_3 as a diluent to keep the elements in suspension until they were analyzed in the ICP-MS. The instrument was calibrated with a multielement standard solution (500, 100, 10, 1, 0.1, and 0 μg L^{-1}; # IV-ICPMS-71A; Agilent).

Multielement analysis was performed with 7700x ICP-MS (Agilent Technologies). After every 10th sample, two of the standards (1 and 100 μg L^{-1}) were measured again to assess instrument stability and avoid drift. ^{103}Rh and ^{115}In were used as internal standards during the measurements. Metal concentrations in each sample were determined according to appropriate standard curves obtained from the calibration of the corresponding metal standards.[42–44] The method is adapted from the EPA Method 3052.

Sample collection

Water samples were taken from different strategic access points of the water systems in this study. The water system for the fish facility at the IA.1 is shown as an example in Figure 1. These points of access included the following: municipal or source water (Fig. 1 (1), pre-ROWS), purified source water (Fig. 1 (2), post-ROWS), water from the bacteriological filter and/or mechanical filters (Fig. 1 (3)), and fish water (Fig. 1 (4)).

All water samples from ZIRC, CCU, NULS, CNIC, KI, and EZRC were delivered to the IA after nitric acid treatment and stored at 4°C until analysis.

Water samples to determine the impact of ROWS filter membrane changes. Triplicates of municipal water and ROWS water samples were collected and analyzed before and 24 h after the change of 2-year-old membranes (#CDRC60202; Merck) at the IA.1. The specific model analyzed was the RiOs Essential 16 water purification system (#ZR0E0160WW; Merck).

Water samples for a temporal quality assessment. Over a 3-month period, fish water samples were collected weekly from fish tanks at the CNIC facility.

Water samples from biological filters. Triplicate samples from the various biological filters were collected at the following fish facilities:

1. Propeller wash bead filter (PWBF) samples: ZIRC
2. FSF samples: ZIRC.
3. Bead moving filter (BMF) samples: CCU, IA.1, KI, and NULS.

TABLE 3. BIOLOGICAL PARAMETERS FROM FISH FACILITIES INCLUDED IN THIS STUDY

Biological parameters							
Institutions	*CCU*	*CNIC*	*EZRC*	*IA.1*	*IA.2*	*KI*	*ZIRC*
Total fish populations	21,500	12,000	350,000	12,000	4000	15,000	52,500
Total no. of single crosses	30	21	19	20	20	14	135
Breeding success (%)	73	70	70	85	75	90	77% ± 15%
Total no. of eggs	3212	2786	671	1050	850	770	—
Fertilization rate (%)	94	86	88	90	90	80	76% ± 19%
Hatching rate: 48–72 hpf (%)	100	100	100	100	100	100	96% ± 2%
Survival rate at the time of entering the CRWS (%)	88	71	—	75	70	90	92.8% ± 7%
Stocking densities (fish/per liter)	10	5	5	5–6	5–6	5	4–7

The table shows different parameters to evaluate husbandry success at zebrafish facilities included in this study. *Institutions:* CCU; CNIC; EZRC; IA.1 and IA.2; KI; and ZIRC. *Breeding success:* number of pairwise crosses that produced eggs divided by the number of all crosses set up. *Fertilization rate:* number of fertilized eggs divided by the number of viable eggs laid. Fertilization is determined by cell divisions, and typically 0–4 h after fertilization. *Hatching rate 48–72 hpf:* number of hatched embryos (around 72 hpf) divided by the number of fertilized eggs. *Survival rate after 30 dpf:* number of larvae that survived the nursery period versus the number of fertilized embryos that were entered into the nursery. *Stocking density:* number of fish per liter.

Table 4. Multielement Data Analysis from Eight Centralized Recirculating Water Systems

