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Assessing the similarity of nanoforms based on the biodegradation of organic surface treatment chemicals

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ABSTRACT

A substance may have one or more nanoforms, defined for regulatory purposes under EU chemicals legislation REACH based on differences in physicochemical properties such as size, shape, specific surface area and surface chemistry including coatings. To reduce the burden of testing each unique nanoform for the environmental risk assessment of nanomaterials, grouping approaches allow simultaneous assessment of multiple nanoforms. Nanoforms with initially different intrinsic properties, could still be considered similar if their environmental fate and effects can be demonstrated to be similar. One hypothesis to group nanoforms with different organic surface modifications is to use parameters linked to biodegradation of the organic surface. The hypothesis contends that nanoforms with a similar core chemistry, but different organic surface treatments may be grouped, if the surface treatment is likely to be lost through biodegradation rapidly upon entering an environmental compartment, such that it no longer modulates fate, exposure and toxicity of the nanoform.

To implement grouping according to surface treatment biodegradability, a robust approach to measure the breakdown of particle surface treatments is needed. We present a tiered testing strategy to assess the biodegradation of organic surface treatments used with nanomaterials that can be implemented as part of an Integrated Approach to Testing and Assessment (IATA) for grouping based on surface treatment stability. The tiered approach consists of an initial pre-screening MT2 colorimetric carbon substrate utilisation assay, to provide a rapid assessment of coating degradation, and a second tier of testing using OECD Test Guideline 301F for assessing organic chemical biodegradability. Six common surface treatment substances are assessed using the tiered testing strategy to refine rules for escalating between tiers. Similarity assessment using absolute Euclidean distances and x-fold difference concluded that the Tier 1 assessment can be used as conservative binary screening for biodegradability (no false positive results in Tier 1), whilst for substances showing intermediate biodegradation (10–60% in OECD 301F, Tier 2), similarity assessments can be informative for grouping surface treatments not considered readily biodegradable. Further validation using higher tier tests (e.g., mesocosms) is needed to define acceptable limits of similarity between intermediately biodegradable substances, where differences in biodegradability of the surface coating lead to negligible differences in fate, behaviour and toxicity of the nanoforms, and this is critically discussed.

1. Introduction

A substance may have one or more nanoforms based on differences in physicochemical properties (REACH, EU Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals, Annex VI, points 2.4.2. – 2.4.5). The surface treatment of nanomaterials with organic substances is an important modulator of their environmental fate and toxicity, resulting in many possible nanoforms of the same core

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constituent material. Indeed, a description of the surface treatment or functionalisation and identification of each agent including IUPAC name and CAS or EC number is required when identifying the nanoform of a substance (ECHA, 2017a; European Commission, 2018). Surface modifications may occur through the intentional addition of coatings, capping agents and other surface bound ligands. A precautionary approach for nanomaterial risk assessment could view particles of the same core, but different coatings, each as distinct nanoforms. Such a discrete approach would require individual testing and assessment of all nanoforms of a material that differ in their surface chemistry, to ensure accurate risk assessment of each nanoform. Not only would this have substantial consequences for the resources required for risk assessment that would make it unfeasible, but in some cases specific testing may be unwarranted and in fact contravene regulations related to reducing animal testing (Directive 2010/63/EU), for example. An effect of surface treatment would not be expected either in cases where the surface treatment has limited effects on fate and toxicity, or where the coating is degraded rapidly in the environment, and so no longer modulates the fate and toxicity of the nanoform. In these cases a similar functional fate of the different nanoforms is expected, meaning that the particles can be operationally grouped (Spurgeon et al., 2020). Given the high potential diversity of nanoforms based on the number of possible core and coating combinations, approaches are needed to address nanomaterial risk assessment decisions in an efficient and evidence based manner.

Grouping is a general approach for streamlining the assessment of multiple chemicals. The approach seeks to identify cases in which "substances whose physicochemical, toxicological and ecotoxicological properties are likely to be similar or follow a regular pattern as a result of structural similarity may be considered as a group" (REACH Annex XI 1.5, 2006). One approach to reduce the burden of testing each and every variant nanoform individually is to use read-across to fill data gaps for one substance (the target) with data from another substance (the source) providing there is scientific justification for the two substances to be considered similar (ECHA, 2016; OECD, 2016). This is known as the analogue approach to read-across in the Read-Across Assessment Framework (ECHA, 2017b). Annex XI to REACH was recently revised to include specific provisions for nanoforms, extending the applicability of grouping and read-across to nanoforms of the same substance (European Commission, 2018). Integrated Approaches to Testing and Assessment (IATA) are a useful tool to guide collection and assessment of data relevant for grouping of nanoforms and may be structured as a series of decision nodes that together form the basis of a grouping justification (Stone et al., 2020). Here we propose loss of the surface treatment to the environment within a specific timeframe, as a component of a wider environmental IATA to justify read-across between nanoforms with different surface treatments.

To group nanoforms of the same core constituent composition, but modified with different organic surface treatments, a structured approach is needed to assess whether the fate and hazard profile of the materials are similar, or are likely to converge as they enter or pass through the environment. There are several mechanisms through which chemical surface treatments are lost to the environment, the most simple of which is diffusion from the particle surface in the case of physisorbed substances, although displacement by environmental macromolecules is more typical. Whilst potentially relevant for aquatic systems, it is perhaps of less relevance for the soil environment, where polymer surface treatments have been found to experience low desorption from nanomaterials (Kim et al., 2009). A precautionary view to grouping would assume surface treatments remain associated with the nanomaterial surface. This can be considered a worst-case assumption when it comes to grouping nanoforms with different surface treatments. Degradation of the surface treatment chemical itself must therefore be demonstrated in these cases, to be confident that the surface treatment is lost to the environment on release of the nanoform.

Biodegradation will be an important pathway for organic surface treatment degradation in both aquatic and soil environments, but also during wastewater treatment, an important reactor for nanomaterials released to the environment through industrial or consumer wastewaters. There is evidence that organic surface treatments for nanomaterials can be utilised for metabolism by microbial communities in situ in the environment. Field trials testing remediation of contaminated ground water using zero valent iron nanoparticles stabilised with a biodegradable non-ionic surfactant (industrial-grade coconut fatty acid diethanolamide), found the surface treatment was accessible for utilisation as a carbon source, stimulating microbial growth (Wei et al., 2012). Likewise, microbial communities in a model wastewater treatment plant adapted over time to CeO_2 nanoparticles treated with the biodegradable surfactant dodecyl benzyl sulfonic acid (Limbach et al., 2008). Utilising the compound for metabolism resulted in loss of the biodegradable coating and destabilisation of the particles, changing their fate within the model treatment plant. Given their role in modifying fate and toxicity, and potential to be removed by degradation, we consider biodegradation of nanomaterial organic surface treatments as a key decision node that can be used as part of a wider environmental IATA to allow for nanoforms with different surface chemistries to be grouped for risk assessment (see Section 2 Rationale and methods for more detail).

There are several methods for evaluating biodegradation by microbial communities, including internationally recognised standard tests. The 28 day OECD 301 Ready Biodegradability test is a standardised, but relatively resource intensive assay to assess biodegradation of organic chemicals. To allow more rapid assessment of coating biodegradation, here we develop and test a benchtop scale, well-plate assay, designed to screen organic surface treatments for their susceptibility to biodegradation. These two tests form a tiered testing strategy that can be implemented within a wider environmental IATA to group nanoforms of different surface organic chemistries. Tier 1 consists of the screening well-plate based assay to identify biodegradable substances and help prioritise those for further testing depending on the purpose of the grouping. The Tier 2 OECD 301F test can identify a pre-defined group of readily biodegradable substances (according to the pass criteria of the OECD 301F test for ready biodegradability), as well as generate data for intermediately biodegradable coatings (i.e., between 10 and 60% of total degraded in 28 days based on the OECD 301F) for which similarity assessment could support further grouping of nanoforms with different organic surface treatments. Pairwise similarity assessment of six common organic surface treatments, based on absolute Euclidean distances and maximal fold differences in biodegradation of these substances, is used to demonstrate how the tiered approach can deliver groups of nanoforms based on the susceptibility of their surface treatment to biodegradation. This assessment is also used to inform the rules for escalation between tiers in the testing strategy.

