Use of fish otoliths as a temporal biomarker of field uranium exposure

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Abstract :

This study aimed to determine uranium (U) pollution over time using otoliths as a marker of fish U contamination. Experiments were performed in field contamination (~20 µg L−1: encaged fish: 15d, 50d and collected wild fish) and in laboratory exposure conditions (20 and 250 µg L−1, 20d). We reported the U seasonal concentrations in field waterborne exposed roach fish (Rutilus rutilus), in organs and otoliths. Otoliths were analyzed by ICPMS and LA-ICP SF MS of the entire growth zone. Concentrations were measured on transects from nucleus to the edge of otoliths to characterize environmental variations of metal accumulation. Results showed a spatial and temporal variation of U contamination in water (from 51 to 9.4 µg L−1 at the surface of the water column), a high and seasonal accumulation in fish organs, mainly the digestive tract (from 1000 to 30,000 ng g−1, fw), the gills (from 1600 to 3200 ng g−1, fw) and the muscle (from 144 to 1054 ng g−1, fw). U was detected throughout the otolith and accumulation varied over the season from 70 to 350 ng g−1, close to the values measured (310 ng g−1) after high exposure levels in laboratory conditions. U in otoliths of encaged fish showed rapid and high U accumulation from 20 to 150 ng g−1. The U accumulation signal was mainly detected on the edge of the otolith, showing two U accumulation peaks, probably correlated to fish age, i.e. 2 years old. Surprisingly, elemental U and Zn signatures followed the same pattern therefore using the same uptake pathways. Laboratory, caging and field experiments indicated that otoliths were able to quickly accumulate U on the surface even for low levels and to store high levels of U. This study is an encouraging first step in using otoliths as a marker of U exposure.

Graphical abstract
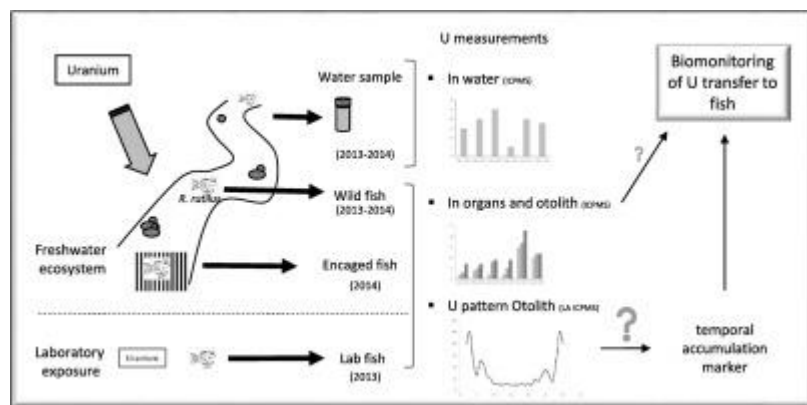
Highlights

► U accumulation signals were detected mainly on the edge of fish otoliths using LA-ICP MS. ► Laboratory, caging and field experiments indicated that otoliths were able to store U. ► Seasonal variation of U levels was measured in the water column and fish organs.

Keywords : Roach, Accumulation, Laser ablation ICP-MS

1 <u>INTRODUCTION</u>

In aquatic ecosystems, heavy metals are considered the most important pollutants, since they are present throughout the ecosystem and can be detected in critical amounts (Jia et al., 2017; Schwarzenbach et al., 2006). Most of these metals accumulate in the tissues of freshwater species, leading to poisoning of fish and effects at different organizational levels, including pathological changes.

Uranium (U) is a radioelement which occurs naturally in the environment. However, industrial processing of nuclear fuel has led to the operation of U mines around the world, thus increasing concentration levels in freshwater ecosystems. Maximum aqueous concentration of total uranium in countries involved in U mining is close to 1350 µg L^{-1} (Goulet et al., 2011; Goulet et al., 2018). Goulet et al. (2011) reviewed the scientific literature on U toxicity in fish but little research has been done to assess the accumulation and toxic effects of U on fish *in situ* (Gagnaire et al., 2014; Goulet et al., 2015; Kraemer et al., 2012; Le Guernic et al., 2016a; Le Guernic et al., 2016b; Pyle et al., 2001) when the concentration in water is higher than the environmental quality guideline (e.g. 0.3 µg.L^{-1} ((INERIS, 2011)) or 0.5 µg.L^{-1}(AA-EQS, (van Herwijnen et al., 2014) and despite the fact that U can have harmful effects on ecosystems and human health, even after the end of mining activities. The data set for the study of accumulation levels and the associated effects in fish should be increased to characterize the ecological risk related to the presence of U.

Indeed, fish are considered to be the most significant biomonitors in aquatic systems for the estimation of metal pollution levels (Shotyk et al., 2019; Van der Oost et al., 2003; Viarengo et al., 2007); studying them offers several specific advantages including the description of the natural characteristics of aquatic systems. The common roach *Rutilus rutilus* is very adaptable and can be observed in any freshwater ecosystem, ranging from small ponds to the largest rivers and lakes. It will feed at any depth (benthic invertebrates, zooplankton, plant material and detritus), although its preferred food sources tend to be in shallower water. *Rutilus rutilus* generally stays in backwaters or close to bottom waters to winter and undertakes short migrations during breeding season when the water temperature exceeds 20°C. They are often included in biomonitoring studies (Blanco et al., 2018; Geraudie et al., 2017; Reynders et al., 2008).

The metal concentration in fish tissues reflects exposure via water and/or food and can be used to assess a current situation of the animals before inducing adverse toxicity effects. However, the mechanisms of excretion alter the metal burden accumulated over time. As accumulation levels are most often measured occasionally, using them as markers cannot be used to build accumulation histories of entire lives. But the toxic action of metals is particularly pronounced in the early stages of fish development, and adversely affects various metabolic processes in developing fish, resulting in developmental retardation, morphological and functional deformities, or death of the most sensitive individuals (Jezierska et al., 2009). High levels of accumulation during the entire life or during the key stages of development can have negative repercussions on the future of populations.