Institutions	*CCU*		*CNIC*		*EZRC*		*IA.1*		*IA.2*		*KI*		*NULS*		*ZIRC*	
Elements	*Mean*	*RSD*	*Mean*	*RSD*	*Mean*	*RSD*	*Mean*	*RSD*	*Mean*	*RSD*	*Mean*	*RSD*	*Mean*	*RSD*	*Mean*	*RSD*
Macronutrients (mg L^{-1})																
Calcium	—	—	16	2.75	—	—	6.11	0.45	—	—	—	—	—	—	16.3	2.02
Magnesium	20.4	0.95	6.51	7.98	—	—	10.9	1.72	—	—	7.94	4.12	5.34	3.92	11	3.07
Potassium	7.57	0.28	4.87	7.34	—	—	7.07	26.22	—	—	3.50	6.79	2.85	5.22	4.3	2.22
Sodium	210	0.25	83.6	6.30	—	—	144	4.66	—	—	110	4.21	56.3	4.50	83.2	2.59
Essential elements (μg L^{-1})																
Chromium	0.18	31.04	0.34		0.03	12.36	0.15	4.11	0.26	36.26	0.06	37.69	0.05	9.96	0.04	15.33
Cobalt	0.09	—	0.02	—	ND	—	0.37	4.83	0.15	44.18	0.01	—	0.70	2.56	ND	—
Copper	0.59	57.60	ND	—	ND	—	3.16	2.59	0.24	40.86	1.54	127.5	0.14	59.95	ND	—
Iron	4.23	43.53	4.21	43.42	1.77	49.32	1.94	7.11	1.49	50.43	1.15	102.3	0.68	22.48	0.95	26.24
Manganese	0.52	87.15	0.17	91.41	0.13	25.87	0.11	11.24	0.19	50.79	ND	—	0.11	4.24	0.05	18.80
Molybdenum	0.70	2.76	0.25	14.54	—	—	0.34	2.95	—	—	—	—	—	—	0.28	12.19
Selenium	ND	—	0.39	18.34	—	—	ND	—	ND	—	ND	—	ND	—	0.52	
Zinc	14.5	36.20	4.31	55.12	13.3	65.86	14.4	29.18	7.11	26.06	ND	—	4.51	6.43	ND	—
Nonessential elements (μg L^{-1})																
Aluminum (pH >6.5)	34.1	6.30	3.67	39.56	25.2	1.52	13.3	2.93	3.51	64.34	ND	—	ND	—	36.9	1.55
Arsenic	0.99	6.03	1.77	24.63	0.38	1.83	0.82	0.12	0.50	33.08	0.23	18.03	0.35	0.67	0.11	21.21
Barium	7.79	1.50	3.78	11.14	45.1	0.22	13.5	0.41	3.37	8.46	0.22	9.50	42.9	0.58	2.67	0.87
Beryllium	ND	—	0.01	—	0.01	—	0.01	—	0.24	286.43	0.01	—	0.01	—	0.04	—
Boron	79.1	3.12	7.38	8.43	5.49	7.40	55.4	4.23	35.09	43.48	12.6	8.16	14.9	3.88	35.6	3.98
Cadmium	0.01	—	0.01	—	ND	—	0.01	—	0.21	43.18	ND	—	0.01	—	ND	—
Cesium	0.15	1.60	0.02	—	0.01	—	0.01	3.43	0.12	35.47	0.01	—	0.01	—	0.05	1.47
Gallium	0.41	2.56	0.01	—	0.01	—	0	—	0.13	77.75	0	—	0.01	—	0.03	4.33
Lead	0.01	—	ND	—	ND	—	0.02	6.71	0.19	34.91	ND	—	0.01	—	ND	—
Mercury	—	—	—	—	—	—	0.02	2.72	0.02	5.44	—	—	—	—	—	—
Nickel	0.25	57	0.16	123.5	0.26	13.07	0.21	1.74	0.38	41.26	ND	—	0.36	0.64	ND	—
Rubidium	1.80	1.14	1.94	15.84	0.69	2.30	1.08	0.41	0.92	25.98	0.27	9.94	0.37	1.18	1.82	0.68
Silicon	1752	0.28	9358	6.16	—	—	—	—	440	4.05	360	13.22	546	0.24	2967	53.2
Silver	0.04	—	ND	—	0.18	102.90	ND	—	0.10	82.30	ND	—	ND	—	0.03	52.19
Strontium	206	0.19	38.2	10.54	132	0.24	37.9	1.58	70.26	18	43.9	3.35	19	2.24	154	0.35
Thallium	0.01	8.65	ND	—	ND	—	ND	—	0.07	67.66	ND	—	ND	—	ND	—
Thorium	0.07	—	0.10	116.87	0.02	5.61	0.03	13.92	0.14	46.69	0.05	—	0.03	—	ND	—
Titanium	0.44	1.22	0.37	25.29	—	—	—	—	0.28	2.76	0.36	15.68	0.42	12.80	0.33	23.64
Uranium	0.03	—	0.01	—	0.44	0.56	0.05	0.70	0.13	41.34	0.01	—	0	—	ND	—
Vanadium	1.40	0.98	0.80	24.71	0.11	6.78	0.41	3.28	0.31	30.16	0.19	11.04	0.13	1.66	0.37	1.84

Mean and RSD of metals and metalloid concentrations from eight CRWS: CCU; CNIC; EZRC; IA.1 and IA.2; KI; NULS; and ZIRC.
ND, not detected; RSD, relative standard deviation.

