Response to "Comment on 'Bioaccumulation of Pharmaceuticals and Other Anthropogenic Waste Indicators in Earthworms from Agricultural Soil Amended with Biosolid or Swine Manure'"

We thank Dr. Cline for her comments (*1*) concerning our research (*2*). As correctly noted, the environmental presence and behavior of pharmaceuticals, synthetic endocrine-disrupting compounds, and other anthropogenic waste indicators (AWIs) has recently garnered substantial attention in public and scientific media. Peer-reviewed publications describing the presence and impacts of AWIs in aquatic and terrestrial biota have increased concomitantly (*3*−*6*). To move this frontier of science forward has required developing new analytical methods or the adaptation of existing methods. An example of adaptation of recently developed methods is our recent proof-of-concept study to determine if earthworms collected from agricultural field sites amended with biosolid or manure accumulate AWIs in their tissues (*2*).

Data Quality and Validity. Dr. Cline (*1*) questions the quality of the analytical work and data we presented (*2*). The analytical methods used for this research have all been previously published and have undergone rigorous, nationally consistent validation procedures (*7*−*9*). Many measures of quality control were incorporated into this study, and are described in the Supporting Information (*2*), including (a) triplicate analysis of all environmental samples and of multiple field blanks, (b) analysis of laboratory blanks and laboratory and matrix spike samples, (c) multiple-ion monitoring of study compounds during gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) measurements, (d) use of multiple method performance surrogates, and (e) quantitation using injection of internal standards. About 10% of the triplicate chemical analyses had a combination of censored (nondetections) and uncensored (detections) values. In these cases, censored concentrations were assigned a zero for the calculation of the reported mean of the triplicate results, a conservative approach to calculating this statistic. Matrix spike samples were used to evaluate the precision and accuracy of the analytical methods for earthworms, which, along with the other quality control data collected, is a recognized tool for this purpose (*10*). Contrary to Dr. Cline's assertion regarding galaxolide (*1*), in no case was a compound reported and quantified when the result was less than ten times a corresponding blank detection. This is clearly stated in the Supporting Information section published as part of this research (*2*).

Another point of contention (*1*) was our decision to include data for compounds that were detected below the previously calculated (*11*) method detection limits (MDLs). The detected compounds reported below the MDLs met all reporting qualifications, namely chromatographic retention time, detection of quantitation and confirmation ions, and ion intensity ratios within acceptable limits compared to those of standard compounds, and therefore qualified as positively identified compounds. These detections were clearly noted as such by a "[b]" in Table 1 (*2*). Adaptations of the methods for tissue, biosolid, and manures have included a move to smaller sample size, as was the case in this study. Although the masses of the analyzed earthworm samples were relatively small, the mass spectral ion intensities of the quantifying ions for 70% of the reported detections above the MDLs in the earthworms were greater than that for the lowest calibration standard. Overall, the inclusion of these data captures a more realistic range of the compounds present, but ultimately did not change the conclusions reached.

We chose to include only the compounds detected in one or more of the samples in Table 1 because this was a study to determine if AWIs present in biosolids and manure can transfer to earthworms; therefore, we focused our discussion on those compounds that were found in environmental samples. Listing the compounds not detected in any of the samples would not enhance the interpretation of the data. A complete list of compounds was provided in the referenced literature (*7, 8*). In light of these factors and editorially imposed limits on manuscript length, including sizes of tables and figures, not including the undetected compounds was determined as the best use of allowed space.

Dr. Cline (*1*) concludes that the substantial spatial separation between the three field sites, differences in crops and farming practices, along with differences in soil characteristics and amendments, could result in the differences in AWI composition observed at each site, a fact we explicitly recognized in our paper. Our study was intentionally designed to demonstrate that uptake of AWIs into earthworms occurs over a range of soil types and conditions. Comparisons of AWI profiles in soils and in earthworms from each site demonstrate these site-specific differences. Furthermore, the profile of AWIs in earthworms generally mirrors that of the soil and soil amendments, which is graphically represented in Figure 1. Dr. Cline asserts that the expression of relative content of broad AWI groups in Figure 1 infers lower concentrations at Site 1. It is unclear how Dr. Cline reached this conclusion as this is contrary to how the figure was used to describe the data in our paper (*2*). The figure illustrates the connection between the relative content of different AWI classes in the different environmental compartments at each site. For example, the presence of personal care products in the biosolid applied to Site 2 is largely reflected in the relatively higher content of personal care products in the soil and earthworms from Site 2. This figure also demonstrates some differences in the relative contributions of AWI classes between samples from different sites; for example, the fractional contribution of personal care products in samples from Site 2 is greater than in samples from Site 3, reflecting the relative importance of human-use compounds in biosolid at Site 2 compared to the swine-manure amended Site 3. This representation of the data is useful for discussing the transfer of some AWIs from source materials into earthworms under field conditions, but cannot be used to infer differences in absolute AWI concentrations between sites.

A consequence of choosing a field reference site is that it is subject to effects outside of the control of the study. As we reported (*2*), the soil and earthworms from Site 1 did contain some of the study compounds that have natural sources as well as compounds that are uniquely anthropogenic. While Dr. Cline (*1*) views this as a shortcoming of the project, it does not invalidate the study or its results, nor does it constitute a misleading presentation of the data. On the contrary, this result confirms the difficulty of finding a true control site in areas subject to human activities for compounds that are so commonly used. We specifically highlighted this finding and provided hypotheses for the results (*2*). For example, select AWIs may have originated from nearby septic systems (*12*) or upgradient biosolid or manure amendments. The presence of trace organic contaminants in seemingly pristine environments and biota in such environments has been reported (*13, 14*). Furthermore,

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others have found that mechanisms such as atmospheric transport and surface runoff can translocate AWIs, even onto tightly maintained control field research sites (*15*). Finally, the lack of detections in field blank samples and the careful assessment of laboratory method blank sample results ((*2*); Supporting Information) that were part of the extensive quality control used to determine potential methodological contributions to the environmental data clearly refutes Dr. Cline's assertion that the presence of some AWIs in the reference site is an artifact of analytical methodology or sample collection.

