Romain DAVID, ARVANITIDIS C., ÇINAR M.E., SARTORETTO S., DOGANA., DUBOIS S., ERGA Z., GUILLEMAIN D., THIERRY DE VILLE D'AVRAY L., ZUBERER F., CHENUIL A., FERAL J.-P.
CNRS- IMBE: Mediterranean Institute of Biodiversity and marine and terrestrial Ecology, Station Marine d'Endoume, Marseille (CNRS, AMU, IRD, Avignon Univ.)
E-mail: romain.david@imbe.fr [www.cigesmed.eu]

CIGESMED PROTOCOLS: HOW TO IMPLEMENT A MULTIDISCIPLINARY APPROACH ON A LARGE SCALE FOR CORALLIGENOUS HABITATS SURVEYS

Abstract
The European program CIGESMED addresses the Good Environmental Status of the coralligenous habitats. Its implementation on the field is firstly attempted by 4 protocols to be applied in France, Greece and Turkey. They have been tested in Marseille's region, since early 2014. These protocols are the following: (i) cartography of chosen coralligenous sites, (ii) spatial variability analysis by means of photo-quadrats and image processing, (iii) population genetics study of two common biobuilding species that may be cryptic (the bryozoan Myriapora truncata, and the rhodophyta Lithophyllum cabiochiae), and (iv) metagenomic approach of benthic species. The ultimate aim of these protocols is to link the results from the population genetics analysis and the spatial variability analysis to the sites' features thanks to the cartography. First results suggest that different clades exist for both complex of the previous species. Cartography forshadows models of repartition for species assemblages; they will then be compared between regions in the second part of the project.

Key-words: Coralligenous habitats, monitoring protocols, cartography, photo-quadrats, population genetics.

Introduction
The term "coralligenous", meaning coral producer, was first used by Marion in 1883 to describe the hard bottoms called *broundo* by the fishermen from Marseilles. But the meaning of this term is nowadays different, including different types of hard bottoms communities in the Mediterranean Sea. In 2006, Ballesteros recommends to use the terms "coralligenous habitats" since there exist many distinct types of coralligenous habitats.
In the current European legislation context, coralligenous habitats are considered habitats of "community interest" (Habitats Directive 92/43/CEE, habitat code: 1170-14) and should be shortly promoted as "priority" habitat. It is currently considered as the second "hotspot" of biodiversity in the Mediterranean Sea (*Posidonia* meadow being the first one), with than 1,600 species hosted by these habitats (Ballesteros, 2006). Since few years, the EU Marine Strategy Framework Directive (MSFD) requires that each country develops a strategy and an action plan in order to reach and maintain a "Good Environmental Status" for its marine habitats.
There are currently a few programs and networks for the monitoring of coralligenous habitats. CIGESMED (Coralligenous Indicators based to Evaluate and Monitor the "Good Environmental Status" of the Mediterranean coastal waters) involves three countries (France, Greece and Turkey), on a collaborative effort from 2013 to 2016. CIGESMED objectives are (1) to fulfil the key gaps in the current scientific knowledge,

(2) to enhance the knowledge on coralligenous populations by deciding on reference states and setting up a network of Mediterranean experts (for the production of long term series), (3) to monitor networks, locally managed and coordinate them on a regional scale, (4) to test population genetics criteria as tools to monitor the GES of the coastal Mediterranean Sea, (5) to implement a "citizen science" network and (6) to use trees of knowledge as tools to sort, organize and illustrate the large heterogeneous sets of produced data. This work includes habitats cartography, population genetics studies to understand species relations and dispersal potential, and the setting up of a monitoring protocol.
A phase of inter-calibration of methods/material/operators has been implemented. This step is essential in order to evaluate the variability related to these experimental parameters. It allow comparability of results obtained by different underwater protocols. Moreover, this phase of test helps to select the best protocol to apply (the easiest, and most reliable), depending on the habitats types. The next phase will be the study of natural variability inter-site or intra-sites.

