Highlights

- new concept proposed for estimating small-area human populations
- Sewage Chemical-Information Mining (SCIM) measures biomarkers in sewage
- real-time estimation of populations (accommodating influx and efflux) is possible
- coprostanol is identified as a candidate biomarker for estimating population size
- composite biomarkers having complementary properties could improve accuracy

Real-Time Estimation of Small-Area Populations with Human Biomarkers in Sewage

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Abstract: A new approach is conceptualized for measuring small-area human populations by using biomarkers in sewage. The basis for the concept (SCIM: Sewage Chemical-Information Mining) is supported by a comprehensive examination and synthesis of data published across several disciplines, including medicine, microbiology, clinical chemistry, and environmental science. Accurate measures of human populations are fundamental to numerous disciplines, including economics, marketing, politics, sociology, public health and safety (e.g., disease management; assessment of natural hazards; disaster prevention and response), quality of life, and the environment. Knowing the size, distribution, and flow of a small-area (local) population facilitates understanding the numerous and complex linkages and interactions between humans and the environment. Examples include material-flow (substance-flow) analysis, determining the magnitude of per capita contribution of pollutant loadings to watersheds, or forecasting future impacts of local populations on the environment or a population's demands on resources. While no definitive approach exists for measuring small-area populations, census-taking is a long-established convention. No approach exists, however, for gauging small-area populations in real-time, as none is able to capture population dynamics, which involve transient changes (e.g., daily influx and efflux) and lasting changes (e.g., births, deaths, change in residence). Accurate measurement of small-area populations in real time has never been possible but is essential for facilitating the design of more sustainable communities. Real-time measurement would provide communities the capability of testing what-if scenarios in design and policy decisions.

After evaluation of a range of biomarkers (including the nitrogenous waste product creatinine, which has been long used in clinical chemistry as a parameter to normalize the concentrations of other urinary excretion products to account for urine dilution), the biomarker with the most potential for the SCIM concept for real-time measurement of population was determined to be coprostanol ─ the major sterol produced by microbial reduction of cholesterol in the colon.

Keywords: small-area populations; biomarkers; sewage; coprostanol; creatinine; excretion; substance-flow analysis

Abbreviations: ASAP-SCIM: analysis of small-area populations by sewage chemical-information mining; BOD: biological oxygen demand; CoP: coprostanol; FEUDS: forensic epidemiology using drugs in sewage; MDL: method detection limit; POCIS: polar organic chemical integrative sampling; SCIM: sewage chemical-information mining; SPMD: solid phase membrane device; STP: sewage treatment plant.

Contents

1. Introduction

1.1. Small-area populations and their measurement

The critical importance of knowing the size of discrete human populations was clear millennia ago with rulers who needed to gauge the pool of those who could pay taxes or serve in militia; the Bible's book of Numbers is but one example. Today the importance continues to grow, driven by questions germane to economics, marketing, politics, sociology, public health and safety (e.g., epidemiology, disease management; assessment of natural hazards; disaster prevention and response), quality of life, and the environment. Population density and its flow play major roles in demands on infrastructure and ecological services, as well as serving as a major environmental stressor itself.

City planning (public requirements for infrastructure, such as land, transportation, schools, communication, drinking water and wastewater treatment and distribution, waste disposal, emergency services, and healthcare), formulation of public policy, and resource allocation often rely on understanding not just the size and distribution, but also the flow of small-area (local) populations. Understanding the size, distribution, and dynamics of local populations is essential for modeling future projections and forecasting. It also facilitates understanding the numerous and complex linkages and interactions between humans and the environment, such as for material-flow (substance-flow) analysis (Baker, 2009) – especially the flow of pollutants – and for the study of sustainability and environmental justice.

As a key denominator term in a wide array of statistics and ratios involved with various calculations, the error associated with population size is often overlooked relative to the numerator (Aickin et al., 1991; Thunhurst, 2009). Given the numerous integral roles played by population statistics, the methodologies fundamental for measuring or estimating small-area populations are surprisingly limited; none is an accepted standard and all can be prone to considerable error. Because of the complexities involved with population estimation, advancements in the range of available tools have been incremental. New paradigms have not emerged. The most recent improvements have been the application of remote-sensing and geographic information system techniques to extant census data (e.g., for small-area populations, where data on housing units and persons per household can be applied). Summary of the existing approaches and their complexity and limitations is widely available (e.g., Deng et al., 2010). The general approaches for census taking – and its associated problems and limitations – have also been summarized in numerous documents (e.g., Smith and Morrison, 2005; Swanson and McKibben, 2010; US Census Bureau, 2011; Wilmoth, 2004).

Population size is clearly a key parameter in a broad spectrum of processes fundamental to governance. Accurate data are required to make sound decisions involved with planning and forecasting, policy making, and resource allocation. Accurate data are necessary for guiding development, assessing demand on services and infrastructure, and informing legislation. Current methodologies are based on public surveys (such as census taking), augmented with a wide array of demographic statistics, such as tourism. Census taking, however, provides estimates that quickly become increasingly outdated and cannot be easily updated to accommodate change – births, deaths, and migration (movement); this is exacerbated by the fact

that census taking is infrequent because of its cost. Complex models (e.g., cohort survival models) relying on numerous assumptions and estimates are therefore required to interpolate or extrapolate in order to generate postcensal or intercensal population estimates (e.g., Dennis et al., 2007).

Census taking uses one of two basic approaches for estimating geographically located populations. These result in data applicable to two different purposes – estimates of de jure population or de facto population. A de jure population comprises all "usual" residents, mainly those with formal residences. A de facto population comprises all those who happen to be present, regardless of the location of their formal or usual residence. A de facto population therefore includes all non-residents (e.g., commuters, visitors, tourists) and excludes all permanent residents who happen to be absent (Bell, 2004; Wu et al., 2005). There can be ambiguity in defining the two types, and delineating between them in practice can become convoluted.

For the purposes of environmental impact, the de facto population can be considered the more significant parameter, as it reflects the actual demands on local services. With an increasingly transient and mobile society, estimating de facto population assumes increasing importance. Furthermore, one particular aspect of measuring population size and distribution has been growing in importance – the need for real-time measurement, which essentially yields estimates of the "true" de facto population. Conventional estimation approaches all rely on existing data (e.g., census) to drive computational and statistical extrapolations and projections. Although accurate measurement of small-area populations in real time is essential for facilitating the

design of more sustainable communities, it is currently not possible. Real-time measurement would provide communities the capability for the first time of quickly testing what-if scenarios in design and policy decisions.

With this said, alternative approaches for measuring population size continue to emerge but have fundamental limitations excluding them from the uses considered here. For example, monitoring of cell (mobile) phone use has been explored as a means of measuring urban population density and flow (e.g., Dan and He, 2010). The utility of this approach is limited by the variable extent of cell phone possession (market penetration) and does not directly correspond with de facto local population (e.g., the very young do not use cell phones); whether a cell phone is actually in use depends on user behavior. With these problems aside, cell phone use would likely measure a sizeable de facto non-local population (such as commuters) that is only momentarily passing through a local area. This would make the resulting population estimates useless for a major application of the concept presented here, which will entail the acquisition of biomarker data from sewage (see Section 7.3).

1.2. Objectives

Presented here is a concept for measuring dynamic, small-area populations in real time. The concept relies on monitoring raw sewage for the combined excretion of a biomarker known to have a relatively stable per capita rate of elimination in urine or feces. The approach ensures that the entire actual (de facto) population is sampled and that the absent portion of the de jure population is excluded. This contrasts sharply with the census approach, which acquires a static snapshot estimate and usually succeeds in only capturing a portion of the population. This new

approach could also provide a vastly improved accounting of transient population mobility. The principles and foundation of the concept are presented, along with its limitations and weaknesses. Preliminary proof-of-principle is also discussed. With follow-on, field-based studies, this measurement methodology could be continually refined. A major objective of this work is to foster discussion and catalyze further investigation designed to evaluate the proposal's merits.

The concept would be suitable for the vast majority of metropolitan and urban small-area populations. It might provide not just real-time estimates, but also require considerably less skill and expense compared with all conventional public-survey approaches such as a census. The concept also has the potential to eventually be widely employed and automated, providing sufficient spatiotemporal resolution so that the dynamics of population movement could be mapped and displayed in real time. Population movements (both transient and permanent) occur in different time frames, ranging from seasonal (e.g., vacations and seasonal-driven migration) to daily (commuting to work, business trips, errands, socializing, visits). The concept could revolutionize the way that population data are visualized – in a way analogous to the implementation of surveillance radar for actively conveying current weather; on-the-fly prediction capabilities from examination of trends might also be possible. Moreover, the concept imposes no concerns regarding confidentiality or privacy since the data never relate to identifiable individuals.

The concept proposed here is based on the mining of the chemical information contained in sewage. A portion of this untapped, rich source of information resides in the numerous

biomarkers of endogenous human biochemical processes. These biomarkers continually undergo urinary or fecal excretion and represent the sum total contributions from the real-time population served by any given sewerage system. The basis of the concept is the measurement of one or more biomarkers whose intra- and inter-individual variation in daily per capita excreted quantities is minimal.

The general approach is termed "analysis of small-area populations by sewage chemical-information mining" (ASAP-SCIM). This paper outlines the general conceptual approach and discusses the criteria for selection of suitable biomarkers whose levels in sewage reflect per capita contributions and can therefore serve as proxies for population counts.

