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Title: Early diagenetic alterations of sterol biomarkers during particle settling and burial in polluted and pristine areas of the Rio de la Plata Basin

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Keywords: Sterols; Sewage markers; settling material; Rio de la Plata

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Abstract: Sources and diagenetic alterations of sterol markers were studied in settling material and sediments near Buenos Aires main sewer (BA), and at a relatively non-polluted northern site at the Uruguay River (N). Vertical particle fluxes were 7-times higher at BA relative to N (34 ± 24 vs. 4.6 ± 3.6 mg/cm2/day; mean ± standard deviation),increasing during rainy months. Total sterol contents were consistently higher at BA, both in settling material (7140 ± 7905 vs. 41 ± 47 µg/g at N) and sediments (708 ± 454 vs. 1.9 ± 0.18 µg/g). This difference was further amplified in the vertical flux of sterols (116 ± 168 vs. 0.070 ± 0.13 mg/cm2/year). At BA, sterol composition of settling material and sediments was dominated by fecal sterols (75-77%), with extreme coprostanol concentrations (3.6 ± 4.8 vs. 0.35 ± 0.28 mg/g at N) which are similar to sewage sludge. In contrast, at N the sterol profile was dominated by plant sterols (57-64%), mainly sitosterol, stigmasterol and campesterol. At BA the discharge of fresh sewage was confirmed by the high coprostanol/(coprostanol + epicoprostanol) ratio. At N, the overwhelming dominance of plant sterols over herbivore fecal sterols was reflected by the high sitosterol/(sitosterol + ethylcoprostanol) ratio and the low coprostanol/(coprostanol + ethylcoprostanol) ratio. The coprostanol/(coprostanol + epicoprostanol) and cholesterol/(cholesterol + cholestanol) ratios were lower in sediments than in settling material, reflecting the sterol degradation at the sediment surface. The accumulation efficiencies, calculated as the difference between trap fluxes and sediment inventories, were 2-7 times higher at BA reflecting strong vertical fluxes and enhanced preservation under anoxic conditions. During diagenetic processes, epicoprostanol (partially produced in situ), cholestanol and plant sterols were the best-preserved sterols, while cholesterol was the most labile during burial.

Cover Letter

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Dear Dr. Mark Yunker,
Associate Editor, Organic Geochemistry

We are pleased to resubmit the revised version of the manuscript “Early diagenetic alterations of sterols biomarkers during particle settling and burial in polluted and pristine areas of the Rio de la Plata basin” by E. D. Speranza, M. Colombo, C.N. Skorupka and J. C. Colombo (Ms. OG-3426). The manuscript has been corrected according to the reviewers’ comments and it has been thoroughly edited by several people in order to check typing and style.

We acknowledge you and both referees for your careful revision which help us to clarify several concepts, to correct some mistakes and to format the manuscript according the journal guidelines. The responses to each comment are listed in the “Response to reviewers” file, uploaded to the EES.

Sincerely yours,

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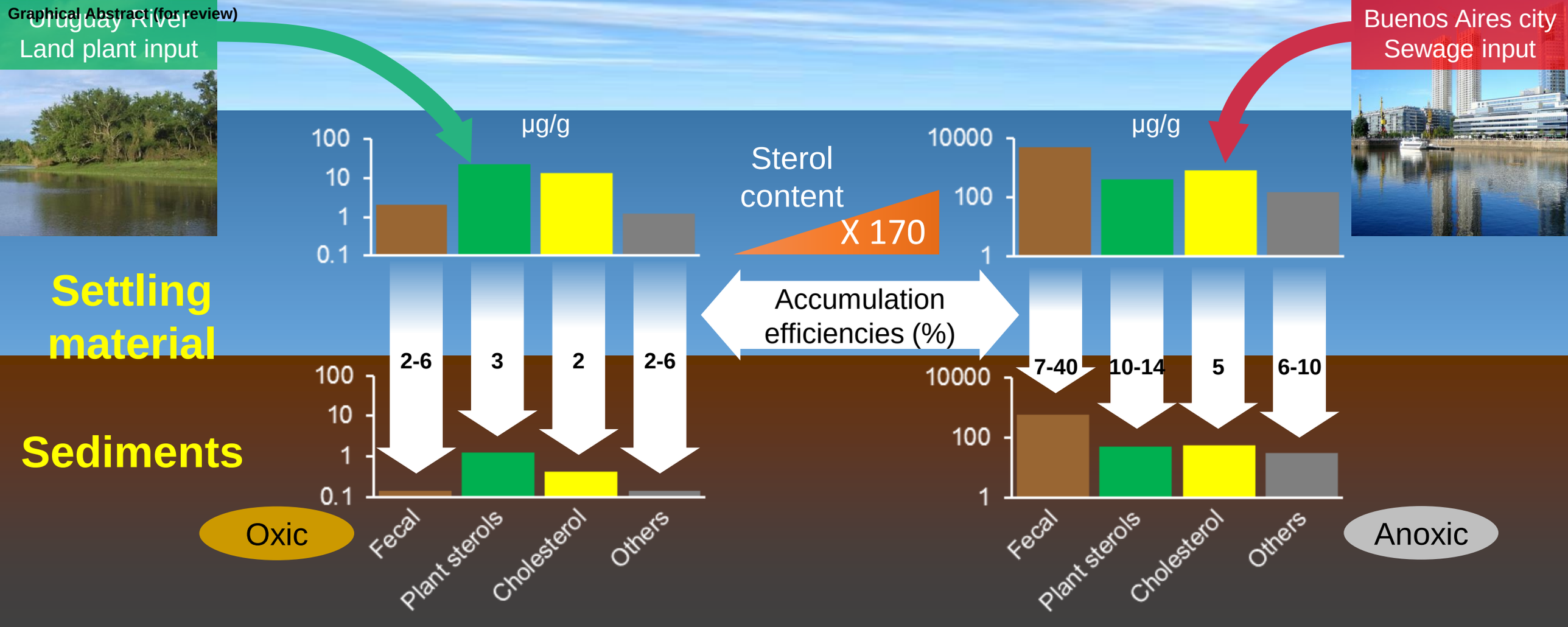
Graphical Abstract (for review)



- Settling particles contained ~10-20 times more sterols than underlying sediments
- Higher runoff during rainy months significantly increased particle and sterol flux
- Buenos Aires sediments contained huge coprostanol levels, similar to sewage sludge
- A non-polluted upstream site showed prevailing plant sterols
- Higher fluxes and anoxic sediments at Buenos Aires favored sterol preservation

Early diagenetic alterations of sterol biomarkers during particle settling and burial in polluted and pristine areas of the Rio de la Plata Basin

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Abstract:

Sources and diagenetic alterations of sterol markers were studied in settling material and sediments near Buenos Aires main sewer (BA), and at a relatively non-polluted northern site at the Uruguay River (N). Vertical particle fluxes were 7-times higher at BA relative to N (34 ± 24 vs. 4.6 ± 3.6 mg/cm2/day; mean ± standard deviation) and increasinged during rainy months. Total sterol contents were econsistentlyconsistently higher at BA, both in settling material (7140 ± 7905 vs. 41 ± 47 µg/g at N) and sediments (708 ± 454 vs. 1.9 ± 0.18 µg/g). Thise difference was further amplified in the vertical flux of sterols (116 ± 168 vs. 0.070 ± 0.13 mg/cm2/year). At BA, sterol composition of settling material and sediments was dominated by fecal sterols (75-77%), with extreme coprostanol concentrations (3.6 ± 4.8 vs. 0.35 ± 0.28 mg/g at N) which are similar to sewage sludge. In contratscontrast, while at N the sterol profile was dominated by plant sterols dominated (57-64%), mainly sitosterol, stigmasterol and campesterol. At BA the discharge of fresh sewage was confirmed by the high fecal sterols/phytosterols and coprostanol/(coprostanol + epicoprostanol) ratios. At N, the overwhelming dominance of plant sterols over herbivore fecal sterols was reflected by the high sitosterol/(sitosterol +24 ethylcoprostanol) ratio and the low coprostanol/(coprostanol + 24 ethylcoprostanol) ratio. The coprostanol/(coprostanol + epicoprostanol) and cholesterol/(cholesterol +

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cholestanol) ratios were lower in sediments than in settling material, reflecting the sterol degradation at the sediment surface. The accumulation efficiencies, calculated as the difference between trap fluxes and sediment inventories, were 2-7 times higher at BA reflecting stronger vertical fluxes and enhanced preservation in under anoxic conditions. During diagenetic processes, epicoprostanol (partially produced in situ), cholestanol and plant sterols were the best-preserved sterols, while cholesterol was the most labile during burial.

Keywords: Sterols; Sewage markers; settling material; Rio de la Plata.

1. Introduction

The molecular composition of lipids from settling material and sediments provides ~~particularly~~ useful information about ~~on~~ the sources and diagenetic alteration~~s~~ of organic matter (Meyers and Ishiwatari, 1993; Canuel and Hardison, 2015). Sterols, present as components of cell membranes in eukaryotes ~~but~~ and also in prokaryotes, are especially suited as biomarker compounds due to their widespread environmental occurrence, stability and structural diversity (Volkman, 2005). The source specificity of sterols range from some rather unspecific sterols (e.g. cholesterol) to several marker sterols associated to particular organisms, such as diatoms, dinoflagellates, plants and fungi (Volkman ~~et al., 1986~~2016; Puglisi et al., 2003). A group of sterols, collectively referred as fecal sterols, have been widely used as sewage tracers. Coprostanol, formed during the biohydrogenation of the Δ5 double bond of cholesterol by bacteria present in the gut of humans or animals, is the primary fecal sterol detected in domestic wastes (<60% total sterols, Bull et al., 2002). ~~In contrast with cholesterol, coprostanol~~ Coprostanol, unlike cholesterol, is barely absorbed by the intestinal epithelium and is massively excreted with feces (Veiga et al., 2005). Although it is degraded under oxic conditions, it can resist relatively unaltered for many years in anoxic sediments (Nishimura and Koyama, 1977).

Discharge of municipal wastewater to rivers and coastal areas is a source of continuing environmental concern. Municipal discharges are~~since this is~~ a major source of organic matter and nutrients that may cause eutrophication, oxygen depletion, turbidity increase, acidification, and trophic structure alterations leading to habitat deterioration (~~Takada et al., 1997;~~ deBruyn et al., 2003; Blanch et al., 2004; ~~deBruyn et al., 2003~~Kress et al., 2016). Moreover, since most sewer systems in Latin America also receive storm drainage and industry inputs, sewage contains many hazardous materials such as organic and inorganic pollutants and pathogens that jeopardize the use of water for human consumption, fishing activities or recreation (Helmer and Hespanhol, 1997). Urban-industrial effluent ~~The~~ discharges ~~of urban-industrial effluents~~ in major ~~the estuarine~~river systems are a key source areas ~~of~~ of anthropogenic material to marine environments. ~~major river systems is particularly relevant since they are an important source of anthropogenic material to marine environments.~~

Among these major river systems worldwide, the Rio de la Plata Basin ranks 5th in terms of drainage area (2.8×10^6 km2), covering nearly 20% of South America surface area (Milliman and Meade, 1983). The main tributary rivers of this basin~~,~~ (the Parana and Uruguay rivers) discharge an average of 22,000 m3/s of water to the Atlantic Ocean through the Rio de la Plata estuary, a large funnel and shallow shaped estuary that receives > 82-129 × 10^6

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tons/year of particulate load making it one of the most turbid estuaries in the world (Milliman and Meade, 1983). The coastal area of metropolitan Buenos Aires is strongly impacted by anthropogenic discharges resulting in high concentrations of hydrocarbons, organochlorine pesticides, PCBs and metals in sediments (Colombo et al., 1989, 2005; Tatone et al., 2009), settling material (Colombo et al. 2007c; Tatone et al., 2012) and biota (Colombo et al., 1997, 2007a, 2007b, 2011). Before the installation of a primary wastewater treatment plant ~~U~~in~~ntil~~ 2015 ~~when a primary wastewater treatment plant began to operate~~, the main Buenos Aires sewer outfall discharged 2.2 × 10^6 m3/day of crude domestic wastes from 6 × 10^6 inhabitants as well as industrial and municipal wastes 2.5 km offshore (www.aysa.com.ar; FREPLATA, 2005). The Riachuelo River, located 20 km upstream the main sewer, also discharges sewage material and industrial waste~~s~~. The combined loads of both effluents ~~make up to~~reach ~~3.8~~4.3 x 10^6 m3/day, which is comparable to the flow of the world's largest sewage outfall in Boston (Roberts and Villegas, 2016).

In this context, ~~-~~the analysis of settling material in rivers systems with~~of~~ high turbidity and organic matter load, ~~the analysis of settling material~~ is particularly relevant. Settling material represents the fresh inputs of organic matter to aquatic environments and is thus useful to assess ~~the~~ its sources as well as the temporal variability. Sediments integrate these signals over a wide temporal range, with a composition ~~biased towards more resistant~~ dominated

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by ~~compounds~~refractory compounds. The comparison between settling material and underlying sediments ~~permits~~ allows for a detailed evaluation of the early diagenetic behavior of organic compounds, mainly ~~which is basically~~ controlled by factors such as sedimentation rate, temperature and redox conditions (Colombo et al., 1996b).

In this paper, we evaluate the sources, vertical fluxes and diagenetic alterations of sterol markers during burial in two contrasting sites of the Rio de la Plata Basin: the highly impacted metropolitan area of the Rio de la Plata estuary and a relatively non-polluted northern site at the Uruguay River. Differences in terms of sterol concentration and composition, vertical fluxes, differential preservation in sediments and temporal variation are discussed.

2. Materials and methods

The sampling strategy comprised two sites with contrasting anthropogenic impact: the heavily polluted Buenos Aires metropolitan area of the Rio de la Plata estuary near the main sewer outfall (BA, 34°43.33' S - 58°10.30' ~~O~~W) and a more pristine site ~200 km upstream on the Uruguay River, the Ñandubaysal Bay (N, 33°05.27' S - 58°21.37' W; Fig.1). Sampling campaigns were carried out seasonally from 2007 to 2014. Settling material

was collected in pre-weighed polypropylene conical Falcon tubes coupled to a fixed 10 cm diameter cylindrical sediment trap deployed at 1.~~5m~~5 m during 1-3 days (BA) or 30-60 days (N). Superficial sediments were collected using a stainless steel Hydro-Bios Van-Veen grab sampler. Samples were immediately refrigerated and transported to the laboratory. Tubes containing the settling material were centrifuged and weighed after discarding supernatant water. Water content was determined gravimetrically after drying in an oven at 40 °C. Total organic carbon determination was carried out on a Thermo Finnigan Flash EA 1112 elemental analyzer. Total particle flux was computed as:

$$\text{Flux (mg/cm2/day)} = \frac{\text{settling matter mass (mg)}}{\text{trap surface (78 cm2)} \times \text{deployment time (days)}}$$

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Sedimentation rate was calculated as:

$$\text{SR(cm/year)} = \frac{\text{Flux} \times 365}{1000 \times \text{density (g/cm3)}}$$

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The discharge of the Uruguay River was calculated as the turbinated plus compensation flow discharged daily by the Salto Grande Dam, located 240 km upstream N station and averaged for each sediment trap deployment period (wholesale electricity market administration company: www.cammesa.com). The discharge of the Rio de la Plata estuary was assumed as the sum of the corresponding monthly discharges of the Uruguay River, measured 90 km upstream N station, and of the Parana River, measured near

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the mouth of its main channels (Paraná Guazú and Paraná de las Palmas; Base de Datos Hidrológica Integrada, bdhi.hidricosargentina.gov.ar; Jaime and Menendez, 2002).

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Lipids were extracted ultrasonically with acetone:dichloromethane:petroleum ether (1:2:2). ~~The extract was~~ dried over anhydrous sodium sulfate and gravimetrically ~~lipid content was~~ determined (Colombo et al. 1996a) ~~gravimetrically~~. Deuterated sterols (deuterocholesterol-D7 and deuterositosterol-D7, Steraloids, Inc., Newport, RI, steraloids.com; Table 1) were ~~added~~ used as internal standards. In order to avoid the interference of fatty acids, lipids (100 mg approx.) were saponified with 1M KOH in methanol and non-saponifiable compounds were extracted with petroleum ether – diethyl ether (4:1 v/v, Christie, 1989). The extracts were concentrated under a nitrogen stream and derivatized with 150 µl of N,O-Bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA:TMCS, 10:1 v/v; AppliChem GmbH, Darmstadt, Germany; Sigma-Aldrich, St. Louis, MO, USA) for 3 hours at ~~60ºC~~60 ºC. The resulting trimethylsilyl derivatives were taken to dryness ~~were concentrated to dryness~~ under nitrogen and resuspended in toluene prior analysis. All solvents used were pesticide residue analysis grade.