List of water systems and technical specifications. Main water quality parameters, compounds added, and some technical specifications of the different water systems that contributed to this study are listed in Table 2.

Fish facilities. The participating facilities were established at least 1 year before our study. The following three criteria characterized the facilities that participated in sampling and water testing. (1) Health: During the duration of our study, specifically at times of sample collections, no disease outbreaks were reported in any of the facilities. The majority of the facilities carry out regular health status monitoring including microbial status. (2) Animal Environment: All facilities were operated under environmental conditions that fell into the range of recently published guidelines for zebrafish husbandry by Aleström *et al.*,[45] for FELASA (the European equivalent of AALAS). These conditions included temperature, pH, and conductivity and are listed in Table 2. (3) Facility Performance: In Table 3, we list the biological parameters that can be considered normal for most facilities for the purposes of this study; these include breeding success, fertilization rates, hatching rates, postnursery survival rates, as well as total population and stocking densities.

Data management and statistics

GraphPad Prism version 7.04 was used for statistical analysis and charting of results (GraphPad Software, Inc., San Diego, CA). Statistical significance was determined using the Holm–Sidak method for multiple *t*-tests and Sidak's multiple comparisons test for two-way analysis of variance, with alpha = 0.05. Raw data have been deposited at Mendeley (10.17632/htdhxbpgpg.3).

Ethical statement

Animals were housed in accordance with bioethical regulations for the use of laboratory animals from the corresponding countries: CCU, Portuguese General Directorate of Veterinary (DGAV); CNIC, the Community of Madrid "Dirección General de Medio Ambiente," Spain; ERZC, the Government of Baden-Württemberg, Regierungspräsidium Karlsruhe, Germany; IA.1 and IA.2, Amt für Veterinärwesen, Canton of Bern, Switzerland; KI, the international and local ethical guidelines; NULS, the Norwegian Food Inspection Authority (NFIA); ZIRC, the University of Oregon Institutional Animal Care and Use Committee.

Results

Metal and metalloid concentrations in eight recirculating water systems

To determine metal and metalloid concentrations in CRWS, we analyzed fish water from eight facilities distributed over seven countries (Germany, Norway, Portugal, Spain, Sweden, Switzerland, and the United States). The highest concentrations were detected for the macronutrients Ca, K, Na, and Mg (Table 4). Some nonessential elements, such as Al, B, Ba, Si, and Sr, were detected at higher concentrations than the essential elements, Fe and Zn. The elements As, Cr, Co, Cu, Mn, Mo, Ni, Pb, Rb, Ti, and V were detected in a range between 0.1 and 2 μg L^{-1}; whereas traces of Be, Cd, Cs, Ga, Hg, Pb, Se, Th, Tl, and U were found below 0.1 μg L^{-1} in most fish facilities (Table 4). Some metal traces—Dy, Er, Eu, Gd, Ho, La, Lu, Nd, Pr, Sm, Tm, and Yb—were detected at very low concentrations or remained undetectable (Table 5).

Our results indicate that the concentration of several nonessential elements (Al, B, Ba, Si, and Sr) was one or several orders of magnitude higher than that of essential elements such as Fe and Zn. Furthermore, we observed below technical threshold of detection, absence, or considerably low concentrations of several essential elements such as Cr, Co, Cu, Mn, Mo, and Se (Table 4).

Reverse osmotic water systems do not remove all elements with equal efficiency from municipal water

Municipal water from seven cities and their corresponding reverse osmosis purified water samples were analyzed for 38 elements to determine the capacity of ionic rejection in

Table 5. Trace Metals in Eight Centralized Recirculating Water Systems

Nonessential elements (μg L^{-1})