Otoliths of teleost fishes are a series of paired calcified structures located in the inner ear cavity, which grow through the fishes' life. They are metabolically inert, so any chemicals elements incorporated into the precipitating surface are permanently retained (Campana, 1999; Casselman, 1990; Gauldie et al., 1990), making it possible to access historical exposure via waterborne and dietary sources, especially for non-essential elements, even when the fish move to find better living conditions or avoid metal pollution. During otolith formation, various elements can be incorporated into either the organic or inorganic portion of otoliths (Campana, 1999; Elsdon et al., 2003; Taddese et al., 2019). Therefore, fish otoliths have long been known as a timekeeper. Ways of using them in stock identification, determination of migration pathways, and reconstruction of temperature and salinity history has been developed (Bareille et al., 2015; Begg et al., 2000; Campana, 1999; Hampton et al., 2018; Hogan et al., 2017; Tabouret et al., 2010; Teichert et al., 2018). In addition, primary growth increments in otoliths can be used as an alternative method of age determination (Luo et al., 2016; Vilizzi, 2018). Otolith element analyses are used extensively to reconstruct

environmental histories of fish (Izzo et al., 2016; Martin et al., 2013; Riou et al., 2016) but few ecotoxicological studies have been focused on their toxic metal binding capacity (Barbee et al., 2014; Barbee et al., 2013; Daverat et al., 2012; Ranaldi et al., 2008; Selleslagh et al., 2016; Shotyk et al., 2019) and therefore their ability to operate as a marker of pollution. Ambient water strontium/calcium (Sr/Ca) and barium/calcium (Ba/Ca) concentration ratios affect composition of triplefin *Forsterygion nigripenne* otoliths linearly and positively (Taddese et al., 2019) and make it possible to reconstruct past environmental characteristics from fish otolith chemistry. Recently, otolith microchemistry of black bream collected from an artificial and contaminated inlet was used to measure levels of copper (Cu), lead (Pb) and iron (Fe) (Andronis et al., 2017). But data are scarce on metals and radioelements likely to be found in freshwater while elements such as U which possess Ca-like properties are good candidates to be retained in otoliths (Izzo et al., 2016).

The objective of this study was to assess the ability of otoliths to accumulate U in *R. rutilus* collected in a contaminated U pond, and use them to build U accumulation histories through the entire life. U levels in water and fish collected over several seasons and after laboratory caged exposure sampling was also measured. The purpose of this study was to explore a novel biomonitoring program using otoliths as tools for assessing the potential transfer of U mining releases on fish over time.

2 <u>MATERIALS AND METHODS</u>

2.1 Ethics statement

Experiments were conducted in accordance with European Commission recommendation 2007/526/EC on revised guidelines for the accommodation and care of animals used for experimental purposes. The registration number for the ethics of the IRSN-PRP ENV/SRTE laboratory is B 13 013-07. Fish were killed within minutes by immersion in melting ice and then by transaction of the spinal cord in accordance with the ethical guidelines displayed and used by the NIH intramural research program (National Institutes of Health, 2011).

2.2 Study site

In France, one of the most important U extraction zones was the Limousin region. The study site is an abandoned open pit U mine located in a small village (Saint Julien au Bois) in the Limousin region (45.114256, 2.152551, France). Ore extraction (110 t) occurred between 1959 and 1981. After the operating period, the mine was naturally flooded without chemical treatment. Today, a small pond (400x80x20m, $[CO_3^{2-}]$ = 11-29 mg.L^{-1}, [IC] = 2-5 mg.L^{-1}, [OC] = 3.6-5.8 mg.L^{-1}) is home to an aquatic ecosystem after fish were added.

2.3 Roach field sampling

Both water and field fish sampling were performed on May 15, July 8 and September 25 in 2013 and on June 6, July 17 and September 23 in 2014. Fish collected in 2014 (20.5 ± 9.2g, 12.63 ± 2.2 cm, n=55) were stored in dry ice during transport, then at -80°C in the laboratory. 30 fish collected in September 2013 (12.12 ± 2.15g, 19.5 ± 19.03 cm) were brought back to the laboratory and introduced into non-contaminated exposure conditions for 5 months.

2.4 Roach laboratory exposure

Roaches were purchased from a fish farm (France) and had not previously been exposed to U levels above the natural background. Fish for laboratory experiments (7.10 ± 2.4g, n=12) were acclimated to soft artificial water (pH=6.5, Ca^{2+}=11.6; Mg^{2+}=27.2; Na^{+}=7.3; K^{+}=5.9, Cl^{-}=32.6; SO$_4^{2-}$=16.5; NO$_3^{-}$=19.22; CO$_3^{2-}$=0.64, in mg.L^{-1}) under 14/10h light/dark regime at 18-20°C. Roaches were fed twice a day with commercial dry food from fish farming. Roaches were exposed to waterborne exposure conditions (20 and 250 µg L^{-1}) for 20 days in soft artificial water. Low pH (6.5)

and ionic composition of artificial water (low hardness and cation concentrations) were chosen to favor waterborne U bioavailability (Barillet et al., 2007; Bucher et al., 2014; Fortin et al., 2007; Markich, 2013; Simon et al., 2014; Simon et al., 2018). The selected U concentration in water (20 µg.L^{-1}) was higher than the environmental quality guideline (e.g. 0.3 µg.L^{-1} (INERIS, 2011) or 0.5 µg.L^{-1}(AA-EQS, (van Herwijnen et Verbrugen, 2014) but close to *in situ* U concentrations measured in French contaminated ponds (Le Guernic et al., 2016b; Simon et al., 2005). Optimal conditions were achieved using a constant-flow peristaltic pump, with U concentration, pH and temperature monitored on a daily basis.

2.5 Roach-filled caging

Roaches were purchased from the same fish farm (France, U in water < 5 µg.L^{-1}). For both exposure conditions, fish weighed 20 ± 7 g, n=15 at 15d (15 days) and 21.4 ± 6.4g, n=20 at 50d. Fish were directly transported from farms to the experiment sites and put in cages (Ø=0.8, h=1m) for 50 days from May 28 to July 17. One cage received water from upstream. The second cage was introduced directly to the contaminated pond (28/05: pH=6.64, Ca^{2+}=2.2; Mg^{2+}=1.2; Na$^+$=3.3; K$^+$=1.2, Cl$^-$=3.0; SO$_4^{2-}$=3.48; NO$_3^-$=0.1; CO$_3^{2-}$=13.8, in mg.L^{-1}). The two cages were placed at a 1m depth with 40 fish per cage. Fish were fed every other day with commercial feed. 35 fish were sampled and dissected immediately in the field. Organs were quickly frozen and stored in dry ice until they reached the laboratory. Organs were then stored at -80°C. Water sampling was performed every 3 days.

2.6 Fish organs and otolith collection

Fish were collected with fishing rods ant their organs (gill epithelium, gill arch, muscle, gonads, liver, kidney, intestine and intestinal content), weighed and stored at -80°C. Sagittal otoliths were extracted using forceps, washed and cleaned of adhering tissue, rinsed with 18.2 MΩ, air dried at room temperature and weighed.

