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Agenda item 4: Progress achieved regarding implementation of the Programme of Work 2020-2021 related to Land-Based Pollution and Governance Themes

Implementation of IMAP Common Indicator 18 on Biomonitoring

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List of Abbreviations / Acronyms

AChE	Acetylcholinesterase activity
BAC	Background Assessment Criteria
Bio Risk	Biological Risk
Chem Risk	Chemical Risk
CI	Common Indicator
Ci	Concentration of the contaminant i in the environmental matrix
COP	Conference of the Parties
CORMON	Correspondence Group on Monitoring
EAC	Environmental Assessment Criteria
EcAp	Ecosystem Approach
EEA	European Environmental Agency
EC	European Commission
Env RI	Environmental Risk Index
EU	European Union
Exp-DSS	Expert Decision Support System
FAO	Food and Agriculture Organization of the United Nation
GES	Good Environmental Status
HELCOM	Baltic Marine Environment Protection Commission - Helsinki Commission
IAEA	International Atomic Energy Agency
IMAP	Integrated Monitoring and Assessment Programme of the Mediterranean Sea and Coast and Related Assessment Criteria
LMS	Lysosomal Membrane Stability
MAP	Mediterranean Action Plan
MED POL	Programme for the Assessment and Control of Marine Pollution in the Mediterranean Sea
MED QSR	Mediterranean Quality Status Report
MESL	Marine Environmental Studies Laboratory
MNi	Micronuclei frequency
OSPAR	Convention for the Protection of the Marine Environment for the North-East Atlantic
QA/QC	Quality Assurance/Quality Control
QSR	Quality Status Report
PAHs	Polycyclic aromatic hydrocarbons
PEC*i*	Probable Effect Concentration of contaminant I, above which negative effects are likely to occur
SoS	Stress on Stress
TEC*i*	Threshold Effect Concentration of contaminant i, below which no effect will probably be evident
TPCitec	Toxic Pressure Contribution of contaminant i
Toxicity unit	TECs of the different contaminants able to start to give toxic effects on the organisms
TV	Toxicity Value
UNEP/MAP	United Nations Environment Programme Mediterranean Action Plan
wF	weighting Factor

1 The intercalibration exercise to support the Quality Assurance related to IMAP Common Indicator 18

1. The Protocols for the Quality Assurance (QA) of IMAP Pollution Cluster Common Indicators, including CI 18 are provided in UNEP/MED WG.492/7. A very important aspect of the Quality Assurance (QA) for CI 18 is the realization of an intercalibration exercise that is fundamental to guarantee the comparability of the biomarker data collected by national competent laboratories. The organization of the intercalibration exercise related to CI 18 must be assigned to a competent reference laboratory.

2. The results of the intercalibration exercise realised in the initial phase of the MEDPOL Biomonitoring programme have been published in the scientific international journal Marine Environmental Research in 2000 (Viarengo et al., 2000). This was the first successful attempt undertaken at international level, to guarantee the comparability of the biomarkers data. The intercalibration practices proposed here, represent the natural evolution of the past activities. They take into account the knowledge acquired during the realization of the MEDPOL Biomonitoring programme, as well from similar international monitoring programmes (e.g. the EU Funded Research Programme realized in 1998 “The Biological Effects Quality Assurance in Monitoring Programmes (BELQUAM)”; Project “Biological Effects of Environmental Pollution in marine coastal ecosystems” (BEEP) supported by EU in 2002; Background document and technical annexes for biological effects monitoring of OSPAR Commission, as updated in 2013).

3. The present document provides details related to realization of the intercalibration exercise for the four biomarkers that were agreed for biomonitoring under CI 18. Due to the differences in the methodologies used for the collection of the data for different biomarkers, the intercalibration activities are elaborated separately for the four different biomarker analysis’.

