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Review

Using biomarkers in sewage to monitor community-wide human health: Isoprostanes as conceptual prototype

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ABSTRACT

Timely assessment of the aggregate health of small-area human populations is essential for guiding the optimal investment of resources needed for preventing, avoiding, controlling, or mitigating exposure risks. Seeking those interventions yielding the greatest benefit with respect to allocation of resources is essential for making progress toward community sustainability, promoting social justice, and maintaining or improving health and well-being. More efficient approaches are needed for revealing cause-effect linkages between environmental stressors and human health and for measuring overall aggregate health of small-area populations. A new concept is presented – community health assessment via Sewage Chemical Information Mining (SCIM) – for quickly gauging overall, aggregate health status or trends for entire small-area populations. The approach – BioSCIM – would monitor raw sewage for specific biomarkers broadly associated with human disease, stress, or health. A wealth of untapped chemical information resides in raw sewage, a portion comprising human biomarkers of exposure and effects. BioSCIM holds potential for capitalizing on the presence of biomarkers in sewage for accomplishing any number of objectives. One of the many potential applications of BioSCIM could use various biomarkers of stress resulting from the collective excretion from all individuals in a local population. A prototype example is presented using a class of biomarkers that measures collective, systemic oxidative stress — the isoprostanes (prostaglandin-like free-radical catalyzed oxidation products from certain polyunsaturated fatty acids). Sampling and analysis of raw sewage hold great potential for quickly determining aggregate biomarker levels for entire communities. Presented are the basic principles of BioSCIM, together with its anticipated limitations, challenges, and potential applications in assessing community-wide health. Community health assessment via BioSCIM could allow rapid assessments and intercomparisons of health status among distinct populations, revealing hidden or emerging trends or disparities and aiding in evaluating correlations (or hypotheses) between stressor exposures and disease.

Contents

Abbreviations: AA, arachidonic acid; BioSCIM, sewage chemical-information mining targeted at biomarkers; BMI, body mass index; BOSS, Biomarkers of Oxidative Stress Study (of the National Institute of Environmental Health Sciences); COX, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FEUDS, Forensic Epidemiology Using Drugs in Sewage; HBM, human biomonitoring; HPLC, high-performance liquid chromatography; IsoP(s), isoprostane(s); MS, mass spectrometry; NSAIDs, non-steroidal anti-inflammatory drugs; PUFAs, polyunsaturated fatty acids; SCIM, sewage chemical-information mining; STPs, sewage treatment plants.

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1. Introduction

An efficient, timely, and holistic approach for revealing cause–effect linkages between environmental stressors and the health of human populations persists as an unmet need. Presented here is a new concept that targets biomarkers of endogenous human metabolism that serve as measures of actual exposure and effects from a wide spectrum of stressors extending beyond the traditional focus limited strictly to chemicals. These biomarkers are normally hidden in a uniquely rich source of chemical information that has been largely ignored — raw (untreated) sewage. The concept – termed *BioSCIM* (sewage chemical information mining targeted at biomarkers) – could make possible the real-time collection of exposure/effects data that reflects the overall, averaged health of entire small-area communities.

Measures of community-wide health are rare. The only means of assessment is currently limited to morbidity and mortality data and conventional demographic data; included in morbidity data are specific sources such as poisonings and epidemiological studies (e.g., human biomonitoring). Morbidity data is not necessarily representative of an entire population (because of the incompleteness in reporting). It is also not possible to correlate any of these data with a defined population (or with stressors) in real-time.

Resource- and time-intensive epidemiological studies have been the primary route by which specific cause–effect linkages can sometimes be revealed. Indirect approaches, such as the CDC's National Biomonitoring Program (CDC, 2010), establish body burdens for a narrow spectrum of pre-selected xenobiotics in various body tissues and fluids (two examples being the analysis of biopsies for PCBs or halogenated pesticides, or urine for phthalates). Biomonitoring for chemical stressors, however, only provides the means for inferring the potential for exposure. Biomonitoring is unable to address questions of linkage with human health effects. The body burden of a chemical may simply be coincidental and of no biological consequence. Although development of new approaches for assessing human exposure is an ongoing task, most rely on advanced technology and are limited by the laborious requirements and hurdles imposed by the need to test individuals (e.g., Borrell, 2011). They are simply not suitable for quickly assessing exposure across entire populations.

A more direct approach for discovering potential linkages between environmental exposure and biological effects is the measurement of endogenous biomarkers. In the context of human health, biomarkers include physiological or behavioral responses, or endogenous biochemicals produced in response to: normal biological processes (known as biomarkers of health), disease processes (as induced by exposure to a biological, physical, radiological, psychological, or chemical stressor), or pharmacological intervention (Biomarkers Definitions Working Group, 2001).

Conventionally, retrospective assessments (e.g., epidemiology; biomonitoring) or prospective assessments (e.g., predictions based on toxicology or in silico methods) have served as guides. But neither of these approaches provides the ability to gauge collective, population-wide health status or trends, nor can they provide measurement in real-time. Consequently, they are not useful for supporting the use of short-term experiments (testing "what-if" scenarios) or trial interventions that are essential for evaluating the outcomes from actions intended to improve or maintain population-wide health. Nor are they suitable for performing comparisons among small-area populations — an especially important need for revealing disparities regarding exposure or health between communities (e.g., environmental justice). In this sense, the BioSCIM concept presented here can be viewed as a convenient means to facilitate or capitalize on conducting so-called "natural experiments". These quasi-experiments are strictly observational, with non-randomized designs that cannot be influenced by the researcher and which occur "as is" in the course of ongoing events. Natural experiments are one of the only approaches for evaluating the health outcomes from community-wide interventions — whether targeted or not at influencing public health. In this sense, they could play important roles in public health research, especially for study of interventions intended to reduce health inequalities (Petticrew et al., 2005).

Adding further complexity is the need to determine the comparative impacts on health resulting from disparate classes or types of stressors among the vast spectrum of possibilities (both anthropogenic and naturally occurring) to which humans are exposed. Currently not possible are meaningful assessments of the relative importance of exposure to the continuum of chemical toxicants (singly or in combination; naturally occurring or anthropogenic), especially at the trace levels normally occurring in the ambient environment. Even more insurmountable obstacles are faced in assessing the relative importance among all stressors — prioritizing across chemical, biological, physical, radiological, and psychological (e.g., social or emotional) stressors. Further discussion of the background and rationale underlying the proposed BioSCIM concept is presented in the Supplementary materials.

The proposed BioSCIM concept could serve as a key indicator in the "Three Pillars" approach for sustainability, a rapidly evolving

paradigm that must account not just for environmental factors, but also social and economic factors in environmental decision making (NRC, 2011). Since specific aspects of human health are impacted by a confluence of interlinked environmental, social, and economic factors, BioSCIM indicators could serve as integrative measures for sustainability. The application of BioSCIM to monitoring collective, population-wide health holds potential for more effectively guiding the development of sustainable communities and in reducing inter-community disparities by facilitating the most effective interventions or corrective actions in greatly reduced time frames. This ability is important in the Environmental Protection Agency's vision, especially for measuring health disparities in vulnerable groups and for demonstrating outcomes from numerous decision, compliance, and enforcement activities (USEPA, 2010). A collective measure of stress would hold the potential for making it possible for the first time to facilitate pinpointing which individual stressors play the most important roles in community-wide disease — whether the stress is chemical or non-chemical.

2. Biomarkers in sewage — *BioSCIM*

Proposed here is a concept for performing "remote" community-wide biomonitoring — a means for monitoring the status or trends in the collective health of small-area populations; the nature of small-area populations and how their size can be measured has been presented (Daughton, 2012). The approach is highly innovative but also conceptually simple — measuring biomarkers collectively excreted by a community into sewage. If successful, it would provide for the first time the ability to derive a measure of population-wide health status in a manner that has potential to be straightforward, fast, inexpensive, widely applicable, amenable to automation, and scalable (applicable to a broad spectrum of population sizes — ranging from local neighborhoods to cities). Status or changes in community-wide health could be quantified and then be used to seek correlations with community-wide risk factors. Intra-community trends could be established, and comparisons made across communities. A new paradigm could emerge for justifying the prioritization of exposure risks and for determining the optimal use of resources for control or minimization of exposure risks.

The potential advantages of the proposed approach include: (i) acquisition of the data on exposure/effects-based biomarker levels representative of entire populations in near real-time, (ii) eliminating the need to obtain samples from individuals (a form of "remote" monitoring), (iii) sufficient time-series community-wide data could be obtained for establishing status and trends, thereby making intra- and inter-community comparisons possible, and (iv) the impacts of risk-reduction actions could be more readily evaluated.

3. Generalized approach to developing a BioSCIM application

Development of a BioSCIM application begins with determining the most promising biomarker to first target in raw sewage. The complete criteria are outlined in Table 1 and summarized in a flow chart (Fig. 1). The major attributes include whether the biomarker is: (i) produced exclusively by disease or stress (i.e., not introduced by unrelated, exogenous mechanisms), (ii) excreted in sufficient quantities (to allow detection in sewage), (iii) sufficiently stable in sewage (not degraded, such as by microbial activity), (iv) amenable to cost-effective analysis in sewage while meeting whatever analytical figures of merit are required, and (v) excreted at levels in stressed/diseased states sufficiently elevated to discern significant differences compared with the narrow baseline range of "normal" basal states (see Section 4: Reference intervals — "normal" versus stressed).

An appropriate monitoring method would include standardized protocols for all steps beginning with sampling the raw sewage and concluding with data reporting (see flow chart, Fig. 1). Because of the anticipated natural variance in biomarker basal (baseline) excretion (a function of both intra- and inter-individual natural variations in excretion), coupled with the many irregularities associated with sewage flow, it would be critical to minimize the analytical variance to ensure that the signals are not obscured from any excursions in biomarker levels resulting from perturbations due to critical exposure events. A final hurdle would be the need to ensure that inter-laboratory variance is minimized — to eventually enable meaningful intercomparisons between sewage treatment plants (STPs).

Importantly, the absolute accuracy of the analytical method would not be critical — as long as the same standardized method is used in all BioSCIM studies; analytical bias would only need to be repeatable. But precision would be essential. The absolute concentration of a biomarker in sewage has little meaning in terms of clinical or epidemiological significance – unless these levels could eventually be used to reconstruct average systemic levels within the individual – in a manner analogous to the use of FEUDS (Forensic Epidemiology Using Drugs in Sewage) for reconstructing community-wide usage of an illicit drug based upon the drug's concentration in sewage (Daughton, 2011). In contrast, the reproducibility (and repeatable bias) of the method is critical for distinguishing statistically significant changes in excreted biomarker levels.

The two major uses for BioSCIM both involve the inter-comparability of per capita-normalized biomarker levels. These two uses would entail intra- and inter-STP relative comparisons. Relative values would rely on time-series data having sufficient precision.

The value of relative levels is illustrated with an emerging approach being implemented in a testing program for drug abuse in athletes. The "Athlete Biological Passport" uses drug monitoring designed only to detect changes (e.g., outliers) relative to the individual athlete's established personal profile of banned substances (those that are also endogenous substances, such as certain anabolic steroids) (Sottas et al., 2010). A major advantage in acquiring data for determining relative changes is that the method of analysis need only be precise — yielding reproducible values. Absolute accuracy is not important.

Intercomparisons would be used for establishing current status and answering questions regarding trends (relative changes over time): has a local per capita biomarker level changed over time? What percentage increase or decrease in per capita level has a community experienced over time? Is a local level trending upward or downward? How do the per capita levels or rates of change compare between local communities?

A relative measure could reveal those communities with the highest or lowest collective biomarker levels, or largest relative rate of increase, and thereby guide subsequent studies to locate the risk factors. A change in biomarker level could be viewed as analogous to a vehicle's "check engine" fault light for a community at large — as an alert to a possible increase in existing stress or emergence of new stress.

Significant differences in inter-community comparisons could be used in conjunction with data-rich geographic information systems and demographic data to test for correlations with unlimited types of geospatial variables — some obvious examples being noise (e.g., communities near airports, freeway interchanges, inner cities, green spaces), population density, proximity to toxic chemicals (e.g., hazardous waste sites) or other community disamenities (e.g., landfills), pesticide exposure (agricultural areas), radiation (high-voltage lines), population age structure, respirable particulates (freeway traffic or regional air quality), and per capita income. In this sense, BioSCIM would serve as a relatively quick screening tool that could then be used to justify and guide more in-depth follow-up examination of community-wide health as correlated with the presence or absence of various exposure sources.

4. Reference intervals — "normal" versus stressed

Even if per capita basal levels of a biomarker for a particular STP exhibited a sufficiently narrow range of variability at any point in

Table 1
Ideal attributes for biomarkers to target with BioSCIM.[a]

Biomarker attribute	Example	Potential limitation
Sensitive to changes in amplitude or duration of stress, disease, or health	Excreted levels must be a function of the amplitude of adverse or beneficial health effect.	Some biomarkers can reflect both adverse and beneficial health effects.
Should be known what physiological system or biochemical pathway the biomarker acts upon or results from.	Range can span from very specific (a particular organ or process) to systemic (whole body).	Biomarkers sometimes result from no endogenous process but rather originate from exogenous sources (e.g., naturally present in diet).
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.
Confounding of biomarker levels by exogenous sources is minimal.	Low occurrence in raw or cooked foods (or nutritional supplements), which are often disposed directly into sewers.	Input possible from other animals where industrial/agricultural sewage is mixed with human sewage (can confound data).
Confounding of biomarker levels by other variables is minimal.	Levels can be perturbed by medications.	Medications can up- or down-regulate biomarker biosynthesis.
Minimal intra-individual variance in daily excretion.	Daily basal levels excreted by an individual vary minimally over time (minimal diurnal or seasonal fluctuations).	Excessive variation will obscure fluctuations due to changes in health.
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).
Daily per capita excretion in sewage is independent of extraneous variables not considered risk factors.	Minimal effect from season, weather, geographic locale, medications, water-use restrictions.	Genetic determinants can be a function of geography.
Occurrence levels in sewage 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.[b]
Levels in raw sewage are well above method detection limits (MDL).	Few analytical interferences; easier implementation of a routine method.	Isobaric biomarker isomers often become common interferences; this is particularly problematic, for example, with isoprostanes; many potential biomarkers with potent physiological action are not useful since they are excreted at extremely low levels.
Minimal potential for exogenous interference from other sources.	Exogenous sources include residues of target analyte on analyst's hands.	Residues of target analytes[b] can sometimes be excreted in sweat.[c]
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; this occurs especially when a biomarker is excreted via the feces.
Minimal degradation of biomarker in sampled sewage (levels persist in stored sewage samples).	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 time-consuming 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.
Amenable to 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;[d] 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] Adapted in part from Daughton (2012).
[b] The challenges associated with obtaining representative samples from an STP are discussed by Ort et al. (2010).
[c] Daughton and Ruhoy (2009).
[d] Examples of EPS (Zabiegała et al., 2010) include polar organic chemical integrative samplers (POCIS) and semipermeable membrane devices (SPMD).

time – making possible the detection of statistically significant trends – a major question is whether it would be possible to assign the community an absolute measure of “health” or “stress”. To answer the question of whether a local per capita biomarker level is “abnormal” is considerably more difficult.