2.7 Total elemental concentrations

Analysis of uranium in water, otoliths and organs was performed using an Agilent ICP-MS 7500 cx (Agilent, Tokyo, Japan). Internal standard (Bi) was added in each sample dilution to check for possible matrix effects on the U signal. Accuracy of the measurement was determined using CRM (SLRS-4 from NRC, TM28.3 from EC) spiked in matrices, recoveries were within 8% of the certified value. Organs were digested using cycles of 3 mL of HNO$_3$ (65%, Merck) and 1 ml H$_2$O$_2$ (33%, Merck) on a sand bath (100 °C). Otoliths were dissolved in HNO$_3$ (65%, Merck). Water, organs and otoliths were then stored in acidified ultra-pure water (2%, v/v) before ICP analysis.

2.8 Determining age of fish with otolith increment technique

For ageing, the sagittal otolith and scales removed from each specimen were used and analyzed using the specialized TNPC software (Traitement Numérique des Pièces Calcifiées/Numerical Treatment of Calcified Structures: www.tnpc.fr). Since growth of *R. rutilus* has not been significantly researched before, ageing calibration was performed. Fish age was determined by counting the number of alternating translucent and opaque bands. Calibration was based on contrasting results from multiple ageing techniques. Ageing techniques comprised readings performed on whole otoliths, sectioned otoliths and scales. Whole otoliths were observed under both transmitted and reflected light. After the otoliths were embedded in epoxy resin, transverse sections (TS) through the nucleus were made using a precision saw with a blade thickness of 0.3 mm. Finally, the TS were ground and polished on both sides until the core was visible (thickness of 0.2 mm). Scales that have regenerated after loss or damage cannot be used for ageing; hence, five scales were extracted from under the pectoral fin where the likelihood of regenerated scales is limited. Finally, for each specimen, polished TS and scales were examined and compared by two readers to control for observer bias. Age observed between the different calcified structures was comparable.

2.9 Otolith preparation and LA-ICP SF MS analysis

Sagittal otoliths were embedded in araldite resin and left to dry for 48 hrs. They were then ground on a sagittal plane to expose their nucleus, polished with diamond paper of decreasing grain diameter (9.0, 3.0 and 0.3 µm), washed with ultrapure water and dried prior to analysis. ^{88}Sr, ^{138}Ba, ^{238}U, ^{64}Zn and ^{42}Ca were analysed using a sector field inductively-coupled plasma mass spectrometer (ICP SF MS, ELEMENT XR Thermo Fisher, Netherlands) coupled to a laser ablation system (UP213, ESI, Freemont, CA). A linear raster scan of 200 µm width passing through the primordia/nucleus (early growth) to the edge (recent growth prior to capture) of the otolith was started about 300 µm before the primordia using a 20 Hz frequency at a speed of 5 µm s^{-1}. The sample was ablated with 100% delivered energy resulting in 6 J.cm^{-2} fluency. Ablated material was carried by a He gas flow at 0.5 L min^{-1} from the laser cell to the ICP SF MS where it was mixed with an Ar sample gas prior to introduction into the dry ICP source. The Ar gas flow rate was optimized to obtain a U/Th ratio close to 1. Prior to ablation, a gas blank measurement of 20s duration was recorded. ^{42}Ca was used as an internal standard for each ablation (Whitledge et al., 2007) to avoid variation in ablation yield and thus improve the reliability of the elemental abundance at each point of the transect. The elemental signal was recorded in low resolution mode to maximize instrumental sensitivity. ^{238}U, ^{88}Sr, ^{138}Ba and ^{64}Zn signals were transformed into concentrations (µg.g^{-1} or ng.g^{-1}) using an external calibration curve by ablating glass standard NIST 610, 612 and 614 with operational conditions identical to the ones used for otolith analysis. The average detection limit based on three standard deviations (SD) of the blank gas was 0.3, 88, 26 and 85 ng g^{-1} for ^{238}U, ^{88}Sr, ^{138}Ba and ^{64}Zn, respectively.

2.10 Statistical analyses.

Results were expressed as mean ± standard deviation. As data were not normal, differences between conditions were assessed using a Kruskal-Wallis test (p<0.05). Statistical tests were performed using Statistical 7.1 software (Statsoft, Chicago, IL, USA).

3 <u>RESULTS</u>

3.1 Seasonal variation of U in the water column

U concentrations ([U] µg L^{-1}) and abiotic parameters (pH, O$_2$ %, temperature °C) were measured at three water depths (0, 2, 15 m) over the 2014 season (Figure 1). High differences of U levels in the water column were observed over time, especially at surface and intermediate water depths (-2m) where U decreased from January (50 µg L^{-1}) to May (10 µg L^{-1}) remaining low until September. This decrease was correlated to variation of all abiotic parameters and mainly the temperature of the water column from January to September. In contrast, U levels remained high in deep water (-15m), close to 30-65 µg L^{-1} over the whole period. Levels of U concentrations at surface water depth measured in May, June and September in 2013 range between 15-20 µg L^{-1} (data not shown). Levels of U in encaged ponds were comparable to surface water.

3.2 U accumulation in fish

   In wild fish

Figure 2.A shows U accumulation levels (ng g^{-1}, fw) in organs of wild fish (2014). Accumulation levels were detected in all organs, with concentrations ranging from 100 to 20 000 ng g^{-1}, fw. Intestinal content, kidney, liver and gonads showed high U accumulation levels. But significant increases of U accumulation over the season were only observed in gill epithelia, gill arch, muscle, and intestine. Accumulation in the intestine was 20 times higher in September (20 000 ng g^{-1}, fw) than in June (1000 ng g^{-1}, fw), while 5 to 7 times higher for muscle in September (1000 ng g^{-1}, fw) than in June (150 ng g^{-1}, fw). Finally, a 2- to 3-fold increase in U levels was found for gill epithelia and arch. The same trend

was observed for the fish collected in May, July and September 2013 (Figure 2.B). The 5 month laboratory depuration experiment shows a significantly high decrease of accumulation (ratio (r) = [U] Sept-2013/[U] sept 2013+ lab 5 month depuration) in the intestine (r=1155), gonads (r=460), gill epithelia (r=225), liver (r=139), gill arch (r=97) muscle (r=85) and kidneys (r=51).

