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**ENVIRONMENT DIRECTORATE**
**JOINT MEETING OF THE CHEMICALS COMMITTEE AND**
**THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY**

# Report of the validation study supporting the development of the draft (new) TG 318 on dispersion behaviour of nanomaterials in different environmental media

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# REPORT OF THE VALIDATION STUDY SUPPORTING THE DEVELOPMENT OF THE DRAFT (NEW) TG 318 ON DISPERSION BEHAVIOR OF NANOMATERIALS IN DIFFERENT ENVIRONMENTAL MEDIA

IOMC INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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**Environment Directorate**
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Paris 2017

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## FOREWORD

This document presents the validation study that supported the development of the OECD Test Guideline 318 on Dispersion Behaviour of Nanomaterials in Different Environmental Media. The project to develop the test guideline was proposed by Germany to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) in 2014.

The purpose of this study was to learn about the applicability and accuracy of proposed experimental routines, determine whether the described procedures are interpreted and implemented correctly, identify the factors that can affect results variability and perform the analysis of statistical variability. The current document reports the design of the round robin test and describes the procedures that were followed. As such, it includes information on:

- Participating Laboratories;
- Study Design;
- Chemicals tested;
- Validation of the results;
- Results; and
- Conclusions

The report was reviewed, commented on, and approved by the OECD *Ad hoc* Expert Group on Environmental Fate of Nanomaterials. The report was subsequently endorsed by the 29th Meeting of the WNT in April 2017. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (hereafter Joint Meeting) agreed to its declassification on July 2017. This document is published under the responsibility of the Joint Meeting.

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## INTRODUCTION

1. During the OECD meeting in Berlin in January 2013 experts agreed on the necessity to develop two new OECD Test Guidelines and a corresponding guidance document required to perform the tests on the agglomeration and dissolution behavior of nanomaterials in the environmental aquatic media. The developed Test Guidelines answer the question if the analyzed materials should be tested under consideration of their nanomaterial specificities in further environmental tests and if so, provide the experimental routine to test the behavior of such materials in aquatic media to estimate their possible fate in and impact on the environment. One of the main activities during the development of the mentioned TG is validation of proposed TG by other interested laboratories worldwide (OECD, 2015).

2. The current document is designed to report[1] the efforts taken during the validation test (round robin test, RRT) of TG on dispersion behaviour of nanomaterials in different environmental media. The RRT was designed to learn about the applicability and accuracy of proposed experimental routines, determine whether the described procedures are interpreted and implemented correctly, identify the factors that can affect result variability and perform the analysis of statistical variability.

## PARTICIPATING LABORATORIES

3. Besides the leading laboratory, seven laboratories were participating in the RRT.

- Fraunhofer-Institute for Molecular Biology and Applied Ecology (Germany)
- Colorado School of Mines (USA)
- Carnegie Mellon University (USA)
- University of Nevada Las Vegas (USA)
- US Army Engineer Research and Development Center (USA)
- Korea Institute of Toxicology (South Korea)
- Birla Institute of Technology and Science (India)
- University of Vienna (Leading Laboratory) (Austria)

4. Only one out of seven laboratories performed the entire set of requested experiments.

5. Considering agglomeration behavior of $TiO_2$ (NM105) material, seven of participating laboratories provided the results regarding influence of electrolyte concentration of agglomeration behavior, three of participating laboratories provided the results on agglomeration behavior in absence of $CO_2$. Five laboratories studied influence of DOM and seven laboratories studied influence of established pH=8.5 on particle agglomeration behavior.

6. Two out of seven laboratories (marked as lab 6 and lab 7 on the plots below) provided the results on $TiO_2$ (NM105) agglomeration behavior that were strongly deviating from the results provided by remaining 5 laboratories.

[1] This Report prepared by Philipp Kozin and Frank von der Kammer from Vienna University, Department of Environmental Geosciences University of Vienna Althanstraße 14 UZAII, 1090 Vienna.

7. Considering agglomeration behavior of Ag (NM300K) material, only three laboratories were able to provide the requested analysis. Thus three laboratories reported the influence of electrolyte, absence of $CO_2$, presence of DOM and effect of established pH=8.5 of the sample media on the agglomeration behavior of the suggested materials.

**STUDY DESIGN**

8. Analysis of particle agglomeration behavior was based on the analysis of particle dispersion stability over 6 h time period. The experimental conditions were therefore set to allow the preparation of samples within this time period. The entire procedure including all preliminary preparations was set to be performed within 8 hour (1 working day). All experimental procedures to follow were described in the document "TG on agglomeration behavior of nanomaterials in different aquatic media" in sections 21-33. Typical experimental design was presented in the Annex 1 to the corresponding test guidelines. Laboratories participating in the RRT were asked to study the agglomeration behavior of nanomaterials through monitoring the dispersion stability of suggested materials in several conditions. Among these conditions was electrolyte [$Ca(NO_3)_2$] concentration, presence of DOM (10 ppm), absence of $CO_2$ in the sample surrounding atmosphere and effect of established pH=8.5 of the sample media.

**CHEMICALS AND TEST**

9. Two nanomaterials were distributed among the participants. These were $TiO_2$ (NM105) powder material and Ag (NM300K) dispersions with the concentration of approximately 20000 ppm. Ag (NM300K) material was considered as a positive reference material, since its dispersions were stable at all applied conditions. On the other dispersions of $TiO_2$ (NM105) material revealed condition-dependent stability. Thus the participants of RRT were asked to pay special attention to the studies of $TiO_2$ (NM105) dispersion stability.

10. The required materials and laboratory equipment included but was not limited to:

Equipment:

(a) Suitable calibrated pipets for sample preparation (5ml, 2ml, 0.1ml volume)
(b) Ultrasonic probe for homogenization of particle dispersion
(c) pH-meter to measure the pH of the dispersion
(d) DLS devise for measuring the particle size with suitable for such measurements cuvettes
(e) 50 ml conical bottom polypropylene tubes (N) to perform the agglomeration experiments
(f) 10 ml polypropylene tubes (N) to prepare the samples for ICP-OES/MS analysis
(g) Standard lab centrifuge, capable of 5000 rpm rotary speed
(h) Inductively Coupled Plasma Mass Spectrometry Device for analysis of AgNP nanoparticle dispersions
(i) Inductively Coupled Plasma Optical Emission Spectrometry Device for analysis of $TiO_2$ nanoparticle dispersions.

Materials:

(a) Water (H2O) – ultrapure DI (18 Ohms resistivity)
(b) Sodium Hydroxide solution (NaOH) – 0.1M solution in Ultrapure DI water, for pH establishing

(c) Hydrochloric acid (HCl) – 0.1M solution in Ultrapure DI water, for pH establishing
(d) NOM solution- solution of Natural Organic Matter (1g/L)
(e) Calcium Nitrate (Ca(NO3)2) – 0.1M solution to establish the needed concentration of electrolyte
(f) TiO2 (NM105) – analyzed powder of titanium dioxide, provided by JRC repository
(g) Ag particles (NM300K) – analyzed dispersion of silver particles, provided by JRC repository

11. Results obtained from analysis of the samples are performed in the form of plots, where X-axis stays for time of sampling (0,1,2,3... to 6 hours) and Y-axis stays for the percentage (%) of the concentration of analyzed material compared to the expected concentration in the samples of interest. Expected concentration is the concentration in assumption that the analyzed dispersion is stable, i.e. the concentration calculated assuming that there are no losses of material during experimental manipulations. Decision of representing the results in such way was taken to be able to provide the statistical data on obtained results. Providing the experimental points are obtained in triplicates, the mean value for each point was calculated and presented as result. Standard deviation of results was calculated based on the triplicate measurements and presented on the plots as confidence intervals (error bars) for each point.

12. Such representation differs from the representation accepted in other documents, where the data is normally set to the concentration of material obtained during 0h measurement. Latter approach allows monitoring the losses of the material during experimental manipulations, as shown in Annex 1 to the corresponding test guidelines. However it does not allow the statistical analysis to be performed, that is highly necessary for the inter laboratory validation test.

## VALIDATION OF RESULTS

13. During the validation of the results the obtained data was visually assessed to assure the similar character of obtained dependencies. If such similarity took place the further statistical analysis was performed.

14. During the statistical analysis the experimental points obtained from independent laboratories were gathered and the mean value of all points obtained during similar sampling time was calculated within one experimental dependency, (i.e. either for dispersion containing 1mM $Ca(NO_3)_2$ or for dispersion containing 10 mM $Ca(NO_3)_2$). After that the standard and the mean deviation values for all the points obtained from independent laboratories were calculated and presented as a result of statistical analysis.

