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# Evaluation of Experimental Design Options in Environmental Nano-Science Research

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## Abstract

As an experimental research design plays a pivotal role in executing a research problem, it is imperative of a researcher to develop a suitable and sound research design. Utilizing robust statistical methods can further enhance the study power and thus allow drawing a logical conclusion. The same holds true for basic environmental science research, including research related to the effects of engineered nanomaterials in the environment. In this paper, we (i) provide a succinct overview of multiple experimental design options that are available to conduct environmental research with focus on emerging nanomaterial science research; (ii) outline the pros and cons of various study designs providing examples as appropriate; (iii) identify and discuss the challenges facing nano-researchers in quantifying and characterizing nanomaterials; and (iv) provide a perspective on how these challenges can be addressed in a situation when instrumentations and protocols that have been used for conventional toxicant characterization are purportedly less suitable for gaining insights into interactions potentially occurring at bio-nano interfaces to explain nanotoxicology.

## Keywords

Experimental design; Nanotoxicology; Nanomaterials; Randomization; Higher level design

## Introduction

The objective of a scientific research is to understand the phenomenon underlying the research problem by using a systematic approach which enables researchers to predict, explain, or determine the causal relationship between the variables by precisely manipulating or controlling the experimental conditions. A research study is valued based upon its completeness and integrity. To meet this goal, it becomes crucially important that the research is carried out using sound experimental design(s), and appropriate statistics be applied to test the hypothesis and draw a logical conclusion. Any empirical data collected using a robust research methodology facilitates higher reproducibility of the results, which provides a logical platform to build upon the hypothesis that is generally accepted. This is the basis of any scientific inquiry which attempts to provide a logical understanding of the properties and processes associated with the subject at hand.

As a researcher develops interest in a research project, thorough literature search is pursued, followed by discussion and brain-storming within the research group to address any shortcomings. This will allow the researchers to refine the project to better accomplish the research goals. The researcher develops hypotheses based on the problem statement, which are then executed following specific experimental design(s) per the need and nature of the research. The success of any scientific research is largely dependent on its experimental design. Many kinds of experimental designs have been practiced to generate empirical data in various research settings.

At the heart of rapidly growing nanotechnology lies the purposely manipulated matter in the atomic scale, called the engineered nanomaterials. Engineered nanomaterials have been the subject of increasing interest for material scientists in recent years as they demonstrate uniquely unusual properties [1-3], which are being harnessed for developing high-value products at low cost [1,3]. Their continual applications in various commercial products have raised significant concern for environmental release [4,5] and subsequent environmental hazard which is beginning to emerge [1,2,6-9]. Potential risk of nanomaterials, however, remains to be assessed due to (i) the lack of standards, protocols, and instrumentations to directly quantify nanomaterials, (ii) batch-wise heterogeneity among nanomaterial samples, (iii) instrument-wise variability in particle sizing [9], and (iv) less understanding of interactions potentially occurring at nano-bio interfaces [8].

In this paper, we provide insights into various experimental designs that are used in environmental nano-science research, address the strengths and weaknesses of these research designs, identify and discuss the challenges facing the nano-researchers in quantifying and characterizing nanomaterials, and offer a perspective on how these challenges can be addressed when existing standard protocols and instrumentations that have been routinely used for characterizing conventional toxicants may be less suitable for gaining insights into interactions potentially occurring at nano-bio interfaces when explaining environmental toxicology of engineered nanomaterials.

## Experimental Designs

### One-shot design

This is a design in which a group of test subjects is exposed to a chemical, a drug, or any other treatment, and then the response is measured. This design does not randomize the subjects, nor does it incorporate a control group for comparison [10]. For example, considering the necessity of understanding potential toxicity of silver nanoparticles – one of the widely used nanomaterials in commercial applications today [11] – in plants, a few seeds of a particular plant species can be treated with a single concentration of silver nanoparticles and observe for certain end points (e.g., germination rate, root/shoot growth, DNA damage) [9]. One-shot design can be particularly useful for screening the potential toxicity of any novel

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nanomaterials against some model organisms, before any detailed study can be performed to elucidate factors or mechanistic basis of toxicity.