In encaged fish

Figure 3 shows U accumulation levels (ng g^{-1}, fw) in organs of encaged fish. Both for fish encaged upstream or encaged in the pond, U accumulation increased with exposure time. After 50 days of exposure, accumulation levels in all organs of encaged pond fish were significantly higher than in encaged fish upstream. Accumulation in encaged fish ranged between 50-150 and 500-1000 ng g^{-1}, fw for upstream and pond, respectively, except for the intestine, intestinal content and muscle. The concentrations in the muscle remained lower than 20 ng g^{-1}, fw, unlike the intestine and intestinal content for which concentrations were higher, *i.e.* 100 to 7000 and 3.000 to 70000 ng g^{-1} (fw) for upstream and pond fish, respectively. At 50d, levels of U accumulation for each organ of encaged pond fish were generally significantly lower than those measured for wild fish (June-July), except for gonads, the intestine and intestinal content. High levels of U in intestinal content were also measured in upstream fish at the end of the exposure period.

In laboratory exposure conditions

Figure 4 shows U accumulation levels (ng g^{-1}, fw) in organs of laboratory exposed fish. Accumulation in intestine, intestinal content, gill epithelia and arch were high, close to 10000 ng g^{-1}, fw for the high waterborne exposure concentration (250 µg L^{-1}, 20d, pH=6.5). Significantly different U accumulation levels between the two U exposure concentrations (20 and 250 µg L^{-1}) were only detected in gill epithelia and arch, gonads and kidney. For muscle, gonads, liver and kidney, the high waterborne exposure concentration led to U accumulation levels close to those measured in wild fish in June and July 2014.

3.3 U in otoliths

Otolith, mass, allometric relationship and age

For all experiments, otolith mass ranged between 0.2 and 9 mg, dry weight (dw). Differences in mass between both otoliths from the same fish was low (1.53% n=38, data not shown). The age of all fish varied between 0+ and 6 years. No significant differences in both otolith mass and age of fish were observed between wild and encaged fish. Moreover, no linear relationship was observed between otolith mass and fish mass (8-30g) (R^2=0.229, n=38, data not shown). Finally, parameters that are easy to determine, such as the mass of the whole fish or otolith did not make it possible to determine the age of the fish.

Total accumulation by ICP MS

Figure 5 shows total accumulation levels (ng g^{-1}, dw) in otoliths from the various exposure conditions, except laboratory exposure conditions (lab-20µg/L-20d). Uranium was detected in all fish whatever the exposure conditions. In wild fish, accumulation levels ranged between 70 and 350 ng g^{-1}, dw. Without taking into account the age of fish, a significant difference (Kurskall-Wallis test, p<0.05, x4.7) was observed between accumulation levels in wild fish from June and July, indicating that otoliths are able to rapidly accumulate U.

Higher levels of U (approximately 3.5-fold) were measured in wild fish otoliths collected in July 2014 (350 ng g^{-1}, dw) compared to those caught in September 2013 and June 2014 (70- 100 ng g^{-1}, dw). U measured in the otoliths of fish collected in September 2013 seemed to have accumulated significantly, since five months of depuration did not lead to a significant decrease of U in otoliths. No significant difference of accumulation levels was observed between September 2013 and June 2014, indicating that U adsorption during this period could be low.

Moreover, laboratory or encaged experiments demonstrated the ability of U to quickly accumulate on the surface and edge of the otolith. U was detected in encaged fish after only 15d of exposure in the field in June 2014 (20 ± 4 ng g^{-1}, dw) increasing over time by a factor 7 after 50d (encaged fish from July 2014: 140± 120 ng g^{-1}, dw). High accumulation levels (310 ± 110 ng g^{-1}, dw) were also measured after 20d laboratory exposure to high levels of U (250 µg L^{-1}).

Finally, accumulation of Sr (440-1050 ng g^{-1}, dw) and Ba (30-120 ng g^{-1}, dw) in field fish otoliths varied over the season and were significantly higher than U accumulation. A linear relationship (R^2=0.73, n=7) between average Sr and Ba accumulation levels was observed for all conditions.

Continuous U profiles in R. rutilus otoliths using LA ICP SF MS

Figure 6 shows the typical patterns of U (ng mg^{-1}) along transects from 300 µm before the primordia to the edge of the otolith, passing through the wild roach *R. rutilus* otolith nucleus. Note that over time, the compounds accreted onto their growing surface are permanently retained. Polished transverse sections (TS) through the nucleus provide a chronological record of information throughout the entire life of the fish (Magnaye et al., 2019). High heterogeneity was observed regarding levels, with the highest peaks measured at the edges of the otoliths. Note that the profile was not perfectly symmetrical around the nucleus. Figure 6A-6B-6C are representative of the profiles obtained for a 2-year-old fish. Two to three peaks were detected depending on the fish, even though they come from the same cohort. The first peak is found after birth, the second around the 1 year old mark and the third at the edge of the otolith. However, the data set did not strictly confirm a relationship between the age of the fish and the number of peaks (data not shown). In older fish, only one, relatively high, peak was detected at the edge (Figure 6E). Note that Sr concentrations also showed seasonal variation.

Figure 7 shows the integrated concentration of U at the edge of the otolith. In wild fish, accumulation levels varied widely from 160 to 3500 ng g^{-1}, n=12. Taking into account all wild fish, the intensity of the peak did not seem to correlate with the age of the fish (n=9). Wild fish collected in July 2014 (1716 ± 1130 ng g^{-1}, n=6) showed higher accumulation levels compared to wild fish from June 2014 (693 ± 514 ng g^{-1}, n=7) and September 2013 + fish with 5 month depuration in the lab (152 ± 87 ng g^{-1}, n=5). For 50d encaged fish, the 2 year-old fish (n=3) showed higher concentrations than 1 year-old fish (n=4) while they were exposed at the same conditions.

4 <u>DISCUSSION</u>

4.1 Accumulation in fish

Field abiotic parameters, levels of U and U bioavailability showed extreme variations over the season.

During the spring and summer, high differences of temperature (T°) between the 3 horizons prevented the mixing of water, leading to large differences in U concentrations in water. Moreover, the pH remained below 6.5 throughout the year, favoring the presence of dissolved bioavailable species of U for waterborne contamination (Denison et al., 2005; Fortin et al., 2007). Waterborne contamination pressure on roaches and their prey therefore varies quite widely over time and space (water depth) in the studied area. From July to September, roaches are exposed to high U concentrations in deep water but lower U levels in surface and intermediate water depths, while during the spring the whole water column exposure is high. In September, the lower pH in deep water should even further promote bioavailability and favor waterborne U transfer. Given the potential temporal variability of the parameters that drive U levels in water and its bioavailability (wind stress and water mixing, temperature rise, pH, etc.), one would expect wide variation in U exposure in the studied system over weeks, months and years, which cannot be assessed by occasional measurements in the environment.