Material and methods

<u>Observations and cartography of coralligenous habitats</u>
<u>Intercalibration methods</u>
The French studied sites are located in Marseilles Bay. They are transects of 10 meters long at 28 meters depth. To date, three variables of the protocol implementation have been studied: the sampling method, the quality of the camera, and the level of knowledge of operators in charge to identify species. The protocol was implemented as follow. Divers made photo-quadrats using a frame of 50 cm by 50 cm. The pictures were analysed by operators using the software Photoquad® (Trygonis & Sini 2012). Hundred points were distributed by stratified randomization. Then the operator assigned each point to one category among these three: (i) higher taxa (such as phyla, orders), (ii) abiotic, (iii) indeterminate. In the first category (i) the sub-categories are lower taxa (such as genus or species). The second category (ii) is subdivided into four sub-categories: sediment, bare rock, organic detritus or debris. In the third one (iii), there are three sub-categories: fuzzy image, shadow/hole, and unidentified taxon.

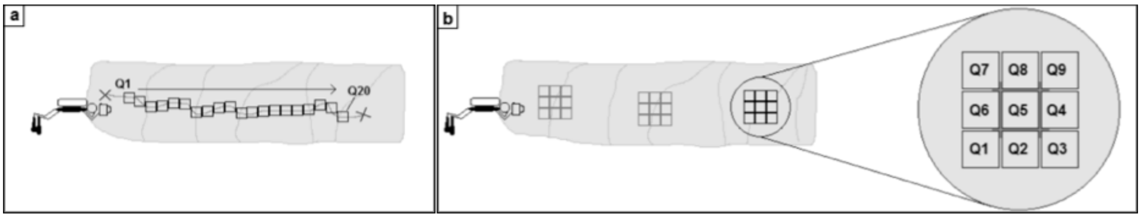

Fig. 1: The two types of transects tested. Linear transect (a): 20 photo-quadrats are taken at a given depth, located by permanent marks. Random patch transect (b): 3 groups of 9 photo-quadrats are taken, following the indicated numbers scheme from 1 to 9, several times at a same depth.

The two sampling methods compared were: (i) the permanent linear transect and (ii) the random patches transect (fig.1). To compare the sampling methods 20 photo-quadrats done by the permanent transect method were compared to 18 photo-quadrats (2 patches) done by the random patches method.
The second variable studied was the quality/performance of the camera. Two cameras have been compared: (i) a camera of medium quality and (ii) a camera of high quality. The models used are (i) GoPro®, and (ii) Nikon® D300s. To compare both cameras, two sets of 8 photo-quadrats done on a permanent transect at the exact same place, were used.
The third variable studied was the level of knowledge of the operators in charge to identify

the taxa on photo-quadrats. Two levels of operators were compared: (i) novice and (ii) experienced. The set of operators participating were: one novice and two experienced operators. Each of them analysed separately the first 5 photo-quadrats of the transect (series 1). Then they met to exchange their results (taxon identification) about this first set and re-do the identification work together to produce "validated data". Again, they studied separately the 2nd series of 5 photo-quadrats. Finally, they met again to exchange their knowledge and produce "validated" data on this 2nd series. Therefore, there is an iteration loop of 4 subsequent cycles to analyse the 20 photo-quadrats of the permanent transect.

Profile characterization and cartography
For each site, two depths were sampled around 28m deep (±1m), and around 45 m deep (±1m). Samples were collected along transects cut into segments of 5m long and 1m wide.

Population genetics studies
The aim here is to understand the population structure of the coralligenous species by studying the intraspecific diversity of demes and their connectivity. For the study of the target species, which are living throughout the Mediterranean Sea, we will use the barcoding method consisting in sequencing a part of the mitochondrial gene COI. We will eventually complete with other alternative or complementary markers. The objective is to test whether the previously-mentioned taxa consist of cryptic species in the Mediterranean.
The two chosen species are (i) the erect-like and tree-like bryozoan *Myriapora truncata*, and (ii) a complex of bioconstructing coralline algae *Lithophyllum stictaeforme /cabiochiae*. Both are identifiable *in situ*. They were selected since they have a widespread occurrence in the coralligenous communities, on all the facies and at all depths (even at very low irradiance). Standard PCR protocols is used to amplify COI fragments for both the bryozoan and the red alga, as well as another marker that is not from the mitochondrial genome, for each species (detailed methods to be published): an intron for *M. truncata* (Chenuil *et al.,* 2010; Gérard *et al*., 2013) and a chloroplast marker for *Lithophyllum sp.* (Broom *et al*., 2008). PCR products are sent to the industry for DNA sequencing, then after alignment, haplotype network reconstruction is made using the Median Joining Network software (Bandelt *et al*., 1999).