Advantages: Because only one group of subjects is involved in the test, it significantly reduces the cost of having many groups of test subjects as required by other research designs; hence, it becomes an economical option. It allows researchers to decide if extensive study is necessary for the research problem. Having a single group makes the test simple and easy to carry out and can also be accomplished in a relatively shorter time period compared to having multiple groups as in other research designs.

Disadvantages: Since the one-shot design only provides information on one treatment, it lacks information on what could happen under different treatments and conditions. Lack of control group(s) in the design further makes it difficult to statistically compare the response; *albeit* some information obtained could be used to make a decision on whether detailed study is necessary. Therefore, it can only serve as a pretest or a screening tool before any robust research design is implemented to understand the potential influence of the treatments and conditions on the response variable.

## Completely randomized design

A completely randomized design is a design in which experimental groups receive treatment(s) at random. Considering potential implications of nanomaterials to human health and safety, it is important to screen for nanomaterial toxicity at various concentrations, ranging from environmentally relevant low dose to high dose, the later signifying an accidental or a spill scenario. Hence, using a completely randomized design, individuals of test organisms can be randomly assigned to treatments or controls. This complete randomization can be achieved either by using random numbers generated by computer or by using some physical means, such as using paper slips and drawing subjects at random. This design can significantly control potential effects of unknown extraneous factors on the response variable [12]. In addition, the completely randomized design can be incorporated with many other types of research designs, especially in the laboratory settings, to strengthen internal validity [13].

Advantages: This design is widely adopted for its simplicity and flexibility. It can include any number of factors and its levels. Simple statistical methods can be applied to test the research hypothesis, even when some treatments have missing observations.

Disadvantages: A completely randomized design requires a high degree of homogeneity, despite that natural populations are often highly heterogeneous. Homogeneity between test subjects increases the likelihood that the test statistics will adequately explain the true relationship and determine that it did not occur only by chance. Although randomization is highly regarded for reducing sequential bias, it is important that the research personnel ensures that there was no significant confounding effect of the size, sex, or strain of the model animals being used in the research so that the response can be strongly linked to other significant factors being evaluated.

## Randomized block design

In a randomized block design, the test subjects are randomly assigned, as explained above, to blocks of several treatments or controls. For example, a brood of *Daphnia* (water flea) can be used to conduct an experiment to investigate the potential toxicity of gold nanoparticles. *Daphnia* can be randomly picked and assigned to the test chambers with or without nanoparticles in them [6,8]. When a test of different concentrations is of interest, each concentration group is considered to be a block. These blocks are, then, randomized in terms of their spatial placement during the experimental period.

Advantages: This design increases the propensity that all blocks receive homogeneous test subjects, and therefore is expected to reduce potential conscious/unconscious bias and variances that would otherwise occur. It is a generally well accepted experimental design due to its potential to provide higher precision in the results. When many factors may contribute to influence the response variable, randomization will equally distribute each factor's effects among the blocks such that the potential effect of the treatments can be identified.

Disadvantages: At times block-treatment interactions may also occur, requiring special attention during statistical analysis of the data collected. If the variances between the blocks are not homogeneous or if any missing observation occurs in one or more blocks, it would then require adjusting the statistics accordingly.

## Factorial design

This design requires having at least two factors or independent variables, each consisting of two levels. This represents a 2 x 2 factorial design in which the levels of each factor interact with the levels of the other factor. However, depending on the research objective, the number of factors and its levels may vary, thus increasing the number of experimental combinations and the logistics that follow.

Consider an experiment testing for potential impact of temperature, moisture content (of the test matrix), and quantum dots on percent seed germination in maize, where each independent variable has three levels. This would be a 3 x 3 factorial design. Having triplicate runs for each combination, it would then necessitate 27 tests to run, in total. However, at times when logistics, time, and other resources are limited, then the number of tests can be reduced following a fractional factorial design, *albeit* its limited statistical validity. In a fractional factorial design, three levels of each dependent variable can be reduced to two. Likewise, samples can be run in duplicates instead of three replicates. This would then reduce the design to 2 x 2 x 2 model, thereby leading to significantly fewer test runs.