## RESULTS

### *Influence of electrolyte presence on the agglomeration behavior of $TiO_2$ (NM105)*

15. The majority of obtained experimental results correlated well with the results obtained in the leading laboratory. Figure 1 illustrates the dependencies of agglomeration behavior of $TiO_2$ (NM105) material from the concentration of electrolyte (0, 1, 10 mM).

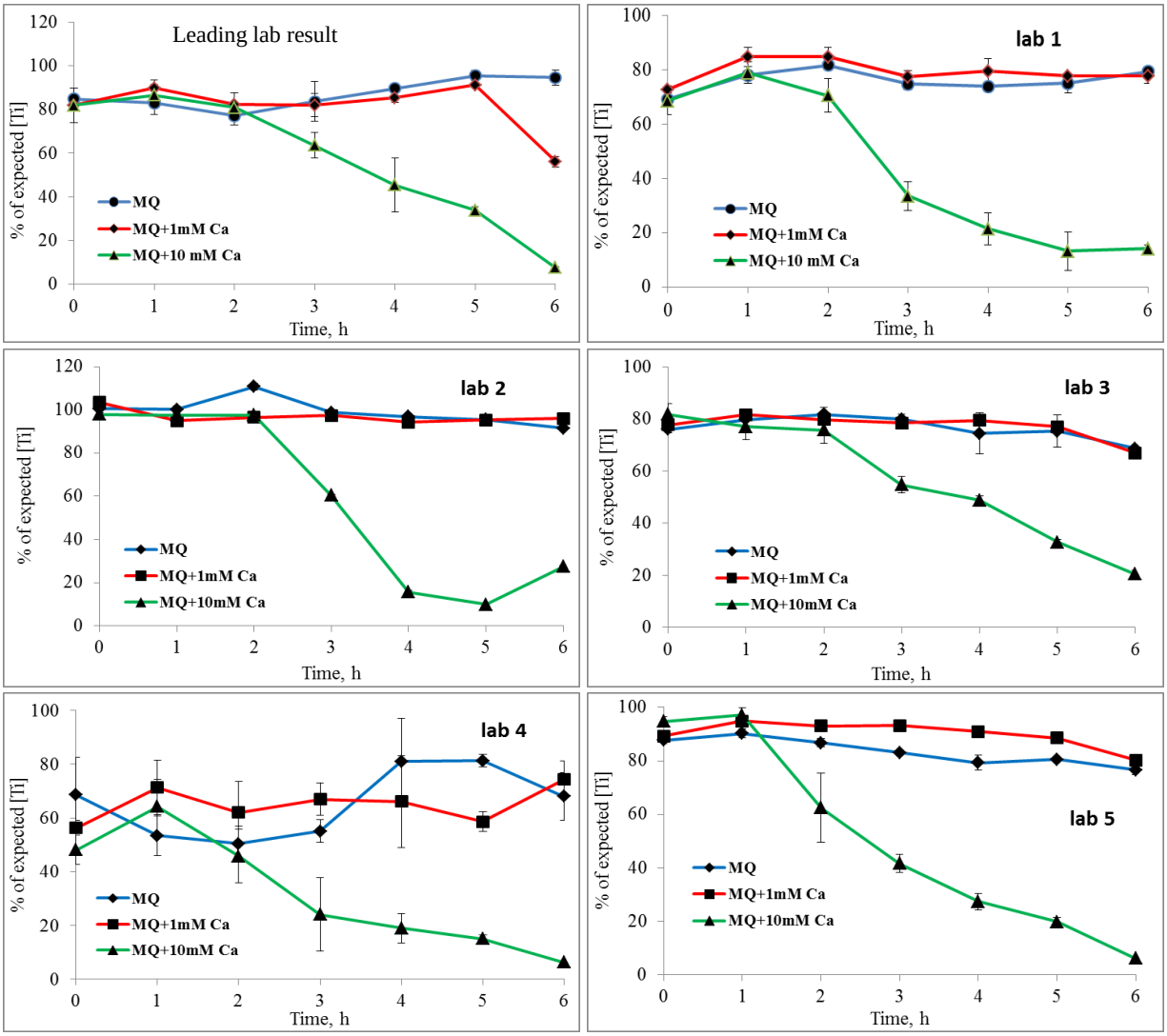



Fig.1. Dependency of agglomeration behavior of $TiO_2$ (NM105) material from electrolyte concentration.

16. As can be seen from the provided results the agglomeration of analyzed material that was observed in 1 mM electrolyte system in the leading laboratory was not observed during RRT in the results of another laboratories. This fact can evidence that presence of 1 mM electrolyte is a very weak destabilizing factor that does not necessarily have to be considered.

17. Based on the obtained results, standard deviation and mean deviation of all experimental points were calculated. The calculated data is shown at figure 2.

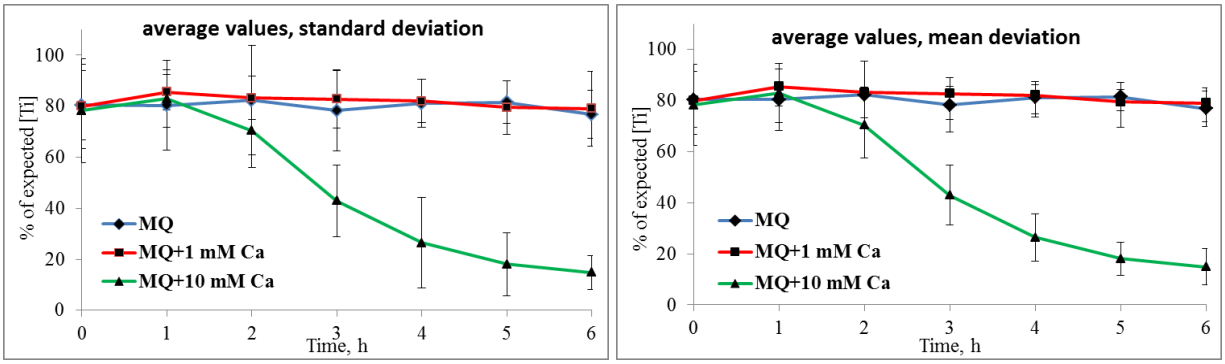



Fig. 2. Average values based on the results obtained from the independent laboratories and calculated standard and mean deviations. Influence of electrolyte presence on $TiO_2$ (NM105) agglomeration behavior.

18. Several laboratories provided results that significantly deviated from the results of other laboratories. During the personal communication with the laboratories providing aforementioned results regarding the possible reasons of such outcome, it was figured out that participants did not manage to perform the analysis of prepared samples within 24 hours as it was prescribed by the provided draft of TG. Such datasets could not be considered for the statistical analysis, but were considered as a proof of errors that could appear when the TG is not followed precisely. Examples of such results are presented at figure 3.

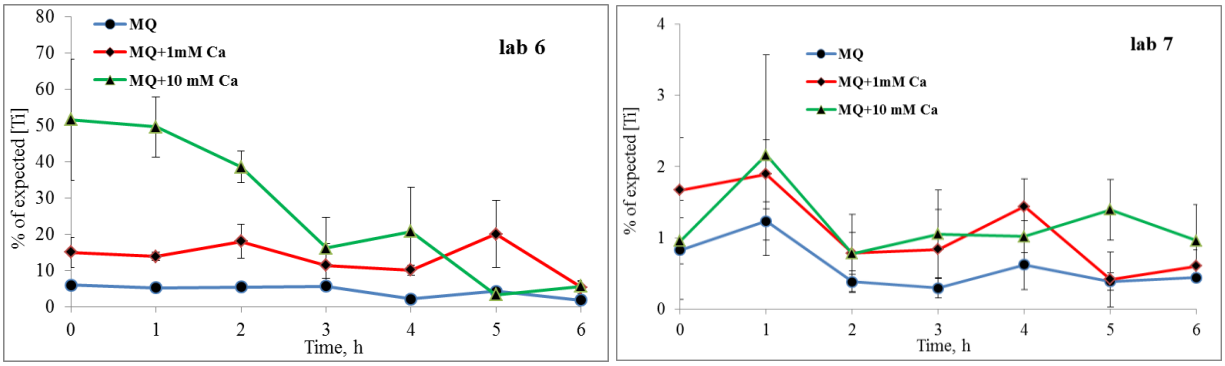



Fig. 3. Results that were not considered for statistical analysis because the large differences between them and the results provided by leading laboratory. Influence of electrolyte presence on $TiO_2$ (NM105) agglomeration behavior.

