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STUDY REPORT ON A TEST FOR REMOVAL IN WASTEWATER TREATMENT PLANTS OF GOLD MANUFACTURED NANOMATERIAL (MN): ACTIVATED SLUDGE SORPTION ISOTHERM

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STUDY REPORT ON A TEST FOR REMOVAL IN WASTEWATER TREATMENT PLANTS OF GOLD MANUFACTURED NANOMATERIAL (MN): ACTIVATED SLUDGE SORPTION ISOTHERM

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT
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FOREWORD

This document presents a draft Study Report on "*Gold Manufactured Nanomaterial (MN) Removal in Wastewater Treatment Plants: Activated Sludge Sorption Isotherm*".

The study report was prepared by the United States, leading the project between 2014 and 2021. The original plan was to develop a Test Guideline. To this end, the lead country conducted experimental work with one type of nanoparticles but ran short of resources to test further nanomaterials. The Expert Group following the project had several teleconferences to provide input. Experts recognised that additional test would be needed for a Test Guidelines but also recognised the value of the experimental work completed by the United States. The Expert Group and the Secretariat recommended to share the outcome of the experimental work in the form of a Study Report intended to be published in the Series on Testing and Assessment.

This report includes: i) the results obtained; ii) the protocol followed; iii) the relationship with other existing OECD Test Guidelines in the area of environmental fate, and iv) an annex containing an Excel spreadsheet to be used as a template for the reporting of data. The Study Report and its associated Excel spreadsheet were approved by the WNT in April 2021. The Study Report is published under the responsibility of the Chemicals and Biotechnology Committee.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 887268. Previous financial contributions from the European Union supported the development of publications referenced here published before 2020.

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1 INTRODUCTION

1. The increased production and a wide range of uses for manufactured nanomaterials (MN) in commercial products and applications would suggest that a portion of these MNs will make their way to waste water treatment plants (WWTP). The primary mechanisms by which chemical contaminants are removed from wastewater treatment include: volatilization, biodegradation, hydrolysis, or association/adsorption with sludge. The association of MN with suspended solids (microbial biomass/bioflocs) in WWTP is likely to be the dominant pathway of their removal (Ganesh, R., et al., 2010; Gomez-Rivera, F., et al., 2012; Kaegi, R., et al., 2011 and 2013; Kiser, M.A., et al., 2009, 2010; Mueller, N.C. and B. Novack, 2008; Westerhoff, P., et al., 2011). While some MNs may undergo dissolution, redox transformations and biodegradation, the extent of transformation reactions will be nanomaterial- and coating-specific and may occur at slower rates than aggregation with suspended solids (i.e. biofloc) (Kaegi, R., et al., 2011 and 2013; Clar, J.G., et al., 2016). When a MN is associated with sludge biomass, it will be removed from the system along with other solids. Only a very small percentage of biomass exits clarifiers as un-settled solids, so most MNs associated with solids will reside in sewage sludge residuals that WWTPs dispose to land applications, landfills and/or incinerators. MNs that have a poor affinity for suspended solids that are not biodegradable, or volatile compounds will pass through a biological treatment system unaffected. Therefore, information on the affinity of MNs for suspended solids is needed to assess the possibility of their removal by wastewater treatment systems.

2. The method applied in this study follows a procedure for estimating the potential of activated sludge solids to remove MNs from the water phase. Many MNs are expected to undergo only aggregation/deposition (e.g. affinity to sludge) and may be expected to associate largely with sludge during wastewater treatment (Ganesh, R., et al., 2010; Kaegi, R., et al., 2011 and 2013; Kiser, M.A., et al., 2009, 2010; Mueller, N.C. and B. Novack, 2008; Westerhoff, P., et al., 2011). Some MNs (e.g., nano-silver materials) may undergo dissolution and/or surface reactions (Kaegi, R., et al., 2011 and 2013; Meier, C., et al., 2016). Testing for affinity of MNs to sludge, including the eventual fate and dissolution by-products, will simulate their potential removal during secondary clarification in a WWTP. While some dissolved organic chemical solutes, follow reversible sorption/desorption mechanisms, other dissolved metal ions and ionized organic chemicals exhibit a strong affinity for sorption onto activated sludge or other suspended materials (i.e., bioflocs). As colloids, MNs behave differently from these dissolved chemicals but still interact with activated sludge or other suspended materials, and nanoparticle interactions with suspended materials are likely to be dominated by heteroaggregation and shear-off or break-up, respectively (Kiser, M.A., et al., 2010; OECD, 2017). The distribution between dispersed MNs and MNs associated with larger bioflocs, evolves over time, and may reach a pseudo steady state between aggregation and break-up. The kinetics and extent of these aggregation / break-up processes depends upon the amount of bioflocs (i.e., total suspended solids (TSS) in the mixed liquor suspended solids (MLSS) of suspended growth biological wastewater treatment systems, such as activated sludge processes) and mixing conditions (e.g., shear forces, duration).

3. Existing OECD Test Guidelines (TG) may apply to some extent to estimate properties such as adsorption to and desorption from sludge (e.g. via TG 106 (OECD, 2000) or TG 121 (OECD, 2001a), or behaviour in wastewater (e.g. removal using wastewater simulation tests in TG 303 (OECD, 2001b) or TG 314 (OECD, 2008)). However, limitations exist, and specific considerations must be addressed

before deciding on the suitability of a TG for MNs. For example, Annex 5 in TG 303 does discuss the application of the method to poorly water-soluble substances, of which MN may be characterized. The method discusses utilizing a solid liquid mass balance approach for determining if the material had been "degraded" or removed from the effluent. However, there is no outlined procedure for monitoring the removal/degradation of inorganic contaminants. OECD Test Guideline TG 318, Dispersion Stability of Nanomaterials in Simulated Environmental Media (OECD, 2017) does provide a detailed over view of the physicochemical properties that will affect the stability of a MN suspensions and provides several methods for quantifying the stability of MN suspensions under varying environmental conditions. However, the focus of the methods discussed is on the stability of the MN suspension in the absence of other colloids or suspended solids.

4. The current report is intended to help bridge the current gap that exists in OECD TGs to quantify the association of MN with biomass in WWTP and to understand the utility of the proposed test method in the context of wastewater treatment screening and testing methodologies. As such the method differs from those TG previously mentioned by focusing on measuring the removal of MN through association with sewage sludge using an approach that differs from both TG 303 and 318. As knowledge and experience increase on the properties and behaviour of various types of MN, understanding on the utility of the method studied in this document will inform its possibility to complement existing OECD TGs, as such, modified, or in an overall strategy.

2 SIGNIFICANCE AND USE

5. This document includes a detailed description of a procedure for measuring the net removal extent (NR) to which a MN distributes between activated sludge and water in wastewater treatment systems. The goal of this test is to provide sufficient information to predict the removal of a test MN in a wastewater treatment facility through association with sludge. The distribution of conventional contaminants between supernatant and biomass is often described by a sorption isotherm (Pagga, U. and K. Taeger, 1994). These isotherms can be used to develop mass balances expressions for WWTP unit processes to estimate the amount of the chemical that will be removed during wastewater treatment. The Activated Sludge Sorption Isotherm, in Fate, Transport, and Transformation Test Guideline (OPPTS 835.1110) method uses freeze-dried biomass and has been validated for neutral and ionized organic chemicals and dissolved metals (USEPA, 1998). However, the OPPTS 835.1110 method has recently been demonstrated ineffective for predicting the removal of a variety of different MNs during wastewater treatment (Ag, Au, Ti) (Kiser, M.A., et al., 2009, 2010; 2012). The freeze-drying process significantly alters the physical size and structure of the biofloc, which reduces interaction with the MNs. The current document provides an update to the existing method that may be used for predicting the removal of MNs during wastewater treatment by employing the use of fresh biomass/biofloc collected from a WWTP or generated under laboratory conditions.

6. MN interactions with biofloc under most WWTP conditions reaches a steady-state condition related to aggregation and/or dissolution, rather than thermodynamic equilibrium that exist for some organic chemicals (e.g., neutral organic molecules). Over time, the processes that bring MNs into association with larger suspended solids (heteroaggregation) and those involved in the release from heteroaggregates (break-up) may reach a steady state. This steady state describes the maximum amount of MNs that may be removed by settling alone. This removal will vary as a function of mixing conditions and the chemistry of the suspended solids and nature of the MNs under testing.

7. Evaluations of the net removal of MNs observed at bench scale conditions provide useful information on what might be expected at full scale. The test procedures presented here are formulated to be used as a screening level assessment for estimating the removal of MN from wastewater due to association with biomass.

3 SCOPE

8. This study report proposes a procedure for evaluating the association of MN with mixed liquor suspended solids (MLSS) to advance a screening level assessment of the removal of MNs during wastewater treatment. The study relies upon measuring the quantity of the MN removed from a suspension following prescribed mixing periods and biomass concentrations to estimate the amount of MN associated with the sludge as a function of biomass concentration.

4 INITIAL CONSIDERATIONS

9. In this study, we propose to evaluate the reproducibility of a protocol designed to estimate the removal in batch bench-scale experiments of MNs under a set of conditions of MLSS, mixing, temperature and residence time using return activated sludge from a variety of WWTPS. The test conditions are selected to simulate removal in the activated sludge and clarifier unit processes of a WWTP that will allow for extrapolation to operating conditions that are outside of the test protocol.

10. By setting the conditions in the test to mimic wastewater treatment (e.g., amount of biomass per volume of water), the measured percentage removal of MN at a small number of concentrations from the water phase will provide the evaluator with a rough estimate of the percentage removal to be anticipated in WWTPs and, together with other information, will justify the release numbers used in the environmental exposure assessment. A 0% WWTP removal is used in assessments unless relevant data is available in the literature or produced by the manufacturer. Research groups have demonstrated the ability to use batch bench-scale MN removal data to model and/or match continuous-flow lab-scale reactors or full-scale WWTP removals of MNs (Kiser, M.A., et al., 2009; Westerhoff, P., et al., 2011). The benefits of the batch bench-scale protocol and simpler tests than continuous-flow reactors are the ability to simulate a larger range of site-specific conditions (temperatures, MLSS levels, hydraulic residence times, etc.) using a smaller mass of MNs.

5 PROPOSED REFERENCED SUBSTANCE

11. A reference MN is defined to ensure the test system is functional and responsive, and that data can be compared across studies using the same reference MN. The reference MN will be defined in a future standardized method or Guideline, as well as the expected outcome of the test. A candidate reference material for use should:

- Be easily detectable by the specific method of detection;
- Have low elemental abundance in the biomass;
- Be non-reactive;
- Have a narrow particle size range;
- Be stable in suspension.

12. In the current study, a gold (Au) MN is used as it fulfils the above criteria. Au MNs may be purchased for a nominal fee, are easily detectable by ICP and UV/Vis analysis, have a low elemental abundance in WWTPs, are non-reactive over short time periods, and may be purchased with a narrow size distribution.

6 PRINCIPLE OF THE TEST

13. The current method evaluates the partitioning of MN to return activated sludge (RAS) collected from a wastewater treatment facility. The method is based on a simple experimental procedure of conducting a sorption isotherm on buffered biomass samples at multiple solid: solution ratios and concentrations of nanomaterials mixed for a set time and then allowed to settle prior to sampling. Analytical techniques for determining the concentration of MN remaining in solution after the test is left to the discretion of the experimenter, as one specific analytical technique is not suitable for all nanomaterials.

