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COMMENT

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COMMENT

Comments on: "Virus adsorption in a complex system: an experimentally designed study" by F. Quignon, F. Thomas, C. Gantzer, A. Huyard and L. Schwartzbrod *Wat Res* **32**(4), 1222–1230 (1998).

This is an unusually ambitious paper. As indicated by its title, it has *two* major purposes, of approximately equal importance. One is to determine, using polioviruses in laboratory aqueous environments similar to those found naturally, how virus adsorption onto clay particles depends on the chemical conditions, which is information with numerous potential applications to water and wastewater engineering. The other is to convince water and wastewater engineering researchers that experiments designed according to principles derived from combinatorial mathematics can determine interaction effects in multiparameter systems that would be overlooked in less sophisticated experiments.

The authors have performed an excellent study and have presented a very clear description of the work. The following questions and comments are intended less as criticisms than as ways of clarifying our own understanding and perhaps that of other readers.

1. Were the Doehlert matrices chosen because they have some clear superiority over other types of combinatorial designs (e.g. other types found in the *CRC Handbook of Probability and Statistics*)? If so, what makes them superior?
2. Has mathematical research in the decades since Doehlert's papers in 1970 and 1972 turned up any newer experimental design principles that would be applicable to these types of experiment? Does the importance of the 'additional experiments' (the ones performed on combinations of parameters that were not part of the Doehlert matrix system) imply a need to find combinatorial designs that would be more comprehensive than the Doehlert matrices?
3. Has there ever been an effort (by virologists or other microbiologists, if not by water and wastewater engineering researchers) to observe viruses adsorbed on clay particles, using scanning electron microscopy or any other imaging technology? Would such imaging applied to particles formed under varying chemical conditions add insight to the kinds of observations recorded in this paper?
4. Do the results in this paper lead to any feasible recommendations for enhancing virus removal in tertiary treatment in wastewater treatment plants? As the authors probably know, microfiltration does an excellent job of removing cellular organisms, but its performance in removing viruses, which are far smaller than the pores of microfiltration media, is highly variable (Jacangelo *et al.*, 1995; Iranpour *et al.*, 1998a, b), and so it has been widely accepted that microfiltration must be followed by a final disinfection step, either chemical, using chlorine or ozone, or physical, using UV. These results raise the possibility of an alternative approach using virus removal by clay adsorption before microfiltration, with the need for final disinfection reduced or eliminated. Do the authors have any comment on this possibility?
5. Tables 3 and 4 show standard deviations of the results of the additional experiments, but there is no indication in Table 2 or Fig. 3 of the variability of the results from the experiments in the Doehlert matrix designs. Although these were all done only in duplicate, and therefore could not simply be fed into the conventional standard deviation formula, we note that each estimated virus concentration was obtained by a titration and counting method that allowed associating a confidence interval with it and that the confidence interval was reduced by a maximum likelihood test, all of this being computed by the methods of Maul (1991). Are we correct in concluding that it would have been possible to indicate a magnitude of uncertainty for these results?
6. Is it realistic to be concerned about the possibility of saturating the adsorption capability of clay samples after prolonged use for virus adsorption? Since the experimental methodology assumed that adsorption need not cause inactivation, and this was confirmed by the results, it seems possible that eventually the available adsorption sites on the clay particles would be filled, and additional viruses would pass through, or have an equivalent effect by displacing previously adsorbed viruses, in a manner somewhat analogous to the way that other filter media become saturated with removed material and must either be replaced or backwashed. As backwashing seems inapplicable in this context, a valu-

able use of the results of this paper might be to help understand the possibilities for manipulating the chemistry of an aqueous environment to promote inactivation of adsorbed viruses.