Fundamental questions surround what is even meant by “normal” (or “healthy”) versus “diseased” — or “non-diseased” versus “non-healthy”. For the purposes of BioSCIM, it would require a comprehensive series of initial monitoring studies across carefully selected STPs — those serving populations with well-documented overall health. These studies would be needed to establish “normal” ranges (baseline physiological levels for a population deemed to be “healthy”) for the targeted biomarker. Data from individual sewage treatment plants (STPs) would serve instead of the data from individual human subjects. Entire communities would be viewed as individual patients (Daughton, 2012).

These studies would be analogous to the clinical studies required for establishing the widely used *reference intervals* or *reference values* for blood or urinary levels of various markers — a concept introduced in 1969 by Gräsbeck (2004). A unified approach for conceptualizing, defining, and developing reference values was begun in 1970 and summarized in a series of papers beginning in 1987 (Solberg, 1987). Despite the widely accepted and routine use of reference intervals in clinical chemistry for a wide spectrum of biochemical parameters,

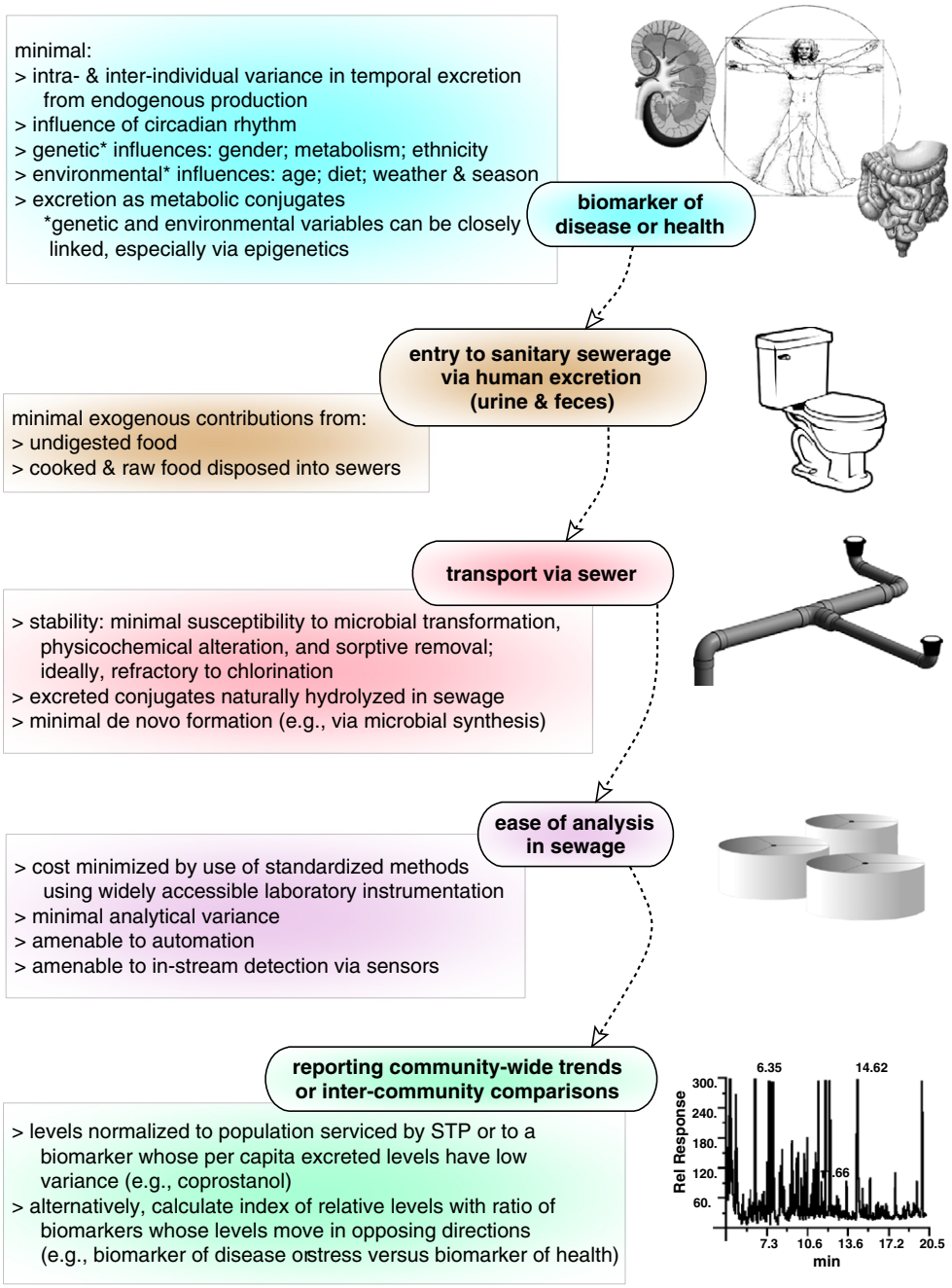

Fig. 1. Characteristics of an ideal biomarker for BioSCIM.

it is an extremely complex and controversial concept. It is one fraught with numerous challenges and far exceeds the scope of this paper. Comprehensive examination of the complexities, limitations, and pitfalls surrounding the development of reference intervals for laboratory medicine, which necessarily must accommodate natural biological variability, is available from a number of authors, including Apostoli et al. (1998), Gräsbeck (2004), Henny and Petersen (2004), Hyltoft-Petersen and Henny (2004).

The idea of examining an entire local community as if it were an individual patient poses many additional challenges than already encountered in establishing clinical reference values for inter-individual comparison. At the least, however, it should be possible for a local community to serve as its own reference (analogous to the clinical practice of "delta checking" based on intra-individual time-series reference values, or the Athlete Biological Passport mentioned above) if the contribution to intra-community variance (i.e., combined inter- and intra-individual variations for "healthy" individuals — due to both analytical variance and natural daily variation) — is sufficiently small. A reference range for BioSCIM would at least only need the power to differentiate between a positive or negative relative change in status.

Regardless of whether it is possible to establish reference values (and intervals) for BioSCIM analytes, it would be possible to establish ranges based on (for example) the upper quintile (or lower quintile depending on whether the desired relative level of the selected biomarker should be high or low) of community-wide values. Values from a given STP could then be expressed relative to the extreme range, which could be termed the "target range" or some such — a range that a community might strive to reach by implementing appropriate measures to improve health or avoid stress.

5. Sewage chemical information mining — *SCIM*

Sewage can be viewed as "chemical litter" emanating from human behavior, actions, and activities. It has long been regarded as economically burdensome waste — only recently viewed as a potential source of nutrients for agriculture or for reclaiming energy (e.g., Heidrich et al., 2010).

BioSCIM makes use of the wealth of chemical information present in sewage and relevant to health and disease. This rich but highly complex reservoir of potential biomarkers has never been mined. It resides in raw sewage in the form of unique signatures from countless chemicals excreted from the body as a result of myriad endogenous biochemical processes. In raw sewage, these substances represent the combined metabolic processes from all individuals composing a small-area population serviced by a discrete STP at any point in time. Applications of SCIM essentially serve as a form of community-wide, *en mass* urinalysis — where single samples (collected over sufficient time) are necessarily representative of the entire population. The potential application of SCIM is flexible in terms of selecting well-defined small-area populations. The physical layout of sewer conveyance lines enables the collection of samples from different spatial locations, representing different segments of a population — spanning discrete hierarchical subgroups ranging from portions of a city, community, neighborhood, or particular street.

One way to apply BioSCIM in quantitative assessments necessitates knowing the size of the real-time population contributing to the sewage. This allows calculation of average per capita contributions (or loadings) of a biomarker. There are many limitations and complexities surrounding the concept of a small-area population. Their ramifications with respect to SCIM are discussed in Daughton (2012), as an application termed ASAP-SCIM (analysis of small-area populations by sewage chemical-information mining), which presents a new concept for estimating small-area population size by use of biomarkers whose constant levels of excretion reflect per capita contributions. An alternative approach for BioSCIM would obviate the need to directly know the population size. This could be done by monitoring for an orthogonal biomarker (such as a biomarker of health rather than stress) whose level the target biomarker level could be normalized against. This would create a dimensionless or normalized index (discussed in Section **11**: Differential Stress Index: increasing the sensitivity of BioSCIM by also measuring biomarkers of health to derive a normalized index of stress or homeostasis).

The BioSCIM approach could also be viewed as a form of remotely sensed, collective HBM, where no active involvement or participation from any individual is needed. Although BioSCIM's economy of scale cannot generate data relevant to the individual, it generates time-series data and would facilitate quick inter-community comparisons. BioSCIM holds the potential for minimizing or eliminating the issues and concerns normally surrounding subject recruitment, retention and participation of recruits ("research fatigue"), informed consent, and confidentiality — all of which are problematic for HBM studies and often even for epidemiology (Bauer, 2008). The non-invasive nature of BioSCIM would negate the need for recruiting and interacting with individual subjects and thereby obviate any requirement for Institutional Review Board approvals. This could greatly accelerate the pace of studies.

The NRC (2009) has noted the need for simpler screening-level tools for assessing cumulative risk — especially at the community-wide scale. BioSCIM would serve as a first attempt at providing such a tool based on a biomarker of effect. As recommended by the NRC, the new concept might also facilitate the conduct of risk assessments by community stakeholders, thereby empowering more involvement in evaluation, design, and management of local remedial or control activities. Access to near real-time data could facilitate the testing of what-if scenarios — one example of many being encouraging change in community-wide behaviors and evaluating possible subsequent changes in BioSCIM biomarker levels. BioSCIM could create the opportunity to view communities from a completely new perspective — where an entire community serves as the patient (Daughton and Ruhoy, 2011).

In addition to developing the framework for the BioSCIM remote biomonitoring concept, initial proof of principle is currently being planned by development of an analytical methodology for measurement of an effects-based biomarker in sewage. This may mark the first time that an effects biomarker has been targeted in the monitoring of sewage. Theoretically, comprehensive arrays of biomarkers suitable for monitoring in sewage could eventually be developed. Chemical information can be derived from the full spectrum of chemicals excreted by humans (including those in urine, feces, and sweat). These markers could range from general to specific in terms of the types and levels of biological effects they indicate.

5.1. Evolution of the SCIM concept: from FEUDS and ASAP-SCIM to BioSCIM

The BioSCIM concept derives from prior work directed at the extraction of chemical information from sewage. The proposed BioSCIM concept represents a new application of a general approach called sewage chemical-information mining – SCIM (Daughton, 2012) – specifically targeted at select biomarkers. The real-time monitoring of sewage for biomarkers as measures of community-wide health and disease was first proposed by Daughton (2011) and shortly thereafter by Thomas and Reid (2011); it has also been mentioned by Fanelli (2011). The work reported here resulted from a U.S. Environmental Protection Agency (EPA) 2010 research proposal on SCIM funded by EPA's ORD Pathfinder Innovation Projects (PIP) internal grants program (Daughton, 2010).

The first application of SCIM for extracting information from community-wide contribution of chemicals to sewage was introduced in 2001 (Daughton, 2001) and was first applied to real-world use in 2005 (Zuccato et al., 2008). This original concept (sometimes loosely referred to as "sewer epidemiology" or "sewage epidemiology") involved analysis of domestic sewage for illicit drugs (or their metabolites) and using pharmacokinetics and sewage flow rates to back-calculate aggregate and per-capita drug usage for an entire community served by the STP. This application represented the first time that aggregate dose-reconstruction could be accomplished at such a large scale. This application of SCIM has since been widely explored internationally. It was later termed Forensic Epidemiology Using Drugs in Sewage (FEUDS); for a review see Daughton (2011).

A second application of SCIM was introduced in 2011 and termed ASAP-SCIM: Analysis of Small-area Populations by Sewage Chemical-Information Mining (Daughton, 2012). This approach was proposed as a means of providing near-real-time estimates of small-area (local) populations by measuring select biomarkers excreted into sewage. ASAP-SCIM may prove useful in the implementation of BioSCIM. BioSCIM represents the third methodology based on SCIM and strives to monitor for biomarkers in sewage as a gauge of community-wide health. It is worth noting that most of the complexities associated with the actual implementation of FEUDS and ASAP-SCIM also apply to BioSCIM. So understanding the first two applications helps with understanding the limitations and challenges faced by BioSCIM.

Important to appreciate is the complexity surrounding the estimation of small-area population size in interpreting population-wide measures of health. When the small-area population (or change in population) is not accurately known, then the significance of absolute changes in health measures (mortality being one of the simplest examples) cannot be evaluated (Daughton, 2012). Any change in incidence can result from changes in any or all of three factors (Thunhurst, 2009): (i) absolute population (constant rate of

incidence in a larger population results in higher occurrence rate), (ii) composition of constant population (changes in disaggregation, resulting in higher percentage of vulnerable individuals — an example being an inverted age structure), or (iii) response of the overall population (e.g., changes in stressor levels). A methodology for easily resolving these factors is currently not possible.

A noteworthy aspect of the proposed BioSCIM approach is that it could theoretically also be extended for use in ecological assessments, especially given the emerging field of "oxidative stress ecology" (Costantini et al., 2010). It might prove particularly applicable, for example, in determining the health status of local aquatic environments — by measuring select biomarkers excreted by aquatic organisms in their native environment; biomarkers of oxidative stress (already used in assessing individual organisms) constitute one example that may prove useful (Isaksson, 2010; Lushchak, 2011; McGraw et al., 2010; Metcalfe and Alonso-Alvarez, 2010).