### *Influence of $CO_2$ absence on agglomeration behavior of $TiO_2$ (NM105)*

19. Similar analysis was performed on the systems that were prepared in absence of $CO_2$. Since the results of experiments were highly similar to those in the presence of $CO_2$, those experiments were not made in such large extent as the ones described above and therefore fewer results are provided. Results can be observed at figure 4.

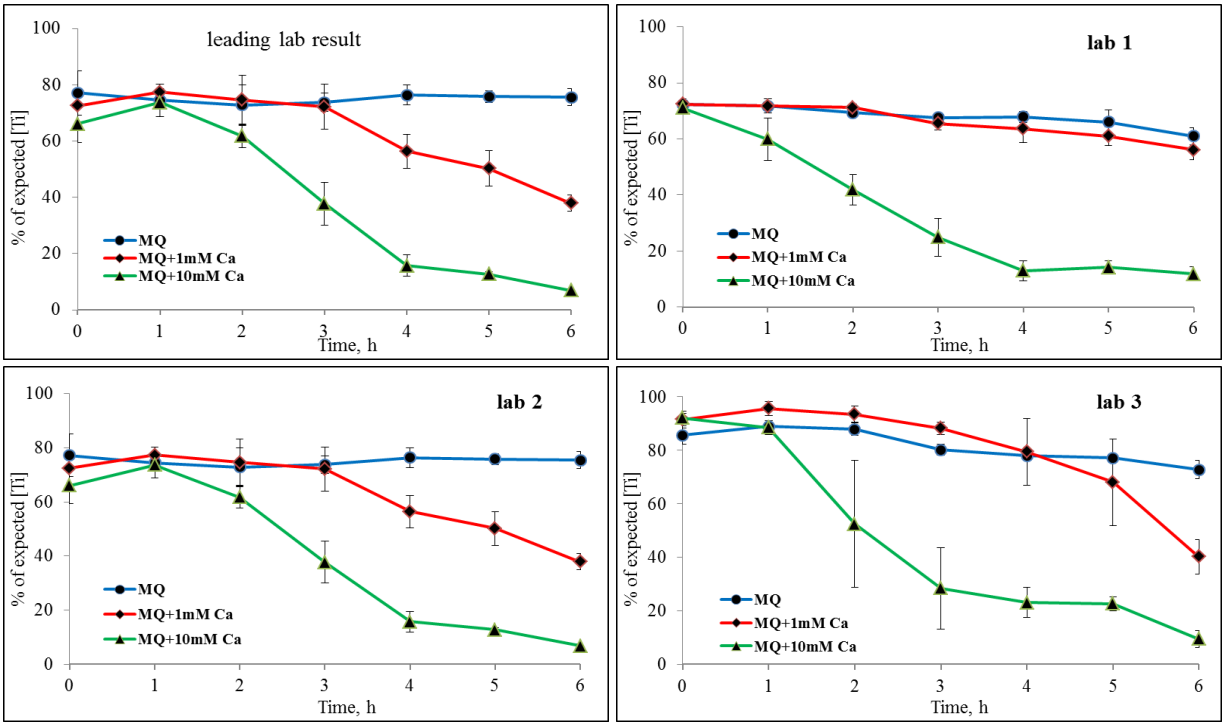



Fig. 4. Absence of $CO_2$ did not play a significant role while investigating the agglomeration behavior of $TiO_2$ (NM105).

20. Based on the obtained results, standard deviation and mean deviation of all experimental points were calculated. The calculated data is shown at figure 5.

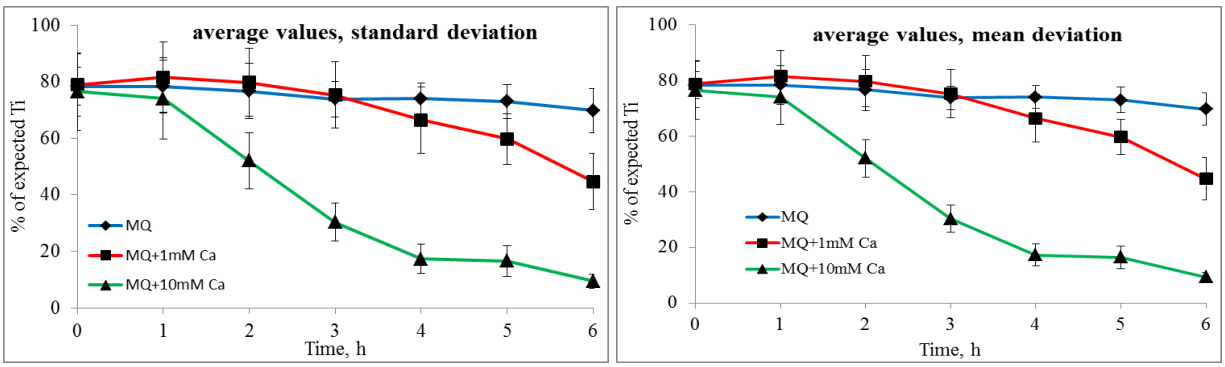



Fig. 5. Average values based on the results obtained from the independent laboratories and calculated standard and mean deviations. Influence of CO2 absence on $TiO_2$ (NM105) agglomeration behavior.

### *Influence of DOC presence on agglomeration behavior of $TiO_2$ (NM105)*

21. Analysis of Dissolved Organic Matter (DOM) containing systems has shown that in case of leading laboratory presence of DOM stabilized the system containing 1 mM electrolyte. Results from other laboratories have shown that the 1mM electrolyte systems remained their stability. Such result shows that presence of 10 ppm DOC is not a crucial stabilizing factor that has to be mandatory considered.

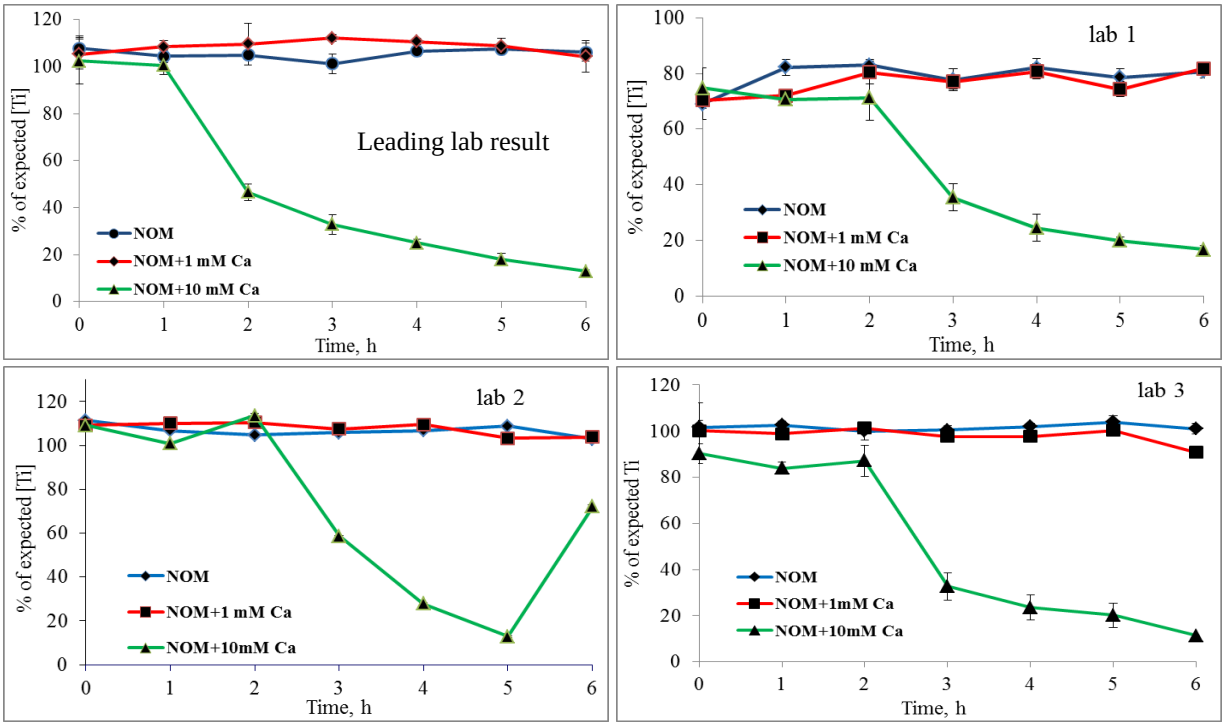



Fig. 6. Influence of 10 ppm DOC media on the agglomeration behavior of $TiO_2$ (NM105) particles.