Accumulation levels in wild fish increased over the season and remained higher than those measured after laboratory experiments.

As expected, gill, liver and kidney showed high levels of U and confirmed U transfer. Seasonal variation of U accumulation must be related to changes in both waterborne and trophic exposure concentrations. All organs showed high accumulation levels, in particular gonads. U accumulation levels measured in wild fish could induce adverse effects on reproduction as observed in *D. rerio* after laboratory exposure conditions (Bourrachot et al., 2014; Bourrachot et al., 2008; Eb-Levadoux et al., 2017; Simon et al., 2014). U concentration in Danio ovaries (5300 ng g^{-1}) led to significant drastic effects on spawning and fecondity numbers (Bourrachot et al., 2014). Long exposure durations at 20 µg L^{-1} altered the reproduction capacity of Danio (Simon et al., 2014). Molecular effects in Danio ovaries were also observed from a 20 µg L^{-1} exspoure ((Bucher et al., 2016; Eb-Levadoux et al., 2017; Simon et al., 2018).

The accumulation in muscle showed the largest range of level, depending on exposure conditions. Therefore the muscle has high U accumulation capabilities, from low levels after encaged experiments to high levels in wild fish in connection with bioavailability seasonal variation. Together with the gill (epithelium and arch), the accumulation level in muscle could be used as a uranium waterborne exposure marker. The highest accumulation levels were measured in intestine and intestinal content and could contribute to wild fish contamination. Previous studies have shown that U accumulation levels in the intestine are also observed after waterborne exposure (Simon et al., 2018). This was confirmed in this study by the laboratory waterborne exposure experiment (even at environmental U exposure concentrations, 20 µg L^{-1}) on roach, in which intestine and intestinal contents showed the highest levels of U, close to those measured in wild fish. In addition, the U accumulation phenomenon is rapidly reversible once the fish are placed in water without U, as shown by the 5 month laboratory depuration experiment.

However, whereas intestinal concentrations are maximum in September, water concentrations tend to decrease, except at a depth of 15m. Therefore it seems that waterborne exposure cannot alone explain the full accumulation level in the intestine. Indeed, roach have an opportunist food regime that varies with growth and with the seasons (Richeux et al., 1992). This food regime also conditions the living depth of roaches. Consumption of contaminated prey could explain the high accumulation in the intestine and contribute to trophic exposure.

Roaches may therefore live at the bottom of the pond during the cold period and alternate between different horizons during the warmer period; the deep zone serves as a protection zone against predators but is also the place of highest waterborne concentration exposure. Characterization of field exposure remains difficult, as it must take into account the seasonal diversity of fish behaviors and associated heterogeneity of exposure. Given the heterogeneity of waterborne exposure (spatial differences of U in water column and bioavailability) and trophic exposure (seasonal diversity of contaminated U prey), knowing the life behavior and food consumption also seems necessary for predicting U exposure and transfer.

Relevance of laboratory versus in field experiments with regard to accumulation.

This study combines experiments conducted in the laboratory and in the field. The two exposure conditions (20 and 250 µg L^{-1}) selected were expected to correspond, respectively, to a low dose, which could be encountered in the field, and a high dose. After laboratory exposures (20d, pH=6.5), accumulation levels in 4 organs (muscle, gonads, liver, kidney) were lower than those measured in wild fish. The ratio between accumulation levels measured after low exposure and wild fish collected in September in these four organs ranged between 11 and 35. After high exposure, the ratio was close to 3-5. Thus, a long exposure duration (2-6 years old) associated with a low exposure concentration leads to accumulation levels in organs higher than those obtained after a short exposure to high doses in laboratory exposure conditions. Therefore, the realism of controlled laboratory experiments is open to question. Note that otoliths seem to better record accumulation levels from long exposure.

Comparison between lab/field data must raise questions about the objectives of experiments carried out under controlled laboratory conditions. The duration of exposure could explain the high difference between encaged and wild fish and therefore seems to play an important role. The observed inter-individual variability in accumulation levels could be related to the heterogeneity of the age of fish collected *in situ* (not easy to determine precisely) but also to the diversity of behaviors among individuals (time spent in different water depths, selectivity of prey, etc.). Indeed, neither fish mass/length nor mass of otolith seem to correlate with fish age. Under these conditions, measuring the levels of accumulation in fish without knowing their age can be confusing.

4.2 Otoliths as a temporal marker of U exposure?

This aragonite structure from the inner ear of fish grows throughout their life with daily increments and is metabolically inert. As the fish grows, chemical elements are incorporated into the otoliths under the influence of the physicochemical conditions of the surrounding water (chemical composition, temperature) and the composition of their diet. Metals may be incorporated as part of the calcium phase, trapped in interstitial spaces with the crystal, or associated with the protein matrix (Miller et al., 2006). This study demonstrates that U can accumulate in otoliths. Here, the U is mainly found at the edge of the otolith, confirming a seasonal U bioaccumulation. Only a small amount of U seems to be able to cross all the biological barriers and accumulate in the otolith. However, otoliths are able to trap and enable the detection of low levels of U exposure, such as those encountered *in situ*. Moreover, in this study, otolith analysis did not provide robust information on U accumulation in early biological stages, which corresponds to a uranium signal close to the nucleus. In addition, the kinetics of formation of the otolith can vary over time and fish age, thus modifying the rate of U deposits in the otolith. The reduction of otolith formation kinetics combined with a relatively low spatial resolution of U accumulation analysis could prevent identification of each annual peak.