Samples were analyzed using a Perkin Elmer Clarus 500 GC-MS (Perkin Elmer, Waltham, MA, USA) fitted with a Quadrex 007-5MS capillary column

(60 m, 0.32 mm i.d., 0.25 µm; Quadrex Corp., Bethany, CT, USA). Helium was used as a carrier gas with a flow rate of 1.2 ml/min and the temperature of injector was set at ~~250ºC~~250 ºC (split-splitless mode). The oven temperature program started at ~~100ºC~~100 ºC with a ramp to 225 ºC at ~~15ºC~~15 ºC/min and to ~~300ºC~~300 ºC at ~~3ºC~~3 ºC/min with a final holding time of 10 min. The transfer line temperature was set at ~~200ºC~~200 ºC and the analytes were ionized by 70 eV electron impact at ~~180ºC~~180 ºC. The mass spectrometer was simultaneously operated in scan mode (60-600 amu) and selective ion monitoring. Data were acquired and processed with TurboMass 5.1 software.

Steroids with their trivial and IUPAC names, molecular weight, retention times and mass-to-charge ratios (*m*/*z*) used for quantification and confirmation are presented in Table 1. Coprostanol, epicoprostanol, coprostanone and ethylcoprostanol were collectively referred to as fecal sterols. Compounds were identified by comparison with ~~authentic~~ standards of ~~14~~ 15 steroids (Brassicasterol, Campesterol, Coprostanone, Deuterocholesterol, Deuterositosterol, Epicoprostanol, Ergosterol and Sitosterol from Steraloids; Cholesterol, Coprostane, Coprostanol, Dehydrocholesterol, Desmosterol, Stigmastanol and Stigmasterol from~~Steraloids,~~ Sigma-Aldrich), literature data and interpretation of mass spectrometric fragmentation patterns. Quantification was performed using a 4-points calibration curve (0.20-50 µg/ml) prepared in dichloromethane from certified ~~with authentic~~ standards

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(Table 1). Peak areas were corrected according internal standard recoveries. Commercially standards were not available for some compounds (~~Cholestenol~~Cholestanol, Dehydrobrassicasterol and~~,~~ ~~24-~~Ethylcoprostanol) which were quantified based on response factors of structurally related sterols.

The limit of detection (LOD) of each steroid was estimated by calculating the signal to-noise ratio (S/N) of triplicate standard solutions in the range of 0.20-50 µg/ml. LODs values averaged 6.5 ± 11 ng/g, ranging from (0.31 ng/g, coprostanol) to (43 ng/g, ergosterol). Reproducibility was assessed by the relative standard deviation (RSD) of triplicate analysis of the same samples in different batches, and averaged 11 ± 3.8 The method was highly linear in the range of concentrations of calibration curves (~~R²~~r2 > 0.99 for all steroids with available authentic standards). Recoveries of deuterated internal standards averaged 96 ± 1.7. Individual recoveries, evaluated by analysis of spiked samples ranged from 82 ± 15% (Ergosterol) to 110 ± 19% (Desmosterol).

Statistical analysis was carried on with Python scripting language~~,~~ (www.python.org), using SciPy~~,~~ (www.scipy.org), NumPy~~,~~ (www.numpy.org), MatPlotlib (matplotlib.org) and pandas (pandas.pydata.org) libraries. Multivariate analyses were executed in *R* language, using RStudio development environment (www.rstudio.com) and ggplot2 and ggbiplot packages~~.~~ (http://ggplot2.org/). Data were expressed as mean ± SD. Relative standard deviation (RSD: [data - mean] × 100/SD) was used to assess

parameter variability. To avoid division by zero errors, the ratios between two sterols, A and B were calculated as: A/(A + B). The accumulation efficiency of sterols from settling material to sediments was estimated as the relationship between the annual vertical flux of the sterol and its corresponding one-year inventory in sediments (sterol concentration in sediment × annual mineral flux). Student's *t* test was used to perform comparisons between two means as well as to evaluate the significance of correlation coefficients. Multivariate analysis was performed by principal component analysis of standardized data (x – X/y, where X = mean and y = SD).

3. Results and discussion

3.1. Total particle flux

The intense discharge of one of the largest sewer ~~outfall~~ worldwide at BA ~~contributes to~~ adds to the natural particle load of the Rio de la Plata resulting in extraordinarily high vertical particle fluxes (34 ± 24 mg/cm2/day) and sedimentation rates (4.7 ± 3.3 cm/year), in agreement with previous measurements in this area (5.5 ± 2.1 cm/year, density: 2.65 g/cm3; Colombo et al., 2007c). These~~is~~ values are ~~is however~~ higher than sedimentation rates reported for nearby sites of this turbid estuary (0.3-1.3 cm/year; Di Gregorio et al., 2007; Bonachea et al., 2010~~), ~~). This suggests ~~suggesting than~~ that most

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234 particles captured by sediment traps at BA are highly organic detritus derived
235 from urban-industrial discharges, as confirmed by the high concentration of
236 lipids (Speranza et al., 2013) and fecal sterols of this material (see below). At N,
237 the total particle flux was 7-times lower (4.6 ± 3.6 mg/cm2/day), comparable to
238 values previously reported for the Uruguay River (2.7 ± 2.3 mg/cm2/day, range:
239 0.73-7.3 mg/cm2/day; Colombo et al., 2015), resulting in a~~and the resulting~~
240 sedimentation rate of ~~(~~0.64 ± 0.49 cm/year~~) was comparable to values~~
241 ~~previously reported for the Uruguay River (Colombo et al., 2015), which also~~
242 ~~showed a high variability (1.0 ± 0.88 cm/year, range: 0.27-2.7 cm/year)~~. In
243 contrast to BA, where the settling material is composed mostly by
244 anthropogenic detritus over the background particle load from Parana River,
245 the settling material at N reflects the ~~smaller~~ lower solid discharge of the
246 Uruguay River (Moreira et al., 2013). The total particle flux was largely
247 dependent on river discharge, which was 6-46 times higher at BA (~~19465-~~
248 ~~46088~~19-46 x 10^3 m3/s) relative to N (~~420-8410~~0.42-8.4 x 10^3 m3/s), fitting an
249 exponential curve (~~R~~^{2}r2 = 0.78, $p < 0.0001$; Fig. 2). This correlation had been
250 previously observed at the Uruguay River and reflects the enhanced transport
251 of eroded material as river flow increases (Colombo et al., 2015).

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253 *3.2. Total sterol concentrations*

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The total sterol concentration in settling material was highly variable (RSD: 113 ~~114~~%) and exhibited a marked geographical difference. At BA, the tendency of hydrophobic sterols to associate to particulate matter is enhanced by the high organic content of settling particles (total organic carbon: 9.6 ± 7.4%), resulting in ~~very~~ high sterol concentrations at this site ~~(7140 ± 7905 µg~~7.1 ± 7.9 mg/g dry weight). Previous research on~~studies dealing with~~ sterols in settling particles primarily conducted ~~were mostly based~~ in ocean waters, relatively deep and clear, which had average sterol concentrations 1-4 orders of magnitude lower than the Rio de la Plata estuary metropolitan area, a ~~compared to this~~ shallow, turbid and polluted ~~freshwater~~ environment (Takada et al., 1994; Colombo et al., 1996; Parrish et al., 2000; Burns et al., 2008). The range of sterol concentrations in settling material published for riverine environments is considerably lower than those measured at BA~~Reports of sterols in settling material from riverine environments are more limited but the concentrations are still much lower than those from BA~~ (1-184 µg/g; Saliot et al., 2001; Li et al., 1995; Jeng and Kao, 2002). In fact, ~~total sterol concentrations in BA settling material~~ the sterol ~~se~~ concentrations at BA are comparable to values reported for sewage sludge from wastewater treatment plants (2-9 mg/g; Venkatesan and Kaplan, 1990; Kelly, 1995; Nguyen et al., 1995). At N, total sterol concentrations in settling material are 2-3 orders of magnitude lower (41 ± 47 µg/g) and comparable to aforementioned values in particulate matter from ~~riverine~~ freshwater environments. Total sterols in

sediments were 10-20 times lower than in settling material and were less variable (RSD: 10-61%), ~~but~~ and also presented a 2-3 orders of magnitude difference between BA and N (708 ± 454 vs. 1.9 ± 0.18 µg/g). The reduction in sterol concentration from settling material to sediments reflects the degradations ~~tendency~~ of sterol ~~to degrade~~ at the ~~water~~ sediment ~~interfase~~-water interface, especially under oxic conditions (Sun and Wakeham, 1998).

3.3. *Temporal variation of particle flux and sterol concentrations in settling material*

The large ~~data~~ variability on settling material data observed for both BA and N, ~~settling material resulted from significant~~ was explained by temporal variations between warm and cold periods. ~~Effectively~~A ~~, a~~ distinctive temporal pattern of higher particle fluxes during warm and rainy months (September to March, 22 ± 2.6°C, 127 ± 18 mm) relative to cold and dry ones (April to August, 13 ± 2.5ºC, 74 ± 23 mm) was observed ~~both~~ at BA (50 ± 25 vs. 20 ± 9.4 mg/cm2/day, $p < 0.005$; Fig. 3) and N (6.2 ± 4.0 vs. 3.2 ± 1.9 mg/cm²/day, respectively, $p < 0.05$). Total sterol concentration at BA was significantly correlated with total particle flux (~~r~~r2 = 0.~~64~~41, $p < 0.05$) ~~and followed~~following its temporal variation, raising during warm months (~~11163 ± 9599 µg~~11 ± 9.6 mg/g) and decreasing significantly during cold ones (~~3564 ± 3711 µg~~3.6 ± 3.7 mg/g; $p < 0.05$, Fig. 3). This increase~~d~~ of sterol flux during the

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rainy period is related to the wash-out of streams and effluents that discharge in this area of the Rio de la Plata, as ~~also~~ previously observed ~~previously~~ for other organic tracers (Colombo et al., 2007c). The reinforcement of total flux and concentration patterns results in an order of magnitude higher sterol vertical fluxes during warm periods (220 ± 202 vs. 23 ± 19 mg/cm2/year in cold months). At N, sterols were also significantly correlated with particle flux (~~r~~r2 = 0.~~00~~36, $p < 0.05$), but there was no significant difference between warm and cold months (45 ± 61 vs. 36 ± 28 µg/g respectively) thus sterol fluxes reflect ~~basically~~ the total particle flux pattern of higher values during the warm period (87 ± 165 vs. 52 ± 63 µg/cm2/year in cold months).

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3.4. Sterol composition

The sterol composition of settling material showed contrasting differences between BA and N (Fig. 4). At BA, fecal sterols predominated (75 ± 5.4% of total sterols), mostly coprostanol (52 ± 11%), followed by cholesterol (12 ± 2.9%) and phytosterols (8.3 ± 3.6%) whereas at N the contribution of plant sterols prevailed (phytosterols: 57 ± 13%, cholesterol: 26 ± 12, fecal sterols: 7.5 ± 7.0%). The fecal signature of BA resembled the composition of human feces (fecal sterols: 85%, phytosterols: 8.8%, cholesterol: 5.2%, others: 1.2%; Leeming et al., 1996), with extremely high concentrations of coprostanol (3.6 ± 4.8 mg/g) similar to sewage sludge and effluents (1-4 mg/g, 50-80% total sterols;

Venkatesan and Kaplan, 1990, Nguyen et al., 1995). The presence of epicoprostanol (9.3 ± 9.6%), originated from coprostanol biodegradation, evidence an incipient alteration which is likely occurring in the long sewer pipeline (9900 km total, main sewers > ~~100km~~100 km, www.aysa.com.ar) rather than in the very shallow (3-5 m) water column. Despite the relative abundance of cholesterol at BA, its utility as biomarker is limited since ~~as indicated previously~~ it is present in multiple organic matter sources (Mudge et al., 1999; Creuzburg and von Elert, 2009). A typical fecal herbivore marker, ~~24-~~ ethylcoprostanol derived by hydrogenation of sitosterol form terrestrial vegetation (Bull et al., 2002), is also relatively abundant at BA (8.5 ± 4.4%), but human feces can also include significant amounts of ethylcoprostanol (Leeming et al., 1996). The significance of coprostanone (5.4 ± 3.3%) is difficult to ascertain since it originates in mammalian gut as an intermediary in coprostanol microbial synthesis, but it can also be produced in sediments as a result of interconversions between this ketone and coprostanol and epicoprostanol (McCalley et al., 1981; Bull et al., 2002). The relatively low proportions phytosterols observed at BA, mainly represented by sitosterol (4.4 ± 1.9%), reflect the minor contribution of vegetal inputs, possibly including ~~possibly also~~ kitchen oil and foodstuff products, at this site.

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Despite being found in some algae, the three major phytosterols found ~~is~~ in settling material from N, sitosterol (19 ± 5.4%), stigmasterol (15 ± 7.9%) and

campesterol (13 ± 11%), are strongly associated with land plants (Huang and Meinschein, 1979, Volkman, 2005) and have been used as biomarkers of paper mill pollution (Lahdelma and Oikari, 2006). The fecal sterols signal at N, dominated by ethylcoprostanol (3.9 ± 4.7%) followed by coprostanol (1.3 ± 1.3%), differs both quantitatively and qualitatively from the sewage signature of BA. The presence of ethylcoprostanol as the main fecal sterol at N probably reflects the contribution of cattle fecal matter from the neighboring livestock establishments. The small concentrations of coprostanol cannot be unambiguously attributed to sewage pollution since small relative amounts of coprostanol can be formed by in situ hydrogenation of cholesterol in sediments not contaminated by fecal pollution (Nishimura and Koyama, 1977).

The change in percentage composition with total sterol concentration and its seasonal variation in settling material also showed geographical differences. At BA, as total sterol concentration increased, coprostanol proportion also raised (~~r~~r2 = 0.~~55~~30; $p < 0.005$) while stigmasterol and campesterol (~~r = -~~r2 = 0.~~56~~31 and 0.~~64~~41; $p < 0.005$) decreased and the remaining sterol proportions were not correlated, confirming that the increase in particulate sterol responds basically to anthropogenic discharges. At N, there was a strong significant correlation of total sterol concentration with cholesterol proportion (~~r = 68~~r2 = 0.46; $p < 0.0001$) and an inverse relationship with ethylcoprostanol and stigmasterol (~~r = -~~r2 = 0.~~39~~15 and -0.~~43~~18

respectively; $p < 0.05$). The sterol composition, on a percentage basis, showed little temporal variation except for the inverse trend of coprostanol and epicoprostanol observed at BA. While coprostanol proportion tended~~s~~ to be higher during warm months (59 ± 9.5 vs 45 ± 8.7 in cold months; $p < 0.01$) and correlates with total particle flux (~~r~~r2: 0.~~38~~15; $p < 0.05$), its epimer increases during the cold period (2.6 ± 2.0 to 15 ± 9.2; $p < 0.005$) and it is inversely correlate~~ds inversely~~ to total particle flux (~~r: -~~r2: 0.~~70~~49; $p < 0.005$). This is in agreement with previous work in this area of Rio de la Plata estuary where the terrestrial runoff results in an enhanced discharge of organic compounds with a fresher signature during warm and rainy periods, in contrast with the less intense and more degraded signal observed during cold and dry months (Colombo et al., 2007c). Similarly, Puerari et al. (2012) observed an enhanced level of sewage degradation in the dry winter period in Brazilian rivers~~,~~ associated with a lower terrestrial runoff.

The sediment sterol profile was similar to that of settling material, with some minor differences related to the sterol degradation at sediment surface. At BA, this degradation is apparent in the relative increase of degradation products such as epicoprostanol, stigmastanol and cholestanol from settling particles (9.3 ± 9.6, 1.6 ± 0.88 and 1.7 ± 1.2%) to underlying sediments (16 ± 4.5, 2.6 ± 1.5 and 2.8 ± 1.1%, respectively, $p < 0.05$), reflecting the microbial reduction of stenols to stanols and coprostanol epimerization at the oxic-anoxic

boundary (Wakeham, 1989). Despite ~~this~~ degradation processes, sediments at BA still have remarkably high sterol concentrations, especially of coprostanol whose concentration (349 ± 282 µg/g) is among the highest reported for surficial sediments severely impacted by sewage discharges (Table 2). Coprostanol highest values were chiefly measured in freshwater locations or in relatively enclosed seawater environments where ocean dilution is reduced. In sediments from the Uruguayan coast of the Rio de la Plata near Montevideo, Venturini et al., (2015) reported 17-400 times lower concentrations of coprostanol (0.05-21 µg/g) and cholesterol (0.48-5.1 µg/g), evidencing that the background levels of these sterols are quite low and that they derive mainly from local urban discharges at BA. Interestingly, the concentrations of phytosterols were only slightly lower to those of BA for stigmasterol and campesterol (0.30-3.14 and 0.13-2.13 µg/g, respectively; Venturini et al., 2015) but not for sitosterol, which was 6-70 times lower (0.43-5.3 µg/g). This suggests that while sewage discharge contributes significantly sitosterol at BA sediments, terrestrial runoff is the main source of stigmasterol and campesterol. ~~This suggests that while sewage discharge contributes significantly sitosterol at BA sediments, the terrestrial runoff is the main source of stigmasterol and campesterol.~~ This is in agreement with previous reports of high concentrations of sitosterol in sewage effluents of domestic origin (e.g. flush of kitchen vegetable oils; Furtula et al., 2011). At N, the sediment sterol profile was dominated by terrestrial plant phytosterols and cholesterol, as observed in settling material but with higher proportions of

epicoprostanol, sitosterol and stigmastanol (2.7 ± 1.2, 25 ± 3.0 and 12 ± 1.9%, respectively, $p < 0.05$). The marginal impact of sewage pollution at N sediments is evidenced by ~~the~~ low coprostanol concentrations, which are well below the threshold values reported as indicative of sewage pollution (0.1-0.7 µg/g; Grimalt et al., 1990; Leeming et al., 1997; Rada et al., 2016) and are comparable to values reported for riverine sites with low to moderate sewage pollution (Table 2).