2. Rationale and methods

2.1. Grouping hypothesis

Nanoforms with different initial physicochemical properties may be operationally grouped where similar functional fate is expected (Spurgeon et al., 2020). The importance of surface treatment stability and durability for nanomaterials fate and hazard is acknowledged in the OECD's report on "Assessment of Biodurability of Nanomaterials and their Surface ligands" (OECD, 2018). A worst-case assumption is that the effect of a surface treatment chemical on the fate and toxicity of the nanoform persists throughout the lifecycle of the material, provided the surface treatment chemical is not lost from the nanomaterial or degraded. A number of processes can lead to loss of the nanomaterial surface treatment in the environment. These may be:

- Physical e.g., diffusion or displacement of weakly interacting coatings with natural organic matter or biomolecules
- Abiotic e.g., photo-degradation, or

- Biotic e.g., through microbial degradation and metabolism of organic compounds.

Each of these can be considered decision nodes in an IATA for grouping of nanoforms based on demonstrating similar durability of their chemical surface treatments. In this paper, we focus on biodegradation as a pathway for the removal of the organic surface treatment from a nanomaterial.

Heterotroph bacteria utilise organic molecules as a carbon and energy source. Oxidation of organic coatings by bacterial communities during aerobic respiration could therefore be a significant driver of coating loss in the environment. Whilst this may feasibly occur in any environment containing aerobically respiring microbial communities, we have chosen to prioritise the assessment of biodegradation in wastewater treatment plants (WWTP) in light of their high microbial population density and diversity and their specific role in the biological degradation of organic material as part of the wastewater treatment process. Nanomaterials from common "down the drain" consumer products will also pass through waste water treatment plants, assigning these communities a potentially active role in the fate of nanomaterial coatings (Svendsen et al., 2020). Activated sludge in WWTPs consists largely of saprotrophic bacteria which release extracellular enzymes to break down organic material into monomers that can then be utilised for respiration. Importantly, this occurs outside of the cell, and so does not require internalisation of the nanoform by bacteria. In addition, whilst inoculum may be derived from a variety of sources, including surface waters and soils, to assess biodegradation of organic chemicals, the standardised test guidelines recommend that activated sludge taken from a treatment plant receiving primarily domestic sewage be utilised, as this should give a more reproducible assessment compared to other sources with lower cell densities which demonstrate higher scattering of results (OECD, 1992).

Within the context of the passage of coated nanomaterials through wastewater treatment plants, the *hypothesis regarding surface treatments* can thus be summarised:

> *"Nanoforms with an organic surface treatment that is lost from the material surface following exposure in WWTP compartment can be grouped: Fate and toxicity of the exposure relevant nanoform can be considered similar to a non-coated analogous nanoform if the coating is lost".*

2.2. Overall approach to assessment and testing of surface treatment biodegradation

The grouping hypothesis can be considered a decision node to be implemented within a wider environmental IATA. Tiered testing guides information gathering in the IATA to support decision making. Where available, existing testing and assessment methods are recommended. To assess surface treatment substance durability, we propose a strategy that utilises the OECD test guideline 301F Ready Biodegradability test (OECD, 1992), and can thus leverage existing data for many organic chemicals. Ready biodegradability tests have long been the central foundation for understanding the biodegradation of chemicals in regulatory frameworks for hazard and environmental risk assessments. They are standardised and conservative regulatory tests that measure the relative biodegradability of chemicals (e.g., OECD 301 or ISO 14593 tests (ISO, 1999)). Fulfilling the pass criteria for the readily biodegradability test is a reliable indicator that the chemical can biodegrade rapidly in a matter of days in most environments under aerobic conditions.

An additional motivation for creating a tiered testing strategy to assess biodegradation of nanomaterial organic surface treatments is to limit the number of OECD 301 Ready Biodegradability tests required if multiple surface treatment substancs are under assessment. The 28 day OECD 301 Ready Biodegradability test is a relatively resource intensive assay to conduct. Thus, the tiered testing strategy presented here includes a pre-screening test, which can be useful to prioritise the further testing of chemicals in a cost-effective manner. This pre-screening Tier 1 test makes use of the Biolog MT2 Microplates™ user defined substrate utilisation assay (Biolog Inc., Hayward, California). The reduced assay time and low cost involved in the performance of this assay makes this useful as a Tier 1 test within the tiered assessment strategy, particularly for the specific purpose of grouping, for example to identify a Safe(r) by Design strategy (SbD). For example, if the SbD strategy was to select a surface treatment that conferred functionality to a nanoform, but also could be degraded quickly in the environment to reduce persistence of the nanoform upon release, this Tier 1 pre-screening assay could screen for viable options at an early stage of the design process, such that only the most promising sub-set of potential coating substances would be further tested in the next tier.

It should be noted that both tiers of testing recommended here are based on assessment of the surface treatment chemical itself, rather than assessment of the nanoform and surface treatment in combination. The MT2 test is based on measurements of optical density, thus limiting the potential for optimisation of the test to allow for testing of nanoforms themselves, as these would significantly interfere with the measurement as a result of scattering. However, biodegradable substances attached to nanomaterials have been demonstrated to remain available for biodegradation by soil (Wei et al., 2012), aquatic (Kirschling et al., 2011) and WWTP microbial communities (Limbach et al., 2008). This supports the use of tests that can be applied to the surface treatment substance alone as an appropriate testing strategy for assessment of the biodegradation of nanomaterial surface treatments. The predictive capacity of biodegradation testing of the "chemical alone" to be representative of biodegradation of nanomaterial surface associated chemicals will be critically discussed later in the article.

To develop the tiered testing strategy, six common chemical surface treatments have been selected that cover the dynamic range of the biodegradation assessment, from the non-biodegradable 10 kDa synthetic polymer polyvinyl pyroloidide (PVP, Sigma Aldrich, UK) to the readily biodegradable stabiliser, citric acid (Sigma Aldrich, UK). These two organic compounds represent end members for the assessment of biodegradation. In addition to these end members, four further substances are also assessed: the non-ionic surfactant Tween 80 (Sigma Aldrich, UK), the anionic surfactant sodium dodecyl sulphate (SDS, Sigma Aldrich, UK), the dispersant Dispex® A4040 (BASF, product 501,128 42) and the biopolymer Dextran (Dextran from Leuconostoc spp. Sigma Aldrich, UK). Selection criteria for these six chemicals was their reported use in the literature as surface treatments for nanomaterials and their varying durability against biodegradation from our preliminary testing of a wide panel of coating agents in the MT2 assay and assessment of their chemical oxygen demand (data generated in H2020 project NanoFASE).

2.3. Tier 1 biodegradation test: MT2 user defined substrate utilisation assay

2.3.1. Principle of the MT2 test

MT2 Microplates™ are designed to allow investigation into the ability of an inoculated microorganism suspension to oxidise a panel of different carbon sources. Each well of the 96 well layout contains a tetrazolium redox dye and a buffered nutrient medium, but does not contain a carbon source. This allows the investigator to define the identity of the carbon sources, in this case organic chemicals, to be tested. Single species or microbial communities extracted from environmental sources are inoculated to the wells and tetrazolium violet is used as a redox dye to colorimetrically indicate utilisation of the selected organic substances (Garland and Mills, 1991). Tetrazolium violet acts as an artificial electron receptor, which when reduced forms an insoluble coloured formazan. Colour development in the wells is, therefore, an indication of cellular respiration and the metabolism of the added

carbon substrate by the microbes. The percentage degradation of a carbon source can be estimated through calibration of colour intensity against known concentrations of a reference compound that is assumed to be fully metabolised within the test duration (typically glucose). These MT2 microplates have been successfully employed for a variety of applications, from screening for lignocellulosic-straw-degrading bacteria from soil, composts and straws (Taha et al., 2015), to identifying and assessing the potential application of polycyclic aromatic hydrocarbon degrading soil isolates for bioremediation (Haleyur et al., 2018).

2.3.2. Isolation of microbial communities from WWTP activated sludge

Microbial communities used for the MT2 assays were isolated from activated sludge sourced from a UK WWTP on the day of testing, following OECD 301F guidance (OECD, 1992). Both the microbial community and the test substances were prepared in OECD 301F mineral medium. The inoculum was diluted to attain a starting OD_{600} of 0.0054 in the well plate, whilst test substance stocks were prepared to obtain 500 mgL^{-1} in the assay. This concentration is optimum for the 48 h duration of the test, as readily biodegradable D(+)-glucose and citric acid reached peak absorbance at 48 h at this concentration, plateauing beyond this point, indicative of complete degradation of the compound. It is also in line with the minimum concentration of 100 mgL^{-1} recommended in the OECD 301F test. Proportional degradation is a ratio of the starting concentration, and so can be biased to underestimation at high concentrations. The concentrations tested are higher than predicted environmental concentrations in WWTPs or receiving surface waters. For example, screening of 1564 surfactants and their transformation products in composite samples from 33 WWTPs generally detected influent concentrations in the low mgL^{-1} (Freeling et al., 2019). Whilst testing at higher concentrations than predicted for the environment may increase the chance for false negative results, it reduces the risk of wrongly concluding ready biodegradability of a substance that could happen if excessively low concentrations are tested. Testing at this concentration is therefore in line with the philosophy of the proposed tiered testing strategy, to be a conservative grouping for biodegradability of the surface treatment substances.