Institutions	*CCU*	*CNIC*	*EZRC*	*IA.1*	*IA.2*	*KI*	*NULS*	*ZIRC*
Elements	*Mean*	*Mean*	*Mean*	*Mean*	*Mean*	*Mean*	*Mean*	*Mean*
Cerium	0.006	0.002	ND	ND	ND	ND	ND	ND
Dysprosium	ND	0.001	ND	ND	ND	ND	ND	ND
Erbium	0.001	ND	ND	ND	ND	ND	ND	ND
Europium	0.002	0.001	0.01	0.002	0.002	ND	0.007	ND
Gadolinium	0.002	ND	ND	ND	ND	ND	ND	ND
Holmium	ND	ND	ND	ND	ND	ND	ND	ND
Lanthanum	0.004	ND	ND	ND	ND	ND	ND	ND
Lutetium	ND	ND	ND	ND	ND	ND	ND	ND
Neodymium	0.003	0.002	ND	ND	ND	ND	ND	ND
Praseodymium	ND	ND	ND	ND	ND	ND	ND	ND
Promethium	ND	ND	ND	ND	ND	ND	ND	ND
Samarium	ND	ND	ND	ND	0.001	ND	ND	ND
Thulium	ND	ND	ND	ND	ND	ND	ND	ND
Ytterbium	0.002	ND	ND	ND	0.002	ND	ND	ND

Mean values of nonessential trace metal concentrations from eight CRWS. *Institutions*: CCU; CNIC; EZRC; IA.1 and IA.2; KI; NULS; ZIRC.