2. Background

Beyond the numerous conventional uses of population estimates, newly emerging applications relevant to measuring interactions at the human-environment interface will require increasingly accurate real-time, de facto population estimates – those capable of resolving the diurnal ebb and flow of small-area populations, such as the daily redistribution from residences to locations outside the defined area. The most common of these newer applications are models aimed at providing normalized per capita estimates of material flows [e.g., "pollutant load per capita" (Tsuzuki, 2006)]; examples include estimating per capita contributions to the environment of pesticides, biocides, drugs, personal care products, nanomaterials, and household chemicals. These models facilitate better understanding of individual contributions to waste streams, thereby permitting assessment of the effectiveness of measures targeted at waste reduction, or with assessing environmental justice. Examples of the problems encountered and the many variables

and factors involved with deriving per capita flows can be seen in a number of papers (e.g., Khan and Nicell, 2010; Ort et al., 2010a; Ort et al., 2010b; Rieckermann et al., 2011).

In general, most of these current applications that make use of chemical loadings in sewage must rely on inaccurate population data derived from aged or incomplete sources such as census surveys or utility customers billed (e.g., Anderson et al., 2004; Banta-Green et al., 2009; Clara et al., 2011; Kasprzyk-Hordern et al., 2009; Neset et al., 2010; Ort et al., 2009; Rowsell et al., 2010; Tsuzuki, 2006).

2.1. Prior approaches for using sewage to estimate population size

Advancements in estimating de facto small-area population size would greatly aid the development of human-environment interface models such as those for material flows. One of the only attempts at developing a non-census based approach for estimating population was conceptualized in the early 1970s. The model attempted to estimate population size simply from an algorithm that used wastewater flow rates. "Demoflush" (a term derived from "demographics" and "flush") was developed for Ocean City, Maryland, to gauge the annual influx of tourists, initially in order to plan for augmented staffing of medical facilities (Goldschmidt and Dahl, 1976); estimates using conventional techniques were providing estimates of tourist influx that varied by five fold.

Although simple in concept, the Demoflush model had to rely on many unverifiable assumptions, a key one being the daily per capita water usage – a value that is subject to many drivers. Confounding its usefulness were additional unknowns such as sewer

infiltration/exfiltration and contributions from industrial water discharge. A very large but indeterminate error associated with the Demoflush approach eventually became evident (Editorial, October 2, 2009; Russo, August 14, 2009) and indicated that wastewater flow cannot be used as a proxy for population. There are too many variables and unknowns, all of which will vary from city to city and depend on variations in local conditions, sewerage infrastructure, periodic changes such as season/weather, and local water usage customs and restrictions. The information content of the physical attributes of sewage is insufficient for estimating population size.

Some material-flow models have tried to make use of approaches conventionally used for estimating population size for the design of sewage treatment plants (STPs). Several terms are used in referring to the population serviced by a particular STP: "population served," "population equivalent," and "effective population." Population served refers to the population residing and paying hook-up fees within the sewage district physically connected to a particular STP. The population served approximates the de jure population. This slice of population does not account for reduction or increase in waste discharge that occurs during resident travel outside the STP's service zone or for non-residents traveling into the zone. Population equivalent (unit per capita loading) is an approach long-used for roughly estimating the serviced population – a statistic relied upon for design of STP capacity. It is based on the assumption that the 5-day biological oxygen demand (BOD_5) of the material excreted from one individual ranges from about 40 to 80 grams per day (Heidrich et al., 2010); variations of this approach use BOD measured over different periods or similar measures such as chemical oxygen demand (COD, which ranges from 60-120 g/day). A major problem with the routine use of BOD is the time required for the

test; this could potentially be solved with new, real-time sensors (e.g., Hur et al., 2010). But with respect to any potential utility for SCIM, BOD does not specifically reflect the residue from human metabolism since it measures any biodegradable substance that enters sewers, which often includes food waste and industry effluents. The effective population simply tries to account for all of those entities contributing BOD to an STP's sewerage, whether originating directly from humans or from other sources. Improvements based on these approaches have tried to incorporate geographic information system techniques or the use of conservative tracers such as boron (but whose presence is not unique to human activity nor is its per capita usage stable) (e.g., Fox et al., 2002; Keller et al., 2006; Keller et al., 2007).

A major confounder to basing population estimates on information mined from centralized sewage systems is the incidence of the resident population that is not hooked up to a system, such as those using septic systems or even the less common practice of straight-piping. The incidence of septic systems can vary greatly with locale. On average, an estimated 20% of total U.S. housing units were served by septic systems in 2007 (USEPA, 2008); the geographic distribution is roughly equal among the rural and suburban US.

The need for accurate measures of a population whose combined sewer discharge comprises the total flow into a single STP was catalyzed with introduction in 2001 (Daughton, 2001) of a material-flow model concept later to be termed FEUDS – "Forensic Epidemiology Using Drugs in Sewage" (Daughton, 2011). The objective of FEUDS is to use the measured concentrations of particular drugs (or metabolites) excreted into sewage to back-calculate the original total usage and per capita usage levels of the aggregate population; the FEUDS approach is being extended

to other substances, such as ethanol (Reid et al., 2011) . The overall usage data are normalized against the presumed total population for the purposes of time-course or inter-population comparisons of community-wide or per capita drug usage.

Since population size is not accurately known, as an alternative to normalizing material-flow data to the estimated population size, the FEUDS concept as originally proposed (Daughton, 2001) suggested that specific biomarkers might be used as surrogate population measures against which to normalize total usage. This could then indirectly generate average per capita consumption rates. At that time, two of the suggested biomarkers (Daughton, 2001) were creatinine (a major urinary metabolite) and coprostanol (a major fecal sterol) (see Figure 1). These biomarkers will serve as the primary focus of the remainder of this paper, although the prospects for several other biomarker possibilities will be briefly discussed.

3. Possible biomarkers for estimating population size via Sewage Chemical-Information Mining (SCIM)

A large expanse of literature from a broad spectrum of disciplines was examined in evaluating the potential utility of excreted biomarkers. Publications were located using SciVerse ScienceDirect and Google Scholar, coupled with locating numerous additional references by reverse and forward citation analysis. Full-text reprints were archived in a bibliographic citation database (EndNote, Thomson Reuters), facilitating full-text keyword Boolean searches.

The many factors or attributes that need to be examined in determining whether a biomarker might be useful for estimating human population size via SCIM are summarized in Table 1. An

essential characteristic for a biomarker to be useful for measuring population is a low population-wide variance in the per capita absolute quantities excreted daily; knowledge of quantities excreted daily ensures that diurnal variations (e.g., resulting from biorhythms) are fully accommodated. Published data for daily excreted quantities of biomarkers, however, are not provided as frequently as excreted concentrations. This is because most biomarker data come from clinical studies, where sampling of subjects usually employs the spot testing of excreta (convenience samples) rather than the much more onerous collection of complete 24-h samples. Therefore, much of the published data on biomarker excretion is reported in terms of concentration levels in spot samples of either urine or feces. To derive the per capita daily excreted rates from the concentration levels in spot samples of urine or fecal material, the rates of elimination of urine (e.g., volume/day) or fecal material (wet or dry mass/day) must be known for each study; these data are often not provided by published studies, limiting the usefulness of the data.

3.1. Creatinine as a possible biomarker for estimating population

A reasonable starting point for selecting possible SCIM biomarkers is the published literature from clinical chemistry. A biomarker widely used in clinical chemistry, and one with extensive published data on excretion, is creatinine. A small portion of creatine (and phosphocreatine), which is stored predominately in skeletal muscle, is continually converted by non-enzymatic dehydration/cyclization to form the endogenous anhydride, creatinine (a nitrogenous waste product cleared via the kidney); the rate of conversion, in males for example, is about 1.6-1.7% per day (Boeniger et al., 1993).

Some historical context is required to understand the important role played by creatinine in clinical chemistry. In 1905, Folin first proposed that urinary creatinine could be used to normalize (adjust) for urine dilution in spot samples, in order to avoid collection of 24-h samples (Edwards et al., 1969). For decades, levels of creatinine in spot urine samples have been routinely used to normalize the levels of other clinical urinary markers. Widely accepted has been the assumption that creatinine is excreted at a constant rate, independent of urine flow/volume (and could serve as a measure of glomerular filtration rate). It could therefore be used to account for the relative dilution of the urine sample (which is largely a function of hydration status) or variation in diuresis (as a function of disease); urinary creatinine concentration is therefore assumed to vary inversely and in a constant manner with respect to urine dilution. Evidence continues to emerge, however, that creatinine may often serve as a biological marker of exposure or effect; one example is its variable output being a strong function of arsenic methylation (Basu et al., 2011). The many factors involved with variability in creatinine output have been summarized by Ryan et al. (2011).

Considerable research has examined the intra- and inter-individual excretion of creatinine in terms of urinary concentrations. For the purposes of using SCIM to estimate population size, however, the question is not whether creatinine excretion varies with respect to its urinary concentration (which is expected), but rather, whether its 24-h per capita mass output is constant – and therefore whether it can serve as a measure of combined contributions from discrete individuals. Important to note, however, is that only a small portion of the creatinine excretion literature provides data on the basis of quantity excreted per day; most is expressed in terms of concentration.

Although creatinine has been long used in clinical chemistry to account for variations in urine output, the underlying principles are not without considerable debate. There are many potential pitfalls that derive from numerous variables. Despite its wide acceptance in clinical chemistry, the published literature (even as far back as the 1950s) shows that intra- and inter-day creatinine excretion is not constant and that daily excreted quantities can have high variance. As one example, a wide range (nearly 3-fold) of creatinine output among individuals was shown in a 1968 study, where mean daily creatinine output over the course of several weeks among four men in apparently good health ranged from 914 to 2,552 mg/day (Scott and Hurley, 1968).