To simultaneously evaluate the contribution of ~~the~~ different sterols to the overall variability observed in settling material and sediments, ~~multiple regression and~~ multivariate analysis (PCA) were performed for major sterols (compounds with < 0.5% abundance were excluded, Fig. ~~6~~5).This model explains 59% of total variability, mainly through principal component 1 (47%), which is loaded in the negative side with fecal coprostanol, coprostanone and epicoprostanol and in the positive side with cholesterol and plant sterols. The second component accounts for 12% of data variability and is negatively loaded with ethylcoprostanol and dehydrocholesterol and positively loaded with cholestanol and epicoprosatanol. ~~The s~~Settling material from BA is clustered on the left side of the PCA, denoting fecal inputs, and is clearly discriminated from N, plotting on the right due to the major contribution of plant sterols to the overall composition. The average sterol composition of human feces plots in the center of the BA cluster, further confirming the sewage origin of settling

material at this site. The sediments segregation was similar to that of settling material, with minor differences reflecting the degradation that takes place at the water-sediment interfassee. BA sediments are scattered on the right, with most samples gathering near the epicoprostanol and cholestanol vectors, reflecting the degradation of coprostanol and cholesterol respectively. N sediments are more homogeneous and plotted on the upper right side of the PCA, close to cholesterol and stigmastanol vectors.

3.5. Sterol ratios

SMany sterol ratios have been routinely used to assess the contribution of different sources of organic matter as well as degradation processes (Jeng and Han, 1994; Takada et al., 1994; Chalaux et al., 1995; Fattore et al., 1996). All the ratios evaluated in this work presented highly significant differences between BA and N (*t*-test; $p < 0.0001$; Fig. 56). In settling material, the high coprostanol/(coprostanol + epicoprostanol) ratio at BA (0.85 ± 0.15) reflects the relatively fresh sewage inputs discharged, in contrast to the weak and extensively highly degraded fecal signature at N (0.48 ± 0.15). The coprostanol/(coprostanol + 24-ethylcoprostanol) ratio is 2 times higher in BA settling material relative to N (0.86 ± 0.064 vs. 0.35 ± 0.19) indicating that the reduced fecal sterols at N are chiefly from herbivore mammal feces. However, despite the overwhelming abundance of coprostanol at BA a small non-human

contribution to the overall fecal signal cannot be disregarded. At ~~BA~~this site, the sitosterol/(sitosterol + ~~24-~~ethylcoprostanol) index was 0.36 ± 0.15, in the range of values proposed by Nash et al., (2005) as typical for feces runoff of herbivore with high ethylcoprostanol proportions, such as cattle and pigs~~, used to assess herbivore fecal pollution, was 0.36 ± 0.15, below the threshold of 1.0 proposed as typical for cow feces runoff (Nash et al., 2005). may lead to erroneously neglect the non-human fecal pollution at this site. At BA, the sitosterol/24-ethylcoprostanol index, used to assess herbivore fecal pollution, was 0.36 ± 0.15, below the threshold of 1.0 proposed as typical for cow feces runoff (Nash et al., 2005). Beside cattle, other animal such as pigs and poultry also have high ethylcoprostanol proportions in their feces and could affect this ratio (Leeming et al., 1996), suggesting a small non-human contribution to the overall fecal signal at BA~~. At N, this ratio (0.84 ± 0.17) was above the limit ~~of 4~~ suggested ~~by Nash et al., (2005)~~ as indicative of non-fecal polluted plant decay inputs (Nash et al., 2005), denoting minimum impact of fecal contamination at this site. The cholesterol/(cholesterol + cholestanol) ratio is useful to assess the microbial reduction of stenols to 5α-stanols that typically takes places under anoxic conditions (Reeves, 2005). At BA, the relatively low values of this ratio (0.85 ± 0.~~043~~036) indicate prevailing reductive conditions in the sewage effluent, which favors sterol preservation. On the contrary, oxic conditions at N favors the sterol degradation over their hydrogenation (Nishimura and

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Koyama, 1977), resulting in proportionally low amounts of cholestanol (ratio: 0.95 ± 0.043).

In the sediments, these ratios exhibited the same geographical differences observed in the settling material but reflected the diagenetic processes that take places at sediment surface. The degradation of coprostanol and cholesterol, which is intensified after particle deposition, resulted~~ing~~ in lower coprostanol/(coprostanol + epicoprostanol) ratio (BA: 0.75 ± 0.064, N: 0.26 ± 0.058) and cholesterol/(cholesterol + cholestanol) ratio~~s~~ (BA: 0.75 ±0.11, N: 0.93 ± 0.025) ~~ratios~~ in sediments relative to settling material.

~~To simultaneously evaluate the contribution of the different sterols to overall variability in settling material and sediments, multiple regression and multivariate analysis (PCA) were performed for major sterols (compounds with < 0.5% abundance were excluded, Fig. 6). This model explains 59% of total variability, mainly through principal component 1 (47%), which is loaded in the negative side with fecal coprostanol and epicoprostanol and in the positive side with cholesterol and plant sterols.~~

3.6. Sterol vertical fluxes and accumulation efficiency

Vertical flux of total sterols was highly variable and averaged 116 ± 168 mg/cm2/year at BA, with coprostanol accounting up to 60% (70 ± 108 mg/cm2/year, Fig. 7). At N, sterol flux was four orders of magnitude lower, 0.070 ± 0.13 mg/~~cm^2~~cm2/year and cholesterol and sitosterol were the sterols with the highest fluxes. The accumulation efficiencies, obtained from the difference between sterol deposition based on trap fluxes and the inventories estimated from the observed sediment concentrations allow an evaluation of the early diagenetic behavior of these compounds. The accumulation efficiencies were 2-7 times higher at BA compared with N but the general pattern of accumulation efficiency of individual sterols was rather similar at both sampling sites. The higher accumulation efficiencies at BA reflect the variation in vertical fluxes and the differences in the oxic-anoxic transition of the sediments and the greater preservation of organic matter at sites with fast~~er~~ burial (Hedges and Keil, 1995). At BA, the high sedimentation rate rapidly removes sterols to anoxic black-colored sediments, favoring their preservation. In contrast, at N the oxic layer is thicker resulting in a greater aerobic degradation of sterols. Epicoprostanol presented the highest accumulation efficiency, especially at BA (BA: 40%, N: 5.9%) probably due to *in-situ* microbial epimerization of coprostanol rather than to an enhanced preservation during deposition. Coprostanone accumulated more efficiently than coprostanol (BA: 10 vs. 6.5%, N: 3.7 vs 2.2%). Since coprostanone and coprostanol belong to the same metabolic pathway and can readily interconvert

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(Grimalt et al., 1990; Bull et al., 2002), the preferential coprostanone preservation in sediments might be related to its higher resistance to biodegradation (Wakeham, 1989; Chaler et al., 2001). Plant sterols were in general well preserved (BA: 9.8-14%, N: 2.9-3.4%), as has been previously observed in the Saint Lawrence estuary (Colombo et al., 1997), possibly as a result of ~~to the~~ enhanced resistance of terrestrial sterols, associated with waxy higher plant material that hinder bacterial degradation (Volkman et al., 1987). Galeron et al., (2015) found that sitosterol have a low susceptibility to biodegradation and most of its decomposition proceeds via autoxidation and photodegradation, a process that is especially intense on land where chlorophyll acts as a sensitizer. Cholesterol was the least preserved sterol (BA: 4.6%, N: 1.6%) reflecting the intense breakdown of this sterol, mostly through biodegradation (Galeron et al., 2015). This explain the high accumulation efficiency of cholestanol (BA: 10%, N: 6.1%), which is originated~~results~~ from in situ microbial reduction of cholesterol rather than from preservation of settling cholestanol.

Despite the large spatial and temporal variability of hydrological parameters and sewage emission, an attempt was made to compare the sediment burden of coprostanol with the expected discharge from BA outfall. The massive vertical flux of coprostanol results in its rapid buildup in superficial sediments, which contain 24 ± 19 g/m2 of this sterol in the top 5-cm

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layer. Human coprostanol excretion depends on multiple factors such as diet, water intake, lifestyle and genetic differences, with~~resulting in~~ daily emission varying from <0.2 to >2 g/day per capita (Walker et al., 1982; Keller and Jahreis, 2004; Daughton et al., 2012). Considering an average coprostanol excretion of 1 g/day per capita and taking into account that the sewer network serves 6×10^6 people (www.aysa.com.ar), the expected sewer discharge of coprostanol can be roughly estimated to be 2200 tons/year. As previously discussed, coprostanol undergoes an extensive degradation at the water-sediment interface, so based its accumulation efficiency estimated in this work (6.5%) from 2200 tons/year only 142 tons/year would be effectively preserved in sediments. Considering an average outfall plume area of 25 km2 (Roberts and Villegas, 2016) in which most of the sewage material would settle down and a sedimentation rate of 4.7 cm/year, the expected coprostanol inventory for the top 5 cm layer ~~(averaged for the whole plume area)~~ would be 6.0 g/m2 ([5 cm / 4.7 cm/year] x [1.42 x 10^8 g/year / 2.5 x 10^7 m2]). This rough estimation, ~~value~~based on a homogenous coprostanol settling over the whole plume area, does not takes into account the rapid coprostanol decrease usually observed with distance from sources (Venkatesan and Kaplan, 1990; LeBlanc et al., 1992; Bachtiar et al, 1996). Therefore, the expected coprostanol inventory (6.0 g/m2) is lower than the one based on our measurements (24 ± 19 g/m2), ~~is in the lower range of the estimated BA inventory (24 ± 19 g/m2)~~ which considers sediments sampled close to the sewer outfall (0.5 km), where most of the

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coprostanol settling takes place . ~~neglecting the rapid coprostanol decrease usually observed with distance from sources (Venkatesan and Kaplan, 1990; LeBlanc et al., 1992; Bachtiar et al, 1996) .~~

4. Conclusions

The simultaneous analysis of sterols in settling material and underlying sediments allowed the ~~source~~ identification of sources, the calculation of vertical fluxes and the evaluation of early diagenetic changes~~study of their early diagenesis.~~. The massive inputs of anthropogenic organic matter at the Buenos Aires (BA) area of the Rio de la Plata estuary cause remarkable alterations in the fluxes and ~~natural~~ signature of particulate sterols. ~~Effectively~~Indeed, h~~, resulting in h~~uge vertical fluxes of highly organic particles enriched in fecal sterols, ~~especially~~i.e. coprostanol, ~~at levels comparable~~comparable ~~to those of a~~ raw sewage sludge are observed at this site. Th~~e~~ese anthropogenic ~~input is~~discharges are further intensified during ~~the~~ warm~~- and~~-rainy periods due to enhanced sewage discharge and terrestrial runoff. In contrast, at ~~the~~a relatively pristine northern site (N), vertical particle fluxes and particulate sterol concentrations are 3-7 orders of magnitude ~~were 7 times~~ lower, with a composition ~~and sterol concentrations both in settling material and sediments were 2-3 orders of magnitude lower,~~

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dominated by plant sterols i.e. ~~such as~~ sitosterol, stigmasterol and campesterol, derived from terrestrial vegetation. The sterols signature of underlying ~~in s~~sediments reflects the early diagenetic alteration ~~are determined by the degradation of particulate organic matter that takes places~~ occurring at the water/sediment interface. T~~, t~~hus, compared to settling material~~particles~~, the~~ir~~ concentrations decrease ~~are~~ 10-20 times ~~lower and~~ and the ~~their~~ composition ~~is enriched~~shows an enrichment of ~~in~~degradation products, i.e. cholestanol, epicoprostanol and stigmastanol. The accumulation efficiency of sterols in sediments varies according to the differential resistance of individual sterols and in situ production. Overall, the combination of higher sedimentation rates and prevailing anoxic conditions in the ~~the~~ highly polluted BA site ~~leads to~~results in ~~an shows~~ enhanced sterol preservation with ~~reflecting higher sedimentation rates and prevailing anoxic conditions leading to~~ a remarkably high ~~At the highly polluted BA site, with prevailing anoxic condition in sediments and high sedimentation rates, the sterol preservation is enhanced, resulting in~~ coprostanol accumulation ~~The massive discharge of crude sewage at Buenos Aires (BA) resulted in huge vertical fluxes of highly organic particles enriched in fecal sterols, which were further intensified during the warm and rainy period due to enhanced terrestrial runoff. Settling material presented a clear predominance of coprostanol, and a high coprostanol/epicoprostanol ratio, comparable to sewage sludge, reflecting high inputs of fresh sewage material. Despite the sterol degradation that takes places at the water/sediment~~

~~interface, coprostanol readily accumulates in surficial sediments, reaching concentrations tha~~which is ~~t are~~ among the highest ever reported in the literature ~~in literature~~. ~~In contrast, at the relatively pristine northern site (N), vertical particle fluxes were 7 times lower and sterol concentrations both in settling material and sediments were 2-3 orders of magnitude lower, dominated by plant sterols such as sitosterol, stigmasterol and campesterol. The higher vertical fluxes and prevailing anoxic conditions near the sewer favored sterol preservation, as indicated by the relatively high accumulation efficiencies compared with N. Nevertheless, at both sites epicoprostanol, cholestanol and plant sterols had the highest accumulation efficiencies reflecting both in situ production and the differential resistance to degradation.~~

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Table 1. Names, formula, molecular weight (MW), retention time (Rt) and mass of ions used for quantification (target ion) and identification (confirmatory ions) of sterols and steroids (coprostane and coprostanone) analysed in this work.

Trivial name	Systematic name*	Formula	MW	Rt	Target ion	Confirmatory ions	
Coprostane	5ß-cholestane	$C_{27}H_{48}$	372.37	30.80	217	357	372
Coprostanol	5ß-cholestan-3ß-ol	$C_{27}H_{48}O$	388.37	35.57	370	355	215
Epicoprostanol	5ß-cholestan-3α-ol	$C_{27}H_{48}O$	388.37	36.08	370	215	355
Cholestanol	5α-cholestan-3α-ol	$C_{27}H_{48}O$	388.37	36.16	215	355	370
Coprostanone	5ß-cholestan-3-one	$C_{27}H_{46}O$	386.35	37.13	386	231	370
Deuterocholesterol	cholest-5-en-3ß-ol-25,26,26,26,27,27,27-D7	~~$C_{27}H_{46}OC_{27}$~~ $H_{39}OD_7$	393.70	37.31	129	336	375
Cholesterol	cholest-5-en-3ß-ol	$C_{27}H_{46}O$	386.35	37.48	329	129	368
Dehydrocholesterol	cholesta-5,22E-dien-3ß-ol	$C_{27}H_{44}O$	384.34	37.73	215	445	355
Brassicasterol	ergosta-5,22E-dien-3ß-ol	$C_{28}H_{46}O$	398.35	38.19	456	129	366
Desmosterol	cholest-5,24-dien-3ß-ol	$C_{27}H_{44}O$	384.34	38.36	129	343	253
Ergosterol	ergosta-5,7,22E-trien-3ß-ol	$C_{28}H_{44}O$	396.65	39.17	343	337	468
Dihydrobrassicasterol	ergost-5-en-3ß-ol	$C_{28}H_{48}O$	400.37	39.75	343	129	384
Campesterol	campest-5-en-3ß-ol	$C_{28}H_{48}O$	400.37	39.92	343	129	382
Ethylcoprostanol	24S-5ß-stigmastan-3ß-ol	$C_{29}H_{52}O$	416.40	40.19	398	215	383
Stigmasterol	stigmasta-5,22E-dien-3ß-ol	$C_{29}H_{48}O$	412.37	40.55	129	255	484
Deuterositosterol	stigmast-5-en-3ß-ol-25,26,26,26,27,27,27 -D7	~~$C_{29}H_{50}OC_{29}$~~ $H_{43}OD_7$	421.75	42.00	129	364	403
Sitosterol	stigmast-5-en-3ß-ol	$C_{29}H_{50}O$	414.39	42.20	129	488	473
Stigmastanol	stigmastan-3ß –ol	$C_{29}H_{52}O$	416.40	42.59	215	473	488

*: according LIPID MAPS classification system (http://www.lipidmaps.org/data/classification).

924 **Table 2.** Coprostanol concentration (µg/g) from surficial sediments throughout the world.