Data Interpretation. Dr. Cline (*1*) suggests that including the terms "Bioaccumulation" and "AWIs" in the paper title is misleading, in part because of the inclusion of biogenic sterols in the study. This is a selective interpretation of the data that ignores the bulk of the results, which include data for many compounds that do have human activity as their only source to the environment. Furthermore, the clear statement in our paper (*2*) that "Biogenic sterols are natural components of fecal materials and may have originated from indigenous terrestrial wildlife and/or soil fauna." addresses the issue. Biogenic sterols were treated as a separate group in the paper to highlight the overwhelming contributions to all of the samples tested. That said, including biogenic sterols such as cholesterol is warranted in AWI studies for several reasons. Biogenic sterols are clearly abundant in the municipal waste stream and biosolids, and therefore serve as good indicators of AWI contributions, especially when coupled with other AWIs. In addition, biogenic sterols can be transformed into sex hormones in the environment, such as the microbial transformation of cholesterol to testosterone (*16*), which clearly justifies the monitoring of such compounds. Dr. Cline maintains that it is inappropriate to monitor cholesterol in the environment and calculate its bioaccumulation factor (BAF). This is inaccurate because biosolid and manure are clear sources of cholesterol to the environment. In addition, recently completed control laboratory work has clearly demonstrated that the concentration of cholesterol in earthworms is related to the concentration of cholesterol in biosolids to which the earthworms were exposed (*17*). These results do not suggest that cholesterol and other biogenic sterols are the most important AWIs to monitor or include in a study of earthworm uptake, but there is unmistakable value in including these compounds as part of such a study to reflect the full range of anthropogenic influence of biosolid application.

Dr. Cline (*1*) provides an incorrect interpretation of the BAF data provided in our paper (*2*) by suggesting that bioaccumulation is not a result of soil amendment with biosolid or swine manure. On the contrary, there is strong evidence that bioaccumulation is occurring as a result of such biosolid and manure amendments. First, BAFs greater than 1 were observed for several synthetic compounds, including triclosan and para-nonylpheonol. Second, BAFs could not be calculated for many of the compounds measured in the earthworms ("NC" in Table 2), because these compounds, including several synthetic compounds, detected in earthworm tissue were below detection levels in the corresponding soil sample. This finding further confirms that bioaccumulation is taking place and demonstrates the utility of using earthworms as sentinel organisms for detecting AWI contamination in terrestrial environments. Dr. Cline's argument (*1*) that comparing the number of BAFs > 0 at each site suggests a lack of difference between the sites is again incorrect. In fact, the BAF values demonstrate a clear difference between the sites and reflect the influence of biosolid and manure applications. A more appropriate comparison would be the number of BAF*s* > 1, which demonstrates accumulation of AWIs relative to the surrounding environment. There are three times the number BAFs > 1 for the biosolid-amended site (site 2) in comparison to the minimally impacted reference site (site 1). There are specific examples of synthetic compounds, such as galaxolide and triclosan, with larger BAFs in the biosolid-amended site compared to the reference site, which may well reflect differences in bioavailability. Therefore, the practice of biosolid or manure application results in different accumulation of AWIs than was observed at the reference site, although for a small subset of compounds there are natural sources and the reference site is subject to the passive influence of these. These data are consistent with previously published research findings (*5, 6*).

Phenol is another compound that, as Dr. Cline points out, has both anthropogenic and natural sources. This fact does not mean that phenol is not an AWI nor that biosolids or manure are not a direct source of phenol to the environment. Not only were high concentrations of phenol present in the biosolid and manure used in this field study, but it has been detected in biosolid samples previously analyzed for the same set of compounds at concentrations significantly greater than might be present in natural sources (*18*).

We appreciate the necessity of critical review of scientific publications and understand that scientific opinions will differ on the methodology and interpretations of our data; however, we take exception to Dr. Cline's statement (*1*) that we "overstated and/or misinterpreted" our study results. Dr. Cline has chosen to take a selective view of our data that ignores the larger data set and its importance. We maintain that the methods and data presented (*2*) are of the highest quality and the results represent an important contribution to the study of AWIs. The conclusions of this study that select AWIs in wastewater biosolids and animal manures, many of which are synthetic, can transfer to and bioaccumulate in earthworms following land application are sound and consistent with the available data.

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Chad A. Kinney

Department of Chemistry, Colorado State University at Pueblo, Pueblo, Colorado 81001

Edward T. Furlong

National Water Quality Laboratory, U.S. Geological Survey, Denver Federal Center, Denver, Colorado 80225-0046

Dana W. Kolpin

U.S. Geological Survey, Iowa City, Iowa 52240-4105

Mark R. Burkhardt

National Water Quality Laboratory, U.S. Geological Survey, Denver Federal Center, Denver, Colorado 80225-0046

Steven D. Zaugg

National Water Quality Laboratory, U.S. Geological Survey, Denver Federal Center, Denver, Colorado 80225-0046

Stephen L. Werner

National Water Quality Laboratory, U.S. Geological Survey, Denver Federal Center, Denver, Colorado 80225-0046

Joseph P. Bossio

Department of Chemistry and Biochemistry, Eastern Washington University, Cheney, Washington 99004

Mark J. Benotti

Southern Nevada Water Authority, Rocky Mountain Water Treatment Facility, Las Vegas, Nevada 89193-9954

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