22. Based on the obtained results, standard deviation and mean deviation of all experimental points were calculated. The calculated data is shown at figure 7.

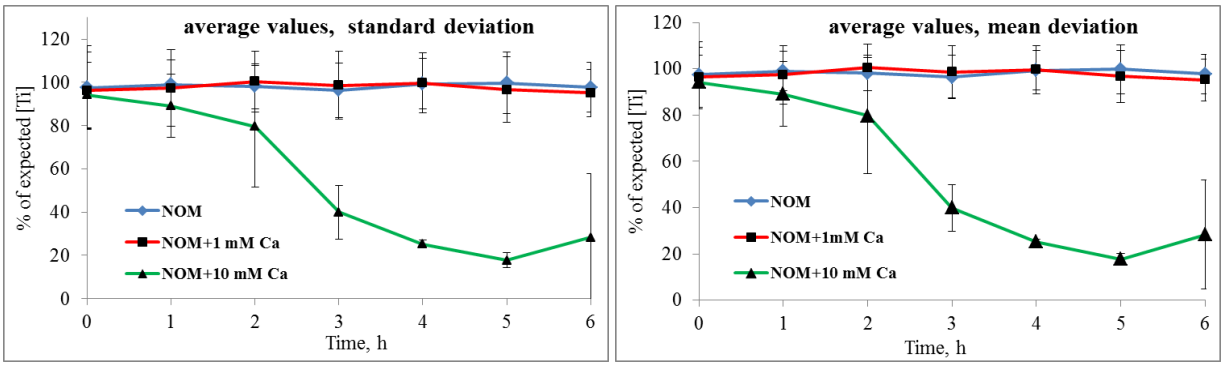



Fig. 7. Average values based on the results obtained from the independent laboratories and calculated standard and mean deviations. Influence of 10 ppm DOC media on $TiO_2$ (NM105) agglomeration behavior.

23. Statistical analysis has shown that the material recovery has increased with addition of DOM to the aquatic media. This fact means that DOM prevents particle precipitation on the walls of experimental tubes.

24. Several obtained results were strongly deviating from majority of other results. As such deviating results were all obtained from the same laboratories, as before, this effect was again explained with the fact that investigators were not able to perform the analysis of prepared samples within 24 hours after their preparation, what led to particle precipitation on the tube walls. As a consequence these results were excluded from statistical analysis.

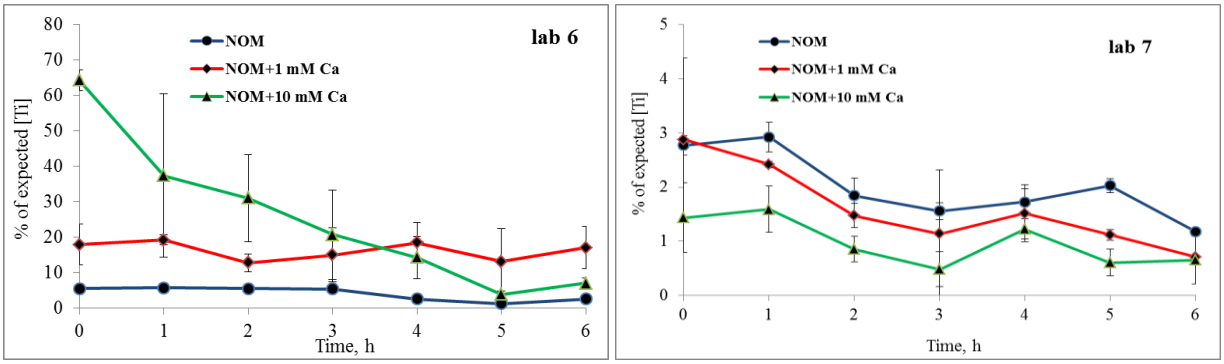
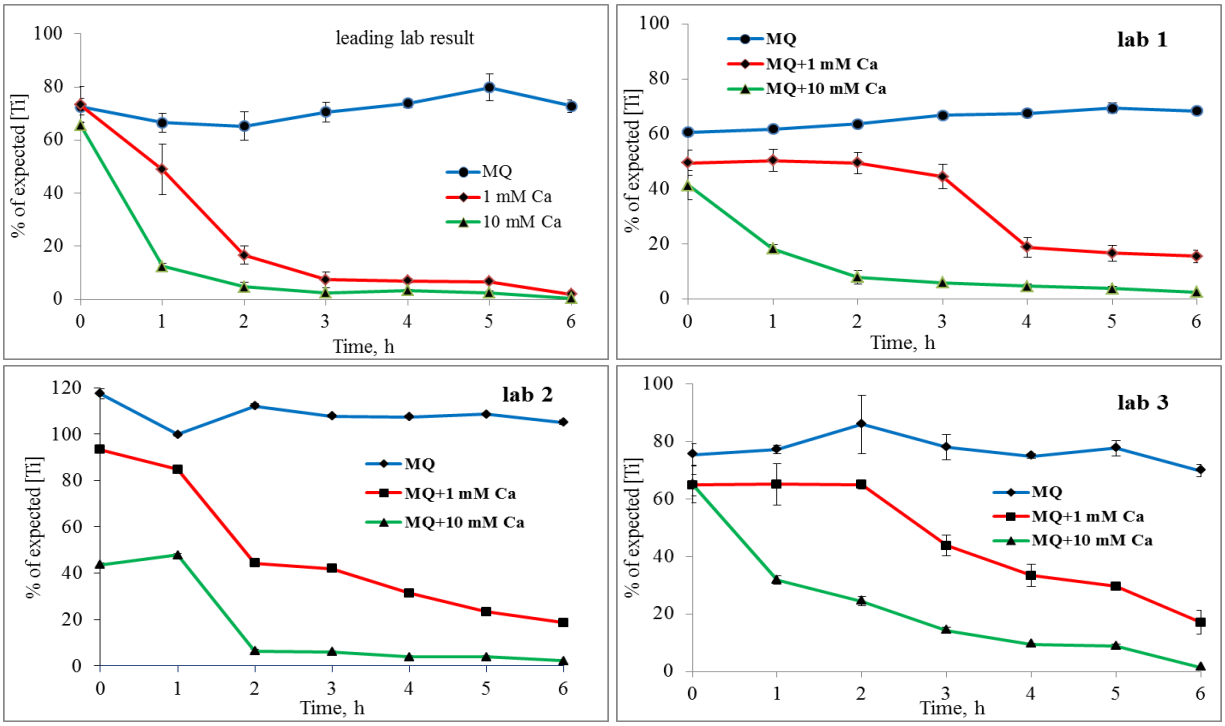



Fig. 8. Results that were not considered for statistical analysis because the large differences between them and the results provided by leading laboratory. Influence of 10 ppm DOC media on $TiO_2$ (NM105) agglomeration behavior.

### *Influence of established pH=8.5 on agglomeration behavior of $TiO_2$ (NM105)*

25. Experiments performed in established pH=8.5 revealed existence of large differences in the initial concentrations of material in 0, 1 and 10 mM electrolyte systems. Such behavior is considered to be a result of samples preparation. The samples were shaken for 24 hours before preparation of samples for the ICP measurements and thereby the loss of material on the tube walls might have occurred. Similar factor might have resulted in quite large standard deviation values in the results of lab 4.



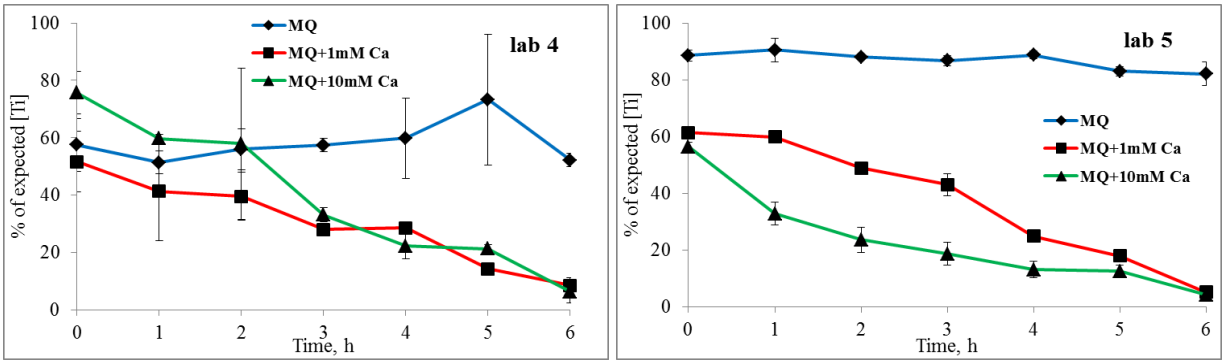



Fig. 9. Dependency of agglomeration behavior of $TiO_2$ (NM105) material from established pH=8.5.