Sampling site	Environment	Concentration	Reference
Highly polluted sediments			
Yucatan Cenotes, Mexico	Underground river	< 1-1690*	Arcega-Cabrera et al., 2014
Northeastern Hamilton Harbour, Canada	Lake	< 1-1600	Coakley et al., 2002
Rio de la Plata, Argentina	**River**	**59-708**	**This study**
Barcelona, Spain	Sea	< 1-390	Grimalt et al., 1990
Iguaçu and Barigui Rivers, Brazil	River	< 1-375	Puerari et al., 2012
Bilbao Estuary, Spain	Estuary	2.2-293	Gonzalez-Oreja and Saiz-Salinas, 1998
Barigui River, Brazil	River	< 1-196	Froehner et al., 2009
Firth of Clyde, Scotland, United Kingdom	Sea	< 1-176	Kelly and Campbell, 1996
Tan-Shui Estuary Taiwan	Estuary	< 1-163	Jeng and Han, 1994
Northeastern Hamilton Harbour, Canada	Lake	< 1-147	Bachtiar et al., 1996
Guanabara Bay, Brazil	Sea	1.4-105	Lima da Costa and Carreira, 2005
Kaoping River, Taiwan	River	< 1-58	Jeng and Han, 1996
Lake of Neuchatel, Switzerland	Lake	6.1-55	Pittet et al., 1990
Ria Formosa, Portugal	Sea lagoon	< 1-42	Mudge and Bebbiano, 1997
Venice Lagoon, Italy	Sea lagoon	< 1-41	Sherwin et al., 1993
Narrangansett Bay, USA	Sea	< 1-39	Le Blanc et al., 1992
Rio de la Plata, Uruguay	River	< 1-21	Venturini et al., 2015
Reference low-moderately polluted river sediments			
Siak River, Indonesia	River	0.050-11	Liebezeit and Wöstmann, 2010
Mississippi River, USA	River	0.10-7.5	Writer et al., 1995
Capibaribe River, Brazil	River	0.52-7.3	Fernandes et al., 1999
Uruguay River, Argentina	**River**	**nd -1.7**	**This study**
Santa Ana River, USA	River	nd-0.49	Noblet et al., 2004
Langat River, Malaysia	River	0.0028-0.42	Adnan et al., 2012

925 *: Sum of fecal sterols.

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Figure captions:

Fig. 1. Sampling stations of settling material and sediments in the metropolitan coastal area of the Rio de la Plata estuary, near Buenos Aires main sewer (BA) and at ~~a northern site~~North (N) in the Uruguay River ~~(N).~~.

Fig. 2. Relationship between river discharge and total particle flux at Buenos Aires (black circles) and North (grey squares) sites during warm (September to March, solid markers) and cold months (April to August, open markers). Note the logarithmic scale on the y-axis.

Fig. 3. Temporal variation of total particle flux (solid line, left axis) and total sterol concentration in settling material (dotted line, right axis) at Buenos Aires (top panel) and North (bottom panel). The boxplot inserts shows the averages for warm months (September to March) and cold months (April to August) for total particle flux (black boxes) and total sterols (white boxes).

Fig. 4. Sterol composition of settling material (top panel) and sediments (bottom panel) at Buenos Aires (BA, black bars, left pie chart) and North (N, grey bars, right pie chart). Pie charts show proportions of cholesterol, fecal sterols, phytosterols and other sterols. Bar graphs show

individual sterols concentrations, in a dry weight basis (note the logarithmic scale).

Fig. 5. Principal component analysis of sterol composition of settling particles (solid circles) and sediments (open squares) from Buenos Aires (black) and North (grey). The black asterisk correspond to the average sterol composition of human feces (according Leeming et al. 1996).

Fig. ~~5~~6. Box plots of different sterol ratios from Buenos Aires (black) and North (grey) in settling material (filled boxes) and sediment (open boxes). Copr/epiCop: coprostanol/(coprostanol + epicoprostanol), Cop/ethylCop: coprostanol/(coprostanol + ethylcoprostanol), Sito/ethylCop: sitosterol/(sitosterol + ethylcoprostanol, Chnol/Chrol: cholesterol/(cholesterol + cholestanol) All ratios were significantly different between Buenos Aires and North ($p < 0.0001$).

~~**Fig. 6.** Principal component analysis of sterol composition of settling particles (solid circles) and sediments (open squares) from Buenos Aires (black) and North (grey).~~

Fig. 7. Accumulation efficiencies of sterols from settling material in superficial sediments (%, bars, left axis) and vertical fluxes (points with standard error bars, right axis) for Buenos Aires (upper panel) and North (bottom panel). Horizontal dotted lines indicate accumulation efficiency of total

969 sterols. Minor sterols (~~<~~(≤ 1% of total sterols) were excluded from
970 calculations.
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Early diagenetic alterations of sterol biomarkers during particle settling and burial in polluted and pristine areas of the Rio de la Plata Basin

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Abstract:

Sources and diagenetic alterations of sterol markers were studied in settling material and sediments near Buenos Aires main sewer (BA), and at a relatively non-polluted northern site at the Uruguay River (N). Vertical particle fluxes were 7-times higher at BA relative to N (34 ± 24 vs. 4.6 ± 3.6 mg/cm2/day; mean ± standard deviation),increasing during rainy months. Total sterol contents were consistently higher at BA, both in settling material (7140 ± 7905 vs. 41 ± 47 µg/g at N) and sediments (708 ± 454 vs. 1.9 ± 0.18 µg/g). This difference was further amplified in the vertical flux of sterols (116 ± 168 vs. 0.070 ± 0.13 mg/cm2/year). At BA, sterol composition of settling material and sediments was dominated by fecal sterols (75-77%), with extreme coprostanol concentrations (3.6 ± 4.8 vs. 0.35 ± 0.28 mg/g at N) which are similar to sewage sludge. In contrast, at N the sterol profile was dominated by plant sterols (57-64%), mainly sitosterol, stigmasterol and campesterol. At BA the discharge of fresh sewage was confirmed by the high coprostanol/(coprostanol + epicoprostanol) ratio. At N, the overwhelming dominance of plant sterols over herbivore fecal sterols was reflected by the high sitosterol/(sitosterol + ethylcoprostanol) ratio and the low coprostanol/(coprostanol + ethylcoprostanol) ratio. The coprostanol/(coprostanol + epicoprostanol) and cholesterol/(cholesterol + cholestanol) ratios were lower in sediments than in settling material, reflecting the sterol degradation at the sediment surface. The

accumulation efficiencies, calculated as the difference between trap fluxes and sediment inventories, were 2-7 times higher at BA reflecting strong vertical fluxes and enhanced preservation under anoxic conditions. During diagenetic processes, epicoprostanol (partially produced in situ), cholestanol and plant sterols were the best-preserved sterols, while cholesterol was the most labile during burial.

Keywords: Sterols; Sewage markers; settling material; Rio de la Plata.

1. Introduction

The molecular composition of lipids from settling material and sediments provides useful information about the sources and diagenetic alteration of organic matter (Meyers and Ishiwatari, 1993; Canuel and Hardison, 2015). Sterols, present as components of cell membranes in eukaryotes and also in prokaryotes, are especially suited as biomarker compounds due to their widespread environmental occurrence, stability and structural diversity (Volkman, 2005). The source specificity of sterols range from some rather unspecific sterols (e.g. cholesterol) to several marker sterols associated to particular organisms, such as diatoms, dinoflagellates, plants and fungi (Volkman 2016; Puglisi et al., 2003). A group of sterols, collectively referred as fecal sterols, have been widely used as sewage tracers. Coprostanol, formed during the biohydrogenation of the Δ5 double bond of cholesterol by bacteria present in the gut of humans or animals, is the primary fecal sterol detected in domestic wastes (<60% total sterols, Bull et al., 2002). Coprostanol, unlike cholesterol, is barely absorbed by the intestinal epithelium and is massively excreted with feces (Veiga et al., 2005). Although it is degraded under oxic conditions, it can resist relatively unaltered for many years in anoxic sediments (Nishimura and Koyama, 1977).

69 Discharge of municipal wastewater to rivers and coastal areas is a source
70 of continuing environmental concern. Municipal discharges are a major source
71 of organic matter and nutrients that may cause eutrophication, oxygen
72 depletion, turbidity increase, acidification, and trophic structure alterations
73 leading to habitat deterioration (deBruyn et al., 2003; Blanch et al., 2004;
74 Kress et al., 2016). Moreover, since most sewer systems in Latin America also
75 receive storm drainage and industry inputs, sewage contains many hazardous
76 materials such as organic and inorganic pollutants and pathogens that
77 jeopardize the use of water for human consumption, fishing activities or
78 recreation (Helmer and Hespanhol, 1997). Urban-industrial effluent discharges
79 in major river systems are a key source areas of anthropogenic material to
80 marine environments.

81 Among these major river systems worldwide, the Rio de la Plata Basin
82 ranks 5th in terms of drainage area (2.8×10^6 km2), covering nearly 20% of
83 South America surface area (Milliman and Meade, 1983). The main tributary
84 rivers of this basin (the Parana and Uruguay rivers) discharge an average
85 of22,000 m3/s of water to the Atlantic Ocean through the Rio de la Plata
86 estuary, a large funnel and shallow shaped estuary that receives > 82-129 $\times 10^6$
87 tons/year of particulate load making it one of the most turbid estuaries in the
88 world (Milliman and Meade, 1983). The coastal area of metropolitan Buenos
89 Aires is strongly impacted by anthropogenic discharges resulting in high

90 concentrations of hydrocarbons, organochlorine pesticides, PCBs and metals in
91 sediments (Colombo et al., 1989, 2005; Tatone et al., 2009), settling material
92 (Colombo et al. 2007c; Tatone et al., 2012) and biota (Colombo et al., 1997,
93 2007a, 2007b, 2011). Before the installation of a primary wastewater treatment
94 plant in 2015, the main Buenos Aires sewer outfall discharged 2.2×10^6 m3/day
95 of crude domestic wastes from 6×10^6 inhabitants as well as industrial and
96 municipal wastes 2.5 km offshore (www.aysa.com.ar; FREPLATA, 2005). The
97 Riachuelo River, located 20 km upstream the main sewer, also discharges
98 sewage material and industrial waste. The combined loads of both effluents
99 reach 4.3 x 10^6 m3/day, which is comparable to the flow of the world's largest
100 sewage outfall in Boston (Roberts and Villegas, 2016).

101 In this context, the analysis of settling material in rivers systems with
102 high turbidity and organic matter load, is particularly relevant. Settling
103 material represents the fresh inputs of organic matter to aquatic environments
104 and is thus useful to assess its sources as well as the temporal variability.
105 Sediments integrate these signals over a wide temporal range, with a
106 composition dominated by refractory compounds. The comparison between
107 settling material and underlying sediments allows for a detailed evaluation of
108 the early diagenetic behavior of organic compounds, mainly controlled by
109 factors such as sedimentation rate, temperature and redox conditions (Colombo
110 et al., 1996b).

In this paper, we evaluate the sources, vertical fluxes and diagenetic alterations of sterol markers during burial in two contrasting sites of the Rio de la Plata Basin: the highly impacted metropolitan area of the Rio de la Plata estuary and a relatively non-polluted northern site at the Uruguay River. Differences in terms of sterol concentration and composition, vertical fluxes, differential preservation in sediments and temporal variation are discussed.

2. Materials and methods

The sampling strategy comprised two sites with contrasting anthropogenic impact: the heavily polluted Buenos Aires metropolitan area of the Rio de la Plata estuary near the main sewer outfall (BA, 34°43.33' S - 58°10.30' W) and a more pristine site ~200 km upstream on the Uruguay River, the Ñandubaysal Bay (N, 33°05.27' S - 58°21.37' W; Fig.1). Sampling campaigns were carried out seasonally from 2007 to 2014. Settling material was collected in pre-weighed polypropylene conical Falcon tubes coupled to a fixed 10 cm diameter cylindrical sediment trap deployed at 1.5 m during 1-3 days (BA) or 30-60 days (N). Superficial sediments were collected using a stainless steel Hydro-Bios Van-Veen grab sampler. Samples were immediately refrigerated and transported to the laboratory. Tubes containing the settling

131 material were centrifuged and weighed after discarding supernatant water.
132 Water content was determined gravimetrically after drying in an oven at 40 °C.
133 Total organic carbon determination was carried out on a Thermo Finnigan
134 Flash EA 1112 elemental analyzer. Total particle flux was computed as:

$$\text{Flux (mg/cm2/day)} = \frac{\text{settling matter mass (mg)}}{\text{trap surface (78 cm2)} \times \text{deployment time (days)}}$$

135 Sedimentation rate was calculated as:

$$\text{SR(cm/year)} = \frac{\text{Flux} \times 365}{1000 \times \text{density (g/cm3)}}$$

136 The discharge of the Uruguay River was calculated as the turbinated
137 plus compensation flow discharged daily by the Salto Grande Dam, located 240
138 km upstream N station and averaged for each sediment trap deployment
139 period (wholesale electricity market administration company:
140 www.cammesa.com). The discharge of the Rio de la Plata estuary was assumed
141 as the sum of the corresponding monthly discharges of the Uruguay River,
142 measured 90 km upstream N station, and of the Parana River, measured near
143 the mouth of its main channels (Paraná Guazú and Paraná de las Palmas;
144 Base de Datos Hidrológica Integrada, bdhi.hidricosargentina.gov.ar; Jaime and
145 Menendez, 2002).

146 Lipids were extracted ultrasonically with
147 acetone:dichloromethane:petroleum ether (1:2:2), dried over anhydrous sodium

148 sulfate and gravimetrically determined (Colombo et al. 1996a). Deuterated
149 sterols (deuterocholesterol-D7 and deuterositosterol-D7, Steraloids, Inc.,
150 Newport, RI, steraloids.com; Table 1) were used as internal standards. In order
151 to avoid the interference of fatty acids, lipids (100 mg approx.) were saponified
152 with 1M KOH in methanol and non-saponifiable compounds were extracted
153 with petroleum ether – diethyl ether (4:1 v/v, Christie, 1989). The extracts were
154 concentrated under a nitrogen stream and derivatized with 150 µl of N,O-
155 Bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane
156 (BSTFA:TMCS, 10:1 v/v; AppliChem GmbH, Darmstadt, Germany; Sigma-
157 Aldrich, St. Louis, MO, USA) for 3 hours at 60 ºC. The resulting trimethylsilyl
158 derivatives were taken to dryness under nitrogen and resuspended in toluene
159 prior analysis. All solvents used were pesticide residue analysis grade.

160 Samples were analyzed using a Perkin Elmer Clarus 500 GC-MS (Perkin
161 Elmer, Waltham, MA, USA) fitted with a Quadrex 007-5MS capillary column
162 (60 m, 0.32 mm i.d., 0.25 µm; Quadrex Corp., Bethany, CT, USA). Helium was
163 used as a carrier gas with a flow rate of 1.2 ml/min and the temperature of
164 injector was set at 250 ºC (split-splitless mode). The oven temperature program
165 started at 100 ºC with a ramp to 225 ºC at 15 ºC/min and to 300 ºC at 3 ºC/min
166 with a final holding time of 10 min. The transfer line temperature was set at
167 200 ºC and the analytes were ionized by 70 eV electron impact at 180 ºC. The
168 mass spectrometer was simultaneously operated in scan mode (60-600 amu)

169 and selective ion monitoring. Data were acquired and processed with
170 TurboMass 5.1 software.

171 Steroids with their trivial and IUPAC names, molecular weight,
172 retention times and mass-to-charge ratios (*m*/*z*) used for quantification and
173 confirmation are presented in Table 1. Coprostanol, epicoprostanol,
174 coprostanone and ethylcoprostanol were collectively referred to as fecal sterols.
175 Compounds were identified by comparison with standards of 15 steroids
176 (Brassicasterol, Campesterol, Coprostanone, Deuterocholesterol,
177 Deuterositosterol, Epicoprostanol, Ergosterol and Sitosterol from Steraloids;
178 Cholesterol, Coprostane, Coprostanol, Dehydrocholesterol, Desmosterol,
179 Stigmastanol and Stigmasterol from Sigma-Aldrich), literature data and
180 interpretation of mass spectrometric fragmentation patterns. Quantification
181 was performed using a 4-points calibration curve (0.20-50 µg/ml) prepared in
182 dichloromethane from certified standards (Table 1). Peak areas were corrected
183 according internal standard recoveries. Commercially standards were not
184 available for some compounds (Cholestanol, Dehydrobrassicasterol and
185 Ethylcoprostanol) which were quantified based on response factors of
186 structurally related sterols.

187 The limit of detection (LOD) of each steroid was estimated by calculating
188 the signal to-noise ratio (S/N) of triplicate standard solutions in the range of
189 0.20-50 µg/ml. LODs values averaged 6.5 ± 11 ng/g, ranging from (0.31 ng/g,

coprostanol) to (43 ng/g, ergosterol). Reproducibility was assessed by the relative standard deviation (RSD) of triplicate analysis of the same samples in different batches, and averaged 11 ± 3.8 The method was highly linear in the range of concentrations of calibration curves ($r2 > 0.99$ for all steroids with available authentic standards). Recoveries of deuterated internal standards averaged 96 ± 1.7. Individual recoveries, evaluated by analysis of spiked samples ranged from 82 ± 15% (Ergosterol) to 110 ± 19% (Desmosterol).