7. Along these same lines, we note that when a space of system responses to varying combinations of input parameters has been surveyed with sufficient thoroughness, the data may be used as inputs for a search for optimum combination, using dynamic programming. Is it realistic to try to use dynamic programming in this context? More specifically, if the Doehlert matrices are derived from the vertices of multidimensional cuboctahedrons, as indicated by the title of the Doehlert and Klee (1972) paper, do the authors know of any attempts to adapt the general strategy of dynamic programming to the particular geometry (edges, faces, etc.) of cubotahedra?
8. Is anything known about the mechanism of virus inactivation by tannic acid, such as by a 'tanning' effect on the coat proteins? Is the inactivating effect specific to tannic acid characteristic of a wider variety of organic materials found in aqueous environments? These questions are prompted by recalling that discussions of UV disinfection frequently mention that irradiation by light in the wavelength range 250–270 nm causes thymine dimerization in nucleic acids, and this is generally believed to be the most important mechanism of inactivation of microorganisms in these systems.
9. Does the type of virus make a difference in the results? For example, if similar experiments were done with coliphage viruses, is it likely that the results would be significantly different? Since water and wastewater engineering are not usually concerned with viruses that are not infectious to humans, repeating at least a few of these tests on coliphages would not directly add to predictions of the health and safety effects of various virus removal processes involving clay. On the other hand, since coliphages are now often used as substitutes for human viruses, because in certain respects their properties are similar, this test would serve as a test of this substitution, which would fit in with the authors' interest in influencing engineering research methodology.
10. How were the ranges of the parameters used in this study chosen? As the Doehlert matrices are independent of the specific conditions of any experimental study, it appears to us that the two-decade ranges of virus concentration, ionic strength, organic material concentration and clay concentration in this paper were based primarily on past experience with natural aqueous environments. However, since we are interested in the possible applicability of these results to wastewater treatment, where values of some of these parameters may fall outside the ranges observed by the authors, we would like to know a little more about the reasons for the choices of ranges.

Let us close by repeating that this is an outstanding paper and that both the specific virus results and vocacy of combinatorial design methods for experiments on complex systems deserve careful attention from water and wastewater engineers.

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AUTHORS' REPLY

It is always a great pleasure to state that other people but the referees have given some attention to one's paper. I am glad to have the opportunity to give additional indications that are intended to help the reader clarify his understanding of the paper.

The responses are organized as often as possible in the same order as used in the questions.

(1) Modeling of the combinatorial process (behavior under the joint influence of several tested parameters) is often performed using a polynome of general degree three or less. But the higher number of coefficients to estimate, the higher amount of experiments. So many combinatorial designs are intended to evaluate a quadratic model.

Among them the best known are the centered composite designs, the simplex designs of Box–Behnken and the uniform shell designs of Doehlert, which are all special equiradial designs, i.e. designs constituted of points that are regularly spaced on concentric circles or spheres or hyperspheres (Box and Hunter, 1961; Montgomery, 1996). The Latin or Greco-Latin squares are also other famous designs (Box *et al.*, 1978; Armitage and Berry, 1995).

Actually no experimental matrix is ideal. As for the Doehlert matrices, a first feature is their competitiveness towards other designs in the number of experiments required to estimate a given amount of parameters (efficiency). Thus, if k parameters are to be tested, then centered composite designs require 2^{k-p} (fractional design) $+ 2{\cdot}k$ (axial design) $+ N_0$ (central points) experiments, simplex-sum designs of Box–Behnken require $2^{k-1}-2+N_0$ experiments, while Doehlert designs require k^2+k+1 experiments. Still, when the number of parameters to be tested is high, then a fractional factorial design is recommended in a screening purpose. Afterwards, research for an optimum for a given process will take advantage of more sophisticated designs.

Doehlerts' designs most interesting features lie in their ability of expansion in both the "variable's space" and the "experiment's space". Expansion in the experiment's space means that the selected range of any tested parameter can be enlarged by carrying out another set of experiments, without having to repeat all of the former experiments (nested matrices). This is useful for the study of an experimental domain of any shape, which can be sequentially explored in any direction. In the same manner, a given part of the initial space can be more deeply explored by reduction in the mesh size. Expansion in the variable's space means that it is possible to first carry out experiments to determine the impact of p variables out of k and then to carry out another set of experiments to evaluate the impact of the remaining $(k-p)$ variables, provided that these $(k-p)$ variables had been fixed to their respective central value during the first set of experiments.

Expansion in the experiment's space is also possible using a sum-simple design of Box–Behnken or equiradial deigns, but with less versatility for displacement of the domain center. Expansion in the variable's space is not possible at such low cost as with a Doehlert design, but another strategy is then adopted for designs that allow for a partitioning of the whole design into several blocks of experiments. In this approach, additional "blocking variables", allow for the evaluation of a "blocking effect", e.g. because of a parameter that would be time dependent (Box *et al.*, 1978).