6. Biomarkers

Perhaps some of the most important information contained in sewage is represented by the countless excreted substances serving as biomarkers — substances having potential as measures of collective community-wide disease, stress, or health. Biomarkers comprise three major categories in terms of what they can indicate (Hagger et al., 2006): exposure (e.g., as measured by dose), effects (e.g., diagnostic indicators, especially toxicity or other biological endpoint such as pathogenicity, or alteration of structure/function), and susceptibility or vulnerability (e.g., prognostic indicators, such as genetic polymorphisms); a summary of the requirements for biomarkers is presented in Breusing and Grune (2010). Biomarkers can therefore serve as diagnostic or prognostic measures of exposure, stress, vulnerability to disease, emerging disease, overt disease, or health. Biomarkers include endogenous biochemicals produced in response to stress or indicative of health. They also include adducts of xenobiotics or endogenous chemicals. And of course, they include metabolites of detoxication or intoxication processes resulting from xenobiotic exposure. Analysis of sewage for biomarkers, as a collective measure of population-wide health status or stress, has never been reported. Such an application would be aligned with the field of "molecular epidemiology" (Bonassi and Au, 2002), especially since a carefully selected biomarker can serve to integrate a diverse array of exposures — regardless of type, source, or route.

Biomarkers are often used as proxy or surrogate measures of the actual biological process of interest (for example, a toxicological or disease endpoint or outcome) but which is too difficult to directly measure itself. Chemical and biochemical biomarkers are generally measured in tissues (including hair and nails) or body fluids. The fidelity with which biomarkers mirror the endpoints of interest varies widely. Well-known examples include the measurement of mercury (or other metals) or drugs in hair or nails (as an indicator of whole-body exposure) and blood pressure and serum cholesterol (as indicators of vulnerability to heart disease). Some biomarkers are prognostic — indicators of the potential for developing disease, or even disease that has yet to manifest with outward signs. For health conditions with linkages to the environment, prognostic biomarkers and vulnerability biomarkers could be used to guide the design of remedial actions in the immediate environment to prevent or lessen the probable onset or worsening of pathologies. The utility of biomarkers is not just in diagnosis, but also in guiding the selection of subsequent assessment studies for measuring the effectiveness and progress of interventions or other remedial action.

Major limitations in the conventional measurement of any biomarker are the demands on clinical and laboratory resources and the time required for obtaining samples from statistically sufficient numbers of individuals in order to extrapolate conclusions to entire communities or populations; additional hurdles are in gaining human-subject testing approvals. Historically, biomarkers clearly have a number of major limitations in studies at the higher levels of populations or communities for establishing (at a minimum) exposure profiles.

The characteristics of useful biomarkers, partly adapted from Hagger et al. (2006), include those summarized in Table 1. The sewage matrix adds considerable complexities to nearly all of these.

Each of these factors and others would eventually need to be examined before a suitable biomarker (or suite of biomarkers) is selected for measuring community-wide stress using BioSCIM. These are summarized in Table 1. These factors place additional restrictions on the utility of a biomarker, further narrowing an already rather restricted field of possibilities. The numbers of bona-fide biomarkers — those with accepted clinical utility — are extremely limited to begin with. Despite the ever-expanding published literature on biomarkers (many resulting from the various fields of omics), the number of biomarkers that have been validated for use in routine clinical practice may be fewer than 100 (Poste, 2011).

A comprehensive examination of the fidelity with which biomarkers actually measure disease is presented by Ioannidis and Panagiotou (2011); also see Bossuyt (2011). This study revealed major shortcomings in the purported utility of a range of select biomarkers (for roughly two dozen risk factors) for use in diagnosis, assessing vulnerability, establishing prognosis, or guiding treatment. The markers examined were all proteins, genes, or chemicals such as estradiol (which have multiple origins). None of the markers, however, included a biomarker that was amenable to measurement in sewage or that reflected general modes of disease development, such as inflammation (with the possible exception of C-reactive protein: CRP). One controversy surrounding the demonstration of biomarker fidelity with disease is whether large, controlled clinical studies are required (Bacchetti et al., 2011).

7. Effects biomarkers suitable for BioSCIM

In the course of this work, over 900 publications were examined for their possible relevance to different aspects of BioSCIM and to extract supporting data. These articles resided primarily in the fields of clinical research, analytical biochemistry, lipid chemistry, exercise physiology, nutrition, toxicology, and free radical research. One of the primary objectives was to identify the best candidate biomarker with which to develop the initial BioSCIM application. Possible candidates were evaluated on the basis of the criteria in Table 1. The other objective was to use this candidate biomarker to highlight the types of information and data needed to assess biomarkers in general for use with BioSCIM.

7.1. The Isoprostanes (IsoPs) — biomarkers of systemic, system-wide oxidative stress

After evaluating a range of biomarkers as candidates for BioSCIM, the biomarker selected to demonstrate proof of principle belongs to the class of prostaglandin-like isomers — the isoprostanes. The isoprostanes (herein referred to generically as *IsoPs*) were first proposed for use as a measure of oxidative stress in 1991 (Morrow and Roberts, 1991) and have since been accepted in clinical chemistry as the biomarkers that provide the best quantitative measure of total systemic oxidative stress, which serves as a general pathway toward cellular dysfunction (Milne et al., 2008). The biosynthesis of IsoPs was first delineated in 1990, but this new series of prostaglandin-like isomers was not named isoprostanes until 1992 (Morrow et al., 1990a, 1990b, 1992).

The IsoPs gained recognition as the most reliable, sensitive, and specific non-invasive measure of in vivo, systemic oxidative stress after a comprehensive series of studies in the early 1990s using rats that were administered carbon tetrachloride. These studies

(Kadiiska et al., 2005a, 2005b) composed the first comprehensive comparative assessment of a spectrum of candidate biomarkers of oxidative stress — the Biomarkers of Oxidative Stress Study (BOSS) of the National Institute of Environmental Health Sciences (Milne and Morrow, 2006). Somewhat analogous studies have since been done with human subjects (Il'yasova et al., 2010).

While the term "oxidative stress" is not rigorously defined, it generally refers to an imbalance in the levels of reactive oxygen, nitrogen, and halogen species relative to the body's defense mechanisms – a perturbation in the prooxidant–antioxidant balance resulting in disruption of redox signaling and control – more recently termed "redox disruption" (Breusing and Grune, 2010; Burgos Alves et al., 2010; de Castro Fernandes et al., 2010; Jones, 2006, 2008); reactive species include both free radical and non-radical oxidants. Indeed, the prevalence of redox disruption versus free-radical oxidation may explain the lack of expected effectiveness of antioxidants reported in many clinical studies.

Oxidative stress originally attracted attention because of the key role it was theorized to play in aging; this theory was an extension of the original "free radical theory" of aging, proposed and advanced in the 1950s by Harmon (see: Kregel and Zhang, 2007). The history of oxidative stress in disease and in maintenance of homeostasis via cellular signal transduction is provided by Hensley and Floyd (2002).

Regardless of its definition, oxidative stress is widely viewed as a hallmark of various acute and chronic diseases, many of which are also part of the normal aging process (Montuschi et al., 2007). Oxidative stress has become recognized as a central factor is the prevalence of chronic inflammatory diseases, including obesity, hypertension, cardiovascular diseases, and type 2 diabetes, among others that define the metabolic syndrome (Jesmin et al., 2010); for all but three of the 15 leading causes of death in the US (Kochanek et al., 2011), inflammatory pathways (including those involving the production of IsoPs) play roles. Note, however, that oxidative stress in itself is not necessarily deleterious, but rather can sometimes be viewed as healthy or necessary — "eustress"; IsoPs may play roles in governing cellular adaptive response to stress via gene expression and cell signal transduction (Niki, 2008; Noguchi, 2008). A certain controlled, background level of oxidative stress probably plays critical roles in adaptive physiological responses and in critical biological defensive functions (Azzi, 2007). The state of what is known regarding the correlation of oxidative stress and disease has been reviewed by Giustarini et al. (2009).

Although IsoPs are isomers of prostaglandins, they are not directly related, as their mechanisms of in vivo synthesis and their biological functions differ dramatically. The prostaglandins are produced directly in free form by a complex cascade of enzymatically catalyzed reactions (via COX — cyclooxygenases) beginning with free arachidonic acid (see Fig. 2) — or any fatty acid with at least three double bonds, notably the essential fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In contrast, the IsoPs are predominantly formed from the non-enzymatic, free-radical peroxidation and cyclization of these same fatty acids only after they have been esterified to cellular membrane phospholipids (at the sn-2 position); note that "peroxidation" generally refers to reactions involving dioxygenation but some oxidative reactions of lipids involve monooxygenation. Oxidation is initiated on the bis-allylic methylene groups (saturated carbon adjacent to two-double-bonded carbons in the *cis* conformation) of the polyunsaturated fatty acid. This nonspecific autooxidation results in numerous isomers — and therefore a complex and often confusing nomenclature.

Structurally, the prostaglandins and IsoPs differ only by conformation (stereochemistry). Each of the three major classes of IsoPs (F-, D- and E-series) can theoretically comprise 64 distinct isomers; for example, attack of arachidonic acid yields four regioisomers (positional isomers), each of which can yield a further eight isomers, together with diastereomers — making possible 64 F_2-IsoPs. The two side chains for the prostaglandins are predominantly in the *trans* conformation relative to the cyclopentane ring, whereas they exist in the

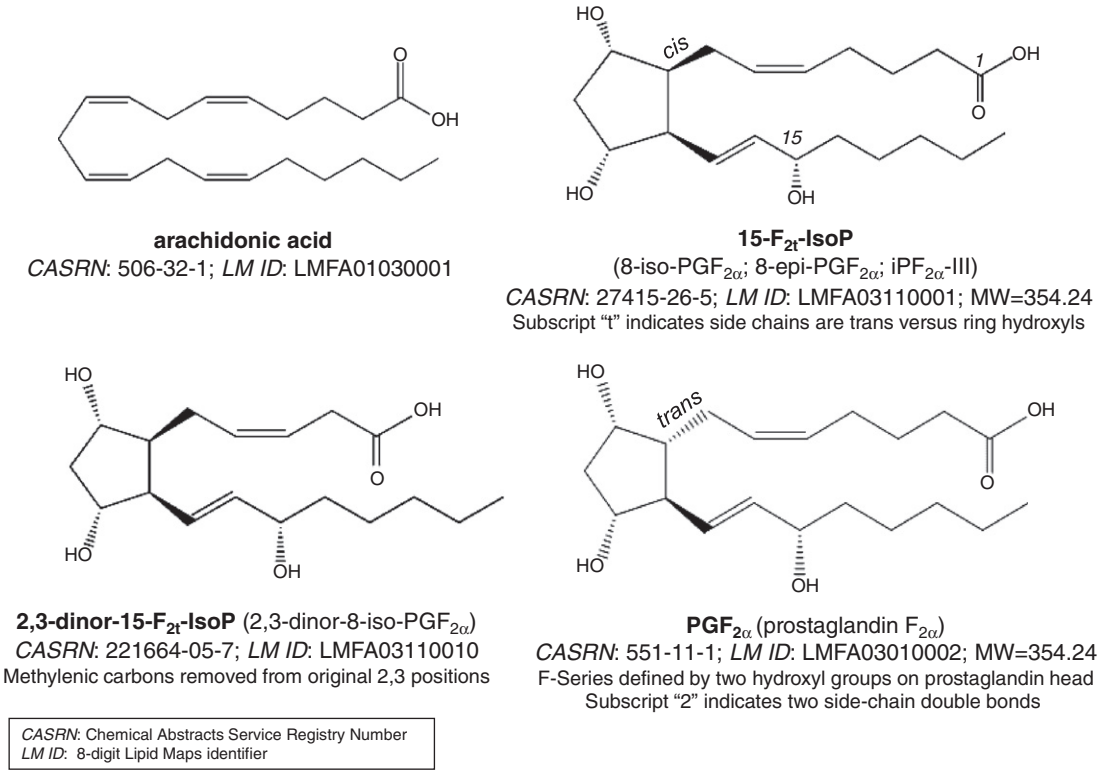

Fig. 2. Example of a common isoprostane (and a prostaglandin isomer), together with a dinor beta-oxidation product, and the parent arachidonic acid.

cis orientation for the IsoPs (see Fig. 2); although the more stable *trans* conformation is favored thermodynamically, the more kinetically favored *cis* results for IsoPs because of the bicycloendoperoxide oxidation intermediate.

IsoPs are released in free form from the esterified membrane lipids upon cleavage by phospholipases. In free form, they can then circulate or be excreted (primarily via urine, but also via bile). They can also undergo further metabolism, most notably yielding dinor products (via beta-oxidation); for example, 8-iso$PGF_{2\alpha}$ can be metabolized to 2,3-dinor-8-iso-$PGF_{2\alpha}$ (see Fig. 2), whose delta-5 double bond can then be reduced to 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (Roberts et al., 1996). The prefix "nor" (*normal*) refers to removal of one carbon and accompanying hydrogen atom (demethylation or removal of a methylene group); for IsoPs, *dinor* metabolites have two methylenic carbons removed from the carboxy-terminus side chain.

The dinor metabolites can be used as markers themselves, as they serve as surrogates for the parent IsoPs. They are sometimes excreted at levels exceeding those of parent IsoPs by several fold (Burke et al., 2000; Nikolaidis et al., 2011). For the purposes of the BioSCIM concept, the dinor metabolites might be suitable for measuring and summing together with IsoPs to achieve a more comprehensive and larger signal of oxidative stress. Some methods capture all dinor-dihydro metabolites in one chromatographic peak (Davies et al., 2006). Urinary excretion of the dinor metabolites varies depending on the overall beta-oxidation activity. In one study, for example, the ratio of urinary dinor-8-epi-$PGF_{2\alpha}$ to 8-epi-$PGF_{2\alpha}$ was 15 (33.72 ± 5.80 nmol/L versus 2.11 ± 0.41 nmol/L), with 2,3-dinor-5,6-dihydro-8-epi-$PGF_{2\alpha}$ at 16.43 ± 5.62 nmol/L. In contrast, in another study involving 845 women serving as controls in the Shanghai Women's Health Study (SWHS), the levels of the dinor metabolites were roughly 30–50% those of the parent F_2-IsoP (Dorjgochoo et al., 2011); but in a related SWHS study, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP was found to be a more sensitive and specific measure of oxidative stress than the parent 15-F_{2t}-IsoP (Dai et al., 2009; Dai and Zhu, 2009). As the level of oxidative stress increases, the relative levels of dinor metabolites can decline if beta-oxidation becomes impaired. This variability supports a rationale for measurement of the parent IsoP together with its dinor metabolites (Nourooz-Zadeh et al., 2006).