1.1 Intercalibration of Lysosomal Membrane Stability (LMS)

a. *Intercalibration of LMS evaluated by the histochemical method of frozen tissue samples*

4. The Reference Laboratory assigned with organizing the intercalibration exercise, as part of the Quality Assurance (QA) for IMAP CI 18, will prepare and analyse samples of uncontaminated controls and contaminant exposed organisms. Subsamples of tissue frozen in liquid nitrogen of both samples will be sent to national competent laboratories participating in the intercalibration exercise. The samples will be coded and the test will be conducted as a blind exercise. This kind of intercalibration activity was successfully used in past years as part of the previous MED POL Biomonitoring Programme (Viarengo et al., 2000).

b. *Interlaboratory Comparison (ILC) of in vivo exposure experiment with subsequent evaluation by the histochemical method*

5. This approach has previously been used in the MED POL Biomonitoring Programme for the intercalibration activity on molluscs. National competent laboratories involved in the intercalibration activity will receive a set of vials containing 10 ml of pure or contaminated seawater from the Reference Laboratory. The vials will be coded and the test will be realised as a blind exercise. The content of the vial has to be added to 20 L of aerated seawater in which a 3 days exposure experiment using 20 mussels maintained at 16 °C should be performed. During the experiments the water must not be changed and the mussels must not be fed. The laboratories are tasked to identify the contaminated samples and to evaluate the change in LMS (in %). The images of the analysed cryostat sections will be sent to the reference laboratory assigned with realization of the ILC to confirm the quality of the results.

c. *Intercalibration of LMS evaluated by the in vivo Neutral Red Retention Time method*

6. Similar to ILC described in *b*., participating national competent laboratories will receive a set of vials containing 10 ml of clean or contaminated seawater. The vials will be coded and the test will be conducted as a blind exercise. The content of each vial must be added to tanks with 20 L of aerated

seawater to perform a 3 days exposure experiment using 20 mussels, maintained at 16 °C. During the experiments, the water must not be changed and the mussels must not be fed.

1.2 Intercalibration of Micronuclei frequency (MNi)

a. Interlaboratory Comparison:

7. The most critical phase of the experimental protocol of the MN test is the slide scoring.

8. An intercalibration scoring exercise is recommended for the national competent laboratories involved in the use of MNi assay for biomonitoring purposes. Microscopic slides from controls and contaminant exposed organisms, prepared and analysed at the Reference Laboratory assigned with the realization of the intercalibration exercise would be sent to participating national competent laboratories. The samples will be coded and the scoring will be executed as a blind exercise.

9. To guarantee the quality of the data during the different biomonitoring activities, it will also be possible to request the laboratories involved to send some slides from a reference and polluted sites to the Reference Laboratory assigned with the realization of the intercalibration exercise. In this way, both the quality of the cells as well as the MNi results will be adequately evaluated.

1.3 Intercalibration of Acetylcholinesterase activity (AChE)

a. Interlaboratory Comparison of AChE on frozen tissue samples

10. As for LMS, the Reference Laboratory assigned with organizing the intercalibration exercise, as part of the Quality Assurance (QA) for IMAP CI 18, will prepare and analyse samples of uncontaminated controls and contaminant exposed organisms. Subsamples of frozen tissue of both samples will be sent to national competent laboratories participating in the intercalibration exercise. The samples will be coded and the test will be executed as a blind exercise. This kind of intercalibration activity was successfully used in previous MEDPOL Biomonitoring Programmes (Viarengo et al., 2000).

b. Interlaboratory Comparison (ILC) of in vivo exposure experiment for Acetylcholinesterase activity (AChE) with subsequent evaluation by the histochemical method

11. This approach has been used in the MEDPOL Biomonitoring Programme for the intercalibration of molluscan biomarkers. National competent laboratories involved in the intercalibration activity will receive a set of vials containing 10 ml of pure or contaminated seawater from the Reference Laboratory. The vials will be coded, and the test will be realised as a blind exercise. The content of the vial has to be added to 20 L of aerated seawater in which a 3 days exposure experiment using 20 mussels maintained at 16 °C should be performed. During the experiments the water must not be changed, and the mussels must not be fed. The laboratories are tasked to identify the contaminated samples and to evaluate the change in AChE (in %).

1.4 Intercalibration of Stress on Stress (SoS)

12. The method is simple and, in general, does not require a complex intercalibration protocol. It is always possible to follow the same procedure used for the intercalibration of the lysosomal membrane stability to ensure that all the steps required for SoS evaluation are implemented correctly. In addition, as for the other biomarkers here described, a set of videos should be made and sent to the Reference laboratory that would be assigned by UNEP/MAP to perform the Quality Assurance (QA) of IMAP Common Indicator 18 for a critical evaluation of the modality of data acquisition.