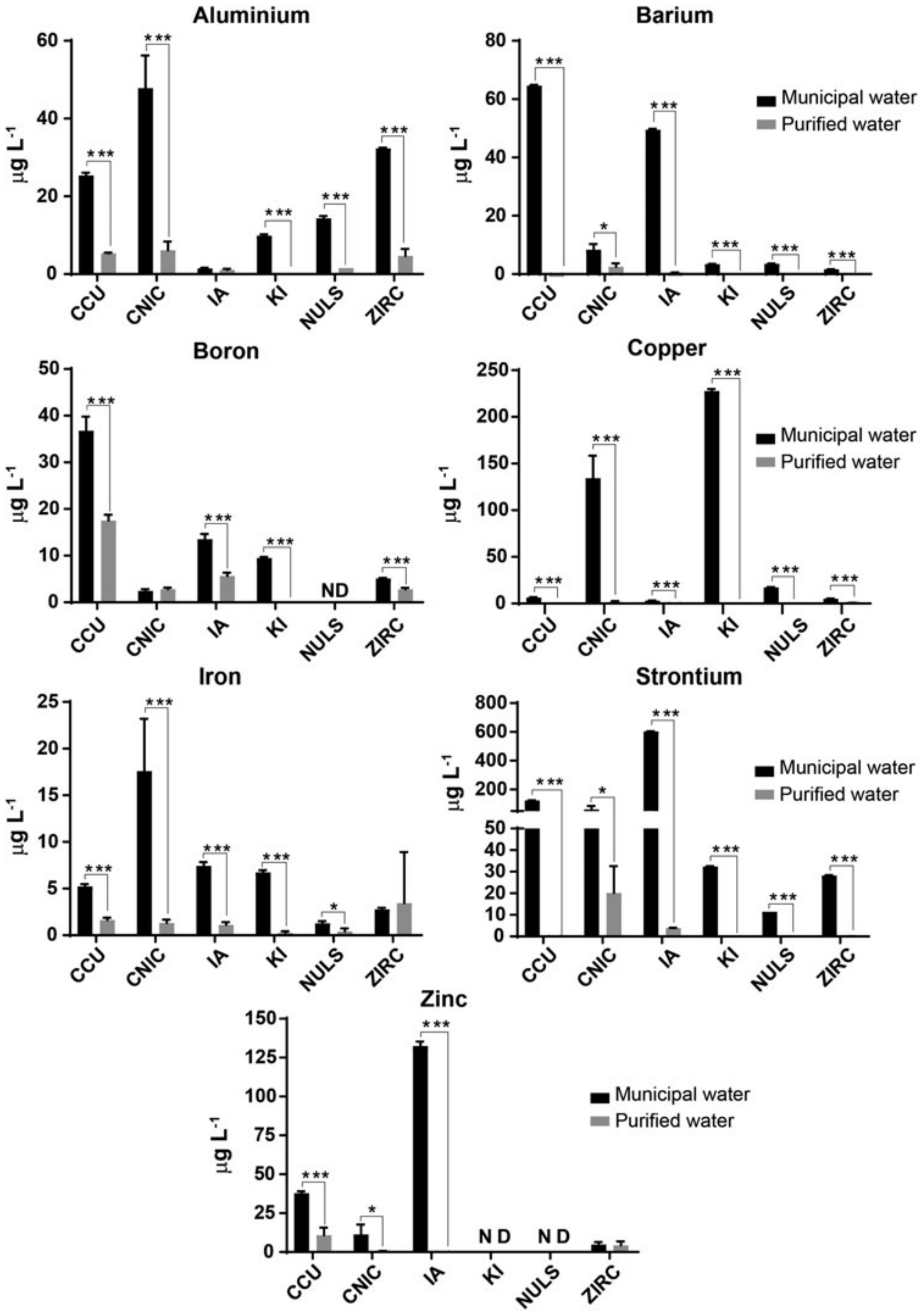


FIG. 2. Metal concentrations before and after reverse osmosis purification of water in six different fish facilities. Municipal water ($n = 3$) and reverse osmosis purified water ($n = 3$) samples were collected at CCU, CNIC, IA.1, KI, NULS, and ZIRC. Data are presented as mean ± SD; $*p < 0.05$; and $***p < 0.001$ according to multiple *t*-tests. CCU, Champalimaud Center for the Unknown; CNIC, Centro Nacional de Investigaciones Cardiovasculares; KI, Karolinska Institutet; NULS, Norwegian University of Life Sciences; ND, not detected; ZIRC, Zebrafish International Resource Center.

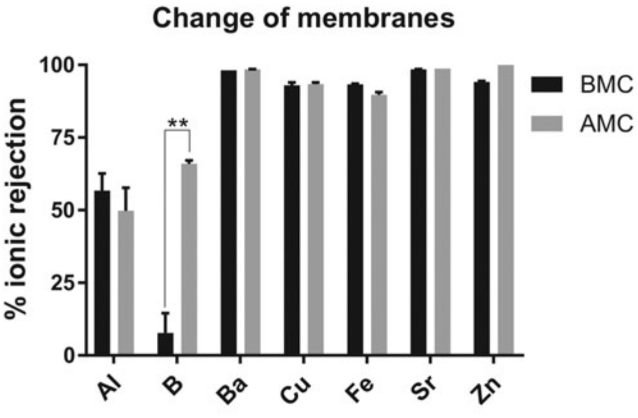
six fish facilities (CCU, CNIC, IA, KI, NULS, and ZIRC). Seven elements were detected in significantly higher concentrations than the other elements we analyzed: Al, B (except at NULS), Ba, Cu, Fe, Sr, and Zn (except at KI and NULS) (Fig. 2). The rest of the elements were detected at low concentrations ($< 0.1\ \mu g\ L^{-1}$) or not traced at all.

To test whether effective ion rejection is associated with the efficiency of reverse osmosis filtration or related to the regular maintenance of the exchange of membranes, we analyzed municipal and purified water samples before and after the change of membranes in one facility. After replacement, we detected efficient ionic rejection only for the removal of B (Fig. 3). For other elements, the membrane change did not appear to make any significant improvement for product water quality.

Our results indicate a considerable presence of Al, B, and Fe after the filtration of municipal water through ROWS and that the replacement of 2-year-old membranes reduced mainly B. Thus, ionic rejection across ROWS is a differential process.

Metal concentrations fluctuate over a period of 3 months

Fish water samples were collected over a 3-month period to evaluate the fluctuation of metals in the CNIC fish facility. While for most elements concentrations remained stable; Al, As, Fe, V, and Zn showed fluctuations higher than 25% (Fig. 4). In contrast, the concentrations of V and As remained consistently low and hardly fluctuated at all.



	BMC	AMC
Temperature (°C)	20.8	21
Conductivity (µS/cm)	20.5	7.8
pH	6.4	6.2

FIG. 3. Percentage of ionic rejection before and after the change of reverse osmosis membranes. Municipal water ($n=3$) and reverse osmosis purified water ($n=3$) samples were collected at IA.1 before and 24 h after the change of 2-year-old membranes. The ROWS is maintained at regular intervals. Data are presented as mean ± SD; $^{**}p<0.01$ according to multiple *t*-tests. AMC, after membrane change; BMC, before membrane change.