Advantages: It allows investigating potential interactions between several variables while conducting a single experiment, instead of several experiments for each independent variable. It minimizes the likelihood of missing a relationship as several candidate variables are included. Furthermore, it also provides flexibility for a researcher to introduce, eliminate, or manipulate independent variables at various levels depending upon the study objectives. Because test subjects also can be assigned randomly to treatments, factorial design has increasingly been practiced in many scientific disciplines, including environmental biology and toxicology. Likewise, when the synthesis of the mono-dispersed (size-controlled) nanoparticle type is desired for evaluating particle size-specific toxicity, the potential influence of several formulation factors such as temperature, concentrations of the precursor molecules and their interactive effects can be conveniently explored using the factorial design to optimize the synthesis protocol.

Disadvantages: Increasing the number of variables and their

interactions at many levels will not only increase the number of tests to be conducted but also introduces complexity in the study. This requires complex statistics to adequately explain the influence of each variable or their combined effects on the dependent variable. Often times the results of complex statistical designs are difficult to interpret. Fractional design further compromises the statistical power as duplicate test runs generate only two data points, which prevents for any statistical hypothesis testing.

### Repeated measures design

In this experimental design, measurements are recorded on the same test subject or a sample in a successive period of time. Because a series of measurements are taken, this design measures the effect of time and the interaction of time with treatment on the dependent variable(s) [14]. For example, in an experiment where a researcher is investigating the effect of nanoparticle treatment on the growth of a plant species at different stages of its lifecycle, seedlings can be assigned randomly to different treatments or controls. Then, the growth of plants can be recorded every week until the plants attain maturity. Suitable test statistics can be applied to test the effect of time and its interaction with treatment [6] on the growth of plants.

Advantages: It minimizes the number of test subjects to be used as the measurements are repeated over time on the same test subject or a group. Potential influence of time on the response variable can be tested at both the individual and the population levels.

Disadvantages: Because measurements are repeated over time on the same sample, it may not be representative of the population and comparison across the groups should be carried out with caution. It should be noted that the repeated measures within subjects are dependent with each other, unlike the measures among different groups which are independent, appropriate test statistics should be applied when testing for statistical significance.

### Response surface design

This research design seeks to understand the relationship between the independent variables (or factors) and the response variable(s) by optimizing the independent variables. Prior to understanding the relationship, it is important to identify what factors are more important than the others and how they can be optimized to obtain the desired response [14]. Factors that are significantly important, such as size, shape, or surface charge of nanoparticles, can be identified using a factorial design as previously explained. Different kinds of suitable statistical methods, including Regression models or General Linear Models (GLM), can be used to assess the strength of relationship between the response and the independent variables [15].

Advantages: With proper understanding of statistics, the researcher will be able to improve significantly the study power by assessing the goodness-of-fit, estimating errors, checking for homogeneity of variance, building higher order designs from simple designs, transforming the variables when needed, and manipulating different treatment combinations. The design can incorporate both quantitative and qualitative factors to improve the predictive power of the model.

Disadvantages: Because it requires higher statistical knowledge, less skilled personnel may not be able to apply this study design in research. It can only be used when factors influencing the response are already known, so that the known factors can be optimized to obtain the best explanatory model. Depending upon the goal of the study, it also requires many iterative runs to achieve minimum, optimum, or maximum response [16].

### Latin squares design

In this design, random assignment of treatments is done once in each row and once in each column to control for variation in rows and columns (or two directions). This design uses Latin letters to illustrate the ways by which the levels of one factor and their combinations with levels of other two factors are assigned. The number of levels is required to be similar to the number of factors in the experimental design, such that the design resembles a square.

Advantages: This design helps to reduce the number of tests as opposed to the maximum possible combinations of the factors and the levels. Suppose, there are 4 different bacterial strains (a, b, c, d), 4 types of nanoparticles (1, 2, 3, 4), and 4 types of bacterial growth media (A, B, C, D). Here, the researchers are interested in understanding the potential influence of the growth media on nanoparticle toxicity to different bacterial strains. The maximum possible combinations in this case would be $4^3 = 64$; however, using Latin squares design the number of combinations can be reduced to 4 x 4 = 16, as shown in Table 1.

