Activity Report
Stazione Zoologica Anton Dohrn
2012-2017

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Preface

This Activity Report presents a synthesis of the researches conducted at SZN in the years 2012-2017. The general aim of SZN researchers is to improve our understanding of the biological systems and the evolution of life. This mission is pursued by investigating different aspects of Marine Biology and Ecology using an interdisciplinary approach.

The scientific activities, their main results and their perspectives are here organized according to twelve scientific themes, which are clearly linked to the research areas included in the SZN Piano Triennale (PTA) 2017-2019 that has been approved by the Italian Ministry of University and Research (MIUR). In particular, themes # 1-7 are framed in the area *Marine organisms: adaptation and evolution,* theme # 12 in the area *Marine biotechnologies*, while themes # 8-11 merge the areas of *Multiscale marine biodiversity* and *Integrated management of marine ecosystems* that are tightly interconnected.

For each scientific theme, the lists of people involved and external collaborators, the background, aims and objectives, main results with corresponding publications and grants are reported along with perspectives and proposed future research. An Appendix containing a short descriptive sheet for each of the external grant projects mentioned in the Activity Report integrates this document.

The researches conducted at SZN are based on various field, laboratory and maintenance activities that represent the initial steps of our work and allow to test hypotheses, develop ideas and pursue objectives. This fundamental work is conducted by the well-qualified technicians of the SZN Departments and Services, whose activities contribute to several and different research topics:

Gaetano Aloe, Alessandro Amoroso, Salvatore Bocchetti, Fabrizio Campili, Marco Cannavacciuolo, Davide Caramiello, Mariapia Ciampa, Fabio Conversano, Federico Corato, Giuseppe Corato, Giovanni De Martino, Luigi De Martino, Iole Di Capua, Francesco Di Liello, Mario Di Pinto, Gabriele Ferrandino, Pasqualina Fiorentino, Maria Francone, Giovanni Gragnaniello, Rita Graziano, Bruno Iacono, Franco Iamunno, Gianpiero Lanzotti, Maurizio Lorenti, Alberto Macina, Alessandro Manfredonia, Pier Paolo Mari, Elvira Mauriello, Luigi Migliaccio, Carmen Minucci, Marco Miralto, Vincenzo Monfrecola, Flora Palumbo, Raimondo Pannone, Augusto Passarelli, Antonio Pedone, Massimo Perna, Alfredo Piccolo, Michele Pischetola, Vincenzo Rando, Raffaele Sepe, Marco Signore, Violante Stefanino, Salvatore Traino, Ferdinando Tramontano, Andrea Travaglini, Gianluca Treglia, Raffaele Trimarco, Cosimo Vestito, Gianluca Zazo.

To all of them the sincere acknowledgement and deep thankfulness of the entire scientific staff.

Moreover, we wish to thank all people of the administrative staff that support all SZN activities.

1. Development and evolution of nervous and sensory systems

1. People involved

Name	*Position*	*Funding*	*Period*
Maria I. Arnone	Staff scientist	SZN	since 2000
Elio Biffali	Staff scientist	SZN	since1994
Marco Borra	Staff scientist	SZN	since 2012
Immacolata Castellano	Staff scientist	SZN	since 2016
Paola Cirino	Staff scientist	SZN	since 1989
Salvatore D'Aniello	Staff scientist	SZN	since 2010
Pasquale De Luca	Staff scientist	SZN	since 2009
Annamaria Locascio	Staff scientist	SZN	since 2009
Rita Marino	Staff scientist	SZN	since 2001
Anna Palumbo	Staff scientist	SZN	since 1980
Filomena Ristoratore	Staff scientist	SZN	since 2000
Paolo Sordino	Staff scientist	SZN	since 2000
Antonietta Spagnuolo	Staff scientist	SZN	since 1989
Elijah Lowe	Postdoctoral fellow	Corbel	2015-2017
Rosa M. Pezzotti	Postdoctoral fellow	SZN	2012-2014
Evgenya Anishchenko	PhD student	SZN	2011-2014
Giovanni Annona	PhD student	CIG	2012-2015
Filomena Caccavale	PhD student	SZN	since 2014
Ugo Coppola	PhD student	SZN	since 2014
Ylenia D'Agostino	PhD/Postdoc	FIRB	since 2013
Elena Ercolesi	PhD student	SZN	2009-2012
Rosaria Esposito	PhD student	SZN	2010-2014
Giulia Fasano	PhD/Postdoc	Sannio Univ/PONa3	since 2013
Ashwani K. Kamal	PhD student	SZN	2012-2015
Valeria Nittoli	PhD/Postdoc	SZN/PONa3	since 2013
Antonio Palladino	PhD student	SZN	2014-2017
Claudia Racioppi	PhD student	SZN	2010-2013
Rosa M. Sepe	PhD student	SZN/PONa3	since 2013
Alberto Valero-Gracia	PhD student	SZN	2013-2017
Quirino Vassalli	PhD/Postdoc	SZN	2013-2017

2. Background, aims and objectives

The research activity on nervous and sensory systems in marine organisms is aligned with the SZN PTA 2017-2019 and Vision 2015-2025 with the goal of a better comprehension of the evolution of life, and the adaptation of marine organisms to environmental niches. Research sets its focus on basic biology – with the study of molecular mechanisms at the base of cellular differentiation - and marine biodiversity for understanding how the environment can mask, release or create genotypic and phenotypic variation.

One of biology's grand challenges is to explain how genes control the growth and development of animal body plans, and how these processes change through evolution. Marine organisms have developed specific and ingenious neural and sensory strategies to cope and thrive with the diversified and variable set of environmental conditions at sea. Research activities aim to study the organization and function of molecular and cellular mechanisms that regulate the development and evolution of different cell types. Research projects include the main experimental models among invertebrates (e.g., cuttlefish, sea urchins, lancelets, sea squirts) through the latest methods in molecular and cell biology, in particular genomics and its derivatives. To understand the origin and the evolution of the neural and sensory systems in marine organisms, the comparison of invertebrate structures with those in the freshwater teleost fish Danio rerio (zebrafish) is of fundamental importance[1]. The possibility of carrying out genetic manipulations also results in the production of transgenic and mutant lines for applicative purposes (Tol2 and CRISPR/Cas9). Complementary and comparative approaches that characterize the field of Evo-Devo interface molecular and chemical systems with environmental and behavioral sciences. Neural and sensory system studies at SZN span a wide range of topics, focusing on the precise roles played by genetic, chemical and cellular properties that (i) regulate synaptic survival and plasticity (Neurotrophins), (ii) define specific cell, tissue and organ identities in chordate neural plate (Gsx), vision system (Onecut, opsins, endocannabinoids), mouth formation and metamorphosis (Nitric Oxide), or (iii) govern adaptive evolution of pigmentation (Tyrosinase). Comparative morphological and molecular approaches are also employed to solve the evolutionary history of planktonic tunicate orders, since distribution patterns of neurotransmitters and neuromodulators have been shown to allow phylogenetic interpretations in numerous taxa. These activities are currently carried out by researchers in BEOM department of the SZN involved in different international, Italian or SZN-funded research projects.

The results obtained during the period of 2012-2017 have been subdivided in eight main research lines with basic and translational aspects.

- Neurotrophin signaling
- Nitric Oxide signaling
- Endocannabinoid signaling
- Dopaminergic neurons
- Thaliacean brain evolution
- Ascidian neural plate formation
- Ascidian eye development
- Evolutionary loss of melanogenesis
- Photoreceptive organ evolution

3. Results

a. Evolutionary conservation and novelties in Neurotrophic pathway (D’Aniello)

Neurotrophins (NT) (BDNF, NGF and NT *sensu stricto*) are growth factors that control development, differentiation, synaptic plasticity and survival of several types of neuronal and glial cells in the embryonic and adult central nervous system and sensory organs. NT binding to specific tyrosine kinase receptors (TRK)[2] and to a pan-neurotrophinic receptor (p75NTR) activates a series of events that, mediated by intracellular signal transduction pathways, modify cytoplasmic and nuclear protein function. Based on the NT role in development and physiology of the nervous system in metazoans, the present project aims at extending our knowledge on the genetic regulatory networks and on the functions exerted by BDNF during the correct development and functioning of embryonic and adult zebrafish brain (**De Felice *et al.*, 2014**). Although the

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[1] Stewart *et al.* Zebrafish models for translational neuroscience research: from tank to bedside. ***Trends Neurosci.*** (2014) 37: 264-78.

[2] D’Aniello *et al.* Gene expansion and retention leads to a diverse Tyrosine kinase superfamily in amphioxus. ***Mol. Biol. Evol.*** (2008) 25: 1841-54.

neurotrophin signaling system in zebrafish is well studied, a comprehensive and exhaustive knowledge on the transcriptional anatomy of the neurotrophin system during early zebrafish development was still missing. For this reason, we completed the expression profile of all NT and NT receptors showing that they have different degrees of cell- and tissue-type specificity (**Nittoli *et al.*, under resubmission**).

A second objective of this project is the generation of a BDNF *knock-out* line in zebrafish through the CRISPR/Cas9 [CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas)] technology (**D'Agostino *et al.*, 2016; D'Agostino and D'Aniello, 2017**) and the characterization of the mutant phenotype from both molecular and behavioral points of view (Figure 1). BDNF-/- mutant zebrafish, unlike what happens in mouse, survive after birth indicating that BDNF protein absence in fish could be compensated by other NTs. Behavioral test in which larvae movements were monitored reveals a significantly lower activity in respect to controls, suggestive of reduced exploratory behavior. Moreover, several anatomical anomalies such as aberrant pharyngeal arch formation and increased apoptotic cell number underline the complex role played by BDNF and its implication in a variety of biological processes. Differential transcriptomics analysis between mutant and wild-type larvae has been performed to identify downstream target coding and non-coding genes regulated by BDNF (**Ventola *et al.*, 2017**). Analysis of the differential RNA-seq data is providing new insights on the role played by BDNF during the development of the central and peripheral nervous systems, eye morphogenesis and circadian rhythm establishment and maintenance.

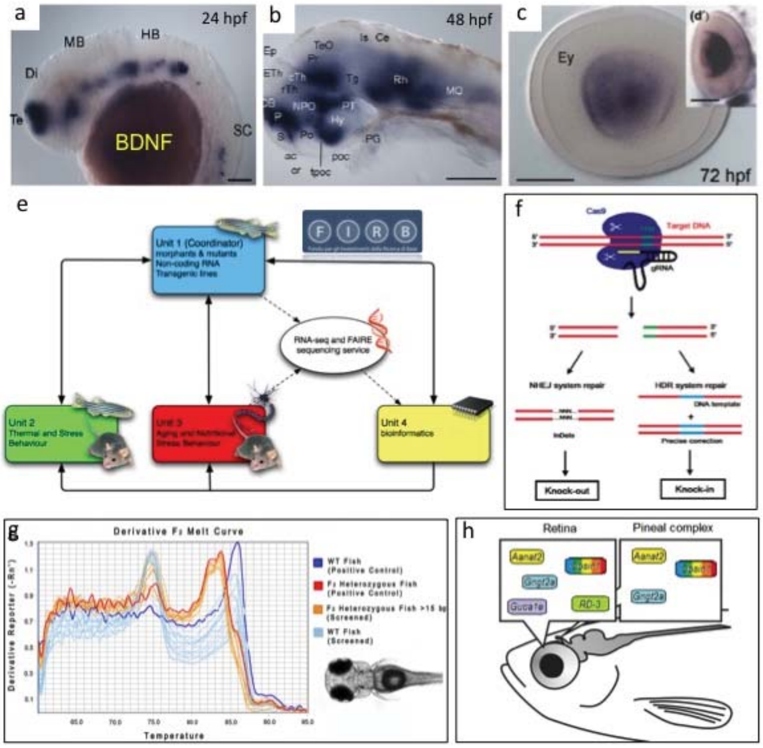
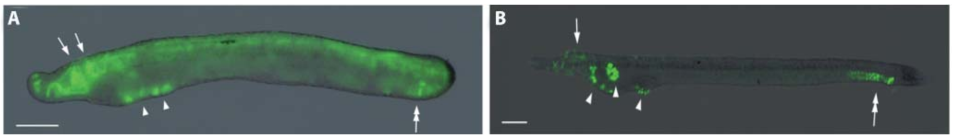


Figure 1. Generation of BDNF knock-out line in zebrafish through the CRISPR/Cas9 technology.

This project is supported by a Futuro In Ricerca (FIRB) grant of Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) to S. D'Aniello, and is in collaboration with A. Locascio, F. Ristoratore, A. Spagnuolo and P. Sordino (SZN).

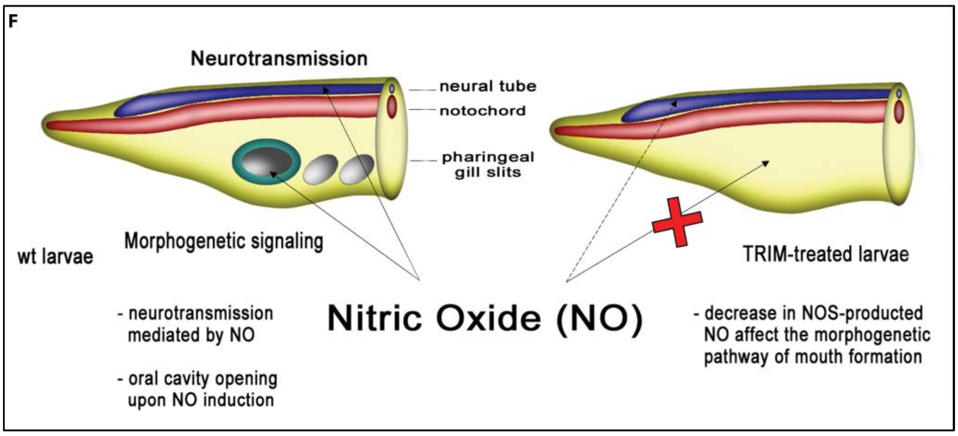
b1. Nitric Oxide signaling in marine invertebrates: neurotransmission and evolution of chordate mouth formation (S. D'Aniello)

Our understanding of Nitric Oxide (NO) signaling has profoundly changed over recent decades. It was originally considered a toxic substance, but nowadays, although harmful at high concentration, NO is believed to be an essential signaling molecule for living organisms. To study the role of NO in the development of European amphioxus, *Branchiostoma lanceolatum,* we set up a live animal culture in our Institute (uniqueness in Italy!) that enable us to obtain live embryos *on demand* in the lab. The biosynthesis of NO is catalyzed by the Nitric Oxide Synthase enzymes (NOS) that are extraordinary highly conserved in metazoan evolution, as previously showed[3]. We isolated three NOS genes that from phylogenetic analysis resulted not orthologous to vertebrate NOS genes, but independently duplicated in the amphioxus lineage. We, therefore, studied NOS expression patterns during amphioxus embryogenesis and found a temporal complementarity (**Annona *et al.*, 2017**). Moreover, functional experiments aimed to inhibit embryonic NOS activity during development demonstrated that, beside its known function as a neurotransmitter and neuromodulator, NO signaling inhibition prevents the formation of mouth and gill slits (Figure 2) (**Annona *et al.*, 2015; Annona *et al.*, 2017**). This suggests that, also in amphioxus, the embryonic origin of the prospective chordate primary mouth is under direct NO control during the neurula stage. We, therefore, added new insights into amphioxus mouth that still represents a longstanding enigma with regard to its evolutionary origin, homology relationships and differences with other chordate mouths.



***In vivo* Nitric Oxide localization in amphioxus larvae**

Inhibition of Nitric Oxide synthesis during development



Role of Nitric Oxide during amphioxus development

Figure 2. Nitric Oxide signaling is necessary for amphioxus mouth development.

We are members of the “European Amphioxus Consortium” that is working to the release of *Branchiostoma lanceolatum* genome scheduled for 2018. This project was supported by the EU Marie Curie Carreer Integration Grant (CIG): “AmphioxusNOS" to S. D’Aniello, and by EU ASSEMBLE Plus to F. Ristoratore.

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[3] Andreakis, D’Aniello *et al.* Evolution of the Nitric Oxide Synthase family in metazoans. ***Mol. Biol. Evol.*** (2011) 28: 163-79.

b2. Nitric Oxide signaling in marine invertebrates: Role of nitric oxide in signal transduction events in marine invertebrates (Palumbo)

Our continuing studies have highlighted the pivotal biological functions of nitric oxide in several fundamental processes in marine organisms, including ink production, color changes[4] and development in the cuttlefish *Sepia officinalis* (**Mattiello *et al.*, 2012; Mattiello *et al.*, 2013**). Recently, we have reported that the signals of Nitric Oxide (NO) and nitric oxide synthase are very dynamic during larval development and metamorphosis in *Ciona robusta* (previous intestinalis), moving rapidly along the larval body, from the anterior part of the trunk to central nervous system, tail and juvenile digestive organs[5]. Moreover, we have demonstrated that changes of endogenous NO levels affect the rate of metamorphosis (**Ercolesi *et al.*, 2012**), and we have identified the molecular mechanisms underpinning this process, that is NO down-regulates MAP kinase phosphatases expression with a positive effect on ERK signaling and on the expression of downstream pivotal developmental genes (Figure 3, **Castellano *et al.*, 2014**).

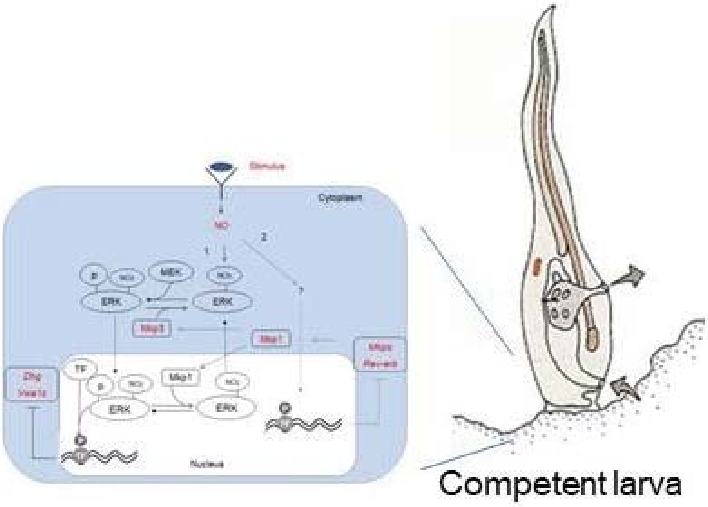

Figure 3. NO induces ERK signaling during *Ciona* metamorphosis.

c. The endocannabinoid system in development and evolution: implications in vision and locomotion (Sordino)

The endocannabinoid system (ECS) comprises neuromodulatory lipids that regulate neuronal excitability through specific receptors in a variety of physiological processes and in specific brain functions, such as nociception, control of movement, memory and neuroendocrine regulation[6]. ECS activity in early stages of brain development suggests a role in the regulation of neuronal progenitor cell differentiation. Because of redundancy and promiscuity characterizing this neuromodulatory system, it is fundamental to establish roles, functions, and contributions of single ECS factors. We revealed a complete ECS system in zebrafish and found that genes coding for enzymes that catalyze 2-AG anabolism, catabolism and brain receptor (*daglα*, *mgll* and *cnr1*/*CB1*, respectively), are co-expressed with axonal growth. By morpholino-induced transient knockdown of *daglα* and its pharmacologic rescue, we found that 2-AG is implicated in the control of axon formation and fasciculation in optic tectum, cerebellum and optic nerve, as well as in stereotyped eye movement and motion perception. Our findings demonstrate for the first time the role of 2-AG

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[4] Mattiello *et al.* Nitric oxide mediates the glutamate-dependent pathway for neurotransmission in *Sepia officinalis* chromatophore organs. ***J. Biol. Chem.*** (2010) 285: 24154-63.

[5] Comes et al. Regulatory roles of nitric oxide during larval development and metamorphosis in *Ciona intestinalis*. ***Dev. Biol.*** (2007) 306: 772-84.

[6] Reisenberg *et al.* The diacylglycerol lipases: structure, regulation and roles in and beyond endocannabinoid signaling. ***Philos. Trans. R. Soc. Lond. B Biol. Sci.*** (2012) 367: 3264-75.

in the regulation of axon growth in distinct areas of the central nervous system, acting as mediator of axonal projections implicated in the control of vision and movement. The 2-AG knocking-down phenotype is a useful model for further insights aimed at the full identification of molecular targets and components of ECS signaling mechanisms and for defining new therapeutic approaches (**Martella *et al.*, 2016**) (Figure 4).

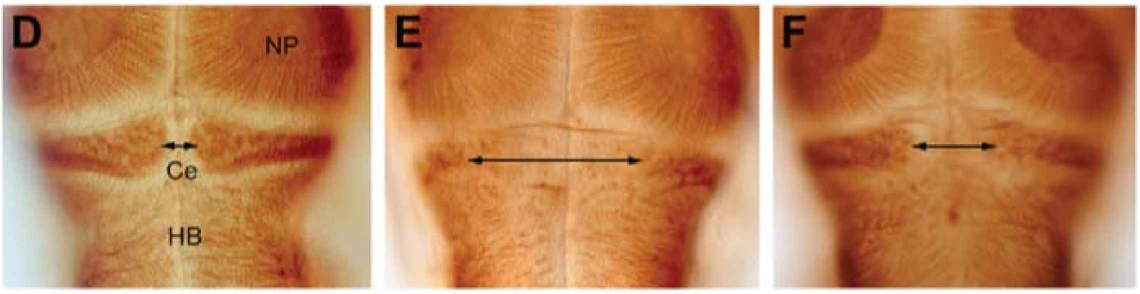


Figure 4. (D *vs* E) Limited extension of cerebellar nerves caused by loss of 2-AG in zebrafish embryos, and (F) phenotype rescue by cannabinoid receptor agonist noladin in zebrafish embryos.

d. Tunicates at the basis of metazoan complexity evolution (Sordino)

The overall pattern of serotonin (5-HT) distribution is an excellent biochemical trait to interpret internal relationships at order level7. Analysis of serotonin distribution in adult thaliacean oozooids shows immunopositive neurons symmetrically distributed in the cerebral ganglion of the examined species. Moreover, we find differences in serotonin-like immunoreactive arrangement in endostyle, digestive system and pyloric gland. These results support the phylogenetic hypothesis representing Pyrosomatida as the first diverged Thaliacean order from the Ascidiacea clade, and Salpida and Doliolida as sister groups (**Valero-Gracia *et al.*, 2016**) (Figure 5).

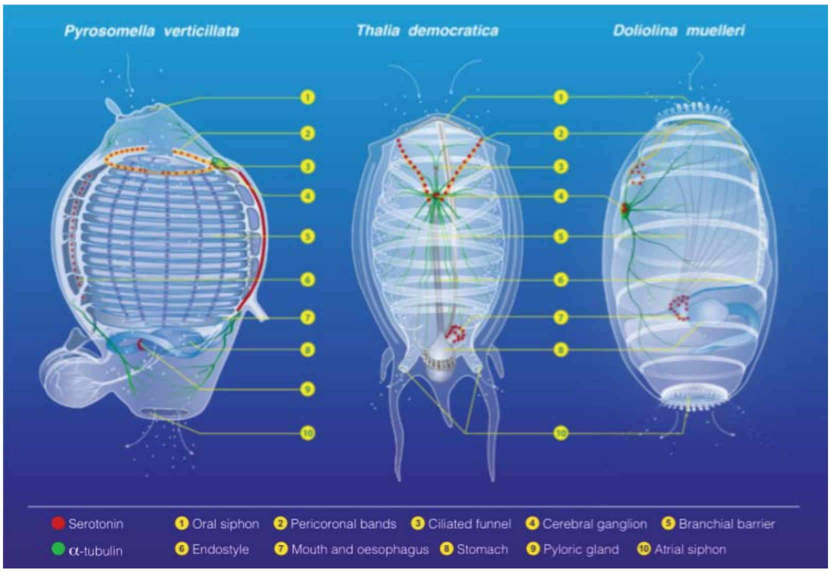


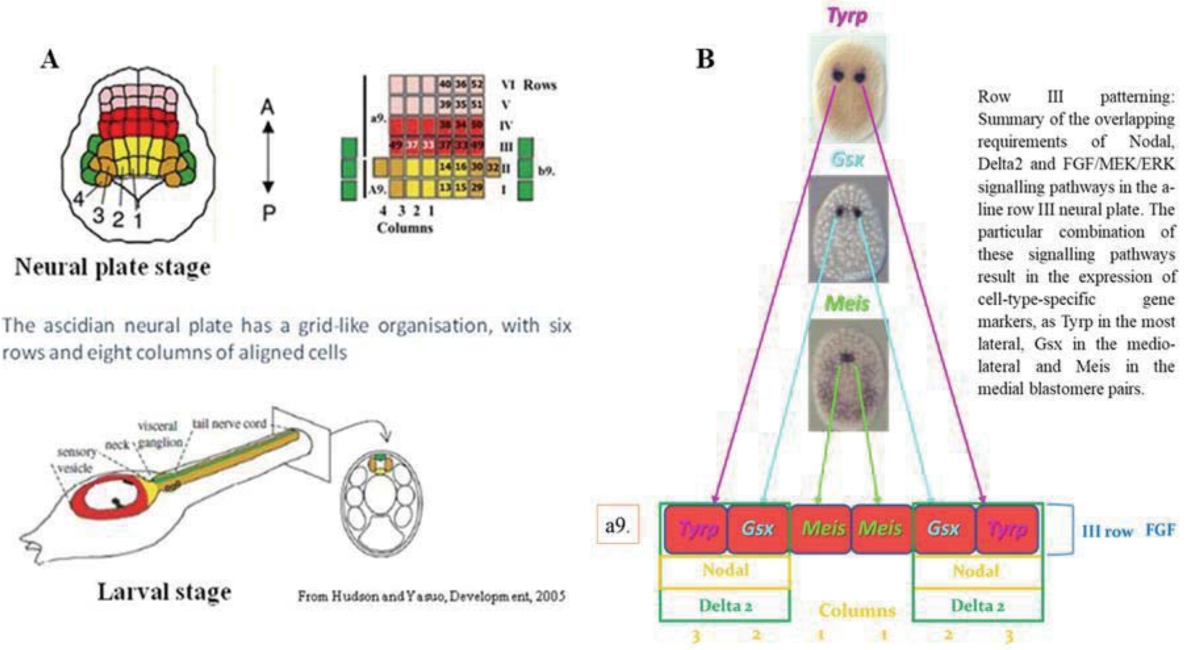
Figure 5. Serotonin-like immunoreactive nervous system in thaliacean orders.

e. Ascidian neural plate formation (Spagnuolo)

The ascidian *Ciona robusta* larva possesses a typical, but simplified, chordate nervous system in miniature (CNS), with around 330 cells. At the neural plate stage *Ciona* CNS shows a grid-like organization in which each cell has its own specific identity and a unique molecular signature, thus permitting to study cell fate diversification at the level of individual cells. This is a huge advantage, compared to the thousands of cells present in vertebrates, to investigate both the precise role played by signaling and transcription pathways and, at the same time, their complex interplay in shaping the CNS. *Ciona* CNS derives from three of the four founder lineages, a-, b- and A-lineages

(Figure 6A). The a-line (red) gives rise to the anterior part of the sensory vesicle, including the characteristic pigmented cells (the two black spots in the larval brain) which are part of two sensory organs, the otolith, used for the perception of gravity, and the ocellus, used for light reception.

Our studies aim at identifying the molecular mechanism defining cell identities in the sensory vesicle, the ascidian 'brain', the most anterior part of *Ciona* CNS. We have previously carried out a detailed study of tyrosinase family proteins (the enzymes involved in pigmentation) in *Ciona* and conducted a deep comparative and evolutionary analysis of these genes amongst metazoan, in order to trace back their origin (**Esposito *et al.*, 2012**). Furthermore, we have reviewed all the information collected so far on the structures and developmental programs of *Ciona* pigmented sensory organs (**Esposito *et al.*, 2015**). More recently, in collaboration with Dr. Yasuo and Hudson groups, we have demonstrated that a particular combination of three signaling pathways, Nodal, Delta2/Notch and FGF/MEK/ERK, defines each of the distinct cell identities that make up the anterior blastomeres of the neural plate, specifically the row III (**Esposito *et al.*, 2017**). In particular, *Nodal* signaling is required for lateral neural plate fates and *Delta/Notch* refines the initial pattern established by *Nodal* and subdivide each of the lateral and medial domains to generate medio-lateral fates. Superimposed on this pattern is the FGF signaling, which induces differences in antero-posterior identities. These cascades work through signal transduction pathways that ultimately result in the transcriptional control of cell-type-specific gene markers. In particular, Nodal, Delta2/Notch and FGF/MEK/ERK control the expression of Trp, Gsx and Meis genes, which label row III cells in columns 3 (lateral), 2 (intermediate), and 1 (medial), respectively (Figure 6B). Therefore, our studies, together with all the data collected so far, further confirm that understanding of the basic and essential mechanisms revealed in the simple ascidian CNS compartments contributes to the understanding of the more complicated vertebrate CNS regionalization.



The ascidian neural plate has a grid-like organisation, with six rows and eight columns of aligned cells

From Hudson and Yasuo, Development, 2005

Row III patterning: Summary of the overlapping requirements of Nodal, Delta2 and FGF/MEK/ERK signalling pathways in the a-line row III neural plate. The particular combination of these signalling pathways result in the expression of cell-type-specific gene markers, as Tyrp in the most lateral, Gsx in the medio-lateral and Meis in the medial blastomere pairs.

Figure 6. Ascidian neural plate formation.

f. Onecut genetic pathway: its role in eye formation and functional conservation during chordate evolution (Locascio)

To understand the origin of the two-dimensional camera-type visual system of vertebrates, the comparison of vertebrate eye with the photoreceptive structures present in extant non-vertebrate chordates is of fundamental importance. *Ciona robusta* photoreceptors are commonly proposed to be ancestral to that of vertebrates, being thus used as a model to investigate the complex molecular mechanisms underlying photoreceptors formation and differentiation (**Esposito *et al.*, 2015; Martinez-Morales and Locascio, 2016**).

With this project, we aim to characterize in *Ciona* the functional role of the Onecut transcription factor. This gene family has been recently implicated as key regulator of specific eye and neural structures but very little is known about its function and mechanism of action. To study the genetic pathway of the Onecut genes during eye formation we extensively characterized the OC upstream *cis*-regulatory regions and found a small region of 262 bp able to reproduce most of the OC expression profile during embryonic development (Figure 7). Bioinformatics and *in vivo* analyses provided evidence that Neurogenin is a direct activator of OC, which, in turn, is responsible for the maintenance of its expression (**Pezzotti *et al.*, 2014**). These studies evidenced the presence of a direct connection between Neurogenin, Onecut and Rx genes and that these genes are responsible for photoreceptors differentiation.