IsoPs are viewed as providing an integrated measure of systemic oxidative stress because they are produced in every tissue of the body — as a direct consequence of numerous processes that generate oxidative radicals; they are even excreted via the breath (Janicka et al., 2010). Large quantities are excreted via urine, where the concentration can be 30–40 times higher than in plasma. The different series and isomers of IsoPs can all be formed in different and varying proportions — and metabolized at different rates. Even though their formation is predominately by free-radical oxidation, this process is influenced by the type and anatomical location of the specific oxidative stress. The spatial distribution of the various isomers in the body is thereby also affected.

Although each of the IsoP classes directly reflects the free-radical oxidation of a single species of polyunsaturated fatty acid (e.g., F_2-IsoPs derive from arachidonic acid), it is widely assumed that polyunsaturated fatty acids (PUFAs) are oxidized indiscriminately, and that formation of a given class of IsoPs from the oxidation of one species of PUFAs serves as a surrogate for other PUFAs.

Since a significant portion of IsoPs are incorporated into structural lipids (e.g., membrane lipid rafts and caveolae), a reservoir exists from which free IsoPs can be continually liberated; it is believed that once IsoPs incorporated in cellular membranes are peroxidized that the membranes become more rigid, compromising permeability and altering signal transduction (Dietrich-Muszalska and Olas, 2009). This moderates the free concentrations, serving to prevent fast fluctuations in urinary levels. This partly contributes to the utility of urinary IsoP levels as a measurement index. Other characteristics of IsoPs contributing to their usefulness as biomarkers includes their chemical stability, comparatively low variation in intra- and inter-individual variations in excretion rates, and relative resistance of excretion rates to changes in diet and time of day (Cracowski et al., 2002; Il'yasova et al., 2004).

IsoPs can serve dual roles — not just as passive biomarkers of exposure but also as active mediators or promoters of effects (Cracowski and Durand, 2006; Crankshaw and Rangachari, 2003; Morrow, 2006). IsoPs may play extremely complex roles as both inhibitors and activators in regulating a wide spectrum of biochemical pathways that feed into numerous physiological outcomes (Niki, 2008, 2009); they can also serve as incidental ligands, such as for eicosanoid receptors (e.g., the thromboxane receptor). While this aspect is far too complex to summarize here, some of the complexity and unknowns surrounding the biochemical functions of IsoPs is illustrated by their complex roles in the functioning of blood platelets (Ting and Khasawneh, 2010).

IsoPs are believed to play major roles in a broad spectrum of acute and chronic inflammatory diseases, all of which may underlie in particular the general process of aging; see Section 8: Factors and variables affecting regulation of IsoP production: disease, lifestyle, and genetics. An overview of the inflammatory response in relation to the eicosanoid cascade is provided by Helmersson (2005). IsoP levels serve as an integrated response from the cumulative exposure to many forms of stress, spanning the range from chemicals to non-chemicals (e.g., psycho-social stress). Indeed, an exposure measure that encompasses psycho-social stress (important especially in assessing environmental justice) persists as another unmet need in cumulative risk assessment (Lewis et al., 2011; NRC, 2011). Moreover, as noted by Lewis et al. (2011), "…it is difficult to imagine how the effectiveness of interventions could be assessed effectively without a metric for estimating targeted, cumulative risk reduction". IsoPs play dual roles, as biomarkers of oxidative stress and as mediators of inflammation. They are both effects-based and stressor-based (see Supplementary materials). They also reflect cumulative exposure. The problems, limitations, and caveats regarding selection of biomarkers of oxidative stress are discussed by Halliwell (2009).

A major postulated strength BioSCIM in general (especially when targeting IsoPs) is that it would serve to integrate the effects from all forms of bona-fide exposure. It would not rely on the conventional approach of compiling recollected (and often subjective or inaccurate) exposures from test subjects via self-reporting and historical records; but the interpretation of BioSCIM results could be aided by such exposure data. A major potential confounder of the approach is whether a few individuals among a population may have extraordinarily high changes in excretion levels of a biomarker sufficient to disproportionately influence the collective data from the entire population.

A major objective in the conceptualization of BioSCIM set forth here is to catalyze discussion, debate, and research regarding its feasibility. IsoPs were selected as candidate biomarkers most likely to yield results because of their established use in clinical research and a sizeable body of published data — a summary of which is presented in this document. In reality, any number of other biomarkers may also prove useful. Ultimately, a spectrum of biomarkers could form the basis of a comprehensive BioSCIM program. These markers could comprise those that integrate exposure/effects across a broad spectrum of biological processes as well as those that reflect unrelated states of more specific stress or disease. IsoPs are presented here in part as an illustration of the evaluation process needed for implementation. The only limitation to incorporating more biomarkers (especially those of more specificity) is whether they meet the criteria in Table 1. IsoPs reflect a widely integrative measure of cumulative and aggregate exposure/effects relative to the whole body.

7.2. Isoprostanes: background

The chemistry and nomenclature of IsoPs are extremely complex. IsoPs also are but a small part of the vast eicosanoid network (those fatty acids containing 20 carbons — and closely related fatty acids). There are three different chemical naming conventions for the 64 different isomers that originate just from arachidonic acid: the systems of Taber et al., Rokach et al., and Mueller (Nikolaidis et al., 2011). This has added considerable confusion to the published literature and perhaps has also sometimes led to misrepresentation (Mueller, 2010; Murphy and Fahy, 2010). As one example, 15-F_{2t}-IsoP reflects the Taber naming convention (Taber et al., 1997); see Fig. 2. The first number refers to the location of the acyl side-chain hydroxyl group (i.e., 5, 8, 12, or 15). The subscript number after "F" refers to the number of unsaturated bonds in the side chains; other descriptors denote the stereochemistry of the ring hydroxyl group(s). The 5- and 15-series tend to be the most abundant IsoPs because the precursors to the 8- and 12-series are more prone to further oxidation.

15-F_{2t}-IsoP is one of the key isoprostanes studied, largely because it was the first available commercially. It is the same as 8-iso$PGF_{2\alpha}$ and 8-epi-$PGF_{2\alpha}$ and is also called isoprostaglandin $F_{2\alpha}$ type III or i$PF_{2\alpha}$-III (Rokach et al., 1997, 2004); see Fig. 2. The different "types" are a function of the PUFA from which they are derived: e.g., arachidonic, eicosapentaenoic, or docosahexaenoic acid. Taber's nomenclature follows conventional chemistry rules, conforms to existing prostaglandin naming conventions, and has been accepted by the IUPAC. Some of the naming conventions do not accommodate commonalities among specific IsoPs with respect to biochemical origin or especially with respect to biological activities (Mueller, 2010). Compared with the prostaglandins, where the unsaturated side chains are all *trans* to the cyclopentane ring, the conformation for the IsoPs is exclusively *cis* (see Fig. 2). Succinct and authoritative discussions of nomenclature are provided by Nikolaidis et al. (2011) and by PiŁacik et al. (2002), among others. Note, however, that throughout the following discussion and Supplementary materials, little attempt was made to standardize the variety of naming conventions used in the articles under discussion.

An extensive listing of over 100 IsoPs (common names, systematic names, synonyms, structures, formulas, molecular mass) can be found at LIPID MAPS (2008, 2011). Other types of IsoPs (and IsoP-like compounds) are produced in vivo (e.g., cyclopentenone-based, neuroprostanes, isofurans), but the focus of the work reported here is on the more stable forms of IsoPs that are extensively excreted via urine — primarily the F_2-series.

Isoprostanes were originally called "iso-prostaglandins" but their origin within the body is totally distinct — as cyclooxygenase is generally not involved in their formation. Their production is therefore not affected by the medications that can regulate prostaglandin production (i.e., non-steroidal anti-inflammatory drugs [NSAIDs] or glucocorticoids). The in vivo formation of IsoPs in humans was first reported by Morrow et al. (1990a, 1990b, 1992). Significantly, while IsoPs act as biomarkers of oxidative stress, they can simultaneously also directly act as pro-inflammatory agents themselves. For example, they are potent broncho- and vaso-constrictors. In this sense, as both products and mediators of disease, they are biomarkers of both exposure and effects (sequelae of oxidative stress — not always harmful and sometimes essential). They may well cause some of the very pathologies that they are used to measure.

While COX-inhibitors (e.g., NSAIDs) repress the formation of prostaglandins, they have no direct effect on the formation of IsoPs, which are produced by a non-enzymatic COX-independent route. IsoPs are formed as racemic diastereomers. An exception regarding prostaglandin formation is urinary $PGF_{2\alpha}$ (as well as ent-$PGF_{2\alpha}$), trans-side-chain products that seem to be formed primarily via the same non-enzymatic pathway used for IsoP rather than from COX. This prostaglandin and its enantiomer are therefore also measures of free-radical induced oxidative stress — as opposed to markers of COX activity (Yin et al., 2007).

The discovery of IsoPs was first recounted by Morrow et al. (1990a, 1990b). Comprehensive reviews that cover all aspects of IsoPs (nomenclature, biosynthesis, chemistry, metabolism, analysis, roles as markers and mediators in oxidative stress and disease, and cell-signaling) began to appear in the 1990s. Among the many excellent reviews are the following, with perhaps one of the more comprehensive being that of Jahn et al. (Basu, 2008; Basu and Helmersson, 2005; Cracowski and Durand, 2006; Dalle-Donne et al., 2006; Fruhwirth et al., 2007; Jahn et al., 2008; Kom, 2007; Milne and Morrow, 2006; Milne et al., 2005, 2008, 2011; Montuschi et al., 2007; Morrow, 2006; Nälsén, 2006; Nikolaidis et al., 2011; Nourooz-Zadeh, 2008; PiŁacik et al., 2002; Praticò, 1999; Praticò et al., 2004; Roberts and Fessel, 2004; Roberts and Milne, 2009; Rokach et al., 2004; Yen, 2010; Yin, 2008). Excellent graphics showing biosynthetic pathways are reprinted in many reviews (e.g., Jahn et al., 2008; Morrow et al., 1990a).

Worth noting is that 15-F_{2t}-IsoP (8-iso$PGF_{2\alpha}$) is the most frequently studied IsoP, not necessarily because of any inherent importance, but because of its early and widespread commercial availability as an analytical reference standard. Indeed, other IsoPs and their dinor metabolites may eventually prove more important as biomarkers. Furthermore, prevalence at local sites or release in specific tissues may not necessarily reflect abundance in urine. Urine provides a measure of lipid peroxidation integrated over a prolonged interval. In this review, emphasis was placed on examining the published literature on urinary excretion rather than on levels in plasma or other tissues. Data on plasma levels may not have relevance with respect to the types and levels of IsoPs that might occur in sewage. Data on fecal excretion (including bile) would also be relevant but is almost non-existent [see Section 10.4: Biliary (and fecal) excretion].

A very brief overview of the larger context of lipidomics in which IsoPs exist is useful in appreciating that they also serve in many respects as surrogate measures for many other oxidized phospholipids (oxylipid) biomarkers (for more discussion, see the Supplementary materials). The roles of IsoPs with respect to oxylipids are covered in the comprehensive review of Bochkov et al. (2010). The diverse roles in disease played by the many products of lipid peroxidation have been comprehensively covered in a number of reviews (e.g., Negre-Salvayre et al., 2010; Niki, 2009; Palmieri and Sblendorio, 2007; Spickett et al., 2010).

Many of the complex aspects of IsoPs are not a focus of this paper, especially since they are amply covered in the reviews cited above. For the purposes of this paper, the most important aspect is the urinary excretion of IsoPs — as it relates to the proposed monitoring of IsoPs in sewage as a fast and non-invasive means to measure the collective contributions of a biomarker of stress from small-area populations.

7.3. Isoprostanes as biomarkers of both exposure and effects

IsoPs serve as retrospective markers of whole-body oxidant injury (e.g., pathophysiological markers of lipid peroxidation) as well as prognostic markers of potential injury (e.g., as mediators of oxidative stress such as involved with cardiovascular diseases). IsoPs serve as both time-integrative indicators and mediators of oxidative stress. They therefore not only have diagnostic value for disease states but also prognostic value for predicting pathologic conditions. IsoPs not only reflect oxidative stress, they are also involved with causing stress (most directly and fundamentally simply by altering the physical structure, integrity, and fluidity of cell membranes by changing steric properties). At the same time, it is important to keep in mind that IsoPs are also involved in a range of normal processes involved with

maintaining health and regulating homeostasis (e.g., activation of p53 and other tumor suppressors required in apoptosis). As such, they can be elevated as a result of non-pathological eustress and seemingly healthy activities (such as pregnancy and exercise), and most basically, by basal metabolism; they can also display reductions in levels during diseased states — as a result of inhibition of protective basal-level production.

IsoPs integrate information from a very wide spectrum of biological events across the entire body. They can be viewed and utilized as biomarkers of: exposure (actual internal dose of a xenobiotic), effect (response, disease, altered homeostasis, structure, or function), and susceptibility or vulnerability (reflecting stress or status or change in homeostasis or physiological dysfunction — e.g., polymorphisms). They can facilitate or assist in diagnosis (in medicine, epidemiology, or forensics), prognosis, and therapeutic intervention (Crimmins and Vasunilashorn, 2011). An advantage of biomarkers of stress such as IsoPs is that a portion of the processes they serve to measure can emanate from disease that has yet to manifest itself as a clinical phenotype and could not otherwise yet be detected by clinical assessment.

A partial listing of pathologies in which IsoPs are elevated is summarized in Basu (2007, Table 1); these include cardiovascular disease, hypertension, diabetes, hepatic cirrhosis, obstructive pulmonary disease, obesity, neurodegenerative diseases, and asthma; inflammation, vasoconstriction, and platelet aggregation are common modes of both acute and chronic action. At least a portion of the biological activity of IsoPs is believed to occur as a result of IsoP acting as incidental ligands for certain inflammatory prostaglandin receptors (Song et al., 2009). IsoPs therefore serve as an integrated measure of a wide spectrum of combined disease states as well as non-disease stress, such as poor nutrition, exposure to smoke (Ahmadzadehfar et al., 2006), or other physicochemical stressors such as drugs that cause idiosyncratic reactions (e.g., Lu, 2006). Their production can be reduced by other stressors such as moderate exercise, or increased, such as by over-training (Margonis et al., 2007). Therefore, not all IsoP production results from disease, and their production can be reduced by certain activities; so data have the potential to become confounded. There has also been insufficient research on IsoP metabolism and excretion as influenced by genetics, gender, age, lifestyle, or other non-disease variables. These factors could all add a layer of complexity and unknowns to the interpretation of IsoP levels in sewage.