Statistical analysis was carried on with Python scripting language (www.python.org), using SciPy (www.scipy.org), NumPy (www.numpy.org), MatPlotlib (matplotlib.org) and pandas (pandas.pydata.org) libraries. Multivariate analyses were executed in *R* language, using RStudio development environment (www.rstudio.com) and ggplot2 and ggbiplot packages (http://ggplot2.org/). Data were expressed as mean ± SD. Relative standard deviation (RSD: [data - mean] × 100/SD) was used to assess parameter variability. To avoid division by zero errors, the ratios between two sterols, A and B were calculated as: A/(A + B). The accumulation efficiency of sterols from settling material to sediments was estimated as the relationship between the annual vertical flux of the sterol and its corresponding one-year inventory in sediments (sterol concentration in sediment × annual mineral flux). Student's *t* test was used to perform comparisons between two means as well as to evaluate the significance of correlation coefficients. Multivariate

211 analysis was performed by principal component analysis of standardized data
212 (x – X/y, where X = mean and y = SD).

213

214 **3. Results and discussion**

215

216 *3.1. Total particle flux*

217 The intense discharge of one of the largest sewer worldwide at BA adds
218 to the natural particle load of the Rio de la Plata resulting in extraordinarily
219 high vertical particle fluxes (34 ± 24 mg/cm2/day) and sedimentation rates (4.7
220 ± 3.3 cm/year), in agreement with previous measurements in this area (5.5 ±
221 2.1 cm/year, density: 2.65 g/cm3; Colombo et al., 2007c). These values are
222 higher than sedimentation rates reported for nearby sites of this turbid estuary
223 (0.3-1.3 cm/year; Di Gregorio et al., 2007; Bonachea et al., 2010). This suggests
224 that most particles captured by sediment traps at BA are highly organic
225 detritus derived from urban-industrial discharges, as confirmed by the high
226 concentration of lipids (Speranza et al., 2013) and fecal sterols of this material
227 (see below). At N, the total particle flux was 7-times lower (4.6 ± 3.6
228 mg/cm2/day), comparable to values previously reported for the Uruguay River
229 (2.7 ± 2.3 mg/cm2/day, range: 0.73-7.3 mg/cm2/day; Colombo et al., 2015),
230 resulting in a sedimentation rate of 0.64 ± 0.49 cm/year. In contrast to BA,
231 where the settling material is composed mostly by anthropogenic detritus over

232 the background particle load from Parana River, the settling material at N
233 reflects the lower solid discharge of the Uruguay River (Moreira et al., 2013).
234 The total particle flux was largely dependent on river discharge, which was 6-
235 46 times higher at BA (19-46 x 10^3 m3/s) relative to N (0.42-8.4 x 10^3 m3/s),
236 fitting an exponential curve (r2 = 0.78, $p < 0.0001$; Fig. 2). This correlation had
237 been previously observed at the Uruguay River and reflects the enhanced
238 transport of eroded material as river flow increases (Colombo et al., 2015).

239

240 *3.2. Total sterol concentrations*

241 The total sterol concentration in settling material was highly variable
242 (RSD: 113%) and exhibited a marked geographical difference. At BA, the
243 tendency of hydrophobic sterols to associate to particulate matter is enhanced
244 by the high organic content of settling particles (total organic carbon: 9.6 ±
245 7.4%), resulting in high sterol concentrations at this site (7.1 ± 7.9 mg/g dry
246 weight). Previous research on sterols in settling particles primarily conducted
247 in ocean waters, relatively deep and clear, which had average sterol
248 concentrations 1-4 orders of magnitude lower than the Rio de la Plata estuary
249 metropolitan area, a shallow, turbid and polluted environment (Takada et al.,
250 1994; Colombo et al., 1996; Parrish et al., 2000; Burns et al., 2008). The range
251 of sterol concentrations in settling material published for riverine
252 environments is considerably lower than those measured at BA (1-184 µg/g;

253 Saliot et al., 2001; Li et al., 1995; Jeng and Kao, 2002). In fact, the sterol
254 concentrations at BA are comparable to values reported for sewage sludge from
255 wastewater treatment plants (2-9 mg/g; Venkatesan and Kaplan, 1990; Kelly,
256 1995; Nguyen et al., 1995). At N, total sterol concentrations in settling material
257 are 2-3 orders of magnitude lower (41 ± 47 µg/g) and comparable to
258 aforementioned values in particulate matter from freshwater environments.
259 Total sterols in sediments were 10-20 times lower than in settling material and
260 were less variable (RSD: 10-61%), and also presented a 2-3 orders of magnitude
261 difference between BA and N (708 ± 454 vs. 1.9 ± 0.18 µg/g). The reduction in
262 sterol concentration from settling material to sediments reflects the
263 degradations of sterol at the sediment-water interface, especially under oxic
264 conditions (Sun and Wakeham, 1998).

265

266 *3.3. Temporal variation of particle flux and sterol concentrations in settling*
267 *material*

268 The large variability on settling material data observed for both BA and
269 N, was explained by temporal variations between warm and cold periods. A
270 distinctive temporal pattern of higher particle fluxes during warm and rainy
271 months (September to March, 22 ± 2.6°C, 127 ± 18 mm) relative to cold and dry
272 ones (April to August, 13 ± 2.5ºC, 74 ± 23 mm) was observed at BA (50 ± 25 vs.
273 20 ± 9.4 mg/cm2/day, $p < 0.005$; Fig. 3) and N (6.2 ± 4.0 vs. 3.2 ± 1.9

mg/cm^2/day, respectively, $p < 0.05$). Total sterol concentration at BA was significantly correlated with total particle flux (r2 = 0.41, $p < 0.05$) following its temporal variation, raising during warm months (11 ± 9.6 mg/g) and decreasing significantly during cold ones (3.6 ± 3.7 mg/g; $p < 0.05$, Fig. 3). This increase of sterol flux during the rainy period is related to the wash-out of streams and effluents that discharge in this area of the Rio de la Plata, as previously observed for other organic tracers (Colombo et al., 2007c). The reinforcement of total flux and concentration patterns results in an order of magnitude higher sterol vertical fluxes during warm periods (220 ± 202 vs. 23 ± 19 mg/cm2/year in cold months). At N, sterols were also significantly correlated with particle flux (r2 = 0.36, $p < 0.05$), but there was no significant difference between warm and cold months (45 ± 61 vs. 36 ± 28 µg/g respectively) thus sterol fluxes reflect the total particle flux pattern of higher values during the warm period (87 ± 165 vs. 52 ± 63 µg/cm2/year in cold months).

3.4. Sterol composition

The sterol composition of settling material showed contrasting differences between BA and N (Fig. 4). At BA, fecal sterols predominated (75 ± 5.4% of total sterols), mostly coprostanol (52 ± 11%), followed by cholesterol (12 ± 2.9%) and phytosterols (8.3 ± 3.6%) whereas at N the contribution of plant sterols prevailed (phytosterols: 57 ± 13%, cholesterol: 26 ± 12, fecal sterols: 7.5

295 ± 7.0%). The fecal signature of BA resembled the composition of human feces
296 (fecal sterols: 85%, phytosterols: 8.8%, cholesterol: 5.2%, others: 1.2%; Leeming
297 et al., 1996), with extremely high concentrations of coprostanol (3.6 ± 4.8 mg/g)
298 similar to sewage sludge and effluents (1-4 mg/g, 50-80% total sterols;
299 Venkatesan and Kaplan, 1990, Nguyen et al., 1995). The presence of
300 epicoprostanol (9.3 ± 9.6%), originated from coprostanol biodegradation,
301 evidence an incipient alteration which is likely occurring in the long sewer
302 pipeline (9900 km total, main sewers > 100 km, www.aysa.com.ar) rather than
303 in the very shallow (3-5 m) water column. Despite the relative abundance of
304 cholesterol at BA, its utility as biomarker is limited since it is present in
305 multiple organic matter sources (Mudge et al., 1999; Creuzburg and von Elert,
306 2009). A typical fecal herbivore marker, ethylcoprostanol derived by
307 hydrogenation of sitosterol form terrestrial vegetation (Bull et al., 2002), is also
308 relatively abundant at BA (8.5 ± 4.4%), but human feces can also include
309 significant amounts of ethylcoprostanol (Leeming et al., 1996). The significance
310 of coprostanone (5.4 ± 3.3%) is difficult to ascertain since it originates in
311 mammalian gut as an intermediary in coprostanol microbial synthesis, but it
312 can also be produced in sediments as a result of interconversions between this
313 ketone and coprostanol and epicoprostanol (McCalley et al., 1981; Bull et al.,
314 2002). The relatively low proportions phytosterols observed at BA, mainly
315 represented by sitosterol (4.4 ± 1.9%), reflect the minor contribution of vegetal
316 inputs, possibly including kitchen oil and foodstuff products, at this site.

317 Despite being found in some algae, the three major phytosterols found in
318 settling material from N, sitosterol (19 ± 5.4%), stigmasterol (15 ± 7.9%) and
319 campesterol (13 ± 11%), are strongly associated with land plants (Huang and
320 Meinschein, 1979, Volkman, 2005) and have been used as biomarkers of paper
321 mill pollution (Lahdelma and Oikari, 2006). The fecal sterols signal at N,
322 dominated by ethylcoprostanol (3.9 ± 4.7%) followed by coprostanol (1.3 ±
323 1.3%), differs both quantitatively and qualitatively from the sewage signature
324 of BA. The presence of ethylcoprostanol as the main fecal sterol at N probably
325 reflects the contribution of cattle fecal matter from the neighboring livestock
326 establishments. The small concentrations of coprostanol cannot be
327 unambiguously attributed to sewage pollution since small relative amounts of
328 coprostanol can be formed by in situ hydrogenation of cholesterol in sediments
329 not contaminated by fecal pollution (Nishimura and Koyama, 1977).

330 The change in percentage composition with total sterol concentration
331 and its seasonal variation in settling material also showed geographical
332 differences. At BA, as total sterol concentration increased, coprostanol
333 proportion also raised ($r2 = 0.30$; $p < 0.005$) while stigmasterol and campesterol
334 ($r2 = 0.31$ and 0.41; $p < 0.005$) decreased and the remaining sterol proportions
335 were not correlated, confirming that the increase in particulate sterol responds
336 basically to anthropogenic discharges. At N, there was a strong significant
337 correlation of total sterol concentration with cholesterol proportion ($r2 = 0.46$; p

338 < 0.0001) and an inverse relationship with ethylcoprostanol and stigmasterol
339 (r2 = 0.15 and 0.18 respectively; $p < 0.05$). The sterol composition, on a
340 percentage basis, showed little temporal variation except for the inverse trend
341 of coprostanol and epicoprostanol observed at BA. While coprostanol proportion
342 tended to be higher during warm months (59 ± 9.5 vs 45 ± 8.7 in cold months; p
343 < 0.01) and correlates with total particle flux (r2: 0.15; $p < 0.05$), its epimer
344 increases during the cold period (2.6 ± 2.0 to 15 ± 9.2; $p < 0.005$) and it is
345 inversely correlated to total particle flux (r2: 0.49; $p < 0.005$). This is in
346 agreement with previous work in this area of Rio de la Plata estuary where the
347 terrestrial runoff results in an enhanced discharge of organic compounds with
348 a fresher signature during warm and rainy periods, in contrast with the less
349 intense and more degraded signal observed during cold and dry months
350 (Colombo et al., 2007c). Similarly, Puerari et al. (2012) observed an enhanced
351 level of sewage degradation in the dry winter period in Brazilian rivers
352 associated with a lower terrestrial runoff.

353 The sediment sterol profile was similar to that of settling material, with
354 some minor differences related to the sterol degradation at sediment surface.
355 At BA, this degradation is apparent in the relative increase of degradation
356 products such as epicoprostanol, stigmastanol and cholestanol from settling
357 particles (9.3 ± 9.6, 1.6 ± 0.88 and 1.7 ± 1.2%) to underlying sediments (16 ± 4.5,
358 2.6 ± 1.5 and 2.8 ± 1.1%, respectively, $p < 0.05$), reflecting the microbial

359 reduction of stenols to stanols and coprostanol epimerization at the oxic-anoxic
360 boundary (Wakeham, 1989). Despite degradation processes, sediments at BA
361 still have remarkably high sterol concentrations, especially of coprostanol
362 whose concentration (349 ± 282 µg/g) is among the highest reported for surficial
363 sediments severely impacted by sewage discharges (Table 2). Coprostanol
364 highest values were chiefly measured in freshwater locations or in relatively
365 enclosed seawater environments where ocean dilution is reduced. In sediments
366 from the Uruguayan coast of the Rio de la Plata near Montevideo, Venturini et
367 al., (2015) reported 17-400 times lower concentrations of coprostanol (0.05-21
368 µg/g) and cholesterol (0.48-5.1 µg/g), evidencing that the background levels of
369 these sterols are quite low and that they derive mainly from local urban
370 discharges at BA. Interestingly, the concentrations of phytosterols were only
371 slightly lower to those of BA for stigmasterol and campesterol (0.30-3.14 and
372 0.13-2.13 µg/g, respectively; Venturini et al., 2015) but not for sitosterol, which
373 was 6-70 times lower (0.43-5.3 µg/g). This suggests that while sewage discharge
374 contributes significantly sitosterol at BA sediments, terrestrial runoff is the
375 main source of stigmasterol and campesterol. This is in agreement with
376 previous reports of high concentrations of sitosterol in sewage effluents of
377 domestic origin (e.g. flush of kitchen vegetable oils; Furtula et al., 2011). At N,
378 the sediment sterol profile was dominated by terrestrial plant phytosterols and
379 cholesterol, as observed in settling material but with higher proportions of
380 epicoprostanol, sitosterol and stigmastanol (2.7 ± 1.2, 25 ± 3.0 and 12 ± 1.9%,

381 respectively, $p < 0.05$). The marginal impact of sewage pollution at N sediments
382 is evidenced by low coprostanol concentrations, which are well below the
383 threshold values reported as indicative of sewage pollution (0.1-0.7 µg/g;
384 Grimalt et al., 1990; Leeming et al., 1997; Rada et al., 2016) and are
385 comparable to values reported for riverine sites with low to moderate sewage
386 pollution (Table 2).

387 To simultaneously evaluate the contribution of different sterols to the
388 overall variability observed in settling material and sediments, multivariate
389 analysis (PCA) were performed for major sterols (compounds with < 0.5%
390 abundance were excluded, Fig.5).This model explains 59% of total variability,
391 mainly through principal component 1 (47%), which is loaded in the negative
392 side with fecal coprostanol, coprostanone and epicoprostanol and in the positive
393 side with cholesterol and plant sterols. The second component accounts for 12%
394 of data variability and is negatively loaded with ethylcoprostanol and
395 dehydrocholesterol and positively loaded with cholestanol and epicoprosatanol.
396 Settling material from BA is clustered on the left side of the PCA, denoting
397 fecal inputs, and is clearly discriminated from N, plotting on the right due to
398 the major contribution of plant sterols to the overall composition. The average
399 sterol composition of human feces plots in the center of the BA cluster, further
400 confirming the sewage origin of settling material at this site. The sediments
401 segregation was similar to that of settling material, with minor differences

reflecting the degradation that takes place at the water-sediment interfase. BA sediments are scattered on the right, with most samples gathering near the epicoprostanol and cholestanol vectors, reflecting the degradation of coprostanol and cholesterol respectively. N sediments are more homogeneous and plotted on the upper right side of the PCA, close to cholesterol and stigmastanol vectors.

3.5. Sterol ratios

Sterol ratios have been routinely used to assess the contribution of different sources of organic matter as well as degradation processes (Jeng and Han, 1994; Takada et al., 1994; Chalaux et al., 1995; Fattore et al., 1996). All the ratios evaluated in this work presented highly significant differences between BA and N (*t*-test; $p < 0.0001$; Fig. 6). In settling material, the high coprostanol/(coprostanol + epicoprostanol) ratio at BA (0.85 ± 0.15) reflects the relatively fresh sewage inputs discharged, in contrast to the weak and highly degraded fecal signature at N (0.48 ± 0.15). The coprostanol/(coprostanol + ethylcoprostanol) ratio is 2 times higher in BA settling material relative to N (0.86 ± 0.064 vs. 0.35 ± 0.19) indicating that the reduced fecal sterols at N are chiefly from herbivore mammal feces. However, despite the overwhelming abundance of coprostanol at BA a small non-human contribution to the overall fecal signal cannot be disregarded. At this site, the sitosterol/(sitosterol +

423 ethylcoprostanol) index was 0.36 ± 0.15, in the range of values proposed by
424 Nash et al., (2005) as typical for feces runoff of herbivore with high
425 ethylcoprostanol proportions, such as cattle and pigs. At N, this ratio (0.84 ±
426 0.17) was above the limit suggested as indicative of non-fecal polluted plant
427 decay inputs (Nash et al., 2005), denoting minimum impact of fecal
428 contamination at this site. The cholesterol/(cholesterol + cholestanol) ratio is
429 useful to assess the microbial reduction of stenols to 5α-stanols that typically
430 takes places under anoxic conditions (Reeves, 2005). At BA, the relatively low
431 values of this ratio (0.85 ± 0.036) indicate prevailing reductive conditions in the
432 sewage effluent, which favors sterol preservation. On the contrary, oxic
433 conditions at N favors the sterol degradation over their hydrogenation
434 (Nishimura and Koyama, 1977), resulting in proportionally low amounts of
435 cholestanol (ratio: 0.95 ± 0.043).