(2) The rationale of the planning of experiments is to acquire the highest amount of information with precision and at lowest cost. Reduction in the number of experiments can be achieved because the information obtained is brought about by a combination of all preceding experiments. This principle obliges us to set a value to each tested parameter. Thus, using a Doehlert matrix, it would also have been possible to set a parameter to a value that is known to have no impact on the response observed. However, it would have implied the use of extended parameters' ranges, which would not have obviously been easily practicable. (For example, how can one determine the clay concentration that has really no impact?)

Actually the question here raises the problem of determination of the parameters' ranges to be tested. It is thus important to recall that ambitious experimental designs are all the most successfully performed that the experimenter has already screened for the relevant parameters by using simple designs such as fractional factorial ones (Box and Hunter, 1961; Goupy, 1993) and that he (she) is aware of the parameters' ranges that are practically reachable.

(3) In river water, most of the viruses are observed within the particulate fraction of size < 250 nm (Payment *et al.*, 1988). Most of the mineral fraction of soils is clay minerals (Schultze, 1989). More

precisely, the clayey fraction of most soils is mainly composed of phyllosilicates (lamellar silicates), which constitute the larger colloidal fraction of soils and waters (Theng, 1979). So virus adsorption to phyllosilicate particles such as kaolinite or montmorillonite should be very common in natural waters.

Using electronic microscopy, Vilker *et al.* (1983) observed phenomena of virus adsorption to clay particles as well as formation of clay aggregates of 1 to 2 μm in diameter in a pH 7 0.03 M phosphate buffer, these aggregates being favored in the presence of viruses. On 144 clay particles observed, the authors depict a mean of 1 virus adsorbed on every 6.5 particles.

However, before further answering to point (3), I would like to carry on virus adsorption capabilities of clay, as wished in point (6).

If one considers an environment of low ionic strength, i.e. where electrostatic interactions between charged particles are not favored, the hindrance to virus adsorption is not, in a virus/clay system, a shortage in available clay adsorption sites, but a low rate of collision efficiency. According to Allison and Valentine, 1960, the proportion of efficient collisions has to be introduced into the equation describing the percentage of viral adsorption to clay particles. In our own experiments, performed with ca. $1{\cdot}10^5$ mpncu/ml of poliovirus-1 and 10 g/l of Na–montmorillonite in milli-Q (Millipore) autoclaved water, we estimated this proportion to be 15–20%. Similarly, Jewett *et al.* (1995) report for *Pseudomonas fluorescens* absorbed onto glass fiber membranes or silica beads, estimations ranging from 1.5 to 18% (ionic strength ranging from 10^{-5} to 10^{-1} M). For our experimental conditions, we calculated that clay particles were actually in huge excess towards viruses, i.e. in the ratio of 1 to $1–5{\cdot}10^5$ (clay sheets of size 1.5 nm$\times$0.2 μm to 1.5 nm$\times$1 μm).

If a clay filter happens to be no longer efficient in removing viruses, it is then more likely because of the other compounds present in the aqueous medium, such as organic matters. For instance, Babich and Stotzky (1980) showed that addition of BSA to various clay minerals prior to that of coliphages or enteroviruses (naked ones) brings about an inhibition of virus adsorption to clay... as well as an enhancement of *Herpes hominis*-1 virus (enveloped virus) adsorption, most likely because of hydrophobic interactions between BSA and viruses.

Besides, I am not sure that a backwashing process of such a filter (e.g. using gas and deionised water) could not be successfully developed.

Back to point (3), I guess SEM can provide very convincing pictures regarding the abundance or removal of viruses, as far as the preparation does not interfere with the sample structure. Cryoprotection might be an answer. Also, novel investigation tools such as surface plasmon resonance could help in gathering precise information in much shorter experimental times (Dubs *et al.*, 1991).

(4) Regarding water treatment and viruses, we are more concerned with virus displacement than with virus inactivation. For instance, the contamination risk is increased after strong precipitation or storms, as far as viruses are desorbed from soil particles and are flushed into water resources. Water treatment has just the opposite goal: removing viruses from the water compartment and inactivating the remaining ones.