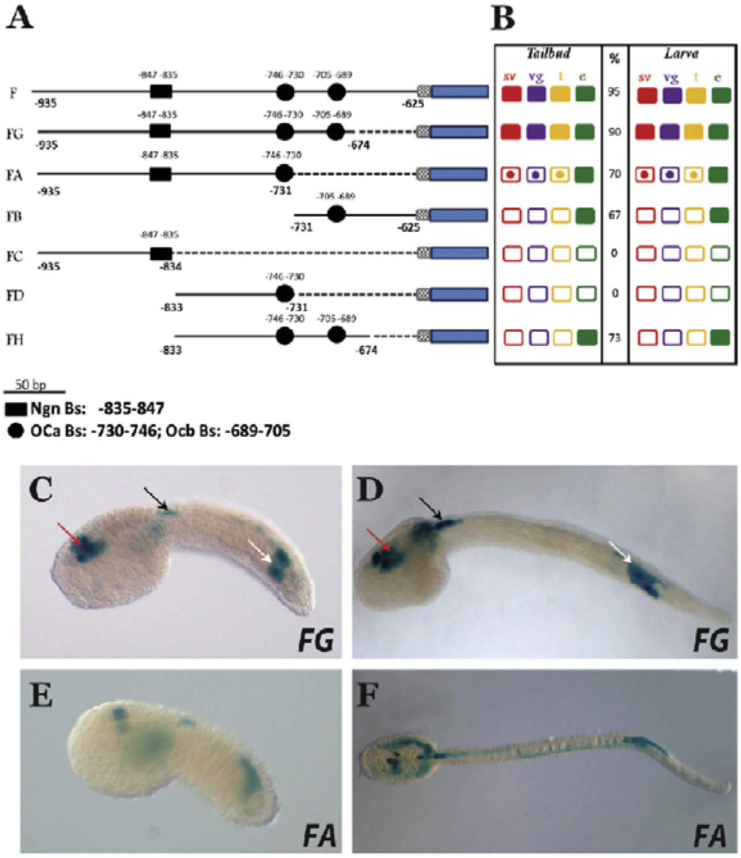


Figure 7. Schematic representation of regulatory sequences (black bars) tested in transgenic embryos (A, B). Reporter gene expression at tailbud and larva stages under the control of Onecut regulatory elements (C-F).

g. Loss of melanogenesis in the ascidian *Molgula occulta* (Ristoratore)

Many examples of trait loss are known in metazoan populations living in dark environments. Probably due to ecological adaptation to distinct habitats, several species of tunicates in the Molgulidae family (ascidians) have tailless (anural) larvae that fail to develop sensory organ-associated melanocytes[7]. We studied the evolution of *Tyrosinase* family genes, indispensible for melanogenesis, in the anural, unpigmented *Molgula occulta* and in the tailed, pigmented *Molgula oculata* by using phylogenetic, developmental and molecular approaches. We found that *M. oculata* (that present a pigmented sensory organ) possesses genes predicted to encode one Tyrosinase (Tyr) and three Tyrosinase-related proteins (Tyrp), while *M. occulta* has only *Tyr* and *Tyrp.a* pseudogenes that are not likely to encode functional proteins (Figure 8). Analysis of *Tyr* sequences from various *M. occulta* individuals indicates that different alleles independently acquired frameshifting short indels and/or larger mobile genetic element insertions, resulting in pseudogenization of the *Tyr* locus. In *M. oculata, Tyr* is expressed in presumptive pigment cell

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[7] Huber *et al.* The evolution of anural larvae in molgulid ascidians. ***Semin. Cell Dev. Biol.*** (2000) 11: 419–26.

precursors as in the model tunicate *Ciona robusta*. In conclusion, we reveal a complex evolutionary history of the melanogenesis pathway in tunicates, characterized by distinct gene duplication and loss events. Our expression and molecular data support a tight correlation between pseudogenization of *Tyr* family members and the absence of pigmentation in the immotile larvae of *M. occulta*. These results suggest that relaxation of purifying selection has resulted in the loss of core genes in the melanogenesis biosynthetic pathway in *M. occulta* with consequent loss of sensory organ-associated melanocytes.

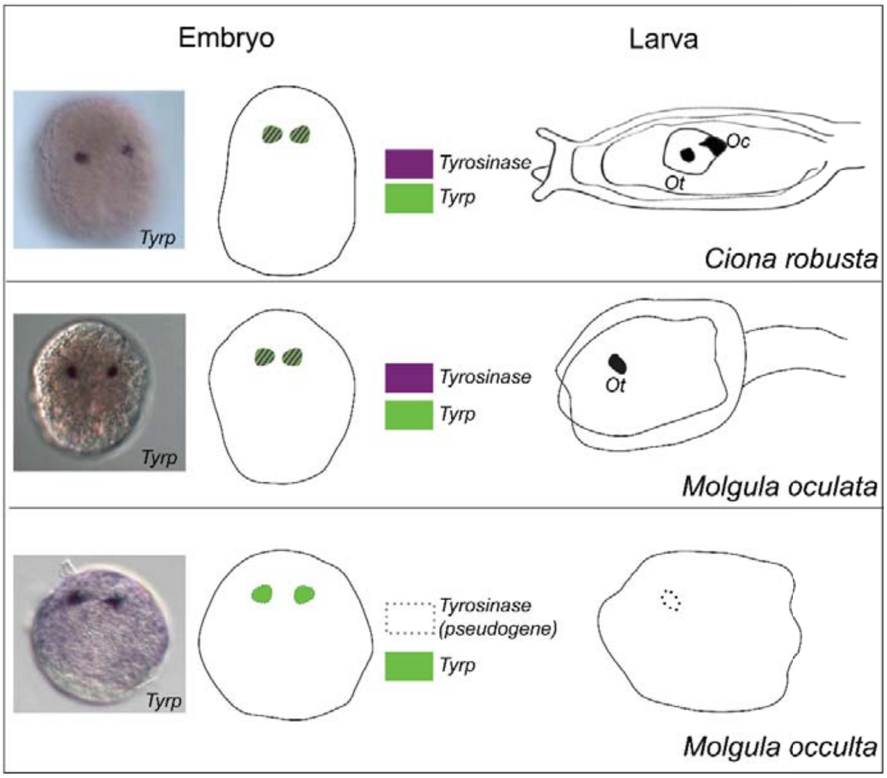

Figure 8. Loss of melanogenesis in the ascidian *Molgula occulta.*

h. Light perception in the oceans and photoreceptor cell (PRC) evolution (Arnone)

How do larvae and adults of sea urchins, despite the lack of a central nervous system and obvious eye-like structures, sense and respond to environmental light? Using a multidisciplinary approach, the Arnone group is studying the morphological structure, molecular composition and physiological properties of photoreceptor cells (PCRs) in echinoderm larvae and adults. Our studies of marine non-chordate deuterostomes thus aim to bridge a gap of knowledge regarding the evolution of deuterostome PRCs prior to the emergence of vertebrates' eyes.

For the study of PRCs in the adult, using antibodies generated against previously identified *Strongylocentrotus purpuratus* opsin sequences[8], we identified putative PRCs in a variety of sea urchin, sea star and brittle star tissues (Figure 9) (**Ullrich-Lüter *et al.*, 2013**). Moreover, we recently demonstrated, in collaboration with Prof. Klein's group, that an orthologous of the mouse Pou4f2 transcription factor is expressed in the sea urchin tube foot PRCs which also express a rhabdomeric opsin and can replace mouse retinal ganglion function (**Mao *et al.*, 2016**).

For the larval PRCs, we identified a Go-opsin expressing pair of cells, which are bilaterally distributed at each the side of the larval apical organ and might be responsible of vertical diel migration in the water column during their planktonic life period (**Valero-Gracia *et al.*, 2016b**). These cells also produce a Thyrotropin-Releasing Hormone (TRH)-like neuropeptide (SpTRH). The role of TRH so far poorly investigated in invertebrates, as well as the one of other echinoderm

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[8] Raible *et al.* Opsins and clusters of sensory G-protein-coupled receptors in the sea urchin genome. ***Dev. Biol.*** (2006) 300: 461-475.

neurpeptides (**Elphick *et al.*, 2015**), is currently under study in the framework of a collaboration with Paola Oliveri's and Maurice Elphick's labs.

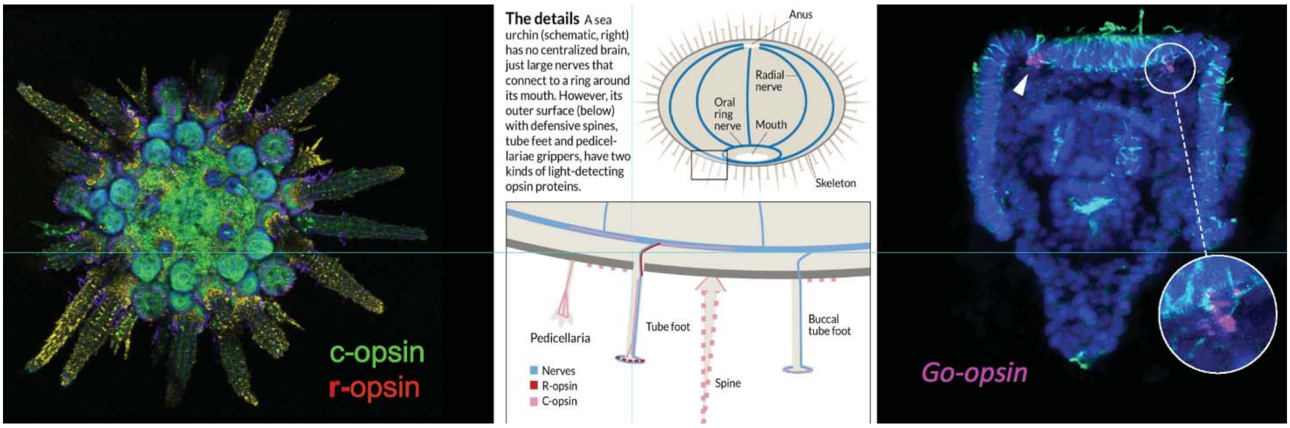
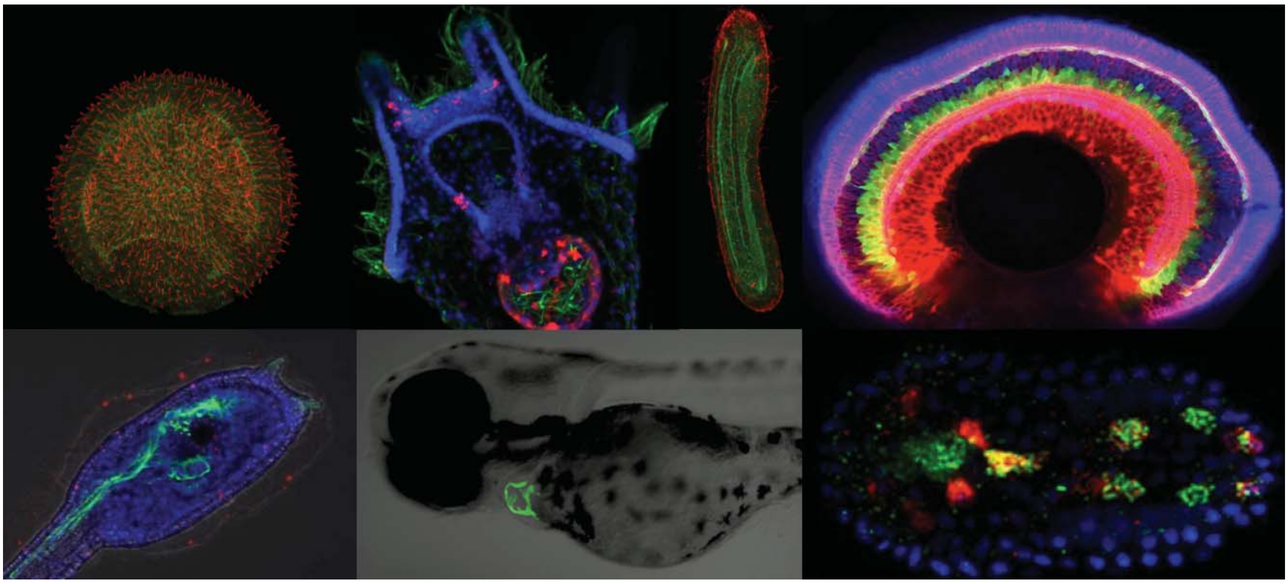


Figure 9. Opsin expression in sea urchin adult (left) and larvae (right).

Using genomics and trascriptomics approaches, with the aim of providing new insights into opsin-based photoreception and photoreceptor cell evolution, we performed a first analysis of opsin sequence data for a major deuterostome clade, the Ambulacraria[9]. We also are investigating opsin diversity in a variety of echiniderms, such the non-visual Europan infaunal brittle star *Amphiura filiformis* (**Delroisse *et al.*, 2014**) and the crown-of-thorns starfish *Acanthaster planci* (**Lowe *et al.*, 2017**), a non-burrowing starfish species which displays well defined eyes at the tip of each arm.
This research was supported by Marie Curie ITN Neptune grant and, partially, by the Corbel Infradev project (support to EK Lowe).

4. Perspectives

Future directions in Research topic 1 point to apply novel technologies and approaches, such as single cell genomics, genome editing (CRISPR/Cas9), and epigenomics (Assay for Transposase-Accessible Chromatin using sequencing, ATACseq) to tackle EvoDevo questions on a wider set of marine organisms than just laboratory models.



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[9] D'Aniello *et al.* Opsin evolution in the Ambulacraria. ***Mar. Gen.*** (2015) 24: 177-183.

a. Evolutionary conservation and novelties in Neurotrophic pathway (S. D'Aniello)

We aim to understand the mechanisms in which BDNF signaling is involved during the development of vertebrate's nervous system and to study the evolutionary importance of NT family members' expansion (including receptors) from marine invertebrates with unduplicated genomes to vertebrates. In future we would like to extend similar studies focusing on other Neurotrophins as well (NGF, NT3, NT4/5).

To complete BDNF$^{-/-}$ mutant characterization:

- We will check the expression level of other NTs belonging to BDNF family (qPCR and Western blots) in order to detect their possible compensating effect after BDNF depletion.
- We will characterize the neuro-morphological defects of dopaminergic, cholinergic, glutamatergic and GABAergic neurons with specific cell markers.
- Preliminary data from differential RNAseq analysis evidencedindirect regulation of BDNF on transcription factors involved in eye development and detection of light stimulus. In particular, we will study in detail the regulation of some opsin genes transcription exerted by BDNF in the developing zebrafish eye.
- We will try to understand how BDNF is involved in the coordination and maintenance of the circadian rhythm through the interaction with one or more down-stream signaling pathways.
- We will study the role of non-coding RNAs, i.e. microRNA and lncRNA, in the transcriptional regulation of mutant fish embryos.

b1. Nitric Oxide signaling in marine invertebrates: neurotransmission and evolution of chordate mouth formation (S. D'Aniello)

Our study points to novel roles of Nitric Oxide in development and physiology of metazoans, particularly in amphioxus as simplified version of more complex vertebrates. We want to add knowledge on the downstream effect of the experimentally induce absence on NO in a developing chordate embryo.

Therefore, to understand which gene pathways depend on endogenous NO during chordate development, we will work in the following directions:

- A differential transcriptomic analysis of NOS-inhibited embryos has been performed and we will experimentally validate the most interesting results. Preliminary results point to novel gene candidates as potential targets of the NO signaling.
- We will explore the conservation of transcriptional regulation of neuronal NOS in amphioxus and compare it with that known from human and mouse. We will perform mVista analysis and test *in vivo* putative enhancers in the locus of nNOS.

c. The endocannabinoid system in development and evolution: implications in vision and locomotion (Sordino)

It is essential to understand the physiological and developmental functions of the ECS during chordate evolution, differentiating between basic ancestral roles from secondary innovations. To the aim, we are studying the organization and developmental roles of the endocannabinoid system in the protochordate species, *Branchiostoma lanceolatum* (Cephalochordata) and *Ciona robusta* (Tunicata), in collaboration with Dr. Salvatore D'Aniello (SZN) and Dr. Antonietta Spagnuolo (SZN).

We will also analyze:

- ECS functions in the differentiation and lamination of vertebrate retina.
- Signal transduction pathways controlled by ECS genes in retina formation.
- Role of eCB receptors (CB1 and CB2) and eCB interacting proteins (CNRIP1a and CNRIP1b) in mediating 2-AG function in the formation of vision- and locomotion-related neural circuits.
- Influence of light spectrum on ECS gene expression, in collaboration with Dr. Cristophe Brunet (SZN).

d. Tunicates at the basis of metazoan complexity evolution (Sordino)

The ‘tubuline code’ is a regulatory mechanism to control the specialization of microtubule cytoskeleton in many physiological processes. It is generated by expression of different α – and ®-tubulin isotypes, and by post-traslation modification (PTM) of tubulin, which generally occur at the C-terminal end of the molecule. Changes in acetylation influence vescicular trafficking along the axon of neurons, detyrosination/tyrosination cycle has a strong effect on the processivity of dynein and has also been shown to influence tumorigenesis and neuronal organization, and glutamylation (such as glycation) is the main post-translational modification of α and ® tubulins found in the neurotubules. Here, we first aim to describe the tubulin code in Tunicata.
- Profiting of the effectiveness of tunicates as model system in evolution studies, we aim to investigate immunoreactivity patterns of tubulin PTMs, representing the most evolutionary conserved and developmentally regulated types of modifications.

e. Ascidian neural plate formation (Spagnuolo)

Thanks to the simplicity of Ciona CNS and the easy to dissect the *cis*-regulation of the genes of interest, we are currently attempting to address the link between Nodal, Delta2/Notch and FGF/MEK/ERK signaling and the expression of the medio/lateral marker gene, *Gsx*. Using a combination of morpholino gene knockdown, expression of dominant-negative forms, window deletion analysis and site directed mutagenesis, we have revealed that FGF signaling is involved in the direct activation of the gene *Gsx.* This data parallels our previous findings, which demonstrate that FGF signaling induces the pigment cell fate in the most anterior-lateral blastomeres of the row III, by directly activating *TCF* gene[10]. We are now attempting to identify further inputs (either positive or negative) involved in the fine regulation of *Gsx* expression in the neural plate. Still concerning Gsx, as development proceeds, a further signal appears more posteriorly, in a region that is likely to be part of the posterior A-lineage descendant, precursors of the photoreceptor cells present in the ocellus sensory organ of *Ciona* brain. On these grounds, we are currently investigating *Gsx* activity in photoreceptor formation, by targeted perturbation of the endogenous *Gsx* function, obtained by expressing constitutively active and repressive forms of *Gsx*, on endogenous Arrestin expression (a marker for terminal differentiation of photoreceptors) and on the activity of Arrestin promoter at the larval stage. This could represent a first evidence of a potential implication of a *Gsx* ParaHox gene in photoreceptor differentiation, thus opening new perspectives on the role, still controversial, of *Gsx* gene in the ancestor of bilaterians.

f. Onecut genetic pathway: its role in eye formation and functional conservation during chordate evolution (Locascio)

We want to study the genetic pathway of the Onecut genes during eye formation and their convergent-divergent evolution in the chordate phylum. A specific interference of Onecut transcription factor activity in the photoreceptor cells lineage has been obtained in *Ciona* transgenic embryos. By using a differential transcriptomic approach, we identified novel genes involved in its genetic pathway and potentially controlling ocellus and photoreceptors formation. We want to validate in *Ciona* embryos the role of these newly identified genes and, finally, study the conservation or divergence of the Onecut genetic pathway in chordate evolution. With this aim, we are characterizing the phenotypes obtained from the downregulation of three Onecut genes in zebrafish embryos, Onecut1, Onecut2 and Onecut-like.

The comparison of the *Ciona* transgenic and zebrafish morphant embryos evidenced a similar function for Onecut, in photoreceptors formation and in the development of specific neural structures. On the light of these results, we are studying the genes expressed not only in photoreceptor cells but also in the visceral ganglion of *Ciona* larvae or in the cerebellum and mid-hind brain boundary of zebrafish embryos, in order to define both the conserved lines and the

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[10] Squarzoni *et al.* FGF/MAPK/Ets signaling renders pigment cell precursors competent to respond to Wnt signal by directly controlling Ci-Tcf transcription. ***Development*** (2011) 138: 1421-32.

evolutionary novelties of this genetic pathway that specifically appeared in ascidians or vertebrates.

g. Light perception in the oceans and photoreceptor cell (PRC) evolution (Arnone)

The evolution of eye-organs including the human eye with its outstanding optical properties has since long been heating scientific debates. To understand the mechanisms and prerequisites that allowed such a sophisticated human sensory system to evolve, we are investigating eye organs featuring a similar origin to those in humans but ideally within a much less complex organization. Sea urchins feature an unusual visual system, which we proposed to work as a giant compound eye with more than 200,000 PRCs distributed over the entire body of the animal[11].
- We will study the molecular, physiological and behavioral properties of 5 different types of PRCs in the sea urchin larvae and adults. This research will be in part performed within a novel MC ITN project, evoCELL, which uses single cell transcriptomics to study evolution of cell types.
- Within evoCELL, we will also study the role of SpTRH and other neuropeptides in the sea urchin larva. Combining in situ hybridisation, whole-mouth immunostaining and knock-down experiments we are currently investigating the role of the SpTRH precursor producing a QYPGamide neuropeptide and its putative receptor (SpTRHR). Preliminary results in collaboration with Paola Oliveri's lab shows that the QYPGamide production in sea urchin larvae is regulated by light/dark cycle and feeding/starving conditions. Furthermore, knock-down experiments of the SpTRH and SpTRHR inhibit the post embryonic arm growth. These results describe for the first time the role of TRH in a non-chordate deuterostome and suggest that the ancestral role of this neuropeptide, which in mammals has a central role on regulation of metabolism and growth by stimulating the secretion of the TSH from the pituitary gland, is indeed on the control of postembryonic growth and reproduction.

h. Manganese role in dopaminergic neuron formation (Sordino)

Alteration of brain manganese (Mn) homeostasis has been linked to neurodegenerative diseases, such as Alzheimer and Parkinson’s disease, raising concerns over long-term exposure to Methylcyclopentadienyl Manganese Trycarbonil (MMT), an organic Mn-containing gasoline additive. Zebrafish is a useful animal model for studying effects of neurotoxicants on the nervous system, by virtue of teleost brain homologies with the mammalian one. Despite evidence of structural and functional damage induced by Mn-based chemicals on dopaminergic (DA) neurons, no *in vivo* studies on the effects on neuronal differentiation following chronic exposure to sub-lethal MMT have been performed. The main objective of this study is to investigate transcriptional, morphological and behavioral alterations caused by sub-lethal MMT exposure during neuronal differentiation.

5. Publications (2012-2017; *corresponding author)

2017

Anishchenko E, Arnone MI* and D’Aniello S*. *SoxB2* in sea urchin development: implications in neurogenesis, ciliogenesis and skeletal patterning. ***EvoDevo*** (submitted the 26/10/2017).

Annona G, Caccavale F, Pascual-Anaya J*, Kuratani S*, De Luca P, Palumbo A, D’Aniello S*. Nitric Oxide regulates mouth development in amphioxus. ***Sci. Reports*** (2017), 7: 8432.

D’Agostino Y and D’Aniello S*. Molecular basis, applications and challenges of CRISPR/Cas9: a continuously evolving tool for genome editing. ***Brief. Funct. Genomics*** (2017), 1-6.

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[11] Ullrich-Lüter E, Dupont S, Arboleda E, Hausen H and Arnone MI. A Unique System of Photoreceptors in Sea Urchin Tube Feet. ***Proc Natl Acad Sci USA.*** (2011) 108: 8367-72.

Esposito R, Yasuo H, Sirour C, Palladino A, Spagnuolo A*, Hudson C*. Patterning of brain precursors in ascidian embryos. ***Development*** (2017), 144: 258-264.

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Pezzotti MR, Locascio A, Racioppi C, Fucci L, Branno M*. Auto and cross regulatory elements control Onecut expression in the ascidian nervous system. ***Dev. Biol.*** (2014), 390: 273-287.

2013

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6. List of grants

- **EU ASSEMBLE Plus "**Association of European Marine Biological Laboratories Expanded" (2018 - 2021). PI: Koistra, **WP JRA3** "Functional Genomics". PI: Ristoratore.
- **EU Marie Curie CIG** "AmphioxusNOS" (2011 - 2015). PI: S. D'Aniello.
- **EU Marie Curie ITN EvoCell** "Animal evolution from a cell type perspective: multidisciplinary training in single-cell genomics, evo-devo and in science outreach" (2018 - 2021). PI: Arnone.
- **EU Marie Curie ITN Neptune** "Multidisciplinarity training in evo-devo and neurobiology of marine animal models" (2013 - 2017). PI: Arnone.
- **Infradev Corbel** " Coordinated Research Infrastructures Building Enduring Life-science Services" (2015-2019). PI: Arnone.
- **MIUR FIRB** "Non-Coding RNA Explosion: Novel Implications in Neurotrophin Biology" (2013 - 2019). PI: S. D'Aniello.
- **MIUR-PONa3_00239 "**Empowering an integrated platform for the study of human diseases with great impact by means of system phenotyping of model animals: mouse and zebrafish clinic (MouZeCLINIC)" (2012-2015). PI: Sordino.

7. List of external collaborators

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2. Gene regulatory networks for development and evolution

1. People involved

Name	*Position*	*Funding*	*Period*
Maria I. Arnone	Staff scientist	SZN	since 1998
Giovanna Benvenuto	Staff scientist	SZN	since 2001
Elio Biffali	Staff scientist	SZN	since1994
Marco Borra	Staff scientist	SZN	since 2012
Salvatore D’Aniello	Staff scientist	SZN	since 2010
Pasquale De Luca	Staff scientist	SZN	since 2009
Filomena Ristoratore	Staff scientist	SZN	since 2000
Elijah K. Lowe	Postdoctoral fellow	Pantrac/Corbel	2015-2017
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Ugo Coppola	PhD student	SZN	2013-2017
Claudia Cuomo	PhD student	SZN	2013-2017
A. K. Kamal	PhD student	SZN	2012-2015
Margherita Perillo	PhD/Postdoc	SZN/MO.DO	2010-2015
Claudia Racioppi	PhD student	SZN	2010-2014
Jovana Randelović	PhD student	SZN	since 2016
Danila Voronov	PhD student	SZN	since 2016

2. Background, aims and objectives

One of the main challenges in Evolutionary Developmental Biology is to understand to which extent developmental changes are driven by regulatory alterations in the genomic sequence. In the recent years, the focus of comparative developmental studies has moved towards a systems biology approach providing a better understanding of the evolution of gene interactions that form the so- called Gene Regulatory Networks (GRN). Marine embryos provide a powerful system to reveal regulatory mechanisms and within the past decade, due to the latest technological innovations, a great number of studies have provided valuable information for comparative GRN analyses. The general aim of this research is to contribute to the understanding of the molecular and morphogenetic processes involved in the development and differentiation of whole embryo, organs and body parts. Two main features of this research topic place it in a unique position within the Italian scientific community in the field of development and evolution (Evo-Devo). First, the advanced technologic "omics" approach, known as *systems biology*, which can be applied to date only to complex but relatively simple systems, as for example the marine organisms considered in this project[12]. Second, the availability, offered by the facilities at Stazione Zoologica Anton Dohrn and by the expertise (*know-how*) of the scientists involved in this project, of several experimentally accessible model organisms including species at different evolutionary positions within deuterostomes, that is the same phylogenetic group to which *Homo sapiens* belongs. These species include: the echinoderms sea urchin (*Strongylocentrotus purpuratus*[13] and *Paracentrotus*

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[12] Davidson EH, Rast JP, Oliveri P, Ransick A, Arnone MI, et al. A genomic regulatory network for development. ***Science*** (2002) 295: 1669 1678.

[13] Sodergren E, Weinstock GM, Davidson EH, Cameron RA, Gibbs RA, Angerer RC, Angerer LM, Arnone MI, et al. The genome of the sea urchin *Strongylocentrotus purpuratus* . ***Science*** (2006) 314: 941 952.

lividus[14]) and sea star (*Patiria miniata*), the hemichordate *Ptychodera flava*[15], the amphioxus (*Branchiostoma lanceolatum*[16]), the protochordate *Ciona robusta*[17] and, for the very important comparison with vertebrates, the zebrafish (*Danio rerio*). We are using these approaches and these organisms to clarify the molecular mechanisms and gene regulatory networks (GRN) that are the basis for the development and function of the structures of the nervous system, digestive tract, pancreas and muscles. The experimental access to these model organisms represents one of the strengths of the project, in particular for Evo-Devo studies related to vertebrates and hence to mankind.

The results obtained during the period of the 2012/2017 have been subdivided in two main research lines:

a. Molecular mechanisms controlling pigmented cell specification (Ristoratore)
b. Structure and evolution of the gene regulatory networks that control organogenesis (Arnone).

3. Results

a. Molecular mechanisms controlling pigmented cell specification (Ristoratore)

Distinct combinations of transcription factors (TFs) and signaling molecules define the complex gene regulatory networks that control patterning and commitment of different cell types during the development of the central nervous system (CNS). Ristoratore's group is interested in characterizing the GNR governing pigment cell formation in the ascidians *C. robusta* with an evolutionary perspectives. Ascidians, belonging to the chordate group, represent an attractive model system to investigate the generation of cell diversity in the central nervous system (CNS) as well as the dichotomy between conserved phenotypes and divergent genomes. The reduced genetic redundancy found in the compact tunicate genome facilitates functional analyses of homologous TFs and signaling molecules in the context of the typical chordate body plan of embryos and larvae. Moreover, the presence of complete information system, ANISEED**,** that includes genomic and anatomical information on ascidian developmental, greatly helps the study on this organism (**Brozovic M *et al*. 2015**).

We are interested in characterizing the GNR governing pigment cell formation in the ascidian *Ciona robusta*. It has been demonstrated that FGF play a key role in this process. To gain insight into the FGF signaling regulatory network, we examined the transcription profiles of pigment cell precursors using a combination of cell sorting and transcriptome analyses. We have shown that FGF signaling functions as successive inputs during induction of PCPs (Fig. 1) controlling early fate specification, the selection of definitive pigment cells within the PCP lineage and the processes that underlie pigment cell differentiation at later stages (Figure 1, **Racioppi *et al*. 2014**).

To identify key effector genes necessary for Ciona PCPs formation and controlled by FGF signaling, we used a 'reverse engineering' approach. This method identifies co-regulated genes, that is, genes that are co-expressed across a set of experimental conditions. We modeled a pigment cell lineage-specific transcriptional network to identify key effector genes involved in the early PCP induction and identified PCP-specific *Rab32/38* as a hub gene (Figure 1). Starting at the early neurula stage, *Rab32/38* results specifically expressed in two cells that correspond to pigment cell precursors as confirmed by the co-localization with *tyrp1/2a*. The specific and early expression of *Ci-Rab32/38* in the pigment cell lineage suggested a role in *Ciona* melanization by regulating vesicle dynamics during pigment cell formation. Specific interference with *Rab32/38* in PCPs altered both otolith and ocellus pigmentation suggesting a specific role for this gene in

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[14] The *P. lividus* genome has been sequenced by Genoscope and is being annotated within the EU Infradev project Corbel with SZN participation.

[15] Hemichordate embryos are obtained through the collaboration with the Academia Sinica of Taiwan, Yi-Hsien Su and Arnone 2012-2014 grant within the NCS-SZN bilateral agreement.