Results

Photo-quadrats inter calibration
The preliminary results are presented on figure 3. It shows that at the phylum level, the two methods give equivalent results. Differences of headcounts are significant only in the phylum *Porifera*. Thus results are comparable.
To compare both cameras, the pictures were not taken at the exact same time; the species *P. clavata* disturbed the observations as it had its polyps spread out or not, depending on the set. To release the experiment from this disturbance, all observations of *P. clavata* were removed from both sets. Figure 4 shows that at the species level both cameras give equivalent results. But the camera of high quality made it possible to reduce the number of "indeterminate" and these observations were assigned to other categories, in the majority at the genus level.
The preliminary results on certain categories are shown on the figure 5. After only one exchange between the three operators, the novice improves a lot his/her capacity of identification. For some categories, the level of knowledge of the three operators gets quickly homogenized: for instance for the categories *Cnidaria* and *Porifera*.

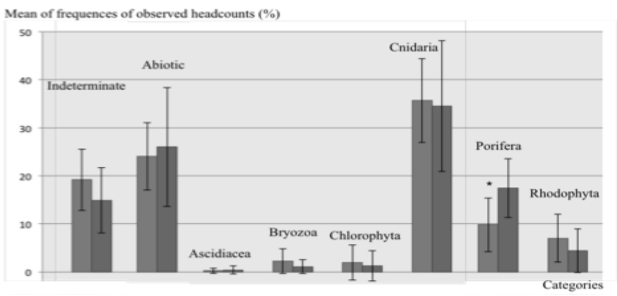

Fig. 3: Comparison of results given by the permanent transect method (light grey) and the random patches method (dark grey). *significant difference according to the Mann-Whitney- Wilcoxon test with a 5 % risk.

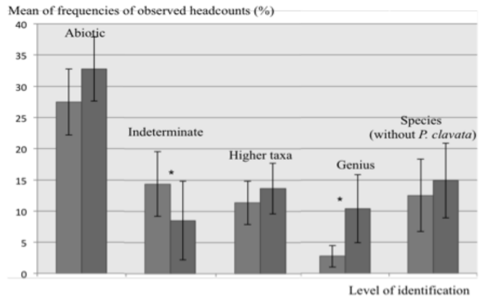

Fig. 4: Comparison of results obtained with a GoPro® (light grey) and a Nikon D300® (dark grey). The star marks a significant difference according to the Mann- Whitney-Wilcoxon test with a risk of 5 %.

They are in most case very hard to identify on photography. From this work, it appears that cnidarian species are easiest to identify on photography by beginners while poriferan species are mostly very hard to identify without specific training.

Analysis of the preliminary cartography results

Using Hierarchical Ascendant Classification (HAC) and Correspondence Factorial Analysis (CFA) on all processed data allowed: (i) to group species according to values of orientation, slope and roughness, and (ii) to pool profile parameters according to species observed per segment of transect. First results shows that cluster of species are better supported using combined factors of slopes and orientations. Roughness less explain the different groups. Using Factorial Correspondence Analysis and Ascending Hierarchical Classifications, preferential profiles of coralligenous species can be determined: bryozoans, encrusting and foliose red algae and foliose green algae occur preferentially on horizontal walls South-oriented with large roughness. *Eunicella cavolinii* is mainly present on inclined walls facing West/North-West with low or medium roughness. Porifera and *P. clavata* are preferentially present on vertical walls facing North. *Codium* genus and encrusting green algae are more present on walls facing South- East and North-East with tiny roughness.