14. The method only evaluates the MN concentration remaining in solution after equilibration with the active biomass. The method does not distinguish between sorption onto biomass or homo/heterogeneous aggregation of particles. The method does not account for specific chemical transformation of the MN being investigated, e.g. dissolution, redox transformation, and/or secondary precipitation. Further details on the test procedure, material, method, analysis and reporting are available in this report in Section 10.

15. The report presents the results from a series of experiments conducted by a variety of researchers within the United States. The purpose of the study was to determine:

- if MN sorption/partitioning to active biomass was similar between different wastewater treatments plants representative of different parts of the country,
- if results from Au-NP removal from the same biomass is reproducible between labs,
- if different wastewater streams e.g. urban or rural result in differences in Au-NP removal, and
- whether Au-NP sorption/partitioning to biomass collected from the same WWTP at different time periods throughout the year vary. For the present study, a gold nanomaterial suspension stabilized with a tannic acid coating was used.

16. Two target concentrations of gold (0.2 and 2.0 mg L^{-1} Au) and up to three different concentrations of biomass (250, 1250, and 2500 mg L^{-1} TSS) were evaluated in each assay.

17. The initial suggestion was to conduct a round robin of experiments using locally procured activated sludge and a tannin coated nano gold reference material (~12 nm) as the first MN. However, resource limitations in the lead country (United States) and OECD member countries did not allow moving to a broader ring-test with more MN tested across multiple laboratories.

7 STUDY DESIGN

18. Five laboratories were recruited to participate in the method evaluation, the laboratories included: Arizona State University (ASU), University of Arizona (UA), University of Kentucky (UK), University of Missouri (U Mizzou), and the U.S. Environmental Protection Agency, Office of Research and Development (EPA). UK provided the engineered nanomaterials for the test, gold nanoparticles 14 ± 3.1 nm in diameter with a tannic acid coating. Complete details of the physicochemical properties of the gold nanomaterials may be found in Judy et al. 2012. Participants were given a copy of the method and a spreadsheet for reporting data. The first page of the spreadsheet gathered basic information about the planned test, the biomass to be used, and the instrument used for analysis (Table 1). The second sheet within the spreadsheet contained a table for recording results from the experiments. Participants only needed to enter results from the analysis of Au remaining in solution after the test period. If the data were entered into the appropriate cells, the spreadsheet would calculate the percent of Au removed from solution and the concentration of Au sorbed to the biomass. Calculation of the biomass affinity constant was not included in the spreadsheet provided, and participants were expected to determine the values based on the equations provide in the method.

Table 1. Physicochemical properties of the Au MNs and biomass for each lab.

Measured Characteristics of NP suspension		
Size (nm)		
Sizing Method		
Stock Concentration (from provider or measured in-house)	mg L^{-1}	
Volume of stock concentration for low-range testing	µL	
Volume of stock concentration for high-range testing	µL	
Experiment starting dose - low-range	mg L^{-1}	
Experiment starting dose - high-range	mg L^{-1}	
Background solution		
Background Composition		
Concentration	mM	
pH		
Measured characteristics of activated sludge		
Biomass stock concentration (Total suspended solids)	mg L^{-1}	
TSS in CONTROL TEST (No Sludge)	mg L^{-1}	
Experiment dose planned - low-range	mg L^{-1}	
Experiment dose actual - low-range	mg L^{-1}	
Experiment dose planned - mid-range	mg L^{-1}	
Experiment dose actual - mid-range	mg L^{-1}	
Experiment dose planned- high-range	mg L^{-1}	
Experiment dose actual- high-range	mg L^{-1}	
Measured pH (CONTROL - no sludge)		
Measured pH (low-range TSS dose) after test		
Measured pH (mid-range TSS dose) after test		
Measured pH (high-range TSS dose) after test		
Measured COD (CONTROL TSS dose)	mg L^{-1}	
Measured COD before test (low-range TSS dose)	mg L^{-1}	
Measured COD before test (mid-range TSS dose)	mg L^{-1}	
Measured COD before test (high-range TSS dose)	mg L^{-1}	
Supernatant COD after test (low-range TSS dose)	mg L^{-1}	
Supernatant COD after test (mid-range TSS dose)	mg L^{-1}	
Supernatant COD after test (high-range TSS dose)	mg L^{-1}	
Experimental Conditions		
Laboratory ware (glass or plastic)	type	
Mixing speed	rpm	
Mixing time	min	
Total matrix volume	mL	
Analytical Instrument		
Type		
Model		
Location		

Participants were asked to provide as much information as possible for each parameter.

8 TEST RESULTS

19. A total of four rounds of testing were conducted. All five labs participated in the first test round. Subsequent rounds of tests were conducted with a reduced number of participants and designed to address specific questions. For the first round, test participants were given a copy of the proposed method, a spreadsheet for recording experimental parameters/results, and a sample of Au MN provided by UK. Participating labs were expected to collect a biomass sample for the method. The experimental conditions utilized by each lab and the results from the biomass sorption are presented in Table 2 and Figures 1 through 3. Examination of the experimental protocols utilized indicated that participating labs followed different procedures for the method (Table 2). The initial method did not specify test parameters such as, volume of biomass, mixing time, settling time prior to sampling or a digestion method to ensure full digestion of the Au MN. Further, it was not clear to all participants that two separate initial Au concentrations were to be evaluated (0.2 and 2.0 mg L^{-1}). As a result, only two of the labs conducted Au sorption experiments on the 0.2 mg L^{-1} concentration, ASU and UK. Additionally, EPA evaluated an isotherm point at 1250 mg L^{-1} in addition to the 250 and 2500 mg L^{-1} TSS.

20. The percent removal of Au from the five different biomass samples is presented in Figure 1. As previously discussed, only two of the five labs conducted experiments at the 0.2 mg L^{-1} concentration, ASU, and UK. The percentage of Au removed was significantly different between the two labs at both biomass concentrations, with the ASU sludge removing twice the amount of Au. For both ASU and UK, the amount of biomass had negligible impact on the percent Au removal. This is surprising for the UK biomass since even an order of magnitude increase in biomass concentration did not result in a significant increase in the amount of Au removed from solution. At the 2.0 mg L^{-1} initial Au concentration, apart from ASU, there was a marked increase in the amount of Au removed based on the biomass concentration. For the UK biomass, the amount of Au removed nearly doubled with an increase in biomass, in contrast to the lower concentration of Au. Among the five biomass samples, similar amounts of Au were removed for the high biomass and high Au condition, with at least 60% of the Au being removed from solution. The difference in the percent removal of Au from the low biomass-high Au condition and the low Au concentration indicate that differences in biomass concentrations of samples impact Au MN removal efficiency and affinity for the surface.

Table 2. Physicochemical properties of Au MNs and Biomass measured by participants for Round 1 of the ring test.

Laboratory-Round	Units	UA-1	ASU-1	UK-1	EPA-1	U Mizzou-1
Size (nm)	nm	12	40	12	12	40
Sizing Method		DLS	DLS	TEM	DLS	DLS
Stock Concentration	mg/L	929	710	712	75	710
Experiment starting dose - low-range	mg/L	--	0.20	0.10	--	--
Experiment starting dose - high-range	mg/L	2.60	1.88	2.70	2.96	2.60
Background solution						
Background Composition		$NaHCO_3$	$NaHCO_3$	$NaHCO_3$	$NaHCO_3$	$NaHCO_3$
Concentration	mM	1.00	1.00	1.00	1.00	
pH		7.88	8	8.12	7.5	
Measured characteristics of activated sludge						
Biomass stock concentration (TSS)	mg/L	13388.0	13400.0	8125.0	6203.3	--
Experiment dose planned - low-range	mg/L	250.00	250.00	250.00	250.00	250.00
Experiment dose actual - low-range	mg/L	--	--	316.00	--	--
Experiment dose planned - mid-range	mg/L					
Experiment dose actual - mid-range	mg/L					
Experiment dose planned- high-range	mg/L	2500.0	2500.0	2500.0	2500.0	2500.0
Experiment dose actual- high-range	mg/L	--	--	3030.0	--	--
Measured pH (CONTROL - no sludge)		7.50	7.76	8.12	7.50	--
Measured pH (low-range TSS dose) after test		7.54	6.85	7.45	--	--
Measured pH (mid-range TSS dose) after test						
Measured pH (high-range TSS dose) after test		7.13	6.51	7.22	--	--
Measured COD (CONTROL TSS dose)	mg/L	--	--	--	--	--
Measured COD (low-range TSS dose)	mg/L	--	1980	394.5	--	--
Measured COD (mid-range TSS dose)	mg/L	--	--	--	--	--
Measured COD (high-range TSS dose)	mg/L	--	18800	3250	--	--
Experimental Conditions						
Glassware/Volume		50 mL	15 mL P	15 mL	50 mL P	250 mL P
Mixing speed	rpm	90	45	<100	50	100
Mixing time	min	60	60	60	60	1
Total matrix volume	mL	40	15	10	50	100

Table 2. continued. Physicochemical properties of Au MNs and Biomass measured by participants for each round of the ring test.

Laboratory- Round	Units	ASU-2	UK-2	EPA-2-LM	EPA-2-TE	EPA-2-UK
Size (nm)	nm	--	12	12	12	12
Sizing Method			TEM	DLS	DLS	DLS
Stock Concentration	mg/L	150.5	50	150.5	150.5	150.5
Experiment starting dose - low-range	mg/L	0.24	0.26	0.24	0.17	0.15
Experiment starting dose - high-range	mg/L	2.17	2.78	2.27	1.62	1.39
Background solution						
Background Composition		$NaHCO_3$	$NaHCO_3$	$NaHCO_3$	$NaHCO_3$	$NaHCO_3$
Concentration	mM	1.00	1.00	1.00	1.00	1.00
pH		6.63	--	6.85	7.08	6.91
Measured characteristics of activated sludge						
Biomass stock concentration (TSS)	mg/L	4832.00	4045.00	31219.00	11610.00	9963.00
Experiment dose planned - low-range	mg/L	250.00	250.00	250.00	250.00	250.00
Experiment dose actual - low-range	mg/L	--	258.00	--	--	--
Experiment dose planned - mid-range	mg/L	--	--	--	--	--
Experiment dose actual - mid-range	mg/L	--	--	--	--	--
Experiment dose planned- high-range	mg/L	2500.0	2500.0	2500.0	2500.0	2500.0
Experiment dose actual- high-range	mg/L	2582.5	2832.0	--	--	--
Measured pH (CONTROL - no sludge)		--	--	6.85	7.08	6.91
Measured pH (low-range TSS dose) after test		7.76	7.58	7.87	8.2	7.1
Measured pH (mid-range TSS dose) after test		--	--	--	--	--
Measured pH (high-range TSS dose) after test		7.29	7.13	7.01	7.25	7.58
Measured COD(CONTROL TSS dose)	mg/L	--	--	--	--	--
Measured COD (low-range TSS dose)	mg/L	--	360	--	70	14
Measured COD (mid-range TSS dose)	mg/L	--	--	--	--	--
Measured COD (high-range TSS dose)	mg/L	--	4395	--	304	69
Experimental Conditions						
Glassware/Volume		50 mL P	50 mL P	50 mL P	50 mL P	50 mL P
Mixing speed	rpm	50	50	50	50	50
Mixing time	min	60	60	60	60	60
Total matrix volume	mL	40	40	50	50	50