[16] Garcia-Fernàndez J, Jiménez-Delgado S, Pascual-Anaya J, Maeso I, D'Aniello S, et al. From the American to the European amphioxus: towards experimental Evo-Devo at the origin of chordates. ***Int J Dev Biol***. (2009) 53: 1359-1366.

[17] Dehal P, Branno M, Spagnuolo A, et al. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. ***Science*** (2002) 298: 2157-2167.

melanosome logistics (Racioppi *et al.* 2014). To gain insight into deuterostome evolution and the possible function of the Rab32 and Rab38 genes, we investigated their molecular evolution and spatio-temporal localization, during development, of two key animal models: the cephalochordate *B. lanceolatum* and the teleost *D. rerio* (**Coppola *et al.* 2016**). This research was supported by SZN FOE.

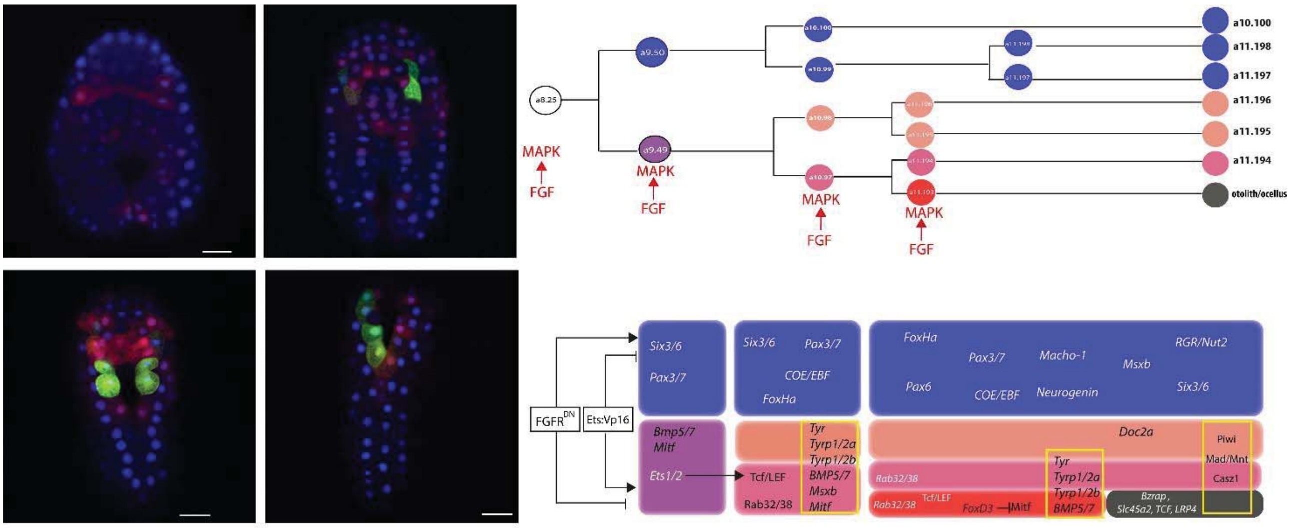

Figure 1. Activation of ERK1/2 (FGF effector) visualized by dpERK1/2 antibody staining (red) at different developmental stage (left side of the figure). Ciona embryos were stained using anti-GFP antibody (green) to label the PCPs from early neurula (a10.97s and a10.98s), late neurula (a10.97s and a10.98s in division) to early tailbud stage (a11.194s, 11.195s and a11.196s). On the right upper side, the bilateral a9.50s, and their descendants, are marked in blue; a9.49 cell pair, and their descendants, in pink. Red arrows indicate FGF/MAPK signaling mediated inductions. Lower part of the figure, a9.49 and a9.50-derived cells with specific gene expression patterns: FGFRDN: dominant-negative form of FGF receptor; Ets:Vp16: constitutive active form of Ets1/2.

b. Structure and evolution of the gene regulatory networks that control organogenesis (Arnone)

The Arnone group aims at studying evolution of organs and body parts by comparison of the Gene Regulatory Networks (GRN) that control the formation and development of such parts in different animals using the sea urchin *S. purpuratus* (Echinodermata) as main model system. Echinoderms, as non-chordate deuterostomes animals, offer an ideal system to study the different factors involved in the morphogenesis of the gut and evolution of organogenesis. After the sequencing of the *S. purpuratus* genome, the process of specification of the endomesodermal territories has been extensively studied in various sea urchin species and has led to the most exhaustive characterization of a GRN for any developmental system (reviewed in Arnone *et al*., 2015[18]). These systems level studies demonstrate the power of developmental GRNs in describing the causal progression of regulatory states (generated by a unique combination of transcription factors, TF) in embryonic space and time, necessary to specify different cell types and ultimately body plans, by providing testable predictions that are not resolvable with simplistic views of gene regulation. They also highlight the potential of comparative GRN approaches to study evolution of specification processes and body plans, providing an explanation of how changes in genome sequence can cause changes in development[19].

In the recent years, sea urchin GRNs have been studied in great detail in the Arnone lab giving insight in gut (**Annunziata *et al*., 2013; Annunziata *et al*., 2013b; Ikuta *et al*., 2013; Arnone and Annunziata, 2014**), muscle (**Andrikou *et al*., 2013; Andrikou *et al*., 2015; Andrikou and**

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[18] Arnone MI, Byrne M, Martinez P Echinodermata. In ***Evolutionary Developmental Biology of Invertebrates*** A. Wanninger ed, Springer 2015 vol. 6: 1-58.

[19] Davidson EH, Erwin DH. Gene regulatory networks and the evolution of animal body plans. ***Science*** (2006) 311:796-800.

Arnone, 2015) and pancreas (**Perillo *et al*., 2014; Perillo *et al*., 2016**) development and evolution, as well as cell survival signaling pathways (**Rizzo *et al*., 2016**). These studies highlighted some important emerging properties of developmental GRNs and their evolution, which have been reviewed in (**Arnone *et al*., 2016**). A striking commonality of the GRNs so far analyzed within deuterostome is that, except for the use of some recurrent sub-circuits (such as the *hnf1, ptx1, notch* sub-circuit controlling pancreatic cell specification and differentiation - shown in Figure 2- which is conserved between sea urchin and mammals) developmental GRNs appear to be subject of considerable rewiring even when they contain the same groups of orthologous genes. Even more prominent, is the fact that these genes often display extreme conservation of topology of expression within similar cell types or body parts. This is well illustrated by the recurrent use of the ParaHox genes *xlox/pdx1* and *cdx* in posterior gut patterning of most bilaterians (see expression in sea urchin and sea star reported in Figure 2). Similarly, a significant number of the same orthologous genes seem to be involved in muscle development within metazoans (*foxc, foxf, myod* among others; see muscle network in Figure 2), but the regulatory connections among them appear poorly conserved. An obvious question that arises is what actually are the constraints that maintain such a strong association between the regulatory genes and the domain of their expression even when the GRNs that connect them get extensively rewired. A broader taxon sampling accompanied by functional systems level comparative approaches are necessary in order to address this problem and further illustrate mechanisms and logics of developmental GRN evolution.

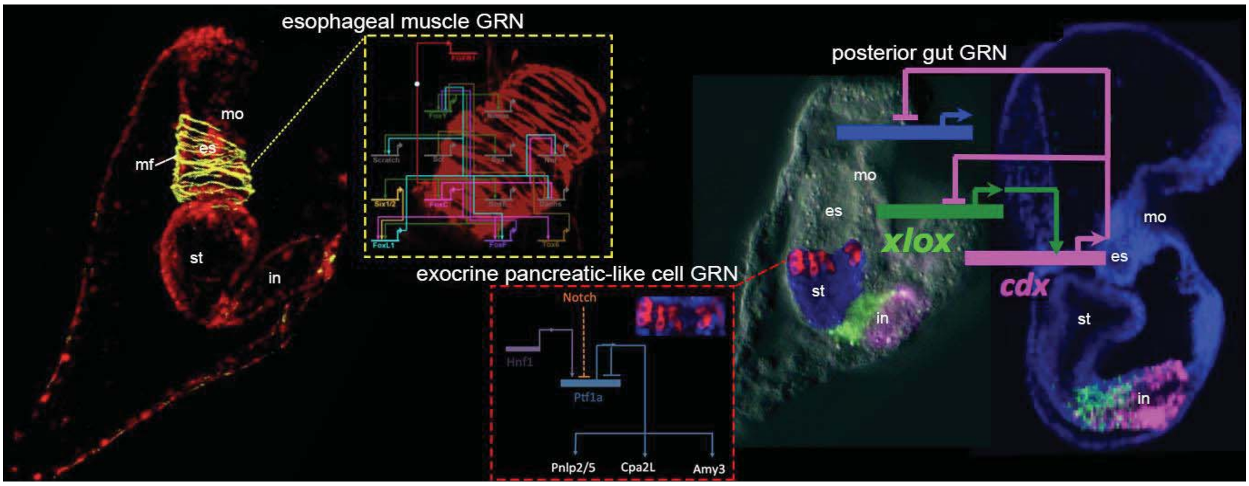

Figure 2. Schematics of regulatory interactions (Gene Regulatory Networks, GRN) controlling the formation of the sea urchin esophageal muscles (stained with phalloidin and depicted in yellow in the sea urchin *S. purpuratus* pluteus larva shown on the left side of the figure), exocrine pancreatic cells (stained by whole mount in situ hybridization using a carboxypeptidase, Cpa2, specific probe and depicted in red in the *S. purpuratus* larva at the center) and posterior gut. The latter is patterned by two ParaHox genes, *xlox* and *cdx*, which in sea urchins are linked by a positive-negative feedback loop, depicted as schematic GRN on the right side of the figure, which does not appear to be conserved in sea star, despite the fact that the topology of expression of these two genes is perfectly conserved between sea urchin and sea star (compare domain of expression of *xlox,* in green, and *cdx*, in magenta, as determined by whole mount in situ hybridization in the *S. purpuratus* pluteus on the center, and the sea star *P. miniata* bipinnaria larva on the right). Abbreviations: es, esophagus; in, intestine; mf, muscle fiber; mo, mouth; st, stomach.

Recently, we established and proposed an integration of multiple NGS technologies for the prediction and validation of gene interactions within sea urchin GRNs (**Lowe *et al*., 2017**). In particular, we used a differential transcriptomic approach to compare target genes of gut specific homologous transcription factors in the sea urchin *S. purpuratus* and the sea star *P. miniata*[20].

Differential transcriptomics coupled with morpholino antisense oligonucleotide mediated loss of gene function has being also used to study, in collaboration with Manuel Irimia lab, the evolution of splicing. Using this approach, we were able to demonstrate evolutionary recruitment of flexible

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[20] Lowe EK, C Cuomo C, Arnone MI. A Differential Transcriptomic Approach to Compare Target Genes of Homologous Transcription Factors in Echinoderm Species. In ***Dynamics of Mathematical Models in Biology*** A. Rogato et al. (eds.) Springer 2016: 55-6.

Esrp-dependent splicing programs into diverse embryonic morphogenetic processes (**Burguera *et al*., in press**). *Esrp* (Epithelial Splicing Regulatory Protein) genes regulate extensive alternative splicing programs associated with cell adhesion and motility in human cells and are essential during mouse organogenesis. Taking advantage of the genetic tools available in different systems, we performed loss-of-function or gain-of-function experiments to shed light into the evolution of Esrp's ontogenetic roles. We found that *Esrp* genes are involved in the development of many structures in deuterostome organisms (we analyzed the sea urchin *S. purpuratus*, the amphioxus *B. lanceolatum,* the protochordate *C. robusta*, and the vertebrate fish *D. rerio*; see expression patterns in Figure 3), often by conferring epithelial-associated cellular properties. However, this common employment of Esrp in morphogenetic functions was not mirrored at the exon level at the largest phylogenetic distances, as no Esrp-dependent exons appeared conserved between phyla.
The work within this research line was supported by SZN FOE, MO.Do, MIUR Premiale Pantrac and the EU Evonet grant.

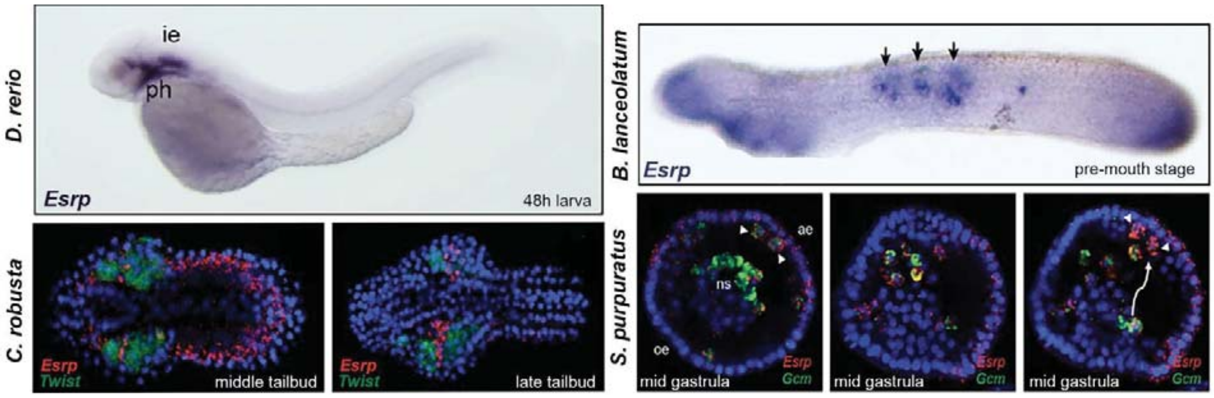

Figure 3. Comparison of *Esrp* (Epithelial Splicing Regulatory Protein) gene expression in fish, ascidian, amphioxus and sea urchin embryos. Both colorimetric (top panels) and fluorescent two color (bottom panels) in situ hybridization were used to assess *ESRP* gene expression (in purple, top panels or red, bottom panels) and other mesodermal markers (in green, bottom panels). Black arrows in the top right panel indicate a few cells that likely corresponding to migrating sensory cells during epidermal incorporation or already integrated into the epithelium. White arrowheads in the sea urchin panel indicate migrating pigment cell precursors that are already in contact with the ectodermal epithelium. The white arrow in the bottom right panel indicates a representative migratory path of pigment cells from mesoderm to ectoderm. Abbreviations are: ae, aboral ectoderm; ie, inner ear epithelium; ns, non-skeletogenic mesoderm; oe, oral ectoderm; ph, pharynx.

4. Perspectives

GRNs describe the interactions for a developmental process at a given time and space. Historically, perturbation experiments represent one of the key methods for analyzing and reconstructing a GRN. Next generation sequencing (NGS) has become a standard experimental technique for genome and transcriptome sequencing, as well as studies of protein-DNA interactions by Chromatin Immunoprecipitation (ChIPSeq) and DNA accessibility by Assay for Transposase-Accessible Chromatin using sequencing[21] (ATACSeq). Using the integrated 'omics' approaches set up in the Arnone lab in the recent years to study gene regulatory networks for development in echinoderms (Lowe et al, 2016 and 2017; see flowchart in Figure 4), we propose for the next few years to pursue on the study of the digestive tract to gain a whole systems biology interpretation of the sea urchin *S. purpuratus* gut GRN, for evolutionary comparison with the sea star *P. miniata* and other deuterostomes (including the cephalochordate *B. lanceolatum*). Using this approach, we are also studying the evolution of the transcription factor Brachyury and its target genes in the gut of two sea urchin species, the Pacific *S. purpuratus* and the Mediterranean *P. lividus*. For the genome of the latter sea urchin species, we are participating within the EU Infradev project Corbel to its annotation and creation of a database expected to be complete by the end of

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[21] Buenrostro JD, Giresi PG, Zaba LC, *et al.* Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. ***Nat Methods*** (2013) 10: 1213–18.

2018. These GRN projects are being performed by the PhD Students Danila Voronov (Project title: Evolution of a Gene Regulatory Network Controlling Gut Patterning: Comparing Upstream Control and Chromatin 3-D Organization Around *xlox/pdx1* and cdx genes in Sea Urchin, Sea Star and Amphioxus) and Jovana Randelović (Project title: Studying Evolution at the Protein Level: Combining Biochemistry and Systems Biology Approaches to Unravel the Mechanisms Underlying the Evolution of the Transcription Factor Brachyury) and will be in part supported by a novel MC ITN project, EvoCELL, which uses single cell transcriptomics to study evolution of cell types.

Ristoratore's group propose, for the next few years, to improve the study of the GNR involved in the pigment cell specification to gain insight on the whole systems underlying cell diversity during Ciona development. Some other genes, specifically expressed in otolith precursors have been already identified (Coppola PhD thesis) and their involvement in pigment cell differentiation will be studied by a combination of regulatory and functional studies (by *CRISP/cas9*).

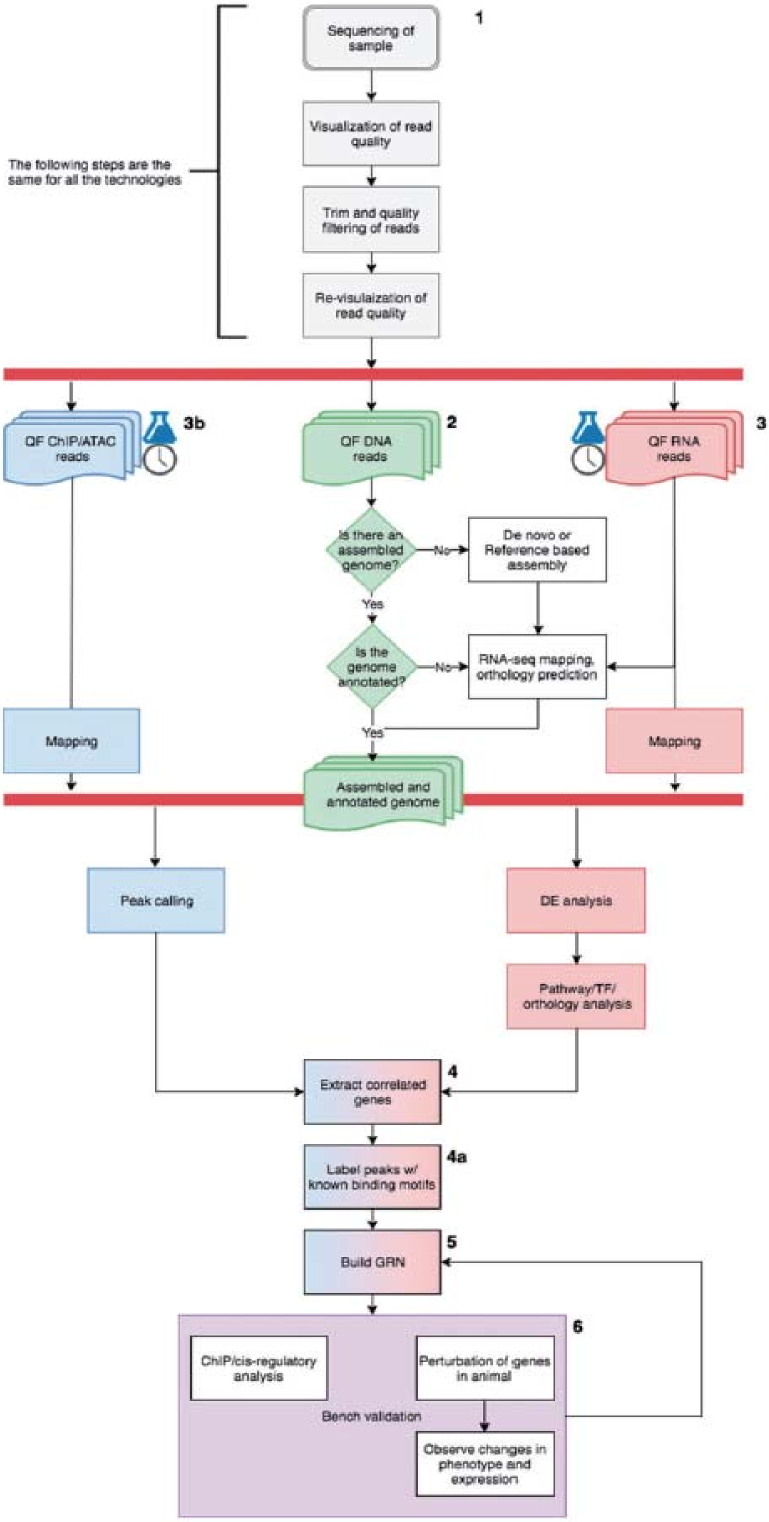

Figure 4. Flow chart summarizing a general scheme for constructing a GRN from omics data. Adapted from Lowe et al, 2017.
1. The first step is always to assess the quality of the data (grey boxes), which should be checked before and after each step.
2. Identify available omics data, and what else is needed for a comprehensive analysis. In cases such as human, mouse, chick, sea urchin, just to name a few, there are available genomes, which are major components of GRN analysis. For many 'non-model' organisms, there may not be a genome available, so the sequencing and assembly will have to be done before a GRN can be reconstructed.
3. Determine the time point (clock symbol) and method of comparison, whether it is perturbation, known-down or knockout (flask symbol). RNA-seq is also used to annotate the genome.
3b. ChIP-seq/ATAC-seq (peak data) read mapping and peak calling are also done in a time-specific manner and can also be tissue specific.
4. Identify the overlapping expression data and peak data for integration. It is best to focus on TF and specific tissues to reduce the information for better interpretation.
4a. After determining the overlapping data, peaks should then be label with binding motifs. These motifs are often called by their human or mouse ortholog, so this must be accounted for.
5. The network can be reconstructed using programs such as BioTapestry.
6. Confirmation of findings by bench validations.

Functional analyses will be improved by the application of the recently developed technique of the CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated proteins) system for targeted genome editing. This prokaryotic immune system functions through short RNAs that guide the nuclease Cas9 to foreign DNA. Modified variants of this system have been used for genome editing applications in various organisms including ascidians[22,23] and sea urchin[24,25]. These studies will be in part supported by the Assemble+ project, recently financed by the EU, especially the WP JRA3 "Functional Genomics" aimed to establish links between genomic information and phenotypes of marine model species.

5. Publications (2012-2017; *corresponding author)

2017

Burguera D, Marquez Y, Racioppi C, Permanyer J, Torres A, Esposito R, Albuixech B, Fanlo L, D'Agostino Y, Navas-Perez E, Riesgo A, Cuomo C, Benvenuto G, Christiaen LA, Martí E, D'Aniello S, Spagnuolo A, Ristoratore F, Arnone MI*, Garcia-Fernàndez J*, Irimia M*. Evolutionary recruitment of flexible Esrp-dependent splicing programs into diverse embryonic morphogenetic processes. ***Nat. Comm.*** (accepted October 2017).

Lowe EK, Cuomo C, Arnone MI*. Omics approaches to study gene regulatory networks for development in echinoderms. ***Brief. Funct. Genomics*** (2017) 16: 299-308.

2016

Arnone MI*, Andrikou C, Annunziata R. Echinoderm systems for gene regulatory studies in evolution and development. ***Curr. Opin. Genet. Dev.*** (2016) 39: 129-137.

Coppola U, Annona G, D'Aniello S*, Ristoratore F*. Rab32 and Rab38 genes in chordate pigmentation: an evolutionary perspective. ***BMC Evol. Biol.*** (2016) 16: 1.

Perillo M, Wang YJ, Leach SD & Arnone MI*. A pancreatic exocrine-like cell regulatory circuit operating in the upper stomach of the sea urchin *Strongylocentrotus purpuratus* larva. ***BMC Evol. Biol.*** (2016) 16: 117.

Rizzo F, Coffman JA, Arnone MI*. An Elk transcription factor is required for Runx-dependent survival signaling in the sea urchin embryo. ***Dev. Biol.*** (2016) 416: 173-86.

2015

Andrikou C and Arnone MI*. Too many ways to make a muscle: evolution of GRNs governing myogenesis. ***Zool. Anz.*** (2015) 256: 2-13.

Andrikou C, Pai CY, Su YH, Arnone MI*. Logics and properties of a genetic regulatory program that drives embryonic muscle development in an echinoderm. ***Elife*** (2015) 4, e07343.

Arnone MI, Hejnol A. Genomics going wild: Marine sampling for studies of evolution and development. ***Mar. Genomics*** (2015) 24: 119-20.

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[22] Stolfi A, Gandhi S, Salek F and Christiaen L. Tissue-specific genome editing in Ciona embryos by CRISPR/Cas9. ***Development*** (2014) 141: 4115-4120.

[23] Gandhi S, Haeussler M, Razy-Krajka F, Christiaen L, Stolfi A. Evaluation and rational design of guide RNAs for efficient CRISPR/Cas9-mediated mutagenesis in Ciona. ***Dev Biol***. (2017) 425: 8-20.

[24] Lin CY, Su YH. Genome editing in sea urchin embryos by using a CRISPR/Cas9 system. ***Dev. Biol.*** (2016) 409: 420-428.

[25] Oulhen, N and Wessel GM. Albinism as a visual, in vivo guide for CRISPR/Cas9 functionality in the sea urchin embryo. ***Mol. Reprod. Dev.*** (2016) 83: 1046-1047.

Brozovic M, Martin C, Dantec C, Dauga D, Mendez M, Simion P, Percher M, Laporte B, Scornavacca C, Di Gregorio A, Fujiwara S, Gineste M, Lowe EK, Piette J, Racioppi C, Ristoratore F, Sasakura Y, Takatori N, Brown TC, Delsuc F, Douzery E, Gissi C, McDougall A, Nishida H, Sawada H, Swalla BJ, Yasuo H, Lemaire P. ANISEED 2015: a digital framework for the comparative developmental biology of ascidians. ***Nucleic Acids Res***. (2015) pii: gkv966.

2014

Annunziata R and Arnone MI*. A dynamic network of regulatory interactions explains ParaHox gene control of gut patterning in the sea urchin embryo. ***Development*** (2014) 141: 2462-72.

Perillo M and Arnone MI*. Characterization of insulin-like peptides (ILPs) in the sea urchin *Strongylocentrotus purpuratus*: insights on the evolution of the insulin family. ***Gen Comp Endocrinol*** (2014) 205: 68-79.

Racioppi C, Kamal AK, Razy-Krajka F, Gambardella G, Zanetti L, di Bernardo D, Sanges R, Christiaen AL*, Ristoratore F*. Fibroblast growth factor signaling controls nervous system patterning and pigment cell formation in *Ciona intestinalis*. **Nat. Comm.** (2014) 5: 4830.

Russo MT, Racioppi C, Zanetti L, Ristoratore F. Expression of a single prominin homolog in the embryo of the model chordate Ciona intestinalis. ***Gene Expr Patterns***. (2014) 15: 38-45.

Stolfi A, Lowe K E, Racioppi C, Ristoratore F, Brown C T, Swalla J B, Christiaen L (2014) Divergent mechanisms regulate conserved cardiopharyngeal development and gene expression in distantly related ascidians. ***eLife*** (2014);3: e03728.

2013

Andrikou C, Iovene E, Rizzo F, Oliveri P and Arnone MI*. Myogenesis in the sea urchin embryo: the molecular fingerprint of the myoblast precursors. ***EvoDevo*** (2013) 4:33.

Annunziata R, Perillo M, Andrikou C, Cole A, Martinez P & Arnone MI*. Pattern and Process During Sea Urchin Gut Morphogenesis: the Regulatory Landscape. ***Genesis*** (2013) 52: 251-68.

Annunziata R, Martinez P and Arnone MI*. Intact cluster and chordate-like expression of ParaHox genes in a sea star. ***BMC Biology*** (2013b) 11: 68.

Ikuta T, Chen Y-C, Annunziata R, Ting H-C, Tung C-H, Koyanagi R, Tagawa Humphreys T, Fujiyama A, Saiga H, Satoh N, Yu Jr-K, Arnone MI and Su Y-H. Identification of an intact ParaHox cluster with temporal colinearity but residual spatial colinearity in the hemichordate *Ptychodera flava*. ***BMC Evol. Biol.*** (2013) 13: 129.

Peterson KJ, Su Y-H, Arnone MI, Swalla B, and King B. MicroRNAs Support the Monophyly of Enteropneust Hemichordates. ***J. Exp. Zool. B Mol. Dev. Evol.*** (2013) 320: 368-74.

6. List of grants

- **EU Infradev Corbel** "Coordinated Research Infrastructures Building Enduring Life-science Services " (2015 - 2019). PI: Arnone.
- **EU ASSEMBLE Plus, "**Association of European Marine Biological Laboratories Expanded" (2018 - 2021). PI: Koistra, **WP JRA3** "Functional Genomics". PI: Ristoratore.
- **EU Marie Curie ITN EvoCell** "Animal evolution from a cell type perspective: multidisciplinary training in single-cell genomics, evo-devo and in science outreach" (2018 - 2021). PI: Arnone.
- **EU Marie Curie ITN EvoNet** "Evolution of Gene Regulatory Networks in Development" (2008 - 2012). PI: Arnone.

- **MIUR Premiale Pantrac** "Gut patterning and PANcreas development in evolution and disease: a TRAnsCriptomic approach" (2014 - 2015). PI: Arnone.

7. List of external collaborators

-Lionel Christiaen, New York University, New York, NY, USA

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-José Luis Gomez-Skarmeta, CSIC/ Universidad Pablo de Olavide, Seville, Spain

-Peter Holland, Department of Zoology, Oxford University, Oxford, UK

-Manuel Irimia, Centre for Genomic Regulation, Barcelona, Spain

-Stephen Leach, Johns Hopkins University, Baltimore, MD, USA

-Pedro Martinez, Universitat de Barcelona, Barcelona, Spain

-Paola Oliveri, University College London, London, UK

-Kevin Peterson, Dartmouth College, Hanover, NH, USA

-Alberto Stolfi, Georgia Institute of Technology, Atlanta, GA, USA

-Yi-Hsien Su, Academia Sinica, Taipei, Taiwan

-Ulrich Technau, University of Vienna, Vienna, Austria

3. Comparative neurophysiology: exploring biological plasticity from cells to behavior

1. People involved

Name	*Position*	*Funding*	*Period*
Graziano Fiorito	Staff scientist	SZN	since 1983
Enrico D’Aniello	Staff scientist–Term contract	SZN	since 2017
Filomena Ristoratore	Staff scientist	SZN	since 2000
Paolo Sordino	Staff scientist	SZN	since 2000
Luigi Caputi	Postdoctoral fellow	SZN	2012-2013
Giulia Fasano	Postdoctoral fellow	University of Sannio	since 2013
Valeria Nittoli	Postdoctoral fellow	SZN	2016-2017
Giovanna Ponte	Postdoctoral fellow	RITMARE	since 2014
Shuichi Shigeno	Postdoctoral fellow	SZN	2016-2017
Ilaria Zarrella	Postdoctoral fellow	MolEcOC	2012-2013
Piero Amodio	PhD student	Cambridge Univ, UK	2016-2020
Elena Baldascino	PhD student	UniCal	2016-2019
Fabio Crocetta	PhD student[26]	SZN	2011-2014
Giulia Di Cristina	PhD student	SZN	2013-2017
Pamela Imperadore	PhD student	UniCal	2013-2017
Paola Manzo	PhD student	UniCal	2016-2019
Francesca Strano	PhD student	SZN/Univ Trieste	2017-2020
Ruth Styfhals	PhD student	SZN/KU Leuven, Belgium	2017-2021
Maria Agnello	Affiliated scientist	University of Palermo	since 2016
Paul L.R. Andrews	Affiliated scientist	St George's Univ, UK	since 2016
Carlo Di Cristo	Affiliated scientist	University of Sannio	since 2016
Alberto Pallavicini	Affiliated scientist	University of Trieste	since 2017
Antonio Terlizzi	Affiliated scientist	University of Trieste	since 2016

2. Background, aims and objectives

Comparative neurophysiology used as the title of this report suggests a comparative approach by utilizing invertebrate and “lower” chordate species to yield new perspectives and insights on the human brain and its functioning. This fits with our *leitmotif*: the understanding of the evolution of physiological and behavioral plasticity.