436 In the sediments, these ratios exhibited the same geographical
437 differences observed in the settling material but reflected the diagenetic
438 processes that take places at sediment surface. The degradation of coprostanol
439 and cholesterol, which is intensified after particle deposition, resulted in lower
440 coprostanol/(coprostanol + epicoprostanol) ratio (BA: 0.75 ± 0.064, N: 0.26 ±
441 0.058) and cholesterol/(cholesterol + cholestanol) ratio (BA: 0.75 ±0.11, N: 0.93
442 ± 0.025) in sediments relative to settling material.

443

3.6. Sterol vertical fluxes and accumulation efficiency

Vertical flux of total sterols was highly variable and averaged 116 ± 168 mg/cm2/year at BA, with coprostanol accounting up to 60% (70 ± 108 mg/cm2/year, Fig. 7). At N, sterol flux was four orders of magnitude lower, 0.070 ± 0.13 mg/cm2/year and cholesterol and sitosterol were the sterols with the highest fluxes. The accumulation efficiencies, obtained from the difference between sterol deposition based on trap fluxes and the inventories estimated from the observed sediment concentrations allow an evaluation of the early diagenetic behavior of these compounds. The accumulation efficiencies were 2-7 times higher at BA compared with N but the general pattern of accumulation efficiency of individual sterols was rather similar at both sampling sites. The higher accumulation efficiencies at BA reflect the variation in vertical fluxes and the differences in the oxic-anoxic transition of the sediments and the greater preservation of organic matter at sites with fast burial (Hedges and Keil, 1995). At BA, the high sedimentation rate rapidly removes sterols to anoxic black-colored sediments, favoring their preservation. In contrast, at N the oxic layer is thicker resulting in a greater aerobic degradation of sterols. Epicoprostanol presented the highest accumulation efficiency, especially at BA (BA: 40%, N: 5.9%) probably due to *in-situ* microbial epimerization of coprostanol rather than to an enhanced preservation during deposition. Coprostanone accumulated more efficiently than coprostanol (BA: 10 vs. 6.5%,

N: 3.7 vs 2.2%). Since coprostanone and coprostanol belong to the same metabolic pathway and can readily interconvert (Grimalt et al., 1990; Bull et al., 2002), the preferential coprostanone preservation in sediments might be related to its higher resistance to biodegradation (Wakeham, 1989; Chaler et al., 2001). Plant sterols were in general well preserved (BA: 9.8-14%, N: 2.9-3.4%), as has been previously observed in the Saint Lawrence estuary (Colombo et al., 1997), possibly as a result of enhanced resistance of terrestrial sterols, associated with waxy higher plant material that hinder bacterial degradation (Volkman et al., 1987). Galeron et al., (2015) found that sitosterol have a low susceptibility to biodegradation and most of its decomposition proceeds via autoxidation and photodegradation, a process that is especially intense on land where chlorophyll acts as a sensitizer. Cholesterol was the least preserved sterol (BA: 4.6%, N: 1.6%) reflecting the intense breakdown of this sterol, mostly through biodegradation (Galeron et al., 2015). This explain the high accumulation efficiency of cholestanol (BA: 10%, N: 6.1%), which is originated from in situ microbial reduction of cholesterol rather than from preservation of settling cholestanol.

Despite the large spatial and temporal variability of hydrological parameters and sewage emission, an attempt was made to compare the sediment burden of coprostanol with the expected discharge from BA outfall. The massive vertical flux of coprostanol results in its rapid buildup in

superficial sediments, which contain 24 ± 19 g/m2 of this sterol in the top 5-cm layer. Human coprostanol excretion depends on multiple factors such as diet, water intake, lifestyle and genetic differences, with daily emission varying from <0.2 to >2 g/day per capita (Walker et al., 1982; Keller and Jahreis, 2004; Daughton et al., 2012). Considering an average coprostanol excretion of 1 g/day per capita and taking into account that the sewer network serves 6×10^6 people (www.aysa.com.ar), the expected sewer discharge of coprostanol can be roughly estimated to be 2200 tons/year. As previously discussed, coprostanol undergoes an extensive degradation at the water-sediment interface, so based its accumulation efficiency estimated in this work (6.5%) from 2200 tons/year only 142 tons/year would be effectively preserved in sediments. Considering an average outfall plume area of 25 km2 (Roberts and Villegas, 2016) in which most of the sewage material would settle down and a sedimentation rate of 4.7 cm/year, the expected coprostanol inventory for the top 5 cm layer would be 6.0 g/m2 ([5 cm / 4.7 cm/year] x [1.42×10^8 g/year / 2.5×10^7 m2]). This rough estimation, based on a homogenous coprostanol settling over the whole plume area, does not takes into account the rapid coprostanol decrease usually observed with distance from sources (Venkatesan and Kaplan, 1990; LeBlanc et al., 1992; Bachtiar et al, 1996). Therefore, the expected coprostanol inventory (6.0 g/m2) is lower than the one based on our measurements (24 ± 19 g/m2), which considers sediments sampled close to the sewer outfall (0.5 km), where most of the coprostanol settling takes place .

4. Conclusions

The simultaneous analysis of sterols in settling material and underlying sediments allowed the identification of sources, the calculation of vertical fluxes and the evaluation of early diagenetic changes. The massive inputs of anthropogenic organic matter at the Buenos Aires (BA) area of the Rio de la Plata estuary cause remarkable alterations in the fluxes and signature of particulate sterols. Indeed, huge vertical fluxes of highly organic particles enriched in fecal sterols, i.e. coprostanol, comparable to raw sewage sludge are observed at this site. These anthropogenic discharges are further intensified during warm-rainy periods due to enhanced sewage discharge and terrestrial runoff. In contrast, at a relatively pristine northern site (N), vertical particle fluxes and particulate sterol concentrations are 3-7 orders of magnitude lower, with a composition dominated by plant sterols i.e. sitosterol, stigmasterol and campesterol, derived from terrestrial vegetation. The sterols signature of underlying sediments reflects the early diagenetic alteration occurring at the water/sediment interface. Thus, compared to settling material, the concentrations decrease 10-20 times and the composition shows an enrichment of degradation products, i.e. cholestanol, epicoprostanol and stigmastanol. The accumulation efficiency of sterols in sediments varies according to the

529 differential resistance of individual sterols and in situ production. Overall, the
530 combination of higher sedimentation rates and prevailing anoxic conditions in
531 the highly polluted BA site results in enhanced sterol preservation with a
532 remarkably high coprostanol accumulation which is among the highest ever
533 reported in the literature.

534

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536

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545

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547

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832 **Table 1.** Names, formula, molecular weight (MW), retention time (Rt) and mass of ions used for
833 quantification (target ion) and identification (confirmatory ions) of sterols and steroids
834 (coprostane and coprostanone) analysed in this work.

Trivial name	Systematic name*	Formula	MW	Rt	Target ion	Confirmatory ions	
Coprostane	5ß-cholestane	$C_{27}H_{48}$	372.37	30.80	217	357	372
Coprostanol	5ß-cholestan-3ß-ol	$C_{27}H_{48}O$	388.37	35.57	370	355	215
Epicoprostanol	5ß-cholestan-3α-ol	$C_{27}H_{48}O$	388.37	36.08	370	215	355
Cholestanol	5α-cholestan-3α-ol	$C_{27}H_{48}O$	388.37	36.16	215	355	370
Coprostanone	5ß-cholestan-3-one	$C_{27}H_{46}O$	386.35	37.13	386	231	370
Deuterocholesterol	cholest-5-en-3ß-ol-25,26,26,26,27,27,27-D7	$C_{27}H_{39}OD_7$	393.70	37.31	129	336	375
Cholesterol	cholest-5-en-3ß-ol	$C_{27}H_{46}O$	386.35	37.48	329	129	368
Dehydrocholesterol	cholesta-5,22E-dien-3ß-ol	$C_{27}H_{44}O$	384.34	37.73	215	445	355
Brassicasterol	ergosta-5,22E-dien-3ß-ol	$C_{28}H_{46}O$	398.35	38.19	456	129	366
Desmosterol	cholest-5,24-dien-3ß-ol	$C_{27}H_{44}O$	384.34	38.36	129	343	253
Ergosterol	ergosta-5,7,22E-trien-3ß-ol	$C_{28}H_{44}O$	396.65	39.17	343	337	468
Dihydrobrassicasterol	ergost-5-en-3ß-ol	$C_{28}H_{48}O$	400.37	39.75	343	129	384
Campesterol	campest-5-en-3ß-ol	$C_{28}H_{48}O$	400.37	39.92	343	129	382
Ethylcoprostanol	24S-5ß-stigmastan-3ß-ol	$C_{29}H_{52}O$	416.40	40.19	398	215	383
Stigmasterol	stigmasta-5,22E-dien-3ß-ol	$C_{29}H_{48}O$	412.37	40.55	129	255	484
Deuterositosterol	stigmast-5-en-3ß-ol-25,26,26,26,27,27,27 -D7	$C_{29}H_{43}OD_7$	421.75	42.00	129	364	403
Sitosterol	stigmast-5-en-3ß-ol	$C_{29}H_{50}O$	414.39	42.20	129	488	473
Stigmastanol	stigmastan-3ß –ol	$C_{29}H_{52}O$	416.40	42.59	215	473	488

835 *: according LIPID MAPS classification system (http://www.lipidmaps.org/data/classification).

836

837 **Table 2.** Coprostanol concentration (µg/g) from surficial sediments throughout the world.

Sampling site	Environment	Concentration	Reference
Highly polluted sediments			
Yucatan Cenotes, Mexico	Underground river	< 1-1690*	Arcega-Cabrera et al., 2014
Northeastern Hamilton Harbour, Canada	Lake	< 1-1600	Coakley et al., 2002
Rio de la Plata, Argentina	**River**	**59-708**	**This study**
Barcelona, Spain	Sea	< 1-390	Grimalt et al., 1990
Iguaçu and Barigui Rivers, Brazil	River	< 1-375	Puerari et al., 2012
Bilbao Estuary, Spain	Estuary	2.2-293	Gonzalez-Oreja and Saiz-Salinas, 1998
Barigui River, Brazil	River	< 1-196	Froehner et al., 2009
Firth of Clyde, Scotland, United Kingdom	Sea	< 1-176	Kelly and Campbell, 1996
Tan-Shui Estuary Taiwan	Estuary	< 1-163	Jeng and Han, 1994
Northeastern Hamilton Harbour, Canada	Lake	< 1-147	Bachtiar et al., 1996
Guanabara Bay, Brazil	Sea	1.4-105	Lima da Costa and Carreira, 2005
Kaoping River, Taiwan	River	< 1-58	Jeng and Han, 1996
Lake of Neuchatel, Switzerland	Lake	6.1-55	Pittet et al., 1990
Ria Formosa, Portugal	Sea lagoon	< 1-42	Mudge and Bebbiano, 1997
Venice Lagoon, Italy	Sea lagoon	< 1-41	Sherwin et al., 1993
Narrangansett Bay, USA	Sea	< 1-39	Le Blanc et al., 1992
Rio de la Plata, Uruguay	River	< 1-21	Venturini et al., 2015
Reference low-moderately polluted river sediments			
Siak River, Indonesia	River	0.050-11	Liebezeit and Wöstmann, 2010
Mississippi River, USA	River	0.10-7.5	Writer et al., 1995
Capibaribe River, Brazil	River	0.52-7.3	Fernandes et al., 1999
Uruguay River, Argentina	**River**	**nd -1.7**	**This study**
Santa Ana River, USA	River	nd-0.49	Noblet et al., 2004
Langat River, Malaysia	River	0.0028-0.42	Adnan et al., 2012

838 *: Sum of fecal sterols.

839

840

841

842 Figure captions:

843

844 **Fig. 1.** Sampling stations of settling material and sediments in the
845 metropolitan coastal area of the Rio de la Plata estuary, near Buenos
846 Aires main sewer (BA) and at North (N) in the Uruguay River.

847 **Fig. 2.** Relationship between river discharge and total particle flux at Buenos
848 Aires (black circles) and North (grey squares) sites during warm
849 (September to March, solid markers) and cold months (April to August,
850 open markers). Note the logarithmic scale on the y-axis.

851 **Fig. 3.** Temporal variation of total particle flux (solid line, left axis) and total
852 sterol concentration in settling material (dotted line, right axis) at
853 Buenos Aires (top panel) and North (bottom panel). The boxplot inserts
854 shows the averages for warm months (September to March) and cold
855 months (April to August) for total particle flux (black boxes) and total
856 sterols (white boxes).

857 **Fig. 4.** Sterol composition of settling material (top panel) and sediments
858 (bottom panel) at Buenos Aires (BA, black bars, left pie chart) and North
859 (N, grey bars, right pie chart). Pie charts show proportions of
860 cholesterol, fecal sterols, phytosterols and other sterols. Bar graphs show
861 individual sterols concentrations, in a dry weight basis (note the
862 logarithmic scale).

Fig. 5. Principal component analysis of sterol composition of settling particles (solid circles) and sediments (open squares) from Buenos Aires (black) and North (grey). The black asterisk correspond to the average sterol composition of human feces (according Leeming et al. 1996).

Fig. 6. Box plots of different sterol ratios from Buenos Aires (black) and North (grey) in settling material (filled boxes) and sediment (open boxes). Copr/epiCop: coprostanol/(coprostanol + epicoprostanol), Cop/ethylCop: coprostanol/(coprostanol + ethylcoprostanol), Sito/ethylCop: sitosterol/(sitosterol + ethylcoprostanol, Chnol/Chrol: cholesterol/(cholesterol + cholestanol) All ratios were significantly different between Buenos Aires and North ($p < 0.0001$).

Fig. 7. Accumulation efficiencies of sterols from settling material in superficial sediments (%, bars, left axis) and vertical fluxes (points with standard error bars, right axis) for Buenos Aires (upper panel) and North (bottom panel). Horizontal dotted lines indicate accumulation efficiency of total sterols. Minor sterols (< 1% of total sterols) were excluded from calculations.

Figure 1

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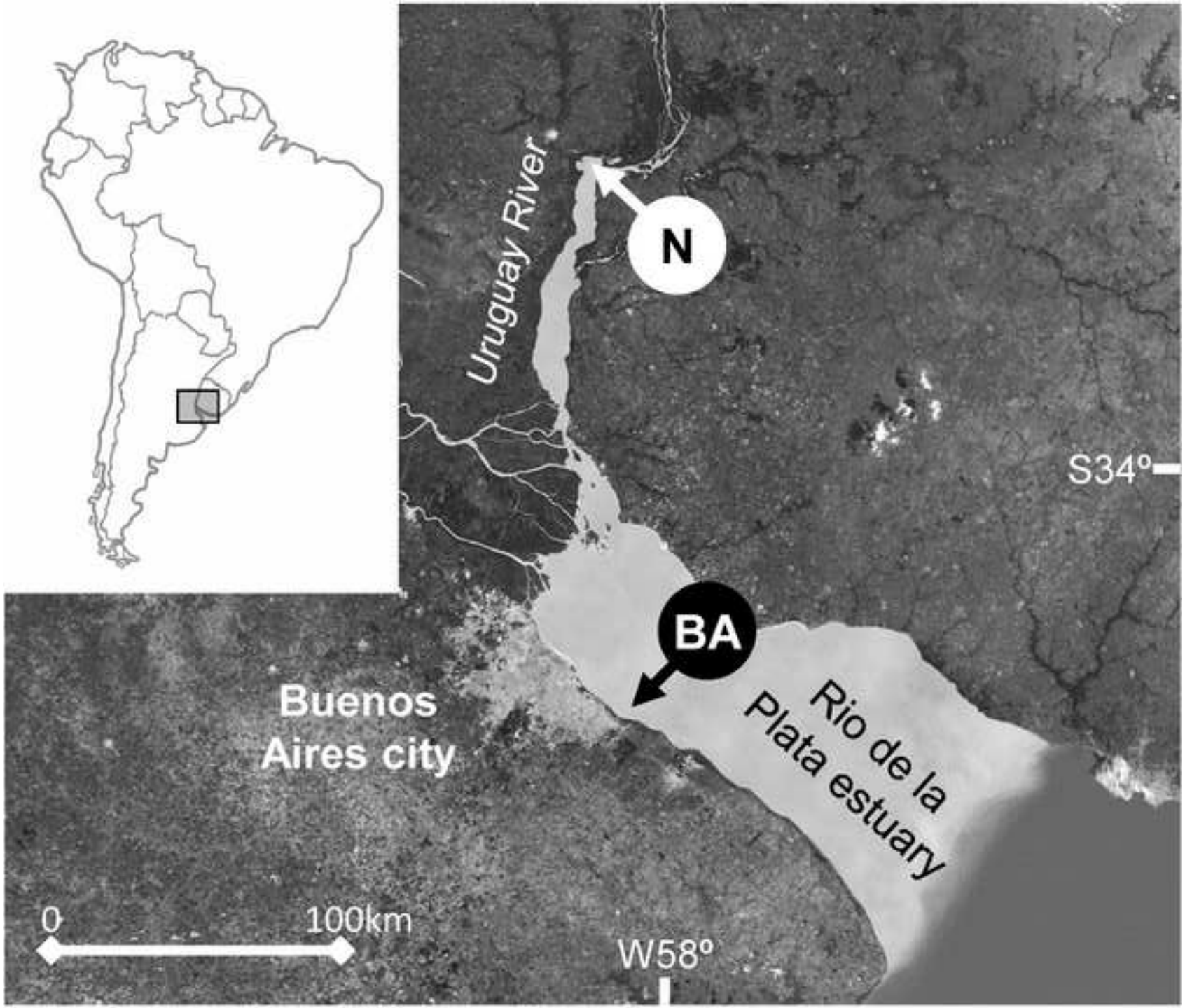