Disadvantages: Because this design does not take into account all possible combinations, it provides limited information about the potential influence of growth media on nanoparticle toxicity.

### Higher level designs

Many kinds of advanced statistical designs are available for testing hypotheses when several predictors including categorical variables (e.g., gender/sex, age class) and their interactions with different combinations are of interest. One such design is the General Linear Model (GLM). GLM is generally considered a robust design as to other basic hypothesis testing procedures (e.g., ANOVA, ANCOVA, or Regression) when multiple predictors, categorical variables, and their interactions at various levels are of research need. Moreover, using different types of sums of squares methods – Type I, Type II, Type III, or Type IV – the potential contribution of independent variables, either alone or in combinations, can be tested by adjusting for the influence of covariates which are already considered in the model [15,17]. When several variables are being studied, testing for a potential multicollinearity problem should allow for discerning if two or more variables have significant correlation with each other. Inclusion of two highly correlated variables in the statistical design is undesirable as it can significantly change the coefficient estimates or inflate the standard errors of other predictors, although it may not necessarily impact the predictive power of the model. Multicollinearity between the variables can be easily detected using simple statistics

**Table 1:** An example of Latin Square Design that can be applied in environmental nano-science research.

| Nanoparticle Type | Bacterial Strain Type | | | |
|---|---|---|---|---|
| | a | b | c | d |
| 1 | D | B | C | A |
| 2 | B | C | A | D |
| 3 | C | A | D | B |
| 4 | A | D | B | C |

Here, the letters A, B, C, and D represent four different types of growth media used, whose potential role in influencing nanoparticle toxicity can be of primary interest of the research project

such as the Condition Index or the Farrar-Glauber Test.

Often, when several morphological or biochemical measurements of the test animals are considered as a response to exposure to nanomaterials (e.g., silver nanoparticles, or carbon nanotubes), such multiple parameters can have some degree of correlation. Thus, several potentially correlated parameters can be reduced into fewer uncorrelated parameters called the Principal Components (PCs) using the Principal Component Analysis (PCA). Further, these PCs can be used to test the hypothesis using a suitable hypothesis testing method. It is, however, important that the data set should meet the conditions for the PCA. Tests such as the Bartlett's test of sphericity or the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy are available to check for appropriateness of the data set for the PCA [15,17].

When a researcher is interested in studying the effect of some environmental contaminants or different types of nanomaterials on the germ cell development or in the fertility of the model organism, then the Generalized Linear Model Nested Design can be a good choice. Because the germ cells or the offspring of an organism are nested within mothers, various biochemical and morphological measurements of the parents and the offspring can then be applied to the nested design to understand the potential effect of nanomaterial (as a model toxicant) dose, its other physicochemical characteristics, including the water quality parameters, and the characteristics of the mother on the body mass or size of the offspring (as a response variable).

Regardless of the type of statistical model developed, it is important that the model be tested for its goodness-of-fit before it is accepted as the parsimonious model. Moreover, observations of the residual plot and the coefficient of determination ($R^2$) of the model can provide added confidence on the power of the model, which can then be utilized to predict similar phenomena or the responses [15].

## Issues of Nanomaterial Toxicity Assessment

As nanotoxicology is in its early stage, it may require modifications in the existing protocols to make it well-suited for nanomaterial characterization in the test media [7,18], which would allow better understanding of the cause-effect relationship to be established on a given model organism for the nanomaterial being studied. Because in many cases the response of an organism generally increases with an increasing dose, correct quantitation of exposure concentration of the tested nanomaterials becomes imperative. Particle size is another important factor known to play a significant role in imparting toxicity, hence size needs to be determined in the test media as agglomeration leads to the formation of larger-sized particles, which can potentially attenuate the toxicity [6-8,19,20].