The comparison between purified water and fish water concentrations (Fig. 2 and Table 4) with levels of fluctuation over the 3-month tracking period (Fig. 4) suggests that most of the variability of these elements reflects addition of factors that varied during a 3-month tracking. For example, we detected Zn at 11.28 μg L^{-1} in the municipal water and 0.38 μg L^{-1} after ROWS filtration at CNIC (Fig. 2). On average, we detected a Zn concentration with a minimal value of 1.54 and a maximum value of 8.24 μg L^{-1} in the fish water samples over the 3-month period. This suggests that the addition of external factors to the water system, such as diet or synthetic salts, which contain Zn and Fe but presumably no or little As or V, contributes to the variability of some metal and metalloid concentrations in fish water.

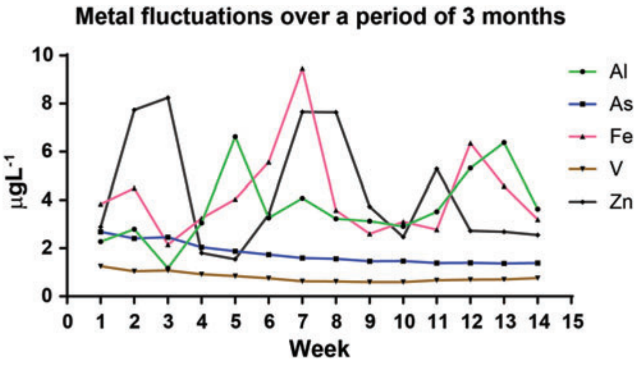


FIG. 4. Fluctuation of metal concentrations over a period of 3 months. Fish water samples ($n=14$) were collected at CNIC. Graph shows the fluctuation of Al, As, Fe, V, and Zn levels (μg L^{-1}) over a period of 3 months in the zebrafish housing system. Color images are available online.

Fine sediment and biological filters can accumulate metals

To test the performance of biological filters and FSF with the capacity to accumulate metals and metalloids, we compared fish water with the sump water from a PWBF (ZIRC), an FSF (ZIRC) and a BMF (CCU, IA.1, KI, and NULS) (Fig. 5). Water from the PWBF showed a significant accumulation of Al, Ba, Cu, Fe, Mn, Sr, and Zn concentrations when compared with fish water (Fig. 5A). Similarly, FSF water also showed a significant increase of Cu, Fe, Mn, and Sr concentrations when compared with fish water (Fig. 5A). In contrast, we did not detect any significant difference in metal concentrations between pre- and post-BMF water samples, except for Al and Mn at the KI facility (Fig. 5B).

Our results indicate a significant accumulation of Al, Ba, Cu, Fe, Mn, Sr, and Zn in PWBF and FSF in the ZIRC water system; while BMF does not show significant accumulation of any element in four fish facilities (CCU, IA.1, KI, and NULS).

Discussion

Controlled environmental conditions for laboratory animals provide a basis for successful animal husbandry and welfare and reproducible results. For zebrafish, husbandry studies included the roles of anesthesia and euthanasia,[46] diseases and pathogens,[47,48] behavior,[20–23,49,50] housing,[39,45,49–51] nutrition,[52,53] and reproduction.[54–56]

In this study, we explored concentrations of metals and metalloids in different CRWS that are used in biomedical zebrafish research facilities. In this regard, our study could serve as a reference for troubleshooting when there are indications of diseases related to deficiency or excess of some elements.

Analysis of macro- and micronutrients and nonessential elements

We detected the macronutrients Ca, K, Mg, and Na at high concentrations, but barely detected the micronutrients Cr, Co, Cu, Fe, Mn, Se, and Zn. The Cu, Mn, and Zn metal content in early embryos has been suggested to be set by the maternal contribution and only increases once zebrafish develop to the stage where they can acquire nutrients through the diet or the environment.[57] The concentrations of Cu, Mn, and Zn reported in the study of Thomason *et al.* indicated that relatively low concentrations are required for fish younger than 30 dpf, suggesting that the concentrations of the CRWS shown in this study might be sufficient for normal biological processes in larval and juvenile zebrafish.