About 60 references with creatinine excretion data, spanning the years 1968-2011, were examined for the work reported here. Numerous studies have shown that daily urinary creatinine excretion can vary widely for individuals and even more among a population. A few of these are summarized here. A review of the literature (1905-1970) on the variability of creatinine daily excretion revealed a coefficient of variation of intra-individual daily excretion rates ranging from about 3 to 20% in different subjects (Curtis and Fogel, 1970). In children, 24-h excreted creatinine can vary by more than an order of magnitude among individuals. For example, in boys with heights spanning 2-fold (90-196 cm), creatinine excretion was found to range over an order of magnitude – from 1.3 to 17.8 mmol/day (Remer et al., 2002). In a study of 8 subjects over 6-10 months, mean daily excretion ranged from 1.54 to 1.87 g/day, and the range of the individual values (representing 54 to 97 daily samples per subject) across all 8 subjects spanned 1.01 to 2.58 g/day (Greenblatt et al., 1976); variance was later shown to derive largely from seasonal influences (Ransil et al., 1977). In a study that collected single 24-h urine samples, levels of

creatine were 1.325±0.525 µg/d for 32 smokers and 1.459±0.470 µg/d for non-smokers; levels were roughly 70-80% higher for males than females (Yan et al., 2007).

One study on reference ranges for creatinine in urine used 288 volunteers across six age groups (spanning ages from birth to over 70) from both genders (Weykamp et al., 1989). For the age groups from 17 to over 70, creatinine excretion ranged from 4.1 to 19.4 mmol/day. For the age groups from 0-16, the creatinine levels ranged from 0.5-15.3 mmol/day. Urinary creatinine from 254 working adults was measured yearly for up to 9 years in a study involving 1,217 samples of 24-h collections (one per year) (James et al., 1988). Daily urinary creatinine excretion (mg/day) for all women was 1,041±291, compared with all men at 1,642±418. Depending on body weight, the range of daily individual excretion over a period of 5-9 years spanned the extremes of roughly 400-600 mg/day (for white women) to 2,000-3,200 mg/day (for black men) – roughly a 5-fold range. Variability for individuals was about 15%. Men excreted about 33% more per day than women. It is important to note that the variance may have been even higher, as outliers were excluded under the possibly erroneous assumption that they resulted from 24-h sample collections that were incomplete; this is a problem that confounds the examination of population variance in many creatinine studies using 24-h collections, as exclusion of outliers was often based partly on the circular assumption that creatinine excretion was supposed to be constant.

A study of 24-h urine samples from 2,075 men and 1,933 women showed creatinine daily levels (mmo1/24 h) of 14.36± 4.1 for men and 10.28±2.7 for women; levels from men were on average 40% higher than women (Kesteloot and Joossens, 1996). This study, like others, showed a continual diminution in excretion with age – decreasing in men (and women) from a maximum of 15.69 (11.04) mmol/day for 35-39 years old to 12.27 (8.86) mmol/day for 70 years and older.

Broad and skewed distributions for daily output of creatinine are a function of gender, presumably because of the differences in skeletal muscle mass. Excretion was observed to vary across the range of 1-2.5 g/day, with tails below 1 and up to 3 or more grams per day. The most frequent values for males were 1.5-2.0 and for females 1.0-1.5 g/day. Day-to-day intra-individual daily ranges (over 4 consecutive days) spanned 9-79% of the maximum values, with the total range among all individuals spanning roughly 0.5-2.4 g/day (Alessio et al., 1985). In 20 healthy subjects, 24-h urinary creatinine averaged 1,638 mg/day with a 3-fold range of 1,114-3,697 mg/day (Newman et al., 2000). In 21 normal adult males and females, urinary creatinine ranged from 910-2,212 mg/d (Forbes and Bruining, 1976). In the same study, with 13 younger males and females but with "short stature", creatinine ranged from 184-956 mg/d; variance over at least three consecutive samplings for each individual ranged up to 19.5%.

Since creatinine is produced in skeletal muscle, it should not be surprising that its daily excretion varies so much across a random population. Indeed, creatinine excretion can be used as a good estimate for lean or skeletal muscle mass (Forbes and Bruining, 1976; Wang et al., 1996).

Urinary creatinine excretion is also highly influenced by diet composition. In one of the first metabolically controlled studies, consumption of protein/creatine-rich foods was found to affect creatinine excretion by roughly 50% (23.4 mg/kg/day versus 15.7 mg/kg/day) (Bleiler and Schedl, 1962). The increased excretion of creatinine (up to 33%) after consuming meat has been extensively documented (e.g., Bingham and Cummings, 1985; Hoogwerf et al., 1986; Kesteloot and Joossens, 1993; Mayersohn et al., 1983; Neubert and Remer, 1998). The growing popularity of creatine as a food and nutritional supplement adds yet another source of potential variation to excretion rates.

This is but a small sampling of studies that demonstrate high variability in daily creatinine excretion. Such high variation should not be surprising given that creatinine excretion is modulated by numerous endogenous and external factors. Excretion is a complex function of diet and food preparation (which serve as exogenous sources of creatine and creatinine), muscle mass (a factor in differences between genders [higher in males] and age [declining with advancing age], as creatine is stored in skeletal muscle), energy expenditure (exercise and activity level), hydration and temperature, and health status (e.g., kidney dysfunction or pathologies). In subjects with a range of kidney diseases, creatinine output can easily span an order of magnitude and more; multi-fold changes can occur over a period of several days for individual patients (Waikar et al., 2010). Wide variance in creatinine excretion has been documented for a number of other diseases as well, including cystic fibrosis (Wagner et al., 2010).

The many and complex factors that affect creatinine formation and excretion and which contribute to extensive variability (including exercise) are discussed in numerous studies (e.g., Barr et al., 2004; Boeniger et al., 1993; Calles-Escandon et al., 1984; Hee, 2010; Heymsfield et al., 1983; Suwazono et al., 2005; Worsfold et al., 1999; Wyss and Kaddurah-Daouk, 2000). Since the body's creatine/phosphocreatine pool is large (about 120 g for a 70-kg man), small variations in its conversion to creatinine can lead to multi-fold variability in intra- and inter-individual daily excretion; roughly 2 g/day of creatine are converted to creatinine.

3.1.1. Confounding of SCIM data by creatine/creatinine in foods

An aspect of creatinine important to its potential for use in this proposed SCIM application is not discussed in the clinical chemistry literature, as it is not relevant to clinical science. Creatinine does not have an exclusive endogenous origin. It also occurs in, or originates from, foods. This could greatly confound interpretation of its presence in sewage. A portion of excreted creatinine is derived directly from exogenous dietary intake. Creatine, and proportionately lower levels of creatinine, occur in certain foods (especially muscle tissues). Heating and cooking serve to convert significant portions of creatine to creatinine. Creatinine is not absorbed from the gut, and dietary intake would therefore pass into the feces. Furthermore, the disposal of leftover prepared foods directly to sewers undoubtedly serves as a source of creatinine of unknown magnitude and one that would have a highly variable correlation with per capita origins. These sources of creatinine would confound interpretation of its levels in sewage for use in SCIM applications.

The exogenous formation of creatinine in foods is shown by the heat treatment of raw milk for the purposes of pasteurization or sterilization. Heating can catalyze extensive conversion of creatine to creatinine, with conversion increasing in direct correlation with temperature and duration of heating. The molar ratio of creatine/creatinine decreased from about 8 (in raw milk) to 2.3 (in sterilized milk). It was estimated that daily per capita consumption of 500 mL of sterilized versus raw milk would increase creatinine daily excretion by 7 mg (Manz et al., 1991).

The levels of creatinine in five pre-cooked meat products ranged from 8.2-15.1% of the total creatine levels (which ranged from about 1-4 mg/g cooked meat); total levels of creatinine ranged up to about 0.7 mg/g, for cooked ham. Creatine levels were linearly related to total protein content (del Campo et al., 1998). Substantial levels of creatinine exist in many raw

meats, and levels increase multi-fold upon cooking (up to nearly 200 mg/100 g dry, fat-free tissue) as a result of dehydration of creatine (Macy et al., 1970).

3.1.2. Creatinine in Sewage

The use of creatinine in sewage as a parameter against which to normalize for obtaining per capita contributions of other excreted chemicals was first proposed in 2001 – for the purposes of illicit drug monitoring (Daughton, 2001; Daughton, 2011). The first attempt at implementing this particular use in sewage was in 2008 (Chiaia et al., 2008; Chiaia Hernandez, 2008). Data regarding the levels of creatinine in sewage are rare. In the work of Chiaia et al. (2008), its levels in sewage from seven STPs were found to range from 220 to 1,500 μg/L, translating in these cases to a range in estimated per capita rates of 120 to 620 mg/day. Such wide variance is consistent with the data previously summarized (section 3.1) from some of the many studies on creatinine excretion. Only a few prior studies exist where creatinine was measured in sewage. One of the first was Alexander and Stevens (1976). Another study measured creatinine in STP influent using two different techniques, yielding values of 311 and 121 μg/L, but several analytical problems were noted regarding its determination in wastewaters (Bisceglia et al., 2010).