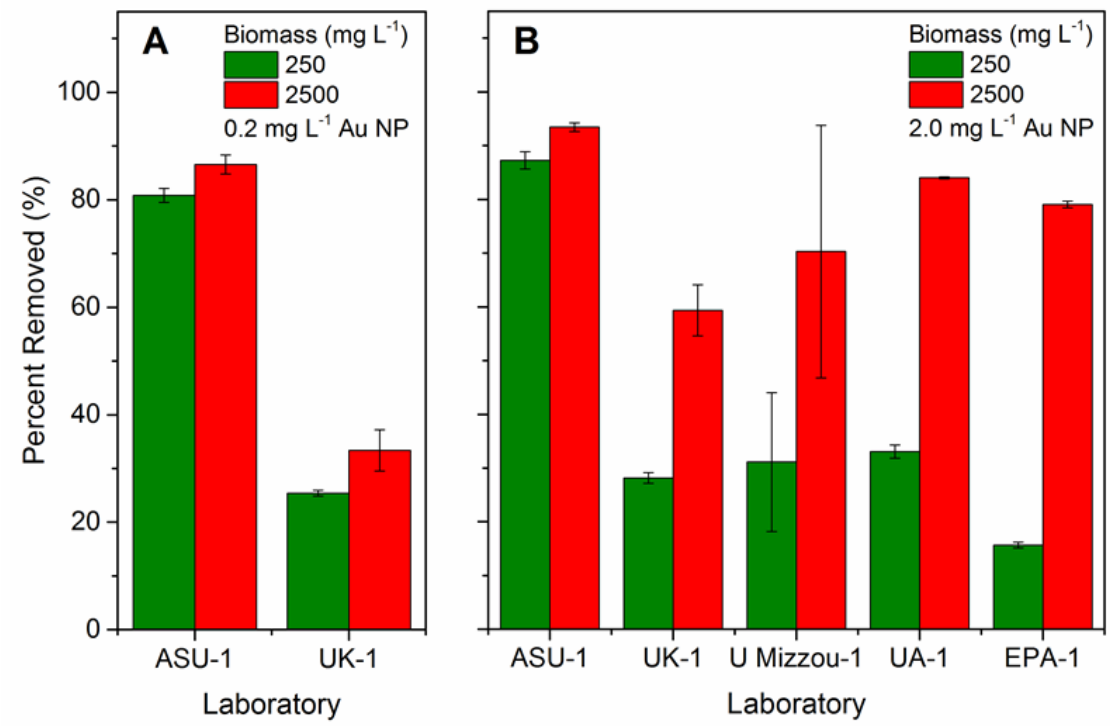
Table 2. continued. Physicochemical properties of Au MNs and Biomass measured by participants for each round of the ring test.

Laboratory Round	Units	EPA-3-ASU	EPA-3-LM	EPA-3-TE
Size (nm)	nm	12	12	12
Sizing Method		DLS	DLS	DLS
Stock Concentration	mg/L	131.93	131.93	131.93
Experiment starting dose - low-range	mg/L	0.19	0.22	0.23
Experiment starting dose - high-range	mg/L	--	2.24	2.19
Background solution				
Background Composition		$NaHCO_3$	$NaHCO_3$	$NaHCO_3$
Concentration	mM	1.00	1.00	1.00
pH		6.80	6.92	7.09
Measured characteristics of activated sludge				
Biomass stock concentration (TSS)	mg/L	12870.00	9740.00	5673.00
Experiment dose planned - low-range	mg/L	250.00	250.00	250.00
Experiment dose actual - low-range	mg/L	257.00	--	--
Experiment dose planned - mid-range	mg/L	1250.00	1250.00	1250.00
Experiment dose actual - mid-range	mg/L	1287.00	--	--
Experiment dose planned- high-range	mg/L	2500.0	2500.0	2500.0
Experiment dose actual- high-range	mg/L	2687.0	--	--
Measured pH (CONTROL - no sludge)		6.80	6.92	7.09
Measured pH (low-range TSS dose) after test		7.06	7.64	7.22
Measured pH (mid-range TSS dose) after test		7.03	7.32	7.24
Measured pH (high-range TSS dose) after test		6.86	7.27	7.57
Measured COD (CONTROL TSS dose)	mg/L	--	--	--
Measured COD (low-range TSS dose)	mg/L	262	582	410
Measured COD (mid-range TSS dose)	mg/L	1355	2562	1965
Measured COD (high-range TSS dose)	mg/L	2799	4968	3372
Experimental Conditions				
Glassware/Volume		50 mL P	50 mL P	50 mL P
Mixing speed	rpm	50	50	50
Mixing time	min	60	60	60
Total matrix volume	mL	50	50	50

Table 2. continued. Physicochemical properties of Au MNs and Biomass measured by participants for each round of the ring test.

Laboratory- Round	Units	ASU-4	EPA-4-ASU	EPA-4-LM	EPA-4-TE	Average	Standard Deviation
Size (nm)	nm	40	12	12	12	17	11
Sizing Method		DLS	DLS	DLS	DLS		
Stock Concentration	mg/L	710	133	131.93	131.93	311	291
Experiment starting dose - low-range	mg/L	0.22	0.15	0.22	0.21	0.20	0.04
Experiment starting dose - high-range	mg/L	--	--	2.16	2.08	2.26	0.43
Background solution							
Background Composition		$NaHCO_3$	$NaHCO_3$	$NaHCO_3$	$NaHCO_3$		
Concentration	mM		1.00	1.00	1.00	1.00	0.00
pH			8.31	6.92	7.09	7.29	0.54
Measured characteristics of activated sludge							
Biomass stock concentration (TSS)	mg/L	--	6800.00	9740.00	5673.00	10218.76	6383.93
Experiment dose planned - low-range	mg/L	250.00	250.00	250.00	250.00		
Experiment dose actual - low-range	mg/L	--	--	--	--	277.00	27.58
Experiment dose planned - mid-range	mg/L	1250.00	1250.00	1250.00	1250.00		
Experiment dose actual - mid-range	mg/L	--	--	--	--	1287.00	0.00
Experiment dose planned- high-range	mg/L	2500.0	2500.0	2500.0	2500.0		
Experiment dose actual- high-range	mg/L					2782.88	167.95
Measured pH (CONTROL - no sludge)			8.31	6.92	7.09	7.30	0.48
Measured pH (low-range TSS dose) after test			7.99	7.64	7.22	7.51	0.37
Measured pH (mid-range TSS dose) after test			7.44	7.32	7.24	7.27	0.12
Measured pH (high-range TSS dose) after test			7.23	7.27	7.57	7.21	0.28
Measured COD (CONTROL TSS dose)	mg/L						
Measured COD (low-range TSS dose)	mg/L		320	582	410	489.50	500.88
Measured COD (mid-range TSS dose)	mg/L		1480	2562	1965	1981.50	468.78
Measured COD (high-range TSS dose)	mg/L		2500	4968	3372	4436.09	4797.11
Experimental Conditions							
Glassware/Volume		50 mL P	50 mL P	50 mL P	50 mL P		
Mixing speed	rpm		50	50	50	56.54	16.57
Mixing time	min		60	60	60	55.79	15.19
Total matrix volume	mL		50	50	50	46.56	18.43

Figure 1. Average percent removal of Au MN for each of the five labs, in the first ring test.



A) Percent removal for the initial Au MN concentration of 0.2 mg L-1. B) Percent removal for the initial Au MN concentration of 2.0 mg L-1. Error bars represent one standard deviation of at least triplicates measurements.

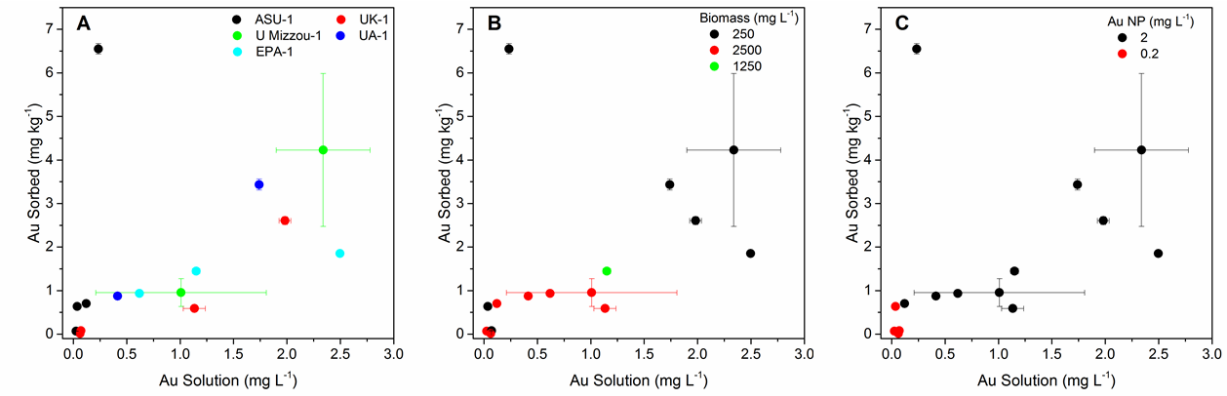
21. Sorption isotherms illustrate the relationship between the bulk solution concentration and the quantity of material adsorbed at equilibria. Sorption isotherms for the five biomass samples are presented in Figure 2. The isotherm data shows more variability in the data compared to the percent removal (Figure 1). Sorption isotherm data indicates the greatest amount of variability in data points were associated with the lowest concentration of biomass and the high initial Au concentration (250 and 2.0 mg L^{-1}, respectively) (Figure 2, 3, and 4). The results are not surprising since the number of potential sorption sites on the biomass surface would likely be limited given the low concentration of biomass. The Biomass Density Model, discussed in the method, was used to determine an affinity constant (*K*) of Au MNs for the WWTP's biomass based on the results from the sorption isotherms. For the purposes of the current ring tests the data was plotted on a log-log plot and Equation 2 (Section 10) was log transformed to create an equation for a straight line.

$$\mathrm{Log}\, BD = \frac{1}{n}\mathrm{Log}\, C_f + \mathrm{Log}\, K$$

22. In the log transformed equation the slope of the line is equal to *1/n* and the *y*-intercept is equal to Log *K* (affinity constant). Values for Log *K* and *1/n* were determined from the linear regression of the log transformed data. Linear regressions were performed on each individual biomass and the entire data set from all five biomass samples (Table 3 and Figure 3). In addition to the linear regression, 95% confidence intervals were calculated and data outliers identified. The linear regression model explained only 47% of the variability within the data set—again highlighting the variability within the data. A comparison of the Log *K* and *1/n* values determined for each individual biomass sample and the entire data set were close in value to the average *K* and *1/n* values with the obvious exception of the ASU data. For the ASU biomass,

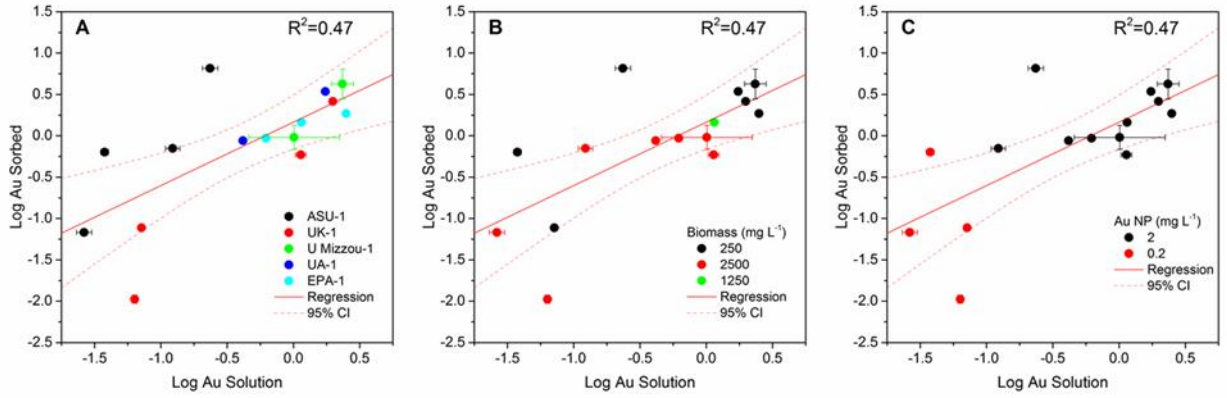
the predicted value for *1/n* was similar to the other biomass samples but the value of *K* was significantly greater (Table 3). With respect to the entire data set, the standard error calculated for *1/n* was reasonable, less than 15% of the value. However, the standard error for Log *K* was 2/3 the value, 0.12 ± 0.08, indicating a very high degree of potential error for a parameter to be used to predict the distribution of a MN between the solid and solution phase.