1.5 Evaluation of the performance of national competent laboratories for the analysis of biomarkers

13. In the analysis of the selected biomarkers, the participating national laboratories should be able to meet the particular criteria described above to be considered compliant with the QA standard.

14. Lysosomal Membrane Stability (LMS): National competent laboratories should be able to identify which of the blind samples were obtained from control animals and which from animals exposed to toxic chemicals. In the case of the evaluation of LMS national competent laboratories should also able to quantify the toxic effects i.e. to verify if the effects in the animals exposed to the

contaminants provide a decrease of the value of the LMS between 20% and 70% or if the decrease is higher than 70%. The laboratories that are not able to correctly evaluate the quantitative changes of the LMS must be trained to reach the required quality standards. The Reference Laboratory should be tasked to provide this training and to assist with the data evaluation.

15. Micronuclei frequency (MNi), Acetylcholinesterase activity (AChE activity) and Stress on Stress (SoS) National competent laboratories should be able to identify which of the samples were obtained from control animals and which were from animals exposed to toxic chemicals. The laboratories that are not able to correctly identify the differences in the values of MNi frequency, AChE activity and SoS in the control and exposed animals must be trained to reach the required quality standards. The data concerning the quantification of the changes of these biomarkers in control and chemical exposed animals will be used by the Reference Laboratory assigned with realization of the intercalibration testing to ensure that the laboratories are analysing the biomarkers in the correct way.

16. National competent laboratories that are unable to recognise controls and contaminant exposed samples, or that find anomalous % variations in some samples from any of the intercalibration/interlaboratory comparison exercises, can send the images taken during analysis to the Reference Laboratory assigned with realization of the exercises, which will expedite the evaluation of the results as an additional service.

17. In the previous MEDPOL biomonitoring programme, most of the participating national laboratories involved were sending back the images and/or videos taken during the respective biomarker analysis, along with the clarification of possible technical problems. This enabled the Reference Laboratory to help them remotely.

18. In the past participating laboratories were given video cameras to record the analysis. Given the availability of new imaging technology, such as smart phones with high quality cameras, this is not necessary anymore and the interfacing of the national competent laboratories with the Reference Laboratory assigned with the task to conduct intercalibration and ICL exercises will be much simplified and at no additional cost for the hardware.

2 Training course aimed at strengthening implementation of IMAP Common Indicator 18

19. A six-day training course should be organised in order to teach the practitioners from national competent laboratories how to implement and execute monitoring and assessment of biomarkers related to CI 18. The realization of this training course should be assigned to a proven competent laboratory, preferably the same one that is responsible for realization of the intercalibration testing for CI 18, as explained above in chapter 1 (i.e. the Reference Laboratory).

20. The training course needs to elaborate both theoretical and practical experimental aspects:

i) Initially, there is a need to demonstrate how to organise the biomonitoring programme for IMAP CI 18. Thereafter, the biological meaning of biomarkers` determination should be explained and discussed. Finally, details of the Quality Assurance should be presented in order to obtain a full participation of all national competent laboratories to this fundamental activity. This initial part should be provided in a lecture style with tutorial;
ii) The second part of the training course should be devoted to the experimental activities. In the laboratory, the researchers should evaluate three selected biomarkers (i.e., LMS, MNi frequency and AChE activity) with the support of internationally acknowledged experts who should clarify all technical problems;
iii) The third part of training course should be related to the use of a set of biomarkers` data. The data should be integrated with different systems and the results discussed. Subsequently, a new approach for the integration of biological and chemical monitoring, as provided in chapter 4 of this document, should be introduced, including an explanation how to optimally report and upload monitoring data related to CI 18 into the IMAP Info System.

21. During the training course, the sessions should be organized in such a way for the researchers so as to encourage discussion among national experts, including the problems and technical concerns

related to use of the various biomarkers. The sustainable networking beyond the duration of the training course should be encouraged.

22. The costs for implementation of the workshop for 12 participants is assesses at 41,000 USD. This covers the costs related to delivery of lectures, hands-on training by the expert laboratory, and travel and accommodation of national experts.

23. The procedures and criteria for appointment of the national experts for participation in the intercalibration exercise and training course should be established upon expected approval of here-elaborated proposals.