In several CRWS, we also detected elements that are described in the literature as nonessential (Al, B, Si, and Sr) and even toxic at excessive levels in concentrations higher than 1 μg L^{-1}. Al, for example, has been reported to increase the acetylcholinesterase activity in the zebrafish brain at 50 μg L^{-1} $AlCl_3$ at pH 5.8[21] and to induce behavioral changes at 6700–26,700 μg L^{-1} $AlCl_3$.[58] In addition, neurotoxic effects have been reported in astroglia at 13,300 μg L^{-1} $AlCl_3$.[59] Sr, as a further example, stimulates the process of bone mineralization at 520 μg L^{-1} $SrCl_2$, and the addition of strontium citrate as a nutritional supplement can increase bone mineral density.[53] However, at concentrations exceeding 52,300 μg L^{-1}, $SrCl_2$ can also lead to the

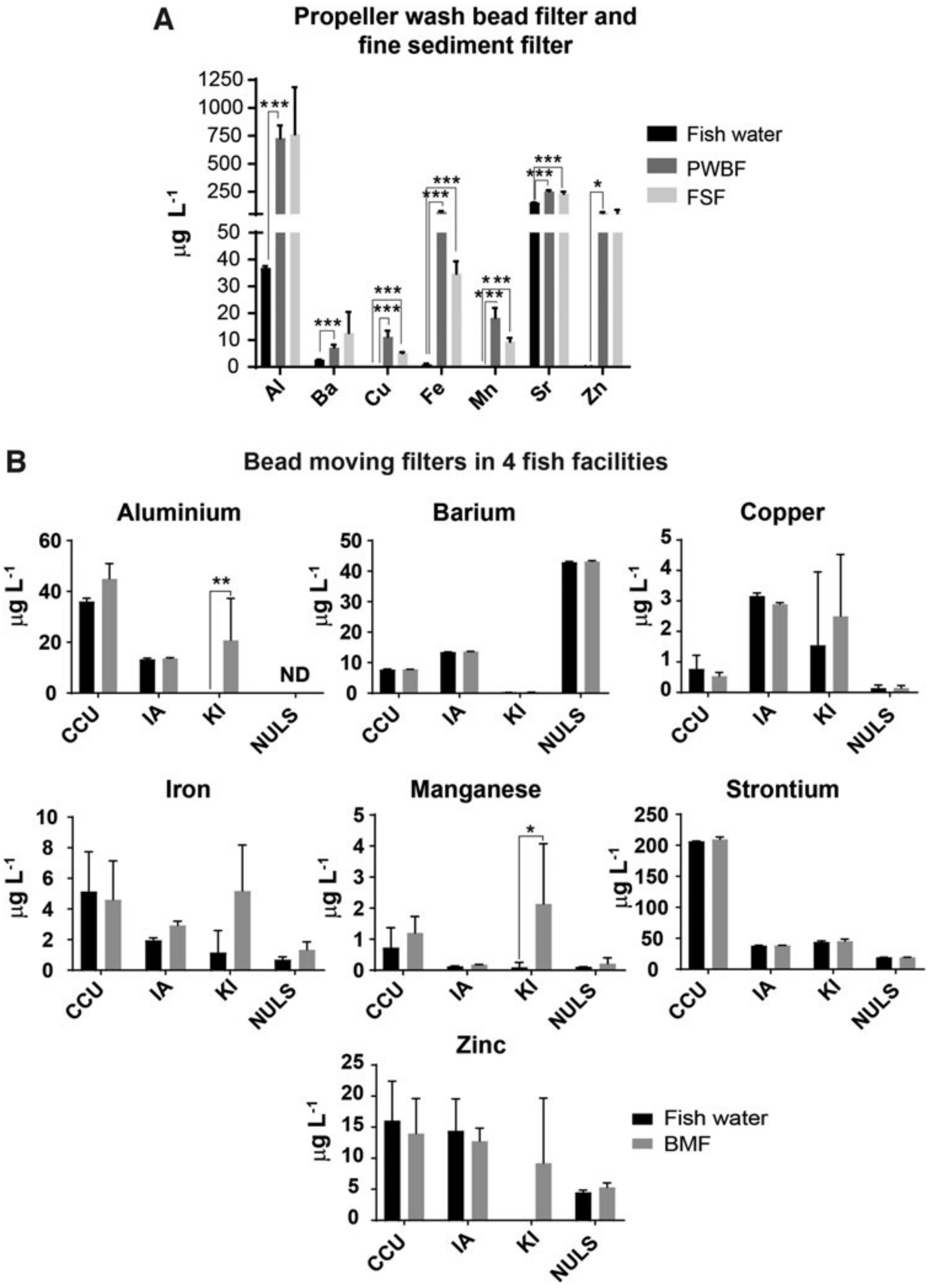


FIG. 5. Metal concentrations are elevated in PWBF and FSF samples relative to system water, but not in BMF samples. **(A)** Water samples from fish tanks ($n=3$), PWBF ($n=3$), and FSF ($n=3$) were collected at ZIRC. **(B)** Water samples from fish tanks ($n=3$) and BMF ($n=3$) were collected at CCU, IA.1, KI, and NULS. Data are presented as mean ± SD ($n=3$); $*p<0.05$; and $***p<0.001$ according to multiple t-tests **(A)** and two-way ANOVA **(B)**. ANOVA, analysis of variance; BMF, bead moving filter; FSF, fine sediment filter; PWBF, propeller wash bead filter.