Figure 2. Gold MN adsorption isotherms from the first ring test as a function of the final Au solution concentration.



A) Results presented for each of the laboratories participating in the test. B) Results presented for each of the initial biomass concentrations investigated. The 1250 mg L^{-1} biomass concentration was only conducted by EPA. C) Results presented for each of the target initial Au gold solution concentrations of 2.0 or 0.2 mg L^{-1}. The 0.2 mg L^{-1} initial Au concentration was conducted by only ASU and UK. Error bars represent one standard deviation of at minimum of triplicate measurements.

Figure 3. Log normalized Au sorption isotherms and linear regression results of the log transformed data from the first round of testing.



A) Results presented for each of the laboratories participating in the test. B) Results presented for each of the initial biomass concentrations investigated. The 1250 mg L-1 biomass concentration was only conducted by EPA. C) Results presented for each of the target initial Au gold solution concentrations of 2.0 or 0.2 mg L-1. The 0.2 mg L-1 initial Au concentration was conducted by only ASU and UK. Error bars represent average difference between the mean value and a minimum of three individual data points.

Table 3. Results from the linear regression of the log normalized sorption data from the first ring test.

The R2 value is only presented for linear regressions that included data combined from multiple laboratories.

Lab	Slope		Intercept		K	R^2
	1/n	Std. Error	Log K	Std. Error		
Round 1	**0.76**	**0.2**	**0.16**	**0.15**	**1.44**	**0.47**
ASU-1	1.61	0.27	1.65	0.33	45	
UK-1	1.23	0.11	-0.12	0.09	0.8	
U Mizzou-1	0.56	0.54	0.2	0.17	1.6	
UA-1	0.95	0.02	0.31	0.01	2	
EPA-1	0.48	0.05	0.1	0.01	1.3	
Lab Ave-1	**0.97***	**0.47****	**0.43***	**0.70****	**10.13***	

*Average of the individual Biomass Density Model results for each separate biomass samples
**Standard deviation of the individual Biomass Density Model results for each separate biomass samples.

23. Based on the first ring test the participating labs (EPA, ASU, UK, UA, and U Mizzou) hypothesized the differences in the total volume used in the test, the mixing time, and settling time impacted the partitioning of Au to the biomass and the total percentage of Au removed. To address the issue, specific instructions on the total volume of the solution used in the test and the specific mixing/settling time were specified in the Sludge Net Removal Experiment section. In addition to establishing consistent methodologies between labs, additional questions were addressed: 1) Is the Au sorption and removal by a specific biomass repeatable between laboratories? 2) Is Au sorption and removal constant for a biomass collected on different dates from the same WWTP?

Second Ring Test

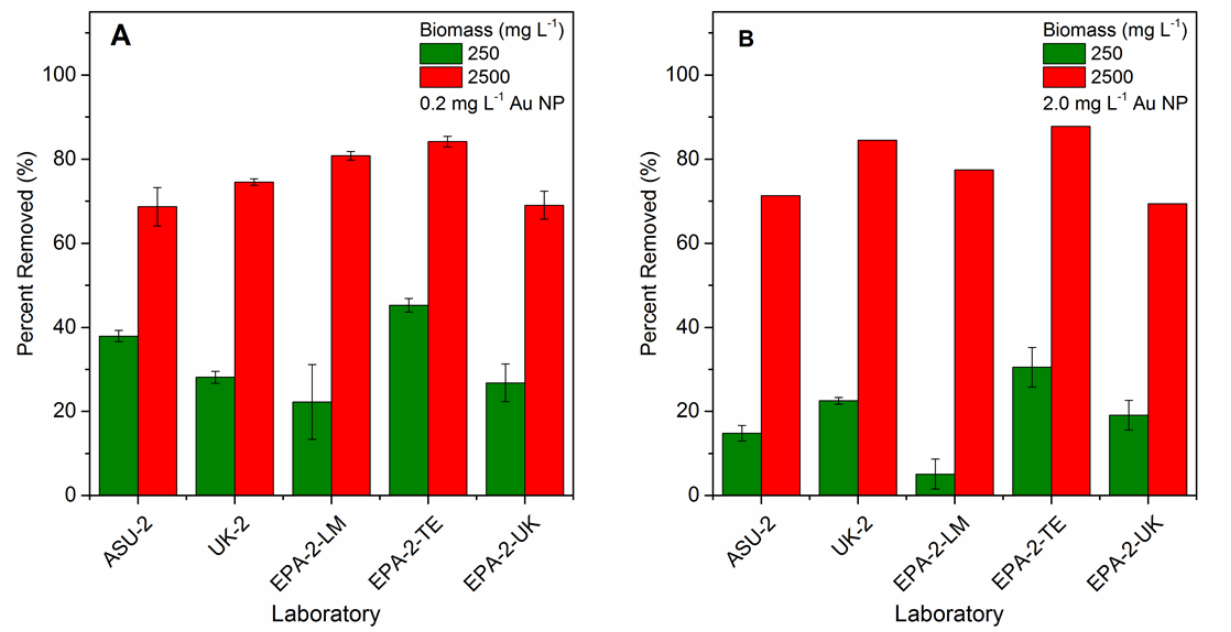
24. For the second ring test only EPA, ASU, and UK participated and only two initial biomass concentrations (250 and 2500 mg L^{-1}) were investigated. The method was updated to reflect specific volumes to use for the assay, time intervals for mixing and settling, and consistent method for digesting the solute prior to analysis. The objective of the second test round was to determine if additional details included within the methodology would reduce the variability in the results. Results of the ring test were analysed to determine if a more consistent methodology resulted in reduced variability. In the second round, two different biomass samples were tested by EPA. One biomass sample was collected from a WWTP that treated exclusively urban effluent (TE), the second biomass sample was collected from a WWTP that serviced a more rural environment (LM). Finally, EPA conducted a third assay on biomass supplied by UK to see how differences in laboratory procedures would influence Au MN removal. In the second round all three labs used two concentrations of Au MN 0.02 and 2.0 mg L^{-1}.

25. Results for the percent removal of Au at two initial concentrations are presented in Figure 4. The percentage of Au removed during the test was more similar between biomass samples tested. For all the biomass samples tested there was significantly more Au removed from solution at the higher biomass concentrations. The average percentage of Au removed for the initial concentration of 2.0 mg L^{-1} and the low biomass concentration across all biomasses was 18% or approximately 0.36 mg L^{-1} of the Au MN. However, at the initial 0.2 mg L^{-1} concentration, no more than 50% of the Au was removed with identical biomass concentrations. These results would suggest that a simple sorption hypothesis for describing the partitioning of Au to biomass may not fully cover the true mechanisms at play.

26. The sorption isotherm data was less variable between labs and within triplicate samples (Figure 5). As with the first round of testing the highest degree of variability within samples and between labs was associated with the 2.0 and 2500 mg L^{-1} concentration for Au and the biomass, respectively. Linear regression results from the log transformed data showed the linear model explained 79% of the variability within the data, up from 47% of the variability in the first round (Figure 6 and Table 4). The biggest

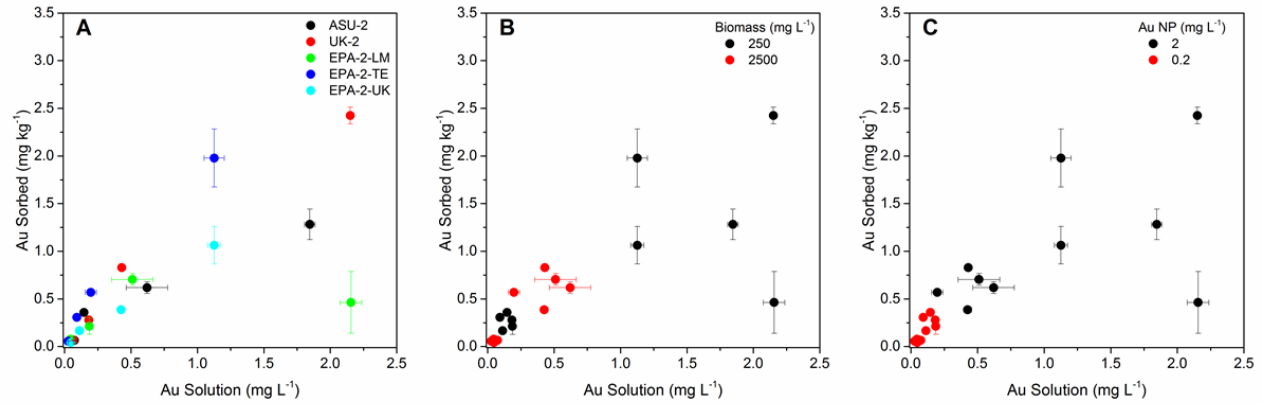
difference in the regression results between the first and second round was the reduced value for Log K indicating a reduction in the affinity of the Au for the biomass surface. This is related to the difference between the Au sorption data for the ASU biomass between the first and second rounds of testing. The percent of Au removed by the biomass was considerably lower in the second round, and the maximum amount of Au sorbed to the biomass was reduced from 6.6 to 1.3 mg_{Au} $kg^{-1}_{biomass}$. The biomass supplied to EPA by UK was shipped unpreserved overnight on wet ice. Upon arrival, the biomass was immediately washed following the method guidelines and the Au sorption studies were conducted the following day. Results from the inter lab comparison (UK-2 versus EPA-2-UK) showed comparable results in the percent of Au removed by the biomass with slightly more Au removed from solution by the biomass sample analysed at UK. The isotherm results were similar as well with an increasing difference between the two labs at higher ratios of Au to biomass. The quantity of Au partitioning to the biomass was greater for the biomass sample that was tested at UK. The reason for the reduced removal and sorption of Au by the biomass that was shipped to EPA from UK is unknown.