Figure 2

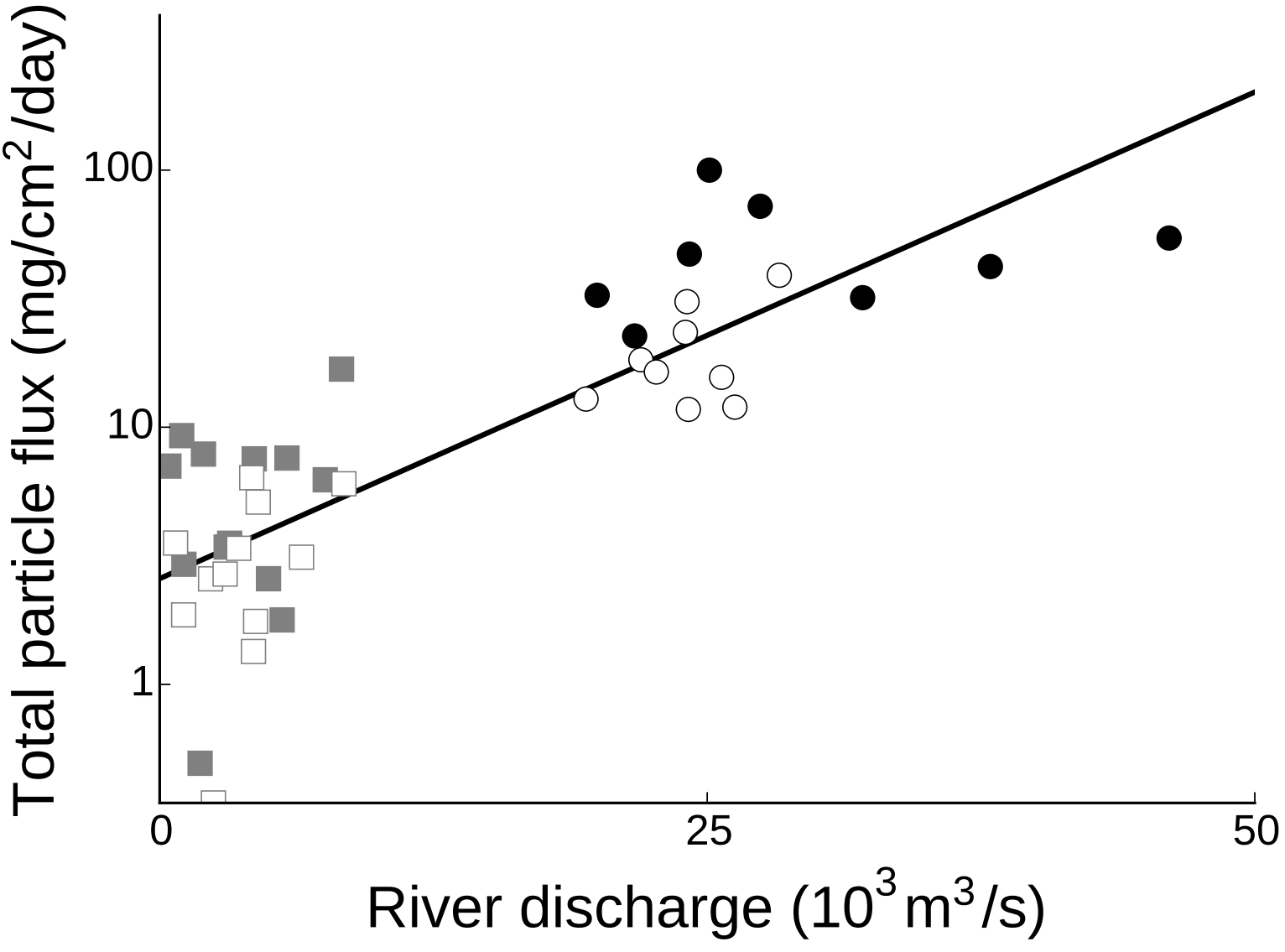

Total particle flux (mg/cm^2/day)
100
10
1
0
25
50
River discharge (10^3 m^3/s)

Figure 3

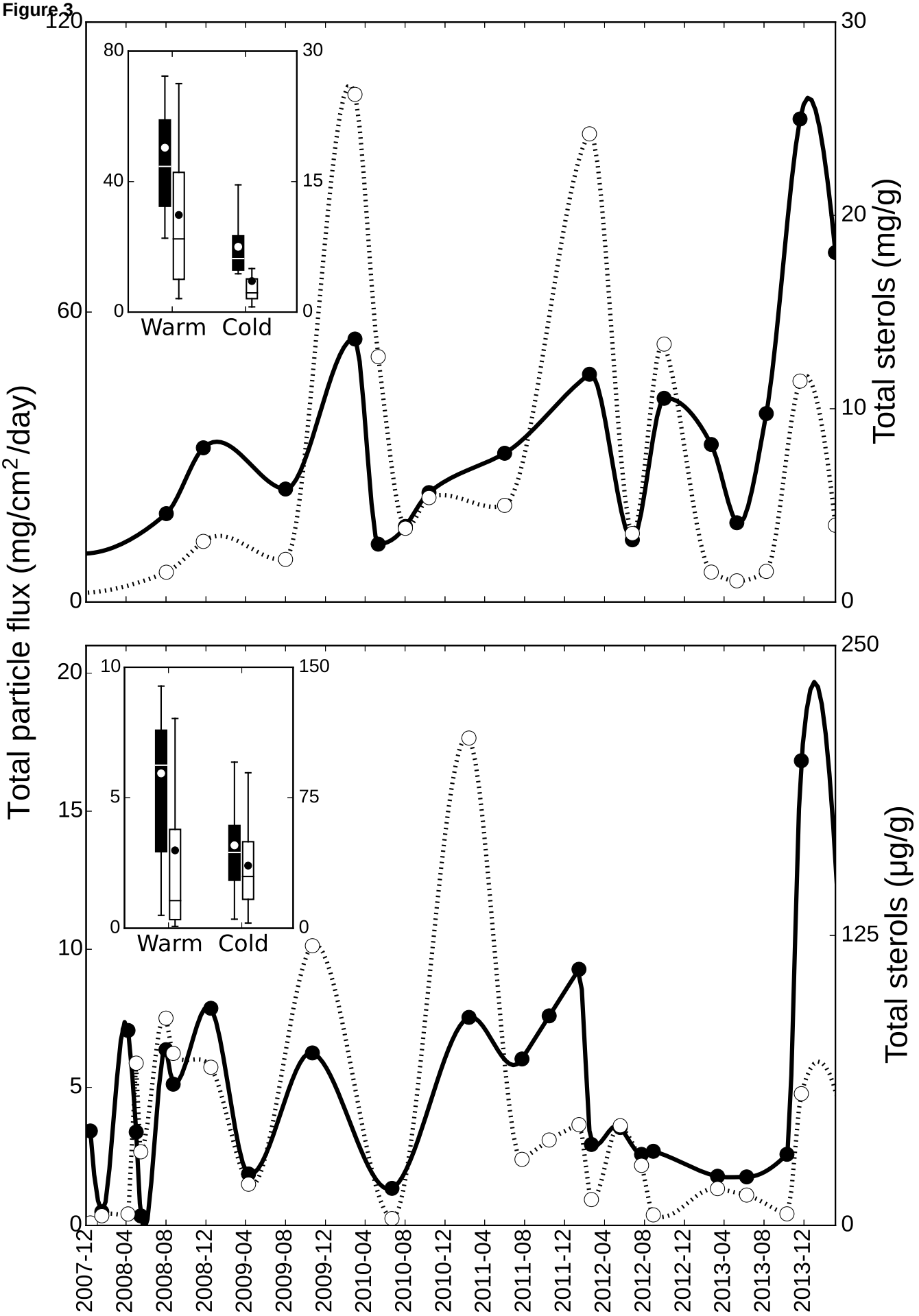

Total particle flux (mg/cm2/day)
Total sterols (mg/g)
Total sterols (µg/g)
Warm
Cold
2007-12
2008-04
2008-08
2008-12
2009-04
2009-08
2009-12
2010-04
2010-08
2010-12
2011-04
2011-08
2011-12
2012-04
2012-08
2012-12
2013-04
2013-08
2013-12

Figure 4

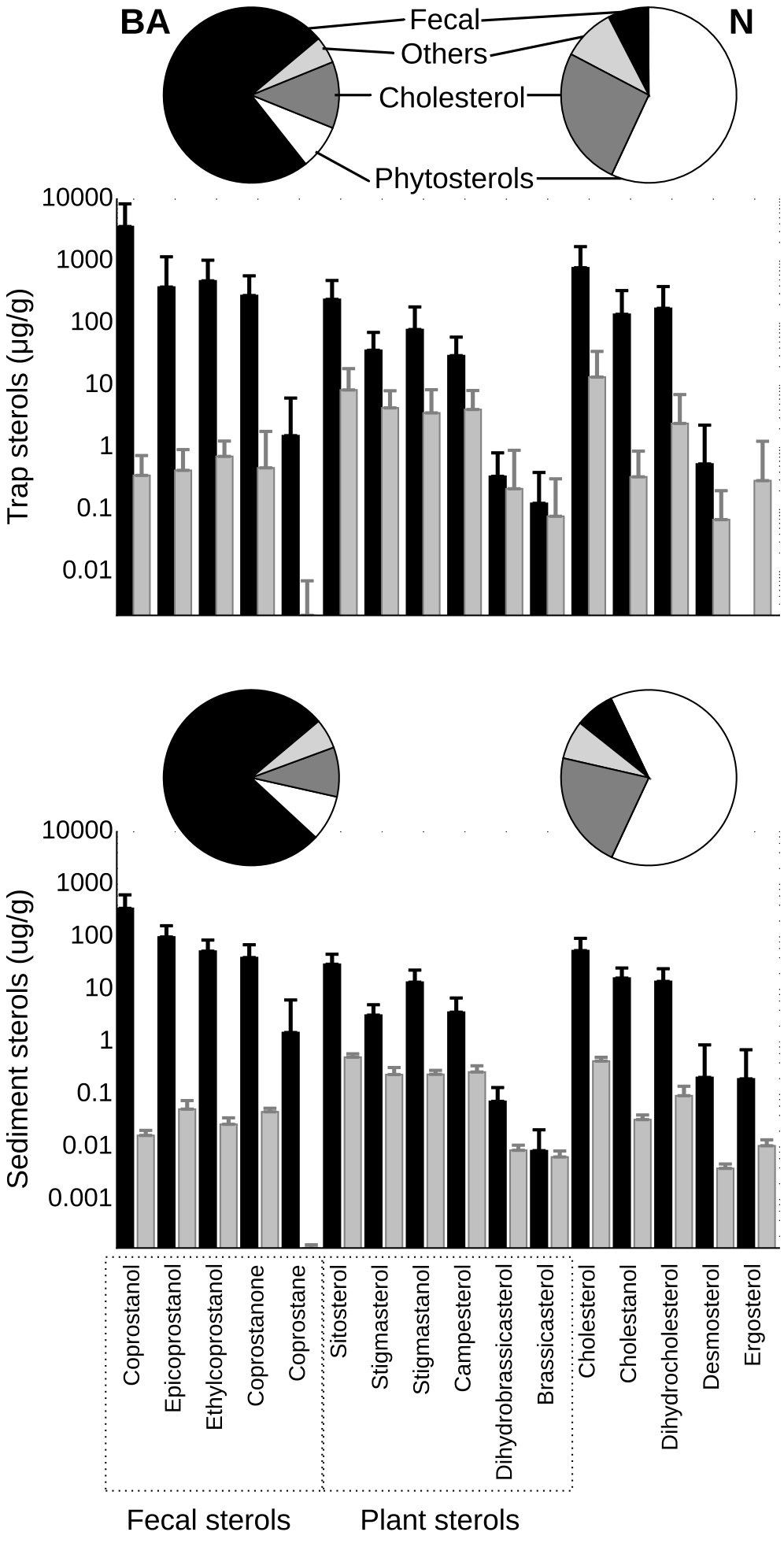

BA
N
Fecal
Others
Cholesterol
Phytosterols
Trap sterols (µg/g)
10000
1000
100
10
1
0.1
0.01
Sediment sterols (ug/g)
10000
1000
100
10
1
0.1
0.01
0.001
Coprostanol
Epicoprostanol
Ethylcoprostanol
Coprostanone
Coprostane
Sitosterol
Stigmasterol
Stigmastanol
Campesterol
Dihydrobrassicasterol
Brassicasterol
Cholesterol
Cholestanol
Dihydrocholesterol
Desmosterol
Ergosterol
Fecal sterols
Plant sterols

Figure 5

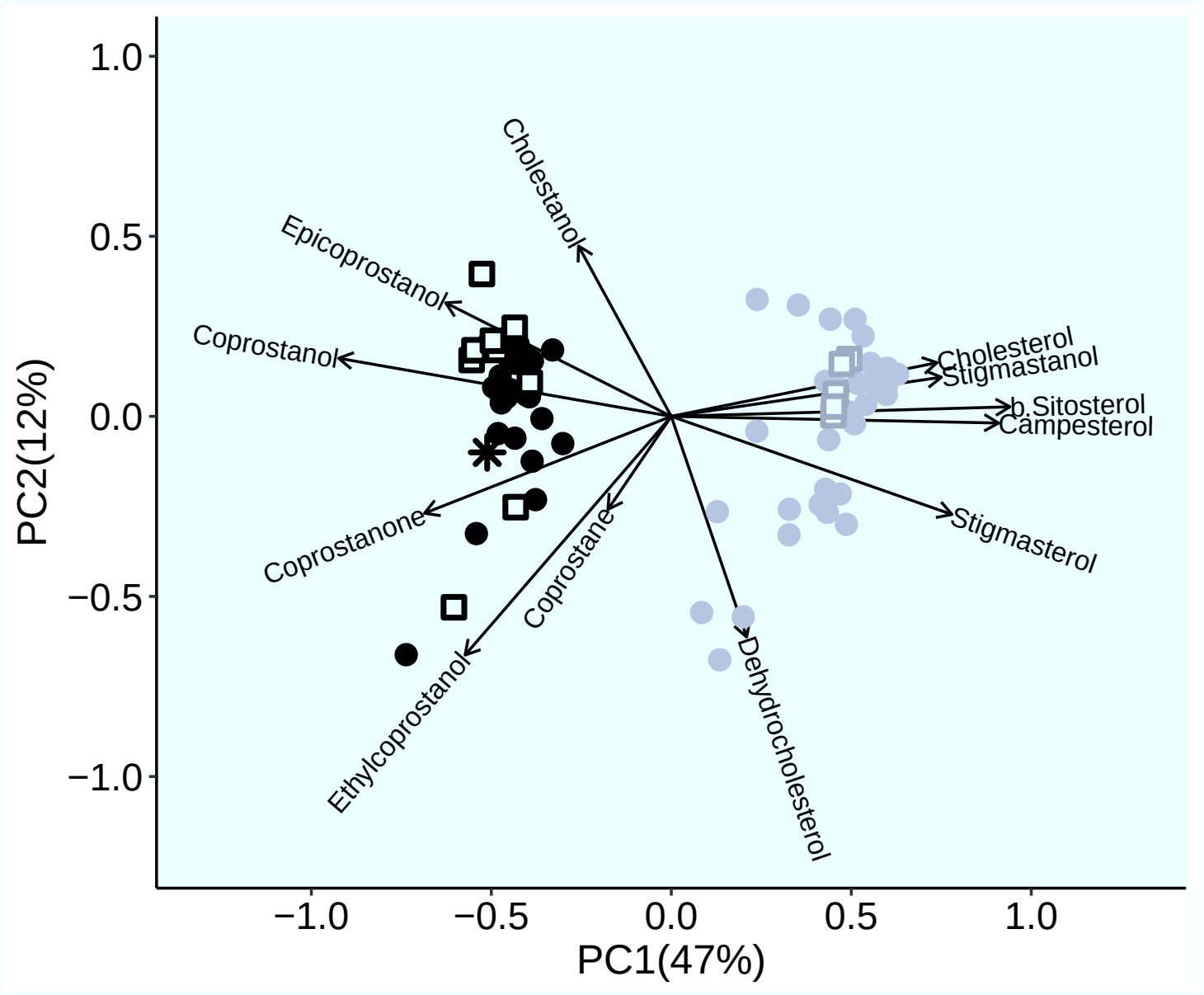

1.0
0.5
0.0
−0.5
−1.0
PC2(12%)
−1.0
−0.5
0.0
0.5
1.0
PC1(47%)
Cholestanol
Epicoprostanol
Coprostanol
Coprostanone
Ethylcoprostanol
Coprostane
Dehydrocholesterol
Cholesterol
Stigmastanol
b.Sitosterol
Campesterol
Stigmasterol