2.3.3. Method

Briefly, triplicate wells were filled for each test substance in a random design across the 96 well plate. Controls with no carbon source were filled with the OECD 301F mineral medium instead. These controls were used to correct for any colour development through endogenous activity of the inoculum. The inoculum was then distributed across all wells, with the exception of the abiotic controls. Abiotic controls contained the test substance and OECD 301F mineral medium but no microbial community. Abiotic controls allowed for any colour formation in the presence of the organic substance alone to be tested independently of the presence of the microbial community. Three independent tests were performed, each with a new activated sludge inoculum from a UK WWTP during winter (February and March 2020). The data reported for the Tier 1 MT2 assay are pooled from these three independent tests, reporting the grand mean and corresponding standard deviation.

Colour development of the tetrazolium dye was monitored at intervals over 48 h; pre-tests showed a plateau of the carbon source reference D(+)-glucose, indicating a full depletion of the substrate. Percentage degradation of each surface treatment was then calculated against a calibration of D(+)-glucose after 48 h incubation in the dark at 20 °C.

2.4. Tier 2 biodegradation test: OECD 301F ready biodegradability test

2.4.1. Selection of OECD 301F test as tier 2 assessment

The IATA uses the OECD 301F ready biodegradability test as the Tier 2 assay. Use of this standardised method as a tier 2 test within the IATA brings regulatory acceptance and can provide additional evidence to reduce uncertainty compared with the well plate Tier 1 screening assay. The use of the OECD 301 test is also valuable to provide supporting assessment in those cases where surface treatment chemicals may show intermediate biodegradability.

The Organisation for Economic Co-operation and development (OECD) Guidelines for the Testing of Chemicals list 7 types of tests for determining the ready biodegradability of chemical compounds, 301A-F (OECD, 1992) and 310 (OECD, 2006). The OECD 301F has been selected as it is most suitable for the assessment of the biodegradability of water-soluble substances, especially for wastewater but also for volatile and hardly soluble substances.

2.4.2. Method

Biodegradation was determined by means of respirometry test, in accordance with the standard OECD 301 F procedure (Ready biodegradability – Manometric respirometry test) and using the OxiTop®-IDS set system (developed and manufactured by WTW, Germany). This standard specifies a method of determining the biochemical oxygen demand (BOD) in a closed respirometer based on the measurement of CO_2 production. The test mixture contains an inorganic medium, the test material (the sole source of carbon and energy) and activated sludge as the inoculum. The mixture is stirred in closed flasks in a respirometer for a period of 28 days. The carbon dioxide evolved is absorbed in a suitable absorber in the headspace of the flasks. The consumption of oxygen (BOD) is determined by measuring the change in pressure as recorded by the OxiTop®-IDS measuring heads and downloaded by the multi-parameter portable meter MultiLine® Multi 3630 IDS. The level of biodegradation is determined by comparing the BOD with the theoretical oxygen demand (ThOD) and expressed in percent. The influence of possible nitrification processes on the BOD was also considered. The test result is the maximum level of biodegradation determined from the plateau phase of the biodegradation curve. The OECD guidelines established that a sample is regarded as “readily biodegradable” if the biodegradation, which is based on dissolved oxygen, passes 60% of the ThOD value of each sample in a 10-day window within the 28-day period (OECD, 1992). The 10-day window starts when biodegradation passes 10%.

2.5. Similarity assessment using Euclidean and x-fold distances

Assessment of similarity between nanoforms is an approach which can be used as part of an analogue read-across, providing justification for read-across between the target nanoform(s) and the source material. The concepts behind when to use a pairwise similarity assessment on individual properties are discussed in detail in Jeliazkova et al., 2021. In some instances, clear biological or environmental cut-offs exist, beyond which similarity assessment is not required. The threshold for ready biodegradability as defined in OECD 301 is one such cut-off. Whilst differences in biodegradation between substances are measurable above the threshold of 60%, all substances which pass this threshold are considered to biodegrade quickly (within months rather than years) in the environment. In this way, measurements beyond this cut-off provide no additional value for grouping. However, where such cut-offs do not exist or are not passed, pairwise similarity assessment on individual properties is required for each decision node in an IATA. Here we employ Euclidean distance and x-fold comparisons as two distinct approaches to similarity assessment of the data derived from the Tier 1 and Tier 2 assessments of organic surface treatment biodegradation.

2.5.1. Euclidean distances

Euclidean distance is equivalent to the length of the line between two points and is widely used as a metric of distance. Percentage biodegradation after 48 h (Tier 1, MT2) and at the plateau phase (Tier 2, OECD 301F) are both scalar descriptors, being a reduction of two dimensions (time and degradation) to a single dimension. The Euclidean distance for scalar descriptors in 1 dimension is therefore the absolute distance between percentage biodegradation for pairs of nanoforms. The distance, $d(p,q)$, between p (the smaller value) and q (the larger value) would

simply be the value for p, subtracted from q. Where two dimensions are compared (such as to calculate a combined Euclidean distance for data from both Tier 1 and Tier 2 assessment of biodegradation), the distance between two points in the two dimensions is calculated by applying Pythagoras's theorem for a right-angled triangle as shown in Eq. (1). The distance, d(p,q), is calculated between each pair of substances, which have two co-ordinates (p_1 and p_2, q_1 and q_2), one in each dimension. In this case the distance is a co-ordinate representing biodegradation in the Tier 1 MT2 and Tier 2 OECD test, respectively. The Euclidean distance in either one or two dimensions can then be calculated from Eq. (1), where if $n = 1$ it is one dimensional whilst $n = 2$ is two dimensional. A detailed example is provided in the Supporting Information (SI Fig. 1).

$$d(\mathrm{p},\mathrm{q}) = \sqrt{\sum_{i=1}^{n}(q_i - p_i)^2} \quad (1)$$

If there is complete agreement between the Tier 1 test outcomes and Tier 2 test outcomes, the pairwise similarity plots should be similar in comparisons of both the one- and two-dimensional Euclidean distances. Where the distances diverge between the one- and two-dimensional comparisons, this is indicative of different test outcomes from the two tiers of testing. This can be easily visually identified by comparison of the two plots.

2.5.2. Maximal fold difference

The maximal fold or x-fold difference approach describes the relative difference between two different nanoforms. The larger value is divided by the smaller (Eq. (2)) to calculate the fold difference, and thus a number >1 is always generated.

$$Fold\ difference = \frac{max(nanoform_a, nanoform_b, etc.)}{min(nanoform_a, nanoform_b, etc.)} \quad (2)$$

All substances that pass the validity criteria of OECD 301F for readily biodegradable substances must be considered perfectly similar when assessing x-fold differences on the Tier 2 data. There is no need to interpret a difference between 60 and 90% biodegradation within the 10 day window, as both substances are considered readily biodegradable, and so are in the same group. To account for this, all substances meeting the ready biodegradability criteria were assigned the same value for calculation of maximal x-fold difference, i.e. the 60% threshold of ready biodegradability. This means that when comparing maximal fold differences, readily biodegradable substances score 1 when compared.

PVP, the non-degradable reference compound, never passed the criteria for reporting a value of biodegradation according to the OECD 301F test, i.e., it never passed 10% degradation in the 28 day period of the test that is needed to start the 10 day window. Such low biodegradation is considered indistinguishable from the background noise in the test. As such, x-fold comparison of PVP against other substances is inappropriate. This is the only substance which did not pass the 10% biodegradation threshold and therefore is considered to be in a different group from the other substances. At present, PVP and any other substances that do not pass the 10% threshold within the 28-day OECD 301 test may be considered as a group of non-biodegrading materials within our tiered testing approach. Maximal fold differences were therefore only evaluated for the remaining 5 substances.

For our overall assessment of similarity, adopting these two statistical methods for similarity assessment allows us to explore how different approaches to numerical similarity assessment may result in different grouping conclusions across the dynamic range of biodegradation, between non- and readily biodegradable substances. The algorithmic similarity assessment can also be viewed in the context of the existing threshold for ready biodegradation in the Tier 2 OECD 301F test and putative thresholds for the Tier 1 screening MT2 assay.