Figure 4. Average percent removal of Au MN for each of the five biomass samples, in second round of testing.



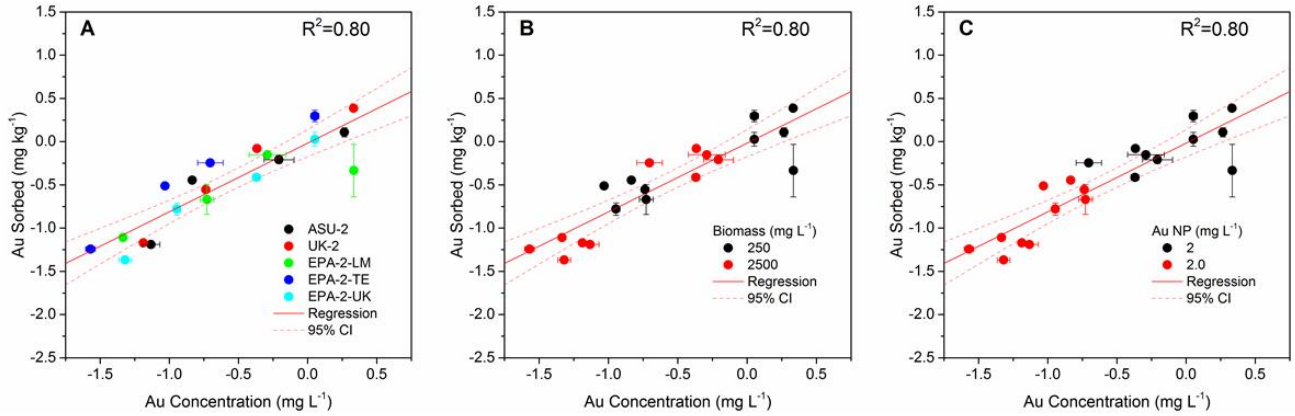
A) Percent removal for the initial Au MN concentration of 0.2 mg L-1. B) Percent removal for the initial Au MN concentration of 2.0 mg L-1. Error bars represent standard deviation of at least triplicates measurements.

Figure 5. Gold MN adsorption isotherms from the second ring test as a function of the final Au solution concentration.



A) Results presented for each of the laboratories participating in the test. B) Results presented for each of the initial biomass concentrations investigated. C) Results presented for each of the initial target Au gold solution concentrations of 2.0 or 0.2 mg L-1. Error bars represent standard deviation of a minimum of triplicate measurements.

Figure 6. Log normalized Au sorption isotherms and linear regression results of the log transformed data from the second round of testing.



A) Results presented for each of the laboratories participating in the test. B) Results presented for each of the initial biomass concentrations. C) Results presented for each of the initial target Au gold solution concentrations of 2.0 or 0.2 mg L-1. Error bars represent standard deviation of a minimum of triplicate measurements.

Table 4. Results from the linear regression of the log normalized sorption data from the second round of testing.

The R^2 value is only presented for linear regressions that included data combined from multiple laboratories.

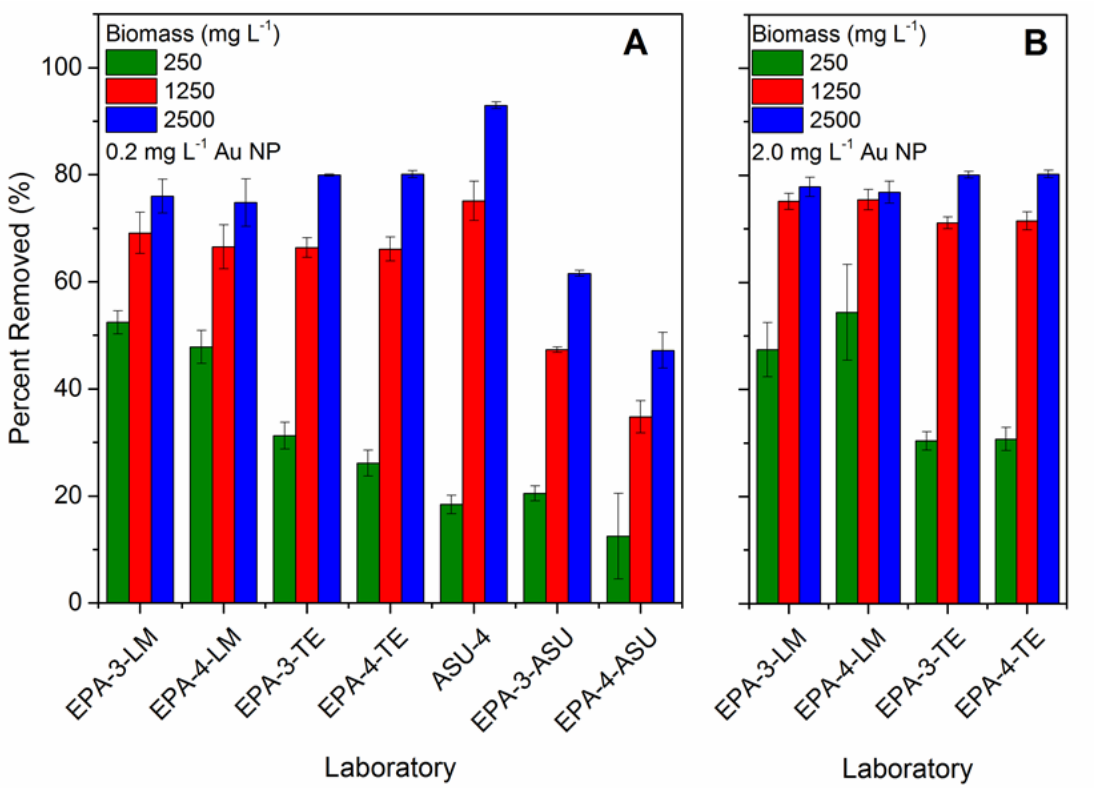
Lab	Slope		Intercept		K	R^2
	1/n	Std. Error	Log K	Std. Error		
Round 1	**0.76**	**0.20**	**0.16**	**0.15**	**1.45**	**0.47**
Round 2	**0.80**	**0.09**	**-0.02**	**0.08**	**0.95**	**0.79**
ASU-2	0.81	0.23	-0.04	0.17	0.91	
UK-2	1.01	0.135	0.14	0.1	1.38	
EPA-2-LM	0.46	0.36	-0.36	0.29	0.44	
EPA-2-TE	0.93	0.12	0.32	0.12	2.09	
EPA-2-UK	0.8	0.11	-0.06	0.06	0.87	
Lab Ave 1 & 2	**0.88***	**0.35****	**0.21***	**0.55****	**5.64***	

*Average of the individual Biomass Density Model results for each separate biomass samples
**Standard deviation of the individual Biomass Density Model results for each separate biomass samples.

27. Results from the second round of testing indicated that harmonizing the protocols used between labs reduced variability between labs and resulted in a more robust biomass sorption model that could be used for predicting Au NP partitioning to biomass. Additional rounds of testing were conducted by EPA and ASU to further validate the results from Round 2 and to see how variable repeated assay measurements were on biomass collected from the same WWTP and different times over a several month period. Results for the Au removal are presented in Figure 7. There is good agreement between results for the same biomass collected at two separate time points. There is good agreement in the results for the low biomass concentration between results obtained by EPA and ASU for biomass samples supplied by ASU. As with the UK-EPA results in Round 2, there was a decrease in the amount of Au removed at higher biomass concentrations. The ASU, LM, and TE biomasses were all tested simultaneously in Round 3 and 4 by EPA. There was little change in the percent removal of Au or the maximum sorption capacity of the LM or TE biomasses in round 3 and 4, indicating the reduction in ASU Au uptake was not related to inherent errors in the experimental protocol. Therefore, the change in Au removal is likely related to changes in the biomass properties either associated with the time after collection or transport of the material.

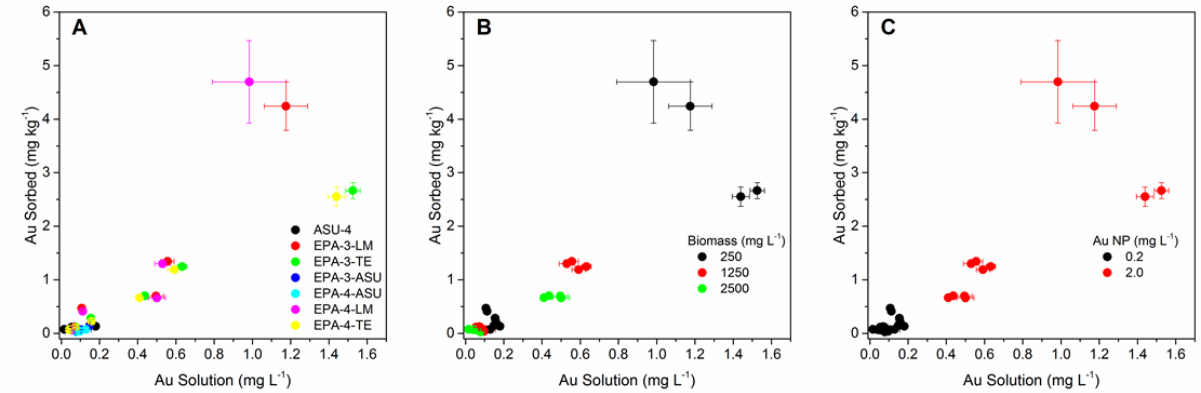
28. Linear regression of the log transformed data provided comparable results for Round 3 and 4 (Table 5), and the linear regression for the combined Round 3 and 4 data sets are presented in Figure 9. As with Round two the regression model explained close to 80% of the variability in the data.

Figure 7. Average percent removal of Au MN for each of the seven biomass samples, in third and fourth round of testing.



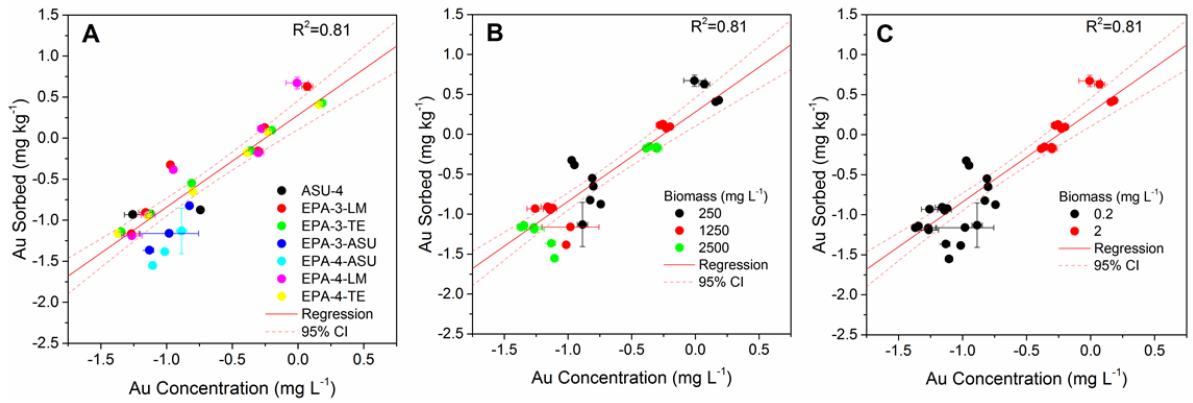
A) Percent removal for the initial Au MN concentration of 0.2 mg L-1. B) Percent removal for the initial Au MN concentration of 2.0 mg L-1. Error bars represent standard deviation of at least triplicate measurements.