Nonetheless, although many options might be available to address the aforementioned issues despite not much discussion available in the literature, time series design could be one of the workable options, which has yet not been realized, in dealing with the existing limitations realized for nanomaterial sizing and toxicity assessment. The variables of interest are measured in a successive period of time in a time series design to investigate for any potential trends. Generally, data collected in close time periods might show stronger relationship than those that are more spaced out in time. However, with nanoparticle propensity to agglomerate or remain stable depending on the test media or the diluent/matrix used for toxicity assessment, the relationship as stated above may not hold, and therefore study needs to be designed in a way that would address such inherent issues identified in the nanotoxicology literature [6,7,18-20]. Application of a time series design might offer better insight in this situation. Considering nanoparticle size as an important variable for toxicity assessment, measurement of size at successive intervals during entire experimental period should provide conclusive information on how (and if) particle size might have varied, and whether it had any role in the toxicity. Likewise, other parameters such as surface charge, state of agglomeration, and solution pH need to be considered in light of time series design.

In the ensuing sections, we present the state-of-the-science about how nanomaterials are quantified and purified, how particles are characterized for size, what the limitations are in nanotoxicology research, and provide additional perspective on how these limitations can be addressed.

### Issues of nanomaterial concentration quantification

Direct measurement of nanomaterial concentration in a suspension has not yet been achieved due to the lack of analytical tools capable of directly quantifying (only) nanomaterials in the suspension. For inorganic nanomaterials, the current state-of-the-science has been to measure total metal concentration by using an Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) or an Atomic Absorption Spectrometer (AAS-flame/furnace), prior to which samples are digested using a concentrated acid (e.g., $HNO_3$, or HCl).

Efforts have, however, been made to quantify nanoparticles alone by isolating dissolved ions and other remnant impurities from the suspension by using ultracentrifugation. As no standard protocol exists for ultra-centrifuging nanoparticles, literature reveals inconsistency in defining the amount of supernatant that would consist of only the ionic/dissolved forms of the test material and therefore the residual volume (at the bottom of the vial) would consist of only the nano sized particles [6,21,22]. Use of low molecular weight (5 kD pore size) filter containing centrifuge tubes may allow separation of larger than 2 nm sized particles from the residual impurities and ions. However, a recent study reported that its separation efficiency was slightly above 50% for silver nanoparticles [21]. Moreover, how would different sized (5 nm versus 50 nm) or polydispersed (with variable size) nanoparticles separate out as supernatant versus residual mass during centrifugation is also unclear. The polydispersed form is generally the most common scenario encountered and used in nanotoxicological studies.

Recently introduced tangential flow filtration (TFF) process, also called diafiltration, has been established as an effective platform to routinely purify residual impurities and ions that might be present in any nanomaterial suspensions. Using hollow fiber membranes, TFF has been shown to adequately preserve the colloidal characteristics (e.g., size, shape, or coating) of the particles and remove the impurities from the nano-suspension, as well [6,7,23,24]. The potential loss of nanoparticle mass within the polysulfone membranes and the tubing (pers. obser. LRP) due to particle adsorption on the walls may render the method less suitable especially when higher concentration of nanomaterial in the purified suspension is of research need. Therefore, analytical methods and devices precisely quantifying and effectively purifying nanomaterials are of paramount importance as accurate quantification of the dose would only allow better estimation

of the risk posed by nanomaterial exposure to the environment and humans [6,21,25-27].

### Issues of particle size characterization

Various methods are available for characterizing particle size of nanomaterials. For example, Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), among others. DLS technique tends to provide larger particle size distribution (PSD) as it measures hydrodynamic diameter (HDD), unlike the TEM or SEM that measures dry particle diameter under high electron energy. Furthermore, with DLS there are three methods of data reporting: intensity weighting (based on the intensity of laser absorbed or refracted by the particles in the suspension), volume weighting (based on the volume of the particles in the suspension), and number weighting (based on the number of particles in the suspension). Generally, each type of weighting provides different average sizing of the particles; hence, the decision about which weighting is preferred over the others should be made by the researcher, which can lead to biased reporting of the size. Also problematic has been to discern the real size of the particles in situation when different instrumentations offer wide variability in measuring size [7,28].