3. Results and discussion

3.1. Summary comparison between tier 1 and tier 2 tests

The comparison between Tier 1 and Tier 2 assessment of biodegradation of the six substances (Table 1) evaluates the capacity of the Tier 1 MT2 assay to predict Tier 2 OECD 301F test results. No false positive results (i.e., cases of degradation in the first tier, but not in the second tier assay) were found using the MT2 assay. Two false negative results were observed in the Tier 1 MT2 test, Dextran and Dispex®, when these results are calibrated against assessment in the Tier 2 assay. Tier 1 assessment is also unable to distinguish between SDS and Tween-80. Tier 2 assessment on the other hand distinguishes SDS as being readily biodegradable (97.77% degradation in the 10 day window) whilst Tween-80 does not meet the criteria for a readily biodegradable substance (degradation of 47.15% in the 10 day window).

3.1.1. Tier 1 MT2 test as a binary assessment for biodegradation

Tier 1 assessment of the six surface treatment substances correctly identified the benchmarks for the non-biodegrading substance PVP (Trimpin et al., 2001; Vanharova et al., 2017) and the readily biodegradable substance citric acid (ECHA, 2011), measuring degradation of 4.3 ± 1.8 and 95.7 ± 5.9% respectively (Table 1). As PVP is resistant to biodegradation, we can use the data to calculate a pass level for the MT2 test, above which biodegradation of the test substance can be interpreted (see Supporting Information Eqs. 1–4). This pass level is based on similar assumptions as calculating a limit of detection, but considers the data for PVP to be representative of the background or noise in the MT2 assay. This assumption is made as we demonstrate that any biodegradation estimated from the MT2 assay for this substance is not truely representative of biodegradation of the substance (calibration against the Tier 2 OECD 301F test), and thus is noise in the test.

We use the reproducibility standard deviation (Supporting Information Eq. 3) and the grand mean across the three independent experiments so that the pass level incorporates the reproducibility of the assay itself (Supporting Information Eq. 4). Based on the measured PVP degradation, a pass level for biodegradation of 14.9% is calculated for the MT2 assay. Thus we propose a cut-off of 15% biodegradation to be observed in the MT2 assay as the threshold to conclude that the test substance is biodegradable, giving <5% chance of a false positive result. Within OECD 301F, a substance is classified as biodegradable if there is unequivocal evidence of biodegradation (primary or ultimate) in any test (OECD, 1992). We propose that identification of biodegradation above the pass level in the MT2 assay would constitute such evidence of

Table 1
summary of the comparison between results from Tier 1 assessment (MT2) and Tier 2 assessment (OECD 301F) of biodegradation for 6 common organic surface treatments. Note that for some molecules, the degradation did not reach a plateau phase during the 28 day period of the test.

Organic surface treatment	Mean biodegradation (%) calculated from Tier 1 MT2 assay (S.D.)	Mean biodegradation (%) at the plateau phase from Tier 2 OECD 301F (S.D.)	Tier 1 outcome from MT2 pre-screening	Tier 2 outcome from OECD 301F
PVP (10 kDa)	4.3% (1.8)	−5.70% (4.89)*	–	–
Citric acid	95.7% (5.9)	66.28% (2.34)	+	++
SDS	31.5% (4.6)	97.77% (3.83)	+	++
Tween-80	27.2% (1.0)	47.15% (2.36)	+	+
Dextran	2.5% (4.5)	56.69% (3.99)*	–	+
Dispex®	6.4% (7.5)	24.48% (2.60)	–	+

These substances are identified with an asterisk (*) in the table and instead represent the mean biodegradation across triplicate tests at the end of the 28 day assay. "–" denotes that no biodegradation was observed, "+" that some biodegradation was observed and "++" that the substance fulfilled the criteria for a readily biodegradable substance.

biodegradation, meaning that the MT2 assay can be used as a binary screen for biodegradability of chemicals (Fig. 1).

It is considered that currently, the MT2 assay can only be used as a binary assessment of biodegradability. No threshold to uniquely identify readily biodegradable substances in the MT2 assay can be inferred from the evidence so far.

3.1.2. Tier 2 OECD 301F test: Defining the applicability range for similarity assessment

Following the same principle as for the Tier 1 MT2 assay, we consider the criteria for readily biodegradable substances in the Tier 2 OECD 301F test in the context of the dynamic, measurable and environmentally relevant range for biodegradation as shown in Fig. 2. In this case, the environmentally relevant range is considered to be limited to biodegradation <60% in the 10-day window. This is because biodegradation greater than this pass level is considered to be readily biodegradable, and thus already a pre-existing group or classification of chemicals. Beyond this level of biodegradation, the environmental fate is considered to be similar between chemicals, and so the measurement technique provides information that is irrelevant for grouping. For example, for the case studies tested, biodegradation of citric acid and SDS reaches 66.28% and 97.77% respectively at the end of the 10-day window. However, this difference is irrelevant for grouping as both are considered readily biodegradable.

Therefore, it follows that it is the region below the threshold for readily biodegradable substances that can be considered the "applicability range" of the hypothesis; the region in which algorithmic assessment can be used to inform on the relative similarity between surface treatment substances on the basis of their biodegradation. This is the applicability range for an analogue approach to similarity assessment (the orange region in Fig. 2), where justification of similarity between a source and target(s) nanoform(s) allows data gaps to be filled from the data rich source material, to the data poor target(s). Similarity assessment in this applicability range is tested in Section 3.2. A lower limit of this applicability range is set at 10% biodegradation. If 10% biodegradation is not reached within 28 days in the OECD 301F test, the 10-day window is never considered to start. Therefore, any organic substance that does not reach 10% biodegradation in the 28 day duration of the test can be considered grouped as non-biodegrading.

3.1.3. When to escalate between tier 1 and tier 2 testing?

As discussed in Section 3.1.1, it would be inappropriate at this stage to define a threshold within the Tier 1 MT2 assay to identify readily biodegradable substances, as this term has a specific regulatory meaning that is operationally defined from the outcomes of the OECD 301F test. We also observe that in the MT2 assay, some readily biodegradable substances may not reach a similar point of degradation within the 48-h period. For example, both SDS and citric acid are considered readily biodegradable, according to the Tier 2 OECD 301F test. However, whilst near complete biodegradation was observed for citric acid in the MT2 assay, only 31.5 ± 4.6% biodegradation was measured for SDS in the MT2 assay. Likewise, for Dextran and Dispex®, if only the Tier 1 MT2 assay is performed, the user would conclude no biodegradation of these substances. However, if the testing was then extended to the Tier 2 test, the two substances biodegradability would be identified but with dissimilar biodegradation, with Dispex® reaching a plateau of 24.48 ± 2.6%, whilst Dextran continued to be degraded throughout the 28 days, reaching 56.69 ± 3.99% after 28 days, albeit still below the threshold for ready biodegradability.

When to escalate between Tier 1 and Tier 2 testing will depend on the purpose of the grouping. Tier 1 assessment provides a rapid and lower cost screening for biodegradable substances. Such an initial screening would be sufficient to answer the decision node for the purpose of safe (r)-by-design to identify substances that have a high probability to undergo degradation (i.e., those showing >15% degradation in the Tier 1 test). For this purpose, the test result from the Tier 1 assessment would be accepted to identify biodegradable substances, whilst escalation to Tier 2 assessment would be optional for those which did not pass the 15% threshold as these may be false negatives.

However, if the grouping is conducted in support of a regulatory decision, escalation to Tier 2 might be desired for those substances identified as biodegradable in the MT2 assay to allow for pairwise similarity assessment of the Tier 2 data as one element of a read-across justification. Importantly, even for a regulatory grouping, screening with the Tier 1 assessment may be desirable if a high number of target nanoforms with different surface treatments are being considered in the grouping. This could streamline further assessment in Tier 2 to only those substances which were found to be biodegradable in the Tier 1 screening assessment, so as to further distinguish readily biodegradable substances (i.e., >60% degradation in the 10-day window), whilst reducing unnecessary Tier 2 testing of substances that are unlikely to be readily biodegradable.

3.2. Similarity assessment using Euclidean distance and maximal fold difference approaches

For regulatory purposes, property-by-property evaluation of similarity between nanoforms is recommended to support grouping decisions (Jeliazkova et al., 2021). Here we use two commonly algorithmic approaches, Euclidean distances and maximal fold differences, to interrogate the robustness of the tiered testing strategy proposed in this article. Expressing the pairwise similarity assessment on Tier 1 and Tier 2 data independently, as well as two dimensional distances, combining results from the two tiers, allows for qualitative assessment of predictivity between Tier 1 and Tier 2 assessment in the testing strategy.

