Within- and among-clutch variation of yolk perfluoroalkyl acids (PFAAs) in a seabird from the northern Adriatic sea

Abstract

Perfluoroalkyl substances (PFAS) are surface-active agents used in diverse industrial and commercial applications. PFAS contaminate both freshwater and marine ecosystems, are highly persistent and accumulate through trophic transfer. Seabirds are exposed to environmental contaminants due of their high trophic position in food webs and relatively long lifespan. We measured levels of ten perfluoroalkyl acids (PFAAs) in egg yolks of yellow-legged gulls (*Larus michahellis*) breeding in the northern Adriatic sea (NE Italy). We examined PFAAs variation within clutches (between eggs of different laying order) and among clutches. Perfluorooctane sulfonate (PFOS) was the most abundant yolk PFAA (mean = 42.0 ng/g wet weight) , followed by perfluorooctanoic acid (PFOA; 3.8 ng/g ww) and perfluorododecanoic acid (PFDoDa; 2.8 ng/g ww). ΣPFAAs averaged57.4 ng/g ww, ranging between 26.5 and 115.0 ng/g ww. PFAA levels varied substantially among clutches (0.29 - 0.79 of total variation), whereas the effects of laying order were considerably weaker (0.01 - 0.13). Egg laying order effects were detected for ΣPFAAs, PFOS, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA) and PFDoDa, whereby the last-laid eggs exhibited lower PFAAs concentrations than early-laid eggs. Our results indicate that seagulls from the northern Adriatic basin deposit measurable amounts of PFAAs in their eggs. The large among-clutches differences in PFAAs suggest that exposure of yellow-legged gull females to these compounds is highly variable.

Keywords: egg yolk; laying order; *Larus michahellis*; perfluoroalkyl acids (PFAAs)

Running head: Levels of PFAAs vary within- and among-clutches in seagull

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a diverse group of synthetic compounds characterized by strong carbon-fluorine bonds. They have been produced and extensively used as protective coatings in commercial products, as additives in fire-fighting foams, and in many other applications since the 1950s (Wang et al. 2017; Lopez-Antia et al. 2019; Singh et al. 2019). Among the thousands of PFAS, perfluoroalkyl acids (PFAAs) are a family of synthetic, organic compounds consisting of a perfluoroalkyl chain with a functional acid group (Buck et al. 2011). As a consequence of their widespread use, PFAAs enter the environment through direct (i.e., industrial production and usage) and indirect (i.e., degradation of precursor compounds) pathways (Martin et al. 2010; Butt et al. 2014). A number of studies have shown the presence of PFAS, and specifically PFAAs, in both abiotic (e.g, De Silva et al. 2011; Houde et al. 2011; Groffen et al. 2018) and biotic matrices (e.g., Smithwick et al. 2005; Braune and Letcher 2013; Gebbink et al. 2009; Gewurtz et al. 2013, 2016), including human tissues and organs (e.g., Monroy et al. 2008; Roosens et al. 2010; Olesen et al. 2016). Because of their widespread presence and potential toxicity, the perfluorooctane sulfonic acid (PFOS) and perfluorooctane sulfonic acid (PFOA) have been classified as Persistent Organic Pollutants (POPs), while others are under consideration to be listed as new POPs (Stockholm Convention 2019). In addition, because of their bioaccumulation potential and toxicity to diverse organisms (Conder et al. 2008; Fang et al. 2014), concerns have been raised towards long-chain perfluoroalkyl sulfonic acids (PFSAs) and perfluoroalkyl carboxylic acids (PFCAs), leading to regulatory measures in their production in Europe and North America (Gebbink et al. 2015; Kim and Oh 2017). Nevertheless, global PFAA levels are still high and even increasing in some countries (Ahrens et al. 2011; Miller et al. 2015; Groffen et al. 2017), suggesting that environmental monitoring of these substances is of high priority. For these reasons, biomonitoring of PFAAs was performed using diverse aquatic (e.g., Giesy and Kannan 2001, 2002; Hong et al. 2015; Khairy et al. 2019; Dong et al. 2020) and terrestrial (e.g., Gewurtz et al. 2018; Groffen et al. 2019) organisms.

A growing number of studies have investigated PFAAs contamination in diverse species of birds, and many focused on seabirds (e.g., Vicente et al. 2012; Bertolero et al. 2015; van der Schyff et al. 2020). Seabirds, as top-predators, are exposed and affected by diverse environmental contaminants, including legacy POPs and PFAS, because of their feeding habits and their longevity (Furness and Camphuysen 1997). Several studies have demonstrated that PFAS are efficiently accumulated in seabirds through their diet (Fisk et al. 2001) and are maternally transferred to offspring via the eggs according to a variety of individual and environmental factors (Pusch et al. 2005). Gull species show a high accumulation potential, mainly due to their high energy requirements and feeding rates (Braune and Norstrom 1989; Duhem et al. 2005). Indeed, high levels of diverse PFAS have been measured in liver, blood and plasma (e.g., Verreault et al. 2005; Gebbink and Letcher 2012), as well as in the eggs of different gull species (e.g., Verboven et al. 2009; Vicente et al. 2012; Bertolero et al. 2015). Gull eggs represent a non-invasive tool that has been successfully used in diverse biomonitoring studies exploring the levels of PFAS (mainly PFAAs) in several geographical areas (Gebbink and Letcher 2012; Vicente et al. 2012; Nordèn et al. 2013; Lopez-Antia et al., 2017; Bertolero et al. 2015). While it has been demonstrated that PFAAs can be maternally transferred to the eggs, information on the within-and among-clutch variation of PFAA levels in bird eggs is limited (Custer et al. 2012; Vicente et al. 2015; Laster et al. 2019). For example, previous studies have demonstrated that PFOS concentrations decreased with order in the laying sequence in eggs of the Audouin's gull (*Larus audouini*; Vicente et al. 2015) and the great tit (*Parus major*; Lasters et al. 2019), but no clear trends were found for other PFAAs. Moreover, previous studies suggested limited variation among females in the amount of PFAAs deposited in their eggs (Lasters et al. 2019). Assessing the within- and among-clutch variation in contaminant concentrations might help in planning in biomonitoring studies (Pastor et al. 1995). If variation among-clutches is considerably greater than variation within-clutches, sampling and assaying contaminants in a single egg (instead of the whole clutch) could provide reliable estimates of contaminant levels in the entire clutch, and possibly of the level of environmental exposure to PFAAs of the hen (Pastor et al. 1995).