The growth of a “comparative neurophysiology” approach at the SZN and of its ‘*related research objects*’ stand mostly on the shoulders and the initiative of the British anatomist and zoologist John Zachary Young (JZ) and the contribution that together with his young fellow, B.B. Boycott, they provided starting just after the World War II. Young integrated himself into a SZ-well-established tradition of exploring the physiology of marine organisms.

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[26] currently, Researcher, Department of Integrative Marine Ecology

JZ and an ever-growing plethora of students and co-workers, was hosted by the Stazione Zoologica for decades. They studied the anatomy and physiology of cephalopods' nervous system with the aim to use octopus as a basis to build a model of the brain[27,28].

Together with Dohrn's family, Young promoted a more systematic study of the physiology of marine organisms and facilitated the growth of the field and the recognition of the diversity of neurophysiological phenomena that characterize marine life[29].

This endeavour contributed to the growth of the Physiology Department at the SZ, where people like Francesco Ghiretti (to name some) acted as Head. It induced an incredible boost into different directions, e.g.: the study of the innate-immune system and its plasticity, and of the characterization of respiratory pigments and their physiological properties, the study of neural conduction and synaptic properties, sensorial capabilities, the analysis of neural circuitry and of the physiological and biological machinery involved in the organismal responses to challenges and stimuli.

It is on the shoulders of these giants that we move our steps.

The scientific discoveries of Young and co-workers were - possibly beyond their intention - a key factor in the "social advancement" of cephalopods. JZ findings provided most of the scientific background for the inclusion of the common octopus, *Octopus vulgaris,* into the protected species in the UK, which since 1993 extended its Animals (Scientific Procedures) Act 1986 to include this single species of invertebrate.

Furthermore, following JZ work, the studies carried out at Caen University (cuttlefish)[30] and at the SZN (octopus)[30] since the late '980s are seen at the basis of the inclusion of cephalopods, the sole invertebrate representatives, in the list of species regulated for scientific purposes by the Directive 2010/63/EU (**Smith *et al*., 2013**).

We aim to contribute to the study of biological plasticity by applying an integrative approach[31]. By studying how complex behaviors, large brains designed on the basis of a relatively simple neural organization, the immune system, developed during evolution in animals very distant from chordates, we investigate the molecular, cellular and behavioral basis of phenotypic (and genomic) plasticity.

3. Results

Over the last five years we focused on the development of tools and approaches aimed to contribute to the study of biological plasticity. In addition, we have been intrigued in evaluating the factors that drive the evolution of systems that guide individual adaptability, i.e. the immune and the nervous systems.

Our major achievements have been:

i. Standardized care, acclimatization (**Amodio *et al*., 2014**; reviews in: **Fiorito *et al*., 2014**; **2015**), training and behavioral protocols for the cephalopod mollusc *Octopus vulgaris*, including the development of novel training paradigms[27,32]. In addition, and to extend the possibilities to use *O. vulgaris* (and other cephalopods) in scientific research, we provided standardized approaches for immunohistochemistry (**Ponte *et al*., 2015**), and are delivering digital brain atlases for *Sepia officinalis* and *O. vulgaris* (in collaboration with Leica Microsystems, CephRes, University of Caen, and SZN).

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[27] Marini G, De Sio F, Ponte G, Fiorito G. (2017) Behavioral Analysis of Learning and Memory in Cephalopods. In: Menzel R. (ed.), *Learning Theory and Behavior, Vol. 1 of Learning and Memory: A comprehensive Reference, 2nd edition,* Byrne JH. (ed.). Oxford: Academic Press, pp. 441-462.

[28] Borrelli L & Fiorito G. Behavioral Analysis of Learning and Memory in Cephalopods. In: *Learning and Memory: A comprehensive reference,* Byrne JH (Ed.). Academic Press, Oxford. (2008) 605-627.

[29] Nardello *et al*. EMBRC-ERIC Business plan (http://www.embrc.eu/embrc-eric-business-plan-nardello-et-al-april-2017).

[30] We are referring to independent research groups that re-initiated the use of cephalopods as organism-of-study in France (Caen University) and Italy (GF @ SZN) starting from 1984.

[31] *sensu* Tada T. (1997). The immune system as a supersystem. ***Annual Review of Immunology*** (1997) 15: 1-13.

[32] Borrelli L and Fiorito G. (2008) Behavioral Analysis of Learning and Memory in Cephalopods. In: *Learning Theory and Behavior,* Vol.1 - *Learning and Memory: A comprehensive reference;* Byrne JH (Ed.). Academic Press, Oxford, UK p. 605-627.

ii. An unprecedented level of accuracy, analysis and representation of the behavioral repertoire of the species (by means of behavioral catalogues)[33] in order to score octopus' behavioral performance, appropriately and consistently over a series of behavioral paradigms (e.g., **Josef *et al*., 2012**; **2014**; **2015**; **2016**; **Zarrella *et al*., 2015**). This serves also to recognize deviation in 'normal' behavior (e.g. as implemented in **Fiorito *et al*.**, **2015** for animals' daily monitoring and care).

iii. The study of the immune system and its plasticity:

  a. By the identification and morphological characterization on haemocytes of the salp *Thalia democratica* (**Cima *et al*., 2014**).

  b. Through the analysis (for the first time) of the behavioral and immune responses to challenges in *Octopus vulgaris* (**Locatello *et al*., 2013**; **Petric *et al*., 2015**[34]). This also represents the first case for attempting cell cultures [see also perspectives].

  c. By characterizing the host/parasite relationship between octopus and *Aggregata*, thereby discovering that the genotypic and morphological characters of the parasite species differentiate in a given area (within the Mediterranean Sea; **Tedesco *et al*., 2017**). These findings raise questions on the effects of the immune responses of octopuses to parasites also at the neural level (**Baldascino *et al*., *under review***), that we explored for the first time.

iv. We contributed to the characterization of genetic diversity and the population structure of *O. vulgaris* (**De Luca *et al*., 2014**; **2015**; **2016**), also through an account of historical records and new genetic data for the assessment of global distribution of the species.

v. We investigated neural systems by extending the capability of describing the localization of neuromodulators in the octopus' nervous system (**Ponte and Fiorito, 2015**), and changes in gene expression following learning (Zarrella *et al*., 2015). This has been recently extended by:

  a. a first attempt to evaluate the relationship between behavioral asymmetries (lateralization) and the neural correlate (**Frasnelli *et al*., 2014**)[35], and

  b. mapping putative nociceptors in the octopus arm (**Di Cristina, 2017**)[36].

vi. We contributed to a comparative analysis of the physiological responses of cephalopods suggesting evolutionary implications by:

  a. Overviewing salivary glands in molluscs (**Ponte & Modica, 2017**);

  b. Proposing *in vivo* non-invasive monitoring of the digestive tract and its physiology (**Ponte *et al*., 2017**; **Sykes *et al*., 2017**)

  c. Evaluating the effect of substances commonly utilized as anaesthetic agents on the cardiac performance of octopus (**Pugliese *et al*., 2016**).

vii. We contributed to the analysis of the impact of Directive 2010/63/EU and European policies on scientific research on cephalopods and invertebrates under regulated contexts (**Ponte et al., 2013**; Fiorito *et al.,* 2014, 2015; **Di Cristina *et al*., 2015**; **Lopes *et al*., 2017**).

viii. We developed the first guidelines for the care of cephalopods in research settings (endorsed by FELASA and AAALAC - Association for Assessment and Accreditation of Laboratory Animal Care; Fiorito et al., 2015).

ix. We extended our contribution to the study of behavioral plasticity in cephalopods, by:

  a. Analysing the camouflage dynamics in cuttlefish (Josef *et al*., 2012; 2014; 2015; 2016);

  b. Studying gene expression changes following learning and behavioral plasticity (Zarrella et al., 2015);

  c. Describing the learning capabilities in octopus (social *vs* individual learning) a species classically considered a solitary living species[37,38];

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[33] Borrelli *et al.* (2006) *A Catalogue of Body Patterning in Cephalopoda*. Stazione Zoologica A. Dohrn & Firenze University Press Italy, 637 pp.

[34] Petríc M, Baldascino E, Lauritano C, Zarella I, Ponte G, Fiorito G. (2015) *Octopus vulgaris* immune response at gene expression level by in vitro immunostimulation of hemocytes. Poster session presented at the 17th EAFP international Conference on Diseases of Fish and Shellfish.

[35] Frasnelli E, Ponte G, Fiorito G, Vallortigara G. (2014) Investigating lateralization in octopuses: first evidence of asymmetry in the optic lobes. Fourth workshop on cognition and evolution, Rovereto, Italy.

[36] Di Cristina G. (2017) Nociception in the cephalopod mollusc *Octopus vulgaris*: a contribution to mapping putative nociceptors in the octopus arm. (Doctoral dissertation, Università degli studi di Napoli Federico II).

   d. Overviewing the behavioral flexibilities of cephalopods as predators (**Villanueva *et al.*, 2017**);
   e. Contributing to an in-depth analysis of the research effort on the study of learning and memory recall in cephalopods[27].

x. We initiated a systematic analysis of the regeneration phenomena in the octopus, by studying wound-healing after amputation of the arm (**Shaw *et al.*, 2016**) and nerve degeneration and regeneration (**Imperadore *et al.*, 2017**).

G. Fiorito studies have been supported by: SZN-FOE, RITMARE Flaship Project, Premiale MolEcOC, Project, MODO – FESR POR Campania.
P. Sordino research has been supported by SZN-FOE, PONa3_00239.

In the following lines the most significant results gained during the last five years are also overviewed as contributions to the analysis of the immune response, the octopus' genome and its plasticity and the octopus neural plasticity. We are not including herein any summary of our recent efforts to better contribute to the understanding of behavioral plasticity; this will be overviewed during the presentation.

3.1. Immune response

The immune system is a multi-cellular network that selectively react to extra-organismal threats. A key feature of the immune response is the fluidity and ability to adapt to challenges. The dynamics of the immune system involve cellular expansion, 'altered' transcription and balancing differentiation with repairing process. The challenge is to unveil how the cascade of immune reactions are orchestrated to contend with the extrinsic information and metabolic demands of cellular differentiation, function and homeostasis in tunicates and cephalopods. Both taxa emerged as compelling 'models' to understand the innate immunity in host-pathogen interactions in the marine environment.

3.1.1 Different types of haemocytes have been identified and characterized in the haemolymph of the oozoids of the salp, *Thalia democratica*. With the exception of lymphocyte-like cells, which are poorly represented in the haemolymph of the adult, we (Sordino and co-workers; supported by SZN-FOE) found distinct categories of circulating haemocyte types: *i)* phagocytic line, contained hyaline amoebocytes and amoebocytes with large vacuoles, suggested to act as sentinel cells, *ii)* mast cell-like line and *iii)* storage cell line (nephrocyte), suggested to be important elements for accumulation and excretion of nitrogenous catabolites. This is the first morphological characterisation and functional classification of the haemocytes of thaliaceans (**Cima *et al.*, 2014**).

3.1.2 Inflammation represents the physiological body defence that attempts to eliminate injurious stimuli and initiates the repair processes. It is part of a complex cascade of events associated with the activation of the immune system. Sordino and coworkers attempted to explore the link between the inflammatory pathways and epilepsy[39], by evaluating the expression of inflammatory molecules and the response of brain immune-resident cells in zebrafish following acute seizure induced by a pro-convulsive agent, the pentylenetetrazole (PTZ). Nittoli and coworkers[40] found that PTZ-induced seizures are associated with an increase of key inflammatory molecules and rapid induction of neuroprotective mechanisms. Moreover, the expression dynamic of inflammatory molecules is accompanied by the activation of microglial cells. These unprecedented finding are corroborated by genetic manipulation and pharmacological treatments that strongly support the hypothesis of a direct role of some inflammatory pathways in seizure activity (Figure 1).

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[37] Amodio P, Fiorito G. Invertebrate Learning and Memory: Observational and Other Types of Learning in Octopus. In: Invertebrate Learning and Memory (Menzel R. & Benjamin P., Eds). Elsevier (2013) Vol 22, Chapter 23.

[38] Tricarico E, Amodio P, Ponte G, Fiorito G. Cognition and recognition in the cephalopod mollusc *Octopus vulgaris*: coordinating interation with environment and conspecifics. In: Biocommunication of Animals. Springer (2014) 337-349.

[39] Vezzani A. Epilepsy and inflammation in the brain: overview and pathophysiology. ***Epilepsy Curr.*** (2014) 14(s2): 3-7.

[40] Nittoli V. (2016) Behavioral, cellular and molecular insights into hyperexcitability using zebrafish model system. (Doctoral dissertation, Università degli studi di Napoli Federico II).

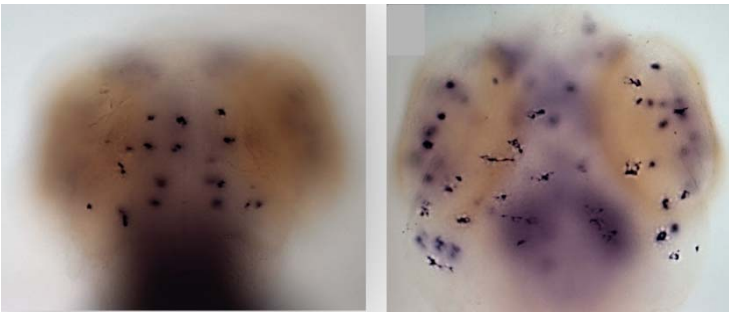

Figure1.Wholemount in situ hybridization of ApoE, a marker of microglia.Increased number of activated microglia in PTZ-treated zebrafish embryos (right) compared to control (left). Dorsal views, anterior to the top.

3.1.3 Studies of the cephalopod immune system have recently become of great importance due to significant changes of policy at EU level and worldwide that increased interest for aquaculture, and to aspects linked to welfare in this taxon. We (G. Fiorito and coworkers; supported by SZN FOE) studied octopus behavioral and immunological (i.e. haemocyte number and serum lysozyme activity) responses to an *in vivo* immune challenge (*Escherichia coli* lipopolysaccharides, LPS). Immune stimulation primarily caused a significant increase in the number of circulating haemocytes after the treatment, thus suggesting an immediate response at the immune level. The simulated infection affected behavioral responses; LPS challenged octopuses were less reactive, and social-avoidance was more marked in LPS challenged octopuses than in the controls (**Locatello *et al.*, 2013**).
We also tested (G. Fiorito and coworkers; supported by RITMARE Flagship Project) immuno-stimulation and the gene expression response of *O. vulgaris* hemocytes[41] in cell culture. Viral, bacterial and fungal imitators challenged octopus hemocytes and activated defense mechanisms including the stress and detoxification related genes (e.g. SOD, GPX and GSH-S; 1-4 hours after stimulation). Responses of immune-related genes followed in time (24-48 hours after; e.g. NFKB, SER and TNF; Petric et al, *in preparation*)[40].

3.2. The genome and its plasticity: a toolkit

The construction of a de-novo *O. vulgaris* transcriptome[42], was a major breakthrough in the field of genomics and molecular biology in cephalopod research. Together with Dr Sanges and his coworkers we greatly extended previously available transcriptomes for the species. Illumina RNA-seq experiments were performed based on a variety of tissues in the adult, but also from different developmental stages of octopus.
The available transcriptome data greatly advanced all research performed in our group and expanded enormously our networking capability. In 2015, the genome of *O. bimaculoides, a* species closely related to *O. vulgaris*, became available and was used as an additional genome resource. A draft genome of *O. vulgaris* has been initiated by the initiative of Dr Fiorito and his coworkers and it is currently on its way, funded by CephRes and BGI[43]. The estimated genome size of *O. vulgaris* is 2.7 Gb, less than the 5.15 Gb previously reported[44].
The transcriptome we developed has been pivotal to a series of studies: mapping of nociceptors, studies of the neural correlate of parasite infection, studies on the immune responses, studies on nerve degeneration and regeneration (to cite some).

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[41] Petríc M, Baldascino E, Lauritano C, Zarrella I, Ponte G, Fiorito G. (2015) *Octopus vulgaris* immune response at gene expression level by *in vitro* immunostimulation of hemocytes. 17th EAFP international Conference on Diseases of Fish and Shellfish.

[42] Petrosino G. (2015) The transcriptional landscape of the nervous system of *Octopus vulgaris*. (Doctoral dissertation, Università degli studi di Napoli Federico II).

[43] Maes G, Simakov O, Fiorito G et al. The genome survey of *O. vulgaris. in embargo and under preparation*; open data

[44] Packard & Albergoni (1970). ***J. Exp. Biol***., 52: 539-552.

Among other experiments we are currently involved we would like to mention the mapping of nociception-related genes/markers identified in the *O. vulgaris* brain and arms for the first time (Di Cristina, 2017). Dr Di Cristina found abundance of genes encoding for proteins involved in sensory perception of pain in the arm (especially of the distal part), while genes related to the consolidation of memory and to the modulation of pain appear to be more expressed in the supraesophageal mass and in the optic lobe in octopus. This study facilitated the collaboration with Dr Rute Fonseca and the merging of a collaborative effort to determine the presence of opiate receptors across phila using transcriptomes. Opiates are some of the most potent analgesic agents, and the presence of such receptors can be indicative that a species is able to respond to opiates for pain management, even when one cannot establish a phenotype for pain. Our ultimate on-going goal is to establish a common base of knowledge that will allow to reach unified consensus regarding the use of opioids to control pain in cephalopod research, an essential aspect animal welfare.

3.3. Neural plasticity

We addressed the study of neural plasticity in cephalopods at different levels of complexity (see also summary above). Our target is to study the visual and tactile multi-sensory integrative systems in the largest invertebrate octopus' brains that are differentially organized in respect to any other known organism. We are interested ***i.*** to explore how the octopus big "brain" centers, the visual and tactile sensory maps[27], are organized; ***ii.*** to attempt to decompose the maps into cellular and molecular levels, aiming to search for mammalian homologous regulatory and transmitters gene expression profiles; ***iii***. to contribute to the analysis of the neural circuitry in the octopus and thus understanding how behavioral plasticity is governed by neural systems.

The octopus brain is the largest non-vertebrate brain structure derived from a non-vertebrate lineage, and considered to have reached relative size and structural complexity rivaling vertebrate orthodoxy[45]. The strong advantages to use octopus brain are: 1) ***Unique anatomy***: the visual/tactile centers and the tractography is more cortically and distinctively arranged rather than mammals. 2) ***Imaging accessibility***: the 'cortico-subcortical' territories can be easily accessed as a whole system in one dimension or thin optical layers on a laser or two photon-confocal microscopy. Brain slice recording is also possible[46] (initiated at the SZN and a long standing collaboration with GF). 3) ***Simple network organization***: the rectilinear and diffused organization of sensory receptive fields are specific and composed of a matrix of fasciculated axonal bundles. It can be considered with other properties of mammals and even to artificial network design such as diffused neural nets in any deep-learning agents.

During the last five years we contributed significantly towards this long journey, and this effort is intended to continue [see also perspectives below].

3.3.1. Neuromodulation

Neuromodulation is a ubiquitous process regulating neural plasticity. Neuromodulators exert their roles by gating of plasticity and by regulating neuronal activity. The largest variety of neuromodulators has been identified in the nervous system of cephalopods with studies carried out (mostly) at the SZN[47,48]. However, a precise distribution (i.e. mapping) of individual molecules within brain lobes in several species of cephalopods is still lacking.

We were able to refine previous knowledge and refined information on the localization of GABA and serotonin[48,49] in the octopus brain. Serotonin resulted to be most abundant in lateral cell layers and fibres, i.e. the lateral gyri of the vertical lobe. A GABAergic network appeared widely

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[45] Packard, A. (1972). ***Biological Reviews***, 47: 241-307.

[46] e.g.: Shomrat et al. (2011) ***Current Biology***, 21: 1773-1782; Hochner et al. (2006). ***Biological Bulletin***, 210: 308-317.

[47] Review in: Messenger (1996). ***Inv. Neurosci*** 2: 95-114; see also Ponte (2012) in #16.

[48] Ponte G, Fiorito G, Edelmann D. (2010). Distribution of GABAergic neuronal populations in the nervous system of *Octopus vulgaris*: an immunofluorescence study. Poster session presented at Society for Neuroscience Annual Meeting, San Diego, CA.

[49] Ponte G. (2012). Distribution and preliminary functional analysis of some modulators in the cephalopod mollusc *Octopus vulgaris*, Doctoral dissertation, Università della Calabria & Stazione Zoologica Anton Dohrn, Napoli, Italy, 110 pp.

distributed, but with with certain regions of the brain reaching distinctive abundance. By applying double labelling, Dr Ponte investigated the reciprocal relation of putatively inhibitory and excitatory populations of neurons in the octopus' brain with unprecedented accuracy.

We also determined the presence and the distribution of biogenic amines in the nervous system of the cephalopod mollusc, *O. vulgaris* (Ponte and Fiorito, 2015). Dopamine, noradrenaline and octopamine were identified visualized and localized in O. vulgaris brain by immunolabelling and *in situ* hybridizations (TH and dopamine receptor) for the first time in any cephalopod[16]. We found these biogenic amines widely distributed throughout the brain, but we identified areas of competence within the brain (Ponte and Fiorito, 2015). An example is provided by the pattern of fibres in the *retina profunda* (optic lobes): we mapped tangential fibres that uniquely identified layers of octopaminergic and dopaminergic competence.

The mapping of octopamine in the octopus brain is unprecedented (Ponte and Fiorito, 2015)[48], despite that the molecule had been identified at the SZN in salivary glands of the octopus by Erspamer[50] in 1948.

By analysing changes in gene expression of several genes involved in neuromodulation we tried to decipher the biological machinery involved in the behavioral plasticity of *O. vulgaris*. The gene expression of Stathmin, tyrosine hydroxylase, dopamine transporter, octopressin and cephalotocin was analysed in response to fear conditioning (learned fear) and social interaction (innate fear)[51]. A differential pattern on down- and up-regulation of gene expression in different regions of the octopus' central nervous system resulted to be correlated to learning and behavioral plasticity (Zarrella *et al*., 2015). For example, Ov-dat and Ov-TH exhibit an opposite pattern in response to fear conditioning and social interaction. The increased expression of Ov-stm in octopuses subjected to innate and learned fear suggests that in octopus this gene plays a role similar to what is known in vertebrate brain. In mammals, it is known that amygdala enriched stathmin is required for the expression of innate fear and the formation of memory for learned fear. Interestingly, Ov-stm undergoes in the octopus to a negative regulation in response to fear conditioning. This suggests that the synaptic architecture may be able to change, and that these changes could be related to variations in microtubule dynamics. This result opens the way to a fascinating working hypothesis that requires further studies to understand the relationship between microtubule dynamics, synapse formation, and plasticity of neurons in the octopus (Zarrella et al., 2015).

3.3.2. Nerve regeneration

We studied the events occurring after nerve injury in the octopus and investigated axon regrowth and nerve degeneration and regeneration using octopus' pallial nerves as a 'model system' (Imperadore *et al*., 2017). Severing of one of the pallial nerves induces scar formation and activates the proliferation of hemocytes, which invade the lesion site. Haemocytes are active in debris removal and seem to produce factors that foster axon re-growth. Dr Imperadore found that connective tissue enveloping axons in the nerve is also involved in driving regenerating fibres facilitating target-reinnervation and contribute to the cell-proliferation by cell-reprogramming. In octopus, injured axons are able to quickly re-grow thus restoring structure and function. Based upon the gene expression data (Imperadore, 2017)[52] we found that after nerve injury an upregulation of several genes involved in DNA methylation occurred, such as DNA- and histon-methyltransferases and polycomb group proteins. Among genes known to be involved during nerve regeneration (RAG), we observed a significant upregulation of RNA-binding protein Musashi homolog 2 (among others). The general picture that emerged from these studies is of a dynamic evolution of gene expression, and of possible interplay between 'epigenetic' and RAGs genes, during octopus' pallial nerve regeneration. Our data are seen as an important starting point to understand the mechanisms involved in nerve regeneration in octopus.

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[50] Erspamer (1948). ***Acta. Pharmalcol***. 4: 224-247

[51] Zarrella I. (2011). Testing changes in gene expression profiles in *Octopus vulgaris* (Mollusca Cephalopoda). (Doctoral dissertation, Open University & Stazione Zoologica).

[52] Imperadore P. (2017). Nerve regeneration in the cephalopod mollusc *Octopus vulgaris*: a journey into morphological, cellular and molecular changes including epigenetic modifications. (Doctoral dissertation, Università della Calabria]).

3.3.3 Neural wiring

We developed a new tool to study neural wiring and neural plasticity in *O. vulgaris* (Imperadore, Ponte and co-workers). Axonal tracing techniques are essential to identify efferent and afferent neural pathways and allow precise elucidation of nervous system structures and connections. Different tracing protocols have been indeed tested in cephalopods in the past, however, they required pledges insertion or repeated injections of dyes in living animals, which may induce alteration of normal behavior and other difficulties. Additionally, faintly and false staining have also been reported in available methods and imaging is difficult to achieve in whole mount preparations and require tissue sectioning for in-depth visualization. We developed a neurobiotin tracer approach (proven to be successful on the pallial and stellar nerves on the mantle and on the axial nerve cord within the arm within *O. vulgaris*). The backfilling method together with optical clearing offered by the use of methyl salicylate, represents a reliable technique for nerve tracking in thick samples of *O. vulgaris* (2-3 mm), and allows whole mount imaging and permits 3D reconstructions of the investigated system.

4. Perspectives

We will further extend our study of biological plasticity in different marine organisms.

4.1 Extending the analysis of behavioral flexibility in octopus (Fiorito)

4.1.1 Complex cognition is classically conceived as an adaptation evolved within the primate lineage. Sophisticated cognition has been shown in non-primate species, particularly in corvids thus suggesting that similar socio-ecological pressures have led to the independent evolution of intelligence in distantly related animals. The study of convergent cognitive evolution is currently constrained by the lack of comparative cognitive data in several taxa. We are investigating [in collaboration with Professor Nicola Clayton; supported by Cambridge University & ABS to PhD candidate Piero Amodio] potential ***convergent physical cognition between corvids and cephalopods*** through comparable experimental paradigms. We are examining tool use and the cognitive mechanisms underpinning this behavior in Eurasian jays and in common octopuses. Experiments are designed to test whether individuals are capable of the spontaneous selection of functional tools on the basis of their physical properties (i.e. size), or whether they flexibly use distinct tools to achieve the same goal. We predict convergent flexibility in the use of tools because the cognitive abilities of these animals were shaped under similar ecological pressures: spatiotemporally dispersed food, dietary generalism, and extractive foraging. By investigating convergent cognition in two cognitively sophisticated species of non-primate animals, we will test the evolutionary hypothesis for the emergence of physical intelligence in a broader comparative framework, thus facilitating a deeper understanding of the general mechanisms of evolution.

4.1.2 We will determine whether ***temperamental differences*** exist between individuals (review in Marini et al., 2017), and if this is linked to different behavioral capabilities/paradigms, with context and over time. We expect that each individual octopus will exhibit distinct temperament types that can be easily categorized into “shy and “bold” continuum across a battery of behavioral assays. In addition, we hypothesize that the “success” of these different temperament types (if present) will differ by context. Specifically, “bold” individuals will demonstrate faster learning, higher reproduction and faster growth in the laboratory assays, but also demonstrate more bodily damage and lower survival in a mesocosm approach [funded by Fyssen Foundation to Dr C. O’Brien @ G. Fiorito starting from 2018]. Our ultimate goal is to contribute to the understanding on evolution of animal behavior.

4.1.3 We are exploring the possibility that social context induces a ***neural-mapping correlate*** [in collaboration with Professor L. Fogassi, Università di Parma] in motor action recognition. Our preliminary evidences (G. Ponte and co-workers) show that octopuses are influenced in the use of their arm by conspecifics, opening the possibilities that octopuses may have a “theory of mind”[53] and therefore a neural-recognition system occurs also in this animal.

4.2. Octopus sensory systems (Ristoratore)

The cephalopods’ sensory system and capabilities are very complex. Recent studies shown the presence of extra ocular photoreception in *O. bimaculoides*. These findings have been also anticipated in *O. vulgaris* by Di Cristina (@ G. Fiorito) that observed opsin expressed in the rim of the suckers.

We initiated a PhD (PhD candidate F. Strano; co-tutor Professor A. Terlizzi, Univ. Trieste) aimed to investigate cephalopod skin light- and mechano- sensory systems. We aim to search for orthologues possibly involved in photoreception and mechanoreception in transcriptome of octopus[54] followed by characterization by *in situ* hybridization. Preliminary analysis revealed the presence of a *Rab32/38* transcript highly expressed in hatching and post-hatching larvae, and in the adult in the epistellar body. Rab32/38 is involved in vesicular trafficking and is expressed in pigment cells associated or not to visual systems in other organisms[55,56].

Cephalopods also possess a mechanoreceptive system considered to be highly sensitive to local water movements. This sensing system is mainly formed by epidermal hair cells located on head, arms and mantle. In hatchlings, the mechanosensory system is suggested to act as sound receptor (Baldascino @ G. Fiorito) [57]. We will also attempt to explore these possibilities.

4.3. Investigating immune system (Sordino)

To expand our knowledge on the cellular and molecular components of the immunological defence mechanisms in pelagic tunicates, we are:

1) Developing enzymatic and immunochemical characterization of the tunicate haemocyte defensive behavior following infections with *Bacillus clausii* spores (in collaboration with Rita Marino);
2) Studying the main ecological functions (particularly biotic and abiotic stress response) of a pelagic tunicate community before, during and after the phytoplankton bloom occurring at the Marquesas Islands (South-East Pacific Ocean; TARA-STEFI), with the aim to highlight functional trait responses to environmental changes (in collaboration with: D. Iudicone, L. Caputi, S. D’Aniello);
3) Investigating the dynamics of inflammatory molecules and the behavior of brain immune-resident cells in the zebrafish model of acute seizures induced by a known pro-convulsive agent, the pentylenetetrazole (PTZ; in collaboration with Marco Borra);
4) Large-scale screening of marine extracts to identify and discovery new molecules with immunomodulatory properties by using the zebrafish and tunicate *Ciona robusta* models of PTZ-induced seizure (in collaboration with Antonietta Spagnuolo).
5) Studying the inflammatory response to exposure to contaminated sediments in ascidian species with different degrees of tolerance to pollutants (in collaboration with Annamaria Locascio, Filomena Ristoratore, and Antonietta Spagnuolo; this research will be supported by the project MIUR-FISR-ABBaCo 2017, WP4 “Holistic approach to the study of multiple stress and risk reduction”).