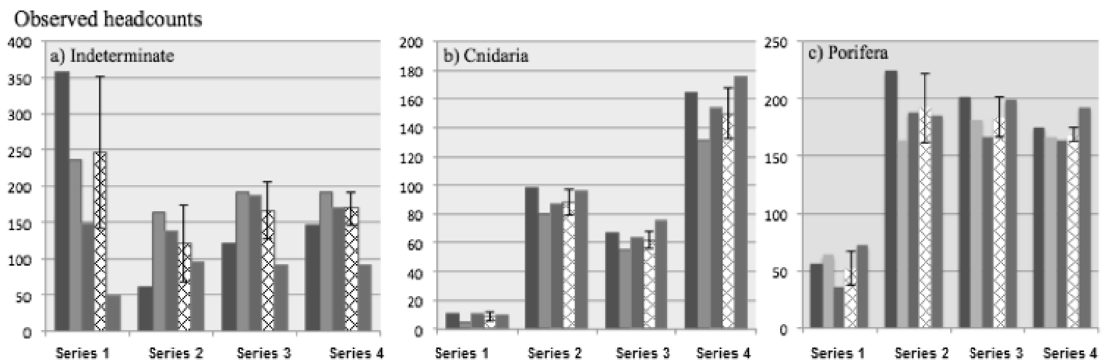

Fig. 5: Comparison of number of headcounts (example for 3phylum) observed by different operators: one novice (1st bar from left) and two experienced (2nd and 3rd bars from left). Mean and standard deviation between the three operators are in white cross-brace. The identifications validated by the three operators are represented by the bar on the right.

A “profile” is thus a combination of orientation parameters combined with inclination and roughness. Further detailed study will be conducted on associations of profile features to determine the preferential profiles of different coralligenous communities.
The cartography details the environmental profiles: depth, orientation, slope, roughness of the coralligenous wall, and main stands. The aim of this protocol is to identify the main species assemblages according to the profile type. The inter-calibration phase of the protocol in order to test the material and methods is done. The study of the variability of the results, linked to the observers and operators implementation, shows which metrics are the most robust. This phase was preliminary in order to make the results workable whatever the country of implementation. The running step permits to identify accessible metrics that are relevant, reliable and efficient to explain the “natural” spatial variability regarding the environmental context. The genetic study of *Myriapora* and *Lithophyllum* used data of sequences and molecular markers to answer taxonomic questions about these species complex and descriptions of connectivity patterns. The preliminary results are promising: the genetic study of *Myriapora* and *Lithophyllum* highlights different clades which strongly suggest that there are most probably mixtures of different species.

Discussion and conclusion

<u>Assessment of the environmental status</u>
The assessment consists of the analysis of the coralligenous megabenthic assemblages by means of direct observation, photographic/video surveys (which are directly influenced by competencies and experience of the operators).
The study of both sampling methods shows that observations made at a phylum level of identification are comparable if they are made according to the one or the other method. To compare Porifera observations from one site to another, it would be recommended to implement the same method in both sites, as it’s shown that there might be significant difference of headcounts according to the applied method. As the random patch method is easier to perform, it should be recommended. Concerning the effect of the quality of the camera on the observations made, it has been proven that the medium quality camera is sufficient to identify as much species as the high quality camera. Difference between the two is observed for species difficult to differentiate at the genus level. Indeed, the high quality camera enables to give a taxonomic level at some individuals that were indeterminate with the medium quality camera. The study of the operator’s knowledge shows that the discussion between operators enables novices to quickly improve their capacity of identification for a given set of taxa. Discussion is very useful at the beginning, and then operators reach a step, and would need a proper training to progress, if more precise identification is needed.

<u>Population studies</u>
The objective of the cartography goes beyond than mapping habitats; it provides information about environmental profiles that will be used to understand species preferences. Population genetics approach which will complete the analyses is essential to investigate species diversity, population structure and connectivity.
The first results about genetic differentiation of demes from distinct localities for each taxon illustrate the fact that gene flow (migration) is limited even at the small scale of Marseilles region for those important coralligenous builders. Genetic barrier were previously evidenced for other species as different as the mysid *Hemimysis margalefi* (Lejeusne *et al*., 2006) or the irregular sea urchin *Echinocardium cordatum* (Egea *et al*., 2011).