In this study we investigated the within- and among-clutch variation of ten PFAAs measured in the yolk from eggs of yellow-legged gulls (*Larus michahellis*) collected at a colony located along the northern Adriatic coast of Italy (Northeastern Italy). The sampling area lies ca. 20 km south of the mouth of the Po River, a highly polluted river that it has been estimated to annually discharge approximately 3 tons of PFAAs (mainly perfluorooctanoic acid, PFOA) into the Adriatic Sea (Loons et al. 2009). Previous studies from the Mediterranean Sea and the south European Atlantic coast have shown broad differences among sampling sites in PFAAs concentrations in seagull eggs from river and industrialized areas showing higher levels of PFAAs (mainly PFOS) contamination (Vicente et al. 2012). In seagulls from the northern Adriatic Sea, close to the mouth of the Po river, we thus expected to detect relatively high concentrations of yolk PFAAs.

2. Materials and Methods

2.1 Egg sampling

The yellow-legged gull (*Larus michahellis*) is a monogamous species inhabiting mostly coastal habitats across the Mediterranean, where it breeds colonially (Cramp 1998). The clutch size ranges between 1 and 3 eggs, which are laid at 1–4 day intervals and hatch 25–31 days after laying (Cramp 1998). The study was conducted during March-May 2016, at large colony of yellow-legged gulls (ca. 400 pairs) settled on small islands located in the Comacchio lagoon (Northeastern Italy; 44° 20′ N, 12° 11′ E). While breeding, birds can forage over a broad area surrounding the colony (up to 30-40 km), exploiting diverse habitats, including pelagic environments, farmland, fresh-, brackish- and salt-water habitats, mudflats, harbours, and landfills (Mendes et al. 2018). At the beginning of the breeding season, the colony was visited at two days intervals. When nests were found, newly laid eggs were marked with a water-proof marker. Eggs were left in the nest until laying of the third (last) egg, when then they were all collected and transported to the lab. Eggs form 15 entire clutches were collected, for a total of 45 eggs (Table S1). First-, second- the third-laid eggs were indicated as a-, b-

100 and c-eggs, respectively. As previous studies showed that PFAA were not detected in the albumen
101 isolated from the eggs of two different *Larus* species, namely the Audouin's gull (*Larus audouini*;
102 Vicente et al. 2015) and the herring gull (*Larus argentatus*; Gebbink and Letcher 2012), yolk of each
103 egg was carefully isolated from the albumen, stored in 50 mL tubes and frozen at -20 °C until the
104 analysis of PFAAs.

105 *2.2 Analysis of PFAAs in egg yolk*

106 The extraction of ten PFAAs, namely Perfluoropentanoic acid (PFPeA), Perfluorohexanoic acid
107 (PFHxA), Perfluoroheptanoic acid (PFHpA), Perfluorooctanoic acid (PFOA), Perfluorononanoic
108 acid (PFNA), Perfluorodecananoic acid (PFDA), Perfluoroundecanoic acid (PFUnA),
109 Perfluorododecanoic acid (PFDoDA), Perfluorohexanesulfonic acid (PFHxS) and Perfluorooctane
110 sulfonic acid (PFOS), from yolk samples was carried out according to the method of Mazzoni and
111 co-authors (2016). Briefly, ca. 1 g of yolk was weighed and spiked with 100 µL of an internal standard
112 methanolic solution (40 µg/L, MPFAC-MXA and M3PFPeA solutions, Wellington Laboratories,
113 Guelph, ON, Canada). A mixture of acidic water-acetonitrile (10:90 v/v) solution was added to the
114 spiked sample. The sample was subjected to ultra-sonication extraction, a treatment with
115 MgSO4/NaCl, freezing and centrifugation. Afterwards, the extracts were partially evaporated and
116 then filtered by HybridSPE®-Phospholipid Ultra cartridges (Merck KGaA, Darmstadt, Germany) to
117 eliminate phospholipids. The final extracts were analyzed by liquid chromatography coupled to mass
118 spectrometry (UHPLC-MS/MS) after an online purification with turbulent flow chromatography
119 (TFC). Quantification was done using the isotopic dilution method. Limits of Detection (LODs) and
120 limits of Quantification (LOQs) in yolk were estimated, according to ISO Standard 6107-2: 2006, as
121 respectively, three-fold and tenfold the standard deviation of an procedural blank. LOD and LOQ
122 values ranged from 0.01 to 0.1 and from 0.03 to 0.3 ng/g yolk wet weight, respectively.
123 Concentrations of PFAAs in first- (a-), second- (b) and third-laid (c-eggs) eggs collected from 15
124 nests were measured. However, values of PFPeA and PFHxA were considered for eggs from only 5

nests because in further analyses we noted blank contamination for these compounds. For these reason, values of PFPeA and PFHxA obtained from the eggs of the other 10 nests were excluded from the dataset. No blank contamination occurred for the other PFAS compounds.

2.3 Statistical analysis

Within- and among-clutch variation in the yolk concentrations of maternally-transferred PFAAs was investigated by linear mixed models (LMMs) with order in the laying sequence as a fixed effect factor and the clutch (maternal) identity as a random intercept effect factor. We estimated the proportion of total variance explained by position in the laying sequence (marginal R^2, R^2_m hereafter), representing within-clutch effects among eggs differing in laying order, the proportion of total variance explained by clutch identity (adjusted intra-class correlation coefficient, ICC_{adj} hereafter), and the proportion of total variance explained by the mixed model (conditional R^2, R^2_c hereafter) (Nakagawa et al. 2017). LMMs were fitted by means of the 'lme4' R package (Bates et al. 2015), while variances were obtained with the R package 'performance' (v. 0.3.0) (https://easystats.github.io/performance/). Significance of the variance explained by the random effect was tested using a likelihood ratio test (Zuur et al. 2009). Statistical analyses were ran in R 3.6.1 (R Core Team 2019). Models were not fitted for PFPeA and PFHxA as these compounds were measured in eggs from 5 nests only and were detected in a limited number of eggs (see Table 1).

3. Results

PFAAs (namely $PFCA_{C7-C12}$, PFHxS and PFOS) were detected in measurable concentrations in all the clutches (Table 1), with the exception of PFPeA and PFHxA. The latter two PFAAs were analysed in only five nests and their yolk concentrations ranged from <LOD to 0.12 ng/g yolk wet weight (ww). The percentage of detection was 67% and 87% for PFPeA and PFHxA, respectively. The ΣPFAAs (mean ± standard deviation) across all samples eggs was 57.4 ± 18.0 ng/g yolk ww. Yolk concentrations of each single PFAAs and ΣPFAAs were not related neither to the egg weight at the

time of laying (Pearson correlation coefficient $r < 0.200$; $p > 0.187$ in all the cases) nor to the yolk weight ($r < 0.216$; $p > 0.154$ in all the cases). On average, PFOS (concentration range: 18.8 – 90.2 ng/g yolk ww) was the principal contributor to the ΣPFAAs (Figure S1), accounting for 73% of the fingerprint, followed by PFOA (concentration range: 1.3 – 15. 4 ng/g yolk ww; 7 %), PFDoDa (concentration range: 0. 9 – 6.1 ng/g yolk ww; 5%), PFDA (concentration range: 1.2 – 5.6 ng/g yolk ww; 5%), PFUnA (concentration range: 1.0 – 5.7 ng/g yolk ww; 5%) and PFNA (concentration range: 0.7 – 3.9 ng/g yolk ww; 3%). PFPeA, PFHxA PFHpA and PFHxS constituted < 3% to the ΣPFAAs (concentration range: <LOD – 4.7 ng/g yolk ww). Variation among clutches was substantial, with the proportion of total variance explained by clutch (i.e., maternal) identity ranging between 0.29 for PFOS to 0.79 for PFOA, with most other PFAAs showing values > 0.50 (Table 2).