26. Based on the obtained results, standard deviation and mean deviation of all experimental points were calculated. The calculated data is shown at figure 10. Statistical analysis revealed the largest standard deviation values in the experiments performed in 0 mM electrolyte systems, which are the less stable systems compared to those of 1 and 10 mM electrolyte systems.

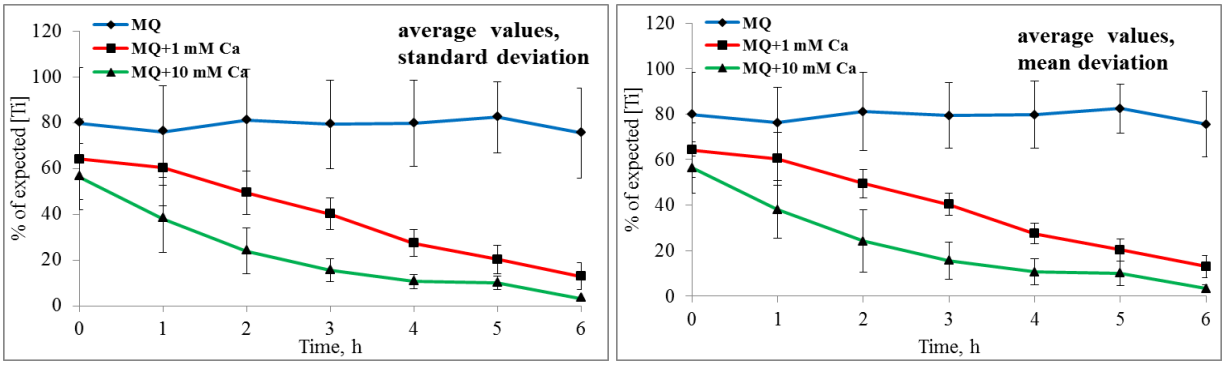



Fig. 10. Average values based on the results obtained from the independent laboratories and calculated standard and mean deviations. Influence of established pH=8.5 on $TiO_2$ (NM105) agglomeration behavior.

27. Strongly deviating results were pointed out and the explanation of these results was found. The reason of such results was the inability to provide the sample analysis within 24 h after sample preparation. These results were as before excluded from the statistical analysis and are presented at figure 11.

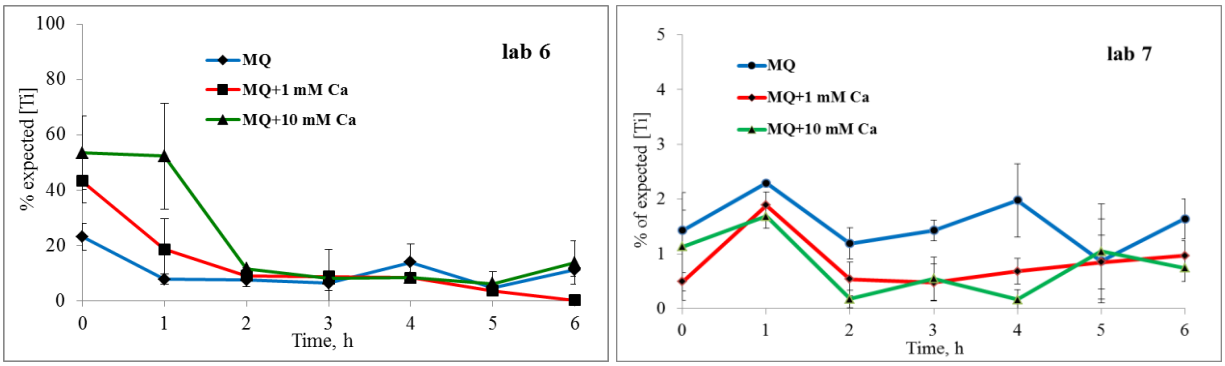



Fig. 11. Results that were not considered for statistical analysis because the large differences between them and the results provided by leading laboratory. Influence of established pH=8.5 on $TiO_2$ (NM105) agglomeration behavior.

***Influence of electrolyte presence on the agglomeration behavior of Ag (NM300K)***

28. Presence of electrolyte did not influence the stability of Ag (NM300K) dispersions as can be observed at figure 12.

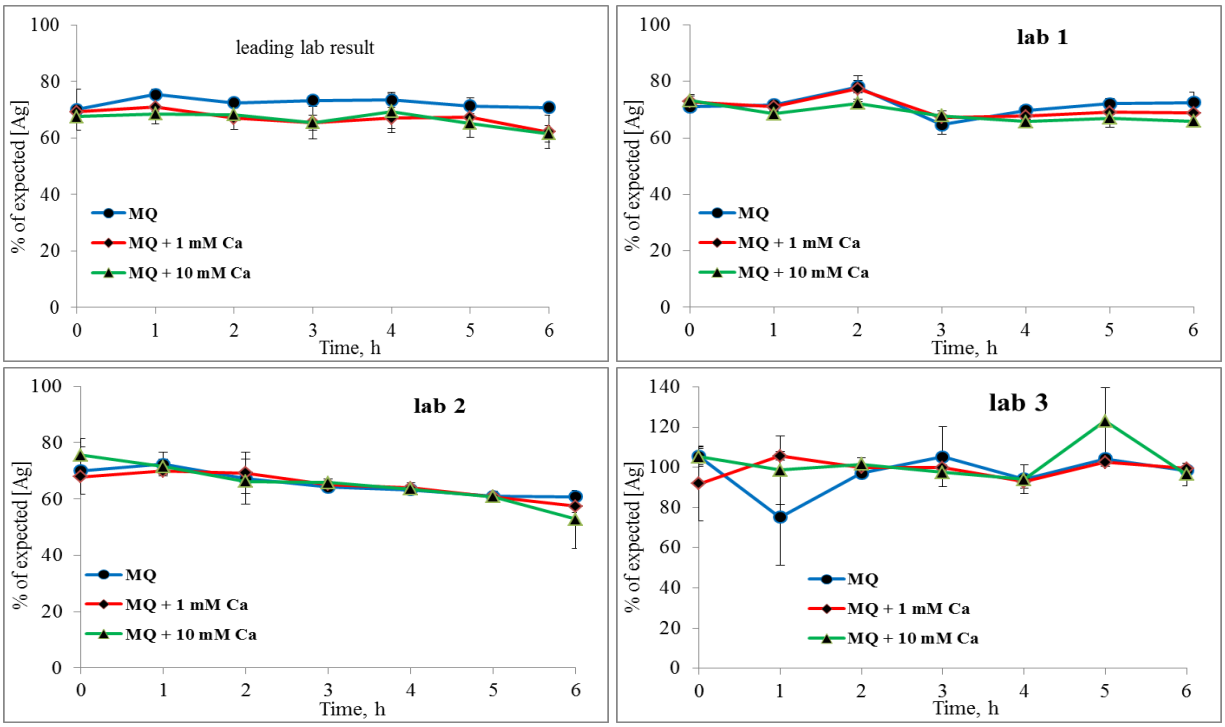



Fig.12. Dependency of agglomeration behavior of Ag (NM300K) material from electrolyte concentration.

29. Based on the obtained results, standard deviation and mean deviation of all experimental points were calculated. The calculated data is shown at figure 13.