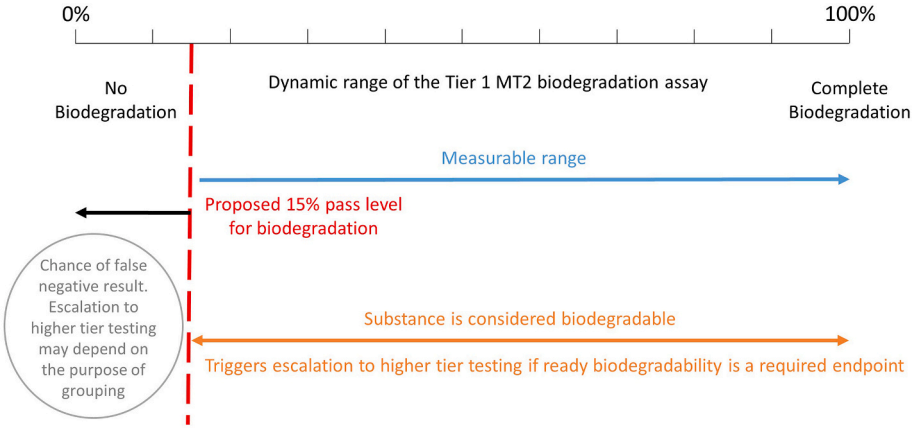

Fig. 1. Schematic adapted from (Jeliazkova et al., 2021) demonstrating how results from the MT2 prescreening assay can be used to attribute biodegradability with confidence above the limit of detection. Screening for biodegradability (the orange range) is demonstrated in the context of the dynamic range (the scale bar) and the measurable range (the blue range). To distinguish between substances where biodegradation is observed and readily biodegradable substances, escalation to higher tier testing would be triggered for substances that fall within the orange range. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

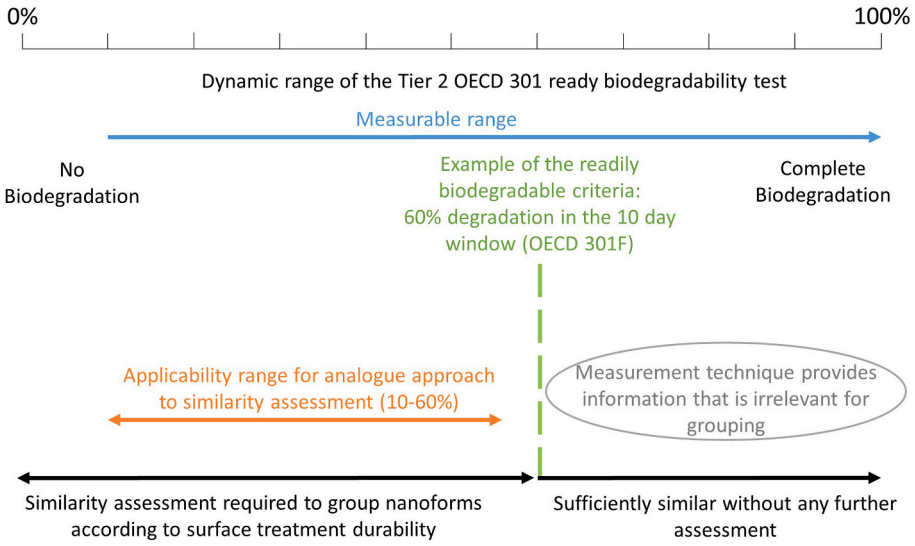

Fig. 2. Schematic adapted from (Jeliazkova et al., 2021) demonstrating how results from the Tier 2 OECD 301F test can be understood in the context of the dynamic range (the scale bar), the measurable range (the blue range) and the threshold for "ready biodegradability" (the green dotted line) beyond which the measurement technique provides information that is irrelevant for grouping, i.e., if substances are classified as readily biodegradable they are considered similar and no numerical assessment of similarity is required. In this way, the "applicability range" of the hypothesis is defined (orange region), wherein, algorithmic pairwise similarity assessment may provide a grouping justification for those substances not considered readily biodegradable. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

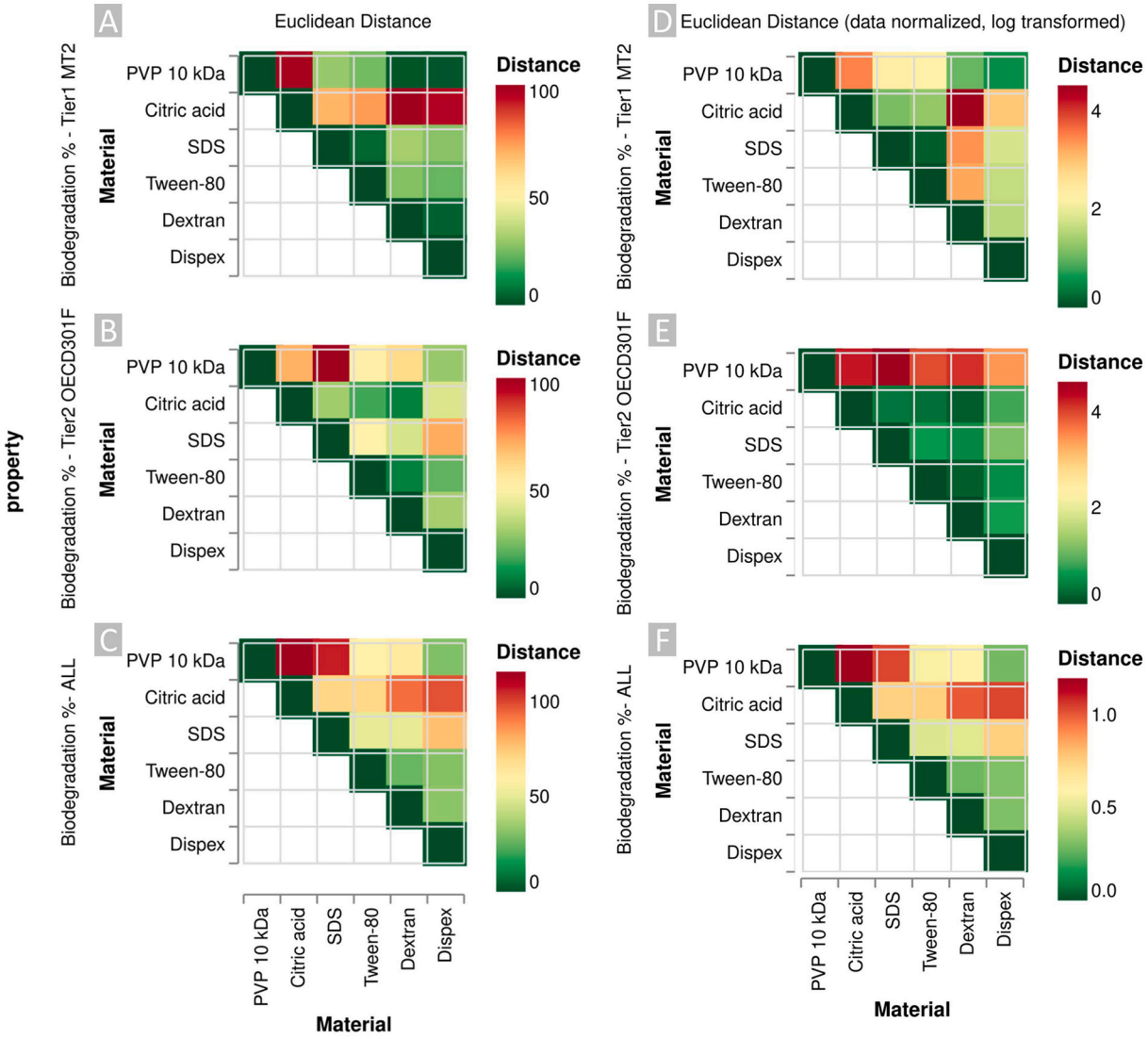

Fig. 3. Pairwise similarity assessment on the single property of biodegradation using Euclidean distance (A – C) or log transformed Euclidean distance (D – F). Tiles A and D represent similarity assessment on the Tier 1 data from the MT2 assay, tiles B and E represent assessment of the Tier 2 OECD 301F data, whilst tiles C and F represent the combined two dimensional Euclidean distance for each pairwise comparison, generating a combined Euclidean distance for Tier 1 and Tier 2 data.