Within-clutch effects, reflecting variation according to order in the laying sequence explained a much lower proportion of variance compared to among-clutch effects (Table 2). Significant changes of PFAA levels throughout the egg-laying sequence were observed for the ΣPFAAs and for some homologues (Table 1 and 2). Concentrations of PFOS and other long-chain PFAAs ($PFCA_{C9-C12}$)

decreased according to the position in the laying sequence (Table 2; Figure 1) (proportion of variance explained: 0.09 - 0.12), while laying-order effects for other PFAAs (i.e., PFHpA, PFOA, PFHxS) were negligible (proportion of variance < 0.05) (Table 2; Figure 2). Post-hoc tests showed that no differences in PFAA concentrations occurred between a- and b-eggs ($P > 0.189$ for all the cases), while levels measured in c-eggs resulted as significantly lower than those of a- and b- eggs. In detail, concentrations present in c-eggs was lower than in a- and b-eggs for the ΣPFAAs (a- vs c-eggs: $P = 0.013$; b- vs c-eggs: $P = 0.028$), PFOS (a- vs c-eggs: $P = 0.011$; b- vs c-eggs: $P = 0.024$), PFDA (a- vs c-eggs: $P = 0.004$; b- vs c-eggs: $P = 0.023$), PFUna (a- vs c-eggs: $P < 0.001$; b- vs c-eggs: $P = 0.020$) and PFDoDa (a- vs c-eggs: $P = 0.002$; b- vs c-eggs: $P = 0.050$).

4. Discussion

The present study demonstrated that different PFAAs were maternally transferred to the eggs of yellow-legged gulls breeding in a colony located in the northern Adriatic Sea. Although the number of measured PFAS, differed among studies (see Table 3), the levels of detected in our survey were in line with those reported in eggs of the same species from other polluted areas of the Mediterranean, close to large rivers of industrialized areas, while they were higher than those measured in eggs of birds breeding in less disturbed areas (e.g., Atlantic islands near the Iberian peninsula; Vicente et al. 2012). Egg levels of PFAAs were however lower than those observed in other gull species from diverse geographical areas. For instance, the levels of five PFAS (PFNA, PFHxS, PFOA, PFBS and PFOS) measured in the yolk of Audouin's gull eggs breeding in the Ebro delta were up to four-fold greater compared to those measured in the present study (Vicente et al. 2015). Similarly, the levels of PFAAs, specifically perfluorinated sulfonates (PFSAs) and perfluorinated carboxylic acids (PFCAs) were lower than those found in the yolk of herring gull (*Larus argentatus*) eggs from the North American Laurentian Great Lakes (Gebbink and Letcher 2012; Letcher et al. 2015; Su et al. 2017), independently of the position in the laying sequence.

Despite differences in levels of PFAAs transferred to the eggs, the fingerprint of eggs collected in a colony from the northern Adriatic sea was similar to that observed in gull eggs from other geographical areas (Verreault et al. 2005; Gebbink et al. 2011a,b; Vicente et al. 2012). As expected, long-chain PFAAs were transferred to the eggs to a greater extent than short-chain homologues because of their greater bioaccumulation potential (Conder et al. 2008; Olesen et al. 2016) and/or higher affinity for certain components of eggs. Focusing on PFOS, which was the more abundant compound found in yellow-legged gull eggs, the levels measured in the present study were lower than those found in eggs of this species collected in the same colony located in the Comacchio lagoon during the breeding season of 2015 (concentration range: 31 – 687 ng/g yolk ww; Parolini et al. 2016; Mazzoni et al. 2016). These results suggest limited PFOS dietary intake and consequent maternal transfer to the eggs or alternatively a decrease in environmental levels, although two time points are

198 not adequate to distinguish temporal trends from inter-annual variability. Moreover, PFOS levels we
199 measured were similar to those found in yellow-legged gull eggs collected in three sites of the
200 northern basin of the Mediterranean Sea, but higher than those measures in southern and western
201 basin (Vicente et al. 2012). Interestingly, the second contributor to the PFAAs fingerprint was PFOA.
202 These results are in contrast with those from previous studies investigating PFAAs contamination in
203 the eggs from the yellow-legged gull and Andouin's gull from the Iberian peninsula that documented
204 limited concentrations of this compound (Vicente et al. 2012; 2015). Thus, our results suggest
205 different sources of contamination between geographical areas and the presence of local sources of
206 PFOA. In fact, a fluoropolymer plant has been individuated to discharge its waste within the Po River
207 basin (Valsecchi et al. 2015; Rusconi et al. 2015), whose mouth is close to the sampling site. Previous
208 monitoring studies of PFAS distribution in surface waters of the Po River (Valsecchi et al. 2015) and
209 in Adriatic seawater off-shore from Venice (Loos et al. 2013) demonstrated that PFOA and PFOS
210 were the predominant PFAS measured in the Po River basin. Thus, although sulfonated PFAS (e.g.,
211 PFOS) are more bioaccumulative than the carboxylated ones (e.g., PFOA and PFNA; Conder et al.
212 2008), the higher levels of PFOA (46 ng/L) compared to PFOS (5 ng/L) measured in surface water
213 of the Po River at basin closure might explain the presence of PFOA in yellow-legged gull eggs in
214 measurable concentrations with respect to other geographical areas.

215 The main finding of our work is related to within- and among-clutches variation of PFAAs
216 concentrations in yellow-legged gull eggs. In spite of substantial among-clutches variation of PFAAs
217 levels, within-clutches variation was noted for yolk concentrations of PFOS and $PFCA_{C9\text{-}C12}$
218 compounds, whose levels decreased according to the position in the laying sequence. This decreasing
219 trend in concentrations of some PFAAs might be due to environmental or physiological constraints
220 experienced by the hens limiting their ability to acquire, and subsequently transfer, contaminants to
221 consecutive eggs (e.g., food availability, condition and age; Rubolini et al. 2011; Parolini et al. 2015;
222 2017). Our results were consistent with a previous study of the Audouin's gull showing a significant

223 decrease of PFOS concentrations, but not of PFNA, PFHxS and PFOA, according to the position in
224 the laying sequence, with marked differences between a- or b-eggs and c-eggs (Vicente et al. 2015).
225 A similar laying-order effect was also noted in a previous study monitoring the within-clutch variation
226 of certain organohalogen compounds (i.e., polychlorinated biphenyls,
227 dichlorodiphenyltrichloroethane and hexachlorobenzene) in the eggs of Audouin's gull from the Ebro
228 delta (Pastor et al. 1995). Our findings are the first to demonstrate a significant laying order effect
229 for long-chain PFAAs such as PFDA, PFUna and PFDoDa. Although a previous investigation
230 demonstrated changes in levels of these compounds in eggs of the great tit depending on the position
231 in the laying sequence, no clear egg order trend was found (Lasters et al. 2019).