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[53] Chiandetti C., De Sio F., Ponte G., Fiorito G. (2017) Challenging cephalopods behavioral and neural plasticity: sociality, territorial behavior and “mind-reading”. In: *The Mind-Reading Brains*, Grasso et al. (Eds), Springer

[54] Petrosino G. (2015) The transcriptional landscape of the nervous system of *Octopus vulgaris*. (Doctoral dissertation, Università degli studi di Napoli Federico II).

[55] Racioppi C, Kamal AK, Razy-Krajka F, Gambardella G, Zanetti L, Di Bernardo D, Sanges R, Christiaen LA, Ristoratore F. Fibroblast growth factor signaling controls nervous system patterning and pigment cell formation in *Ciona intestinalis*. ***Nat. Comm.*** (2014) Sep 5: 5..

[56] Coppola U, Annona G, D’Aniello S, Ristoratore F. Rab32 and Rab38 genes in chordate pigmentation: an evolutionary perspective. ***BMC Evol. Biol***. Jan 27 (2016) 16(1): 26..

[57] Baldascino E, Almansa E, Invitto S, Terlizzi A, De Martino G, Fiorito G. (2016). *Octopus vulgaris* paralarvae may use ‘sound’ to orient in space. Poster at the 10th Forum of Neuroscience - FENS, Copenhagen, Denmark

4.4 Exploring the potential of stem cells in octopus (Fiorito)

We initiated a systematic analysis for the discovering of the nature of stem cells in the adult nervous system of *O. vulgaris*. Preliminary data using the cell division marker phospho-histone3 on brain sections (P. Imperadore & G. Ponte @ G. Fiorito) indicate the presence of a considerable amount of actively dividing cells. In collaboration with KU Leuven (Professor E. Seuntjens and coworkers) we are characterizing these putative stem cells and studying their potential to generate (different) neurons. We molecularly define the different cell types present in the octopus' brain and search for candidate stem cells. We will make use of a potentially indefinite supply of adult cells, the white bodies. In *O. vulgaris*, cells from the blood seem to re-enter the cell cycle upon axonal damage and engage in the nerve repair (Imperadore et al., 2017). Cells from the hematopoietic system might therefore potentially be triggered to transdifferentiate into neurons upon an active need. We hypothesize that the factors that stimulate neurogenesis at that moment might be similar to the factors triggering neurogenesis during embryogenesis. To test this hypothesis, we will isolate, characterize and maintain *in vitro* white body cells in cell culture (A. Deryckere PhD candidate @ KU Leuven; G. Fiorito advisor). We will therefore analyse the types of cell in the white bodies by FACS sorting using cell morphological characteristics combined with nuclear dyes to sort the actively dividing cells from the other cell types. RNA of these cell populations will be isolated and sequenced. This analysis will give us a concrete list of genes that determine "stemcellness" in the white body tissue. By overlaying the "brain stem cell" gene list obtained and the "white body stem cell" gene list, we can identify common, general stem cell determinants and genes that are restricted to a certain tissue. We are collaborating with Dr. C. Verfaillie (Stem Cell Institute, KU Leuven), who possesses the necessary know-how, constructs and tools to transfect primary cells.

4.5. The role of epigenetic changes during development, regeneration and plasticity in octopus (D'Aniello E., Fiorito)

Many studies carried out over the last decades, have led biologists to hypothesize the existence of an "epigenetic code", which appears to be essential for cellular development and synaptic plasticity. The specific epigenetic modifications, mainly DNA methylation, chromatin modifications (through histone proteins) and prion-like mechanisms, which set a sort of specific code that is able to mediate long-term memory formation and learning.

We are interested in studying the role of DNA methylation and histone acetylation during development of *O. vulgaris* (E. D'Aniello). Given the availability of *O. vulgaris* transcriptome, E. D'Aniello will identify by *in silico* analyses evolutionary conserved genes functioning in the modification of the chromatin structure during development. Then, qPCR and whole mount *in situ* hybridization will be utilized to investigate differential expression pattern in various embryonic stages. Understanding how genes responsible of epigenetic changes are expressed during *O. vulgaris* development will lead to new insight of the early life of this organism and will certainly help to identify new mechanisms that ultimately will improve the efficiency of husbandry of this species.

In a preliminary study we have (Fiorito and coworkers) identified candidate methyltransferases in the transcriptome sequences and ESTs libraries of three cephalopod species: *O. vulgaris, Sepia officinalis* and *Euprymna scolopes*, which led us to directly investigate the extent of DNA methylation in the octopus. Our results indicate that the level of DNA methylation in *O. vulgaris* is intermediate between the levels of the human (highly methylated) and *D. melanogaster* (poorly methylated) genomes (A. Riccio & G. Fiorito, unpublished). In addition, our preliminary data strongly support that Polycomb group (PcG) proteins-regulated mechanisms involving local dedifferentiation and re-differentiation could mediate the regeneration in the octopus' arm (Baldascino & Imperadore @ G. Fiorito). Polycomb group proteins associate with DNA methyltransferases and we aim to disclose the role of PcGs in octopus' arm regeneration (in collaboration with Dr. Chiara Lanzuolo). To unravel the molecular mechanisms driving muscle regeneration, a bi-dimensional RNA-seq analysis will be applied extracting regenerating tissue in space (different arm location: tips and proximal part) and time (different timing after the first cut). Expression and activity of PcGs as well as other processes (e.g. stemness, cell proliferation, etc.) will be monitored by tissue immunofluorescence.

Both DNA methylation and histone modifications are proved to be required in the central nervous system (CNS) for memory formation[58]*.* Strikingly it has also been suggested that some behavioral experiences can induce this kind of modifications on DNA. For instance, contextual fear conditioning has been shown to induce DNA methylation and demethylation, as well as histone acetylation, phosphorylation and methylation of several memory-associated genes in the hippocampus. We are working (P. Manzo @ G. Fiorito) to determine whether inter-individual variability in learning can effectively be linked to inter-individual diversity depending on epigenetic modifications.

4.6 The octopus' connectome (Fiorito)

One of our main aims for the future is to establish a cephalopod connectome. We expect that advances in mapping of neural connections will yield insights in how the nervous system is controlled and what the mechanisms are that drive neuron function and activity[59]. Despite the great comparative interest in how these large non-vertebrate neural systems might be organized, few studies of the cephalopod neural systems utilized the modern post-genomic cellular, molecular and imaging methods employed in studies of large vertebrate brains. Although the use of this technology in cephalopods is new and highly exploratory, it would open doors for further novel genome editing technologies.

We aim to study how the octopus' visual and tactile multi-sensory integrative systems are organized, whether in the brain specialized layers or a columnar organization exists. Preliminary data using live brain slice imaging reveals cell morphology and novel spatial organization. We observed a columnar and layered organization of the vertical lobe, which was identified by the use of lyophilic, mitochondrial, nuclear, and lysosome potentiometric markers (G. Ponte & S. Shigeno @ G. Fiorito).

We also progressed in making brain tissue transparent; 'Clarity' has never been attempted in invertebrates. This technique allows for highly detailed pictures of the protein and nucleic acid structure of organs such as the brain. We succeeded in developing a protocol for *O. vulgaris* (G. Ponte & P. Imperadore @ G. Fiorito).

To further understand the molecular basis of neural wiring, we studying a certain type of cell adhesion molecules: the protocadherins (PCDH). These appear essential for creating complex brains in vertebrates. It is the expansion in the octopus of this gene family that is seen as a vertebrate innovation. For instance, *Drosophila* and other invertebrates have a very limited set of PCDHs; in the fruit fly the neuronal diversity is achieved through Dscam proteins (Down syndrome cell adhesion molecules). These proteins possess variable exons, which by alternative splicing can generate over 18,000 different isoforms which are essential for neural circuit formation.

Recently, over 168 protocadherins were found within the genome of *O. bimaculoides*[60], which suggests that this organism uses a vertebrate-like system to create its complex brain.

By mapping the expression patterns of protocadherins within the brain of *O. vulgaris* we aim to gain insight into the function of these molecules, both during development and in the adult individual (R. Styfhals PhD candidate - G. Fiorito; SZN & KU Leuven).

We will also continue to explore wiring, neural circuitry and mapping of the octopus' brain; we will increase our level of accuracy and detail by extending the number of "neural-markers" (i.e. genes and neuro-modulators) and progressing towards the analysis of octopus connectome (Dr. Ponte and Dr Imperadore; collaboration with: University of Cologne, University of Southampton; Leica-Microsystems). The expansion of the mapping of neuromodulators, receptors and transporters (G. Ponte @ G. Fiorito) will significantly increase our knowledge on cellular and molecular diversity within the nervous system of *O. vulgaris*. Since protocadherins are involved in neural identity, overlaying the expression patterns of protocadherins with, for instance the localisation of the dopaminergic system (R. Styfhals & G. Ponte @ G. Fiorito), would provide important insights on the functioning and plasticity of the brain of *O. vulgaris*.

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5. Publications (2012-2017; *corresponding author)

2017

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Imperadore P*, Shah SB, Makarenkova HP, Fiorito G. Nerve degeneration and regeneration in the cephalopod mollusc *Octopus vulgaris*: the case of the pallial nerve. ***Sci. Rep.*** (2017) 7.

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Ponte G, Modica MV*. Salivary Glands in Predatory Mollusks: Evolutionary Considerations. ***Front. Phys.*** (2017) 8: 580.

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Tedesco P*, Gestal C, Begić K, Mladineo I, Castellanos-Martínez S, Catanese G, Terlizzi A, Fiorito G*. Morphological and Molecular Characterization of Aggregata spp. Frenzel 1885 (Apicomplexa: Aggregatidae) in *Octopus vulgaris* Cuvier 1797 (Mollusca: Cephalopoda) from Central Mediterranean. ***Protist*** (2017) 168 (5): 636-648.

Sykes AV, Almansa E, Cooke GM, Ponte G, Andrews PLR*. The Digestive Tract of Cephalopods: a Neglected Topic of Relevance to Animal Welfare in the Laboratory and Aquaculture. ***Front. Phys.*** (2017) 8.

Villanueva R*, Perricone V, Fiorito G. Cephalopods as Predators: A Short Journey among Behavioral Flexibilities, Adaptions and Feeding Habits. ***Front. Neurosci.*** (2017) 8:598.

2016

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2015

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2013

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6. List of grants

- **MO.DO - Model Organisms** POR Campania FESR (2011 - 2014). PI: G. Fiorito.

- **MIUR Premiale MolEcOC** "Approaching inter- and intra-individual variability by molecular ecology for the technology transfer of basic research on marine model organisms (*Octopus* and *Caretta*)". (2013 - 2014). PI: G. Fiorito & S. Hochscheid
- **RITMARE**: The Italian research for the sea. Italian Flagship Project (2013-2018).PI for SZN: M. Ribera d'Alcalà

7. List of external collaborators

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-Ennio del Giudice, Università di Napoli "Federico II", Napoli, *Italy*

-Carlo Di Cristo, Università del Sannio, Benevento, *Italy*

-Leonardo Fogassi, Department of Neuroscience, University of Parma, *Italy*

-Rute Fonseca, Department of Biology, University of Copenhagen, *Denmark*

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-Eve Seuntjen, Department of Biology, Katholic University of Leuven, *Belgium*

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-Gaetano Terrone, IRCCS-Mario Negri, Milano, *Italy*

4. Reproductive biology: mechanisms of gamete maturation, fertilization and early development

1. People involved

Name	*Position*	*Funding*	*Period*
Luigia Santella	Staff scientist	SZN	Since 1989
Elisabetta Tosti	Staff scientist	SZN	since 1989
Jong Tai Chun	Postdoctoral fellow	SZN	since 1999
Alessandra Gallo	Postdoctoral fellow	SZN/ABBaCo	since 2015
Nunzia Limatola	Postdoctoral fellow	SZN	2016-2017
Filip Vasilev	Postdoctoral fellow	SZN/StarTregg	2013-2017
GianLuigi Russo	Affiliated scientist	CNR	since 2016

2. Background, aims and objectives

Marine animals whose reproduction depends on ‘external fertilization’ provide us with advantageous opportunities to study many aspects of gametes activation and fusion, as well as the subsequent embryonic development. Due to the large amount of eggs that are easily available and handled, invertebrate animals such as starfish, sea urchins, and ascidians have been chosen as favourable animal models. The focus of the research activity on this topic at the SZN has been directed mainly towards the molecular mechanisms of gamete maturation and fertilization.

Oocyte maturation is the last phase of oogenesis during which immature oocytes, arrested at the prophase I of meiosis, are unblocked by a chemical/hormonal stimulus to initiate meiotic resumption that involves coordinated nuclear and cytoplasmic modifications. Whereas nuclear maturation starts with the germinal vesicle breakdown and ends with extrusion of the two polar bodies (exit from meiosis), cytoplasmic maturation involves structural and functional modifications of ion channels, organelles and cytoskeleton to produce mature eggs for fertilization. Sperm maturation is accompanied by structural and cytoplasmic modification, which underlie fertilization competence[61,62,63,64,65,66].

The ultimate goal of our study is to understand the molecular events taking place at the plasma membrane and the cortex during meiotic maturation and fertilization. As excitable cells, oocytes and eggs manifest ion currents across the membrane during these transition periods of meiotic maturation, fertilization, and cleavages. Movement of Ca^{2+} ions from extracellular space or from internal stores bears special importance, as the transient increase of intracellular Ca^{2+} takes place in fertilized eggs of all animal species and serves as a second messenger triggering egg activation and subsequent development [67,68,69,70,71,72,73,74,75,76,77,78].

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The research activities on this topic during the period of 2012-2017 are subdivided into two main research lines.

a. Imaging, biochemical and cell biological approach (Santella)

We have studied the roles of the plasma membrane-cytoskeleton interface in the generation and propagation of the Ca^{2+} influxes and waves. To corroborate and extend the results of the earlier studies[67-76] showing that intracellular Ca^{2+} signals are modulated by suboolemmal actin cytoskeleton and vesicles, we have explored and developed new methodologies.

b. Physiological approach (Tosti)

Characterization of morphological, molecular and structural events underlying gamete fertilization competence.

3. Results

a. Roles of the plasma membrane-cytoskeleton interface in the generation and propagation of the Ca^{2+} influxes and waves (Santella)

a1. Modulation of intracellular Ca^{2+} signaling by the actin cytoskeleton

The plasma membrane and internal Ca^{2+} stores such as endoplasmic reticulum (ER) are intimately associated with the actin cytoskeleton, which provides both rigidity and plasticity for the membranes. The actin cytoskeleton and its accessory proteins not only mobilize ER and vesicles but also set the tone to the micro-environment of the ion channels studded on the membranes. Actin is also a Ca^{2+}-binding protein with extremely high affinity (Kd = 10^{-9}M), which could serve as an abundant Ca^{2+} buffer in the cytosol. For these reasons, polymerization status of the actin filaments and the dynamics of their rearrangements were expected to influence the efficiency of ion channel activities. Using starfish oocytes and eggs, we have demonstrated that this is not just a theoretical notion, but a phenomenon which can be experimentally proved. The optimization process that renders maturing oocytes more sensitive to the Ca^{2+}-releasing second messenger $InsP_3$ is actin-dependent, as judged by its inhibition by latrunculin A (LAT-A)[69,70]. Since then,

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besides actin drugs, several methodological strategies have been adopted to alter the F-actin structure and dynamics in starfish eggs and oocytes, e.g. microinjection of actin-binding protein cofilin[71] or metabolically stable guanidine nucleotide[72], interference of endogenous actin-binding proteins by PIP2 sequestration[13], etc. In each case, certain aspects of Ca^{2+} influx (cortical flash) and intracellular Ca^{2+} release have been observed to be significantly changed by the alteration of the actin cytoskeleton[74,75].

In 2013-2014, we put our earlier observations into a test. As exogenously added cofilin, which binds and severs actin filaments, significantly increased the peak amplitude of the Ca^{2+} wave in fertilized eggs of starfish (*Asterina pectinifera*)[71], microinjection of its function-blocking antibody was expected to suppress the Ca^{2+} response. Supporting the idea that the previous observation was indeed attributable to the changes of actin filaments, microinjected antibody against depactin (starfish version of cofilin) expectedly suppressed the Ca^{2+} release in the fertilized eggs (**Chun *et al.,* 2013**, see the Reference). In addition, we have tested if the aforementioned modulation of intracellular Ca^{2+} signaling by the actin cytoskeleton is a peculiar phenomenon restricted to starfish or is also observed in other species. To this end, sea urchin eggs (*Paracentrotus lividus*) were treated with actin drugs prior to fertilization. We observed that the two actin drugs LAT-A and cytochalasin B, which promote depolymerization but by different mechanisms, displayed similar effects. In both cases, the fertilized eggs required longer time lag to generate the Ca^{2+} wave after firing normal cortical flashes. The Ca^{2+} waves also propagated slowly and manifested lower amplitudes in these eggs compared to the control. In addition, the eggs pretreated with actin drugs were less receptive of sperm, as judged by the number of egg-incorporated spermatozoa (**Chun *et al.,* 2014**).

a2. Roles of the cortical actin cytoskeleton in controlling sperm entry

In view of the fact that the fertilization Ca^{2+} signals play critical roles in egg activation and the subsequent development, the compromised Ca^{2+} response in the eggs with the altered actin cytoskeleton advocates the idea that integrity of the actin cytoskeleton is indicative of oocyte quality (**Santella *et al.*, 2015**). In line with this, we have demonstrated that overripe eggs of starfish have tendency of polyspermy (**Santella *et al.,* 2014**). Similarly, the sea urchin eggs exposed to nicotine displayed high rate of polyspermy at fertilization. We have collected the data indicating that the actin cytoskeleton of the overripe starfish eggs and nicotine-exposed sea urchin eggs are hyperpolymerized in the cortex, and we will conclude these studies after corroborating the preliminary results.

a3. Discovery of a novel Ca^{2+} signal at the later stage of meiotic maturation

In 2015, we reported that maturing oocytes of starfish (*Asterina pectinifera*) displays a series of Ca^{2+} spikes at the time of the germinal vesicle breakdown. Occurrence of intracellular Ca^{2+} increase at the time of nuclear envelope dissolution during mitosis was previously described, but its detection during the dissolution of meiotic nuclear membrane was unprecedented. This is due to the calcium influx from outside (Figure 1) through the L-type Ca^{2+} channel, as judged by its inhibition by diltiazem and verapamil (**Limatola *el al.,* 2015**). Again, pretreatment of the oocytes with the actin drugs significantly changed the pattern of Ca^{2+} spikes. While LAT-A increased the average amplitude of the Ca^{2+} spikes (amplitude modulation), the count of the spikes during the given time frame was significantly reduced (frequency modulation). By contrast, the microfilament-stabilizing drug jasplakinolide exerted exactly the opposite effects.

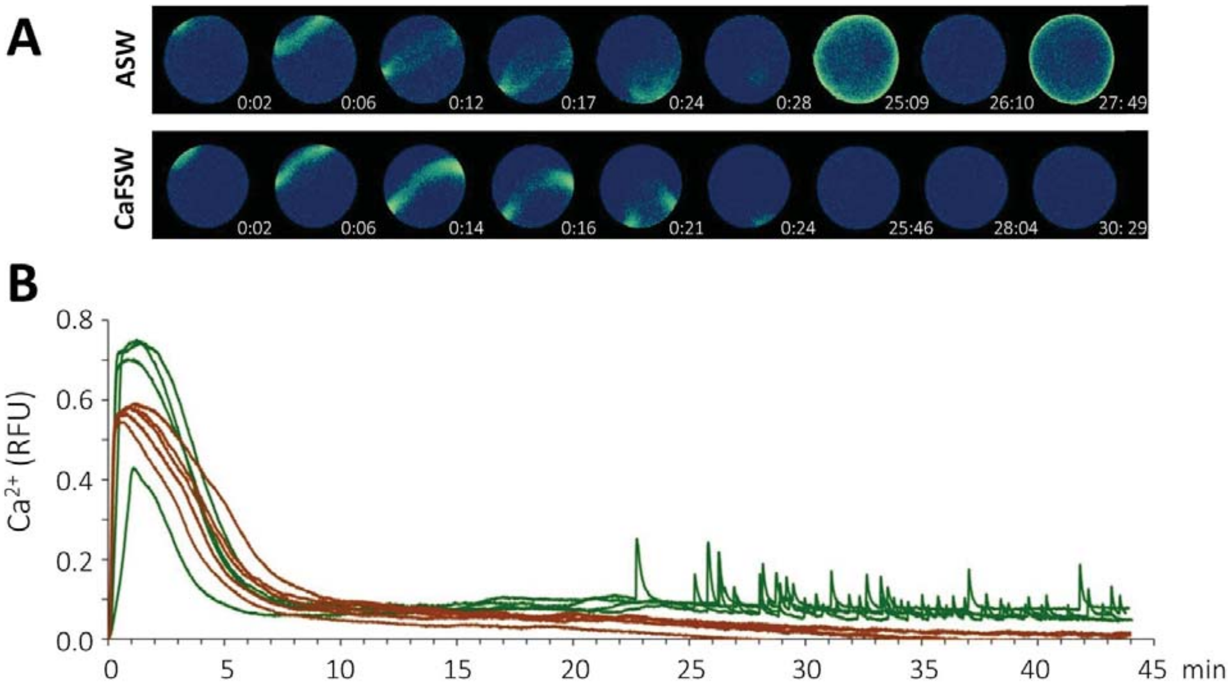

Figure 1. Changes of the intracellular Ca^{2+} levels in the maturing oocytes of starfish. *A. pectinifera* oocytes microinjected with calcium dyes were exposed to 1 µM 1-MA either in artificial seawater ASW or in calcium-free seawater CaFSW. (A) Relative fluorescence images representing the sites of momentary Ca^{2+} increases during meiotic maturation. The Ca^{2+} response initiated 1–2 min after the addition of 1-MA, and the time point immediately before the first detectable Ca^{2+} signal was set to t = 0 for the sake of comparison. (B) The changes of the cytosolic Ca^{2+} levels in the oocytes during the maturation in ASW (green curves) or in CaFSW (brown curves). The maturing oocytes in ASW display dual Ca^{2+} responses to 1-MA: a fast propagating wave at the early phase and the short-lived Ca^{2+} flashes near the plasma membrane (arrow) at the late phase. Note that the latter Ca^{2+} response is totally absent in the oocytes maturing in CaFSW (This research was financially supported by SZN PhD fellowship to N. Limatola).

a4. Effect of cortical vesicles disruption on egg activation

In some practice of *in vitro* fertilization, Ca^{2+} ionophores like ionomycin are utilized to activate oocytes after intracytoplasmic sperm injection to resolve problems related to male infertility. In 2012, we showed that a brief exposure of starfish (*A. aranciacus*) oocytes to ionomycin disrupts cortical granules and actin dynamics. When the drug was added even after monospermic zygotes were already formed, the subsequent early embryonic development was often impaired (**Vasilev *et al.,* 2012**; **Santella and Dale, 2015**). This observation warns against the use of ionomycin for clinical purpose, and indicates that the fully elevated fertilization envelope may protect the early embryos from mechanical strain, but not from chemical stresses.

a5. Effects of lithium on the reproductive programs of sea urchin

Lithium is an alkali metal smaller than sodium and potassium. While its action in the cell is far less known than the two physiologically important ions, lithium has been used as a psychiatric medicine from ancient times. As one of the lithium's actions is known to be depletion of inositol, it may influence the PIP2 metabolism, which in turn is likely to affect actin dynamics. In 2016, in collaboration with Maria Costantini of SZN, we demonstrated that application of 1 mM LiCl to *P. lividus* zygotes before or after fertilization increases the rate of abnormal embryonic development and deregulates the gene regulatory program, as judged by RT-PCR of the developmental marker genes (**Ruocco *et al.,* 2016**). Since the deleterious effect on the developmental programing of the embryo takes place at the therapeutic dosage of lithium in clinical medicine, application of the therapy to pregnant woman calls for special caution. While our preliminary data indicate that LiCl interferes with actin dynamics in sea urchin eggs, we have to confirm the changes of oolemmal actin cytoskeleton and fertilization Ca^{2+} patterns at fertilization in these eggs in the coming breeding season.

a6. Effect of climate and environmental changes on the reproductive system of echinoderms

Since the gametes of external fertilization are released directly into environment, the experimental model system of fertilization could be used as a 'biosensor' that detects subtle warning signs in the living environment. As global warming and increased CO_2 emission are suspected to cause unusual rainfall such as El Niño and ocean acidification, we have examined how dilution of seawater and decreases in pH affect the oolemmal ultrastructure and functions. As was presented in the last Scientific Council Meeting (Limatola, 2014), diluted seawater dislodges cortical granules from the plasma membrane and changes the structure of microvilli. As a result, cortical flash is totally eliminated, whereas acidic pH has milder repression on cortical flash and Ca^{2+} wave. Based on the preliminary results, we wrote the research grant proposal for 2016-2018 Flagship Research Program at the SZN, but it was not funded and the project was tentatively on hold.

a7. *De novo* assembly of the transcriptome from the eggs and early embryos of starfish (*A. aranciacus*)

In the previous Scientific Council Meetings, the committee suggested that our research should adopt a more specific gene-oriented approaches rather than utilizing pharmacological drugs. While the genomic sequence of this favourable animal model of ours is not available, we took the advantage of the fact that the eggs and early embryos are the cell types that have the highest RNA sequence complexity, and assembled its first transcriptome by use of the RNA-seq data collected in collaboration with Remo Sanges and Francesco Musacchia. Thus, over 32,000 transcripts were annotated including the ones that encode virtually all the expected Ca^{2+} signaling toolkits, 13 distinct cyclins, and as many cyclin-dependent kinases. The faithful presentation of the cyclin gene family in reference to the sea urchin genome attests the overall completeness of our transcriptome (**Musacchia *et al.*, 2017**). The results of this study are expected to facilitate our future research in targeting the candidate molecules involved in this research topic. This work was financially supported by MIUR Progetti Premiali StarTregg.

b. Characterization of morphological, molecular and structural events underlying gamete fertilization competence (Tosti)

Calcium increase plays a pivotal role in meiosis re-initiation. It is well established that, during this process, calcium is released from the intracellular stores, whereas less is known on the role of external calcium entering the cell through the plasma membrane ion channels. Among voltage-dependent currents underlying reproductive events (**Gallo and Tosti, 2015; Tosti *et al.*, 2013; Tosti *et al.*, 2016**), L-type calcium currents play peculiar roles during oocyte maturation, fertilization and embryo development, whereas T-type calcium currents are mainly involved in sperm physiology.

By using electrophysiological and pharmacological approaches, we characterized for the first time T-type calcium currents on the plasma membrane of growing immature oocytes of the ascidian *Styela plicata*. We classified three subtypes of immature oocytes at the germinal vesicle stage on the basis of their size, morphology and accessory cellular structures. These stages were clearly associated with an increased activity of T-type calcium currents and hyperpolarization of the plasma membrane. We also observed that T-type calcium currents oscillate in the post-fertilization embryonic stages, with minimal amplitude of the current in the zygote and maximal at 8-cell stage.

In addition, chemical inhibition of T-type calcium currents, obtained by applying specific antagonists, induced a significant reduction in the rate of cleavage and absence of larval formation. We suggested that calcium entry via T-type calcium channels may act as a potential pacemaker in regulating cytosolic calcium involved in fertilization and early developmental events (**Gallo *et al.*, 2013**).

Oocyte mitochondria play a functional role for the acquisition of fertilization and developmental competence. By using electron and confocal microscopy we evaluated the mitochondria distribution pattern and activity during the same three stages of immature oocytes of the ascidian *Styela plicata*. We showed that mitochondria resulted to be spread throughout the cytoplasm in the smallest oocyte stage and to gradually migrate to the periphery of the sub-cortical cytoplasm in the intermediate stage. In the fully-grown oocyte stage, mitochondria resulted to be aggregated in the subcortical cytoplasm. This pattern of polarized mitochondrial distribution significantly correlated with an increase of mitochondrial potential and activity suggesting a potential role on the acquisition of oocyte developmental competence (**Bezzaouia *et al.*, 2014**) (Figure 2). In a comparative work on mammalian models, we elucidated the pattern of bovine oocyte calcium entry through the external layers confirming the role of hormone-induced calcium rise in the oocyte cytoplasmic maturation and competence acquisition (**Silvestre *et al.*, 2012**). Furthermore, it was demonstrated a role of organelles and chromatin decondensation on fertilizing capability of spermatozoa (**Junca *et al.*, 2012; Fortunato *et al.*, 2016**).

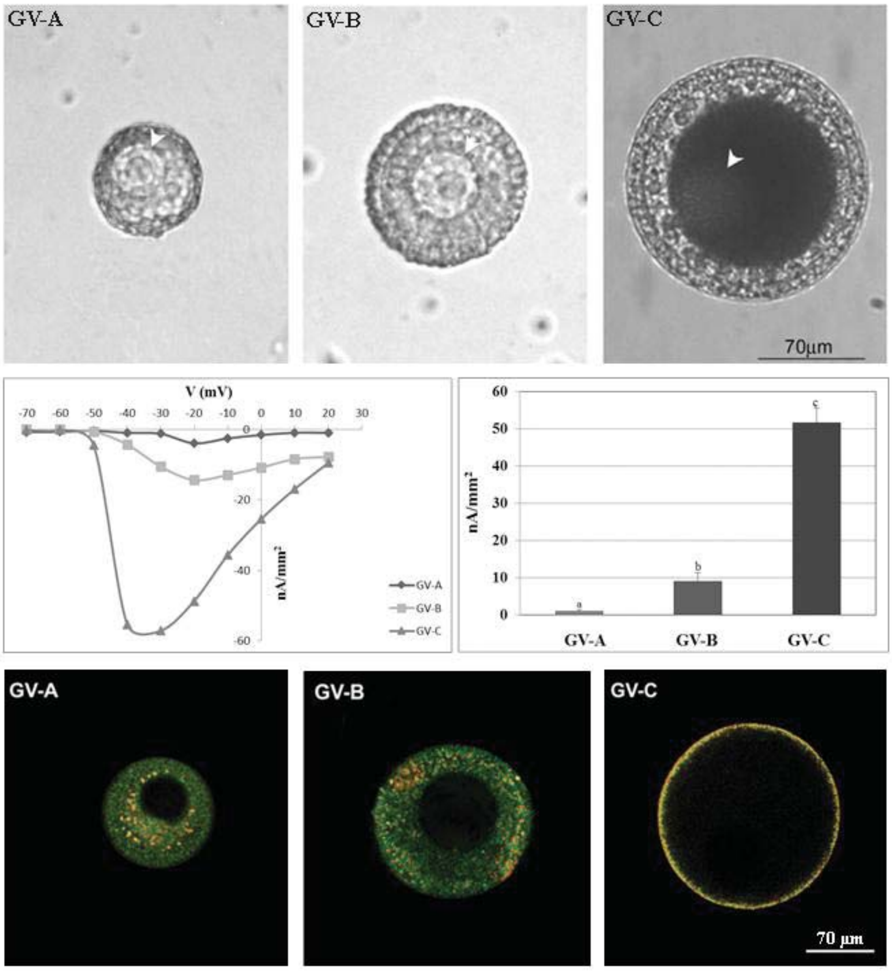

Figure 2. Immature oocytes of *Styela plicata* are characterized by T-type calcium currents activity that increase during oocyte growth and different mitochondrial distribution pattern.