3.2. Coprostanol (CoP) as a possible biomarker for estimating population

We have seen that even though creatinine is a urinary biomarker noted in clinical chemistry for its purported exceptionally stable excretion rate, its daily variance in quantity excreted is probably too large to serve as a potential surrogate measure for per capita population contributions to sewage. Its excretion is a function of many variables, each having considerable

variance among the general population. A reasonable conclusion might be that other possible biomarkers would have even greater variance since creatinine is used so widely in clinical chemistry solely because of its comparatively stable excretion rate.

One biomarker, however, has distinctly different physiological origins. Some of the sterol metabolites of cholesterol originate not from human biochemical pathways, but rather from microbial metabolism in the gut. Published data indicate that the production of at least one of these sterol metabolites – coprostanol – might have a multi-modal inter-individual distribution, but variance in its intra-individual production rate is comparatively low and stable with time [e.g., daily variation less than 5% (de Leon et al., 1987)].

Of the 512 possible stereoisomers of reduced cholesterol, only two occur widely in nature – cholestanol and coprostanol (Walker et al., 1982). Coprostanol (5β-cholestan-3β-ol; CAS RN 360-68-9; MW= 388.67) is a 5β-stanol and differs from cholesterol only by saturation of the C5-6 double bond (see Figure 1); in the older literature it is sometimes referred to as coprosterol. Coprostanol (CoP) is the preponderant 5β-stanol in human feces, comprising roughly 60% of the overall sterol content, although this percentage can vary widely among individuals. A fully saturated microbial metabolite of cholesterol, CoP is poorly absorbed from the gut (it does not undergo enterohepatic circulation) and is therefore fully excreted in the feces; this is believed to serve as a mechanism for lowering serum cholesterol levels.

Given the large published literature on CoP, there have been few comprehensive reviews. One of these was published 30 years ago (Walker et al., 1982). Another review on the general topic of

biomarkers compiled much of the pre-1998 published data on CoP occurrence in feces, wastewater, and sludge (Takada and Eganhouse, 1998).

The published literature surrounding coprostanol is extremely large and cannot be comprehensively summarized. For the work reported here, over 200 papers published between 1934 and 2011 having data related to concentrations in sewage or in urine or feces, or to excretion rates, were examined; the most relevant data from only a portion of these were mined. This literature spans a number of non-intersecting fields, a result of CoP being studied in the fields of medicine and clinical science, microbiology, geochemistry, nutrition, agronomy, archeology (e.g., Birk et al., 2011; Bull et al., 2003; Evershed and Bethell, 1996), analytical chemistry, and environmental science, among others. Most of this literature is not relevant to the proposed use of CoP for SCIM. The only relevant aspects are the variables that influence the synthesis and excretion of CoP in the human gut and the overall levels of CoP in excrement (fecal material) and in raw (unsettled) sewage. Interest in the topic of CoP in fecal material declined prior to 2000, while interest in the use of CoP for source tracking for sewage has increased since 2000.

CoP has a long history of use as a marker of sewage contamination (Hatcher and McGillivary, 1979), especially in sediments (e.g., Pratt et al., 2007) and surface waters (Tabak and Bunch, 1970). In contrast, its analysis in raw and treated sewage has been comparatively infrequent, primarily because such data had no apparent use. One of the earliest studies to propose the use of CoP in environmental monitoring was Murtaugh and Bunch (1967). Early work by the US Environmental Protection Agency pioneered the use of CoP as an indicator of ambient water

contamination by sewage (Tabak et al., 1972). In the 1970s, CoP was first proposed for use in an index for contamination of sediments by sewage (Goodfellow et al., 1977).

Environmental monitoring studies have routinely detected CoP in surface waters and especially sediments. Given its origins with fecal material, and its presence at relatively high concentrations, it is often used as a marker for the degree of fecal contamination in ambient waters or as a surrogate measure for human bacterial levels in waters (Leeming and Nichols, 1996). It can also be used to gauge the degree of dilution of raw or treated sewage in receiving streams (Takada and Eganhouse, 1998). CoP (together with other sterols) has been used as a marker in sediment cores to perform detailed examinations of historic contributions of sewage to marine systems (Venkatesan and Kaplan, 1990).

CoP is excreted by different vertebrates in differing absolute and relative quantities. By monitoring for various associated co-metabolites (such as other stanols), ratio indices can be used for source tracking or for distinguishing between contamination from humans versus wildlife or domestic animals (Bull et al., 2002). Sterols have also found use as markers for detecting fecal contamination of urine in waste handling systems designed to separate urine prior to waste treatment (Börjesson et al., 1998; Höglund, 2001; Schönning et al., 2002).

3.2.1. Biological origins of CoP

Clinical research has been the driver for much of the research on the biosynthesis of CoP in humans. A major question motivating most of this work has been whether bacterial metabolism

of cholesterol in the colon is associated with a variety of diseased states – as both a marker and as a causative or protective factor (Lichtenstein, 1990).

Some of the first experimental evidence that CoP is formed from the hydrogenation of cholesterol by specific bacterial action in the human gut was published in 1934 (Dam, 1934). While CoP is the predominant reduced sterol formed in the human gut, it can also be produced in substantial quantities (mg/g wet fecal mass) by other vertebrates, such as pigs, cows, horses, rabbits, and limited avian species (e.g., chickens) – but usually not as the dominant sterol (a major distinguishing factor from humans) (Leeming et al., 1996; Shah et al., 2007; Tyagi et al., 2008); CoP has been identified, however, as the dominant sterol in swine slurry (Jaffrezic et al., 2011). Worth noting is that animal inputs to combined sewers could serve as confounders in the interpretation of ASAP-SCIM data based on CoP.

3.2.2. Gut microbiome composition as the primary driver in human coprostanol production (converters versus non-converters)

CoP production by the human gut microbiome is characterized by a particular attribute that impacts its utility as a biomarker of per capita population. While the daily variation in intra-individual production is comparatively low, the efficiency with which it is converted from cholesterol seems to depend on the species composition of the microbial consortia in the gut. Evidence indicates that the efficiency of its production (conversion) does not span a continuum, but rather displays two to three discrete intervals of conversion efficiency – often loosely categorized in the literature as nil, low, and high. Significantly, these categories usually do not

seem to change for an individual, so the overall short-term rate of production across a given population is not altered.

The fact that the efficiency of inter-individual cholesterol metabolism (from nil to high) is distributed across two or three distinct sub-populations is consistent with the growing evidence for the existence of gut microbiomes of distinct and stable community compositions – referred to as "enterotypes". A recent study reveals that the gut microbiome is dominated by abundant species from two phyla – Firmicutes (e.g., Bacilli and Clostria) and Bacteroidetes (e.g., Bacteroides) – with numerous low-abundance species contributing to a significant long-tail distribution, which possibly contribute highly specialized functions. Like CoP production, the possibility exists that the compositions of gut microbiomes are not distributed over a continuum, but rather exist as discrete entities. Three enterotypes may exist, each comprising defined consortia of distinct, co-occurring species. Moreover, these enterotypes, while differing in function, appear to resist influence by any number of variables, including composition of diet (especially regarding the intake of phytosterols), nutritional status, gender, age, nationality, or body mass index (BMI) (Arumugam et al., 2011).

There is no evidence that CoP is produced by a few, particular bacterial species. Rather, it seems to result from the combined metabolism of ill-defined consortia whose populations must reach a critical level. At least 10^6 cells/g fresh fecal material may be required, with 10^8 cells/g required for complete conversion of cholesterol to CoP. Those individuals having too few coprostanoligenic bacteria are very inefficient converters and seem to comprise a small but significant percentage of at least some continental populations. Frequently cited for the bi- or tri-

modal population distributions is that roughly 20-25% of various populations studied are "non-converters," where CoP comprises less than a third of their total fecal neutral sterols (Veiga et al., 2005).

Bacterial conversion seems to occur via two pathways – direct (from cholesterol) and indirect (via coprostanone as an intermediate) (Gérard, 2010; Gérard et al., 2007). Although esterified CoP conjugates can also be formed, the evidence is conflicted as to whether they compose a significant portion of CoP. Regardless, it is believed that they undergo facile hydrolysis and therefore a saponification step may not prove to be important in analysis of sewage for total CoP (Kirchmer, 1971; Rosenfeld, 1964; Rosenfeld and Hellman, 1971).

3.2.3. Inter- and intra-individual variation in cholesterol-CoP conversion

Variation in CoP excretion within and between individuals comprises two aspects. One involves the multi-modal inter-individual distribution of CoP microbial production from cholesterol – loosely referred to as low- and high-conversion, with an extreme of non-conversion; the distribution of conversion efficiency among a population seems to be a function in part of age and gender and is driven by the composition of the gut microbiome (Benno et al., 2005). The second involves the intra-individual daily variation (or constancy) in CoP excretion.

The first major study of the bi-modal distribution of population-wide conversion efficiency for CoP was Wilkins and Hackman (1974). The high- and low-conversion traits (an arbitrary cut-off for the two groups was defined at the 50% conversion level) were stable over long periods of time (at least up to 22 months), but isolated, transient instances occurred when a high- or low-

converter inverted; sporadic inversion between low and high conversion has also been noted after cessation of antibiotic therapy (Midtvedt et al., 1990). The range of conversion efficiencies, while not a continuum, can span from nil to 99%. In a test group of 31 North American adults, there were 23 high-converters, with CoP levels of 21.3±8.6 mg/g dry feces (CoP composing 61% of total steroids). In the remaining eight low-converters, CoP levels were 4.1±4.3 mg/g dry feces (8% of total steroids). The low-converters represented roughly 25% of this small limited test population (Wilkins and Hackman, 1974). The incidence of low-converters in North America is often given as 20-25%, but this is a rather rough approximation. The bimodal distribution of high- and low-converters has been recently discussed by Keller (2010).