232 Seagulls are income breeders and mainly use exogenous resources ingested at the breeding sites for
233 the egg formation rather than those stored in their body (Hobson 1995). Thus, the allocation of
234 exogenous resources to egg formation might alter the egg composition and, at the same time, the
235 burden of pollutants (Verreault et al. 2006). Consequently, large variations in PFAA concentrations,
236 which are associated with these resources might be expected and explain the high among-clutch
237 variation. In addition, the among-clutch variation observed for most of PFAA compounds could
238 depend on individual difference in the foraging areas and/or the diet experienced by females coming
239 into lay, which may affect their level of exposure to PFAAs. Indeed, feeding on fishing discards, at
240 landfills or consuming terrestrial preys or other organisms can affect the levels of PFAAs
241 accumulated in maternal tissues, and consequently transferred to the eggs (Vicente et al. 2012). In
242 addition, as suggested by a previous study by Verrault and coauthors (2006), organohalogen
243 contaminants are differentially retained in the body of herring gull females depending on chemico-
244 physical features of each specific compound, potentially affecting their transfer to the eggs. Previous
245 studies demonstrated that PFOS and other PFAS are transferred to eggs during laying in
246 concentrations differing between eggs that can reflect differences in adult body burden (Gebbink and
247 Letcher 2012; Bertolero et al. 2015). Although limited information for the vast majority of PFAS is

248 currently available, PFOS tends to associate with low density lipoproteins and other egg-yolk proteins
249 synthesized in the liver (Bertolero et al. 2015). These complexes are then transferred *via* the blood to
250 the ovary and into the eggs (Yoo et al. 2009, Vicente et al. 2015). As heavier eggs presumably have
251 higher lipoprotein concentrations, they might contain greater higher PFOS concentrations (Lasters et
252 al. 2019). Considering that the weight of yellow-legged gull eggs declines with laying order (marked
253 decrease between the second and the third egg; Rubolini et al. 2011), the decrease in concentrations
254 of PFOS and other long-chain PFAAs in the final egg laid might suggest a decrease in female body
255 burden of these contaminants or a lower rate of maternal transfer. However, these hypotheses need to
256 be further evaluated as no studies to date have investigated changes in body burden of PFAAs in
257 females during the laying period and compared the maternal levels with those transferred to the eggs.

258 Embryonic exposure to PFAAs might adversely affect development through various pathways, e.g.
259 by altering thyroid hormone function and immune system maturation (Mattsson et al. 2019), although
260 a previous of the yellow-legged gull showed that the *in-ovo* injection of environmentally realistic
261 doses of PFOS did not affect the development and the oxidative status of yellow-legged gull embryos
262 (Parolini et al. 2016). *In vitro* studies have demonstrated that the exposure to long-chain PFAS
263 inhibited aromatase activity and alterated of cellular lipid profile in human placental cells
264 (Gorrochategui et al. 2014a), as well as caused changes in lipidome of human placental
265 choriocarcinoma (JEG-3) cells (Gorrochategui et al. 2014b), suggesting potential endocrine effects
266 of fluorinated compounds could occur in other vertebrate species. Despite limited information adverse
267 effects of PFAS towards embryonic or early post-natal development, considering the lower amount
268 of PFAAs transferred to the last laid eggs, future studies may investigate the potential differential
269 effects of these compounds for offspring pre- and post-natal development according to laying order.
270 In fact, chicks hatched from a- or b-eggs containing higher levels of contaminants may experience
271 stronger detrimental effects from PFAAs exposure than their siblings hatched from the final egg laid.
272 In the yellow-legged gull, differences in the quality of the eggs depending on the order in the laying

sequence occur, whereby the concentrations of vitamin E and carotenoids decrease, while those of androgens increase along the laying sequence (Rubolini et al. 2011). Last laid c-eggs are typically smaller and almost invariably hatch later resulting in the final chick experiencing severe competitive handicap compared to their older siblings (Rubolini et al. 2011). Thus, if the allocation of less vitamins and carotenoids to c-eggs could exacerbate the fitness disadvantage of c-chicks, the transfer of lower amount of contaminants could minimize disadvantage. Such potential differential effects may have far-reaching consequences for the phenotypic composition of gull populations, as chicks from early laid eggs are generally of higher phenotypic quality than those from last-laid eggs (Rubolini et al. 2011; Parolini et al. 2015). For this reason, in studies exploring the toxicity of contaminants in pre- and post-natal life stages of the yellow-legged gull and other avian species, the laying order effect needs to be carefully considered.

5. Conclusions

Our results demonstrated large among-clutches variability of PFAA levels, whereas the effect of laying order was much weaker. There was a trend for some compounds and ΣPFAAs to be at lower concentrations in the last- compared to first- and second-laid eggs. Whilst the decrease of PFOS concentrations in last-laid eggs was expected and supported previous findings for other gull species, this is the first study to report a laying order effect for long-chain PFCAs, namely PFDA, PFUnA and PFDoDa.

Our results might have important implications in PFAAs biomonitoring of coastal areas using seagull eggs. In fact, despite the weak within-clutch variation, sampling and analyzing the levels of PFAAs in the first- or second-laid eggs is recommended to assess the contamination levels of a certain area, while the use of the last-laid egg might lead to underestimation of the contamination levels. This is particularly true for long-chain PFAAs, whose levels were significantly lower in last-laid eggs compared to first- and second-laid ones. This precaution should allow to reduce the number of eggs sampled in biomonitoring surveys and, at the same time, to present a novel accurate picture of the

PFAAs contamination. Despite the large among-clutch variation and the high repeatability of contaminant levels within a given clutch, randomly sampling one egg per clutch could not allow a reliable assessment of PFAAs egg concentrations. In fact, considering the differences of PFAS concentrations depending on the order in the laying sequence, sampling consistently the first-laid or second-laid egg would be preferable in long term studies performed over different years and/or to compare contamination levels among colonies because it permits a better comparison among results. Finally, the substantial among-clutch variation suggests that egg PFAAs levels could represent a reliable proxy to assess the environmental exposure to PFAAs by individual females. Large differences among individual females in feeding habits, diet and spatial behavior during breeding and non-breeding seasons may indeed result in differing exposure among variably contaminated environments (e.g., Gentes et al. 2015). Egg PFAAs levels may thus integrate exposure across different spatial and temporal scales. Once egg contaminant levels are coupled with detailed information on individual movements (e.g., by GPS tracking) and diet analyses (e.g., via isotope analyses), they may assist in identifying possible contamination sources (Gentes et al. 2015). For all these reasons, further research is a priority to for enhancing knowledge on distribution of PFAAs, as well as on maternal transfer and potential adverse effects of these fluorinated compounds towards offspring.