Figure 8. Gold MN adsorption isotherms from the second ring test as a function of the final Au solution concentration.



A) Results presented for each of the laboratories participating in the test. B) Results presented for each of the initial biomass concentrations investigated. C) Results presented for each of the initial target Au gold solution concentrations of 2.0 or 0.2 mg L-1. Error bars represent standard deviation of a minimum of triplicate measurements.

Figure 9. Log normalized Au sorption isotherms and linear regression results of the log transformed data from the Third and Fourth Round of testing.



A) Results presented for each of the laboratories participating in the test. B) Results presented for each of the initial biomass concentrations. C) Results presented for each of the initial target Au gold solution concentrations of 2.0 or 0.2 mg L-1. Error bars represent standard deviation of a minimum of triplicate measurements

29. The 4 rounds of testing conducted identified initial issues with the methodology outlined in the original method. Subsequent rounds showed reduced variability between biomass samples tested and a more robust biomass density model. Table A2-5 (Annex) presents the biomass density model results from all four rounds along with the average of 17 individual biomass assays that were conducted. Results from the linear regression of the entire data set are presented in Figure 10. The data presented are well described by the model with 70% of the variability described. Looking at the average and standard deviation of individual regressions for each biomass shows a high degree of variability in the parameters for *1/n* and Log *K*, 1.01±0.46 and 0.24±0.49, respectively. While overall the chemical composition and reactivity of sludge does not vary widely, results for the removal of Au MNs from individual WWTPs did vary widely.

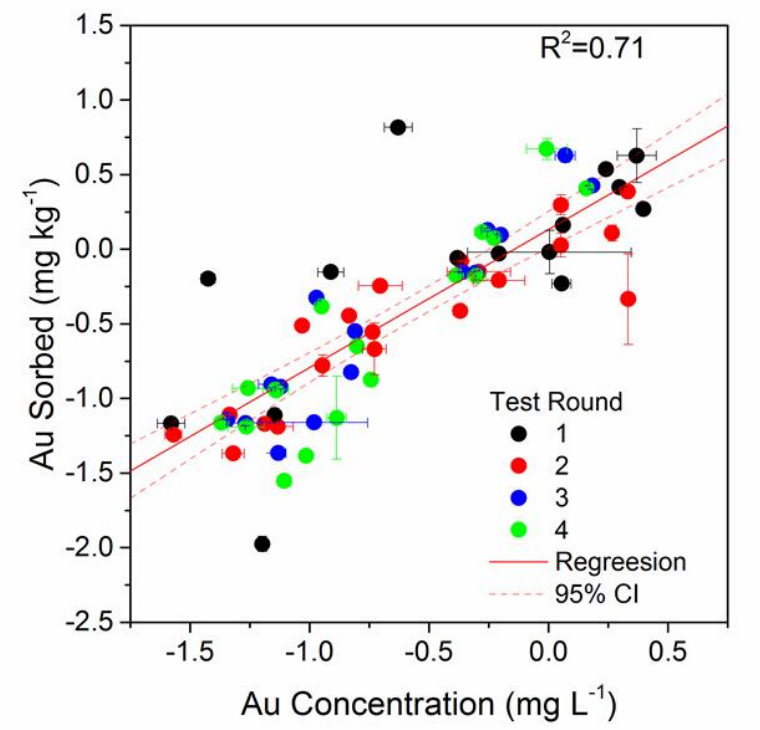
Table 5. Results from the linear regression of the log normalized sorption data from the Third and Fourth Round of testing.

The R^2 value is only presented for linear regressions that included data combined from multiple laboratories.

Lab	Slope		Intercept		K	R^2
	1/n	Std. Error	Log K	Std. Error		
Round 1	**0.76**	**0.2**	**0.16**	**0.15**	**1.45**	**0.47**
Round 2	**0.8**	**0.091**	**-0.02**	**0.08**	**0.95**	**0.79**
Round 3	**1.17**	**0.12**	**0.33**	**0.1**	**2.14**	**0.88**
Round 4	**1.07**	**0.15**	**0.23**	**0.14**	**1.70**	**0.74**
EPA-3-ASU	1.78	0.23	0.62	0.23	4.17	
EPA-3-LM	1.13	0.19	0.43	0.15	2.69	
EPA-3-TE	1.04	0.03	0.26	0.02	1.82	
ASU-4	0.23	0.07	-0.68	0.09	0.21	
EPA-4-ASU	1.93	0.05	0.58	0.05	3.80	
EPA-4-LM	1.23	0.2	0.49	0.17	3.09	
EPA-4-TE	1.05	0.04	0.25	0.03	1.78	
Round 3 & 4	**1.12**	**0.1**	**0.28**	**0.09**	**1.91**	**0.81**
Round 1-4	**0.92**	**0.07**	**0.13**	**0.06**	**1.35**	**0.71**
Round 2-4	**0.96**	**0.07**	**0.13**	**0.06**	**1.35**	**0.78**
Lab Ave 1-4	**1.01***	**0.46****	**0.24***	**0.49****	**1.74***	

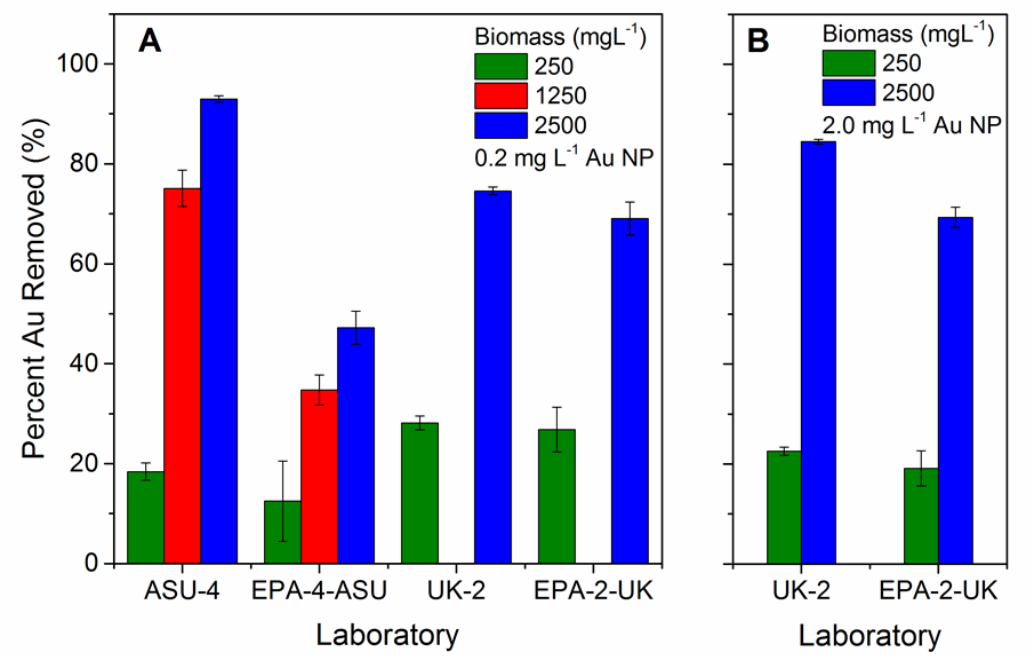
*Average of the individual Biomass Density Model results for each separate biomass samples
**Standard deviation of the individual Biomass Density Model results for each separate biomass samples.

Figure 10 Log normalized Au sorption isotherms and linear regression results of the log transformed data from all four rounds of testing. Results are presented for each round of testing.



30. In testing rounds 2 through 4 the EPA lab repeated the Au sorption experiments using biomass collected by UK and ASU. For both samples the percent of Au removed decreased (Figure 11). The reduction in percent removal of Au was greater for the ASU then UK biomass tested by EPA. The reason for the reduction related to the aging of the biomass. The EPA-2-UK assay was conducted 1 day after the UK-2 assay and the EPA-4-ASU experiment was run 2 days after the ASU-4 experiment. Further research on the role of aging would be of key interest in furthering the method development.

Figure 11. Average percent removal of Au MN for replicate biomass samples analysed by two laboratories.



A) Percent removal for the initial Au MN concentration of 0.2 mg L-1. B) Percent removal for the initial Au MN concentration of 2.0 mg L-1. Error bars represent standard deviation of at least triplicate measurements.

9 STUDY SUMMARY AND CONCLUSIONS

31. Initial results from the ring test after the first round showed a high degree of variability between participating laboratories in the study. A review of the experimental parameters used by each lab revealed a wide discrepancy in how the method was conducted. A subsequent update to the method and ring test by a smaller test group showed reduced variability in the data between labs indicating the primary source of variability in the first round was method related. Subsequent rounds of testing, and inter-laboratory comparisons, and a time series analysis of two biomass samples demonstrated the method was robust, and that variations in the composition and chemistry of the biomass did not result in large differences between labs. However, inter-laboratory comparisons using the same biomass always showed reduced affinity of the Au for the biomass that was transported to another laboratory. Every attempt was made to ensure biomass samples remained cold (> 4^{o}C) during transport and experiments were conducted at both laboratories within 48 h of collection. The immediate reason for the difference in behaviour is unknown. However, it is suspected the age of the biomass suspension may be related.

32. Based on the results of the four rounds of testing the test participants believe the results are promising and the method will be an effective method for screening the potential for MN to be removed during water treatment. The test participants would recommend the lowest biomass level (250 mg/L) be dropped from the test. The method should consider higher biomass concentrations that are more representative of WWTPs. Additionally, the test participants would recommend additional testing on other MN to ensure the method is robust for a wide variety of materials. However, the participants recognize that working with more soluble/reactive materials (Ag, CeO_2, transition metal oxides) or hydrophobic material (CNT and graphene) will introduce additional variables for consideration.

33. In addition to estimating removal of MN during wastewater treatment, the test has the potential to be used to quantify the removal of micro or nano-plastics that may be present. The concepts discussed and presented would allow for a broader array of materials to be considered. The main difference between using the method to estimate removal on inorganic MN and plastics the would be the analytical method chosen to quantify the amount of material remaining in solution after settling of the biomass.

10 ANNEX- DETAILED TEST PROCEDURE APPLIED IN THE STUDY

Definitions and units

34. Definitions and units are set out in Appendix 1.

Principle of the Test

35. This test describes a procedure to estimate the net removal (NR) of manufactured nanomaterials (MN). The range of concentrations investigated for the Rinsed Activated Sludge (RAS) and the MN of interest is up to the user to define. It is suggested that at a minimum two RAS and two MN concentrations are investigated whose concentrations would differ by an order of magnitude. The method described below uses sample biomass and MN concentrations. The test is designed to quantify the extent to which a MN distributes between two phases of the MN, activated sludge and water in wastewater treatment systems. Most WWTPs have hydraulic retention times longer than this period necessary to reach steady-state conditions. Therefore, a fixed percentage removal of MNs under a given set of conditions (Mixed liquor suspended solids [MLSS] level (*i*), temperature, mixing time) calculated as follows:

$$Net\ Removal\ (NR_i) = \left[1 - {C_f}/{C_0}\right] \times 100\%$$

Equation 1

where C_f, is the concentration of MN_i remaining in the supernatant at the end of a batch test with a single MLSS level (mg/L), and C_0 is the initial MN concentration.