3 First reflection on possible further development of the Biomonitoring related to IMAP Common Indicator 18

24. An application of new biomarkers should be explored to support strengthening of monitoring and assessment of CI 18, including the following:

i) *Application of biomarkers of oxidative stress*: In addition to the biomarkers already agreed for CI18, the application of the biomarkers of oxidative stress should be tested. In that respect, application of the Protein Carbonylation and the Lysosomal Lipofuscin accumulation is recommended. The Protein Carbonylation is a biomarker that is able to highlight proteins damaged by oxidative stress, most of which will be unable to perform their normal biochemical role, therefore affecting the physiological status of the cells. The Lysosomal Lipofuscin accumulation represents the end products of the oxidative damage of the cellular components, which is accumulated in the lysosomes, as the first step before their elimination from the cells by exocytosis, represents an excellent index of the level of oxidative damage in the cells of different tissues of the organisms exposed to toxic chemicals. Lipofuscin also binds transition metals such as iron, which will continue to exacerbate oxidative damage to cell constituents. Consequently, lysosomal accumulation of lipofuscin is a harmful process.

ii) *New molecular "OMICS" technologies:* The introduction of these technologies in the Biomonitoring programme for CI 18, in particular of the transcriptomics approach, should be tested. Transcriptomics have been used for twenty years now, both for medicine and environmental applications, therefore Mediterranean laboratories should explore their further application within implementation of CI 18.

iii) *The changes in expression of the genes related to fundamental biological processes*: The results of research in the field of the transcriptomics in marine organisms render possible means for selecting the batteries of genes that are known to be highly responsive to the stress caused by toxic chemicals in the animals. These genes are related to fundamental biological processes, such as protein synthesis, autophagy, energy metabolism, cytoskeleton organisation, oxidative stress, DNA damage, cell signalling pathways (e.g., PI3K-Akt-mTOR) and the responses to specific classes of contaminants such as heavy metals and aromatic organic xenobiotics (PAHs, PCBs etc). The changes in expression of these genes are known to be related to particular biomarker alterations and, therefore, to the stress level of the organisms. To that purpose, application of the most robust and simplest transcriptomic methodology is recommended, such as the qRT-PCR approach for the analysis of selected differentially expressed genes. Several advantages justify the application of this approach, i.e. it is able to produce quantitative data; it is a standardised methodology at international level; it is used on DNA fragments; it is a basal step for transcriptomic analysis; and it can also be easily intercalibrated. Furthermore, most of the laboratories possess the necessary equipment for its application and the costs for a single analysis are low.

25. The present document brings the above listed new biomarkers to the attention of the Parties in order to trigger discussion to guide their further scientific elaboration within implementation of CI 18.

4 Possible approach towards integrated assessment of biological and chemical monitoring data of IMAP Common Indicators 17 and 18

26. It is well known that numerous contaminants present in marine environment may cause a decrease of the ecosystem integrity (i.e. the health of marine environment by causing the negative effects on individual organism health), as well as the longer-term consequences affecting biodiversity.

27. The numerous toxic chemicals present in the environment may have additive or synergistic effects (or, sometimes, at low concentrations, antagonistic effects) on the living organisms; however, until now it has been impossible to give a predictive evaluation of the possible noxious effects of complex contaminant mixtures on the organisms that populate the contaminated ecosystem (Barranger et al., 2019a, b; Moore et al., 2013, 2021).

28. The legislation of most countries uses legal toxicity limits for the single pollutants to describe environmental quality, although it is well known that the effects of the chemicals are often additive and sometimes synergistic. Within the legislation of numerous countries, however, the legal limit of some classes of toxic chemicals, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated dibenzodioxins (and dibenzofurans), are often expressed as the summation of the concentrations of the components of the contaminant class.

29. The present approach explains a two-pronged procedure that combines chemical and ecotoxicological data to support the evaluation of the risk related to marine organisms exposed to contaminated waters and sediments. The main purpose of this procedure is to facilitate scientists and environmental managers in planning future actions and interventions for marine coastal management by clearly determining the potential environmental risks associated with the exposure of the organisms to such contamination.