Data availability: Data are available in Supporting Information (Table S2)

Conflict of interest: the authors declare to have no conflict of interest.

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Table 1: Concentrations (mean ± SD and range) of PFAAs measured in the yolk isolated from yellow-legged gull eggs collected in the northern Adriatic. Concentrations detected in first- (a-), second- (b) and third-laid (c-eggs) eggs from 15 nests are reported. Levels of PFPeA and PFHxA were measured only in eggs from 5 nests. Number of eggs in which PFAA homologues were quantified is reported (N eggs). Concentrations are expressed as ng/g yolk wet weight. ΣPFAAs calculated as the sum of $PFCA_{C7-C12}$, PFHxS and PFOS. To calculate the mean of PFPeA and PFHxA ½ LOD was included as a surrogate when values were <LOD.

Compound	Laying order	Mean ± SD (N eggs)	Range
PFPeA	a-eggs	0.03 ± 0.02 (5)	<LOD - 0.09
	b-eggs	0.03 ± 0.05 (5)	<LOD - 0.12
	c-eggs	0.04 ± 0.03 (5)	<LOD - 0.02
PFHxA	a-eggs	0.08 ± 0.03 (5)	0.06 - 0.124
	b-eggs	0.07 ± 0.03 (5)	<LOD - 0.12
	c-eggs	0.07 ± 0.02 (5)	<LOD - 0.08
PFHpA	a-eggs	0.6 ± 0.2 (15)	0.2 - 1.1
	b-eggs	0.7 ± 0.4 (15)	0.3 - 1.8
	c-eggs	0.7 ± 0.4 (15)	0.3 - 1.9
PFOA	a-eggs	3.6 ± 1.5 (15)	1.9 - 7.8
	b-eggs	4.0 ± 3.0 (15)	1.4 - 14.4
	c-eggs	3.8 ± 3.3 (15)	1.3 - 15.4
PFNA	a-eggs	2.0 ± 0.7 (15)	0.8 - 3.9
	b-eggs	1.9 ± 0.8 (15)	0.9 - 3.9
	c-eggs	1.7 ± 0.7 (15)	0.7 - 3.4
PFDA	a-eggs	3.0 ± 1.0 (15)	1.6 - 5.0
	b-eggs	2.9 ± 1.0 (15)	1.6 - 5.6
	c-eggs	2.4 ± 0.8 (15)	1.2 - 4.4
PFUnA	a-eggs	3.1 ± 1.3 (15)	1.1 - 5.5
	b-eggs	2.7 ± 1.1 (15)	1.3 - 5.7
	c-eggs	2.1 ± 0.7 (15)	1.0 - 3.7
PFDoDA	a-eggs	3.2 ± 1.4 (15)	1.0 - 6.1
	b-eggs	2.9 ± 1.1 (15)	1.2 - 5.5
	c-eggs	2.3 ± 0.8 (15)	0.9 - 3.5
PFHxS	a-eggs	0.8 ± 0.5 (15)	0.3 - 1.9
	b-eggs	0.8 ± 0.4 (15)	0.1 - 1.4
	c-eggs	0.8 ± 0.5 (15)	0.2 - 2.0
PFOS	a-eggs	46.2 ± 15.0 (15)	18.8 - 76.9
	b-eggs	44.8 ± 16.2 (15)	24.7 - 90.2
	c-eggs	35.0 ± 6.9 (15)	22.1 - 46.3
ΣPFAAs	a-eggs	62.5 ± 18.7 (15)	26.5 - 95.3

	b-eggs	60.8 ± 20.4 (15)	35.1 - 114.9
	c-eggs	48.8 ± 11.6 (15)	27.9 - 77.4

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Table 2: Linear mixed models of the effect of order in the laying sequence on the concentration of different PFAAs measured in yellow-legged gull egg yolks. Clutch identity was included as a random intercept effect. The proportion of the total variance explained by the model (R^2_c) is reported, together with the proportion of variance explained by the fixed effect (variance within-clutches, explained by laying sequence effects) (R^2_m) and the proportion of variance explained by the random effect (variance among-clutches, or adjusted repeatability, ICC_{adj}). Statistically significant effects are bolded. Degrees of freedom for *F*-tests were estimated according to the Sattertwhaite's approximation, while significance of the random effect of clutch identity was tested by a likelihood ratio test.

Compound	**Effect of laying sequence**			**Effect of clutch identity**		**Variance components**		
	F	df	*P*	χ^2	*P*	R^2_c	R^2_m	ICC_{adj}
PFHpA	1.27	2, 28	0.30	25.19	< **0.001**	0.73	0.02	**0.72**
PFOA	0.45	2, 28	0.64	32.38	< **0.001**	0.79	0.01	**0.79**
PFNA	2.83	2, 28	0.08	20.44	< **0.001**	0.68	0.04	**0.67**
PFDA	5.34	2, 28	**0.011**	18.14	< **0.001**	0.67	**0.09**	**0.64**
PFUnA	7.40	2, 28	**0.003**	14.99	< **0.001**	0.64	**0.13**	**0.59**
PFDoDA	5.64	2, 28	**0.009**	12.38	**0.004**	0.59	**0.11**	**0.54**
PFHxS	0.04	2, 28	0.96	9.09	**0.003**	0.47	0.01	**0.47**
PFOS	4.38	2, 28	**0.022**	3.36	0.07	0.38	**0.12**	0.29
ΣPFAAs	4.15	2, 28	**0.026**	4.21	**0.040**	0.40	**0.12**	**0.32**

556 **Table 3**: Mean levels of different PFAS measured in eggs of diverse seagull species collected in different locations worldwide.