Often time comparison can be made of HDD with the electron microscopy's PSD; however, the general understanding that under the electron microscopy the particle dries out and shrinks [29,30], giving rise to smaller particle dimension, may not suffice with all types of nanomaterials (pers. obser. LRP). Particle sizing in the dry/powder form provides some information, but does not provide information of the real time sizing in the suspension or in the media used for toxicity assessment. Interestingly, it appears that a recent progress made in microscopy can provide better estimation of the particle characteristics, including its size, in a hydrated sample using the Liquid TEM (http://www.picosci.com/technology). The data thus obtained can be more comparable to that of the DLS measurements, but this will take some time for its adoption as a routine characterization tool.

### Issues of nanoparticle stability

When nanoparticles are suspended in a medium (e.g., moderately hard water, hydroponic medium, agar) which is used for toxicity assessment, they may or may not remain the same with regard to their physicochemical characteristics. For instance, change in particle size from an initial 10 nm (diameter) to 200 nm in reconstituted hard water matrix indicates particle agglomeration and therefore may not be accounted as nanoparticle, should the generally accepted criterion defining nanoparticle (defined as < 100 nm in at least one dimension) be adhered to [31]. Generally, micron-sized particles or agglomerates show lower toxicity than the nano-sized particles of the same chemical make-up [20]. While considering agar as the test medium used in bacterial or plant growth bioassay, an introduction of stable nanoparticle suspension or powder into the agar medium may immediately result into agglomerates due to nanoparticle interactions with several types of mono- and divalent cations present in agar medium [22,23,32]. However, studies show that natural organic matters (NOM such as humic acids, fulvic acids) in an aqueous matrix can reduce particle agglomeration [33]. Considering an aquatic environment where NOMs generally occur, it seems likely that the particles can retain their nanosize; but taking the hardness of the natural waters into account, studies suggest that particles tend to agglomerate and precipitate due to the cations that are ubiquitous in the natural waters [22,23,33-35]. Comparative toxicity evaluation of nanomaterials in the laboratory assays versus the natural water samples and including the soil/sediment as the test matrices should offer better understanding of the potential risk of nanomaterials to the microbial, aquatic, and terrestrial organisms inhabiting the natural environments [6,7,27,32,34-37]. A schematic depicting the aforementioned perspective is presented in Figure 1.

## The Path Forward

Among a plethora of experimental designs available for environmental nano-science research, it is important to identify and apply the design that suits well for the research objective to be investigated. Hence, having an adequate knowledge of the basic and higher level designs should enable one to choose better research methodology, which should offer productive research outcome. With regard to environmental nano-science research, the focus has been to identify important factors explaining nanotoxicity, and the potential mechanisms of bioactivity. Sound characterization of nanomaterials in the test media can provide more information than obtained only for the original nano-stock suspension. However, with the lack of analytical tools and standard protocols, the need to develop them is of utmost priority at the current time. Furthermore, an application of the environmentally relevant exposure concentrations and the use of natural water/soil/sediment samples to compare the toxicity with the laboratory buffered samples should offer meaningful understanding and assessment of the potential risk in biotic organisms from exposure to nanomaterials in the environment.

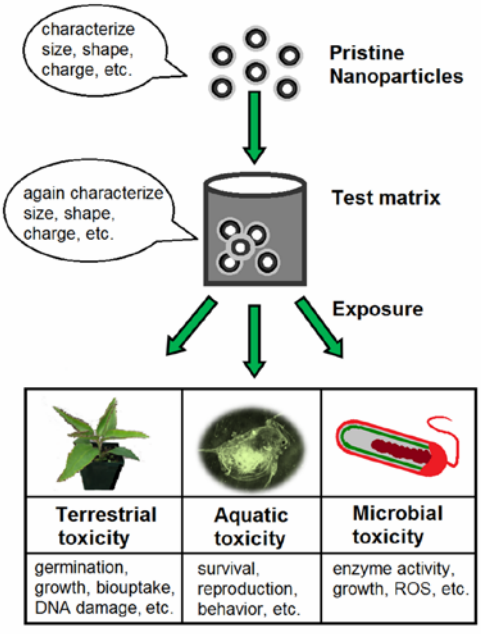


**Figure 1:** Sequence of events in environmental toxicity assessment of engineered nanomaterials in various biological models; ROS: Reactive Oxygen Species.

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