36. Experiments conducted at different MLSS levels (*i*) can be used to fit removal data to a simple biomass density (BD) exponential model shown in equation 2, where BD is the MN concentration associated with the biosolids (e.g., mg MN / g TSS), K is an empirical value related to the affinity of the MN to undergo hetero-aggregation with the biomass., and 1/n is a fitted constant specific to temperature, mixing condition, etc.). This model can be used to estimate removals over a range of MLSS concentrations.

$$BD = K\ C_f^{1/n}$$

Equation 2

37. Preliminary knowledge of the dispersion stability of the test MN is useful before undertaking a sludge association test. If the test chemical will not maintain a stable dispersion, it is possible the material will not be associate/interact with the sludge, and instead deposit along the bottom and surfaces of the container. Dispersion is affected by characteristics of the media, requiring consideration of characteristics of influent. Additional details on generating a stable MN suspension are provided in the section Preparing Nanoparticle Suspension. A stable suspension in the context of the current method would be no detectable aggregation or settling for the time period of the test (4h). Generating a stable suspension of MN is critical for the test. If the MN does not form a stable suspension the current test as described would not be suitable. The current method determines partitioning of the MN to the biomass by measuring the quantity of on MN remaining in solution after settling and dividing that quantity by the initial concentration of MN (Equation 1). If there is uncertainty in the initial MN concentration due to an unstable MN suspension it will not be possible to determine with a high degree of accuracy the quantity of MN removed. OECD TG 318 should be consulted to determine if the test MN forms a stable suspension. If the MN utilized does not form a stable suspension a complete mass balance of the system is required which would include quantifying the MN in solution and in the settled solid phase. The mass of MM in solution and in the solid phase would be used to calculate C_o in Equation 1 using the following equation

$$C_0 = C_f + \left(q_f + TSS\right)$$

Equation 3

Where C_f is the concentration of MN (ug L^{-1}), q_f is the solid phase concentration (μg mg^{-1}), and *TSS* is the total suspended solids (mg L^{-1})

38. If the MN has functional groups that may biodegrade or undergo other transformation processes these should be considered before beginning the test. If the submitter considers that during processing, formulation, use, or release the MN will undergo transformation before arriving at a wastewater treatment plant, it is advised to consider testing of the most relevant MN species, which may not be the original MN as manufactured. The test method is based on the procedures developed during the work described in the references (Ganesh, R., et al., 2010; Kiser, M.A., et al., 2010, 2012; Pagga, U. and K. Taeger, 1994; USEPA, 1998; Hyung, H. and J.H. Kim, 2009).

39. The key parameters introduced above should be chosen to represent a wide range of biomass concentrations that may be present within a WWTP. The ionic strength of the system and pH is buffered with 1 mM $NaCO_3$ to provide a consistent background between varying MLSS obtained at local sewage treatment plants. The goal of this testing is to provide sufficient information to help predict the removal of a test MNs to sludge in wastewater treatment through association with sludge.

40. A critical component of the test is the ability to quantify the MN remaining in solution after settling of the biomass. The method used to quantify the MN remaining in solution will be based upon the MN tested and the desired results. If the total elemental abundance of a MN remaining in solution after partitioning is of interest, the sample may be chemically digested and analysed by the appropriate technique. For inorganic MN (metals, metal oxides, or quantum dots) an acid assisted digestion may be appropriate for quantification using an emission or mass-based spectrometry. If physicochemical properties on the particles remaining in solution is of interest (particle size, aggregation state, particle concentration, dissolution extent) preservation techniques should be employed to preserve the suspension prior to analysis by the technique of interest.

Apparatus and Chemical Reagents

41. Standard laboratory equipment, including but not limited to:

a. 50 mL graduated plastic centrifuge tubes and racks (testing metal and metal oxide MNs).
b. 40mL borosilicate glass vails and racks (for testing carbonaceous nanomaterials).
c. 1-L Bottles.
d. 2-L glass volumetric flask.
e. Centrifuge.
f. Magnetic stir bar and plate.
g. Oven/Muffle Furnace capable of reaching 105^{o} C.
h. Balance 0.01 mg resolution.
i. Glass fiber filter, nominal pore size 1.5 micron (μm) (Whatman #8) and filtration setup.
j. pH probe.
k. Chemical oxygen demand test kit.
l. Hotplate/Block for sample digestion capable of maintaining 95^{o} C.
m. Nitrocellulose filter membrane 0.45 micron

42. Materials:

a. Distilled Deionized Water (18 MΩ DI water).
b. Minimum of 1-L Return Activated Sludge (RAS) from local water treatment municipality.
c. Buffer Matrix Solution 1-mM $NaHCO_3$. Approximately 1 to 2 L of the buffer/matrix solution will be required, depending on the number of samples and controls.
d. Dissolve 1.68 g of $NaHCO_3$ in Distilled Deionized water and dilute to 2 L.

43. A Microsoft® Excel worksheet to be used for entering data in from the experiment and to calculate the net removal in Equation 1 and the affinity constant (K) in Equation 2 is available on the OECD website

General Conditions and Quality Check Measure

44. The concentration of the biomass (e.g., gTSS/L) used in the testing should be relevant to wastewater treatment systems. A control sample (no MN added) should be considered as a quality control check. A non-reactive and well-dispersed MN that is stable in suspension should be included for a consistency or quality control check. Due to a low background of gold, a well-dispersed and stable suspension of gold nanoparticle is recommended as a quality check due to its low solubility and reactivity.

Preparation of Test Nanoparticle Suspension

45. Prior to preparation of the biomass stock solution, the specific biomass and nanoparticles concentrations to be evaluated need to be identified in Table 6. This is critical in order to appropriately prepare the biomass suspension for the test. If the nanomaterial to be tested is supplied/produced as liquid suspension, this stock dispersion is directly diluted into the required test concentrations. The dispersion stability of the nanomaterial should be determined, as described in OECD Test Guideline 318 (OECD, 2017). As the state of dispersion can influence the rate of dissolution, and where practical, should be monitored throughout the test. In case of high degree of agglomeration in the stock dispersion

(agglomerate size >> primary particle size) it may be considered to add some energy into the system for example by sonication or agitation. If sonication is performed the potential influence of sonication on dissolution should be considered and the energy input should be quantified and reported.

46. If the nanomaterial to be tested is supplied/produced as a powder it must be dispersed in the aqueous media prior to testing. In this case, the stock dispersion should be freshly prepared (<1 hour) prior to making the working suspensions (diluting the stock dispersion in the selected test media).

47. To prepare a nanomaterial stock dispersion from powder, reference the protocol described in OECD Test Guideline No 318 (OECD, 2017). Very briefly, this method includes the following steps (Figure 12):

a. The nanomaterial is pre-wetted in deionized water and left as a wet paste for 24 h to insure the proper interaction of material surface with water.

b. The wet paste is dispersed into known volume of ultrapure water, thus providing a stock suspension with a known material concentration.

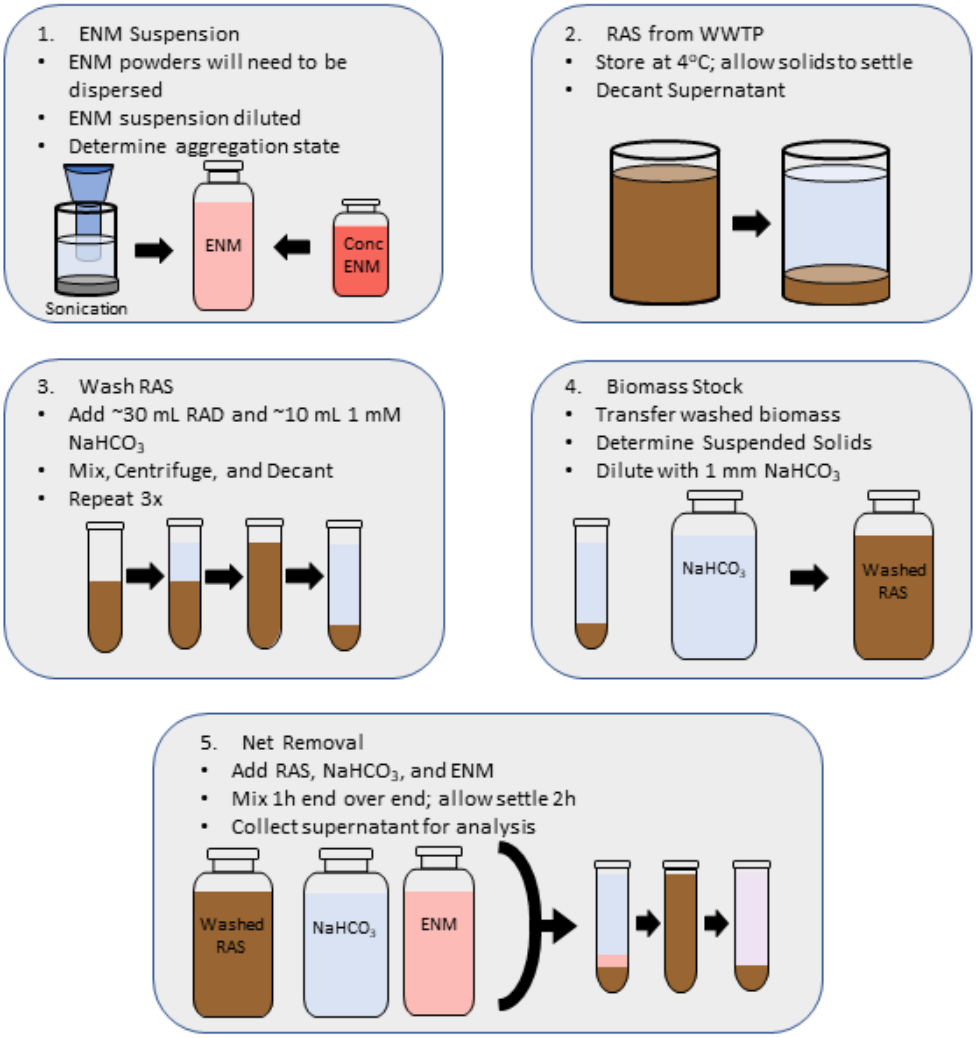
c. The concentration of stock dispersion shall be sufficient to allow the further dilution to the desired test concentrations but should not exceed the concentration of nanomaterial within test samples more than 20 times. Hence, if 1 mg/L is the highest tested concentration, the stock dispersion should not exceed 20 mg/L.

d. Usage of sonication probe is mandatory. The energy input to the dispersion from the sonication probe should be calibrated (supporting information and tools can be found in TG 318).

e. 125 mL stock dispersion is prepared in a 250-mL glass beaker placed in an >1L ice bath and sonicated using an ultrasonic probe ½" or 13 mm diameter, tip placed in the center, 2.5 cm below surface. It is recommended to begin at 40 W output power for 10 min, although a higher power output may be needed based on material type. Users should consider the tradeoff of dispersing materials versus changing the materials and utilize the lowest W possible to enable dispersion.

f. The resulting stock dispersion is stored in an amber glass container at 4°C until use within a 24 h period.