Top panel: Representative images of the three immature oocyte at GV stage (A,B,C) discriminated on the basis of size, morphology and accessory cells structure. Middle panel 2): Electrophysiological characterization of ion currents during oocytes growth. Left: Average current-voltage relationship (I/V curve) generated from depolarizing voltage steps between −70 and +20 mV from a holding potential of −80 mV. The current was maximally activated by voltage step to −20 mV in the GV-A and GV-B and to −30 mV in GV-C. All I/V curves are obtained by averaging the peak current for fifteen oocytes. Error bars indicate S.E. right: The maximum peak inward current (means ± S.E.) recorded at the activation step of −20 mV in GV-A and GV-B, while the value was −30 mV in GV-C (a vs b vs c P<0.01).Bottom panel: Scanning laser confocal images of germinal vesicle (GV) stage oocytes labelled with JC1. In GV-A stage oocytes high polarized mitochondria are grouped in clumps in the perinuclear region and in low –polarized mitochondria are diffuse in the cytoplasm. In GV-B oocytes, high and low polarized mitochondria are scattered within the pericortical cytoplasm, but are co-localized in the subcortical cytoplasm in GV-C stage oocyte.

4. Perspectives

a. Roles of the plasma membrane-cytoskeleton interface in the generation and propagation of the Ca^{2+} influxes and waves (Santella)

a1. To elaborate the model of actin as a scaffold protein in the signal transducing mechanism within the suboolemmal region

We recently revisited the intriguing phenomenon that was first observed in our lab: when mature eggs of *A. aranciacus* is exposed to LAT-A, it starts to exhibit repetitive waves of fertilization-like Ca^{2+} signals and cortical flashes after 5-10 min time lags, but without the aid of sperm or exogenous Ca^{2+}-releasing second messengers[69,70]. The results of our nearly finished ongoing studies indicated that this apparent parthenogenetic egg activation is an actin-dependent process, as it is inhibited by agents stabilizing actin filaments. Further studies revealed that the LAT-A-induced Ca^{2+} waves did not arise from actin-dependent hypersensitization of $InsP_3$ receptor, but is due to the modulation of PLCɣ enzyme activity, as judged by the combined results that: i) the amount of Ca^{2+} liberated by $InsP_3$ uncaging is not increased with the progress of actin depolymerization, ii) LAT-A-induced Ca^{2+} wave is totally blocked by a dominant negative fusion protein containing the tandem repeats of SH2 domains of *A. aranciacus* PLCɣ, iii) the PH domain of PLCδ1 binding to PIP2 is falling off the plasma membrane during the time of Ca^{2+} increase (5-10 min after LAT-A administration, and iv) the cell contents of $InsP_3$ is increased by LAT-A on the same time schedule (Figure 3). These findings suggest that actin filaments may also modulate Ca^{2+} signaling also by controlling the activity of the enzyme that produces Ca^{2+}-mobilizing second messenger $InsP_3$.

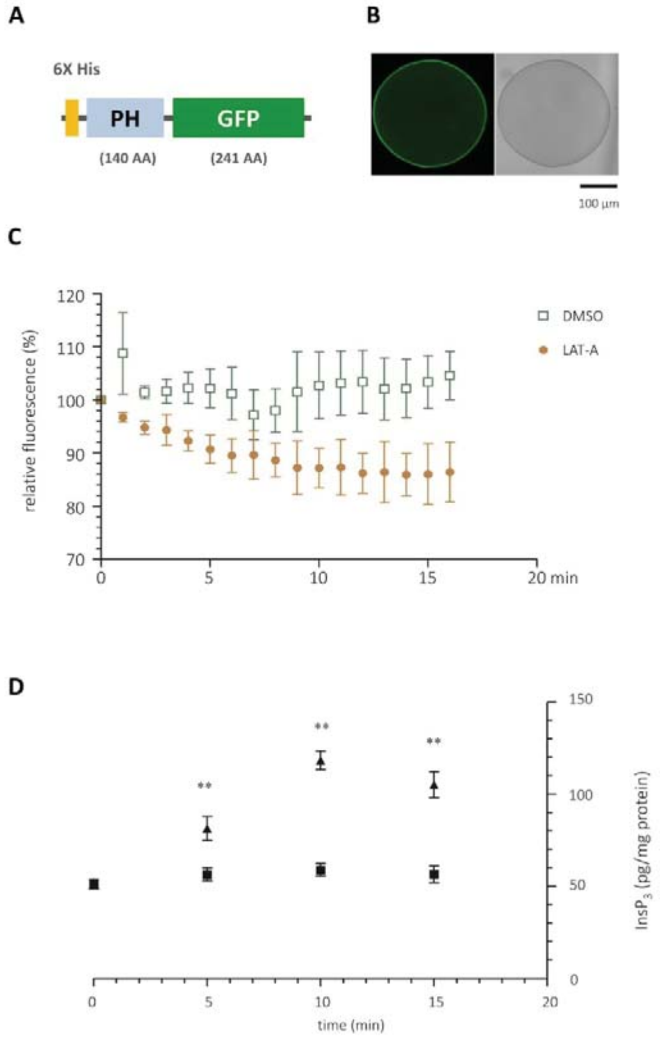

Figure 3. LAT-A induces PIP2 hydrolysis to synthesize $InsP_3$. (A-C) Translocation of PH-GFP from the plasma membrane of starfish eggs as a result of PIP2 hydrolysis. *A. aranciacus* eggs were microinjected with PH-GFP (150 µM, pipette concentration) that specifically binds to PIP2 on the inner leaf of plasma membrane. (A) Configuration of the fluorescent probe for PIP2. (B) A confocal microscopic image and the transmission view of the egg showing the specific localization of PH-GFP on the plasma membrane. (C) Changes of the fluorescence level on the plasma membrane following the exposure of the eggs to 6 µM LAT-A or 0.1% DMSO (control). Relative scores at each time point were averaged from 4 eggs for each treatment: filled circles (eggs exposed to LAT-A) and open squares (control eggs treated with DMSO). Error bars indicate standard deviation (n=4). (D) Quantification of intracellular $InsP_3$ by ELISA (collaboration with Prof. UH Kim in Korea). Eight batches of *A. aranciacus* eggs were incubated with 6 µM LAT-A (triangles) or 0.1% DMSO (control, closed squares), and aliquots were collected for analysis at 5 min intervals. Eggs immediately before adding drug was set as t=0. Post-hoc analysis: $^{**}P<0.01$ in comparison with the values at t=0.

a2. Actin-based control of sperm entry

The notion that monospermic sperm entry is guided by coordinated reorganization and movement of suboolemmal actin filaments following sperm fusion is currently being tested in the experimental models using sea urchin eggs exposed to nicotine, low sodium seawater, and overripe starfish eggs.

a3. Roles of ‘sperm actin’ in the induction of fertilization Ca^{2+} and egg penetration

In the spermatozoa of echinoderms, formation of the acrosomal process involves extensive actin polymerization. Our ongoing experiments indicate that a brief pulse expose of sea urchin sperm (*P. lividus*) to LAT-A prolonged the time interval between the cortical flash and the generation of the Ca^{2+} wave in fertilized eggs. This result is similar to the ones obtained with the eggs pre-exposed to LAT-A and cytochalsin B (Chun *et al.*, 2014). Interestingly, fluorescent polyamine provided by Prof. Jean-Marie Lehn effectively stained the head, tail, and acrosomal processes of starfish and sea urchin spermatozoa. Combined with other methods of staining egg actin filaments (Life-Act and SiR Actin), the trace of the sperm-borne actin is to be studied in the fertilized eggs.

a4. Search for the upstream signaling molecules for actin dynamics

Actin filaments in the suboolemmal region are readily reorganized following the hormonal stimulation (maturing oocytes) and the fusion with fertilizing sperm. However, the signaling pathway leading to that has not been much explored. To date, our experimental strategy for altering actin dynamics was to use actin drugs or actin-binding proteins, but we plan to dissect the components in the upstream pathway that modulate the actin cytoskeletal changes. The potential candidates include Rho, Rac, and cdc42 (Rho family), LIM kinase, ARP2/3 complex proteins, and Exportin 6, whose transcripts are found in the recently assembled *A. aranciacus* transcriptome in moderate abundance. Microinjection of bacterially expressed target domains of these proteins will be carefully designed and analyzed in pursuit of their effects on Ca^{2+} signaling and development.

a5. Roles of suboolemmal vesicles in Ca^{2+} signaling

As a follow-up study of Vasilev et al 2012, the potential role of suboolemmal acidic vesicles has been examined in the context of fertilization Ca^{2+} signaling. We found that treatment with lysosome-disrupting cathepsin C substrate, GPN, led to Ca^{2+} spill in the cytosol of sea urchin eggs, which does not develop into a wave. When fertilized, the GPN-pretreated eggs displayed compromised cortical flash but nearly normal global Ca^{2+} wave. When the cortical granules and vesicles were simply dislodged away from their natural positions near the plasma membrane by a milder treatment with procaine, urethane, and NH_4Cl, the cortical flash was also compromised. While it is not clear how changes of the internal structures like acidic vesicles can influence the Ca^{2+} influx through L-type channels, all these treatments also change the morphology of microvilli. Thus, the collected data will be interpreted also in view of the actin-based modulation of plasma membrane microvilli.

b. Characterization of morphological, molecular and structural events underlying gamete fertilization competence in new model species (Tosti)

Within the partnership to the Flagship research program of the SZN, ModRes (Dr. V. Zupo, PI) we will characterize morphological and functional parameters of oocytes and spermatozoa collected from new model species. Mainly, we will identify different maturation and growth stages by comparing the pattern of voltage dependent currents on the plasma membrane. We will also evaluate the sperm quality parameters that are functional to the fertilizing capability. The main processes of oocyte-sperm interaction, such as fertilization and the following embryo development, will be also investigated by performing *in vitro* fertilization and timing the embryo developmental stages up to the larval formation.

5. Publications (2012-2017 *corresponding author)

2017

Musacchia F, Vasilev F, Borra M, Biffali E, Sanges R, Santella L, Chun JT*. *De novo* assembly of a transcriptome from the eggs and early embryos of *Astropecten aranciacus*. ***PLoS One*** (2017) 12: e0184090.

Tosti E, Boni R, Gallo A*. μ-Conotoxins Modulating Sodium Currents in Pain Perception and Transmission: A Therapeutic Potential. ***Mar. Drugs***. (2017) 15: 295.

2016

Ruocco N, Costantini M*, Santella L*. New insights into negative effects of lithium on sea urchin Paracentrotus lividus embryos. ***Sci. Rep***. (2016) 6: 32157.

Tosti E*, Boni R, Gallo A. Ion currents in embryo development. ***Birth Defects Research Part C: Embryo Today: Reviews***. (2016) 108: 6-18.

Tosti E*. Menezo Y. Gamete activation: basic knowledge and clinical applications: ***Hum. Repr. Update***. (2016) 22(4):420-439.

Fortunato A, Boni R, Leo R, Nacchia G, Liguori F, Casale S, Bonassisa P, Tosti E.* Vacuoles in sperm head are not associated with head morphology, DNA damage and reproductive success. ***Reprod. BioMed. Online*** (2016) 32: 154-161.

2015

Gallo A, Tosti E*. Ion currents involved in gamete physiology. ***Int. J. Dev. Biol***. (2015) 59: 261-270.

Limatola N, Chun JT*, Kyozuka K, Santella L. Novel Ca^{2+} increases in the maturing oocytes of starfish during the germinal vesicle breakdown. ***Cell Calcium***. (2015) 58: 500-510.

Santella L, Dale B*. Assisted yes, but where do we draw the line? ***Reprod. Biomed. Online***. (2015) 31: 476-478.

Santella L*, Limatola N, Chun JT. Calcium and actin in the saga of awakening oocytes. ***Biochem. Biophys. Res. Commun***. (2015) 460: 104-113.

Yazaki I*, Tsurugaya T, Santella L, Chun JT, Amore G, Kusunoki S, Asada A, Togo T, Akasaka K. Ca^{2+} influx-linked protein kinase C activity regulates the β-catenin localization, micromere induction signaling and the oral-aboral axis formation in early sea urchin embryos. ***Zygote*** (2015) 23: 426-46.

2014

Bezzaouia A, Gallo A, Silvestre F, Tekaya S, Tosti E*. Distribution pattern and activity of mitochondria during oocyte growth and maturation in the ascidian *Styela plicata*. ***Zygote*** (2014) 22: 462-469.

Chun JT*, Limatola N, Vasilev F, Santella L. Early events of fertilization in sea urchin eggs are sensitive to actin-binding organic molecules. ***Biochem. Biophys. Res. Commun***. (2014) 450: 1166-1174.

Lennarz WJ*, Santella L*. Introduction. Fertilization and early development. ***Biochem. Biophys. Res. Commun***. (2014) 450: 1133-1134.

2013

Chun JT*, Vasilev F, Santella L. Antibody against the actin-binding protein depactin attenuates Ca^{2+} signaling in starfish eggs. ***Biochem. Biophys. Res. Commun***. (2013) 441: 301-307.

Tosti E*, Boni R, Gallo A, Silvestre F. Ion currents modulating oocyte maturation in animals. ***Systems Biol. in Reprod. Med***. (2013) 59: 61-68.

Gallo A, Russo GL, Tosti E *. T-type Ca^{2+} current activity during oocyte growth and maturation in the ascidian Styela plicata. ***PLoS One***. (2013) 8: 54e604.

2012

Santella L*, Vasilev F, Chun JT. Fertilization in echinoderms. ***Biochem. Biophys. Res. Commun***. (2012) 425: 588-594.

Junca A, Gonzalez Marti B, Tosti E, Cohen M, De la Fontaine D, Benkhalifa M, Ménézo Y*. Sperm nucleus decondensation, hyaluronic acid (HA) binding and oocyte activation capacity: different markers of sperm immaturity? Case reports. ***J. Ass. Repr. Genet***. (2012) 29:353–355.

Silvestre F. Fissore RA, Tosti E, Boni R.* Ca^{2+} rise at *in vitro* maturation in bovine cumulus-oocyte complexes. ***Mol. Repr. Dev***. (2012) 79: 369-379.

Vasilev F, Chun JT, Gragnaniello G, Garante E, Santella L*. Effects of ionomycin on egg activation and early development in starfish. ***PLoS One***. (2012) 7: e39231.

6. List of grants

- **MIUR Progetti Premiale: StarTregg** "Molecular mechanisms controlling fertilization in *Astropecten aranciacus* starfish eggs" (2013 –2014). PI: L. Santella.
- **Flagship Research Programs: ModRes** “New model organisms for the scientific research: culture, ecology, physiology and genomic characterization” (2017- 2018). PI: V. Zupo.

7. List of external collaborators

-Raffaele Boni, University of Basilicata, Potenza, Italy

-Uh-Hyun Kim, Chonbuk National University Medical School, Jeonju, Korea

-Keiichiro Kyozuka, Tohoku University, Aomori, Japan

-Jean-Marie Lehn, Inst. de Science et d’Ingénierie Supramoléculair, Strasbourg, France

-Yves Menezo, London Fertility Associates, London, UK

5. Genomics to understand the molecular basis of evolution

1. People involved

Name	*Position*	*Funding*	*Period*
Elio Biffali	Staff scientist	SZN	since1994
Marco Borra	Staff scientist	SZN	since 2012
Maria Costantini	Staff scientist	SZN	since 2010
Salvatore D'Aniello	Staff scientist	SZN	since 2010
Pasquale De Luca	Staff scientist	SZN	since 2009
Giuseppe D'Onofrio	Staff scientist	SZN	since 1989
Maria I. Ferrante	Staff scientist	SZN	since 2010
Graziano Fiorito	Staff scientist	SZN	since 1983
Annamaria Locascio	Staff scientist	SZN	since 2009
Francesco Paolo Patti	Staff scientist	SZN	since 2010
Remo Sanges	Staff scientist	SZN	since 2010
Paolo Sordino	Staff scientist	SZN	since 2000
Sergio Stefanni	Staff scientist-Term contract	SZN	since 2016
Elijah K. Lowe	Postdoctoral fellow	Corbel	2014-2017
Francesco Musacchia	Postdoctoral fellow	StarTregg/RITMARE	2012-2016
Giovanna Ponte	Postdoctoral fellow	SZN	2013-2017
Ilaria Zarrella	Postdoctoral fellow	MolEcOC	2012-2013
Giovanni Annona	PhD student	SZN	2012-2015
Swaraj Basu	PhD student	SZN	2011-2014
Emanuela Buschi	PhD student	SZN/UNIVPM	2016-2019
Ugo Coppola	PhD student	SZN	2014-2017
Celestina Mascolo	PhD student	SZN	2015-2018
Giuseppe Petrosino	PhD student	SZN	2012-2016
Andrea Tarallo	PhD student	SZN	2013-2016
Quirino A. Vassalli	PhD student	SZN	2013-2017
Massimiliano Volpe	PhD student	SZN	since 2015
Erik Garrison	Guest PhD student	Cambridge University	since 2016
Maria L. Chiusano	Affiliated scientist	University of Naples	since 2016
Alberto Pallavicini	Affiliated scientist	University of Trieste	since 2017
Marco Taviani	Affiliated scientist	ISMAR-CNR	since 2016
Cinzia Verde	Affiliated scientist	BBR-CNR	since 2016

2. Background, aims and objectives

Our research interests are centered on genomes and transcriptomes of marine organisms, their composition, complexity, plasticity, conservation and involvement in determination of adaptations. In agreement with the legacy of SZN, everything we do is done from an evolutionary perspective. Exploiting and contributing to the development of cutting-edge genomics technologies, we aim at expanding our knowledge on the molecular determinants of evolutionary transitions that allowed living organisms to colonize the most diverse environments producing an astonishing

diversification. The existence of reference genomes and transcriptomes for models used in our research is propaedeutic to high impact findings and therefore we are involved in sequencing, assembly, annotation and data mining of genomes and transcriptomes of diverse species. Given that comparisons among species are at the basis of evolutionary studies, the exploitation of functional genomics approaches allow us to execute comparisons at the level of entire genomes permitting the observation of evolutionary patterns at global scales.
The following lines characterize our research interests:

a. Phylogeny, population genetic and adaptation to the deep (Stefanni)

We aim at understanding distribution patterns and connectivity among species from littoral to deep-sea areas. The primary research interests focus on phylogeography as well as phylogeny for closely related species.

b. Genomic compartmentalization and evolution (Costantini)

Many genomes appear to be mosaics of isochores, megabase-size DNA sequences that are endowed with fairly homogeneous base composition (Costantini 2015a,b; Costantini and Musto 2017). Isochores are distributed in a small number of families that cover a broad compositional range characterized by different average GC levels, gene concentration, chromatin structures, replication timing and distribution of mobile elements. We aim at understanding how far back in evolution the compartmentalized organization of the eukaryotic genomes arose and how this impacted the evolutionary integration of mobile elements.

c. Complex evolutionary scenarios of key gene families in Metazoans (S. D'Aniello)

We are interested in the evolutionary history of specific gene families involved in development, nervous system functioning, metabolism, signaling and fertilization. Our interest so far has been focused towards the following families: opsins, selenoproteins and Hox.

d. Genomes, transcriptomes, variations and the evolution of complexity (Sanges)

Transposable elements (TEs) are genomic elements that duplicate in different positions within the genome mediating variations. Although mutagenic, they have provided fresh genomic matter that was shaped by host genomes to evolve genetic novelties. Researchers are beginning to demonstrate that there is a link between the complexity of an organism and the TEs content of its genome. TEs have been shown to mediate somatic variations in the brain. We couple bioinformatics, comparative and functional genomics approaches together within collaborative efforts with researchers involved in field- and bench-work. Because of recent findings showing that TEs are not simply junk DNA, our focus on regulatory and gene expression developmental dynamics, long noncoding RNAs (lncRNAs) and brain functioning has now extended towards the aim to understand the contribution of TEs to the evolution of these features and of complexity.

e. Identification and characterization of conserved non-coding elements (Locascio)

Regions from several dozen to several hundred base pairs of extreme conservation have been found in non-coding regions of all metazoan genomes. The distribution of these elements (CNE) has suggested that many have roles as transcriptional regulatory elements. We develop new comparative approaches to facilitate the identification of CNEs among divergent species and to experimentally demonstrate their functional significance.

f. Mitogenomic analysis of Sparidae fishes (Sordino)

Mitochondrial DNA is a species identity marker to study evolution and to prevent fraudulent substitutions in market products especially regarding fishes. The analysis of the complete mtDNA allows the exploration of alternative mitochondrial barcoding regions and the investigation of evolutionary patterns.

g. Relationships between genotype and phenotype (D'Onofrio)

We aim at identifying whether the composition of metazoan genomes has an impact on the metabolic phenotypes lifestyles of different species.

h. Technological development (Sanges, Sordino, Stefanni)

In order to expand and adapt our research to non-model or emerging model organisms and trying to answer specific questions not easily addressable with standard protocols we are actively involved in designing and developing new approaches taking advantage of functional genomics platforms.

3. Results

a. Phylogeny, population genetic and adaptation to the deep (Stefanni)

New zoantharians associated with cold-water corals (**Carreiro Silva *et al.*, 2017**) as well as the associated fishes (**Gomes-Pereira *et al.*, 2017**) have been described in the Atlantic deep-sea. Other studies contributed to provide further insights on the genetic structure and role of the Strait of Gibraltar on Chondrichthyes (**Catarino *et al.*, 2017**a) and Teleosts (**Catarino *et al.*, 2017b**).

b. Genomic compartmentalization and evolution (Costantini)

By studying the compositional organization of the genomes from unicellular eukaryotes (including marine algae and diatoms) with available genomes, our analysis revealed that their average GC range distribution is very broad (as broad as that of prokaryotes) and individual compositional patterns cover a very broad range from very narrow to very complex. Our analysis also permitted to better understand the dynamics of retroelement insertions in isochore families. We demonstrated that 1) the open state of chromatin structure plays a crucial role in allowing not only the initial integration of retroviral sequences but also that of the youngest retroelement sequences, and 2) that the distribution of old retroelement can be explained as due to insertions being unstable in the GC-poor isochores but stable in the compositionally matching GC-rich isochores, again in line with what happens in the case of retroviral sequences (**Costantini *et al.*, 2013; Costantini *et al.*, 2016; Costantini *et al.*, 2017**).

c. Complex evolutionary scenarios of key gene families in Metazoans (S. D'Aniello)

We provided new insights into opsin-based photoreception and photoreceptor cell evolution of a major deuterostome clade, the Ambulacraria. Systematic data analysis, including for the first time hemichordate opsin sequences and an expanded echinoderm dataset, led to a robust opsin phylogeny for this superphylum. In total, 119 ambulacrarian opsin sequences were found, 22 new sequences in hemichordates and 97 in echinoderms (including 67 new sequences). Our findings corroborate the presence of all major ancestral bilaterian opsin groups in Ambulacraria, and importantly, we identified two opsin groups specific to echinoderms that need to be studies in deep in future (**D'Aniello *et al.*, 2015**).

Selenoproteins are proteins that incorporate selenocysteine (Sec), a nonstandard amino acid encoded by UGA, normally a stop codon. Sec synthesis requires the enzyme Selenophosphate synthetase (SPS), conserved in all prokaryotic and eukaryotic genomes encoding selenoproteins. We studied the evolutionary history of SPS genes, providing a map of selenoprotein function spanning the whole tree of life. Parallel duplications and subsequent convergent subfunctionalization have resulted in the segregation to different loci of functions initially carried by a single gene. This evolutionary history constitutes a remarkable example of emergence and evolution of gene function (**Mariotti *et al.*, 2015**).

Hox genes retain similar roles during the development of animals as evolutionarily distant as humans and flies. During the last two decades, reports on Hox genes from a still growing number of eumetazoan species have increased our knowledge on the Hox gene contents of a wide range of animal groups. We reported the current Hox inventory among deuterostomes, not only in the well-known teleosts and tetrapods, but also in the earlier vertebrate and invertebrate groups. We draw an updated picture of the ancestral repertoires of the different lineages. This scenario allows us to infer differential gene or cluster losses and gains that occurred during deuterostome evolution, which might be causally linked to the morphological changes that led to these widely diverse animal taxa. Finally, we focused on the challenging family of posterior Hox genes, which probably originated through independent tandem duplication events at the origin of each of the ambulacrarian, cephalochordate and vertebrate/urochordate lineages (**Pascual-Anaya *et al.*, 2013**).

d. Genomes, transcriptomes, variations and the evolution of complexity (Sanges)

We have used genomic conservation, synteny and transcriptomic as an instrument to identify functional coding and noncoding portions of the genome in different species and developmental stages. We have characterized the conservation and developmental activities of conserved noncoding regions and lncRNAs (Basu et al, 2013). Transposable elements resulted to be an important potential player for both these types of noncoding regions. Indeed, by sequencing the developmental transcriptome of *Tetraodon nigroviridis* we identified a set of lncRNAs enriched for specific transposable elements that could act as functional domains (Basu et al, 2016). In addition, we identified for the first time the existence of conserved noncoding regions shared between vertebrates and tunicates, these are enriched for ultraconserved elements, are active as transcriptional enhancers during the development and can be transcribed therefore belonging to the class of enhancer-RNAs. Surprisingly, they are shuffled between the two groups of species making retrotransposition a possible mechanism at the basis of their evolution (**Sanges *et al.*, 2013**), (Figure 1).

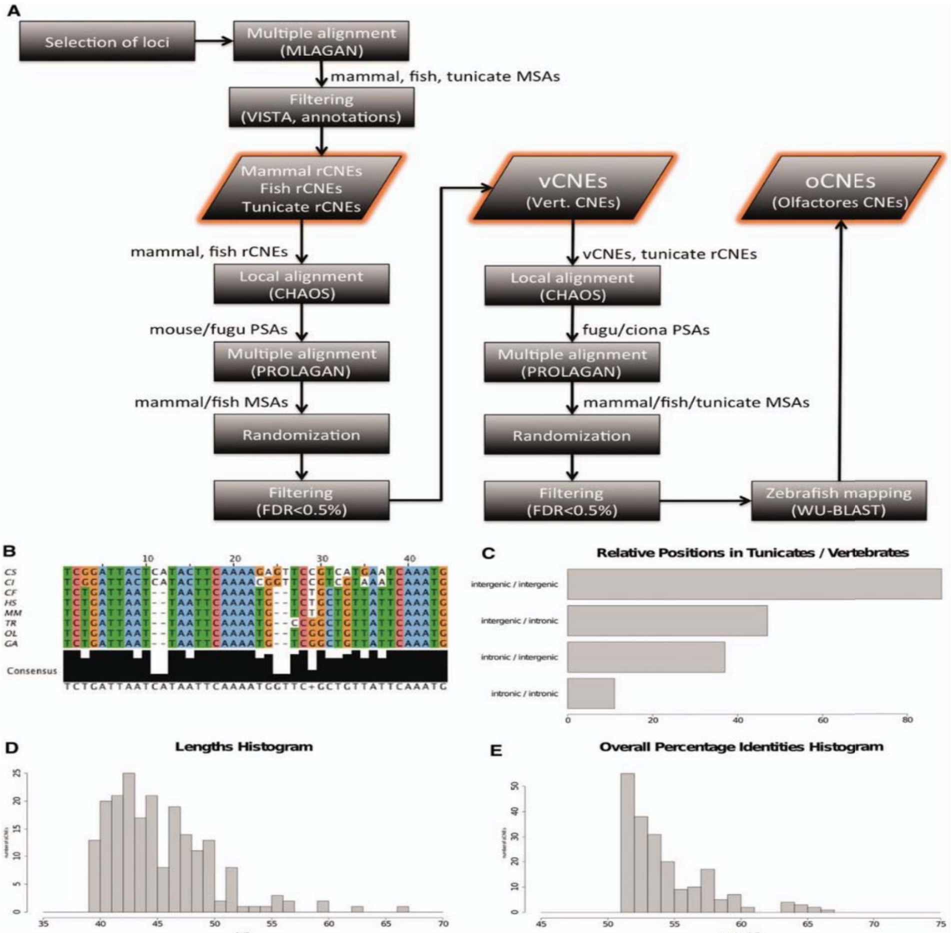

Figure 1. A) Pipeline for the identification of conserved regions. B) Example of a conserved region. C) Reciprocal location of the transcribed enhancers between vertebrates and tunicates. Adapted from Sanges et al, 2013.

We have also investigated variations and variability at the basis of life into the wild. Over the last five years we have assembled, annotated and analysed the genome and the transcriptomes of different emerging model organisms contributing to the establishment of new models. Among them we have published the genome of the diatoms *Pseudo-nitzschia multistriata* and *Fragilariopsis cylindrus* (**Mock *et al.*, 2017**) and a cyanobacterium (**Dvořák *et al*., 2014**). We contributed with both analysis and transcriptomic data from *Pseudo-nitzschia multistriata* to the international consortium for the assembly and annotation of the genome of *Fragilariopsis cylindrus*, a polar diatom. Our contribution was crucial for discovering that about 20% of the genes in the polar diatom present very divergent alleles. These divergent alleles are conserved in the population and among individuals and functional and expression analysis demonstrated that their retainment is needed to thrive with highly diverse conditions such as those occurring at the pole over the year. The diatom is indeed capable to specifically switch the expression of specific alleles in response to different environmental conditions (Mock *et al.*, 2017).

e. Identification and characterization of conserved non-coding elements (Locascio)

A combination of different methodologies, based not only on high sequence identity, can collectively be used to identify CNEs with regulatory activity in phylogenetically distant species. For our analysis, we selected *Homo sapiens* and *Danio rerio* for vertebrates, *Ciona intestinalis* and *Ciona savignyi* for urochordates and *Branchiostoma floridae* for cephalochordates. A low sequence constraints approach was used to search orthologous chordate gene regions for cross-species conserved regulatory elements that control developmental genes. To test if these different bioinformatics parameters could be useful to identify CNEs between tunicates and other chordates, we, first of all, searched various chordate genomes for the CNEs present in the gene loci of several homeobox-containing genes with a functional role during embryonic development. Out of ten analysed genes, we identified six putative CNEs present in all the examined chordate species. Then, we analysed the cis-regulatory activity of these newly identified CNEs in *Ciona intestinalis* transgenic embryos. We obtained positive results with the Hox5, Ipf1, and Otx Ciona CNEs. Once demonstrated the cis-regulatory activity of these Ciona enhancers, we tested their cross-species functional conservation in Ciona and zebrafish transgenic embryos obtaining positive result with the Hox5 CNEs in both chordate species (**Vassalli *et al.*, 2015**), (Figure 2).