Sophisticated potabilization lines allow for an (almost) complete virus removal at the treatment plant outlet (Rose *et al.*, 1986; Payment *et al.*, 1991; Stetler *et al.*, 1992). However, virus removal is progressive along with the various treatment steps, and the efficiency of a tertiary treatment for virus removal is actually dependent on the efficiency of former steps not only in removing viruses thanks to adsorption phenomena but also in removing fine particles that can provide viruses with a protective effect towards disinfection.

If filtration is intended to help remove microbial contaminants, then the mesh size must be chosen according to the contaminant size and flexibility. For viruses, nanofiltration seems to be successful, but microfiltration, as Dr Iranpour *et al.* mentioned, leads to unsatisfactory results. Even for *Cryptosporidium*, whose oocysts are 4–5 μm in diameter, microfiltration would not always be able to avoid a certain flowthrough, because of the flexibility of this microorganism and of imperfections in available filters (Dr C. Drozd, personal communication).

(5) Performing each viral determination in genuine duplicate allows for a reduction in the associated 0.95 confidence interval from 48% down to 32% (29% for triplicates). This interval is thus obtained by multiplying or dividing the final estimate by 1.32 (40 wells by dilution level). So the answer to question (5) is: yes.

(6) See point (3)

(7) Refinement of the modeling would certainly require further improvements to reduce the model variance. Such refinements are already implemented in a PC-based program (called NEMROD) developed by the University of Aix-Marseille, France*. This team, headed by Pr Phan-Tan-Luu, is specialized in the use of experimental designs and of Doehlert ones in particular. They have inspired our work.

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In these later years another kind of "black box" model has been widespread: the artificial neural networks (ANN). I do not know of any research using ANN applied to viruses, but their use is already documented in many other fields.

When the behavior of the studied system is not known, then, for a given number of tested variables, the fact of adding a neuron to the ANN implies adding less coefficients to the model than the fact of incrementing a polynome with one degree (Viennet, 1997). A more detailed information is reported in comparisons between classical optimization designs and ANN, applied for example to fermentation processes (Kennedy and Spooner, 1996) or HPLC's mobile phase composition (Metting and Coenegracht, 1996).

(8) Tannic acid is a complex organic structure containing a variety of chemical groups, hence its use as a model for organic matter. The potentiation by salts of its action on viruses is similar to what Berg *et al*. (1989, 1990) observed for chlorine. So it is possible that for tannic acid too, oxidative processes are involved and lead to virus inactivation. Salts could for instance allow for a closer vicinity between viruses and the various inactivating agents.

(9) A *Leviviridae* (coliphage MS2, f2 or Qβ) or a *Microviridae* (coliphage ΦX174) can be selected for its structural similarities with non-enveloped small enteric viruses (icosahedral shape, 20–30 nm size in diameter, proteinaceous capsid). While these models do not always provide the same sensitivity to various inactivating factors (as far as their respective infectious capacities or genomic damages can be compared), they often demonstrate a similar behavior relating to adsorption phenomena, though with expected different magnitudes. So I am very confident in expecting similar behavior for bacteriophages, as far as the same interactions are involved (mainly electrostatic and van der Waals ones). Hydrophobic interactions should also demonstrate a higher influence with enveloped viruses. Indeed the methodology of experimental designs could benefit from a selected entity (e.g. a phage) for which responses could be obtained with more ease than with enteroviruses. Conversely, this methodology can help perform a higher amount of experiments and obtain results with reduced variance, in order to gain insight within the various phenomena occurring simultaneously in a complex system.

(10) If an experiment is performed under such specific conditions that any result is very difficult to obtain, it will always be the case, whether an experimental design is used or not. Still, lack of a response for a particular point in the classical methodology of "modifying-one-factor-at-a-time" has no other consequence. Using the methodology of experimental designs, this lack of response can impede one to get any estimation at all, as the coefficients of the polynomial model are evaluated on the basis of the whole set of experiments (thereby also allowing for a reduction of variance). This is why it is a prerequisite to have good knowledge of the techniques used and of their intrinsic limitations. Nonetheless an unexpected interaction between various tested variables can occur, which leads to a lack of answer for a particular combination. In this case, algorithms exist that help fix the "injured" matrix. They generally involve testing a few new combinations around the disabling point. Again, a good specific computer program should benefit from such features.

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