Study area	Species	Investigated PFAS	Mean concentration (ng/g ww)	Reference
Ebro Delta (Spain)	Yellow-legged gull (*Larus michahellis*)	PFOS	75.1 ± 31 (SD)[a]	Bertolero et al. 2015
Ebro Delta (Spain)	Audouin's gull (*Larus audouini*)	PFOS	87.9 ± 23 (SD) [a]	Bertolero et al. 2015
Chantry Island, Lake Huron (Canada)	Herring gull (*Larus argentatus*)	PFSAs (PFBS, PFHxS, PFOS,PFDS) and PFCAs (PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA)	ΣPFSA = 258 ± 39 (SE) [a] ΣPFCA = 88 ± 9 (SE) [a]	Gebbink and Letcher 2012
North American Great Lakes (USA and Canada)	Herring gull (*Larus argentatus*)	PFSAs (PFBS, PFHxS, PFOS,PFDS) and PFCAs (PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA)	ΣPFSA = 44.4 – 740 ΣPFCA = 15.3 - 118	Letcher et al. 2015
North American Great Lakes (USA and Canada)	Herring gull (*Larus argentatus*)	PFSAs (PFBS, PFHxS, PFOS,PFDS) and PFCAs (PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA)	ΣPFSA = 36 - 664 ΣPFCA = 8.69 – 63.1	Su et al. 2017
Ebro Delta (Spain)	Audouin's gull (*Larus audouini*)	PFNA, PFHxS, PFOA, PFBS and PFOS	ΣPFAS = 169.6	Vicente et al. 2015
National or Natural Parks from the Iberian Peninsula (Spain and Portugal)	Yellow-legged gull (*Larus michahellis*)	PFNA, PFHxS, PFOA, PFBS and PFOS	10 - 54 [b]	Vicente et al. 2012
Different colonies across Canada	Glaucous-winged gull (*Larus glaucescens*) California gull (*Larus californicus*)	PFSAs (PFHxS, PFOS, PFDS) and PFCAs (PFHxA,	ΣPFSA = 7.8 - 486 ΣPFCA = 1.4 - 82	Gebbink et al. 2011a

	Ring-billed gull (*Larus delawarensis*) Herring gull (*Larus argentatus*)	PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, PFPA)		
Northern Norway	Herring gull (Larus argentatus)	PFSAs (PFBS, PFHxS, PFOS, PFDS) and PFCAs (PFOA, PFNA, PFDcA, PFUnA, PFDoA, PFTriA, PFTeA, PFPeA)	ΣPFSA = 21.7 – 43.4 ΣPFCA = 2.7 – 10.2	Verrault et al., 2007
Norwegian Arctic	Glaucous Gulls (*Larus hyperboreus*)	PFHxS, PFOS, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, PFPA	41.8 ± 5.27 (SE)[a]	Verrault et al., 2005
Northern Adriatic sea (Italy)	Yellow-legged gull (*Larus michahellis*)	PFOS	165.9 ± 36.9 (SD) [a]	Parolini et al. 2016
Northern Adriatic sea (Italy)	Yellow-legged gull (*Larus michahellis*)	PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoDA, PFHxS, PFOS	57.4 ± 18.0 (SD) [a]	Present study

557 [a]SD = standard deviation; SE = standard error. SD or SE were included when available.

558 [b]PFOS was the only PFAS detected in this survey. The levels refer to PFOS only.

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Figures and figure captions

Figure 1: Variation of PFOS (a), PFDa, PFUnA and PFDoDa (b) concentrations (mean ± standard error of the mean) according to the order in the laying sequence in yolk isolated from yellow-legged gull eggs collected in the northern Adriatic. The a-, b- and c-eggs correspond to the first-, second- and third-laid eggs, respectively. Asterisks above the histograms indicate pairwise significant differences between groups at post-hoc tests (* $P < 0.05$; ** $P < 0.01$).

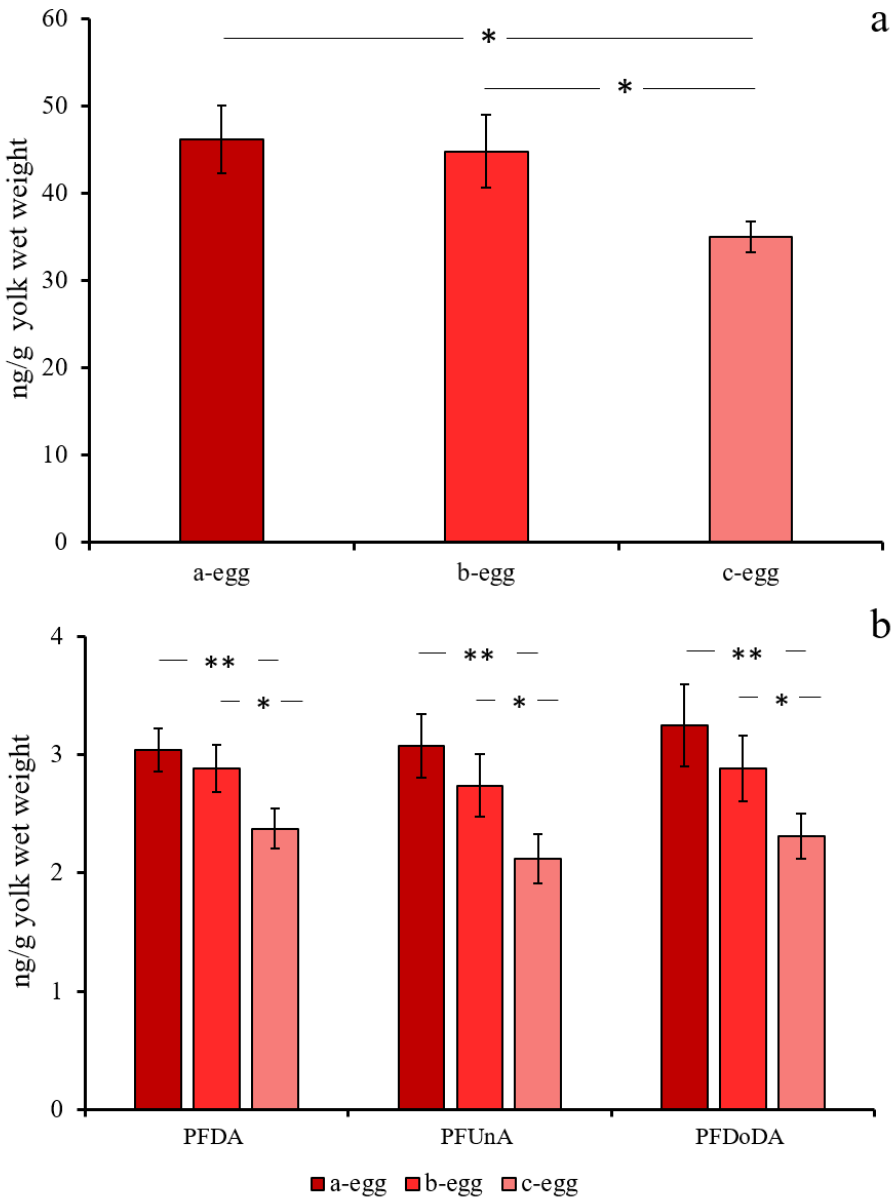


Figure 2: Variation of PFHpA, PFOA, PFNA and PFHxS concentrations (mean ± standard error of the mean) according to the order in the laying sequence in yolk isolated from yellow-legged gull eggs collected in the northern Adriatic. The a-, b- and c-eggs correspond to the first-, second- and third-laid eggs, respectively. No significant laying-order effect in concentrations of these compounds was found (see Table 2).