Establishment of the gut bacteria responsible for conversion begins about 6 months after birth in those who become converters; conversion efficiency then increases into the late teens (de Leon et al., 1987; Midtvedt and Midtvedt, 1993). The factor that consistently reduces CoP production the most (for unknown reasons) is probably age; note that urinary creatinine also declines with age, but for a different reason (i.e., loss of skeletal muscle mass). For the purposes of the ASAP-SCIM concept, age corrections could possibly be derived from existing demographics already in use for existing population modeling.

The percentage conversion of cholesterol to CoP across four countries (France, Germany, Italy, and Sweden) ranged from 55-76% in males and 40-66% in females (in a study involving 94 subjects) (Norin, 2008); an additional 13 subjects (12% of the total subjects) were non-converters. The range among elderly was 61-74%. The "normal" level of CoP conversion in feces may vary geographically.

Diet, drugs, and other interventions that reduce cholesterol (or alter the gut microbiome, such as antibiotics) can also result in changes in coprostanol levels formed and excreted (Benno et al., 2005; Keller et al., 2008; Korpela and Adlercreutz, 1985; Midtvedt and Frederichsen, 1977; Midtvedt et al., 1990; Norin, 2008). Antibiotics in particular tend to lead to reduced conversion to CoP, usually after treatment with those targeting anaerobic, Gram-positive bacteria rather than those targeting aerobic, Gram-negative bacteria (Midtvedt et al., 1990).

3.2.4. Per capita total daily excretion of CoP

The published literature on the human excretion of CoP and other sterols is extensive. Most of this literature comprises clinical or epidemiological studies examining correlations with diet or disease, especially colonic cancers. For a short review, see Nair (1988). Unfortunately, the data from these many studies is reported on the basis of a disparate spectrum of approaches making intercomparisons very difficult. For example, CoP levels are most frequently reported as concentrations (presented on the basis of mass per unit mass, or mass per unit volume) in fecal water, fecal wet mass, or fecal dry mass (e.g., Glatz et al., 1985; Keller and Jahreis, 2004; Lipkin et al., 1981; Shah et al., 2007).

Much less frequent are data presented in terms of total daily excreted mass. The only basis having direct relevance to the concept proposed here is the per capita flux in terms of total excreted daily quantity (mass or moles). Data are usually reported in terms of fecal concentrations because studies are often designed to test whether CoP levels correlate with diseases of the colon. But CoP concentrations alone are not useful for evaluating the potential of the SCIM concept unless the total daily excreted fecal mass is also known; even then, sometimes

the daily excreted amounts are normalized against body mass (e.g., mg/day/kg body weight), but the body masses are not provided. This is because the daily excreted fecal mass excreted can vary widely as a function of the individual and diet (a major determinant is consumption of indigestible fiber); the fecal concentration of CoP is often lower when the fecal mass eliminated increases.

Dietary factors that influence CoP fecal concentrations are complex (Kay, 1981). Major factors are intake of meat (increases CoP concentrations) and fiber (reduces CoP concentrations). The difference in extremes is often a factor of 2 (e.g., Jenkins et al., 1975; Korpela and Adlercreutz, 1985; Reddy et al., 1975; Reddy et al., 1998; Ullrich et al., 1981; van Faassen et al., 1987; Weststrate et al., 1999).

Another major factor can be colon disease. For example, the fecal CoP concentrations (mg/day/g dry fecal mass) for those with polyposis of the colon and familial colon cancer are 20-50% those of healthy subjects (Bone et al., 1975; Lipkin et al., 1981; Moskovitz et al., 1979; Reddy et al., 1976; Watne et al., 1976); the frequency of low-converters is also much higher. Some data, however, are contradictory. Two studies, for example, show colonic cancer correlating with fecal CoP levels up to several fold higher than healthy controls (Korpela et al., 1988; Peuchant et al., 1987).

Most of the published data on per capita excretion rates for CoP (usually expressed in mg/day) have been summarized in Table 2. Of over the 200 studies examined (inclusive of studies published in 2011), only 13 (all published between 1964 and 2002) provided daily CoP excretion rates or data from which rates could be easily derived; as mentioned above, numerous other

studies are available, but they report only CoP fecal concentrations. A few provide data that can be used to indirectly estimate per capita excretion rates. For example, using sterol loadings in sewage influent for three STPs in France, one study calculated estimated fluxes of total sterols of 0.5-0.6 g/day/capita (Quéméneur and Marty, 1994). CoP was reported to comprise 37-48% of particulate sterols and 13-34% of the dissolved sterols in the influent sewage. Using these extremes, an estimated per capita CoP flux can be deduced as perhaps having a range of 65-288 mg/day.

In 1972, Tabak et al. (1972) stated that humans on average excrete CoP at a rate of 800-1,000 mg/day per person. Even though most of the data on CoP excretion rates were published after 1972, this estimate for a range for human excretion rate seems to rest within the high end of the range of values reported in the studies published since. The frequency of low-converters (including non-converters) brings the average down.

A visual inspection of the CoP excretion data summarized in Table 2 reveals that a **range of 200-700 mg/day perhaps encompasses the bulk of the published data**. While the extremes of the total ranges of daily CoP excretion rates from all of the studies combined is somewhat broad, a key point is that the range for any given study is much narrower, especially if the data for low- and high-converters (when reported) are averaged. This undoubtedly reflects differences in the analytical methodologies, as a wide variety of analytical methods have been used in CoP excretion studies. Given the extremely broad array of variables and analytical approaches used in these published studies, the data are remarkably consistent. This contrasts with creatinine, whose

excretion data has presumably benefited from the use of long-standardized clinical analytical methods.

3.2.5. Analysis for CoP

Analytical methodology (including the sampling process) probably drives a considerable portion of uncertainty in CoP data. Despite the broad targeting of CoP in numerous studies over the decades, there has been no attempt at standardizing the analytical methodology – especially for feces and sewage. The limit of detection is not an issue given the high levels of CoP in sewage. Speed and sample throughput are probably the limiting factors, as sample clean-up poses challenges. Another challenge (a possible issue in sewage analysis but not in analysis of human fecal material) is distinguishing CoP from epicoprostanol – its epimer 5β-cholestan-3α-ol (Eganhouse et al., 1988). Distinguishing CoP epimers, however, has since become a routine aspect of analysis, especially in monitoring programs designed to distinguish human fecal sources from contributions by wildlife (e.g., Hughes and Thompson, 2004).

Improvements in various aspects of analysis have been published over the years, including improved extraction (Moliner-Martinez et al., 2010) and derivatization, for gas chromatographic separations (Wu et al., 2010). For a given methodology, inter-sample and inter-analyst precision can be high. For example, 10 subsamples from a 24-h composite sample of STP influent in Italy were divided among two technicians, yielding mean CoP levels of 34.3±1.7 µg/L and 33.4±2.2 µg/L (Gilli et al., 2006).

For the purposes of the SCIM application presented here, a major challenge would probably be development of methodology for sampling and quantifying CoP in sewage solids (including suspended particulates), as the hydrophobicity of CoP limits its solubility in the aqueous phase, which has served as the focus for most of the published sewage-monitoring studies. This topic is discussed in Section 5 (The challenge of representative sampling).

3.2.6. Stability of CoP

The stability of CoP (dictated primarily by its relative susceptibility to biodegradation) has relevance with respect to how far down an STP's process stream sampling can take place and what precautions need to be addressed when storing or shipping samples. Because of mixing, the homogeneity of STP influent increases further downstream, reducing the challenges for discrete sampling, but the impact of biodegradation can rise.

Oxygen partial pressure plays an important role in the biodegradation of CoP. Microbial degradation proceeds faster under lower oxygen tensions but is inhibited in anoxic conditions. The highest average rate of biodegradation (0.438 μg/g/day) was found to occur in sediment from non-aerated coastal water (Bachtiar et al., 2004). Although CoP appears to degrade exponentially in raw primary sewage sludge (Bartlett, 1987), it has shown stability for up to 118 days in urine with feces present (Sundin et al., 1999). These data indicate that CoP would best be sampled early in an STP influent stream.

3.2.7. CoP in raw sewage

Although the daily per capita excretion of CoP seems to have considerably less intra- and inter-individual variance than the widely used clinical urinary biomarker creatinine, its analytical determination in sewage may prove more challenging – primarily because of its hydrophobicity. The log K_{ow} for CoP is estimated at 6.3-7.6, and its aqueous solubility is estimated at 0.7 µM (ca. 272 µg/L) (Takada and Eganhouse, 1998). CoP is therefore expected to preferentially partition to solids. Indeed, a significant portion of the CoP that enters an STP is eventually associated with sewage sludge; but since the overall volume of sludge is small compared with the aqueous phase, the levels of dissolved CoP could possibly provide an estimate of overall CoP loadings. Unless the particulates and suspended solids in raw sewage are included during sample acquisition and analysis, only the dissolved portion of CoP will be determined. As one example, CoP was detected at roughly a level of only 10 µg/L in water decanted from a solids separation step at an STP (Chaler et al., 2001).