3.2.1. Euclidean distance pairwise similarity assessment

Pairwise one dimensional Euclidean distances are intuitive as they are the absolute distances between pairs of substances for each of the two tiers of testing. They scale from 0 to 100%, as this is the dynamic range of the assessment (Fig. 3), from no biodegradation to complete biodegradation. Thus, the pairwise similarity plots of Euclidean distance for Tier 1 (Fig. 3.A) and Tier 2 (Fig. 3.B) assessment of biodegradation allow us to visualise the similarity between pairs of substances assessed by these two tiers of testing. In addition, the two-dimensional Euclidean distances are presented for each pairwise comparison (Fig. 3.C). For non-normally distributed data, log transformations have been demonstrated to improve compatibility of similarity assessment using Euclidean distances with other approaches including maximal fold difference, Arsinh-OWA and Bayes factor algorithms (Jeliazkova et al., 2021). Therefore, we explore the implications of log transforming the data for Euclidean distance assessment (Fig. 3 D – F).

The pattern of similarity differs across Tier 1, Tier 2 and the two-dimensional Euclidean distance, indicating that the two tiers of testing are not perfectly predictive of one another (Fig. 3.). Tier 1 assessment of non-transformed data would class PVP, Dextran and Dispex® as highly similar, SDS and Tween-80 as moderately similar, and Citric acid as dissimilar to all other substances. The false negative result for Dextran in the Tier 1 assessment wrongly classes it as similar to PVP. This supports the conclusion that Tier 1 data should only be interpreted as a binary assessment of biodegradability or not, rather than used for pairwise similarity assessment between surface treatment chemicals.

For the Tier 1 data, log transformation initially appears to improve the pairwise similarity assessment of substances, for example, designating citric acid as more similar to SDS and Tween-80 (two other biodegradable substances) than in the non-transformed assessment. However, transformation of the Tier 2 OECD 301F data, is inappropriate for the assessment (Fig. 3E), as it would classify all substances as similar with the exception of PVP. This highlights the importance of carefully considering the environmental context when interpreting the results of similarity assessment. The hydraulic retention time within a WWTP will vary according to the size and requirements of the works, but it is usually <5 days (for example, Johnson et al., 2005). Therefore, it is useful to consider the differences in biodegradation for those substances not classed as readily biodegradable in this context. Here, it is clear that PVP and Dispex® (biodegradation after 28 days of −5.7% and 24.48% respectively after 28 days), would be considered more similar than Dispex® and citric acid, where citric acid was readily biodegradable. Thus, we can conclude that data transformation should not be applied to the Tier 2 data. This illustrates the importance of carefully considering appropriate algorithmic approaches to similarity assessment that take into consideration the biological or environmental context of the assessment, as very different results may arise, even when using the same algorithm but different data normalisation approaches. Such issues are discussed in more detail in (Jeliazkova et al., 2021).

3.2.2. Maximal fold difference

Maximal x-fold differences are another commonly employed algorithm for similarity assessment. This approach has been used to define acceptable limits of similarity for sets of nanoforms on single properties such as median size (D50, Park et al., 2018) and other physicochemical properties (Janer et al., 2021a, 2021b), including in complex interactions between dissolution rates in different physiological media and the implications for in vivo inhalation toxicity (Keller et al., 2021). Here, we evaluate its application for the two tiers of testing for biodegradation of common nanoform organic surface treatment substances.

As described in Section 2.5.2, PVP is not considered in the maximal fold difference pairwise similarity assessment as it never passed the 10% threshold of biodegradation in the 28-day Tier 2 test (Table 1). Readily biodegradable substances (Citric acid and SDS) are assigned a biodegradation of 60% in the Tier 2 test for the fold difference assessment. They are considered to be sufficiently similar without requiring further assessment, thus score as perfectly similar in the fold difference assessment (Fig. 4).

Once again, pairwise similarity assessment of the Tier 1 data does not match the pattern observed for the Tier 2 assessment and so Tier 1 is best used as a binary screen for biodegradation. The pairwise assessment on Tier 2 data provides a useful visualization of the fold difference distances between pairs of substances. Transforming the Tier 2 data to account for the threshold for readily biodegradable substances (Section 2.5.2) correctly identifies closer similarity between readily biodegradable substances such as citric acid and SDS, and Dextran, which did not pass the validity criteria for readily biodegradable during the test but was found to experience 56.69% biodegradation at the end of the 28 day period. These differences should be considered in relation to the residence times in specific environments. For example, if the residence time was a matter of days, this difference between readily biodegradable substances would be of greater importance than during an exposure that may last months and surpasses the 28 day period evaluated here. It is important that pairwise similarity assessment is only performed with the Tier 2 data, as Fig. 3 and Fig. 4 demonstrate that the Tier 1 assessment does not lead to the 'true' similarities observed in Tier 2. Tier 1 in its current form should be used only to screen as a binary assessment for biodegradation.

3.2.3. Outlook for acceptable limits of similarity for grouping

Acceptable limits of similarity are the level to which nanoforms may differ on a property whilst still being considered to experience a similar fate or (eco)toxicological response. Acceptable limits of similarity have not yet been defined for biodegradation of nanoform surface treatment chemicals. When developed, such limits should be benchmarked against higher tier data. This verification is beyond the scope of this study, but the tiered testing strategy presented here can be the basis upon which data from these two tiers of testing could be used to develop a grouping hypothesis.

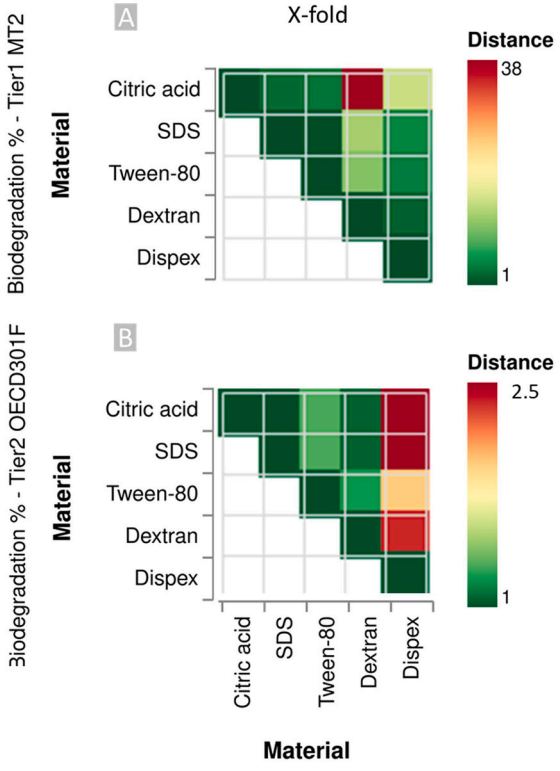

Fig. 4. Pairwise similarity assessment on the single property of biodegradation using maximal fold difference for Tier 1 MT2 assessment (A) and the Tier 2 OECD 301F test (B).

Typically, acceptable limits used for similarity are a function of several factors including the scale type (whether logarithmic, linear or other), the dynamic range of the assay and considerations of the biological and environmental relevance of differences must all be taken into account (Loosli et al., 2021). It is important that the acceptable limits of similarity are wider than the achievable accuracy of the measurement technique. If this is not the case, then the measurement technique will not be sufficiently accurate for use in the similarity assessment, as it will not be able to distinguish meaningful differences between nanoforms. Details on how to estimate the achievable accuracy of an analytical method, based on reproducibility assessments, are considered in the context of defining different nanoforms of a substance in (Cross et al., 2022).

Whilst Tier 2 tests were only performed at a single laboratory (meaning that a reproducibility assessment of the method cannot be made), we can still gain insight into the achievable accuracy as a first step in defining acceptable limits of similarity in biodegradation between chemicals. The absolute repeatability standard deviation (S_r) in biodegradation measured in the Tier 2 OECD 301F test across all five substances and three independent experiments is calculated as 7.1%, using Eq. (1) in the supporting information. This suggests that differences in biodegradation smaller than 7.1% should not be interpreted as a true difference between paired substances. Such differences cannot be confidently attributed to true differences in the biodegradability of substances as opposed to the natural variability of the test. All pairwise differences between surface treatments tested here surpass this 7.1% threshold and so should be interpreted as real differences between samples. It remains to be seen whether the acceptable limits of similarity in biodegradation of substances is wider than this achievable accuracy of the method. This should be a focus of future work.

Whilst the S_r can indicate absolute limits on acceptable similarity using Euclidean distances, considering maximal fold difference assessment, a single absolute repeatability standard deviation cannot be applied. S_r will be a function of the percentage biodegradation observed. As a result, achievable accuracy for less biodegradable substances will be poorer than for more biodegradable substances. This pattern will make it harder to distinguish reliably between the less biodegradable substances. For example, if comparing Dispex® to an alternative poorly biodegradable substance, the difference would need to be >1.3-fold to conclude that the biodegradability was really different (Supporting Information, Table S2). On the other hand, readily biodegradable substances such as SDS and citrate, would be distinguishable based on a minimum of 1.07 fold differences, meaning substances in this region would be easier to distinguish differences between each other.