30. In this approach the Threshold Effect Concentration (TEC) is used as toxicity unit for the various chemicals in order to estimate their additive effects. In this case the term “toxicity unit” is used to indicate the concentration of the different chemicals that are able to start giving toxic effects on the organisms. This calculation may underestimate the possible toxicity due to synergistic effects of the chemicals present in the contaminated environment, but, this situation is compensated by the presence of the biological data: in fact, the possible synergistic effects of the contaminants will be revealed by a higher noxious effect on the organism’s health status. The higher weighting of the biological data (2:1 with respect to the chemical data) will reduce the possible underestimation of the synergistic effects of the pollutants.

31. Threshold Effect Concentration (TECs) and Probable Effect Concentrations (PECs) are target concentrations selected as thresholds for each contaminant in order to guarantee the protection of the environment. TECs are the concentrations below which no effect will probably be evident; and PECs are the concentrations above which negative effects are likely to occur.

32. Therefore, adoption of this model for the implementation of IMAP Common Indicator 18 should be explored as an integrative approach to quantifying the overall effect of contaminants on sentinel species, in order to assess the environmental quality.

33. The procedure here reported basically consists of three modules: (i) a chemical module for integration of the data concerning the concentration of the pollutants; (ii) an ecotoxicological module that integrates data of the biological effects in marine organisms; and (iii) an integration module that combines the two lines of evidence in an Environmental Risk Index (EnvRI).

4.1 Chemical Module

34. The integration of the chemical and the biological data is based on the investigations reported by Dagnino et al. (2008), Dagnino et al. (2013), and Dagnino & Viarengo (2014). The main difference is that, initially, the value of the contribution of every chemical to the matrix toxicity is considered separately.

35. It is based on the calculation of the Chemical Risk Index (ChemRI) that is determined on the basis of the data for the concentrations of selected chemicals in waters, sediments or both, related to their Threshold Effect Concentrations (TECs) and of their Probable Effect Concentrations (PECs) on biota.

36. The integration framework of the Chemical Module is based on three main points:

i) comparison of the measured concentration of each chemical (Ci) with its TEC and evaluation for each chemical of a Toxicity Value (TV) using its TEC specific thresholds, and

ii) calculation of its Toxic Pressure Contribution (TPCitec) using its PEC value; the TPCitec will be used to calculate the contribution of the toxic chemical i to the ChemRI; and finally,

iii) the Toxic Pressure Contribution (TPCitec) of the different toxic chemicals are then added to obtain the values of ChemRI that will range between 0 and 1. In organising the data to obtain a value ranging between 0 and 1, it is essential to use the chemical data, together with the biological data (health status of the sentinel organisms) that will also be determined to lie with the range of 0 and 1.

37. The rationale for separately calculating the toxic contribution of the different chemicals is that the ratio between TEC and PEC of the different chemicals may vary from 2 to 10 or higher (e.g. for Hg: TEC = 130 and PEC = 700 resulting in TEC:PEC = 5; and for Cd: TEC = 68 and PEC = 4210 resulting in TEC:PEC = 62).

38. BAC (Biological Assessment Criteria) values defined in the IMAP Decision 23/6 represent the values of a particular contaminant in unpolluted Mediterranean areas; and, therefore, it should be the lower of the TEC values that represents the values for the chemical, which should be not be exceeded in order to avoid toxic effects on the organisms.

39. EAC (Environmental Assessment Criteria) values defined in the IMAP Decision 23/6 represent the values of a particular contaminant in a contaminated area in which toxic effects on the biota are present; therefore, this value can be higher than the PEC that indicates the concentration value at which it is possible to find adverse alterations in the health status of the organisms.

40. In the proposed Chemical Module if the concentration of a contaminant reaches its EAC (Environmental Assessment Criteria) value, the Chem Risk Index (ChemRI) is 1.

4.2 Biological Module

41. This module is based on the approach for integration of biomarker data that is used in the Mussel Expert System (Dagnino et al., 2007). Due to the fact that in IMAP CI 18, the use of four biomarkers has been agreed, the integration of the data for the evaluation of the biological risk (Bio Risk) has been simplified.

42. The IMAP CI 18 biomarkers are: (i) three biomarkers at the cellular level (early warning), namely Lysosomal Membrane Stability (LMS), Acetylcholinesterase (AChE) activity, Micronuclei (MNi) frequency; and (ii) one biomarker at organism level such as Stress on Stress (SoS).