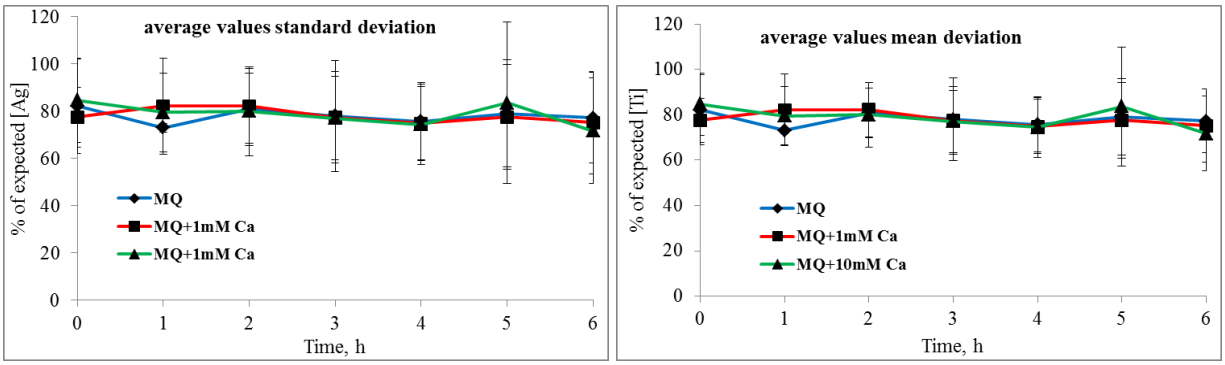



Fig. 13. Average values based on the results obtained from the independent laboratories and calculated standard and mean deviations. Influence of electrolyte concentration on Ag (NM300K) agglomeration behavior.

***Influence of $CO_2$ absence on agglomeration behavior of Ag (NM300K)***

30. Absence of $CO_2$ either did not influence the stability of Ag (NM300K) dispersions at any electrolyte concentrations as can be observed at figure 14.

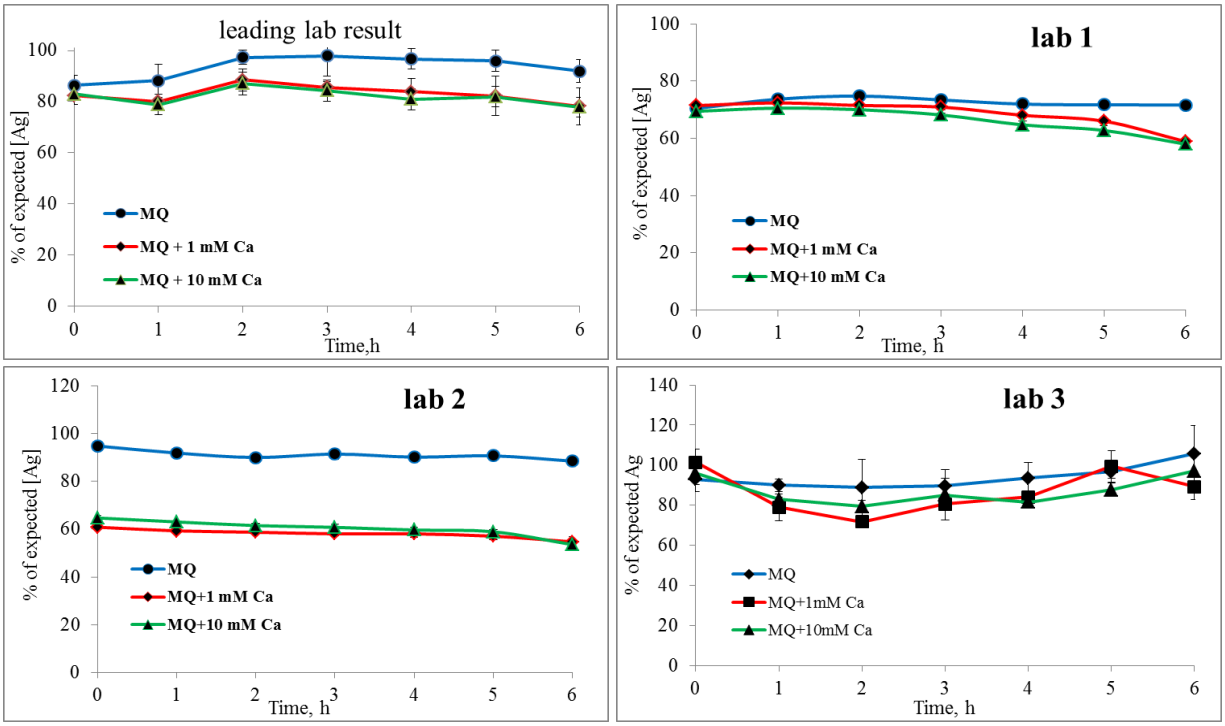



Fig. 14. Absence of $CO_2$ did not play a significant role while investigating the agglomeration behavior of Ag (NM300K).

31. Based on the obtained results, standard deviation and mean deviation of all experimental points were calculated. The calculated data is shown at figure 15.

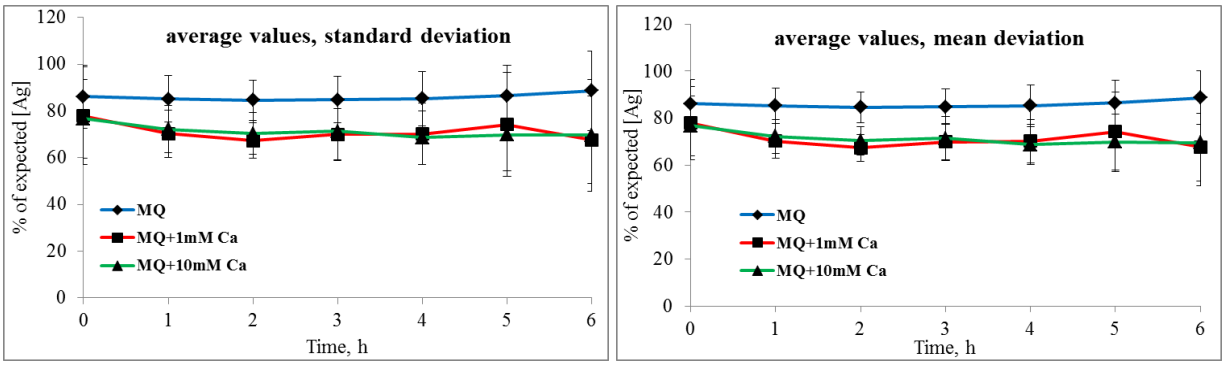



Fig. 15. Average values based on the results obtained from the independent laboratories and calculated standard and mean deviations. Influence of $CO_2$ absence on Ag (NM300K) agglomeration behavior.

### *Influence of DOM presence on agglomeration behavior of Ag (NM300K)*

32. Presence of DOM (10 ppm) did not influence the stability of Ag (NM300K) dispersions either. All dispersions remained stable at all conditions as can be observed at figure 16.

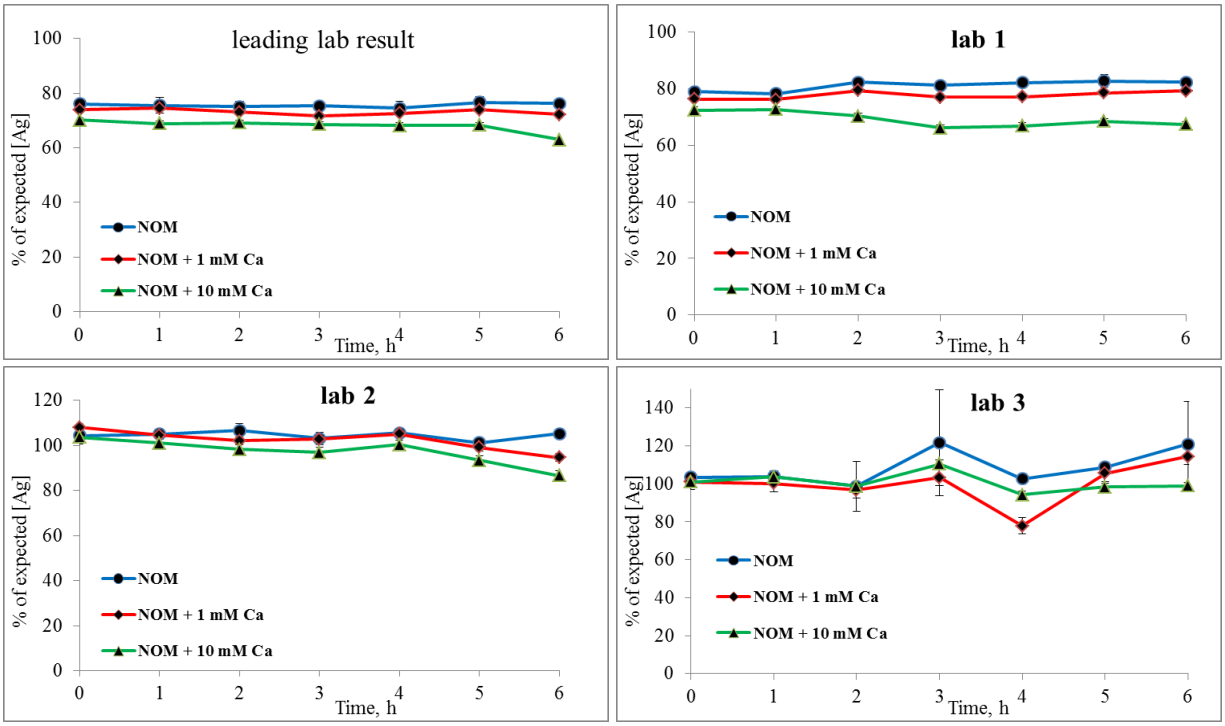



Fig. 16. Influence of DOM on the agglomeration behavior of Ag (NM300K) particles.