By targeting a biomarker of general oxidative stress, the following variables are automatically fully accommodated. In risk assessments, each of these variables must normally be modeled, and in the process must account for numerous unknowns: (i) exposure to ALL stressors (chemical and non-chemical), (ii) actual dose from each exposure event, (iii) additive and interactive effects from all stressors (additivity, synergism, antagonism), (iv) metabolic accommodation (induction or upregulation of detoxication pathways), (v) metabolic activation (induction or upregulation of intoxication pathways), (vi) intra-individual and inter-individual variability (genetic, such as single-nucleotide polymorphisms [SNPs], and epigenetic), (vii) prior exposure history, (viii) windows of vulnerability, and (ix) diurnal fluctuations in metabolism and gene expression — among others.

8. Factors and variables affecting regulation of IsoP production: disease, lifestyle, and genetics

An understanding of a biomarker's synthesis pathways and mechanism of control is needed to appreciate the limitations in interpreting BioSCIM data. Halliwell and Lee (2010) discuss some of the many factors that regulate IsoP production. Various junctures along the pathways of IsoP formation can be affected by different endogenous biochemical processes or exogenous stressors. For example, if esterified IsoP is being continually created by oxidative assault but the phospholipases have been inhibited, then IsoP levels in plasma (and therefore presumably urine) could decline (with a concomitant accumulation of lipid-bound IsoPs in cell membranes). Likewise, if beta-oxidation processes were to be inhibited (thereby inhibiting the formation of dinor metabolites), free IsoP levels could rise. The major question is whether the rate of IsoP production accurately reflects the rate of lipid peroxidation. In reality, the free IsoP production rate may vary wildly among individuals as a function of a broad spectrum of variables — not the least of which is what type of stress each is sustaining and whether the stress is acute or chronic.

Studies have examined possible associations of IsoPs with a wide spectrum of diseases — as both passive and active markers. A number of articles provide summaries (e.g., Schwedhelm et al., 2007). Many studies, especially large epidemiological studies, have targeted multiple biomarkers and multiple risk factors. This makes it difficult to provide straightforward summary of the data on the association of IsoP levels with particular diseases. An example of a larger-scale study comes from the Framingham Heart Study, where urinary 8-epi-$PGF_{2\alpha}$ levels (normalized to creatinine) for 2828 subjects were found to most strongly correlate with smoking, diabetes, and body mass index (Keaney et al., 2003). Corroborating many other studies, a definitive association with gender was shown, where urinary 8-epi-$PGF_{2\alpha}$ levels for women were higher than in men — by 16%. The strongest association, as also seen in many other studies, was for smoking, where smokers had 65% higher mean levels than nonsmokers. One factor that has shown variable correlations was age, which in this case appeared to be negatively associated. Another example comes from a 299-subject cohort of the Insulin Resistance Atherosclerosis Study (IRAS), where four urinary F_2-IsoPs were correlated with gender, ethnicity, smoking, physical activity, BMI, waist circumference, weight change, and diabetes (Il'yasova et al., 2011).

Understanding the action of IsoPs as facilitators of disease may be hampered by the possibility that their actions are mediated in concert (or in series) with numerous other IsoPs at a variety of receptors and at requisite absolute and relative concentrations at disparate cellular locations. IsoP excretion levels sometimes seem counterintuitive or paradoxical — increasing as a result of activities generally associated with healthy lifestyles or activities, and other times decreasing as a result of seemingly unhealthy states. One example shows an association between urinary F_2-IsoP and BMI following a U-shaped curve, with the highest urinary IsoP levels resulting at both the lowest and highest BMI values (Narukawa et al., 2011); with the IRAS cohort, urinary F_2-IsoPs were inversely associated with weight gain (Il'yasova et al., 2011). Also worth noting is that randomized, double-blind, placebo-controlled intervention clinical trials that follow IsoP levels are very rare; most studies are observational. In general, even when statistically significant changes in IsoP levels are seen in clinical studies, they are rarely dramatic.

Some representative studies targeted at correlating specific diseases or stress with IsoP levels are summarized in Table 2 and discussed in more depth in the Supplementary materials. The emphasis has been placed on studies that monitored urine levels rather than plasma levels. Also summarized are studies of those variables that may serve to confound the interpretation of BioSCIM data. Many factors can interact, sometimes serving to thwart generalizations; contradictory studies are not infrequent. Correlations with given factors can also vary among the various parent IsoPs and their metabolites. IsoP levels not only can correlate with disease activity or severity, but also act as mediators of disease progression.

9. IsoP analysis and complications in comparing published data

Of all the factors involved with developing a BioSCIM approach based on IsoPs, the one that would contribute some of the greatest

Table 2
Disease or health factors that correlate with IsoP levels.[a]

Disease or health factor	Relative urinary IsoP levels (versus controls)
Metabolic syndrome (multiple risk factor syndrome)	
• Obesity (and higher BMI and waste circumference)	Several fold to an order of magnitude higher; can be a function or baseline status; levels can reverse with dieting.
• Diabetes	Roughly 3-fold higher; even higher levels with glycemic excursions.
• Cardiovascular disease (CVD)	Elevated for most forms of CVD; also correlate with other markers of CVD and with severity
Smoking	Several fold higher and strongly correlated; gender differences occur; also higher with passive smoking; sensitive to starting and stopping.
Pulmonary disease and asthma	Levels are often higher, but strongest correlations are in exhaled breath condensate (EBC).
Psychological stress and depression	Levels seem to vary inversely with positive mood scores.
Cognitive decline and Alzheimer's disease	Evidence points to possible correlation.
Alcohol consumption	One of the strongest correlations; levels can increase many-fold; most data, however, derive from plasma.
Cancer	Increased levels with some cancers (e.g., liver, lung, prostate), but not all (e.g., breast)
Associations with other diseases or conditions	Sometimes dramatic correlations with: rheumatoid arthritis, Lyme disease, chronic fatigue syndrome, autism, pregnancy, Dengue fever, ischemic stroke.
Exercise	Complex correlations (both positive and negative) depending on level and duration of exertion.
Drug usage correlations	Levels can be elevated or depressed during treatment with certain drugs (e.g., valproic acid) or consumption of illicit drugs; many studies show mixed correlations.
Associations with various non-stressor variables	
• Gender	Significant gender differences are often seen but can reverse among studies.
• Age	Levels of parent IsoPs often increase with age (beginning at age 5).
• Genetics/heritability	Environmental factors seem to have a larger influence than genetics.

[a] See Supplementary materials for discussion and supporting references.

uncertainty is the methodology used for monitoring and chemical analysis. Several factors contribute to this uncertainty, primarily by adversely affecting variance in both accuracy and precision, as well as in impacting decisions as to exactly what chemical species of IsoP should be targeted for monitoring. As emphasized earlier, minimizing analytical variance will prove to be the key in being able to distinguish stress signals from "normal" or "baseline" levels.

Significantly, of the numerous methods reported in the literature and developed for a wide spectrum of tissues and fluids from humans, animals, and plants, none has ever been applied to sewage, which will undoubtedly pose yet additional challenges for sampling and analysis; some of these challenges have already been discussed (Daughton, 2012). *Important to recognize is that no biomarker of stress, disease, or health has ever been targeted in any chemical characterization study of sewage.*

IsoPs are quantified primarily with the use of either mass spectrometry (coupled with liquid or gas chromatography — LC/MS or GC/MS) or competitive immunoassay (RIA or ELISA); representative LC/ESI-MS/MS chromatograms for some isoprostanes (among others for various prostanoids and dihydroprostaglandins) can be seen in Masoodi and Nicolaou (2006). While the analytical results from these various methods correlate well in some studies (e.g., Carraro et al., 2010; Devaraj et al., 2001), they do not in others (e.g., Callewaert, 2004; Liang et al., 2003). Since ELISA has clearly been the method of choice in terms of cost and speed (a critical attribute of any method intended for widespread, routine use), it would eventually need to be corroborated by an orthogonal approach, probably involving MS, especially given the bias in immunoassay that can be introduced by cross-reactivity.

The IsoP most targeted in the literature for analysis in urine is 15-F_{2t}-IsoP. The attributes of the F_2-class of IsoPs that make them ideal as biomarkers are summarized by Montuschi et al. (2004), and include: (i) chemical stability, (ii) generation specifically in vivo by peroxidation and little artefactual formation in urine, (iii) low limits of detection can be achieved in various tissues and fluids, (iv) levels increase substantially during oxidative stress, and (v) levels are little affected by lipid content of diet. They also undergo rather fast elimination from the body (Cracowski et al., 2002), and artefactual formation in urine is minimal because of the absence of arachidonic acid (Kom, 2007). The F_2-IsoPs have been shown to be stable in urine for over a week at room temperature (Praticò et al., 1998) or for up to 3–6 months when stored at −20 to −80 °C (Ohashi and Yoshikawa, 2000). While these studies indicate that sample transport and storage might not pose problems, the variables added by the presence of fecal materials leaves open the question of IsoP stability is stored sewage (especially with the potential for microbial degradation). This topic has never been evaluated and marks one of the priorities for establishing IsoPs as a target biomarker suitable for BioSCIM.

Acquisition of accurate and reproducible analytical data is complicated by the fact that 15-F_{2t}-IsoP (as well as probably all other IsoPs) undergoes extensive but variable inter-individual glucuronidation during metabolism. To eliminate this source of variability, samples might first need to be treated with glucuronidase prior to analysis in order to release the free parent 15-F_{2t}-IsoP. Enzymatic cleavage of the glucuronide has been shown to yield up to 80% more free 15-F_{2t}-IsoP in urine (Oxford Biomedical Research, 2008); the role of IsoP conjugates has been addressed in more detail in Section 10: Isoprostane excretion data. Another challenge, especially in comparing data across published studies, is the unknown degree to which various IsoP isomers (and isobaric analogs such as the prostaglandins) are resolved during mass spectrometric analysis. Some data are undoubtedly biased high because of the overlap of multiple isomers in a chromatographic peak comprising what might be mistakenly thought to represent a single isomer.

Yet another factor that complicates the intercomparison of published data on urinary levels of IsoPs is the numerous dimensional measurements used to express concentrations. These include both mass and molar dimensions, expressed in terms of either absolute levels (such as mass or moles per unit volume) or relative, dimensionless levels (generally normalized to creatinine); the use of creatinine in clinical chemistry poses a variety of its own problems and would introduce yet more variability for BioSCIM data (Daughton, 2012). Unlike clinical use, where a biomarker's urinary concentration is often normalized to creatinine (to compensate for urine dilution — a problem caused solely by reliance on spot urine samples instead of 24-h samples), the value relevant to modeling anticipated levels of a biomarker in sewage is the *total* per capita excreted *quantity*, expressed on the basis of absolute mass or moles. Values normalized to creatinine are problematic to accurately translate into absolute rates of daily excretion. This problem can be most readily seen with the gender discrepancies often noted for urinary IsoP levels, where levels normalized to creatinine are usually higher in women. This may actually represent an inherent bias introduced by the fact that women usually possess less lean muscle and therefore excrete less creatinine (Daughton, 2012). Unfortunately, most studies express urinary IsoP excretion normalized to creatinine rather than on a volume-concentration basis; this confounds the interpretation of IsoP excretion data (as well as data for endogenous biomarkers in general) for the purposes of BioSCIM.

A major problem confronting IsoP analysis is the lack of a validated, standardized method, although a method standardized for urine could possibly be modified and adopted for raw sewage. Method standardization is an unmet need with most biomarkers. An example of a major aspect of standardization (inter-laboratory, inter-method comparison) is shown by the work of the European Standards Committee on Urinary (DNA) Lesion Analysis (ESCULA) for the urinary biomarker of oxidative stress derived from DNA — 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) (Evans et al., 2010).

Currently, two major approaches for IsoP analysis involve immunoassay and hyphenated versions of mass spectrometry. A number of commercial immunoassays are available (such as those from Cayman Chemical, Detroit R&D, Enzo Life Sciences, Kronos Science Laboratory, Northwest Life Science Specialties, Oxford Biomedical Research, and OxisResearch), few of which have been intercompared or compared versus mass spectrometric methods. Numerous methods using GC/MS or LC/MS have been published, but all have been separately developed for disparate research programs in a variety of fields. Nearly all methods rely on some form of prior separation or cleanup, with a wide spectrum of specificities — ranging from solid phase extraction or thin-layer chromatography to immunoaffinity chromatography (IAC). IAC for IsoP analysis was first explored in the 1990s (Bachi et al., 1996). IAC has shown potential in sample preparation for providing highly standardized, intercomparable data as a result of its very high specificity for targeted IsoP analytes (Sircar and Subbaiah, 2007; Tsikas, 2010b; Tsikas et al., 2003). Artefactual interferences are problematic for all IsoP methods not using IAC, even when using internal standards (e.g., Mas et al., 2010a). Only more recently has effort been devoted to developing methods (generally based on HPLC) requiring minimal sample preparation (e.g., Saenger et al., 2007).

Comprehensive inter-method or inter-laboratory comparisons are rare. Comparisons of methods based on immunoassay with those based on mass spectrometry are particularly lacking (e.g., Tsikas, 2010a). Poor correlation or high bias has been discussed in a number of studies (Bessard et al., 2001; Huang et al., 2002; Il'yasova et al., 2004; Klawitter et al., 2011; Liang et al., 2003; Saenger et al., 2007; Smith et al., 2011). Some of the possible causes of variability and some caveats regarding sampling, sample preparation, and analysis have been discussed (e.g., Callewaert, 2004; Jones, 2005).

The questionable precision and accuracy of the commercial immunoassay kits (versus MS-based methods) greatly diminishes the possible utility of a large portion of the published literature, especially clinical research that has heavily relied on the convenience of these kits. Inadequate sample purification and cross-reaction among IsoPs are major limitations. Even when good correlations between immunoassay and MS methods are reported, the immunoassays are always found to yield results biased high — by at least 100% (Carraro et al., 2010; Yan et al., 2007).