Figure 12. Flow diagram of the laboratory steps involved in preparing nanomaterial and biomass samples for determination of net removal.



Collection and Preparation of Biomass

48. Collect RAS (Return Activated Sludge, solids separated from the treated wastewater) from a local WWTP. Collect RAS in 3 to 5 sets 1-L bottles. Immediately after collection, 1-L bottles must be preserved at 4° C for transport to the lab and stored at 4° C until stock is washed. Within 24 hours of collection, rinse the RAS with the matrix solution:

a. Allow the solids to settle in the collection bottles for a minimum of 4 hours at 4° C. Once settled, decant the wastewater liquid.

b. Pour up to 30 mL of solids into multiple 50-mL plastic centrifuge vials.

c. Add matrix solution (1mM $NaHCO_3$) to bring volume up to ~ 40 mL; shake well to completely re-suspend solids.

d. Centrifuge at 2000 F (rcf) for 5 min.

e. Decant liquid.

f. Repeat steps (c) through (e) for a total of three rinses (three volume changes).

g. Consolidate prepared biomass in a clean high density polypropylene (HDPP) bottle and use within 24 hours.

h. Store washed biomass at 4° C.

Total Suspended Solids (TSS) of Washed Biomass

49. The total suspended solids (TSS) of washed biomass can be achieved in the following

a. Pre-weigh and record the mass of the glass fiber filter using a balance with at least 0.01 mg resolution.

b. Place rinsed biomass suspension on a magnetic stir plate, add a magnetic stir bar, and mix well.

c. Filter 20 mL of the well mixed rinsed biomass through the filter. (If the filtration process takes more than 10 minutes reduce the volume of biomass to be filtered and repeat.)

d. After filtration dry the glass fiber filter and biomass at 105° C for 1 hour and reweigh the filter.

I. The mass of biosolids retained on the filter should be between 100 and 200 mg.

II. If the mass retained on the filter is less than 20 mg repeat using a larger volume of biomass.

III. If the value is greater than 200 mg repeat using a smaller volume of biomass

e. Calculate and record the suspension density of the biomass using Equation 4.

$$TSS = \frac{Final\ Filter\ Mass\ (mg) - Intial\ Filter\ Mass(mg)}{Filtered\ Volume\ (L)}$$

Equation 4

f. In addition to TSS other biomass properties may be useful interpreting the results and comparing results of multiple MN. Other properties of interest include but are not limited to: chemical oxygen demand (COD), dissolved organic carbon (DOC), specific ultraviolet absorbance at 254 nm (SUVA-254), floc size distribution, and elemental composition.

Biomass Stock Preparation

50. The biomass stock can be prepared by the following.

a. Transfer all the washed biomass to a clean 1-L HDPP bottle.

b. Dilute the biomass to a suspension density 500 mg L^{-1} greater than the maximum test concentration by pouring all the rinsed solids into one bottle and diluting to the appropriate suspension density using the matrix solution (1 mM $NaHCO_3$), e.g. if the maximum test concentration is 2,500 mg L^{-1} the proper suspension density would be 3,000 mg L^{-1}.

c. Measure the total suspended solid concentration (TSS), chemical oxygen demand (COD), and pH of the biomass suspension. To make biomass stock for experiments, the rinsed biomass suspension must be diluted with matrix solution to achieve the desired final TSS concentration minus the volume of NM solution to be added to the vessel.

d. Based on the TSS measured for the Biomass Stock calculate the volume required to achieve the desired concentration for a total volume of 40 mL.

Sludge Net Removal Experiment

51. Calculate the TSS of the Biomass Stock solution, the volumes of rinsed biomass stock(s), and the volume of nanoparticle suspension to be added to each sample to achieve a certain sample total volume with final TSS and nanoparticle concentrations. This is the "sample recipe." In addition, make recipes for controls. For nanoparticle-only controls, replace the biomass stock volume used in the samples with matrix solution and add nanoparticle suspension. For biomass-only controls, replace the nanoparticle suspension with matrix solution (1 mM $NaHCO_3$) and add biomass stock. Sample Table 6 is provided below for a total volume of 40 mL. The attached worksheet (3.11) has contains a interactive spreadsheet for the calculations

Table 6. Sample table outlining the test conditions to be evaluated in the net removal experiment

Sample Label	Target Biomass Conc.	Target NP Conc.	NP Stock Conc.	Volume of NP	Stock Biomass Conc.	Volume of Biomass Stock	Volume of Matrix/ Buffer
	(mg L^{-1})	(mg L^{-1})	(mg_{NP} L^{-1})	(mL)	(mg_{TSS} L^{-1})	(mL)	(mL)
NR*-2500	2500	0.2	150	0.053	3000	33.3	6.65
NR-1250	1250	0.2	150	0.053	3000	16.7	23.25
NR-250	250	0.2	150	0.053	3000	3.3	36.65
NR-0	0	0.2	150	0.053	3000	0	39.95
MN-Blank	2500	0	150	0	3000	33.3	6.7

*NR = Nano-Removal

52. Store the biomass stock(s) at 4° C until beginning the experiment. Try to do the experiment within 24 hours of rinsing the RAS. The test must be performed within this 24-hour window; storage of the biomass is not an option.

NP-Biomass Batch Interaction Experiment: Net Removal

Table 7. Net Removal Experimental Matrix (*n=15*)

Treatment	MN	MN Concentrations mg L^{-1}	Replicates	Biomass Concentrations mg L^{-1}	Mixing Times hour	Total Samples
No MN Control	No	0	3	2500	1	3
No Sludge Control	Yes	0.2 and 2.0	3	0	1	3
Net Removal	Yes	0.2 and 2.0	3	250, 1250, 2500	1	9

53. Once properties of the biomass stock(s) have been measured and sample recipes have been made, put a magnetic stir bar in the biomass stock bottle(s) and put the bottle on a stirring plate (30 RPM). The biomass must be kept completely mixed while being used to make the samples.

54. Arrange labelled vials, a beaker of matrix solution, sonicated MN suspensions, and biomass stock on the bench space. Try to arrange these components to make addition of ingredients simple and to minimize mistakes.

55. Prepare the vials by following your sample recipe for the appropriate volumes of biomass stock, matrix solution, and MN suspension to each sample vial. The biomass concentrations are indicated in the table above (Table 7) and are measured after mixing for 1 hour. The zero-biomass concentration vial is a control for dispersion stability of the MN (homo-aggregation).

56. After samples have been prepared and sealed, place them in an end over end rotational mixer for 1 hour. The speed of rotation should be recorded and not exceed 50 rpm

57. After the prescribed mixing conditions, remove samples from mixer and allow to settle for minimum 2 hours.

58. After biomass has settled, collect 3 to 5 separate 5-mL aliquots of the supernatant from each sample in separate clean vials. The supernatant samples will be digested in the sample vials to prevent loss of MNs to flocculation or sorption onto vessel surfaces.

59. Store samples at 4 ^{o}C until analysis. In many cases, only the supernatant will need to be analysed. If mass balance is sought, the biosolids will need to be digested and analysed. Samples should be analysed within 5 days

MN Quantification

60. Quantification of the MN remaining in solution (C_f) is critical. The specific technique employed will be based on the MN tested. The applicability of the chosen digestion method may be checked by comparing the results of the spike blanks with the initial concentration of MN added. The specific digestion method selected should be appropriate for the MN of interest. It is suggested the user consult the scientific literature to determine what method would be most appropriate.

Analysis

Sample Analysis Tips

61. For quality control, select at least 1 out of 20 samples to perform duplicates and spike recovery. Be sure to check the calibration curve against an independent certified standard If internal standard intensity is below the intensity in the calibration blank by more than 15%, the samples will need to be diluted to avoid matrix interferences.

Reporting

62. After the test has been performed and analysis for test compound is complete, tabulate the data generated using the following format:

63. Report the following information in the Excel Document Spreadsheet[1] (see paragraph 43):

a. Test conditions: Temperature and pH at which the test was conducted. Provide the duration of each step of the testing. Physicochemical properties of the MN tested including composition, particle size, technique used for particle size determination, presence of coating, and chemical composition of coating.

b. Detailed description of the analytical techniques used in the recovery and quantitative analysis for test compound. Testing that analytically measures the MN in the water phase and associated to the sludge is preferred. The method by which the biomass was separated from the supernatant should be recorded (e.g. settling, filtering, or centrifugation).

c. Amount of test compound dosed (C_0 solution volume), and the amount recovered in each reaction vessel (C_e solution volume).

d. Volume of each reaction vessel and total volume of the sorbent (Biomass) and sorbate (MN) mixture.

e. QA/QC data such as duplicate analyses, background interferences, spikes, and matrix spikes, etc.

f. Data analysis would be conducted at two levels, if desired. First, for each MLSS concentration and fixed mixing time representative of a WWTP (t = 3 hours) the net removal can be calculated via Equation 1. The steady-state biomass density of MNs (BD, mgMN/gTSS) is calculated for each MLSS level as C_0-C_f (mgMN/L) divided by the MLSS concentration (gTSS/L). A non-linear regression analysis of C_f (x-variable) against BD (y-variable) yields the parameters K and 1/n (Equation 2)

g. Sludge solids information: Sampling location, observations, age of the biomass used in the test, calculations for volume sampled, sample custody. Reference samples of unused sludge should be maintained for potential testing of TOC and metals.

h. Any unusual observations made during the experiments.

[1] A Microsoft® Excel worksheet to be used for entering data in from the experiment and to calculate the net removal in Equation 1 and the affinity constant (K) in Equation 2 is available on the OECD website.

Example Application of Experimental Data

64. To predict MN net removals under near steady-state conditions at a typical WWTP either the experimental bench-scale data for the MLSS level that most closely represents the MLSS levels in the full-scale WWTP can be selected, or Equation 3 can be used to estimate removals under different MLSS levels.

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12 APPENDIX - GLOSSARY

Aggregation – The formation of a number of particles into a cluster, happening due to interparticle interactions.

Concentrations – concentrations of the particles and NOM in samples are reported in g/L, mg/L (ppm) and μg/L (ppb). Concentrations of salts ($NaHCO_3$) present in the samples are reported in mM.

Hydraulic Residence Time, Hydraulic Retention Time – is a measure of the average length of time in which a compound remains in a storage unit.

Manufactured Nanomaterial (MN) – A engineered particle with at least one dimension between 1 and 100 nm.

MLSS – Mixed Liquor Suspended Solids, refers to the concentration of suspended solids, in an aeration tank during the activated sludge process, which occurs during the treatment of wastewater. MLSS consists mostly of microorganisms and non-biodegradable suspended matter and is mostly expressed as milligrams per liter (mg/L).

MN- Manufactured Nanomaterials

NOM - Natural Organic Matter, refers to the organic material present in surface or ground water.

RAS – Return Activated Sludge, refers to a tank, known as the clarifier, where the solids settle and are separated from treated wastewater.

Size – Size of the particles are reported in micrometers (μm) or nanometers (nm) and can be determined using TEM imaging, or in (d.nm) using DLS size measurements.

TOC – Total Organic Carbon, refers to the amount of carbon found in an organic compound.

TSS – Total Suspended Solids, refers to the dry-weight of particles trapped by a filter. It is a water quality parameter used to assess the quality of wastewater after treatment in a wastewater treatment facility.