Much of the published literature on the occurrence of CoP in sewage therefore excludes an unknown portion of CoP. Some occurrence data, however, indicate that CoP may be present at total levels closer to its aqueous solubility, minimizing the portion sorbed to solids. Sometimes the published data on CoP levels in sewage may therefore be close to the actual levels (i.e., when they are low). Otherwise, many of the reported apparent levels of CoP dispersed in the aqueous phase of sewage are probably due to CoP sorbed to dissolved organic material or colloids.

Despite the limited aqueous solubility of CoP, the large absolute quantities continually excreted into sewage make it one of the major aqueous-phase organic constituents – exceeded only by the

heavily used surfactants (linear alkylbenzenesulfonates) and the chelator nitrilotriacetic acid (Nguyen et al., 1994). The absolute quantities of CoP excreted by metropolitan regions can indeed be large, as shown by the combined annual mass emission rate of coprostanol and epicoprostanol from municipal wastewater treatment plants into the southern California Bight (which includes coastal southern California) – an estimated 260 metric tons/year, in 1987 (Venkatesan and Kaplan, 1990). The limit of detection is therefore not an issue with respect to analytical methodology.

Homogeneity via adequate mixing is central to ensuring measurement of representative CoP loadings in raw sewage. In one of many similar studies, over 70% of CoP was shown to be associated with centrifugable particulates. Ensuring adequate mixing is therefore important. In five sub-samples of the same well-mixed raw sewage sample, CoP values spanned the range of 200-270 μg/L (mean 228±31.14) (Switzer-Howse and Dutka, 1978). Other studies have collected suspended particulates (e.g., by physical filtration) and compared solvent extracts with those of the filtrates. One study, for example, showed that over 95% of the sterols (including CoP) in sewage influent and effluent were associated with particulates (Isobe et al., 2002). And another reported over 84% of CoP in treated sewage was associated with particulates (Brown and Wade, 1984). Two samples of sewage sludge contained CoP at 1.96 and 3.3 mg/g suspended material (or an equivalent of 41.16-25.08 mg/L) with only 0.22-0.34 mg/L dissolved (Takada et al., 1994). CoP was present in U.S. municipal sewage sludges at 2.6-55 mg/L; the coefficient of variation for replicate samples ranged from 1-10% (Eganhouse et al., 1988). Its partitioning to sludge (at part-per-million levels) (Takada and Eganhouse, 1998) explains why CoP is

apparently so easily removed during sewage treatment (McCalley et al., 1981). Extensive data on the preferential partitioning of CoP to particulates are available (e.g., LeBlanc et al., 1992).

Although numerous papers report on CoP levels in sewage, no attempt has been made to summarize the data here because the levels of CoP in sewage are useful for the SCIM concept only when the flux of CoP is known. This means that the levels must be integrated over time (for example, over the course of 24 hours). This necessitates knowing the total volume of sewage sampled from during the sampling period. Fluctuations in CoP levels in sewage influent can result from: (i) changes in per capita daily excretion (e.g., as a result of changes in diet or health), (ii) change in population (e.g., transient inward or outward migration from the STP service area), or (iii) changes in sewage flow (e.g., wet-weather events; episodic industrial discharges; infiltration/exfiltration). Knowing the flux serves to normalize for the changes in flow. Otherwise, no conclusions can be drawn whether CoP concentrations vary or are the same across different STPs.

Significantly, in no case were CoP concentrations in sewage available together with both daily sewage flow rates and estimated population served by the STP. This would have allowed calculation of estimated daily per capita excretion rates to see if they were consistent with the data summarized in Table 2. Furthermore, much of the published data does not include adequate explanation as to how particulates and suspended solids were handled during sampling or sample preparation. So it is unclear if the reported sewage levels reflected total or dissolved CoP levels (or portions of both).

Only a brief overview of some of the data on CoP in sewage will be summarized below. One indication of CoP's potential utility as a surrogate measure of population would be the variance in sewage levels over time. Given the three major drivers for variance, if time-course samples from a given STP were to show low variance, this would support the potential for CoP as a surrogate measure.

There have been few time-course studies of CoP in sewage. One of the first was published in 1970 (Tabak and Bunch, 1970). Weekly sampling over 6 weeks for the STP influent and effluent streams for a Burlington (Iowa) STP gave CoP influent levels of 245-394 μg/L (mean = 316) and effluent levels of 160-315 μg/L (mean = 240); effluent levels, however, would be of limited use for ASAP-SCIM (see Section 3.2.8). A subsequent 1972 study was one of the most extensive time-course studies (Tabak et al., 1972). It followed CoP levels in STP effluents discharging on three to six dates from five STPs along the Mississippi River. To illustrate the surprisingly small intra-STP variance, the ranges and means [μg/L] were: Sioux City (636-793, 709), Omaha (743-864, 797; 250-363, 312), St. Joseph (391-484, 436; 465-573, 508; 365-498, 424; 424-535, 491), Kansas City (496-587, 535; 259-319, 290; 328-419, 381), and Burlington (245-394, 316; 160-315, 240). Dual-date sampling from single effluents from four STPs along the Ohio River yielded mean CoP levels (μg/L) for: Little Miami (209), Mill Creek (633), Bromley, KY (355), and Muddy Creek (291). The grand mean was 433 μg/L.

Another time-course study followed the weekly variation in CoP in raw sewage from an STP in Honolulu. CoP ranged from 90-381 μg/L (mean of 217) over an 11-month period (Brostrom, 2005); CoP in the treated effluent averaged 138 μg/L. A recent 1-year time-course study

followed CoP in the influent from two STPs in Hungary (Andrási et al., 2011). Levels for one STP were 180, 302, and 45 µg/L and for the other were 188, 100, 144, 44, 31, and 20 µg/L, giving an overall combined range of 20-302 µg/L.

Numerous studies report CoP levels from single samples. These studies illustrate the extremes in the CoP levels encountered in STPs from different countries and from the use of different sampling and analysis methodologies. CoP in the influent for five STPs in Tokyo averaged 327 µg/L (Isobe et al., 2004). Influent to a small rural STP in France (capacity of 1,800 equivalent-inhabitants) had a CoP concentration of 98.7 µg/L (Jeanneau et al., 2011). Ten wastewater samples from urban STPs in Hungary had CoP levels ranging from 930-5,360 µg/L (with a mean of 3,010±1,690 µg/L) (Szucs et al., 2006).

A back-of-the-envelope calculation provides some perspective for comparison of measured CoP levels in sewage with what might be predicted from known excretion rates. Using the general range for per capita CoP excretion of 200-700 mg/day (derived from Table 2) and using a very rough estimate of per capita daily water usage of 100 gal/day [379 L/day (USEPA, 2011)], a projected range of CoP loadings in sewage influent could be 527-1,850 µg/L (assuming influent comprises solely domestic waste). This is consistent with the published data (Table 2) and again points to the possible utility of CoP as a biomarker for the ASAP-SCIM concept.

These levels are of course sensitive to a broad spectrum of variables, including water usage (which can vary dramatically by geographic locale, season, and family), water wastage (e.g., plumbing leaks), combined sewer inputs (e.g., industry, agriculture, wet weather, runoff of

animal feces), infrastructure deterioration resulting in alterations in sewer flows (e.g., infiltration/exfiltration) (e.g., Shelton et al., 2011), and CoP losses during transit to the STP (e.g., biodegradation). This shows the importance of knowing the actual flux of sewage for each STP.

3.2.8. CoP in treated sewage

Numerous studies provide data on CoP (and sterols in general) in treated sewage. These data are often collected as part of a study assessing the occurrence and distribution of CoP in surrounding waterways and sediments – primarily to assess sewage incursion or bacterial source tracking. In general, CoP levels in treated sewage are substantially reduced compared with raw influent, partly as a result of biodegradation but primarily because of removal by sorption to sludge. Although these data are much less useful to the SCIM application, they do serve to emphasize the need for sampling the influent to STPs as early in the process stream as possible.

In a survey of effluents from 10 STPs in the U.S., CoP was detected in only 82% of samples. The median level was 1.3 µg/L and the maximum was 5.9 µg/L (Glassmeyer et al., 2005). In 10 effluents from different STPs, CoP was detected in only one sample, at a level of 140±10 µg/L (Moliner-Martinez et al., 2010).

4. Other potential markers

Any number of other chemical substances exist that could be considered as proxy markers for population size. Possible candidates come from the universe of both naturally occurring and synthetic xenobiotics (and their metabolites or formulation impurities) as well as products of endogenous metabolism. Most markers have been pursued as a means of source tracking for

sewage or as an overall index for human activity or impact. Caffeine is a well-known example (e.g., Froehner et al., 2010). But a variety of other chemicals have also been assessed, including drugs [e.g., carbamazepine (Gasser et al., 2011)], biocides [e.g., triclosan (Singh et al., 2010)], chemicals in household cleaning agents [e.g., fluorescent whiteners, trialkylamines (Managaki et al., 2006; Valls et al., 1989)], and food additives [e.g., sucralose (Oppenheimer et al., 2011)].

All consumer chemicals, however, lack at least several of the attributes listed in Table 1, serving to either confound data interpretation or render data excessively inaccurate. Sucralose is an example that illustrates the problems also posed by other consumer chemicals, including pharmaceuticals. Sucralose is a tri-chlorinated disaccharide artificial sweetener that is refractory to metabolism and initial microbial degradation. Although this would make sucralose an excellent stable sewage tracer, its loadings into sewage are a function of variables that contribute excessive uncertainties. Among these are local/regional culinary and nutritional preferences and customs. The usefulness of synthetic markers such as sucralose would depend on the consistency of their market penetration; but the popularity of any commercial product can vary widely. Its consumption can vary widely not just geographically but also over time. Per capita consumption must be known in advance, and consumption rates are subject to change as the chemical's popularity waxes and wanes. Although these chemicals may prove useful for tracking contamination of open waters back to the sewage sources and for deducing per capita consumption (assuming the population is already known) (e.g., Lai et al., 2011; Oppenheimer et al., 2011), they are not useful for ab initio estimates of population size.