A number of comprehensive reviews on IsoP analysis are available (e.g., Berdeaux et al., 2006; Davies, 2009; Lawson and FitzGerald, 2002; Liang et al., 2003; Liu et al., 2009; Milne et al., 2007; Nikolaidis et al., 2011; Yin, 2008). Advancements in the trace analysis of IsoPs in biological matrices have been infrequent. One analytical approach, developed by Eggink et al. and further developed by Kretschmer et al., makes use of a new derivatization reagent for HPLC (Eggink et al., 2010; Kretschmer et al., 2011). Progress is being made toward automating IsoP analysis, via HPLC/MS (Bai et al., 2011). Others are working on increasing the sample throughput and accuracy of methods (e.g., Dahl and van Breemen, 2010; Langhorst et al., 2010).

Regardless of the dearth of research on method comparability, the various F_2-IsoPs seem to be able to proxy for each other. This is important with respect to another complication associated with analysis — namely, the separatory resolution of the numerous F_2-isomers possibly present. The actual scope of an F_2-IsoP method is often not clear — it is sometimes unknown whether a method might be specific for a single isomer or whether it is capturing multiple isobaric species. With that said, it has often been proposed that it might prove more useful to purposefully capture multiple F_2-isomers together (or better, all F_2-IsoPs in toto, or perhaps even include F_2-dinor metabolites) in a single analysis rather than target discrete isomers. This approach would greatly improve method sensitivity and lessen variability. This important topic has been emphasized in many papers (e.g., Cracowski et al., 2002; Halliwell and Lee, 2010; Mori et al., 1999a; Nikolaidis et al., 2011; Schwedhelm et al., 2007; Taylor et al., 2008; Tsikas et al., 2003).

Uncertainty in exactly which isomers are captured by a method is often indicated with the use of generic expressions such as "F_2-isomers". A conflated class-wide measure would increase the sensitivity of the method and ensure that the origins of oxidative stress are more widely represented. This is especially true since current knowledge is insufficient to determine which isomer(s) best reflects oxidative stress, which is the most potent mediator of stress, or which is most abundant in urine (Cracowski et al., 2002). Moreover, the relative contributions from individual isomers may change with time, depending on the health status of an individual (individual F_2-IsoP isomers may originate from different diseases and may have different biological effects), and the different isomers may experience different metabolic clearance rates. Regardless, the fact that different methods capture different types and numbers of F_2-IsoP isomers can serve to inflate or deflate the apparent range of urinary IsoP values far beyond what would otherwise be achievable with a standardized method.

An extremely broad array of methodologies and QA/QC has been employed in IsoP analysis. The most important insight to gain from the extensive published literature involving IsoP analysis is that the data from different studies (even those using the same basic analytical approach) should probably not be inter-compared with respect to absolute concentrations. The value of these data with respect to BioSCIM currently resides in the perspective they provide on the precision (and the maximum range of values) that might be expected from any given method.

10. IsoP excretion data

Despite the disparities in the way published data on urinary IsoP levels are reported, these data are valuable for determining the potential utility of IsoP in a BioSCIM application. These data address two major unknowns: determining (i) how broad a reference interval might be for urinary IsoP levels from a "normal" population, and (ii) the magnitude of the excursions outside the reference interval for urinary IsoP levels from "non-normal" (e.g., diseased or stressed) individuals. The latter must be significantly greater than the former to be detected.

While the magnitude of the range of the data from an individual study is useful, the magnitude of the range for data compiled across studies, which would probably be very large, is not meaningful because of significant differences in analytical methodologies. The broad range in inter-study data distribution is primarily biased by the inclusion of different IsoP isomers in different methods; this is especially evident in the discrepancies between the data from immunoassay and mass spectrometric methods.

Much of the IsoP data collected from clinical and epidemiological studies has used immunoassay. These data may be biased high because of cross-reactivity with numerous other isomers and may have larger variance because of intra- and inter-method variability.

A large portion of the IsoP studies conducted with human subjects targeted IsoP levels in blood (generally plasma or serum). IsoPs in blood could be viewed as mirroring acute (real-time) overall stress, while urinary levels serve to integrate stress over time (both acute and chronic). Blood levels are not necessarily relevant with respect to predicting the levels or variance of IsoP excretion in urine (or

feces), as the distribution and metabolism of IsoP is complex. Plasma levels are not necessarily reflected in urinary levels (Halliwell and Lee, 2010). Therefore, the focus here is almost solely on urinary excretion of IsoPs. Another limitation of the published data is that most are obtained from studies that collected spot (convenience) samples, as true 24-h samples are difficult to obtain. Diurnal variability in excretion will mean that spot sample data is more difficult to extrapolate to levels that might be expected in sewage.

As previously emphasized, the actual identity of the IsoP(s) targeted in various studies is often unclear and can range from one to multiple isomers among one or more series of IsoPs. For this reason, the term isoprostane (or IsoP) or other informal abbreviations (e.g., F_2-IsoP) are often used in this document in a generic manner, while fully realizing the lack of rigor. Failure to distinguish IsoP isomers could be a source of considerable variability when comparing data across studies. As one example, in a study that targeted five individual IsoP isomers, the range in urinary levels across the isomers spanned nearly an order of magnitude: from 0.532 μg/day (for 15-epi-iPF$_{2\alpha}$-III) or 0.592 μg/day (for iPF$_{2\alpha}$-III) up to 5.169 μg/day (for iPF$_{2\alpha}$-VI) (Yan et al., 2007).

Another factor treated irregularly across studies is the importance of IsoP conjugates in urine. Excretion of conjugates apparently varies between individuals — ranging from little up to 80% of the total IsoP present (Kadiiska et al., 2005b; Yan et al., 2010). Although some studies report the use of a pretreatment hydrolysis step using glucuronidase, others make no mention of whether measurement was made for free or total IsoP. Conjugates, however, may not actually have much impact with respect to acquiring data for BioSCIM. Even if substantial portions of IsoP are excreted as glucuronide conjugates, it is possible these will undergo in situ hydrolysis by the glucuronidase activity present in sewage. While most studies show glucuronidase activity in activated sludge, little attention has been paid to raw sewage (on during transit to an STP) or even once feces and urine mix in the toilet. One study, however, shows that estrogen conjugates undergo hydrolysis while in transit to an STP (D'Ascenzo et al., 2003).

The published urinary data from "healthy" subjects (e.g., controls) conclusively show the continual presence of IsoPs in all urine samples. These basal levels may not exclusively represent oxidative damage per se, but rather perhaps serve as lipid messengers involved in myriad endogenous biochemical processes. A certain low basal level of oxidative stress is probably required to maintain homeostasis and health; this is one hypothesis often put forth to explain the "antioxidant paradox" — the failure of antioxidant supplementation to reduce oxidative stress in healthy individuals (Sheikh-Ali et al., 2011). For example, in the Shanghai Women's Health Study (nearly 75,000 women), supplementation with antioxidants reduced IsoPs only for those with higher BMIs (Dai et al., 2009; Dai and Zhu, 2009).

The production of elevated IsoPs is often a function of current health status and the combined stress level. For example, healthy young adults often show no reduction in IsoP levels when administered fruit/vegetables or antioxidants. This is likely because their IsoP levels are already at the necessary basal maintenance level. Likewise, they often also show no increase in IsoP levels under mild stress (smoking, moderate exercise) because their oxidant defense systems are robust. This means that with a sufficiently low limit of detection, IsoPs should always be detectable in sewage. The critical question is whether the increased IsoP levels in sewage, resulting from oxidative stress, will be sufficiently elevated from basal levels to be able to discern community-wide stress. While any given level of IsoP in sewage most likely results from the combined contributions from many different stressors and causes, a change in trend might more probably result from just a few or perhaps a single cause.

Some studies do not reveal a correlation between known oxidative stress and IsoPs in urine. This is perhaps because the levels in urine are an integrative measure of oxidative stress across the entire body. Localized stress will not always result in increased urinary levels. For example, while exhaled breath condensate shows elevated IsoP levels among certain asthmatic children, IsoP in urine may show no correlation (Carraro et al., 2010).

Despite the considerable published data on urinary levels of IsoPs (for both healthy and diseased subjects), the only values that can be applied directly to predicting the levels of IsoPs that might occur in raw sewage are the 24-h levels (expressed in terms of mass excreted per day). Most data on the urinary excretion of IsoPs are reported in units that cannot be reliably converted to total daily mass (for example, because of normalization against creatinine or because of reporting on the basis of concentration). *With this said, at least seven studies have reported on the basis of total mass excreted daily*. These urinary excretion data represent basal levels of IsoP production from healthy individuals and span a combined range from roughly 500–5000 ng/day, depending on the IsoP isomers captured by analysis; most are in the range of 1000–2000 ng/day (see in particular: Frost-Pineda et al., 2011; Mori et al., 1999b; Shi et al., 2007; Stein and Leskiw, 2000; Tsikas et al., 1998; Yan et al., 2007, 2010). Since this range comprises extremes from different methods, it could be assumed that use of a standard method would have probably yielded a narrower range.

Sporadically, some data fall outside the commonly reported range. One example is the reported daily urinary excretion for 8-isoprostaglandin $F_{2\alpha}$ at 10 pmol/24 h (3.5 ng/day) (Tsai et al., 2009), which is over 2 orders of magnitude below the low end of the range compiled from the seven studies reported above (i.e., 500–5000 ng/day).

On the basis of creatinine output, levels of F_2-IsoPs in normal human urine are reported to range from 500 to 4000 pg/mg of creatinine (Durand et al., 2011), but these data cannot be reliably translated into total daily outputs. They do, however, reflect an inter-method combined range in the distribution of excretion levels similar in magnitude to that for total daily excreted mass (i.e., 500–5000 ng/day). This shows that the published data have internal consistency.

One study is unique in that it reports excretion data on the commonly used basis (normalized against creatinine) as well as both in terms of urinary concentration (pg/mL) and in terms of mass excreted per day (μg/day). These unique data serve to illustrate an important point. For certain IsoPs, correlations that might not be evident when excretion is normalized against creatinine (e.g., similar levels for smokers versus nonsmokers) become apparent when the data are expressed on another basis, such as in terms of mass excreted per day (Yan et al., 2007).

10.1. Examples of the maximum magnitude of urinary IsoP levels

The conditions under which the maximum urinary IsoP levels have been reported may have value in delineating the upper limits to variation in BioSCIM data resulting from changes in a community's health status. These reported, enhanced IsoP levels can be roughly one to two orders of magnitude over basal levels.

Perhaps one indication of a potential maximum magnitude of change in urinary IsoP levels that might result from oxidative stress was shown in the original BOSS report, where urinary 8-iso-PGF$_{2\alpha}$ and 8,12-iso-iPF$_{2\alpha}$-VI increased 10 and 17 fold, respectively, 7 h after treatment with the highest carbon tetrachloride dose (Kadiiska et al., 2005a). Such a dramatic response, albeit in rats, might make detection possible even with a high level of baseline (basal) variability.

Probably the highest reported rates or changes in rates for IsoP excretion in humans have been documented for genetic disorders. These excretion rates might serve to describe the absolute upper limit on IsoP excretion across the numerous other disease states known to influence urinary IsoP. One example studied children with Zellweger syndrome — a rare genetic disorder preventing the proper assembly of peroxisomes and leading to the accumulation of very long chain fatty acids in the blood. Five Zellweger children were shown to excrete 8-Iso-PGF$_{2\alpha}$ at rates over a 100-fold higher than

healthy controls: 63.3 ± 16.6 ng/mg creatinine versus 0.51 ± 0.16 ng/mg creatinine (Tsikas et al., 1998).

Although considerable data have been obtained on the correlation of IsoP production in humans with various disease states, few data are available on the production of IsoP as a result of induced stress such as with the rodent models used in the BOSS assessment. One of the few occasions for acquiring such data is during chemotherapy infusion. One study followed the urinary production of four isomers of F_2-IsoPs [iPF(2α)-III, 2,3-dinor-iPF(2α)-III, iPF(2α)-VI, and 8,12-iso-iPF(2α)-VI] during doxorubicin infusion. After 1 h, the increase in levels ranged from 41% [iPF(2α)-VI] to 62% [iPF(2α)-III]; all levels returned to baseline after 24 h (Il'yasova et al., 2010). This type of study is valuable in that it gives insight regarding the magnitude of increase in urinary IsoPs that can occur after a significant oxidative insult. It also provides insight on the speed and sensitivity of IsoP production and rate of return to baseline. The fact that IsoP returned to baseline levels before doxorubicin had been cleared from the body perhaps meant that antioxidant defense mechanisms had been quickly up-regulated. In a follow-up doxorubicin study involving 18 women, the levels of the four isomers in 10 subjects nearly doubled. It was also shown that for others who began the study with already elevated IsoP levels, doxorubicin had little effect (Il'yasova et al., 2011).

Of all common stressors, one responsible for some of the largest increases in urinary IsoP levels is chronic alcohol consumption associated with liver disease. For healthy adults, urinary excretion of $iPF_{2\alpha}$-III initially increased about 4-fold from baseline [from 116 pg/mg creatinine to 491 (0–6 h) and 349 (6–12 h)] and then declined to 202 (12–24 h) after dosing with 0.9 g/kg alcohol. The levels were also a linear function of dose, increasing from baseline (50 pg/mg creatinine) to 102 at a dose of 0.2 g/kg alcohol, and to 402 for 0.9 g/kg alcohol (Meagher et al., 1999). Urinary levels were higher in patients with acute and chronic alcohol-induced liver disease (ALD) (657 pg/mg creatinine) and higher yet in patients with combined hepatitis C cirrhosis and ALD (922 pg/mg creatinine versus a baseline of 127 for healthy controls). The highest levels were from those with acute alcoholic hepatitis (2205 pg/mg creatinine). This represented roughly a 20-fold increase over controls — one of the largest increases published to date. Excretion of $iPF_{2\alpha}$-III was found to be highly correlated with $iPF_{2\alpha}$-VI, which occurred at much higher levels. The metabolite of $iPF_{2\alpha}$-III (2,3-dinor-5,6-dihydro-$iPF_{2\alpha}$-III) was also markedly elevated.

10.2. Example urinary IsoP ranges for apparently healthy populations

Few studies have been devoted to establishing urinary IsoP levels for "healthy subjects" or perhaps more appropriately termed "apparently healthy" or "otherwise healthy" subjects. The primary source of the data for apparently healthy individuals must usually be derived from their use as controls in clinical studies. Once again, the primary relevance of these studies for a BioSCIM application is not the absolute levels reported, but rather the variance that is revealed. But even with studies of apparently healthy individuals, considerable variance is undoubtedly introduced simply by the prevalence of common factors known to influence IsoP levels — the very factors that would ordinarily be reflected by BioSCIM — especially smoking, alcohol consumption, exercise, BMI, and age — as well as any of numerous potential sources of hidden stress. No published studies enlisting otherwise healthy individuals were found that controlled for all of these major variance factors, either during recruitment or by statistical means.