It may also be linked to ecological conditions (distribution of divergent groups of each species depending on currents and ecologic profiles to be carried out).
This preliminary work yet enables to better understand the importance of the skill and training of operators (human factor rarely taken into account) and of the implemented sampling. Indicators, from communities to infra-specific level, will be co-constructed by scientists and PMA managers, and through the implementation of a "citizen science" network. The outcome will be an integrative assessment of the GES within the MSFD.
To build the network, the community is developing a metadata catalogue and shared typologies; we are working on (i) harmonization of data collection methods and normalization of data access (European standards) (ii) initiation/animation of thematic network about coralligenous habitats in Mediterranean Sea gathering all competent actors. This network is meant to be perennial, open, and fully decentralized (to allow for continuous update) at local, regional, national and international scales. This organisation will permit data diffusion and upper accessibility and will ensure continuous improvement.

Acknowledgements

We are grateful to the organizing committee who invites us to present the first results of the project. We thank A. Haguenauer, S. Chenesseau, F. Zuberer and all the underwater diving team for their participation to the sampling in Marseilles and T. Dailianis for samples from Crete. We thank M. Verlaque who helps us for the identification of Corallinales and A. Le Gall for helpful advices. A special thanks to the SIP (Computing Service of the OSU Pytheas) who provides all the hardware part and assistance for developments. This project was funded by the SeasEra program CIGESMED (CNRS- ANR convention n° 12-SEAS-0001-01).

Bibliography

BALLESTEROS E. (2006) - Mediterranean coralligenous assemblages: a synthesis of present knowledge. *Oceanogr. Mar. Biol.: Ann. Review*, 44: 123-195.

BANDELT H. J., FORSTER P., ROH*L A. (1999)* – Median-joining networks for inferring intraspecific phylogenies, *Mol. Biol. Evol*. 16, 37-48.

BROOM J. E. S., HART D. R., FARR T. J., NELSON W. A., NEILL K. F., HARVEY A. S., WOELKERLING W. J. (2008) - Utility of psbA and nSSU for phylogenetic reconstruction in the Corallinales based on New Zealand taxa, *Mol. Phylogenet . Evol*. 46, 958 – 973.

CHENUIL A. , HOAREAU T. B., EGEA E., PENANT G., ROCHER C., AURELLE D., MOKHTAR-JAMAI K., BISHOP J. D. D., BOISSIN E., DIAZ A., KRAKAU M., LUTTIKHUIZEN P. C., PATTI F. P. , BLAVET N., MOUSSET S. (2010) – An essicient method to find potentially universal population genetic markers, applied to metazoans, *BMC Evol. Biol.* 10, 276.

EGEA E (2011) - *H*istoire évolutive, structures génétique, morphologique et écologique comparées dans un complexe d'espèces jumelles: *Echinocardium cordatum* (Echinoidea, Irregularia). Aix-Marseille Université, Marseille.

GERARD K., GUILLOTON E., ARNAUD-HAOND S., AURELLE D., BASTROP R., CHEVALDONNE P., DERYCKE S., HANEL R., LAPEGUE S., LEJEUSNE C., MOUSSET S., RAMSAK A., REMERIE T., VIARD F., FERAL J.-P., CHENUIL A. (2013) – PCR survey of 50 introns in animals: Cross-amplification of homologous EPIC loci in eight non-bilaterian, protostome and deuterostome phyla, *Mar. Genomics*. 12, 1 – 8.

LEJEUSNE C., CHEVALDONNE P. (2006) - Brooding crustaceans in a highly fragmented habitat: the genetic structure of Mediterranean marine cave-dwelling mysid populations. *Mol. Ecol.* 15, 4123–40

MARION A. F. (1883) – Esquisse d'une topographie zoologique du Golfe de Marseille. *Ann. Mus. Hist.* Marseille, Marseille.

TRYGONIS V. & SINI M. (2012) – Photoquad: a dedicated seabed image processing software, and a comparative error analysis of four photoquadrat methods. J. Esp. Mar. Biol. Ecol. 424, 99-108.