To refine acceptable limits of parameters for grouping, studies to link functional measured properties (i.e., coating status) to key nanomaterial fate parameters are needed. In particular, the assumptions that biodegradation of surface treatments in isolation is predictive of biodegradability of these surface treatment chemicals when bound to nanoforms, and that degradation leads to complete loss of the influence of the surface treatment on nanoform behaviour, need to be robustly assessed. We have presented existing evidence (Section 2.2) that biodegradable substances can still be metabolised by microbial communities in a range of environments when part of a stabilised surface treated nanoform, but verification for the substances tested here could improve acceptance of the proposed tiered testing strategy. Such a calibration strategy is usual for alternative methods, and the principles of such a strategy have been described (Jeliazkova et al., 2021). This calibration strategy is beyond the scope of this article, but will be important in further validation of this decision node in a wider environmental IATA for grouping of nanoforms. Further work is also needed to map the magnitude of these fold change differences onto relevant determinants of fate within environmentally relevant timescales.

3.3. A tiered testing strategy for assessing biodegradation of organic surface treatments

The qualitative comparison between Tier 1 and Tier 2 data and algorithmic similarity assessment of six substances commonly used as surface treatments for nanomaterials has led to the proposal of a tiered testing strategy for assessment of the biodegradation of substances used to modify or treat the surfaces of nanomaterials. This decision node may be used as part of a wider environmental IATA to group nanoforms according to their environmental fate and hazard. The tiered testing strategy, set out in Table 2, indicates how decisions are made within each tier and the rules for escalation between the tiers.

3.4. Critical outlook for tier 1 MT2 test as part of a wider IATA for grouping nanoforms according to their environmental fate and hazard

There are some known interferences with the MT2 assay that should be considered when using the test. High calcium content >100 mgL^{-1} in the test media is known to interfere, causing precipitation of the dye and should be avoided to prevent false positives (Pierce et al., 2014). Calcium content of the test medium used in this study was <1 mgL^{-1}, so unlikely to affect the assay. Some test substances themselves may also be incompatible with the test through preventing reduction of the tetrazolium dye, resulting in false negatives. No evidence of any such effect was found for substances tested here. Further, the design of the tiered testing strategy still allows for the negative result for these substances in Tier 1 to be substantiated through further testing. In this way, even though the MT2 test may not be compatible with all substances, the tiered testing strategy can guide the user to the most appropriate test for such substances, e.g., by escalating to Tier 2.

The absence of any false positive results from the MT2 test indicates that this assay can be employed as a conservative binary assessment for biodegradability. To improve confidence in this conclusion, expansion to a wider set of chemical substances would be required to further demonstrate a low false positive error rate for the MT2 assay. Of the 6 substances tested, two potential false negative results were observed, for the biopolymer Dextran and the synthetic polymeric dispersant Dispex®. Biodegradation did not reach the pass level within 48 h in the MT2 test, but escalation to Tier 2 OECD 301F test would identify both of these substances as being potentially biodegradable.

False negatives could occur if the concentration of the test substance is inhibitory. This is not expected for either Dextran or Dispex®, with biodegradation observed for both chemicals in the Tier 2 OECD 301F assessment, at similar concentrations to that in the MT2 assay of 500 mgL^{-1}, being 320 and 259 mgL^{-1} respectively. Rather, a lag phase of >48 h was observed for both chemicals in the Tier 2 test before biodegradation reached 10%. This lag could explain the false negative result in the 48 h Tier 1 assessment. Future optimisation of the MT2 assay extend the test beyond the current short-term (48 h) duration may make the test more sensitive to such substances and reduce false negative results. This should be considered for future refinement of the tiered testing strategy presented in this article.

A pre-screening toxicity assessment for chemicals of unknown toxicity could also be included in the tiered testing strategy. This step could follow the same rationale as presented in paragraph 25 of the OECD 301 test guidance (OECD, 1992). Toxicity controls for substances could be run alongside the plates, in which both the test substance and a known biodegradable reference are co-incubated in triplicate wells with the inoculum and colour formation monitored for the duration of the test. In the OECD 301 guidance, if <25% biodegradation of the reference substance occurs in 14 days, the test substance is considered inhibitory and either lower concentrations of the substance, or higher concentrations of inoculum are recommended. Similar limits could be developed for the MT2 assay as part of further optimisation of the tiered testing strategy to allow for more hazardous substances to be tested.

Table 2
The tiered testing strategy for a decision node on the biodegradation of organic nanomaterials surface treatments in the environment. The first column describes the tests required for each tier to answer the decision node. The second column describes how to interpret the data and when to escalate between tiers to improve certainty in the decision based on these assessments. Each tier starts with a review of existing data, as it may not always be necessary to perform a test if relevant data are available.

DN1: Is the organic surface treatment considered to be lost quickly from the nanoform surface?	How to interpret data and escalate between tiers.
TIER 1 Review existing data sets Biodegradation screening using the MT2 assay, using relevant microbial community.	• If biodegradation >90% at the end of the test, the material is considered to be completely biodegraded within the test duration. The MT2 assay is shorter in duration than the 10-day window of the Tier 2 tests. Therefore, this assay is considered suitably conservative to reach a decision that the surface treatment is lost quickly. This is not a surrogate for the regulatory definition of a readily biodegradable substance. • If biodegradation is observed >15% at the end of the test, the substance is classified as "biodegradation observed". Escalation to Tier 2 test can be used to assess whether the surface treatment is readily biodegradable if that is required for the purpose of the grouping. • If biodegradation is ≤15%, the user cannot conclude on the biodegradability of the organic surface treatment and escalation to higher tier testing is recommended. For the purpose of SbD, results ≤15% might be useful for reducing the burden of testing in higher tiers, by removing these substances from the list of candidates for surface treatment.
TIER 2 Review existing data sets Regulatory accepted screening tests for biodegradation in surface waters (e.g. OECD 301 A – F)	• The surface treatment is considered "readily biodegradable" if biodegradation passes the 60% of ThOD in a 10-d window within the 28-d period (OECD 301). All surface treatments that pass the criteria for readily biodegradable are considered similar. • If biodegradation is observed, but it does not meet the criteria for "readily biodegradable" these surface treatments can be assessed for similarity through pairwise comparison of biodegradation. If surface treatments are demonstrated to be sufficiently similar in their biodegradation, this may justify read-across between nanoforms based on similarity of surface treatment. Justification for similarity of differently coated nanoforms requires evidence that coating presence does not change dissolution rates and dispersion stability is similarly affected by surface treatment. These considerations are addressed in other IATA in the GRACIOUS Framework Guidance Document (Hunt, 2021). • If no biodegradation is observed, the surface treatments are considered similar in terms of their biodegradability. The nanoform as manufactured with its surface treatment, and the role of this surface treatment on fate and hazard, will need to be considered to assess any potential for grouping.

3.5. Critical outlook for tier 2 OECD 301F test as part of a wider IATA for grouping nanoforms according to their environmental fate and hazard

While the threshold for ready biodegradability has been accepted for a long time, the justification of the 10-day time-window criterion has been a matter of debate for a number of years. Richterich and Steber (2001) address the problems associated with the application of the time-window concept for the ready biodegradability evaluation of surfactants (European Commission, 1999). More recently, European Chemicals Agency (ECHA) guidance on microplastic polymers under REACH (Annex XV restriction report), includes a biodegradability criterion for polymeric compounds. The pass criteria proposed by ECHA for assessing the biodegradability of polymers in the OECD 301F test is 60% consumed oxygen in 28 days but excludes the 10-day time window criterion (ECHA, 2019). For the purpose of this assessment, we use the existing pass criteria in OECD 301 for ready biodegradability but because many of the nanoform surface treatments are polymers (such as PVP and Dextran) it is important to keep in mind for future nanoform grouping efforts the proposal to exclude the 10 day pass window for OECD 301F data.