Some example data from various studies are briefly summarized here to provide perspective on the magnitude of variance encountered. In 323 apparently healthy Japanese, mean urinary 8-IsoP was 0.74 ± 0.03 ng/mg creatinine. Levels were significantly higher in males than females and increased with BMI and frequency of alcohol consumption (Sakano et al., 2009). A study specifically designed to determine a "reference interval" for urinary 8-iso-$PGF_{2\alpha}$ enlisted 34 healthy subjects. The range was 57 to 390 ng/g creatinine with a mean of 221 ng/g creatinine (Saenger et al., 2007). In a study of 72 healthy young women and men (36 each), urinary F_2-IsoPs were found to be invariant (2.26 ± 0.9 μg/g creatinine) (Burgos Alves et al., 2010). In a study of 1647 women (Study of Women's Health Across the Nation — SWAN) spanning the menopause transition, mean urinary $F_{2\alpha}$-IsoP levels in non-smokers did not differ for pre- and post-menopause: 343 ± 12.4 pg/mL versus 379 ± 19.5 pg/mL (Sowers et al., 2008). In the SWAN study, 1610 participants (multirace/ethnic sample of midlife women) had an overall median concentration of urinary $F_{2\alpha}$-IsoPs of 433 ng/L, with an overall total range spanning over an order of magnitude: 167—2074 ng/L (Tomey et al., 2007). Urinary 15-F_{2t}-IsoP from 16 apparently healthy individuals ranged from 55 to 348 ng/g creatinine (Haschke et al., 2007).

One study compared 30 subjects with untreated metabolic syndrome against 30 age- and gender-matched controls. Mean urinary F_2-IsoPs (pmol/mmol creatinine) were 808 (695–943) versus 664 (590–749), respectively (Tsai et al., 2009). In a study using 11 clinical urine samples (unspecified origin), the distribution range for 8-isoPF_2 was wider and higher than for eight samples from healthy controls: mean and median of 0.118 and 0.092 ng/mL (range 0.029 to 0.240 ng/mL) for clinical samples versus 0.048 and 0.039 ng/mL (range 0.017 to 0.084 ng/mL) for controls (Bai et al., 2011). Just for perspective, of the numerous studies reporting on blood levels, "normal" concentrations of F_2-IsoPs in human plasma were reported as 35 ± 6 pg/mL (0.035 ng/L), versus 1.6 ± 0.6 ng/mL in human urine (Milne et al., 2007).

Some studies, however, yield rather large ranges. In a study of 246 women, the range for baseline urinary levels of 8-iso-PGF_2 was 192–4873 pg/mg creatinine (Thompson et al., 2005).

A multi-country study used 588 subjects from Sweden (n = 220), Italy (n = 203), and Poland (n = 165). Modestly higher levels were found for smokers. Mean levels (and ranges) for urinary F_2-IsoPs (pmol/mmol creatinine) among all subjects were 200 (64–1235) [n = 588]. For individual groups, the levels were: 182 (64–1235) [n = 217] non-smoking males; 213 (70–677) [n = 89] smoking males; 204 (68–449) [n = 195] non-smoking females; and 245 (116–752) [n = 81] smoking females (Basu et al., 2009).

10.3. Intra- and inter-individual variations in urinary IsoP levels

Studies on temporal variation of urinary IsoP levels have examined intra- and inter-personal excretion over periods ranging from days to seasons. Among the first studies to examine intra-day variation in urinary IsoP excretion in healthy subjects were those of Helmerson and Basu. Urinary 8-iso-$PGF_{2\alpha}$ levels (determined by immunoassay) were indistinguishable during a 24-h sampling for each of 10 healthy subjects, with a mean level of 0.44 ± 0.23 nmol/mmol creatinine (Helmersson and Basu, 1999). For 13 healthy males and females, mean urinary 8-iso-$PGF_{2\alpha}$ on 10 consecutive days was 0.27 ± 0.11 nmol/mmol creatinine (Helmersson and Basu, 2001).

Using a method developed specifically for an 8-iso-prostaglandin $F_{2\alpha}$ dinor metabolite (2,3-dinor-$iPF_{2\alpha}$-III), a study of 21 subjects measured intra- and inter-individual variations in urinary levels (Zhang et al., 2010). The overall mean was 4.3 ± 0.3 μg/g creatinine. As with many studies, the mean levels were significantly higher for females than males (5.0 ± 0.6 versus 3.6 ± 0.3 μg/g creatinine). Respective intra- and inter-individual contributions to total variation were 40% and 60% (Zhang et al., 2010).

One of the few and most significant studies on time-course variability of urinary IsoPs was a year-long study of 48 randomly selected middle-aged and elderly Chinese men (Wu et al., 2010). Spot urinary samples were collected over 4 seasons. Among the markers targeted were F_2-IsoPs and 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (15-F_{2t}-IsoP-M). The mean levels (expressed as the mean interquartile range) for

each of the four seasons were 2.21, 1.88, 1.89, and 1.80 ng/mg creatinine for F_2-IsoPs; the grand mean was 1.90. For 15-F_{2t}-IsoP-M, the mean interquartile ranges were 0.55, 0.53, 0.51, and 0.52 ng/mg creatinine (grand mean of 0.53). These data reflected surprisingly low variation and high stability among the seasonal levels, especially given the use of spot instead of 24-h samples.

10.4. Biliary (and fecal) excretion

In examining published data for the levels of a biomarker projected to be excreted from healthy and diseased individuals, urine is usually the primary route considered — primarily because of the ease of sampling urine in clinical studies. But the parallel route of fecal excretion (via bile and possibly by intestinal excretion [diffusion into the lumen]) also needs to be examined for BioSCIM. The possibility of two routes of excretion (urinary and fecal) could also dictate the need to design different approaches for sewage analysis depending on whether a portion of the targeted biomarker is not dissolved or suspended in the aqueous phase or raw sewage, but rather remains sorbed to the solids. With some biomarkers – coprostanol being one example (Daughton, 2012) – excretion via the feces can be substantial. For IsoPs, however, few data are available on fecal excretion. It is also not known whether feces could also harbor IsoPs that are created within (but not absorbed from) the gut (as with coprostanol).

In the first study of biliary excretion in humans, esterified F_2-IsoPs were found to be excreted in the bile of healthy individuals at levels of 188 ± 27 pg/mL. In subjects with bile duct stones and various diseases of the pancreas, the levels were nearly 3-fold higher — 523 ± 129 pg/mL and 545 ± 112 pg/mL, respectively (Leo et al., 1997). Assuming an average daily bile production rate of 600 mL, the total daily elimination of F_2-IsoP via the feces for healthy individuals could amount to roughly 113 ng/day. This amount may prove to be significant compared with urinary excretion — contributing from 23% to 2% of the combined urinary range reported earlier (500–5000 ng/day); see Section 10: IsoP excretion data. The only prior study on biliary excretion used rats, where biliary excretion was found to greatly increase upon dosing with carbon tetrachloride, and most IsoP was excreted in esterified form rather than free (Awad and Morrow, 1995).

11. *Differential Stress Index*: increasing the sensitivity of BioSCIM by also measuring biomarkers of health to derive a normalized index of stress or homeostasis

One of the major challenges facing the use of BioSCIM for assessing community health is the need to accurately know the size of the contributing population. The population size allows calculation of per capita contributions to biomarker levels measured in sewage. Sufficiently accurate estimation of population size, however, is fraught with difficulties. One possible approach (ASAP-SCIM: see Daughton, 2012), which for the first time makes use of biomarkers in sewage, has been published as the prelude to this article, but the approach still poses a number of challenges for successful verification and implementation; this published paper also discusses the many limitations to the existing approaches for estimating population size. Any additional sources of error beyond that involved in biomarker analysis and calculation of sewage flow could obscure the ability to detect otherwise significant variations in biomarker levels.

Since the need to calculate community-wide contributions of biomarkers on a per capita basis is problematic, an alternative approach would greatly help. One possible approach (referred to here as the *Differential Stress Index*) would nullify the need to know the specific population size responsible for a measured biomarker level. This approach would create a dimensionless ratio by normalizing the levels of the targeted biomarker of stress (such as IsoPs) against a second biomarker with orthogonal characteristics. The second biomarker (the denominator) would be selected so that its excreted levels would move in opposition to the level of the biomarker of stress (the numerator). To maximize the sensitivity of the Differential Stress Index, the second marker would ideally serve as a positive measure of health.

Dimensionless ratios are routinely used in testing for abused drugs in sports, an example being testosterone/epitestosterone (Van Renterghem et al., 2010). Another example is a proposed "index of endogenous anti-inflammatory potential", which uses the ratio of two oxylipids having opposite effects on inflammation (Gangemi et al., 2005).

Excretion of this second "normalizing" biomarker would have to meet one of two criteria. Its excreted levels would need to be either: (1) a constant function of per capita excretion (and therefore serving as a surrogate measure of per capita population), or (2) a variable function of a physiological state whose level is an inverse, orthogonal function of the targeted biomarker.

Two examples of the first criterion are creatinine and the fecal sterol coprostanol. Creatinine, although long used in clinical chemistry for avoiding the problems created by the extent of dilution of urine (which is problematic for interpreting levels in spot samples), has a number of problems when applied to sewage (Daughton, 2012). A more likely candidate is the use of coprostanol as a proxy for population size, as proposed by Daughton (2012).

An example of the second criterion is easy to conceptualize but identifying a real-world candidate from the universe of known biomarkers proves difficult. The ideal candidate for an inverse, orthogonal biomarker would be one that measures "good health" or "positive health" — or an array of biomarkers that correlate with the lowest risk of morbidity and mortality.

Biomarkers are often classified according to three purposes: measuring dose (exposure), effects, or susceptibility to effect or risk. Surprisingly absent are biomarkers that directly measure or confirm health or maintenance of homeostasis. The *absence* of disease is invariably used as an indirect surrogate for health; but such a signal is necessarily one of ever-declining magnitude. With very few exceptions, nearly all known biomarkers measure some attribute of disease or stress. The few possibilities of positive markers of health (which increase in magnitude) tend to not meet one or more of the three major criteria for use with BioSCIM (Table 1). In particular, they must be: (1) sufficiently stable to be extensively excreted – preferably into urine (or possibly feces) – and persist in sewage, (2) excreted in the absence of stress at levels with low intra- and inter-individual daily basal variation (including not being influenced by normal dietary changes), (3) immune to confounding — introduced into sewage predominantly as a result of endogenous human metabolism (with minimal exogenous contribution, such as by raw or cooked foods), and (4) have a chemical structure amenable to detection in the complex matrix of sewage.

A biomarker of health would provide an independent variable against which to normalize the levels of the biomarker targeted for measuring disease or stress. The per capita contributions of the two biomarkers (from opposite ends of the health-disease continuum) in sewage would vary in opposing directions. Normalizing a measure of negative health against a measure of positive health (creating a dimensionless index) would not just eliminate the need to know the population size, it would also serve to greatly amplify the signal that would otherwise be provided solely by measuring a biomarker of stress (as a result of the numerator and denominator being inversely related).

11.1. Biomarkers of health — anti-inflammatory eicosanoids

A clear example of one of the few documented biomarkers of positive health would be one of the many eicosanoids that serve as counter-regulators to the inflammatory oxylipids. The chemistry

and biochemical pathways involving eicosanoids are extraordinarily complex and intricately inter-connected. They are also incompletely understood, with new knowledge continually emerging. The counter-regulators of inflammation are involved not just with down-regulating inflammation, but also with "resolving" (or repairing) inflammatory damage (catabasis). This group of anti-inflammatory, pro-resolving eicosanoids comprises the specialized classes of proactive, lipid mediators known as lipoxins, resolvins, protectins, and maresins, many of which are biosynthesized from the omega-3 essential fatty acids eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). Even though these anti-inflammatory pro-resolution eicosanoids are produced at highest levels during the recovery phase (resolution) of chronic disease, their presence reflects a healthy immune system; their levels are notably suppressed in those who are unable to resolve chronic inflammation. Overviews are available (Das, 2010; Levy, 2010; Maderna and Godson, 2009; Serhan and Petasis, 2011).

These pro-resolving, hydroxylated, polyunsaturated fatty acids are produced in localized areas of tissues at extremely low concentrations (pM–nM), reflecting their hormonal-like potency. These oxylipids would make ideal biomarkers of health. But unfortunately, little is known about their excretion in urine, and their low levels might challenge analysis. Limited data, however, indicate that certain drugs (e.g., COX-2 and LOX inhibitors) might inhibit the resolution process, whereas others might allow it (e.g., aspirin, statins, and glucocorticoids) (Serhan and Petasis, 2011).

One of the only studies published to date on the urinary excretion of pro-resolving mediators involves a lipoxin, LXA4. Its urinary levels were roughly 2 orders of magnitude lower than those commonly reported for IsoPs — roughly 0.02 ng/mg creatinine (Gangemi et al., 2005). The only published method for lipoxins in urine is an immunoassay, and it is unknown whether its sensitivity and specificity would suffice for sewage.

11.2. Other potential markers of positive health for use as denominators in the Differential Stress Index

In addition to the pro-resolver oxylipids, several other potential biomarkers of "positive health" exist but for a variety of reasons would not work as the denominator in a differential stress index. The production of some biomarkers reflects dual actions — serving as indicators of both positive health attributes and negative aspects of disease. Two sometimes paradoxical outcomes can come about from the same mode of action — sometimes as a function of the concentration or other times as a function of site or timing of production. One example is the production of an array of halogenated lipids from the oxidative stress created by endogenous hypohalous acids. This can result, for example, from collateral damage from immune response in fighting an infection (e.g., Spickett, 2007).

11.2.1. F_3-isoprostanes

While the major focus of IsoP research has been on the F_2-series, many other IsoPs can be formed from various polyunsaturated fatty acids other than arachidonate. In 1997, the IsoP F_3-series was reported to form from the non-enzymatic free-radical peroxidation of eicosapentaenoic acid (EPA), an essential omega-3 fatty acid whose primarily dietary source is fish oil (Nourooz-Zadeh et al., 1997); additional amounts of F_3-IsoP could also be contributed by beta-oxidation of F_4-IsoP generated by analogous peroxidation of the related omega-3 docosahexaenoic acid (DHA). The in vivo formation of F_3-IsoP was first shown by Gao et al. (2006).