Figure 6

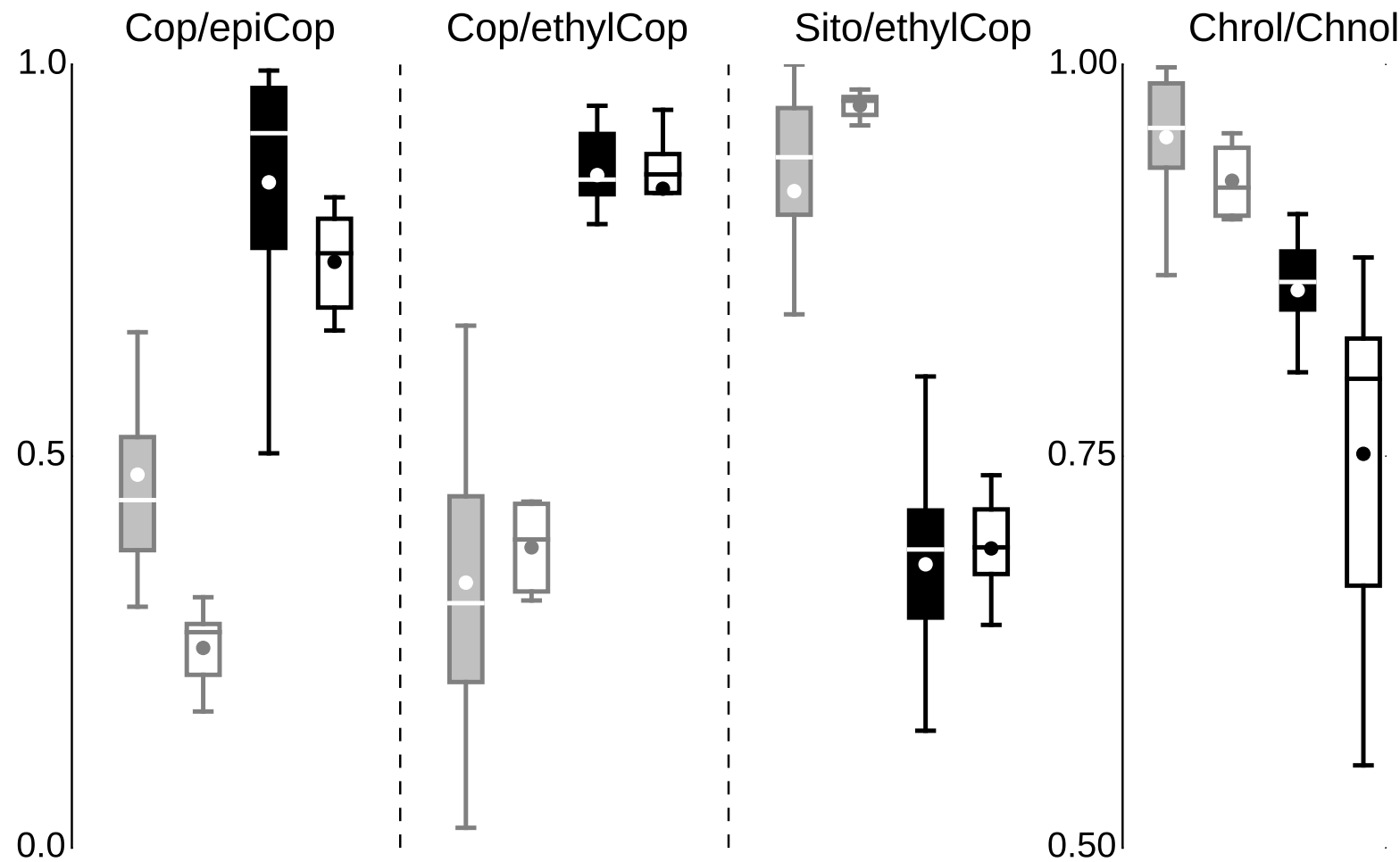
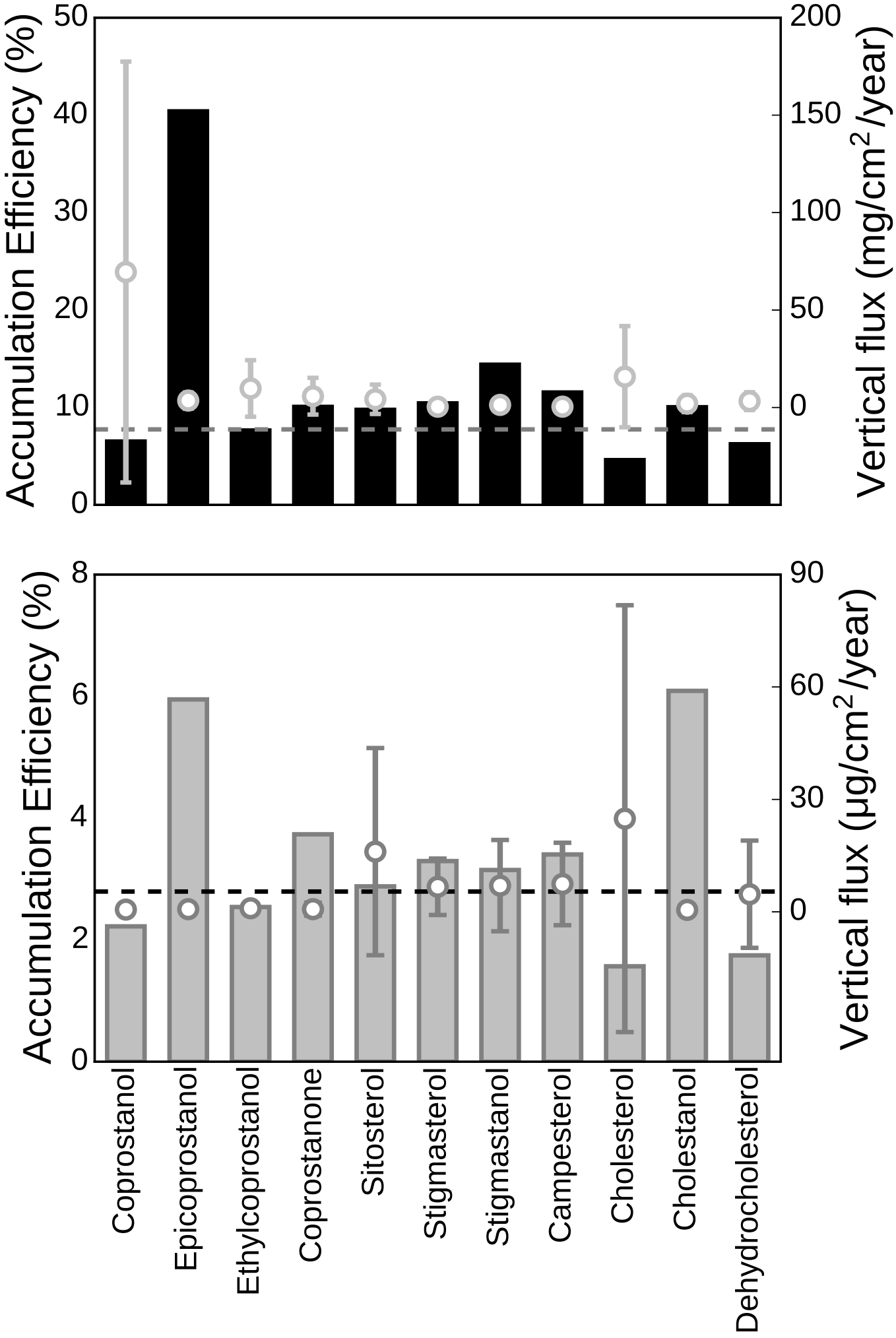

Cop/epiCop
Cop/ethylCop
Sito/ethylCop
Chrol/Chnol
1.0
0.5
0.0
1.00
0.75
0.50

Figure 7


Accumulation Efficiency (%)
Vertical flux (mg/cm2/year)
Accumulation Efficiency (%)
Vertical flux (µg/cm2/year)
Coprostanol
Epicoprostanol
Ethylcoprostanol
Coprostanone
Sitosterol
Stigmasterol
Stigmastanol
Campesterol
Cholesterol
Cholestanol
Dehydrocholesterol

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Responses to reviewers (Line numbers provided in the responses correspond to the revised version of the manuscript):

Associate editor's comments:

- Abstract, Line 22. The ± uncertainties are defined in the Methods, but they also need to be defined at first mention in the Abstract.

  Response: The ± uncertainties have been defined as "mean ± standard deviation" in the abstract. (line 23)

- Line 120: The abbreviation for longitude should be W for west.

  Response: Corrected.

- Line 124. There should be a space between numbers and units (i.e. 1.5 m). Please correct throughout.

  Response: Done.

- Line 146. Specify the number of deuterium substituents for each standard. (A reference to the exact name in Table 1 should also be given. Note that the formulae in Table 1 need to be corrected to show 7 deuterium atoms.)

  Response: Done (lines 150-152).

- Line 189 and following. Provide references or suppliers for all libraries and packages. Also, more detail is needed on the principal component analysis method and program source.

  Response: The URL for all the Python libraries and R packages have been supplied (lines 199-204).

- Line 224 and 266. Five significant figures are given for the BA river discharges and four and five for sterol concentrations. Three figures are likely exceed that which could be supported by the discharge and concentration measurements. Please round off to a maximum of three figures throughout (i.e. only one decimal for numbers larger than 10).

Response: Values were rounded to two or three significant figures throughout the text.

- Line 253. … of sterols to degrade at the sediment-water interface, …

  Response: Corrected.

- Lines 225 and 265. Correlations are given as R2 and r, for the coefficient of determination and correlation coefficient, respectively. Please be consistent. (Correlation is most commonly presented as the coefficient of determination with the abbreviation r2.) (Note that superscripts do not copy properly into the Elsevier system.)

  Response: All correlations were expressed as r2. The superscripts were avoided except for numbers in exponential notation.

- Figure captions. The definition of N as the Uruguay River in the text and Fig. 1 changes to North in Fig. 3 to 7 captions. Use one definition for N throughout.

  Response: Done.

Reviewer #1 comments:

The manuscript of Speranza and co-authors describe a comparative study of sterols composition in suspended particles and sediments as well as the flux from water to sediments between contaminated and pristine sites in an estuarine region. Overall, the research is well designed, the analytical approach is adequate and the data discussion is consistent, which make the manuscript worthy publishing.

The authors should address some minor points, as highlighted below:

- pg6, line 97: the outfall flux was set as 3.8 m3/day, which is a quite low value. For instance, an outfall in Rio de Janeiro city has a flux of 8 m3/s... please, confirm if the value for the BA outfall is correct?

  Response: Indeed, the exponent was omitted. The value was corrected according to the reference (50 m3/s) and expressed in m3/day, in order to be consistent with previous values (line 99).

- pg8, line 132: what value for density was used? Did the authors measure the densities of the particles collected in the two sites? Based on the distinct nature of the particles collected in each site, their densities are probably quite different, and if so the calculated fluxes would be significantly affected.

  Response: Since fluxes were calculated as particle mass settling over surface unit (see equation in material and methods section), they are independent from density. Nevertheless, sedimentation rates derived from fluxes clearly depends on density. At BA density measurements ranged between 2.2 and 2.7, therefore the typical value for sediments, 2.65 g/cm3, has been used (Colombo et al. 2007). At N the sedimentation rate was also estimated using this value. Anyway, the 4 orders of magnitude difference between BA and N in terms of flux would largely exceeds any variation in density

among sites. Furthermore, calculation of coprostanol burdens in top sediment layers, using the abovementioned density, were made only for BA, as it was irrelevant for a little polluted site such as N.

- pg 11, line 194: the authors mentioned the consideration of the A/A+B ratio of sterols. However, in the Results and Discussion section, only ratios calculated as A/B were presented.

  Response: All ratios presented in the Results and Discussion section were calculated as A/(A+B) and has been named with this format in the text.

- pg12, line 215: the authors should list the compounds grouped as 'fecal sterols'. I could not find such definition throughout the text.

  Response: A definition has been added to the Materials and Method section (lines 175-176).

- pg 21, lines 396-397: it was mentioned that warm-blooded animals other than humans have high ethylcoprostanol concentration that could affect the ratio sitosterol/ethylcoprostanol. In this sense, values around 1 would be typical of cow fecal material. Perhaps, the right statement should be that other animal have LOW ethylcoprostanol related to sitosterol when compared to humans. This would explain the ratio of 0.36 ± 0.15 found for BA and the conclusion that this outfall delivers basically human feces.

  Response: According Nash et al. 2005, the value of the sitosterol/ethylcoprostanol ratio for cattle feces is typically lower than 1. At BA, this ratio is below this threshold, indicative of cow feces pollution. The paragraph has been modified to clarify the idea that a small non-human fecal pollution cannot be disregarded (lines 422-430).

- pg 21, lines 407-413: the discussion about the changes is sterols ratios and their relation to hydrogenation/degration reactions in the suspended particles and sediments could be rethinked, as the differences reported for the selected ratios seems not significant

  Response: Despite the difference between BA and N is pretty small (around 0.1) this was highly significant according t-test ($p > 0.0001$) due to the very low dispersion of data (relative standard deviation were below 5% for both sites). A minor typo error in the BA value has been corrected (0.85 ± 0.036 instead of 0.85 ± 0.043).

- pg 22, first paragraph: the discussion about PCA results is quite limited. Perhaps, it should be placed earlier in the discussion, in order to support the sterols source assignments.

  Response: The PCA analysis has been further discussed. As suggested, the paragraph has been expanded and moved to the “Sterol composition” subsection where this multivariate analysis serve to integrate the discussion of the difference in

terms of individual sterols between BA and N in settling material and sediments (lines 389-409).

- pg 25, first paragraph: it was not clear the significance of the discussion abouth the change in the efficiency of sterol preservation in the restricted and a large area influenced by the outfall discharges.

  Response: The paragraph has been modified to clarify the discussion about the observed coprostanol in sediments and the expected discharge according available data (lines 501-509).

- Conclusion: it should be rethought, because in its current format it is more an abstract than a description of the relevance of the findings of this study.

  Response: The conclusion was thoroughly reworked, changing the previous descriptive approach by a more integrative summary focused in the main findings of this work. Special emphasis was taken to underline the contrast between a severely polluted metropolitan area and a relatively pristine site in terms of sterol biogeochemical dynamics, as well as the magnitude of sewage pollution observed at BA. Additionally, the importance of settling material in aquatic lipids dynamic was emphasized.

Reviewer #2 comments:

This is an interesting manuscript that discuss diagenetic alterations of sterols biomarkers in polluted and pristine áreas of the Rio de La Plata. I think that materials and method are not fully informative and need to be improved for publication. I suggest some alterations, as listed below:

Introduction

- Include current references (above 2015).

  Response: The introduction has been thoroughly revised and an exhaustive literature search has been conducted in order to provide newer references (Canuel and Hardison 2015, Volkman 2016, Kress et al., 2016, Roberts and Villegas, 2016). Nevertheless, several relatively old reference are conserved since they are landmark papers or they could not be substituted with recent literature.

- L107: It is missing the end point.

  Response: Corrected.

Materials and method

- Did you optimize the method for lipid extraction? If not, include the reference you used.

  Response: The reference has been added to the text (line 150).

- Did you check the extraction efficiency?

  Response: The extraction efficiency was evaluated through the recovery analysis. Individual recoveries were high, ranging between 82 and 110% (lines 197-198).

- What is the volume of BSTFA used? Please specify.

  Response: The BSTFA volume used has been specified in the text (line 156).

- Information about the solvent purity is missing.

  Response: Added to the text (line 161).

- Is it possible to operate the mass spectrometer in scan mode and SIM mode simultaneously?

  Response: The mass spectrometer used for sterol analyses, a Perkin Elmer Clarus 500 allow to perform multiple simultaneous mass functions, including a full scan and selective ion scanning. This functionality is particularly useful to check the identity of each compound, otherwise only identified by the retention time and a couple of characteristic ions.

- L175: Which standards were used?

  Response: The standard used have been detailed in the text (lines 177-181).

Results and Discussion

- L232: (RSD: 113-114%) I did not understand what it means.

  Response: The relative standard deviation (RSD), used to measure data dispersion, was defined in the Material and Methods section as the ratio of the standard deviation to the mean. Since these values, corresponding to BA and N respectively, were almost identical, they have been replaced by a common average in the text.

- PCA analysis was not discussed in the text. It can be improved.

  Response: The PCA discussion has been corrected and expanded. It has been moved to the “Sterol composition” subsection, in order to summarize the contribution of individual sterols to the settling material and sediments composition (lines 389-409).

Supplementary Material

Click here to download Supplementary Material: Supplementary Material.docx

Interactive Map file (.kml or .kmz)

Click here to download Interactive Map file (.kml or .kmz): 1508841746534.kmz