Whilst the current testing strategy assesses the chemical surface treatment alone, it is possible to jointly assess the nanoform with its organic surface treatment when applying OECD 301F. A limiting factor in assessing surface treated nanoforms in the test would be the concentration of nanoform required to deliver a concentration of the organic surface treatment sufficient to meet the validity criteria of the test, of a minimum of 100 mgL^{-1}. The organic surface treatment in many cases would be expected to only be a small fraction of the total mass of solids in the nanoform dispersion. It should also be considered that if the contribution of surface layers to the overall mass of the nanoform is >20% (*w*/w), this would normally trigger a separate registration obligations for the substance under REACH (ECHA, 2017a). Taking this example of a nanoform modified with 20% w/w organic surface treatments, a minimum 500 mgL^{-1} concentration of the nanoform is needed to deliver sufficient concentration of the surface treatment to be valid for the OECD 301F test. Such high concentrations of added nanomaterials are likely to result in inhibitory effects or interference with the test and so are likely to be unfeasible for most cases. Future efforts may be better placed evaluating the success of the proposed tiered testing strategy in correctly predicting nanoforms for which the surface treatment would be lost quickly in the environment through biodegradation (and thus fate become analogous to non-surface treated nanoforms).

3.6. Considering biodegradation of organic surface treatments as a decision node for a wider IATA for grouping nanoforms in the environment

Here we demonstrate implementation of the tiered testing strategy to assess biodegradation of nanomaterial organic surface treatments by microbial communities isolated from WWTP. There is, however, no barrier to applying the testing strategy to other environmental compartments. Indeed, the OECD 301 test guideline allows for the inoculum to be derived from a variety of sources. A substance found to be readily biodegradable in the OECD 301 test is concluded to "rapidly and completely biodegrade in aquatic environments under aerobic conditions" (OECD, 1992), and so the result can be considered applicable both to WWTPs and aerobic aquatic environments. This conclusion may not hold true for other environments such as soils or sediments where oxygen is less available. Should these be the focus of any assessment, higher tier testing or environment specific assessment may be needed. The tiered testing strategy could be expanded in the future to include tests beyond the scope of this article, such as higher tier simulation tests for specific instances within the wastewater cycle such as OECD 314 "Simulation tests to assess the biodegradability of chemicals discharged in wastewater" (OECD, 2011), or additional Tier 2 tests such as biodegradation in soils (ISO, 2019; OECD, 1981).

Degradation of surface treatments is only one route to convergence in surface properties between nanoforms manufactured with different surface chemistries. It is also true that surface properties of nanoforms can be modified by other means, not only by coating with organic surface treatments, for example conferring specific charged surfaces for polystyrene nanoparticles through functionalisation of the polymer with carboxyl or amino groups. Biodegradation of surface treatments does not inform on convergence in the fate of such nanoforms in the environment.

Over coating by natural ligands such as proteins or humic and fulvic acids in the environment to form an "eco-corona" (Nasser et al., 2020) could also mitigate differences in the fate and toxicity of different nanoforms. One could suggest that demonstration of loss of the surface treatment is not necessary, if it is likely that natural ligands will overcoat the particles and passivate different particle surfaces in a similar manner. However, we suggest that demonstrating the loss of the surface treatment through biodegradation is a conservative approach to justify similarity between nanoforms with different surface treatments. Differences in toxicity between aminated positively charged nanopolystyrene particles compared to the negatively charged carboxylated nanoform can be reduced when the surface charge of these particles is masked by the absorption of dissolved organic matter i.e., formation of an eco-corona (Schultz et al., 2021). However, the difference between forms was not completely eliminated, and indeed the acquired organic coating can be degraded easily revealing the charges again. Whilst the difference in toxicity in this example was due to functionalisation of the polymer, rather than conferred by an organic surface treatment, a similar lesson might apply to coated nanoforms, where demonstrating formation of an eco-corona may not be sufficient to conclude that the fate and toxicity of nanoforms manufactured with different surface treatments converge. We consider loss of the surface treatment as a more lasting mechanism than over-coating, and thus consider it a key step when deciding whether differently surface-treated nanoforms have similar fate in the environment.

The current tiered testing strategy focuses on aerobic biodegradation as a mechanism through which organic surface treatment substances may be lost from the nanoform, but other degradation pathways may exist. It is established that methanogenic bacteria, also isolated from sewage sludge, can be effective at degrading polymers (such as polyethylene glycol), with degradation rates inversely related to the molecular weight of the compound (Dwyer and Tiedje, 1983). Further, biodegradation is also just one of several environmental processes which may act to degrade nanoform surface treatments. Extension of the principles described here could equally apply to photolysis of organic compounds for example, for which standardised tests also exist, e.g. OECD 316 on direct photolysis (OECD, 2008).

For those nanoforms for which degradation of the surface treatment is not achieved, nanoforms could still be grouped into a "set of nanoforms", if it is demonstrated that other elements of the particles fate and toxicity are similar. A wider environmental IATA (e.g. concerning the stability and dissolution behaviour of nanoforms) may still allow for grouping across nanoforms with different non-biodegradable surface treatments in cases where surface treatment does not sufficiently change the fate and behaviour. For example, bare zinc oxide (ZnO) and ZnO modified with a hydrophobic surface treatment experience reduced accumulation and toxicity to the marine copepod *Tigriopus japonicas* in acute 96 h exposures as compared to hydrophilic surface treated ZnO (Lai et al., 2021). However over chronic exposures of 21 days, differences in the toxicity between these nanoforms were reduced compared to those in the acute study. This could be explained by a similar dissolution behaviour of the different nanoforms over longer time scales of days compared to hours. Dispersion stability and dissolution rates of nanoforms have been proposed as key properties driving the predicted environmental concentrations of nanoparticles in the environment (Meesters et al., 2019). Such an IATA based on these properties should also consider the context of compartment specific residence times for grouping and as part of environmental risk assessments (Svendsen et al., 2020).

4. Conclusions

Herein we demonstrate an application of the Biolog MT2 colorimetric well-plate assay as a Tier 1 level screening test for biodegradability of chemicals used as organic surface treatments for nanomaterials. A tiered testing strategy is proposed consisting of the Tier 1 MT2 assay and a Tier 2 standard test for ready biodegradability (OECD 301F). This strategy is refined based on results for 6 commonly used surface treatment substances from these two tiers of testing, to guide the user in the interpretation of results from each tier and to formalise rules for escalation between tiers, based on the purpose of the grouping. The tiered testing design allows for its incorporation as a decision node into wider IATAs for grouping of nanoforms according to their environmental fate. Existing evidence in support of this decision node has been critically discussed and the next steps for further validation of this decision node are outlined. Importantly this includes the need to demonstrate that the biodegradation observed for these chemicals in isolation is predictive of their biodegradation when associated with the nanomaterial core, and that nanoforms which demonstrate similar biodegradation in the tiered testing strategy demonstrate convergence in their fate and behaviour under simulated or real environmental conditions.

Similarity assessment using two algorithmic approaches, the Euclidean distance, and maximal fold difference, provides insights into the applicability of these two approaches on the data derived from Tier 1 and Tier 2 assessment in this tiered testing strategy. We conclude that the Tier 1 MT2 assay should be used as a binary assessment to identify biodegradable substances, but that without further optimisation of the assay to reduce the false negative error rate, algorithmic similarity assessments are not recommended for test data from this Tier currently. For the data derived from Tier 2 assessment of biodegradation (OECD 301F test), both Euclidean distances and maximal fold difference similarity assessments provide useful insights into acceptable limits of similarity for this property. Further calibration against additional substances using the OECD 301F test and higher tier data (e.g., from mesocosm studies) would be needed to refine acceptable limits of similarity. In the light of the ready biodegradability criteria that exist for the OECD 301 test, we recommend that fold difference similarity assessment is constrained to within the applicability range of the hypothesis only. The applicability range is defined as the range between substances which reached 10% biodegradation within the 28-day period of the test, and those which pass the 60% biodegradation threshold within the 10-day window of the OECD 301F test. Substances which do not reach 10% biodegradation within the 28-day period of the test may be considered non-biodegrading. For those that pass the criteria for readily biodegradable substances, it is assumed that these compounds are similar and will rapidly and completely biodegrade in aquatic environments under aerobic conditions. For substances within the applicability range (i.e. between 10 and 60% degradation), algorithmic assessments of similarity may be used as part of a read-across justification.

CRediT authorship contribution statement

Richard Cross: Writing – original draft, Methodology, Visualization, Formal analysis, Conceptualization. **Marianne Matzke:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Dave Surgeon:** Writing – review & editing. **María Diez:** Conceptualization, Supervision, Writing – original draft. **Veronica Gonzalez Andres:** Investigation. **Elena Cerro Galvez:** Investigation. **Maria Fernanda Esponda:** Investigation. **Marie-France Belinga-Desaunay-Nault:** Conceptualization. **Iseult Lynch:** Conceptualization, Writing – review & editing. **Nina Jeliazkova:** Formal analysis, Writing – review & editing. **Claus Svendsen:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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