Chemicals involved in endogenous metabolism (products of biosynthesis or catabolism) avoid many of the problems of xenobiotics for use as proxy measures since their association with per capita activities has higher fidelity. The main problems are those of excessive intra- and inter-individual variation in excretion. This was documented in Section 3.1 (Creatinine as a possible biomarker for estimating population) for creatinine, a marker long assumed to have a relatively stable excretion rate across entire populations.

Problems other than variation in excretion rate confront possible markers originating from endogenous metabolism. An example is a biochemical relatively unique to human metabolism and which at first might appear to be a good candidate proxy marker – namely, 1-aminopropan-2-one (1-aminopropanone: APR; 1-aminoketone). APR, via 1-aminopropan-2-ol, serves as a precursor to vitamin B-12 (Fitzsimons and Belt, 2005) and is very water soluble; it is excreted in urine but in daily quantities much lower than CoP (e.g., Dawit et al., 2001; Fitzsimons et al., 1995). The data regarding its levels in sewage, although limited, are contradictory. It is sometimes found in sewage at levels higher than in urine, with implications of perhaps de novo microbial formation in sewage (Dawit et al., 2001; Fitzsimons and Belt, 2005; Fitzsimons et al., 1995); but other times it cannot be detected (Singh and Gardinali, 2006).

5. The challenge of representative sampling

The focus of this paper is on the rationale for selecting appropriate biomarkers excreted into sewage as a means for gauging the size of small-area populations. Chief among the selection factors are minimal intra- and inter-individual variance in excretion (Table 1). It cannot be overemphasized, however, that many other parameters unrelated to physiology and metabolism

can contribute to possibly large short-term and longer-term variance, especially in the sampling and analytical processes required for measuring the daily excreted quantities of a given biomarker.

The uncertainty surrounding the sampling process itself could prove to be the largest source of variance in measuring biomarker loadings in sewage. This variance can originate from two critical needs: (i) obtaining sewage samples that are representative in space and time, and (ii) measuring the total flow of sewage across the time span over which the sampling occurs. These two factors pose considerable challenges. Because of their complexity, they will not be examined in any depth here. Indeed, the complexity of these challenges has prevented nearly all published studies on quantifying pollutant loadings in sewage from settling on comprehensive sampling approaches applicable to the wide spectrum of sewer system designs and hydraulics (e.g., varied extents of pressurized connections, associated pumping and retention volumes, and mixing dynamics, and their influence on transient, intermittent pulsatile flows such as from the flushing of individual toilets). To accommodate for this considerable uncertainty, a conservative approach would be the use of high-frequency, flow-proportional sampling for acquiring daily composite samples (Ort et al., 2010b). A possible alternative would be continuous passive samplers, such solid phase membrane devices (SPMDs) or the polar organic chemical integrative sampler (POCIS) (Harman et al., 2011). It is unknown, however, as to whether highly hydrophobic biomarkers such as coprostanol would remain sorbed to suspended solids rather than partition to the sampling device; very few studies using SPMD or POCIS have reported on the extraction of coprostanol, so it is unknown whether this approach would work in the presence of suspended solids (e.g., Moliner-Martinez et al., 2010; Rujiralai et al., 2011).

One sewage sampling problem would be somewhat ameliorated with respect to monitoring an endogenous biomarker versus a xenobiotic (e.g., drugs or other so-called emerging contaminants) – the latter of which have been the subject of most published studies. It would be expected that the occurrence frequency for biomarkers would be a higher, as they would originate from a larger percentage of toilet flushes than would xenobiotics; this is because biomarkers undergo continual excretion across a larger percentage of the population. Therefore, the sampling frequency should be less affected by the need to ensure complete capture of pulsatile flows.

Published studies have also limited themselves almost exclusively to those chemicals that would be expected to be fully dissolved in the aqueous phase – to avoid the considerable additional problems associated with sampling suspended solids. Extensive examination of the problems surrounding sampling uncertainty as applied to water-soluble contaminants (but not hydrophobic analytes) in sewage has been published by Ort et al. (2010a; 2010b).

Yet another problem would be in locating the point of sampling. If suspended solids must be captured, the sampling location would need to be in the influent, prior to clarifying. Although obtaining a representative 24-h sample of the dissolved phase of sewage faces many challenges itself, the problem is greatly magnified by needing to also ensure that suspended solids are captured representatively – as might be required for a biomarker such as coprostanol. This problem results primarily from the possibility of cross-sectional non-homogeneity of flow in sewers, especially in open-channel sewers. Problems associated with suspended solids are

discussed by Larrarte (2008). For these reasons, the ASAP-SCIM concept presented here might require considerable further development before reliable implementation could begin.

Another problem relates to the reality that any given spatial segment of sewage flow captures excreted materials from only a portion of the population served during that period of time – and this portion is not known. But assuming that each individual excretes a particular quantity of CoP over a 24-h period, then by monitoring CoP in the sewage stream for an STP over successive 24-h periods, the flux (concentration multiplied by flow rate: ng/L x L/day) gives a series of daily values for mass per day (e.g., ng/day). The running average then devolves to the per capita level.

Finally, with respect to the problems associated with measuring sewer flows, the effect of flow pulses will depend in part on whether a pipe is operated under pressure and how full non-pressurized pipes are. Spatiotemporal variabilities in sewerage systems and flows, coupled with the heterogeneity introduced not just by the pulsatile nature of sewage flow but also by wet-weather flows in combined-sewers, pose great challenges to flow measurement. The use of in-line flow sensors may help in this regard (Larrarte, 2008). Other emerging technologies may eventually contribute to the better estimation of flows. One example is the development of real-time measurement of local rainfall at higher spatial resolutions than currently possible – with the use of mobile phone networks (Goldshtein et al., 2009; Ryser and Bryner, 2010).

6. Next steps

To further develop the SCIM concept for estimating population by monitoring for coprostanol, a series of possible milestones can be foreseen. These are summarized in Table 3.

7. ASAP-SCIM and the future

Three emerging areas of advancement can be seen as greatly improving the potential utility of the ASAP-SCIM concept; these are presented in the following sections.

7.1. Automated, in-line, real-time measurement

If ASAP-SCIM using a suitable biomarker proves successful for estimating small-area population size, its utility could be greatly extended with in-line sensors (for measuring both biomarker levels and sewage flow rate). These outputs could be used to calculate population in real time. Placed at strategic locations in a sewage collection system, a network of sensors could provide data on increasingly finer scales. The ebb and flow (efflux and influx) of small-area populations could even be visualized – in a format analogous to weather maps. Successively larger, city-wide and regional populations could be automatically estimated simply by summing the constituent small-area populations.

7.2. Composite index of de facto population via multiple biomarkers

Uncertainty in measured levels of any biomarker (including coprostanol) will always exist because of the numerous variables driving the variance in biological systems. This uncertainty could possibly be reduced by measuring additional biomarkers. An algorithm could be developed for generating a composite population-proxy index based on multiple biomarkers. This approach could be guided by selecting biomarkers that are biased by different variables (complementary) or biased in different directions by the same variable (orthogonal). For example, two biomarkers could be selected whose excretion rates are known to change in opposite directions as a function

of a given variable, such as age, gender, or diet. This might serve to smooth the errors associated with their independent per capita excretion rates.

7.3. Facilitating new uses for de facto population estimates – gauging the health of communities

Finally, the availability of real-time population levels for discrete geographic locales could be used to provide real-time estimates of per capita consumption and production statistics for any number of chemicals or non-chemical environmental stressors of human origin and which can be measured in sewage. Examples include per capita consumption (or disposal) of household chemicals, pharmaceuticals (including illicit drugs), and personal care products, or per capita production of endogenous biomarkers indicative of disease or health.

An emerging example of the need for better population estimates is the growing number of studies applying the FEUDS methodology for gauging community-wide consumption of illicit drugs. Inaccurate population estimates are a major source of error in estimating average per capita usage (Daughton, 2011).

Although FEUDS represents the first use of sewage monitoring for mining useful chemical information attributable to the collective contributions from individuals in defined populations, countless other applications can be foreseen whose value would be enhanced with the ability to allocate, apportion, or attribute on a per capita basis. First noted by Daughton (2011), SCIM as a general approach holds the potential for mining a wealth of information from sewage in the form of biomarkers that reflect general or specific aspects of health or disease. These data would require normalization on a per capita basis, in a manner analogous to the conventional

measurement of urinary markers as implemented in clinical chemistry. This new measurement ability would create the first opportunity to view and treat communities from a new perspective – by defining the community at large as the patient. Such an application would represent the first true implementation of "sewer epidemiology" (Daughton, 2011).