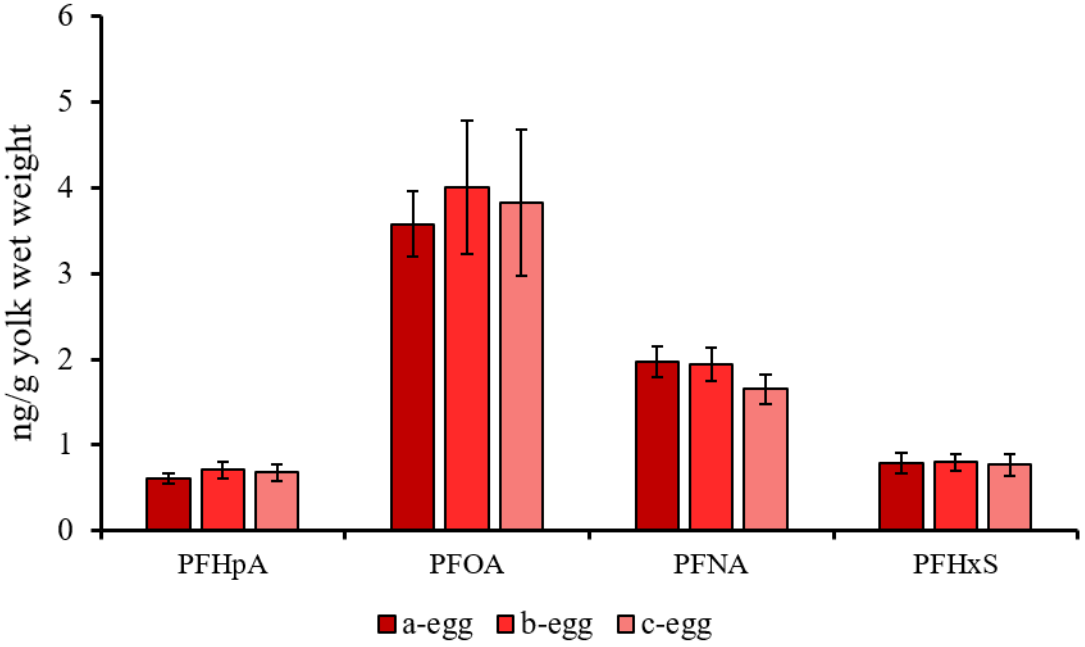
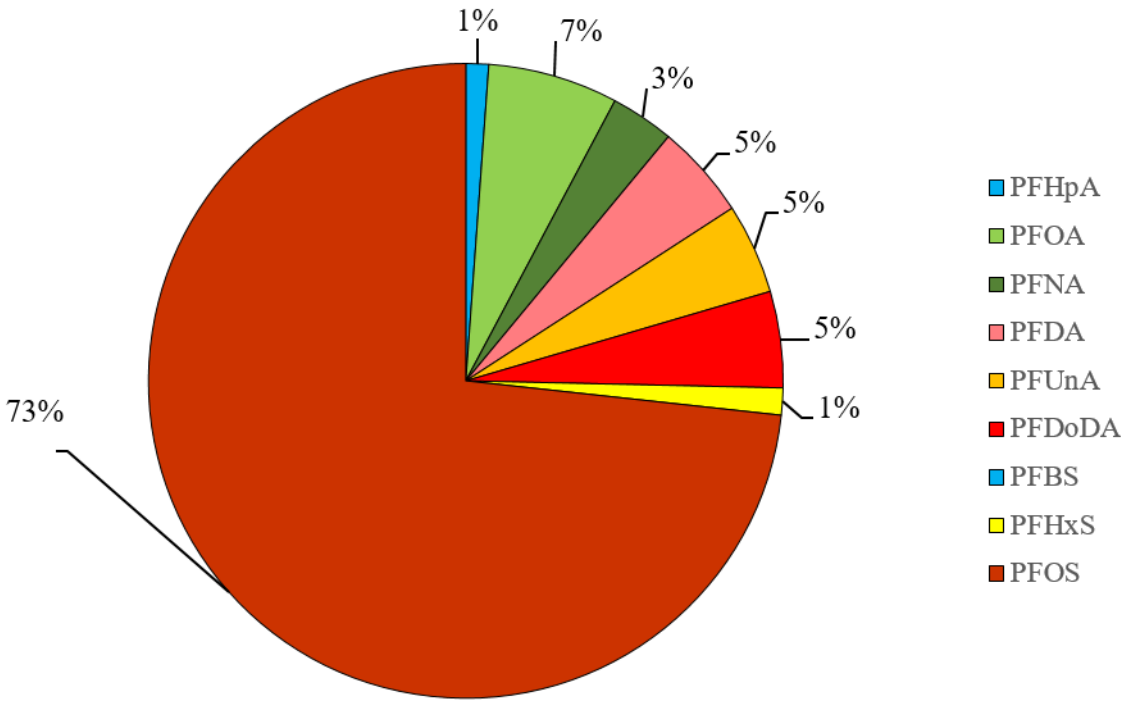


Figure S1: Percentage contribution of each single homologue to the ΣPFAAs in yellow-legged gull eggs collected in the northern Adriatic. PFPeA and PFHxA were not considered because of their negligible contribution.



590 **Table S1**: Laying date, weight at the time of laying, yolk and albumen weight of eggs from the 15
591 nests considered in the present study.

Nest	Laying order	Laying date (Julian date: 1 = 21st March 2016)	Egg weight at laying (g)	Yolk weight (g)	Albumen weight (g)
1	a-	29	81.4	20.4	50.5
	b-	31	77.2	21.7	46.3
	c-	33	72.8	22.6	41.1
2	a-	30	85.2	28.1	47.7
	b-	32	77.4	21.0	45.9
	c-	34	73.6	20.3	38.4
3	a-	34	80.4	23.2	48.0
	b-	36	80.2	21.9	46.8
	c-	38	73.3	21.9	42.2
4	a-	38	87.8	24.9	49.7
	b-	40	87.1	23.0	54.4
	c-	42	77.4	20.0	47.9
5	a-	38	93.5	28.3	53.7
	b-	40	88.3	20.9	56.1
	c-	42	83.2	23.7	50.2
6	a-	38	92.5	25.3	56.7
	b-	40	73.2	26.2	37.2
	c-	43	84.2	24.0	50.7
7	a-	38	83.0	30.1	41.7
	b-	40	81.2	22.6	48.6
	c-	43	75.2	20.0	45.3
8	a-	38	93.7	26.6	55.7
	b-	40	96.5	25.2	60.3
	c-	42	94.7	23.5	60.0
9	a-	38	95.5	36.7	47.6
	b-	40	95.7	24.0	60.4
	c-	42	85.8	20.7	55.9
10	a-	38	78.7	25.6	44.8
	b-	40	74.5	24.8	41.2
	c-	42	73.2	21.7	43.2
11	a-	38	89.4	24.7	54.8
	b-	40	80.6	23.9	45.9
	c-	42	79.4	20.1	48.8
12	a-	38	84.1	24.7	49.4
	b-	40	82.5	27.7	44.1
	c-	42	74.5	20.2	45.2
13	a-	38	80.4	24.3	46.2
	b-	40	65.7	23.1	46.4
	c-	43	66.7	21.5	36.8
14	a-	38	87.5	24.1	53.8
	b-	40	78.2	20.9	46.3

	c-	42	73.0	18.6	46.2
15	a-	38	71.1	22.4	39.7
	b-	40	64.1	22.2	32.9
	c-	42	64.5	18.2	37.2