33. Based on the obtained results, standard deviation and mean deviation of all experimental points were calculated. The calculated data is shown at figure 17. Presence of DOM in the aquatic media slightly increased the recovery of Ag material from approx. 80% in the previous experiments to about 90-100% in current experiments.

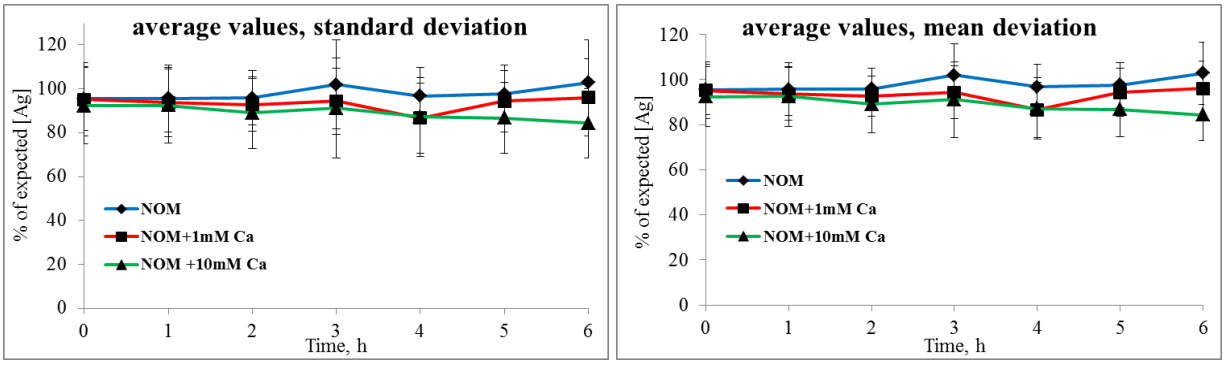



Fig. 17. Average values based on the results obtained from the independent laboratories and calculated standard and mean deviations. Influence of DOM presence on Ag (NM300K) agglomeration behavior.

***Influence of established pH=8.5 on agglomeration behavior of Ag (NM300K)***

34. Results obtained from the current experiments were divided into 2 groups: comparable with results of leading laboratory and incomparable. Results comparable to those obtained in the leading laboratory are presented at figure 18.

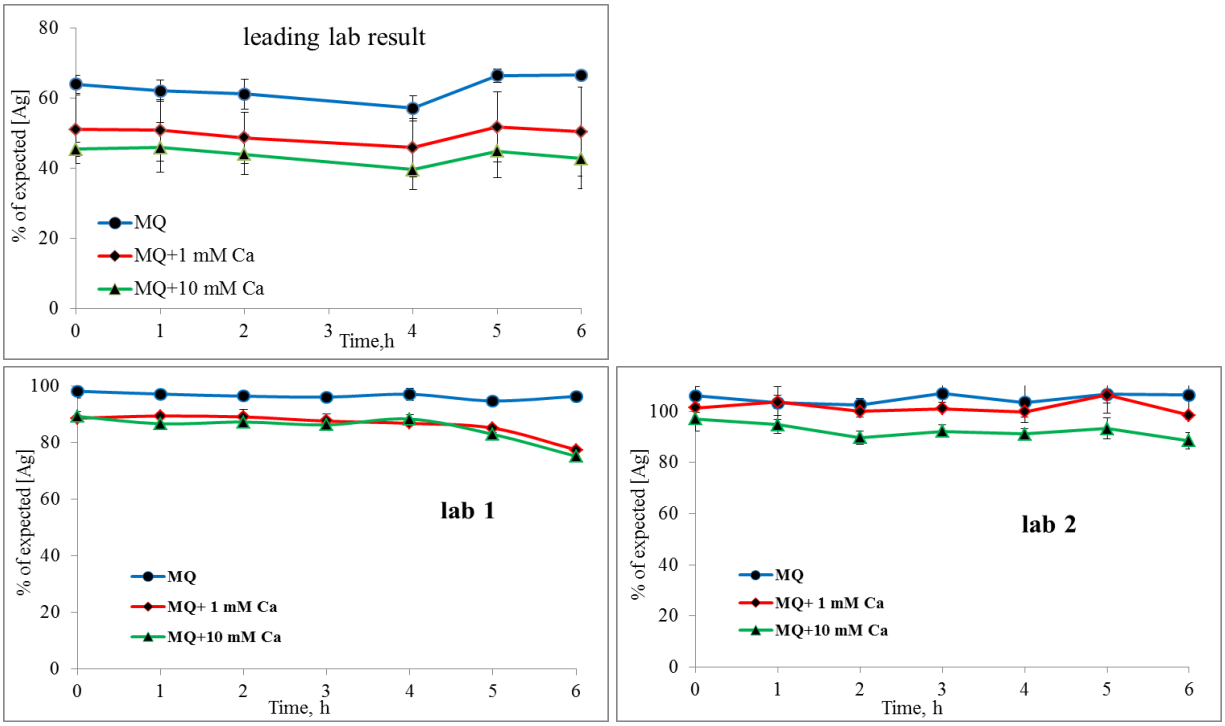



Fig. 18. Dependency of agglomeration behavior of Ag (NM300K) material from established pH=8.5.

35. Based on the obtained results, standard deviation and mean deviation of all experimental points were calculated. The calculated data is shown at figure 19.

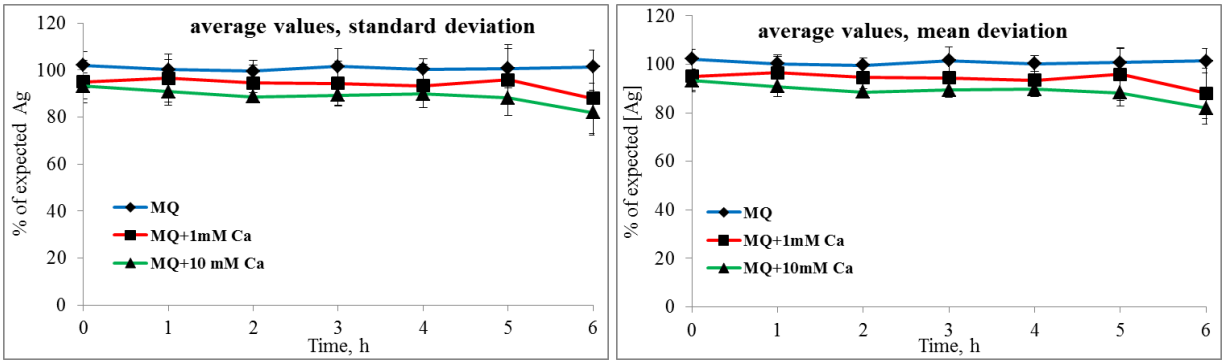



Fig.19. Average values based on the results obtained from the independent laboratories and calculated standard and mean deviations. Influence of established pH=8.5 on Ag (NM300K) agglomeration behavior.

36. Results that significantly deviated from those presented above can be observed at figure 20.

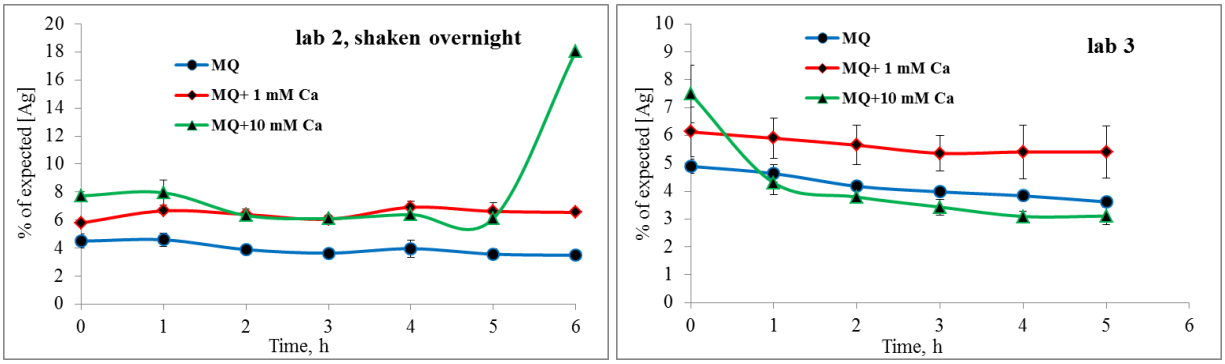



Fig. 20. Dependency of agglomeration behavior of Ag (NM300K) material from established pH=8.5.Results that were not taken in account for statistical analysis due to the large differences with the results of leading laboratory.