Table 1. Ideal attributes for SCIM biomarkers targeted for estimating population

attribute	example	potential problem
must be excreted into sewage	excretion via urine rather than feces poses fewer sampling and analytical challenges	excretion via feces creates a non-homogenous sample stream and requires more comprehensive sample preparation
uniqueness to human metabolism	coprostanol (CoP) is produced in significant quantities primarily by higher vertebrates that synthesize cholesterol	input possible from other animals where industrial/agricultural sewage is mixed with human sewage or runoff carrying animal feces into combined sewers (both would serve to confound data)
exogenous sources are minimal	low occurrence in raw or cooked foods	creatinine occurs in meats and can be created from creatine during cooking (can confound data)
minimal intra-individual variance in daily excretion	daily levels excreted by an individual vary minimally over time	stress or disease can affect the excretion of all biomarkers
minimal inter-individual variance in daily excretion	per capita daily excretion across a population varies minimally	a wide spectrum of physiological variables can dictate the excretion of biomarkers (age, gender, genetics, stress)
daily per capita excretion in sewage is independent of extraneous variables	minimal effect from season, weather, geographic locale, water-use restrictions, medication usage, diet	diet can influence excretion variance for both creatinine and CoP
occurrence levels independent of design and usage of sewerage system	length of sewerage distribution pipes and residence time of sewage in pipes	time-dependent degradation by microorganisms during sewage transit can lead to variable reductions in biomarker levels
minimal degradation of biomarker in flowing sewage (levels persist in sewage)	slow degradation allows sampling raw sewage further downstream, permitting better mixing of influent "pulses"; ensures minimal loses during transit through sewer connections of varied lengths and residence times	sewage pulses with widely varying biomarker levels (compounded by changing pulse frequency) greatly increase the required frequency of sampling [a]
levels in raw sewage well above method detection limit (MDL)	few analytical interferences; easier implementation of a routine method	isobaric biomarker isomers often become common interferences with methods using mass spectrometry
minimal potential for exogenous interference from other sources	exogenous sources include residues of target analyte on analyst's hands	residues of target analytes can sometimes be excreted as sweat (e.g., drugs) or remain from prior dermal contact or direct application (e.g., personal care products) (Daughton and Ruhoy, 2009)

homogenous distribution; biomarker preferably partitions to aqueous phase	minimal partitioning to dissolved or suspended solids or sludge	partitioning to solids increases the complexity of sampling and sample preparation
minimal degradation of analyte in sampled sewage (levels persist in sewage)	refractory to microbial degradation or to further physicochemical degradation during sample shipment or storage	preservatives may be required to inhibit microbial degradation in stored or shipped samples
minimal de novo, ex vivo formation of analyte in sewage	minimal formation by microbial activity during sewage transit and during sewage treatment	sampling of raw sewage as early as possible in influent stream may be necessary
minimal sample clean-up and sample preparation	requires minimal pre-concentration to meet MDL	excretion of biomarker in the form of conjugates may require additional hydrolysis step
analytical determination uses instrumentation routinely available; analytical methodology amenable to standardization	conventional GS/MS, LC/MS, or immunoassay	innovative "research grade" methodologies are too costly or complex for wide implementation
minimal capital investment in instrumentation; minimal analyst time	allows for high-frequency sampling	"research grade" methodologies are too costly for wide implementation
high sample through-put	amenable to automation; reduces cost	analyst intervention reduces timeliness of results
potential for in-stream continuous sampling or monitoring	equilibrium passive samplers (EPS) allow for passive, time-integrated sampling;[b] in-stream sensors facilitate real-time data	discrete sampling gives biased results because of stream heterogeneity and sewage pulses
minimal occupational hazards for technicians	minimal hazards from samples, and from analytical reagents or reactions	handling raw sewage poses risks associated with pathogen exposure

[a] The challenges associated with obtaining representative samples from an STP are discussed by Ort et al. (2010b).

[b] Examples of EPS (Zabiegała et al., 2010) include polar organic chemical integrative samplers (POCIS) and semipermeable membrane devices (SPMD).

Table 2. Selected data on per capita total daily excretion of CoP [a]

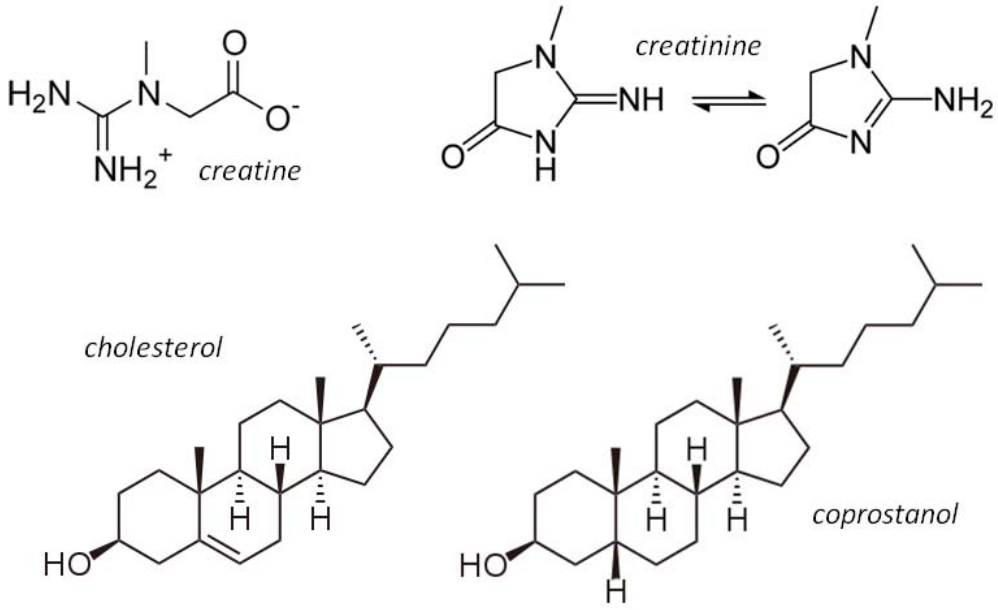
per capita CoP excretion rate	study size	reference
270 ± 62 mg/day	12 healthy adults	(Bartram et al., 1991)
567 ± 214 mg/day	6 healthy subjects	(Batta et al., 2002)
nil	8 subjects fed a high-carbohydrate diet free of fat and fiber for 12 days	(DenBesten et al., 1973)
346 ± 45 mg/day	38 healthy adults; none was a low-converter (all showed greater than 89% conversion)	(de Leon et al., 1987)
420 (301-662) [129-704] mg/day <butter-supplemented diet> 417 (228-666) [128-1305] mg/day <corn oil-supplemented diet>	mean (and range) of individual averages, and [total range of individual values] for 6 subjects	(Eneroth et al., 1964)
222-740 mg/day [b]	22 subjects	(Férézou et al., 1978)
500-1,500 mg/day (total fecal neutral sterols) [CoP rate would be roughly 40-80%]	preponderance of data from 15 studies published from 1957-1965	(Miettinen et al., 1965)
155.2-345.3 mg/day	range in averages for 5 patients on a constant fat diet monitored for 3 weeks	(Mitchell and Diver, 1967)
80.9 ± 21.9 mg/day 150.4 ± 21.2 mg/day 152.9 ± 24.1 mg/day 182.8 ± 40.7 mg/day	- 18 Seventh-Day Adventist pure vegetarians - 50 SDA lacto-ovo vegetarians - 50 SDA non-vegetarians - 50 general population non-vegetarians	(Nair et al., 1984)
266.4 mg/day [b] 634.5 mg/day [b] 357.1 mg/day [b]	22 Indian vegetarians 22 white omnivores 18 white vegetarian premenopausal women	(Reddy et al., 1998)
3.65 mg/day/kg body weight 7.24-7.45 mg/day/kg body weight (for a 70 kg individual, these translate to 256 mg/day and 507-522 mg/day)	- existing mixed western diet - high-fat, high-beef diet 30 subjects; excretion rate returned to 3.84 mg/day/kg body weight post test diet	(Reddy et al., 1980)
794-995 mg/day (5 subjects) [c] 241-499 mg/day (2 subjects)	two groups possibly displaying high and low conversion; data represent total CoP (including conjugates)	(Rosenfeld, 1964)
294-668 mg/day (7 subjects) [c] 5-6 mg/day (2 subjects) 100 mg/day (1 subject)	10 subjects (7 healthy and 3 hospitalized)	(Sekimoto et al., 1983)

[a] These data exclude all reports that provided CoP excretion data solely on the basis of fecal concentrations with no indication of fecal mass excreted per day.
[b] Ranges and averages of excretion rates derived from data in cited reference.
[c] Some studies, with multiple ranges, often provide data for sub-populations with diseases or having high-, low-, and nil-conversion efficiencies.

Table 3. Major steps required for validating SCIM concept for estimating population size by monitoring for coprostanol or other biomarkers

Milestone	Rationale
Adopt/develop sewage sampling methodology	Must accommodate solids. Preferably flow-proportional and time-integrated to acquire 24-h samples
Adopt/develop and standardize analytical methodology	Method would ideally be suitable for automation; determine analytical figures of merit and whether parameters such as metabolic conjugation are important to accommodate
Identify suitable number of STPs in a variety of geographic locales for acquiring test samples	STPs must span a broad range of populations served, and the error associated with the established measures of the population sizes must be well known and understood
Acquire CoP flux data from each STP	Using the monitored CoP fluxes, calculate the estimated per capita contributions for each STP using known population sizes. Determine the variance among STPs
Acquire time-series data from select individual STPs	Evaluate variation in data with time (daily, weekly, monthly). A portion of data variance will originate from the measurement methodology and another portion from actual de facto population fluctuations
Establish "reference ranges" of per capita daily excretion	This will entail an iterative process of repeated sampling at a sufficient number and diversity of STPs serving established populations
Perform sensitivity and uncertainty analyses	Establish those variables contributing the most error and take measures to reduce them

Figure 1. Chemical structures of select biomarkers



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