Of the six possible series of F_3-IsoPs (5-, 8-, 11-, 12-, 15-, and 18-series), the most prevalent in urine seem to be 5-epi-8,12-iso-iPF_3-VI and 8,12-iso-iPF_3-VI, whose ratio is relatively fixed (Song et al., 2009).

F_3-IsoPs tend to be produced more efficiently than F_2-IsoPs — probably being more easily formed thermodynamically since EPA and DHA (having 5 double bonds) are more unsaturated than arachidonic acid (AA) and therefore are more readily oxidized. When EPA and DHA are present, they might therefore serve to competitively reduce the oxidation of AA, resulting in lower production of inflammatory F_2-IsoPs. Indeed, clinical data show that EPA and DHA can reduce F_2-IsoP levels (Mas et al., 2010b).

Evidence also indicates that the F_3-IsoPs possess their own anti-inflammatory actions (Roberts and Milne, 2009). Although the generation of F_3-IsoP from EPA is much more efficient than F_2-IsoP from AA, urinary levels of F_2 are substantially higher than F_3, probably because of the much higher levels of AA than EPA (or DHA), whose dietary source is limited primarily to fish oil. Moreover, the intra-individual excretion rates of F_3-IsoPs seem to be much more variable than for F_2-IsoPs. Nonetheless, F_3-levels, if detectable in sewage, might have potential as orthogonal biomarkers against which to normalize F_2 levels.

Other factors can disqualify a biomarker from use with BioSCIM. Some of these are illustrated by the adrenal androgen dehydroepiandrosterone (DHEA), which is one of the few biomarkers tracked in existing clinical tests that purportedly reflects positive health (see the Supplementary materials section "Aspects of biomarkers not suitable for BioSCIM").

12. Consideration of other biomarkers for SCIM

Other biomarkers were considered for use in an initial (proof of principle) BioSCIM application but were determined to have one or more negative attributes compared with IsoPs; many of these also have extensive histories of use in clinical research, such as prostate specific antigen (PSA) and various hormones. Among these are not just other markers of lipid oxidation [nitrated fatty acids; plasmalogens (Leßig and Fuchs, 2009), halohydrins (Spalteholz et al., 2004), and other chlorinated lipids (Spickett, 2007)], but also products of protein oxidation [*o,o′*-dityrosine, nitro-tyrosine, and halo-tyrosines (Mohiuddin et al., 2006)], DNA (oxidized guanine derivatives such as 8-oxoguanine) (Andreoli et al., 2011; Winnik and Kitchin, 2008), and uric acid (metabolized to allantoin) (Il'yasova et al., 2010); for an overview, see Jain (2010). Many of the halogenated biomarkers are formed from the action of myeloperoxidase (MPO), which uniquely generates hypohalous acids from halides at physiological concentrations (Klebanoff, 2005).

While it might seem that halogenated products could serve to facilitate detection in sewage, the ubiquitous occurrence of myriad organohalogen natural products, particularly in marine life, might confound the use of a halogenated biomarker for SCIM. The occurrence of organohalogens in food sources might serve as a significant exogenous source (Gribble, 2010).

13. Biomarker profiles and community-wide allostasis

The utility of BioSCIM could be enhanced with the incorporation of a select array of biomarkers — preferably reflecting a wide spectrum of biological regulatory processes and providing complementary, orthogonal measures of disease (or health). It is now widely recognized that aggregate exposure to multiple stressors sharing the same mechanism or mode of action – but at individual levels below any known effects levels – can yield adverse effects as a result of their combined contributions. But much less is known with regard to stressors acting by unrelated pathways and yielding unrelated effects (mixed-outcome effects). The question is whether individual effects, which would otherwise not prove adverse in isolation from others, when combined with the unrelated effects elicited by orthogonal-acting stressors, might serve to exceed the allostatic load.

An array of carefully selected biomarkers would better reflect the effects from combined cumulative exposures. Such a composite picture – or profile – would more closely align the BioSCIM concept with the *biological passport* approach used in sports random drug testing (referenced earlier). Perhaps the first and most comprehensive attempt at designing a composite measure of how the body responds to cumulative exposure to all stressors is the concept of allostasis and its quantitative measure referred to as allostatic load (McEwen and Stellar, 1993).

In practice, allostasis is measured by the combined levels of roughly a dozen or more biomarkers whose increasing (or decreasing) levels are associated with an increased risk of mortality. Conceptually, allostasis reflects the body's overall status in maintaining homeostasis and indicates when perturbations exceed that capacity. The allostatic load accounts for the two forms of stress: quotidian (ordinary, recurring, persistent, chronic, episodic, daily stress, which tends to be perceived as minor), and infrequent, intense, acute forms of stress. Significantly, allostasis would also account for health disparities caused by stress resulting from origins usually not reflected by traditional chemical-exposure monitoring — stressors such as socioeconomic status, ethnicity, race, and psychological stress (Djuric et al., 2008).

A recent review on allostasis is available from Juster et al. (2010), and a study examining the weaknesses of the allostatic load concept is presented by Dowd et al. (2009). A variety of analogous approaches intended for practical clinical application have been proposed (Ochi and Cutler, 2003; Southern, 2010; Veglia et al., 2010).

14. Potential role for BioSCIM in revealing health disparities (via IsoPs)

Health disparities reflect the disproportionate morbidity, mortality, stress, and degraded productivity and sense of well being experienced as a result of differential exposure by racial and ethnic minorities and by the disadvantaged/under-served, such as rural and poor populations. These factors coalescence into greater need for healthcare but which is often not available. Health disparities often result from combined actions of genetic and epigenetic factors, social setting, psychological stress (and impact on well-being), environmental stressors (including diet), lifestyle choices, and insufficient access to medical care (Djuric et al., 2008). Traditionally, an overwrought focus has been devoted to chemical pollutants at the exclusion of numerous other stressors (Morello-Frosch et al., 2011) — many of which have outcomes measurable by oxidative stress.

The need to assess cumulative impacts of aggregate stressors at the community level is certainly an emerging interest and need. To date, the approach used in assessing cumulative impacts focuses on assessing proximity to known hazards, social vulnerability indicators, land use, demographics, and other signs or measures of possible disproportionate exposures. The variety of methodologies employed have been summarized (Alexeeff et al., 2010; Jakubowski and Frumkin, 2010; Medina-Vera et al., 2010; Zartarian and Schultz, 2010). Some of the many projects underway have been summarized (SEHN/CHE, 2011). BioSCIM could help as a new tool in assessing cumulative exposure and risk in communities, such as with EPA's Community-Focused Exposure and Risk Screening Tool (C-FERST) (Zartarian et al., 2011).

These methodologies are not quantitative, but rather designed to facilitate relative rankings of communities (e.g., from disproportionate exposure to one or more documented stressors). While conventional measures of stress or disease are used (e.g., known incidence of disease such as cancer), none of the approaches relies on biomarkers of exposure or effect.

An emerging appreciation for health disparities and environmental justice led to the creation of what is now the National Institute on Minority Health and Health Disparities (NIMHD). Needed are quick and inexpensive measures that account for the multi-factorial nature of health disparities — including those of psychosocial origin (e.g., emotional stress, such as brought about by socioeconomic status). A biomarker or suite of biomarkers that reflects cumulative stress from all sources would be extremely useful. It appears that no research has ever been published on determining cumulative exposures to any class of stressor via biomarkers; a major impediment has been the lack of an approach for collecting samples on a community-wide scale (Lewis et al., 2011). Sexton and Linder discuss the complexities of assessing cumulative risk and the additional challenges posed by non-chemical stressors — in particular psychosocial stress (Sexton and Linder, 2011).

With the appropriate biomarker(s), BioSCIM holds potential for helping to reveal hidden or emerging disparities, and to assess the effects of attempted interventions. It could help accelerate and improve our understanding of community-wide health disparities. Conventional monitoring of biomarkers in individuals requires considerable resources, and major challenges and limitations are posed by field work (especially in rural areas), all adding to delay in dissemination of data. Conventional health disparities research can suffer from: bias introduced by selection of a limited study population, by accessibility of clinics or in-home clinicians, or by sample processing, storage, and shipping (Djuric et al., 2008).

In an overview of biomarkers (Djuric et al., 2008), a range of biomarkers was examined for assessing health disparities. In the data examined for the work reported here, only IsoPs were deemed suitable for use in BioSCIM because of shortcomings with one or more of the criteria in Table 1. For example, although baseline plasma levels of 15-F_{2t}-IsoP were reported to not differ between African Americans and white Americans, levels of 15-F_{2t}-IsoP increased more in African Americans in response to acute hyperlipidemia, which is a simulation for postprandial oxidative stress and which may be a factor in ethnic differences in cardiovascular and renal risk (Lopes et al., 2003). In a study of cognitive aging, Caucasians were reported to have lower levels of urinary IsoPs than Hispanics (Insel et al., 2011).

15. Limitations of BioSCIM

A wide array of potential problems could limit the application of BioSCIM, regardless of the targeted biomarker. Foremost among these are problems with the sampling of sewage streams representative of the entire population served by the STP. This aspect has been discussed (Daughton, 2012). The sewage influent stream to an STP at any point in time serves as a sampling of combined excretion from a random sub-population of the total population served by the STP. The relative size of this random sub-population is also unknown. This necessitates the use of continuous in-stream sampling designed to collect the total flux of the biomarker over a sufficiently long time (probably at least a day). This poses a number of challenges with respect to the technologies used in sampling. Moreover, however, despite the sophistication of whatever sampling approach might be used, additional hurdles are faced with respect to the representativeness of the data. These result from the fact that diurnal variations in the excretion of urine and fecal matter, coupled with possible diurnal variations in biomarker excretion, cannot be fully represented in any continuous sampling process simply because a certain portion of the population served by the STP will be absent from the service area at any point in time.

Several additional limitations include factors having the potential for confounding BioSCIM data based on IsoP measurement. These variables range from those with the potential to alter IsoP formation and excretion (in the absence of exposure to a stressor) to those that create analytical artefact. Major variables include age, medications and food supplements, dieting (e.g., fasting), nutrition, sewage contributions from healthcare facilities (such as long-term care facilities and hospitals, where excreted levels could be expected to be

unusually elevated), and exogenous (ex vivo) sources of IsoPs that would inflate the levels resulting from endogenous stress. Corrections could be implemented for some of these; an example would be the age structure of the local population, where perhaps demographics could be used to account for age. Several examples of confounding factors are provided in Table 3; these are discussed in more detail in the Supplementary materials (see section on "Potential Confounders").

16. The future

Even though a BioSCIM application remains to be reduced to practice (whether based on IsoPs or alternative biomarkers of stress or health), foreseeable advancements in various technologies could clearly add further power to the approach. These could range from the mundane and obvious to the esoteric. Most simply, better biomarkers of oxidative stress might well exist (e.g., those with greater excretion rates or more easily analyzed) but perhaps have simply not yet been recognized. The development of a certified reference material (e.g., IsoPs in a matrix simulating urine or sewage) could help eliminate a large portion of the uncertainty deriving from analytical measurements between labs (especially when using differing methodologies and instrumentation).

More advanced developments include development of in-line, automated sewage monitoring capability. With the development of in-stream biomarker sensors that could function in raw, untreated sewage, data could be streamed from sewage distribution lines to web-based community dashboards displaying continuous real-time information showing absolute status or trends. The data generated by BioSCIM could be validated by using the voluminous health data being made available via the Community Health Data Initiative (CHDI), launched in 2010 by the US Department of Health & Human Services (HHS, 2010). These capabilities could eventually also provide the type of instant feedback that has proved so effective in motivating individuals to begin changing their behaviors in ways conducive to achieving continual improvements in health or well-being. For example, the use of feedback and peer-to-peer comparisons (such as via social networking sites) has been shown effective in reducing household energy usage (Foster et al., 2010). BioSCIM data revealing a clear time trend for a given STP could be used as positive reenforcement for communities possessing healthy lifestyles, or as a means to flag those communities where certain factors may be degrading health; an ability to predict declining community health allows for development of interventions.

The availability of inexpensive sensors that could target IsoPs and other biomarkers would clearly advance the implementation of BioSCIM. Recent advances in sensor technology are bringing this closer to reality. One example is the adoption and reengineering of the ubiquitous personal glucose monitor (used by diabetics) for the detection and quantitation of a broad array of new analytes via the use of functional-DNA conjugated to invertase (which hydrolyzes sucrose to yield glucose) (Xiang and Lu, 2011). Inexpensive monitors could empower local communities to participate in the implementation of BioSCIM.

A more esoteric future possibility would be the development of what might be called "health checks" for entire communities. One approach would be the use of an exogenous chemical probe specially designed to reflect the level of active stress when taken orally — for example, by way of producing unique metabolites when subject to peroxidation. If a sufficient portion of a community's population participated, then sewage could simply be analyzed for a metabolite unique to a chemical probe and which is indicative of oxidative stress. Indeed, such chemical probes have been proposed for use in clinical medicine (Khatib et al., 2007; Szuchman et al., 2008; Vaya, 2008; Vaya and Tamir, 2008).

Table 3
Factors with potential to confound IsoP levels for use in BioSCIM.[a]

Confounding factor	Examples
Raw and cooked foods as possible exogenous source of IsoPs	Lipid oxidation during frying (e.g., foods high in polyunsaturated fatty acids); however, calculations show that this source could be a minor contributor to IsoP levels in sewage (compared with levels originating from endogenous metabolism). IsoPs seem to occur only at low levels in raw foods.
Dietary lipid influence on IsoP production	Dietary omega-3 fatty acids (e.g., EPA and DHA) can lead to reductions in IsoP formation (possibly by competing with arachidonic acid during oxidative stress); in this sense, omega-3 fatty acids could be viewed as factors protective of oxidative stress. Certain unsaturated fatty acids, however, can inhibit the metabolism of IsoP, leading to enhanced levels.
Exogenous antioxidants as inhibitors of IsoP production	Antioxidants in foods and in nutritional supplements can be viewed either as potential confounding factors or as a reflection of a behavior that can improve health. Studies of the influence of antioxidants on reducing IsoP levels are often inconsistent and sometimes contradictory. Dosage and overall baseline health status are determinants in outcomes.
Other dietary influences on IsoPs	Caloric restriction increases IsoP excretion in obese subjects. Hyperglycemia can increase IsoP levels several fold.

[a] See Supplementary materials for detailed examples and discussion, along with supporting references.

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Appendix A. Supplementary data

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