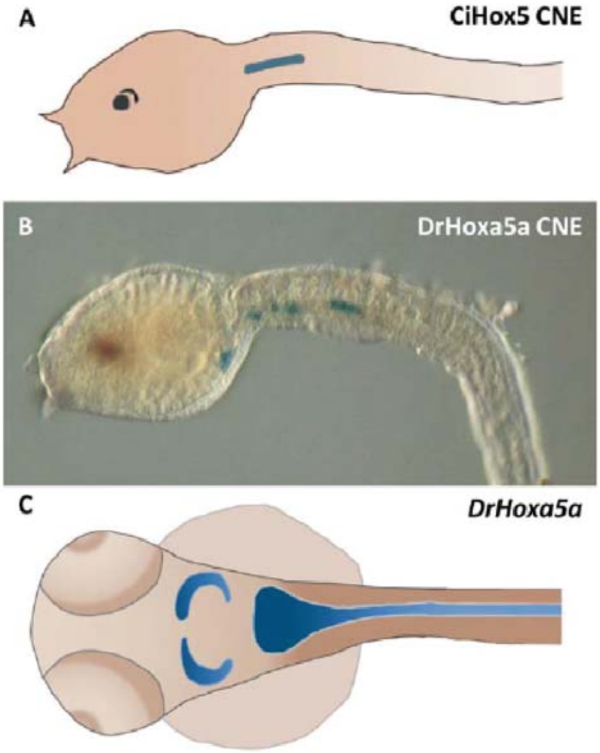

Figure 2. Cross-species regulatory activity of the Ciona Hox5 and zebrafish Hoxa5a CNEs in Ciona embryos.

f. Mitogenomic analysis of Sparidae fishes (Sordino)

The Sparidae family is among the most valuable fish resources in the world and it is the most diverse coastal family of Sparoida. Although several phylogenetic analyses were conducted based on specific molecular markers, their classification remains unresolved. We determined the mitogenome of *Pagellus erythrinus* and *Dentex gibbosus*, finding that the organization of mtDNA fits well with that of the common vertebrate mitogenome. Our study confirms that the analysis of the entire mitochondrial DNA sequence is a primary key step to 1) understand sparid fish phylogeny, 2) identify barcoding markers and 3) investigate mtDNA evolution (**Mascolo *et al.*, in press**).

g. Relationships between genotype and phenotype (D'Onofrio)

The research aim of the last years was devoted to fully demonstrate the correlation between the metabolic phenotype (i.e. the routine metabolic rate) versus the genome phenotype (i.e. the molar ratio of the genomic GC content). The analysis of the lifestyle of organisms belonging to different taxa (both invertebrates and vertebrates) strongly supported the above-mentioned correlation (**Tarallo *et al.*, 2016**).

h. Technological development (Sanges, Sordino, Stefanni)

We also contributed to technological development releasing bioinformatics tools to annotate new transcriptomes also identifying lncRNAs (**Musacchia *et al.*, 2015**) as well as developing new protocols to perform coinjection in zebrafish to study the developmental function of enhancers (**Roberts *et al.*, 2014**) and to efficiently produce ready to sequence mitochondrial DNA from emerging model organisms (**Mascolo *et al.*, 2017**).

All the described research was mainly supported by SZN FOE and by RITMARE Flagship Project, Premiale StarTrEgg and Premiale MolEcOC.

4. Perspectives

a. Phylogeny, population genetic and adaptation to the deep (Stefanni)

Our interest is focused on fishing pressure, climate change, human impact that might be linked to changes in distributional shifts, induce new adaptation responses and alter interaction on ecological niches. This task is tackled using multidisciplinary approaches integrating data from batteries of molecular markers to traditional taxonomy and ecology of the species. In addition, we are contributing to the transcriptome research activities exploring genes that are associated to the adaptation to extreme environments. This is continuation of studies started before being enrolled at SZN and includes the use of fish from deep-sea and mussels from the hydrothermal vents . We have also started the investigation of the diversity and functions of microbial assemblages (i.e., bacteria, archaea and eukaryotes such as fungi) associated with the deep-sea benthic taxa (from meio- to megafauna) by using metagenetics and metatranscriptomics. This line of research has started thanks to the 3 years project DEMBAI "Diversity and Evolution of marine Microbial assemblages associated with Benthic Antarctic Invertebrates" funded the Italian Ministry of Education (MIUR) under the National Antarctic Programme (PNRA16_00173). It also sees the contribution by the PhD student Emanuela Buschi (co-funded SZN–Polytechnic University of Marche, Italy) started in 2016.

b. Genomic compartmentalization and evolution (Costantini)

We will aim to further extend to other marine organisms (for which the assembled genome sequences will be available), the study on compositional compartmentalization and epigenetic modifications of their genomes.

c. Complex evolutionary scenarios of key gene families in Metazoans (S. D'Aniello)

We are working on the evolution of Nitric Oxide Synthase family in novel teleosts genomes (*Lepisosteus oculatus* and *Amia calva*) and cyclostomes (lamprey *Lampetra japonicum* and hagfish *Eptatretus burgeri*). In addition, we are surveying metazoan genomes to study Rab proteins (Ras-related in brain), a family of small GTPases known as key players in the control of membrane transport in Eukarya kingdom. We are surveying the genome of 11 metazoans: *Nematostella vectensis*, *Caenorhabditis elegans*, *Capitella teleta*, *Lottia gigantea*, *Saccoglossus kowalevskii*, *Strongylocentrotus purpuratus*, *Branchiostoma lanceolatum*, *Ciona robusta*, *Oikopleura dioica*, *Anolis carolinensis*, *Homo sapiens*. Finally, we are working a project called PROTARA (PRoject Opsin TARA) in search of new and ancestral light-sensitive opsins from the Oceans to infer evolutionary insights. We are systematically collecting potential Opsins by blasting against the Meta transcriptome (MetaT) of the international TARA OCEANS project. The finding of novel putative functional opsins from unexplored environment could tell us about how this specific sub-family of G protein–coupled receptors (GPCRs), also known as seven-transmembrane domain receptors, was selected for the fundamental light sensing capability.

d. Genomes, transcriptomes, variations and the evolution of complexity (Sanges)

Broadening our focus on TEs and their involvement in the evolution of complexity, we have identified an active transposable element active in the portions of the *Octopus vulgaris* brain associated to cognitive capabilities. Computational analysis exploiting the already sequenced genome of the *Octopus bimaculoides* and gene expression transcriptomic data from different tissues of the same individual allowed us to find the homolog of the *Octopus vulgaris* element and demonstrate the ability of this element in mediating somatic retrotransposition into the brain.

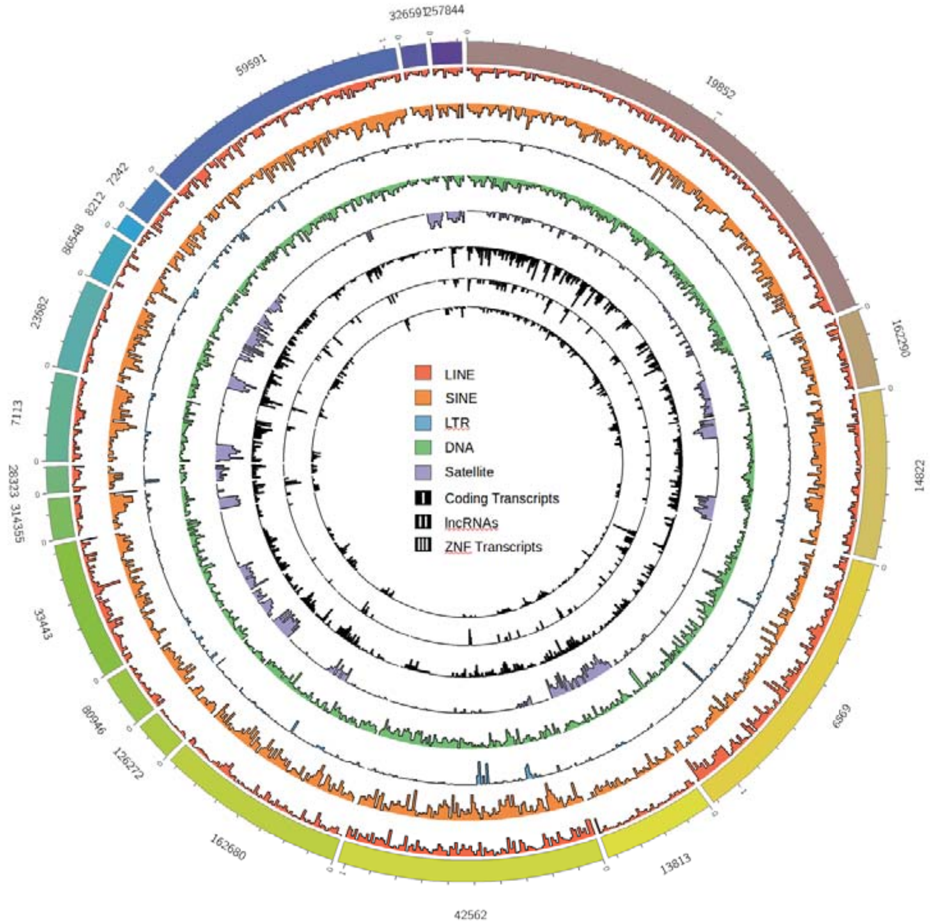

Figure 3. Circos showing the relationship between satellite DNA, TEs and genes divided in protein coding, lncRNAs and zinc fingers (ZnF) in the scaffolds of the *Octopus bimaculoides* genome containing ZnF clusters.

In addition, we are analysing the genomic distribution of TEs and functional domains in relation to coding and noncoding genes; our preliminary observation show a non-random pattern (Figure 3). We are also working at identifying how variations between individuals have impact on the expression of flanking genes. Our results on diatom genomes show that the promoters of diatom genes are significantly enriched for short tandem repeats with specific sequence composition and suggest that these genomic elements might be important in the regulation of transcription of downstream genes (Figure 4). Finally, we are sequencing the genome of the *Octopus vulgaris* as well as we are collaborating to the genome sequencing projects of different marine species such as the tunicates *Phallusia mammillata* and *Halocynthia roretzi* and the diatom *Skeletonema marinoi*.

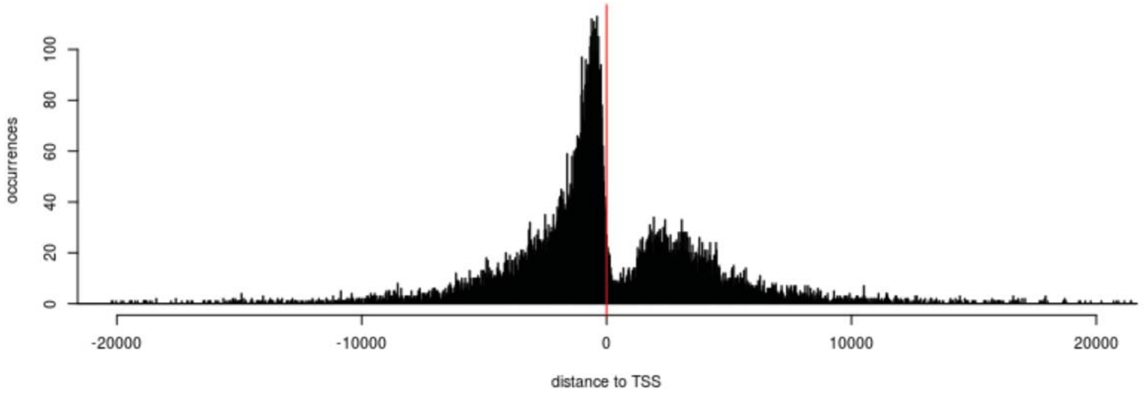

Figure 4: Genomewide distribution of a specific short tandem repeat motif with respect to the transcriptional start sites (TSS) in the genome of a diatom.

e. Identification and characterization of conserved non-coding elements (Locascio)

Eight tunicate genomes have been published in the last years, including one solitary thaliacean *Oikopleura dioica* and seven ascidian species: three solitary phlebobranch Ciona species; three solitary stolidobranch Molgula species; and the colonial stolidobranch *Botryllus schlosseri*. Five additional genomes have been sequenced, including two solitary stolidobranch Halocynthia species and two solitary phlebobranch Phallussia species that are currently in assembly, as well as the draft genome of the colonial aplousobranch *Didemnum vexillum*. We are using the CNEs of *Ciona intestinalis* that we previously characterized in comparison with these other tunicate genomes in order to improve our computational approach and demonstrate its usefulness in the comparison of highly divergent or extant models. This analysis will provide key resources for further investigations into the evolution of gene regulation among Tunicates and even more among chordates.

f. Mitogenomic analysis of Sparidae fishes (Sordino)

We are sequencing the mitochondrial genomes of several sparid species in our laboratory.

g. Relationships between genotype and phenotype (D'Onofrio)

We are extending our analysis to invertebrate chordate genomes, as well as pre-duplicative fishes, in order to understand the frame about the emergence of the evolutive scenario we have observed in vertebrates.

h. Technological development (Sanges, Sordino, Stefanni)

We are working at the design of an autonomous soft robot with an automated unmanned device capable of collecting samples (organisms living in the sediment or in the water column), extract their genomic material, construct DNA libraries (unless we manage to skip this step) and sequence the DNA/RNA fragments using the cutting edge High Throughput Sequencing (HTS) Oxford nanopore technology. This project sees the contribution of the PhD student Mrudul Chellapurath (co-funded SZN–Scuola Superiore Sant'Anna, Italy). In addition, we are setting up a fast and cost effective method to isolate mtDNA for NGS by comparing different methods to extract total genomic DNA and to isolate complete mtDNA for Illumina MiSeq. Our contribution is also being crucial in the development of a specific sequencing protocol capable to amplify and sequence all the integration sites for a given TE in DNA samples. Finally, we are completing the development of two bioinformatics pipelines, one to identify gene clusters in newly sequenced genomes and one to identify polymorphisms associated to TEs from whole genome sequencing data.

In the framework of the SZN Vision 2015-2025 document the Institute has expressed interest in funding an internal project named “100 Mediterranean Genomes Project”. The rationale behind this choice is in the identification, for the years 2015-2025, of the challenge to complete genomes of 100 species from the Mediterranean in order to discover and value their characteristics and potentials. This highly transversal project would hopefully also support the sequencing efforts already initiated from SZN researchers using core and/or external budget and/or contribution to external consortia to realize the reference genomes of several Mediterranean species such as *Skeletonema marinoi*, *Thalassiosira rotula*, *Posidonia oceanica*, *Cymodocea nodosa*, *Octopus vulgaris*, *Paracentrotus lividus*, *Branchiostoma lanceolatum*.

5. Publications (2012-2017; *corresponding author)

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6. List of grants

- **MIUR Premiale MolEcOC** “Approaching inter- and intra-individual variability by molecular ecology for the technology transfer of basic research on marine model organisms (Octopus vulgaris and Caretta caretta)” (2012-2013). PI: Fiorito, Hochscheid, Sanges.
- **EU INFRADEV BioMedBridges** “Building data bridges between biological and medical infrastructures in Europe” (2012-2015). PI: Sanges.

7. List of external collaborators

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-Andrea Carpentieri, University of Naples “Federico II”, Naples, Italy

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-Stefano Gustincich, Department of Neuroscience Italian Institute of Technology, Genova, Italy

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6. Ecotoxicological impact of environmental stressors on marine biota

1. People involved

Name	*Position*	*Funding*	*Period*
Giovanna Benvenuto	Staff scientist	SZN	since 2010
Maria Cristina Buia	Staff scientist	SZN	since 1989
Maria Costantini	Staff scientist	SZN	since 2010
Anna Palumbo	Staff scientist	SZN	since 1980
Giovanna Romano	Staff scientist	SZN	since 1989
Paolo Sordino	Staff scientist	SZN	Since 2000
Antonietta Spagnuolo	Staff scientist	SZN	since 1989
Elisabetta Tosti	Staff scientist	SZN	since 1989
Valerio Zupo	Staffscientist	SZN	since 1994
Enrico D'Aniello	Staff scientist-Term contract	SZN	since 2017
Immacolata Castellano	Staff scientist-Term contract	SZN	since 2016
Enrico D'Aniello	Staff scientist-Term contract	SZN	since 2017
Alessandra Gallo	Postdoctoral fellow	SZN/ABBaCo	since 2015
Rosaria Esposito	PhD student	SZN	2010-2014
Anna Lettieri	PhD student	SZN	2010-2014
Oriana Migliaccio	PhD/Postdoc	SZN/RITMARE	2012-2016
Antonio Palladino	PhD student	SZN	2014-2017
Manuela Piccardo	PhD student	SZN/Univ Trieste	2017-2020
Nadia Ruocco	PhD student	SZN	since 2015
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Giovanni Pagano	Affiliated scientist	University of Naples	since 2016
Alberto Pallavicini	Affiliated scientist	University of Trieste	since 2017
Antonio Terlizzi	Affiliated scientist	University of Trieste	since 2016

2. Background, aims and objectives

A range of environmental stressors is continuously threatening marine ecosystems. Anthropogenic activities can produce different stressors including pollution and climate changes. Chemical contamination caused by hazardous substances is one form of pollution of marine environment and a major environmental concern. A vast array of commonly used chemical contaminants, such as metals, antifoulants, nanoparticles and microplastics, found in marine environment can cause serious damage to its functioning adversely affecting marine organisms[79,80]. Effects varied according to the type of contaminant and the biological endpoints measured in sentinel species. These biological endpoints include molecular, biochemical and physiological markers (i.e. biomarkers) that are used as indicators of exposure and/or effects[81].

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Hazardous substances deriving from natural sources represent another form of marine pollution. Natural toxins as those deriving from microalgae during bloom termination demonstrated to exert adverse effects on marine organisms. The impact of these toxins on various stages of development of the sea urchin *Paracentrotus lividus* and the tunicate *Ciona robusta* (also known as *Ciona intestinalis*) was assessed. Among microalgae toxins, attention was focused on oxylipins (including polyunsaturated aldehydes [PUAs], hydroxyacids [HEPEs], and epoxyalcohols), that are end-products of a lipoxygenase/hydroperoxide lyase metabolic pathway in diatoms. Although the effect of these molecules has been extensively studied on copepods, the mechanism of action was not completely deciphered. Moreover, most of the studies have been conducted with the "model" PUA decadienal, whereas very little information is available on the other most common oxylipins produced by diatoms, the PUAs eptadienal and octadienal and the HEPEs. The molecular mechanisms underlying the effect of PUAs have also been studied in *Ciona robusta* development and larval transition.

Climate change is predicted to affect structure and functioning of marine ecosystems. The Intergovernmental Panel on Climate Change has identified four principal climate drivers such as pH, temperature, oxygen concentration and food availability. All four are subject to substantial perturbations in projections of future climate change scenarios. Literature on climate change impacts in marine environment is growing exponentially. Temperature affects physiological processes ranging from protein damage to membrane fluidity to organ function, so its increase may negatively affect marine organism performance and survival. Researches on the biological impacts of ocean acidification span a broad diversity of marine organisms and reveals an even broader range of species' responses such as decreased survival, calcification, growth, development and abundance[82].

In view of the present scenario of contamination of the marine environment, it has become an urgent need to assess the adverse effects on marine biota. Ecotoxicological studies will aim at presenting fundamental advances on the effects of chemo-physical stressors and natural toxins on marine organisms. The biological responses will be investigated at different levels of complexity from molecules to cells, from tissues to whole organisms, in order to elucidate acute and chronic toxicities, to shed light on the mechanism of toxic action at various levels of biological organizations and to identify bioassays, biomarkers and genomic tools that could be considered for use in ecological risk and natural resource damage assessment.

3. Results

The results obtained during the period of the 2012/2017 have been subdivided in two main research lines focusing on the ecotoxicological impact of chemo-physical pollutants and natural toxins on marine organisms.

Chemo-physical stressors impact: sublethal, such as reproduction, development and bacteria culturability, and lethal endpoints were evaluated.

Reproduction is the most used endpoint in ecotoxicological risk assessment. Evaluation of gamete quality in marine species is an important issue due to the increase of ecotoxicological studies looking at the impacts of marine pollutants on reproductive health.

The influence of environmental stressors on oocyte quality can be mediated through changes of electrical properties of the oocyte plasma membrane. In this line, by using an electrophysiological approach, Tosti's group evaluated the effects of heavy metals (lead and zinc) and antifouling biocides[83] (tributyltin, diuron and chlorothanoil) on oocyte physiology of the ascidian Ciona *intestinalis* (**Gallo, 2017; Gallo and Tosti, 2013; Gallo and Tosti, 2015**). The impact of these contaminants on sodium currents and fertilization events was evaluated. Results showed that all tested compounds reduced in concentration dependent manner sodium current amplitude while some of them affected fertilization current (FC). In particular, tributiltin reduced FC frequency and

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[82] Gallo A, Tosti E. Adverse Effect of Ocean Acidification on Marine Organisms. ***J. Marine Sci. Res. Dev***. (2016) 6: e139.

[83] Gallo A, Tosti E. Antifouling Compounds Endangering Marine Invertebrates Reproduction. ***J. Marine Sci. Res. Dev***. (2015) 5: e133.

amplitude, whereas cholorothanoil was shown only to decrease FC amplitude. Among the two tested metals, only zinc exerted an adverse effect by totally inhibiting FC. Furthermore, TBT and zinc affected oocyte competence by causing a significant decrease of fertilization rate, whereas offspring quality was impaired by TBT and chlorothalonil that induced malformations of the tails and absence of sensory organ pigmentation.

Sperm quality is a measure of the ability to successfully fertilize an oocyte and subsequently to allow the development of normal embryo. Tosti's group developed a rapid and sensitive method to evaluate different parameters strictly related to fertilizing capability to assess sperm quality impairment after exposure to emerging contaminants such nanoparticles (NPs) and stress by temperature increase. By using a combination approach of florescent probe and spectrofluorimetric analysis it has demonstrated that NPs affected sperm quality and consequently fertilizing capability of marine animals (Figure 1**; Gallo *et al***.**, 2017 in press**). Exposure of *Ciona robusta* spermatozoa to nickel NPs (Ni NPs) generated oxidative stress, which induces lipid peroxidation and DNA fragmentation, and altered mitochondrial activity and morphology. Furthermore, Ni NPs exposure reduced fertilization rate and caused developmental anomalies in the offspring (**Gallo et al, 2016**).

This in-depth investigation shed light on the specific reprotoxicity pathways expanding the perspective on the potential role of the ascidian *C. robusta* as a bioindicator of marine pollution levels and seawater quality[84].

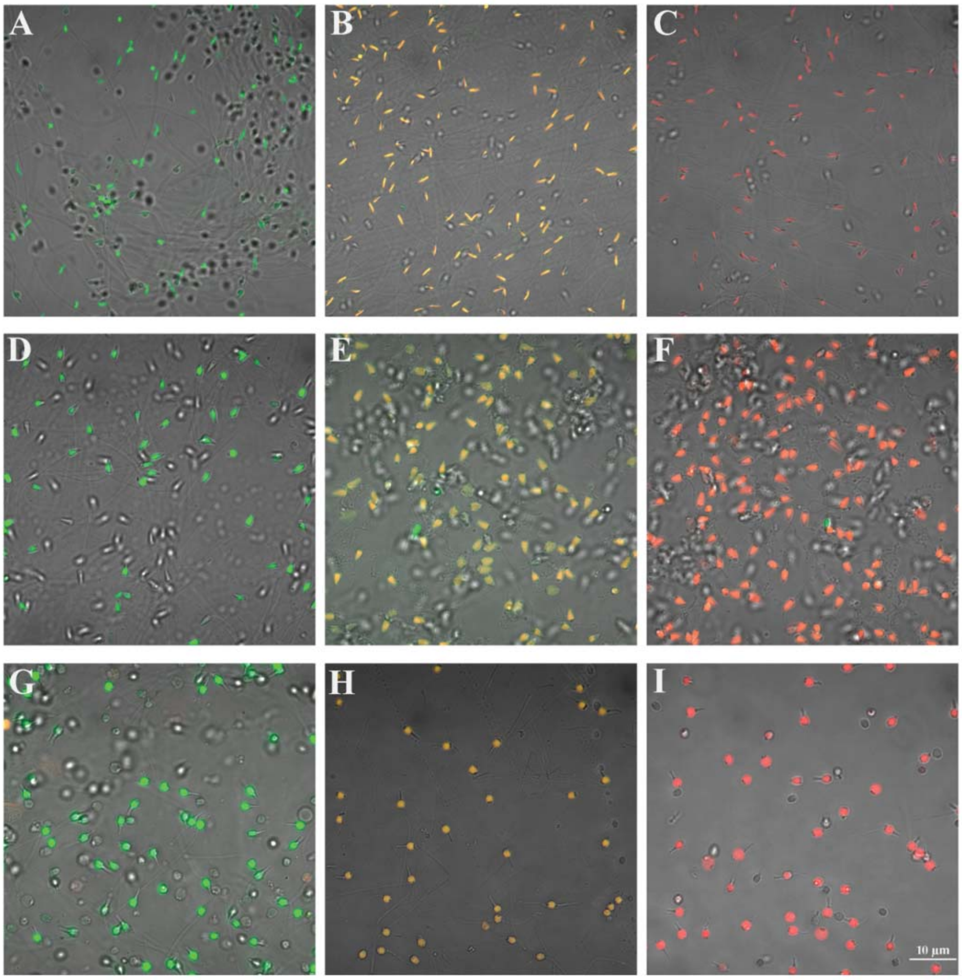

Figure 1. Fluorescence images of *Ciona robusta* (A, B, C), *Paracentrotus lividus* (D, E, F) and *Mytilus galloprovincialis* (G, H, I) spermatozoa dual stained with SYBR-14 and propidium iodide (PI). SYBR-14 stained the live spermatozoa with intact plasma membrane (A, D, G) fluoresced bright green; PI stained dead ones with disrupted plasma membrane fluoresced red; and dying spermatozoa doubly staining with both dyes showed yellow/orange fluorescence.

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[84] Gallo A, Tosti E. The ascidian *Ciona intestinalis* as model organism for ecotoxicological bioassays. ***J. Marine Sci. Res. Dev***. (2015) 5: e138.

In the sea urchin *Paracentrotus lividus*, CuO NPs has been shown to induce spermiotoxicity by affecting fertilization success (**Rotini *et al.*, 2017 in press**); this research was supported by NanoBioTech Ambient Project.

Water temperature is the most pervasive environmental factor to influence gamete quality of marine invertebrates. Global warming are causing the oceans to warm so understanding the impacts of rising temperature on sperm quality is an important matter of investigation. Tosti's group found a strong evidence for sperm quality impairment in response to paternal exposure to thermal stress in the mollusc *Mytilus galloprovincialis*. Temperature increase reduced sperm concentration while increase motility, it also affected mitochondrial activity and lipid peroxidation. Furthermore, DNA fragmentation as well as morphological abnormalities after long-term exposure has been observed (Figure 2**; Boni *et al.*, 2016**).

All together, these researches demonstrated that marine animal gametes are highly sensitive to different environmental insults supporting the use of gamete quality parameters as new potential biomarkers in ecotoxicological risk assessment[85].

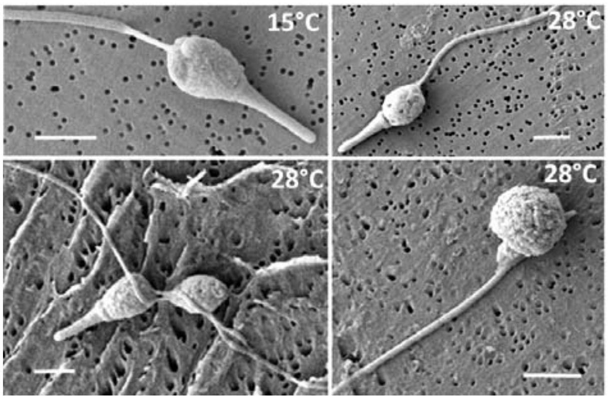

Figure 2. Scanning electron microscopy of *Mytilus galloprovincialis* spermatozoa by parental exposure to thermal stress. After long-term exposure to 28 °C spermatozoa were characterized by asymmetrical tails, globular head and absence of acrosome. Scale bars are 1.5 mm.

Embryo development is recognized as suitable endpoint to assess the biological effects of contamination by heavy metals. Mercury (Hg) is one of the most toxic and persistent elements in the environment, deriving both from natural and anthropogenic sources. Romano's group studied the effect of this heavy metal on the larval development of two different sea urchin species: *Echinometra mathaei*, the most ubiquitous and abundant shallow-water sea urchin in tropical and subtropical regions, and the mediterranean sea urchin *P. lividus* (Figure 3). Two-photon(TP), second (SHG) and third harmonic generation (THG) microscopy techniques, TUNEL staining, propidium iodide (PI) and Hoechst 33342 probes were used to detect skeletal malformations or to stain apoptotic and necrotic cells in fixed and alive plutei. Results showed that the two sea urchin species activate different mechanisms in response to mercury exposure; the less-sensitive temperate species lost selective permeability of the membrane, and did not induced the apoptotic mechanism at the tested concentrations. In the more sensitive tropical species, plutei showed an increase in apoptotic-like signals (**Buttino *et al*., 2016**).

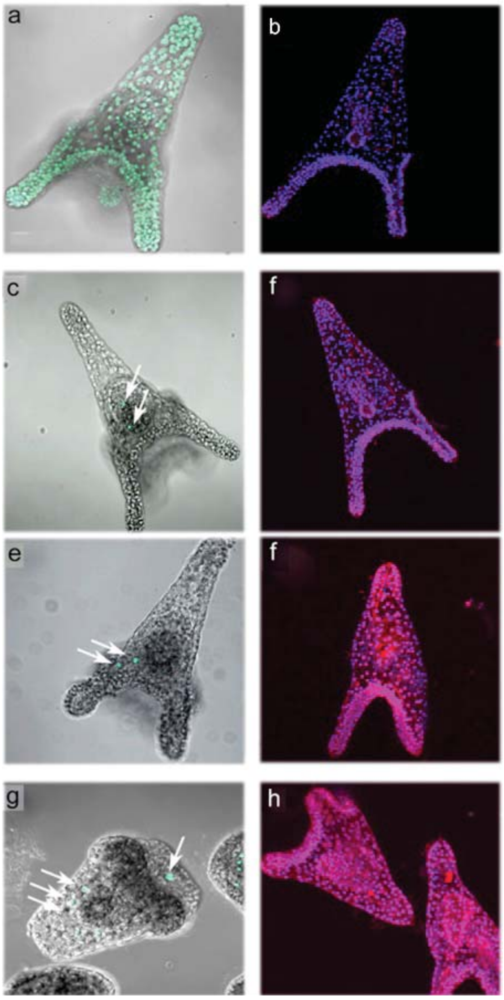

Figure 3. *Paracentrotus lividus* plutei exposed at increasing concentration of mercury chloride (B-H) observed with confocal laser scanning microscopy. Apoptosis was detected using the TUNEL staining (in green); A and B are positive control naupli. Hoechst 33342 was used to detect nucleic acids (in violet) and propidium iodide to detect membrane integrity (in red, appearing pink due to the presence of violet staining).