37. Such results were obtained due to the fact that samples established pH=8.5 were equilibrated on the rotary shaker during 24 h to ensure the stable pH value. Such equilibration procedure resulted in the precipitation of the material on the walls of experimental tubes. The results, significantly deviated from majority of obtained datasets were not considered for statistical analysis.

38. To check the hypothesis that overnight shaking of the sample leads to the losses of material on the walls of experimental tubes, one of RRT participants (lab 2) tested the agglomeration of Ag (NM300K) material in aqueous dispersions with established pH=5. Experiment was presented in 2 replicates, where in first case dispersion was left intact over a period of 24 h, while in the second case it was shaken for the same time-period. Observed difference proves the validity of suggested hypothesis. Obtained results can be observed at figure 21.

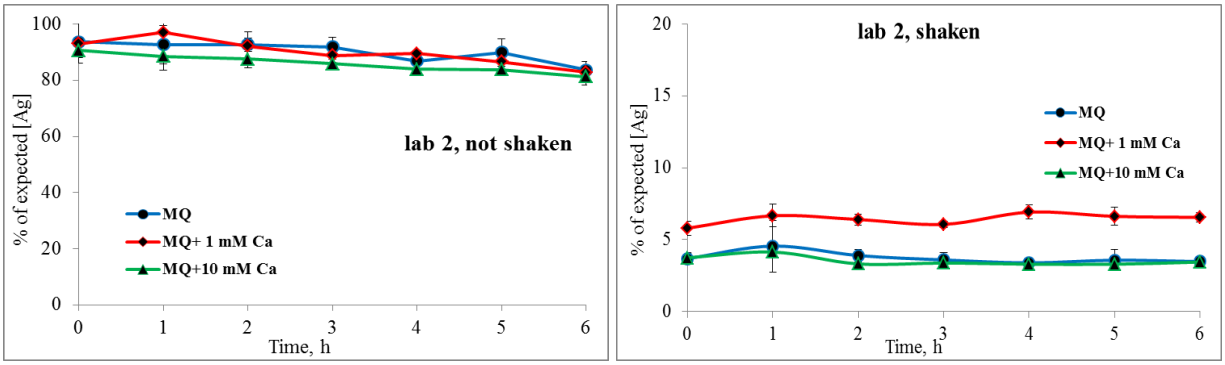



Fig. 21. Ag (NM300K) material losses occur due to precipitation of particles on the walls of experimental tubes during shaking the samples for equilibration purposes at pH=5.

## CONCLUSIONS

39. Obtained results have shown that the character of statistically analyzed experimental dependencies is highly similar. Thus initial expectations for the similarity of obtained experimental results were fulfilled. Largest standard and mean deviations for each test condition and corresponding material are presented in table 1 and table 2. These numbers were gathered from statistical analysis of results provided by laboratories participating in RRT. Calculated mean and standard deviations remained within 25% and 29% of total material concentration respectively in case of $TiO_2$ (NM105) (table 1) and within 26% and 34% of total material concentration respectively in case of Ag (NM300K) (table 2). Such values can be considered as fully acceptable providing the fact that the losses of material due to experimental

manipulations stayed within the magnitude of provided values. Values for the material losses due to experimental manipulations can be found in Annex 1 to the corresponding test guidelines.

Table 1. Largest standard and mean deviations for experiments on $TiO_2$ (NM105)

| TiO2 (NM105) | Max. mean dev. (%) | Max. standard dev.(%) |
|---|---|---|
| electrolyte | 15,85 | 21,48 |
| CO2 | 10,45 | 14,32 |
| NOM | 25,11 | 29,32 |
| pH | 18,47 | 24,37 |

Table 2. Largest standard and mean deviations for experiments on Ag (NM300K)

| Ag (NM300K) | Max. mean dev. (%) | Max. standard dev.(%) |
|---|---|---|
| electrolyte | 26,27 | 34,25 |
| CO2 | 18,38 | 23,98 |
| NOM | 13,89 | 22,81 |
| pH | 10,64 | 15,05 |

40. It was found that to obtain the correct results the analysis of prepared samples shall be performed within 24 nearest hours, as outlined in the corresponding TG. Analysis within 24 hours is strictly recommended for cases, when slurry analysis is applied to stabilized particle dispersions. In case slurry analysis cannot be performed within 24 hours, full digestion of prepared samples shall be applied. Inability to fulfil this requirement can lead to particle sedimentation on the walls of experimental tubes and incorrect measurement readings, as was demonstrated on the example of $TiO_2$ (NM105) particles.

41. Manipulations with the samples have to be performed carefully, avoiding unnecessary disturbance, such as placing the samples on the shakers, etc. for significant periods of time. Such conditions will likely lead to the particle precipitation on the walls of experimental tubes as was demonstrated on the example of Ag (NM300K) particles.

**QUESTIONS RAISED AND ANSWERED DURING RRT**

42. There were a number of questions that appeared and were solved during the RRT. Full list of those questions and answers for them is presented below.

1. ***Is it only a bottle-top filter that can be used for NOM filtration?***
   Any filter can be used, but the filter pore size shall be similar to the one requested in the corresponding TG. Type of filter and pore cross-section shall be reported.
2. ***Is there any digestion of particles needed, or slurry analysis would be OK?***
   When the analysis of prepared samples can be performed within 24 hours application of slurry analysis is feasible. If there is not such possibility, the digestion of sample shall be performed.
3. ***Shall I establish pH with HCl, or other acid is suitable too?***
   In principle any strong acid/ base can be used to establish the pH of aqueous media. One however shall keep in mind the possible interactions of acid/base with analyzed material.
4. ***How to disperse the samples after formulating them (just by shaking)?***

Hand shaking for several seconds in enough to disperse material in the aquatic media. No other mechanical shaking for longer time-period shall be applied.

5. ***Specify more precisely the concentration of NOM for the experiments of the TG.***

The concentration of NOM shall be calculated based on the assumption that 1 molecule of DOM per 1 nm2 of material is required to uniformly cover the entire surface area of analyzed material. Assistance in such calculations provided in annex 2 of corresponding TG. For primary assessment 10 ppm DOM concentration that was calculated from the previous assumption shall be used as suggested in the current TG.

6. ***Would the bath sonicator work to disperse the particles?***

Leading laboratory strongly recommends using tip sonicator to disperse the particles. Using tip sonicator allows calibrating the energy input made to the sample during sonication. Tip sonication is more effective than any other way of sonication, because the sonication tip interacts directly with the dispersion. In addition in case of tip sonication calibration of energy input is possible.

7. ***When stock dispersions are prepared, for how long can they be stored?***

It is not recommended to keep stock dispersions for more than a day, additional sonication might be needed to re-disperse the material.

8. ***When taking the sample, one decreases the volume of sample: does it influence the effect on particle agglomeration?***

The volume of aliquots is very small – thereby the decrease of the volume of the sample does not provide any effect on particle agglomeration.

9. ***Can we substitute SRNOW with another type of natural organic matter?***

Yes, such substitution can be done. However used natural organic matter shall be the same level of purity regarding electrolyte and ash content as SRNOM.

10. ***Can we use pH = 4,7 and 9 instead of suggested pH= 5,7 and 8.5.***

Yes, the new levels of pH are acceptable and included in the text of corresponding TG instead of former proposed.

11. ***Why NaHCO3 is used as a buffer, if we investigate such a wide range of pH?***

NaHCO3 is used as a buffer because it provides the ions that are commonly found in the natural environment and weakly adsorb to the material surface, thus almost not influencing the agglomeration behavior of nanomaterials.

12. ***Too many ICP measurements. Can the number of measurements be reduced?***

Yes, the number of measurements can be reduced. Corresponding changes are included in the text of TG and scheme of decision tree.

## REFERENCE

43. OECD (2005), Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment, OECD Environment, Health and Safety Publications Series on Testing and Assessment No. 34.