Izzo et al. (2016), Brophy et al. (2004) and Miller et al. (2006) indicate that in otoliths, Ca-like elements, *i.e.* Sr, Ba, Al, Pb and Cd tend to substitute Ca and bind the crystalline otolith matrix. They are then suitable candidates for retrospective analyses because they are less prone to post-depositional alteration. On the contrary, more than 40% of Zn seems to be incorporated into the protein matrix (Avigliano et al., 2015). U, in its UO_2^{2+} chemical form, is known to be likely to disturb Ca homeostasis *via* possible competition with Ca^{2+}; in this way it could act as a Ca analog (Annamalai et al., 2017a, b; Pardoux et al., 2012; Prat et al., 2005), like Sr. Taking into account the fact that the atomic radius of U (240 pm) is close to that of Ca (231 pm), U should be expected to bind within the mineral crystalline matrix (Izzo et al., 2016). But, surprisingly, the U signature in otoliths strictly followed that of Zn. There is little data on this essential metal. The concentration of Zn in otoliths from *Percophis brasiliensis* is known to be associated with fish diet (Avigliano et al., 2015) and 40% to 60% of Zn found in whole otoliths from pacific-habitat cod (*Gadus morhua*) was associated with the soluble portion of the protein matrix (Miller et al., 2006). Miller et al. (2006) concluded that the concentration of Zn in otoliths was likely to be a poor indicator of waterborne Zn exposure. Co-localization of Zn and U as observed in this study could therefore be linked to internalization via the trophic pathway. Diet contribution should help understand the wide variability of U accumulation in otoliths and organs between individuals. Roaches' diet varies according to age and season (Richeux et al., 1992). Juveniles forage on detritus, phytoplankton (diatoms), zooplankton and macroinvertebrates depending on the season, whilst older fish are essentially detritivores. We can therefore assume that the peak of U measured at the edge of the otoliths would be caused by the consumption of contaminated U food leading also to an increase of U in organs. This seasonal diet U would be supported and transported by protein to the edge without being incorporated definitively into the calcium matrix. Nevertheless, part of the U is sequestered, confirming that the measurement of U in otoliths can be a good indicator of the presence of uranium (even at low concentrations and after short term of exposure of U, (Figure 6)) in the medium compared to soft organs that have a strong ability to eliminate U when exposure ends. From this observation, one potential way of reconstructing temporal U exposure in fish is the study of U signals in fossil fish otoliths deposited in sediment.

4.3 Conclusions

In environmental exposure conditions, U is able to be transferred to fish. Seasonal variation of U levels in water and possibly associated with varying uptake pathways led to high accumulation levels in fish organs. Finally, this study is an encouraging first step in using otoliths as an integrative temporal marker of U accumulation, in long- or short-term exposure conditions. A first ecological risk assessment step, the analysis of U at the whole otolith level by ICP-MS, may be sufficient for using otoliths as a U exposure marker. Expanding to other fish species could provide insights into the relationship between fish physiology, especially diet and U accumulation. Further study could determine the U distribution between protein matrices and crystalline lattices, and refine the link between the numbers of peaks and the age of the fish.

5 <u>ACKNOWLEDGEMENTS</u>

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Figure 1. Uranium levels ([U] µg L^{-1}) and abiotic parameters (pH, O$_2$ %, temperature °C) in field water horizons (0, 2, 15m) over 2014 season.

Figure 2. A. Accumulation levels (ng g^{-1}, fw, ± SD, n=10) in organs of wild fish collected in June, July and September 2014. B. Accumulation levels (ng g^{-1}, fw, ± SD, n=10) in organs of wild fish collected in May, July, September 2013 and in fish collected in September 2013 introduced after no U exposure for 5 months.

Figure 3. Accumulation levels (ng g^{-1}, fw, ± SD, n=10) in organs of encaged fish collected (June/15d –July/50d 2014).

Figure 4. Accumulation levels (ng g^{-1}, fw, ± SD, n=6) in organs of fish after 20 days of laboratory waterborne exposure at 20 and 250 µgU L^{-1}.

Figure 5. Average U concentration (ng.mg^{-1}, dw, ±SD, A) and mass (mg, dw, B) in otoliths. Brackets "()" indicate the number of replicates (n). Kruskall-Wallis test.

Figure 6. Elemental signature of U, Zn and Sr concentration ratios (ng mg^{-1}, LA-ICP SF MS) along line transects from 300µm before the nucleus to the edge of the otolith from wild roach *R. rutilus* (A/June, B/September, C/July, D/September, E/July). (F) Photograph of polished otolith after the LA ICP SF MS analysis. Arrow: Nucleus.

Figure 7. Individual U concentration (ng g^{-1}, LA-ICP SF MS) at the edge of the otolith of *R. rutilus* over exposure conditions, coming from integration of the main peak. Age (number of years) is given above the histograms. nd: undetermined.

Highlights:

- U accumulation signals were detected mainly on the edge of fish otoliths using LA-ICP MS.
- Laboratory, caging and field experiments indicated that otoliths were able to store U.
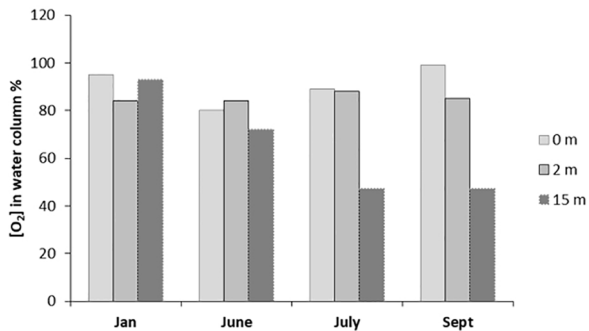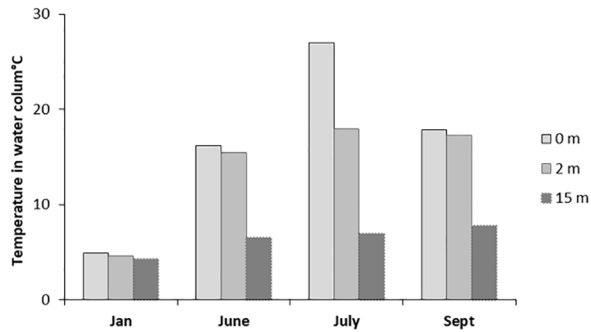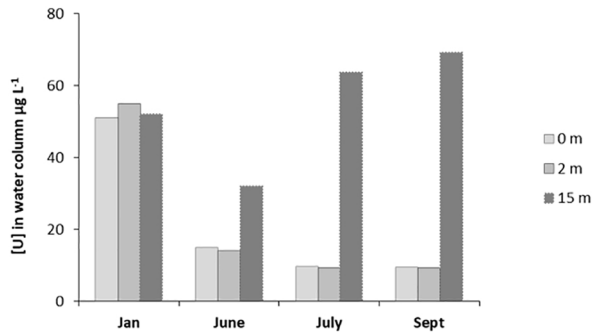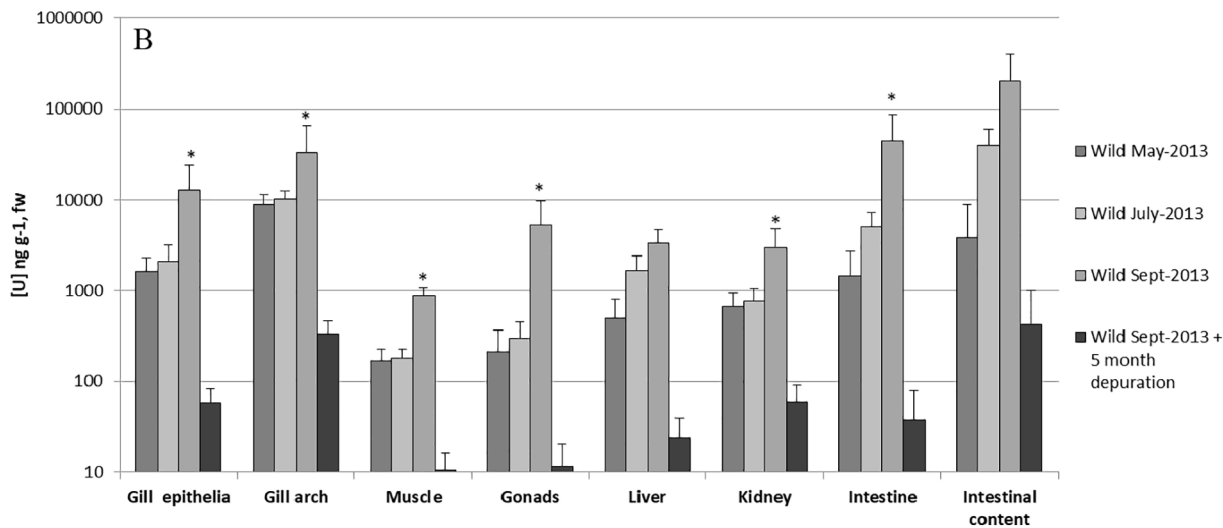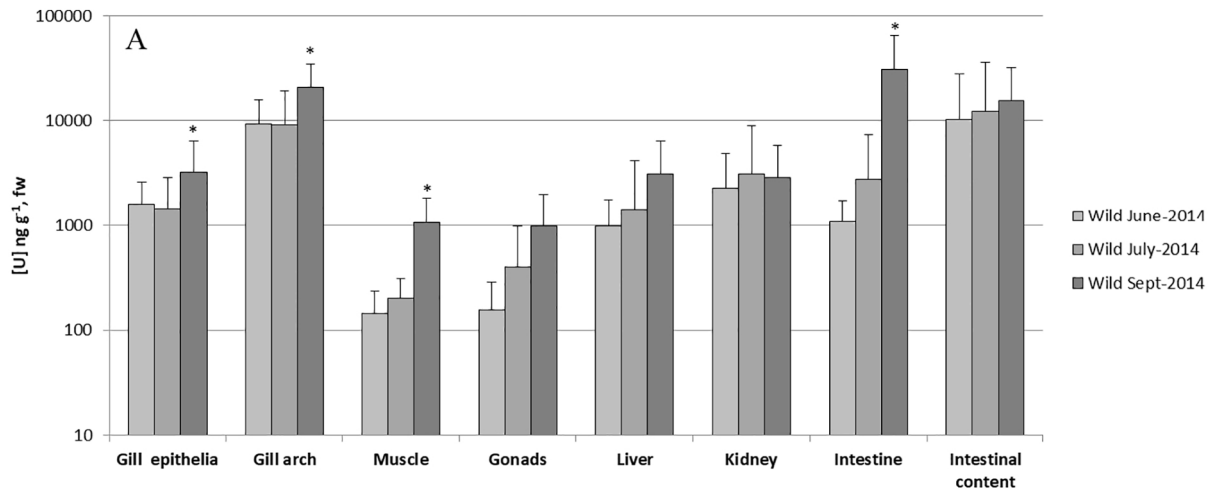