43. The rules for the use of the biomarker data are outlined below. As shown, particular importance is given to changes in the value for LMS: indeed, this biomarker is not only diagnostic for a stress syndrome in the organism, but it is also prognostic for possible effects at population/community level (Moore et al., 2006a & b). A reduction in LMS equates to an increase in lysosomal membrane permeability resulting in the release of lysosomal iron and in severe instances the release of degradative lysosomal enzymes (hydrolases) (Stern et al., 2012). Release of lysosomal iron results in the generation of harmful reactive oxygen species (ROS): the ROS can lead to oxidative stress and pathology in the animals (Moore et al., 2020). The LMS, therefore, can be defined as a “prognostic biomarker”. In fact, it was demonstrated that a drastic decrease in LMS is associated with radical alterations at the tissue level (due to a reduction in the cytoplasmic volume of the cells and loss of some of their physiological functions) with deleterious consequences in the Scope for Growth of the organism (Moore et al., 2006a & b, 2008, 2013).

44. Biological risk evaluation (Bio Risk): A ‘Change’ of a biomarker is defined as the variation of the biomarker by more than 20% compared to the control value, with the variation being statistically significant by using a non-parametric statistical test. Different Bio Risk factors are defined as follows:

i. If there is a change of 1 biomarker at cellular level, the Bio Risk is 0.2;

ii. If there is a change of 2 biomarkers at cellular level, the Bio Risk is 0.4;

iii. If there is a change of 3 biomarkers at cellular level, the Bio Risk is 0.6;

iv. If there is a change of 1 biomarker at cellular level AND a significant decrease of the value of the cellular prognostic biomarker LMS by 70 % or more, the Bio Risk is 0.8.

v. If there is a change in all 3 cellular biomarkers tested,

   a. INCLUDING a decrease in the value of LMS by 70 % or more,

   b. OR a change of 1 biomarker at organism level (SoS)

   the Bio Risk is 1.

vi. If the mortality of caged molluscs is more than 20 % compared to the control value the Bio Risk is also 1.

45. If more than 3 biomarkers at the cellular level are used, it is possible to integrate the biomarker data using a new version of the Mussel Expert System (Dagnino et al., 2007) that incorporates the rules used for the calculation of the Bio Risk.

4.3 Integration Module

46. In the Integrated Module the Environmental Risk Index is defined as follows:

$$\text{EnvRI} = \frac{wF_{ChemRI} \times ChemRI + wF_{BioRI} \times BioRI}{wF_{ChemRI} + wF_{BioRI}}$$

with

wF = weighting Factor

$wF_{\text{Chem Risk}} = 1$

$wF_{\text{Bio Risk}} = 2$

47. The weighting factors (wFs) suggested are arbitrary values, selected to emphasise the importance of the effects on the marine organisms of the toxic contaminants present in the ecosystem. The higher value of the weighting Factor assigned to Bio Risk is important, in order to clearly highlight the possible synergistic effects of the chemicals present in the contaminated environment.

4.4 Advantage of integrative approach for quantification of the overall effects of contaminants for assessment of marine environment

48. Evaluation of the environmental risk is usually obtained by an “expert judgement” of a panel of qualified environmental scientists; however, as has often happened in the past, that the results of the evaluation may vary greatly depending on the specific national and regional scientific background of the experts. The risk evaluation procedure presented here may, therefore, represent an important tool for the environmental managers; indeed, it allows managers to obtain a more objective and quantitative evaluation of the Environmental Risk derived from the biological effects of the contaminants present in the marine ecosystems. As shown in the text, this procedure takes into account the additive effects of the toxic chemicals; and can also highlight the presence of synergistic and toxic biological effects of the pollutants based on analyses of biomarkers in the sentinel organisms deployed in the biomonitoring programme.

49. The acceptable range of the marine Environmental Risk Index, based on biomarkers, must still be agreed on by the Parties upon experts’ recommendations.

50. Applying the Environmental Risk Index will result in objective risk values which allow national and regional policy makers and environmental managers to decide on required actions to decrease marine contamination, or to remediate a polluted area. The effectiveness of these actions can subsequently be monitored and quantified until an acceptable Environmental Risk Index is achieved.

Annex I
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