inhibition of the mineralization process during embryonic osteogenesis.[60] For the two other elements, B[61] and Si, little is known, for example, about the tolerance levels, or acute and chronic toxicity in zebrafish. Thus, depending on the concentrations, nonessential elements can have beneficial or detrimental effects on zebrafish health.

Despite RO, RWFSs form dynamic animal environments

Evidence for differences of chemical composition of fish water can be gained from two facilities at the same institution (IA.1 and IA.2; see Table 4) that share the same ROWS source of water, but the overall infrastructure, synthetic sea salts, and diets differ between the two facilities. These data can be valuable and taken into account when conducting comparative studies between institutions that work in the same field of research.

Few studies have shown whether or not fluctuating environmental conditions such as temperature, conductivity, pH, hardness, and oxygen, parameters that play a role for the speciation of metal ions, influence metal and metalloid toxicities.[2,4,27,62] Therefore, additional information obtained by careful control of environmental parameters for each of these elements is needed to better understand under which conditions environmental changes and metal concentrations might cause chronic, acute, subpathological, or apparent disorders of behavior, metabolism, reproduction, health, and development in zebrafish.[16,19–22,26,27,31,33,58,59,63–66]

Seven of the analyzed CRWS use municipal water as their water source that is purified by ROWS and reconstituted with synthetic sea salts and buffers to reach freshwater conditions. The efficiency of ROWS to filter water appears to be similar between facilities based on the relative concentrations of dissolved elements present in their municipal water. However, our observations suggest that some elements are removed more efficiently (Ba and Sr) than others (mainly Al, B, and Fe) by ROWS filter membranes. The causes of the observed differences in ionic rejection between elements may be due to excessive convective transport, poor size exclusion, or ineffective charge repulsion.[67] The concentration of particular elements after ROWS filtration can be used as an indication that membranes need to be replaced periodically. Previous studies already reported about the efficiency of ROWS for removing heavy metals,[68,69] but there is lack of and occasionally confounding information regarding the most adequate timing to replace ROWS filtration membranes. Therefore, we suggest more long-term studies to better understand optimal, facility-specific frequencies for filter and membrane changes and the impact of seasonal fluctuations on CRWS. Such measurements will contribute to more stable water composition, and thus, a reduction of costs for water system consumables, environmental sustainability, and the increased knowledge about the chemical composition of CRWS water. Despite ROWS and reconstituted freshwater conditions, we observed fluctuations of Al, Fe, and Zn concentrations in one of the water systems over a 3-month period. This suggests that even with relatively tight control of source water quality, fish diets, fluctuation of the number of fish on the system, and buffering agents may introduce significant variations in system water quality.[70–72]

Possible role of filtration components for system water composition and stability

Biological filters represent crucial infrastructural components of RWFS for the oxidation of ammonia into nitrite and subsequently to less toxic nitrate.[38,39] Moreover, bacteria, algae, and fungi have the capacity to chelate metals.[73,74] However, so far there are no reports indicating which elements are accumulated by microorganisms in fish facilities. In addition, biofilm appears to be more effective in trapping metals in PWBF in comparison with BMF. We suggest that the more abrasive backwashing in BMF knocks off and removes microorganism-containing biofilm that is beneficial for the elimination of metals from immediate contact with fish. Moreover, it has not been reported yet, whether or not filter systems may also function as metal traps in RWFS. Specific studies are required to better understand which microorganisms might be involved in this process, which specific metals they accumulate, and how they impact the overall water quality in CRWS.[75] Several factors might affect metal accumulation in filter components: the population growth phase of the bacterial population, the type of materials used in the filters (beads, minerals, pads, etc.), the total biomass of the fish population relative to the total water volume of the system, and the level of its pollutant equilibrium.

In conclusion, our study sheds light on 46 elements that can be expected in CRWS. This information might be helpful for the research community as a troubleshooting aid to solve potential husbandry problems related to changes in water quality.

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Disclosure Statement

No competing financial interests exist.

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