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608 **Table S2**: Concentrations of PFAAs measured in the yolk isolated from yellow-legged gull eggs. Concentrations measured in first- (a-), second- (b)
609 and third-laid (c-eggs) eggs from 15 nests are reported. Concentrations are expressed as ng/g yolk wet weight. n.d. = not determined. ΣPFAAs
610 calculated as the sum of PFCAC7-C12, PFHxS and PFOS.

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Nest	Laying order	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoDA	PFHxS	PFOS	ΣPFAAs
1	a-	0.02	0.06	0.2	2.7	3.9	5.0	5.3	4.9	0.4	56.7	79.1
	b-	0.12	0.05	0.4	3.2	3.9	5.6	5.7	5.5	0.6	90.2	114.9
	c-	0.06	0.08	0.3	2.4	2.7	4.4	3.7	3.5	0.6	40.0	57.4
2	a-	n.d.	n.d.	0.6	2.3	1.5	2.7	2.7	2.2	0.3	50.2	62.6
	b-	n.d.	n.d.	0.4	1.4	0.9	1.6	1.5	1.4	0.1	31.5	38.9
	c-	n.d.	n.d.	0.4	1.3	0.7	1.2	1.0	0.9	0.2	22.1	27.9
3	a-	n.d.	n.d.	0.6	2.1	0.8	1.6	1.1	1.0	0.4	18.8	26.5
	b-	n.d.	n.d.	1.0	3.0	1.0	1.7	1.3	1.2	1.2	24.7	35.1
	c-	n.d.	n.d.	0.9	3.2	1.0	1.6	1.2	1.2	1.6	24.2	35.0
4	a-	n.d.	n.d.	0.8	4.5	2.4	4.3	5.3	6.1	1.0	61.0	85.6
	b-	n.d.	n.d.	0.7	3.3	1.5	2.5	2.8	3.1	0.9	35.4	50.1
	c-	n.d.	n.d.	0.7	3.2	1.4	2.7	2.9	3.2	0.9	36.8	51.8
5	a-	n.d.	n.d.	0.5	2.6	2.0	3.5	2.6	2.3	0.5	45.6	59.5
	b-	n.d.	n.d.	0.6	2.7	2.0	3.2	2.4	2.2	0.4	44.2	57.6
	c-	n.d.	n.d.	0.6	2.6	1.7	2.8	1.6	1.9	0.5	43.9	55.6
6	a-	n.d.	n.d.	1.1	7.8	2.0	2.3	1.6	2.1	0.9	31.2	48.9
	b-	n.d.	n.d.	1.8	14.4	3.3	3.9	2.6	3.2	1.4	41.6	72.2
	c-	n.d.	n.d.	1.9	15.4	3.4	3.3	2.3	3.0	2.0	46.3	77.4
7	a-	n.d.	n.d.	0.6	3.7	1.9	2.6	3.0	3.5	0.6	43.0	58.8
	b-	n.d.	n.d.	0.7	4.1	2.1	3.1	3.1	3.7	1.2	53.5	71.6
	c-	n.d.	n.d.	0.6	3.5	1.4	1.7	1.7	2.0	0.7	35.6	47.3
8	a-	n.d.	n.d.	0.7	4.1	2.2	2.7	2.6	2.9	1.0	43.9	60.1
	b-	n.d.	n.d.	0.8	3.5	1.4	1.8	1.7	1.9	0.8	35.7	47.6
	c-	n.d.	n.d.	1.0	4.6	1.8	2.0	1.9	2.2	0.7	41.6	55.7

9	a-	n.d.	n.d.	0.8	3.9	1.6	2.5	2.5	2.8	0.7	44.9	59.7
	b-	n.d.	n.d.	1.2	4.8	2.1	3.2	3.2	3.6	0.7	61.9	80.6
	c-	n.d.	n.d.	0.7	4.4	1.9	2.8	2.7	3.2	0.7	35.1	51.5
10	a-	0.02	0.08	0.5	4.0	1.7	1.9	2.1	2.2	0.5	31.1	44.0
	b-	<LOD	0.12	0.6	5.4	2.1	2.4	2.7	2.7	0.5	40.4	56.7
	c-	0.02	0.08	0.3	2.8	1.1	1.3	1.3	1.4	0.3	27.2	35.8
11	a-	n.d.	n.d.	1.0	4.6	2.6	5.0	5.5	6.0	1.9	68.6	95.3
	b-	n.d.	n.d.	0.6	2.6	1.9	3.4	3.4	3.5	1.2	50.5	67.0
	c-	n.d.	n.d.	0.6	2.2	1.5	2.5	2.2	2.4	1.1	40.4	52.9
12	a-	n.d.	n.d.	0.6	2.9	1.5	2.5	2.9	2.8	1.7	36.3	51.1
	b-	n.d.	n.d.	0.7	3.3	1.9	3.4	3.8	3.8	1.2	49.5	67.4
	c-	n.d.	n.d.	0.7	3.3	1.6	2.6	2.8	3.0	0.5	35.2	49.9
13	a-	0.06	0.12	0.5	3.9	2.3	3.5	3.0	3.6	0.9	43.5	61.2
	b-	0.02	0.07	0.5	3.4	1.9	2.8	2.3	2.6	0.5	36.3	50.2
	c-	0.08	0.10	0.6	3.2	1.7	2.3	2.1	2.5	0.5	33.0	46.0
14	a-	<LOD	0.06	0.3	1.9	1.2	2.3	2.4	2.5	0.6	41.5	52.8
	b-	<LOD	0.06	0.3	2.2	1.4	2.0	1.8	2.0	0.5	26.8	37.0
	c-	0.01	0.06	0.4	2.7	1.6	2.6	2.6	2.4	0.6	32.6	45.6
15	a-	0.09	0.09	0.3	2.7	2.0	3.1	3.4	3.6	0.7	76.9	92.8
	b-	<LOD	<LOD	0.5	2.9	1.7	2.8	2.9	3.0	0.8	50.2	64.8
	c-	<LOD	<LOD	0.6	2.4	1.3	1.9	1.7	1.8	0.5	31.4	41.6

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