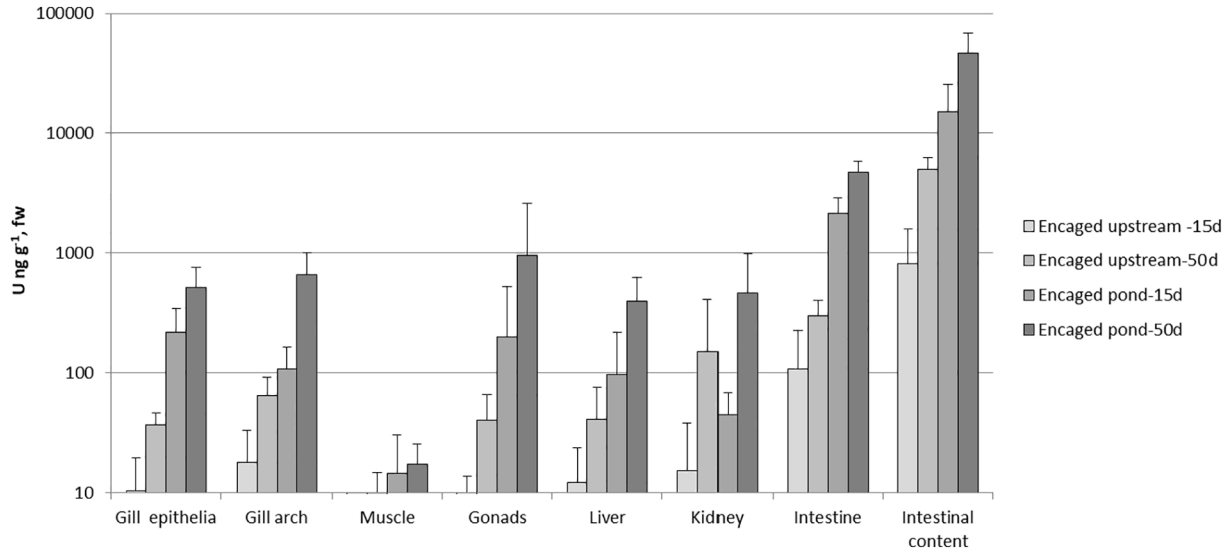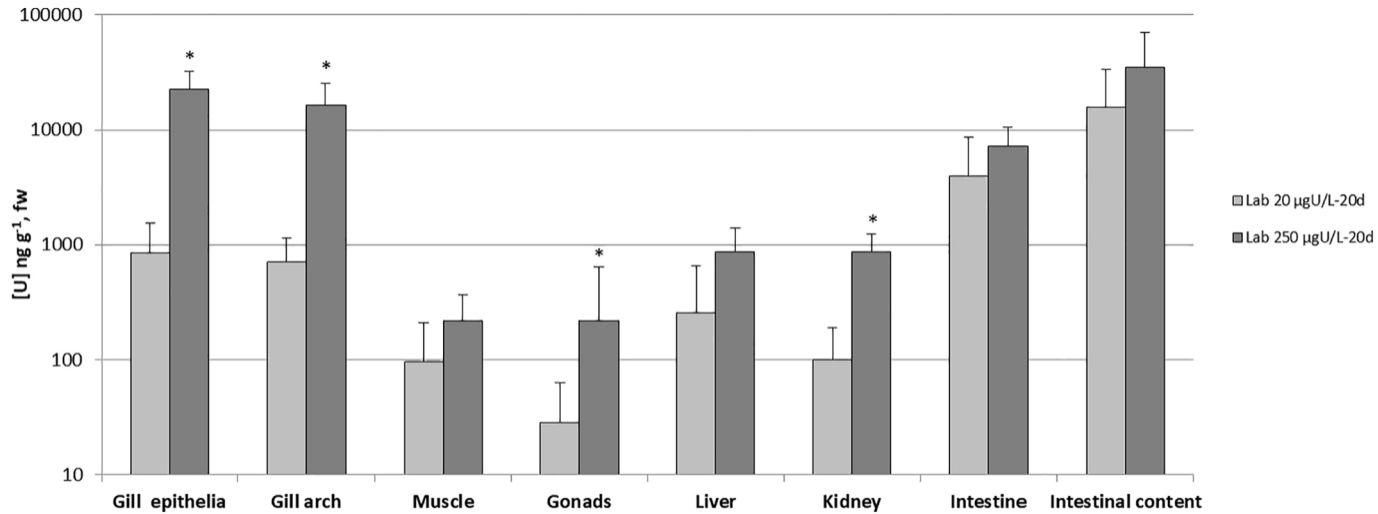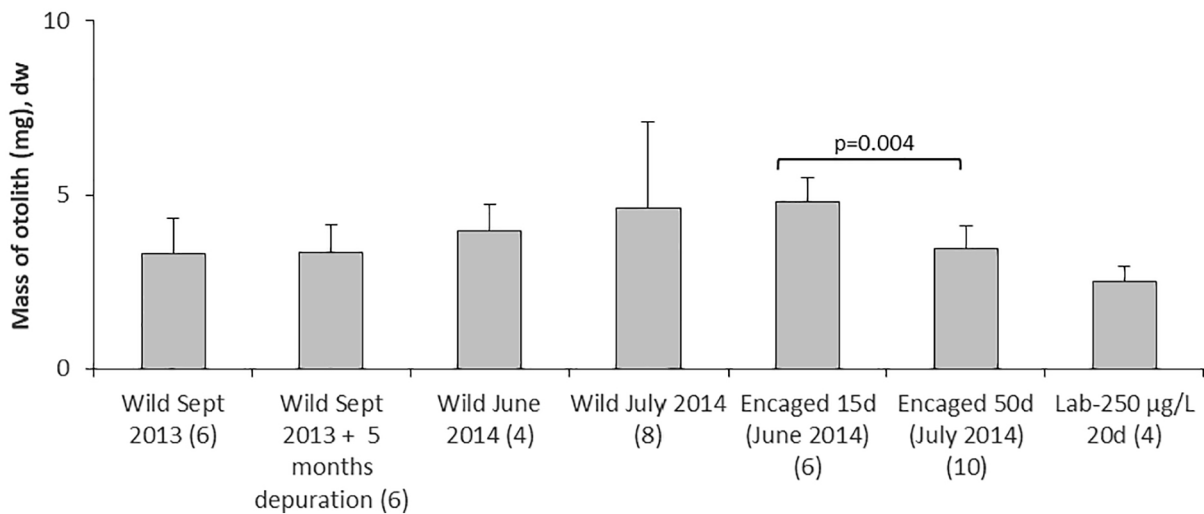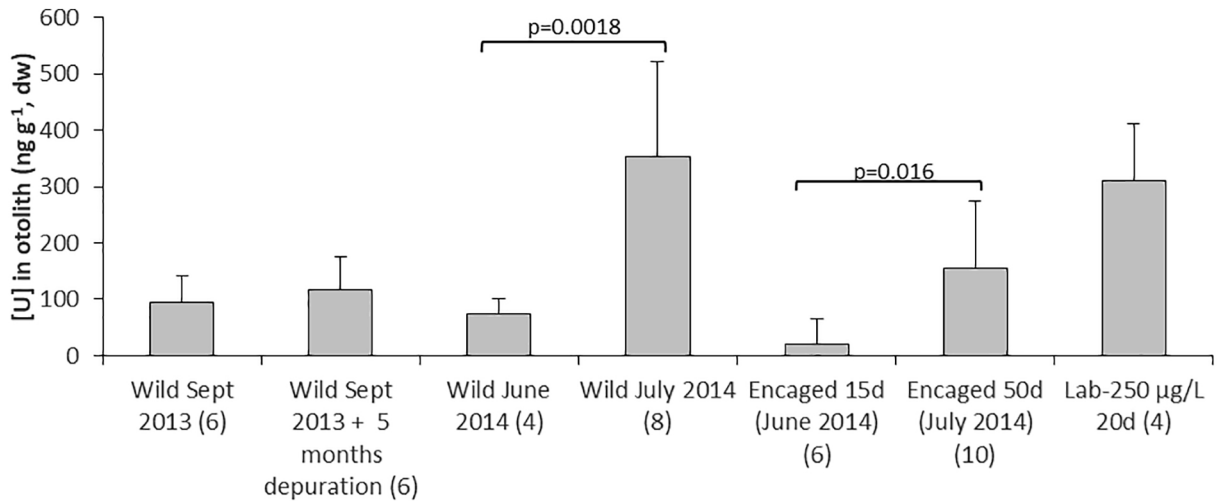- Seasonal variation of U levels were measured in the water column and fish organs.
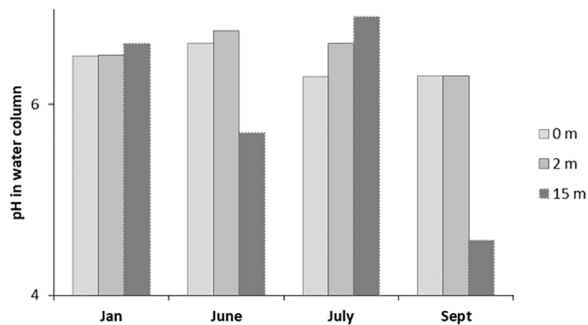
Figure 1

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