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[85] Gallo A, Tosti E. (2017) Reproductive processes of marine animals as biomarker for environmental stress impact. In Encyclopedia of Marine Biotechnology (Se -Kwon Kim editor), Wiley-Blackwell. In press.

Palumbo's group investigated the mechanisms by which the toxic heavy metal cadmium and the potential emerging contaminant manganese induce developmental delay and abnormalities in the sea urchin *Paracentrotus lividus*. Results showed the key role played by nitric oxide in the stress response of developing embryos to these metal ions (**Migliaccio *et al.*, 2014**). Indeed, the concentration of nitric oxide, the physiological messenger produced by L-arginine oxidation by nitric oxide synthase, increases in sea urchin embryos after metal treatments and its levels have been shown to regulate directly or indirectly the transcriptional expression of some metal-induced genes involved in stress response, skeletogenesis, as well as in detoxification and multi drug efflux processes (Figure 4A). Moreover, maternal exposure experiments to ecologically relevant cadmium and manganese concentrations were performed. The effects of these metal ions were examined on both *P. lividus* adults and their offspring following reproductive state, morphology of embryos, nitric oxide production and differential gene expression. The authors demonstrated that both metals differentially impair the fertilization processes of the treated female sea urchins, causing modifications in the reproductive state and resulting in a progeny with a high percentage of abnormal embryos and alterations in gene expression (**Migliaccio *et al.*, 2015,** Figure 4 B). The abnormalities in the progeny are associated to an increase in the endogenous nitric oxide levels. The comparison between the effects of these metals on adults and their offspring (Migliaccio et al., 2015) and on embryos (Migliaccio et al., 2014) reveal that toxicity transmitted to the progeny is exacerbated by maternal treatment. The developmental impairment caused by these pollutants likely has consequences on adult populations with possible reverberation on the whole benthic system.

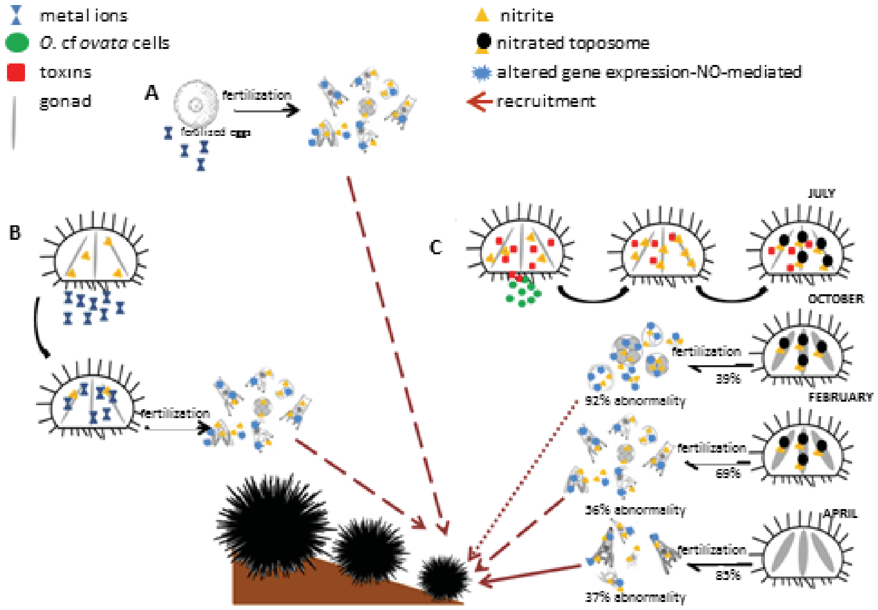

Figure 4. Response of sea urchin *Paracentrotus lividus* to environmental constrains (A, B: metal ions; C: *O.* cf *ovata* bloom).

A systematic study on the effect of heavy and light rare earth elements has been performed on sea urchin development in collaboration with Dr. Giovanni Pagano, treating fertilized eggs or sperm with different concentrations of these elements. These elements affect embryogenesis, fertilization, cytogenetic and redox end-points (**Oral *et al.*, 2017, Pagano *et al.*, 2016**). A difference in species sensitivity has been observed (**Trifluoggi *et al.*, 2017**).

Culturability of marine bacteria, in term of the capability to replicate and form colonies, was used as endpoint by Benvenuto-Manfra' group to evaluate the biological response of *Vibrio anguillarum* at CuO NPs exposure. The toxicity of CuO NPs at different salinities was evaluated using CuSO4 5H2O and CuO bulk as solubility and size control, respectively. The size of CuO NP aggregates increased with salinity and, concurrently, their toxicity decreased. Results confirmed the salinity-dependent toxicity of CuO NPs, showing modest Cu2+ dissolution and no evidence of CuO NP internalization or induction of bacterial morphological alterations (Figure 5**, *Rotini* et *al.*, 2017**).

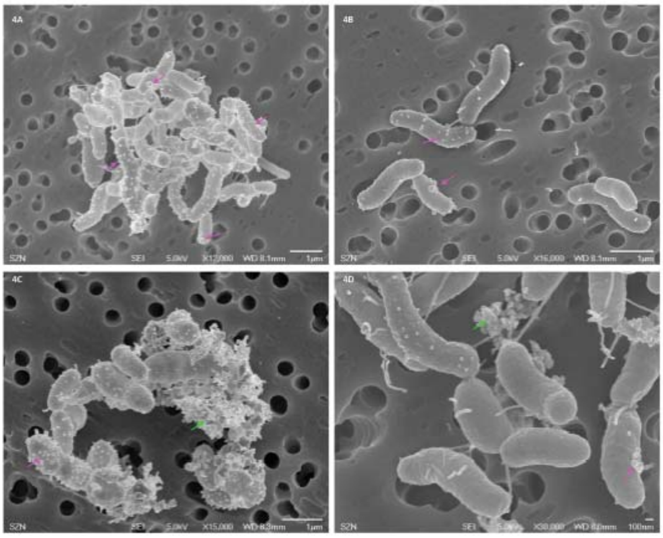

Figure 5. SEM images of *Vibrio anguillarum* after toxicity testing exposure in 2% NaCl saline solution: control (A, B) and CuO NP dispersion (40 mg/l, C,D). Blebs are indicated by pink arrows, nanoparticle aggregates by green arrows.

Moult failure has been investigated, in this study, for the first time on marine copepod *T. fulvus* exposed to CuO NPs. It appeared a suitable sublethal endpoint for testing NPs toxicity, comparable to fertilization rate in *P. lividus*.

Mortality is also a reliable ecotoxicological endpoint. Tosti-Manfra's group performed mortality tests to evaluate the potential toxicity of CuO NPs and ionic Cu to suitable indicator species, belonging to the ecologically relevant level of marine consumers: the rotifer *Brachionus plicatilis*, the shrimp *Artemia franciscana* and the copepod *Tigriopus fulvus*. The results showed a concentration-dependent response for all species; however, copper salts showed higher toxicity than CuO NPs. All together these results demonstrated that the successful application of ecotoxicological reference protocols allowed to produce reliable L(E)C data useful to identify thresholds and assess potential environmental hazard due to NPs (**Rotini *et al.*, 2017 in press**).

Natural toxin impact: Costantini's group studied the effects of diatom-derived oxylipins on the sea urchin *Paracentrotus lividus*. They showed that the two PUAs heptadienal and octadienal were able to induce malformations in sea urchin embryos at concentrations lower when compared with HEPEs (**Marrone *et al.,* 2012**; **Varrella *et al.*, 2014; Varrella *et al.*, 2016b**). Interestingly, HEPEs also induced a marked developmental delay in sea urchin embryos, which has not hitherto been reported for PUAs (Varrella *et al*., 2016b). Costantini's group also studied the oxylipins' effects from the molecular point of view, reporting the variation of the expression levels of fifty genes, having a key role in a broad range of functional responses, such as stress, development, differentiation, skeletogenesis and detoxification processes (Figure 6).

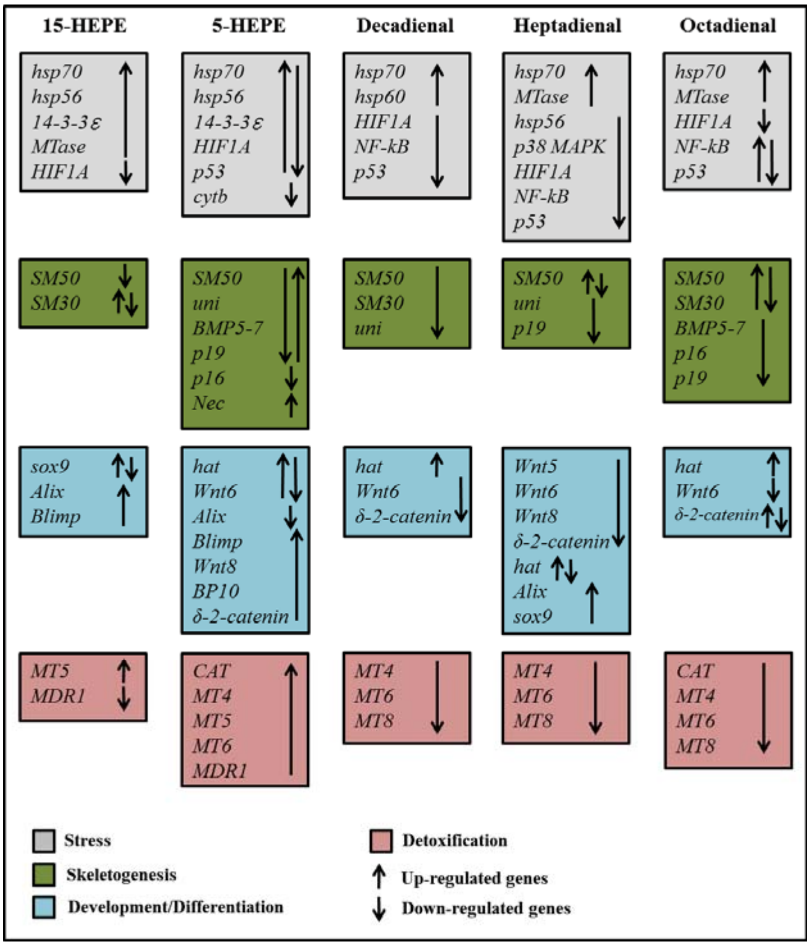

Figure 6. Synopsis of the patterns of up- and down-regulation by Real Time qPCR of different classes of genes in the sea urchin, *P. lividus*, in the presence of different oxylipins: hydroxyacids (5- and 15-HEPEs) and PUAs (decadienal, heptadienal and octadienal).

In this way the molecular targets affected by oxylipins have been identified and their correlation with morphological abnormalities. We then performed an interactomic analysis on modulated genes revealing that they were inter-correlated and involved in specific gene networks (**Varrella *et al.,* 2016a; Ruocco *et al.,* 2017a**).

Moreover, Costantini's group performed experiments at higher concentrations of oxylipins, revealing that PUAs and HEPEs induced apoptosis in sea urchin embryos, detected by microscopic observation and through the activation of caspase-3/7 and caspase-8 measured by luminescent assays (**Ruocco *et al*., 2016**). This study also opens new perspectives for understanding the cellular mechanisms underlying the response of benthic organisms to diatom exposure. In fact, the results showed how changes in gene expression levels may be used as an early indicator of stressful conditions in the marine environment. The genes identified in this work as targets for PUAs may represent possible biomarkers to detect exposure to pollutants that may include microbial products, heavy metals and phytotoxins.

In the last two years, Costantini's group in collaboration with Zupo's group have extended the interest to benthic diatoms, for which the chemical ecology is not well documented, due to difficulties in the collection, quantification and massive culturing. For the first time, they detected the effects of feeding on two bloom-forming benthic diatoms, *Nanofrustulum shiloi* and *Cylindrotheca closterium,* isolated from the leaves of the seagrass *Posidonia oceanica*, on the sea urchin *P. lividus*. Adult *P. lividus* were fed for one month on diets of either one of the two diatoms and on the green alga *Ulva rigida*, used as a feeding control. The results demonstrated that embryos generated by females fed with these benthic diatoms were strongly malformed. Preliminary molecular experiments have been also performed, firstly setting-up a protocol for high-quality RNA extraction for Next Generation Sequencing approaches (**Ruocco *et al.* 2017b**), aiming to identify the gene pathways affected by these benthic diatoms.

Spagnuolo's group demonstrated that the diatom PUA 2-trans-4-trans-decadienal (DD), the most toxic secondary metabolite produced by some diatoms, can induce developmental aberrations also in Ciona larvae in a dose-dependent manner (Lettieri et al, 2015). Moreover, through a preliminary analysis, DD was shown to affect the expression level of genes involved in stress response and developmental processes. A summary of the results obtained is represented schematically in Figure 7.

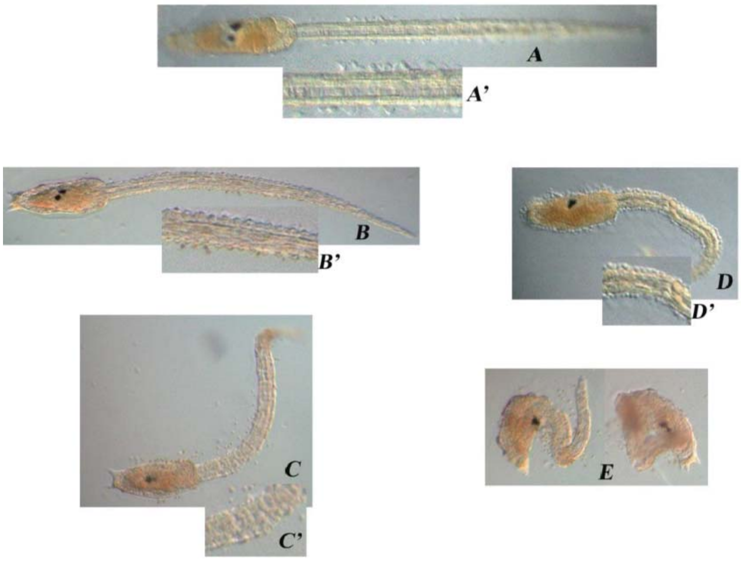

Figure 7. Aberrant *Ciona* phenotypes. Hatched larvae after DD treatment on fertilized *Ciona* eggs ((A) CTRL; (B) 0.30 μg·mL−1; (C) 0.35 μg·mL−1; (D) 0.40 μg·mL−1; (E) 0.45 μg·mL−1). (A'–D') Magnification of the tails showing the progressively altered structure (B'–D') compared to the control (A').

Palumbo's group has discovered that DD also affects a key checkpoint of *Ciona robusta* development that is the larval transition from the vegetative to the reproductive life stage. Treatment of larvae at competence stage with DD resulted in a delay of metamorphosis. The signaling network affected by DD was identified, including decreases of total glutathione and nitric oxide levels, the upregulation of ERK phosphatase mkp1 and the consequent reduction of ERK phosphorylation, with final changes in the expression of downstream ERK target genes (**Castellano *et al*., 2015**).

Among harmful algal bloom, there are blooms of species of the benthic dinoflagellate genus *Ostreopsis*, which produces ovatoxins, i.e. palytoxin- like molecules that affect marine organisms and are also responsible for a number of health problems in humans. *Ostreopsis* cf. *ovata*, first reported in subtropical waters, has been increasingly recorded also in temperate seas and in the Mediterranean Sea, with intense blooms in July/August. Palumbo's group, in collaboration with Adriana Zingone, examined the reproductive ability of apparently healthy *P. lividus* population from a marine protected area affected by toxic blooms of *Ostreospsis* cf. *ovata* in the Gulf of Naples prior to and during the bloom (July) as well as at several times thereafter, during the reproductive season (October, February and April) (Figure 4C). Spawning, fertilization and development were followed in the laboratory. Our approach also included biochemical analysis of the gonads, reproductive success, morphological and biochemical analysis of the sea urchin progeny along with selected gene expression profile. Subtle but significant reproductive flaws are transmitted from the female gonads to the offspring with the nitric oxide involvement. Indeed, adults show a low fertilization rate, along with high nitric oxide levels in the gonads and the nitration of the major yolk protein toposome, an important player in sea urchin development. Serious developmental anomalies are observed in the progeny, which persist several months after the bloom without a complete recovery. Developing embryos show high nitric oxide levels, and variations in the transcription of several genes that are found to be directly or indirectly modulated by nitric oxide (**Migliaccio *et al*. 2016**). Moreover, changes in the structure and function of toposome have been associated with such an environmental stress (Castellano *et al*., under revision, submitted Sci. Rep. 29th August 2017).

4. Perspectives

Tosti's group are studying the impact of physical stressors such as seawater acidification on gamete physiology, fertilization and development on the ascidian *C. intestinalis* and the mollusc *M. galloprovincialis* exposed to low pH (7.7) either in mesocosms and natural acidified areas along the Ischia island coasts. Preliminary data show that ocean acidification exerts an adverse effect on the male gamete morphology and functionality affecting in turn the fertilization capability. In addition, the reprotoxicity of emerging contaminants such as other NPs, nano and microplastics will be evaluated in marine animals. Preliminary results have shown that CuO NPs impair sea urchin *P. lividus* sperm functionality via oxidative stress. Within the partnership to the flagship research program of the SZN, ModRes (Dr. V. Zupo, PI), Tosti's group will characterize morphological and functional parameters of oocytes and spermatozoa collected from new model species and will assess the impact of chemo-physical stressors on their reproductive processes in order to propose these specie as a new bioindicator of marine environmental stress. These studies will contribute to closing a gap in the knowledge related to the toxicity of chemo-physical stressors in marine environments. However, this research goes beyond the studying of simple toxic impact but may provide new and useful tools for a fundamental aspect of marine biology represented by worldwide marine biomonitoring programs and ecological risk assessment.

On-going ecotoxicology research of Palumbo's group with Immacolata Castellano is related to the study of the impact of the colonized microplastics, collected from coastal areas with contrasting anthropogenic pressure, on the sea urchin *P. lividus*. The mechanism of uptake of microplastics in sea urchin will be investigated and cells and tissues differing for microplastics content will be characterized by measuring redox endpoints and stress markers. This study is in the frame of the flagship project: MICROplastics in the MARine Environment: colonization by microbes and their effect on benthic model organisms (MicroMARE) with Raffaella Casotti.

The group of P. Sordino has initiated a PhD (PhD candidate M. Piccardo; co-tutor Professor A. Terlizzi, Univ. Trieste) aimed to investigate the fate and effects of microplastics in wild fish populations and laboratory fish models. They will study mechanisms of microplastic translocation across the gut, search for adverse effects of polyethylene microplastics in *Danio rerio* (zebrafish), and test the impact of microplastics in liver on the nutritional value of fish flesh.

Enrico D'Aniello, a new researcher in the BEOM department will focus his studies on putative epigenetic alterations in the mollusc *M. galloprovincialis* after experimental exposure to specific emerging contaminants. Initially, he will investigate potential changes in the global DNA methylation and on differential expression of those genes responsible of epigenetic changes after exposure to contaminants. Later, taking advantage of his past interest on Nuclear Receptors (NRs) and given their relation with environmental pollutants, he will also seek for potential epigenetic mechanisms linking the regulation of Retinoic Acid Receptors and Peroxisome Proliferator Activated Receptors signaling to the effects of environmental pollution on marine invertebrates.

Spagnuolo's group is currently analysing DD effects on *Ciona* using a microarray approach, one of the most feasible methods to rapidly inquire into the entire biological response of a whole organism to chemical and drug exposure (data not published yet). At the same time the group is exploring the effects of iodine and the endocrine disruptor potassium perchlorate on the advancement of metamorphosis in *Ciona* larvae (manuscript in preparation), determining the impact of an impaired thyroid hormone biosynthetic pathway on this developmental process. Still in the perspective, this group is planning to study the effects of selected legacy contaminants, as PAHs (i.e. benzo(a)pyrene) and emerging contaminants, such as dispersants (i.e. anionic surfactants used for cleaning up the petroleum hydrocarbon contamination in case of accidental oil spills at sea) and nanomaterials, including nanoplastics alone and in combination as resembling their usage during environmental remediation of oil spill.

Romano's group have identified a panel of 10 genes, having a key role in the induction of Programmed Cell Death (PCD), in the sea urchin *P. lividus*. They investigated the expression level of those genes in response to aldehyde exposure, in order to identify PCD pathways induced by the treatment. The group will test these genes also in response to classical pollutants with the final aim to identify new markers as early warning system of toxic environments.

Costantini's groups will aim to further analyze the toxic effects of diatom-derived oxylipins, also testing mixture toxicity of them using always *P. lividus* as model organisms. Furthermore, Costantini's group will intend to continue to test the toxigenic effects of benthic diatoms *Nanofrustulum shiloi* and *Cylindrotheca closterium* by Next Generation Sequencing, also extending these analyses to other benthic diatoms.

Within ABBaCo Project, ecotoxicological studies will be performed in order to assess the impact of contaminated sediments collected from Coroglio-Bagnoli Bay on marine biota.

5. Publications (2012-2017; *corresponding author)

2017

Bergami E*, Pugnalini S, Vannuccini ML, Manfra L, Faleri C, Dawson KA, Corsi I. Long-term toxicity of surface charged polystyrene nanoplastics to marine planktonic species *Dunaliella tertiolecta* and *Artemia franciscana*. ***Aquat. Toxicol***. (2017) 189: 159-169.

Gallo A, Boni R, Tosti E*. Sperm viability assessment in marine invertebrates by fluorescent staining and spectrofluorimetry: A promising tool for assessing marine pollution impact. ***Ecotox. Environ. Saf.*** (2017) in press, doi: 10.1016/j.ecoenv.2017.07.069.

Galdiero E, Falanga A, Siciliano A, Maselli V, Guida M, Carotenuto R, Tussellino M, Lombardi L, Benvenuto G, Galdiero S*. *Daphnia magna* and *Xenopus laevis* as in vivo models to probe toxicity and uptake of quantum dots functionalized with gH625. ***Int. J. Nanomedicine*** (2017) 12:2717-2731.

Gallo A.* Reprotoxicological evaluation of marine pollutants in ascidians. ***Zygote*** (2017) in press

Manfra L*, Rotini A, Bergami E, Grassi G, Faleri C, Cicero AM, Corsi I. Comparative toxicity of polystyrene nanoparticles in natural seawater and reconstituted seawater using the rotifer *Brachionus plicatilis*. ***Ecotox. Environ. Saf***. (2017) 145: 557-563.

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Oral R, Pagano G*, Siciliano A, Gravina M, Palumbo A, Castellano I, Migliaccio O, Thomas P, Guida M, Tommasi F, Trifuoggi M. Heavy rare earth elements affect early life stages in *Paracentrotus lividus* and *Arbacia lixula* sea urchins. ***Environ. Res.*** (2017) 154:240– 246.

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Pagano G*, Thomas P, Guida M, Palumbo A, Romano G, Oral R, Trifuoggi M, Sea Urchin Bioassays in Toxicity Testing: II. Sediment Evaluation. ***Expert. Opin. Environ. Biol.*** (2017) 6:1. DOI: 10.4172/2325-9655.1000141.

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Ruocco N, Fedele AM, Costantini S, Romano G, Ianora A, Costantini M.* New inter-correlated genes targeted by diatom-derived polyunsaturated aldehydes in the sea urchin *Paracentrotus lividus*. ***Ecotox. Environ. Saf.*** (2017a) 142: 355-362.

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2013

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6. List of grants

- **ABBaCo**: regional project for bioremediation. MIUR (2017- 2020)
- **MicroMARE** "MICROplastics in the MARine Environment: colonization by microbes and their effect on benthic model organisms" SZN FOE (2017-2019). PI: A. Palumbo, R. Casotti.
- **ModRes**: New model organisms for the scientific research: culture, ecology, physiology and genomic characterization Progetto Bandiera (2017- 2018). PI: V. Zupo.
- **Monitoring of *Ostreopsis ovata*** funded by Campania Region (2007-2019). PI: A. Zingone
- **NanoBioTech Ambient Project:** development and optimization of ecotoxicological protocols for nanosized materials. Regione Lazio-Consorzio Hypatia (2015- 2017).

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-Francesco Regoli, Polytechnic University of Marche, Ancona, Italy

-Alice Rotini, University of Tor Vergata, Roma, Italy

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7. Plankton variability in space and time

1. People involved

Name	*Position*	*Funding*	*Period*
Elio Biffali	Staff scientist	SZN	since1994
Marco Borra	Staff scientist	SZN	since 2012
Christophe Brunet	Staff scientist	SZN	since 2000
Ylenia Carotenuto	Staff scientist	SZN	since 2010
Raffaella Casotti	Staff scientist	SZN	since 2000
Isabella D’Ambra	Staff scientist	SZN	since 2017
Daniele Iudicone	Staff scientist	SZN	since 2009
Wiebe Kooistra	Staff scientist	SZN	since 2008
Francesca Margiotta	Staff scientist	SZN	since 2009
Maria Grazia Mazzocchi	Staff scientist	SZN	since 1989
Marina Montresor	Staff scientist	SZN	since 1989
Milva Pepi	Staff scientist-Term contract	SZN	since 2017
Grazia Marina Quero	Staff scientist-Term contract	SZN	since 2017
Eugenio Rastelli	Staff scientist	SZN	since 2017
Maurizio Ribera d’Alcalà	Staff scientist	SZN	since 1981
Diana Sarno	Staff scientist	SZN	since 1994
Maria Saggiomo	Staff scientist	SZN	since 2012
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Sergio Stefanni	Staff scientist-Term contract	SZN	since 2016
Francesco Terlizzi	Staff scientist	SZN	since 2016
Marco Uttieri	Staff scientist-Term contract	SZN	since 2017
Adriana Zingone	Staff scientist	SZN	since 1989
Cecilia Balestra	Postdoctoral fellow	MarMIC/RITM./SZN	since 2007
Luigi Caputi	Postdoctoral fellow	RITMARE	since 2013
Domenico D’Alelio	Postdoctoral fellow	RITMARE	since 2013
Laura Escalera	Postdoctoral fellow	SZN	2016-2018
Florian Kokoszka	Postdoctoral fellow	RITMARE	since 2017
Christophe Lejeusne	Postdoctoral fellow	RITMARE	since 2015
Isabella Percopo	Postdoctoral fellow	RITMARE/ABBaCo	since 2012
Roberta Piredda	Postdoctoral fellow	FIRB/RITMARE	since 2013
Luciana Sabia	Postdoctoral fellow	SZN/Terna	since 2016
Eleonora Scalco	Postdoctoral fellow	MicroB3/RITM./Lincei	2013-2018
Maria Paola Tomasino	Postdoctoral fellow	CNR/LifeWatch	2014-2016
Mark Van Dijk	Postdoctoral fellow	S&TMed/RITM./IN-OGS	since 2014
Romain Watteaux	Postdoctoral fellow	RITMARE	since 2013
Francesco Bolinesi	PhD student	SZN/Naples University	since 2017
Greta Busseni	PhD student	SZN/RITMARE	since 2015

Lorenzo Longobardi	PhD student	SZN	since 2017
Gauri Mahadik	PhD/Postdoc	SZN/RITMARE	2010-2014
Aikaterini Pargana	PhD student	SZN	2013-2016
Kumari Richa	PhD student	SZN	2012-2016
Krzysztof F. Stec	PhD student	SZN/RITMARE	2012-2015
Anna Chiara Trano	PhD student	Campania Region	2015-2016
Heather Ann Wright	MPhil student	SZN	2010-2013

2. Background, aims and objectives

Plankton are a fundamental element of marine life. They are characterised by high taxonomic, biological and ecological trait diversity, which determines complex non-linear interactions with the environment across a vast range of spatial and temporal scales, from seconds to geological times and from local to global. The general objective of our research is to understand the mechanisms that underlie the distribution of planktonic organisms in space and time and their adaptations to different sets of biotic and abiotic environmental factors, including their response to climatic and anthropogenic pressures.

Our research is based on field observations and encompasses numerous aspects of plankton ecology, with focus on different components of the pelagic system, from bacteria to zooplankton, at different levels of organization, from communities to species, in relation with the abiotic context. The recent advancements in oceanography, imaging, genomics and technology have paved the way to overcome the prior limitations and now allow a deeper and more detailed analysis of biotic responses in the natural environment. In line with these advances, our multidisciplinary approach spans from automatized instrumental recording and classical microscopic analysis to the most recent molecular, genomic and modelling tools. Our field observations take place in different marine areas, from the Gulf of Naples and other Mediterranean regions to other temperate and high-latitude basins.

To address temporal variability,_we investigate the seasonal, interannual and decadal plankton time course within the *MareChiara* project, a long-term ecological research project conducted at the fixed station LTER-MC in the Gulf of Naples. This station is one of the very few sites of Mediterranean plankton monitoring and it is unique in terms of sampling frequency and detail of taxonomic identification. Beyond the single sampling point, research at LTER-MC is conducted in the context of the entire Gulf of Naples and integrated with spatial research in the whole area, thus contributing to the regional marine observatory. In the Gulf of Naples, bacteria and protists are analysed also with molecular methods, including advanced HTS-metabarcoding and metagenomics techniques, which is an important step forward within our broader aim of establishing a genomic observatory in the area.

Besides the Gulf of Naples, the factors and processes that drive plankton distribution are also investigated in terms of structure and activity across the global ocean and in peculiar or extreme environmental conditions. For instance, transport processes ruling plankton biogeography are elucidated within the TARA-Oceans and Tara Arctic surveys, while the effect of iron fertilization and ice characteristics on phytoplankton physiology and community structure are studied in the Southern Ocean. Finally, the response of plankton to the occurrence of microplastics is the focus of researches conducted in a mesocosm experiment (INPUT) in the inner Gulf of Naples as well as in the course of a sampling cruise at the basin-wide scale (Tara-Mediterranean). These studies ultimately aim at assessing the impact of microplastics on the whole ecosystem functioning with a focus on the Mediterranean Sea, which has been recently proposed as one of the most impacted regions of the world for what plastic pollution is concerned.

The results obtained during the period 2012-2017 have been arranged in four main research lines:

a. Plankton ecology in the Gulf of Naples
b. Plankton molecular ecology
c. Plankton responses to specific environmental and anthropic factors
d. Plankton dynamics at global scale.

3. Results

a. Plankton ecology in the Gulf of Naples

In the last years, the analysis of the large dataset acquired so far at station LTER-MC has been focused on specific topics as outlined below.

a1. Trophic webs (Ribera d'Alcalà)

Lagrangian models showed that the inner, shallower Gulf of Naples was mainly affected by coastal circulation and the horizontal water-transport was the major environmental constraint acting on plankton at LTER-MC, determining oligotrophic or eutrophic conditions. The alternation of these nutrient input regimes allows the growth of opportunistic species but also of rarer species, therefore promoting plankton diversity (**Cianelli *et al*., 2017**). In summer, the eutrophic and oligotrophic regimes were characterized by the same plankton taxa but with different trophic links (**D'Alelio *et al*., 2015**). While phytoplankton showed quantitative variations between conditions, zooplankton were more resilient to trophic intermittency. A network analysis highlighted different linkage topology in the two trophic states, with a predominant vertical topology (i.e., phytoplankton => herbivorous zooplankton => carnivorous zooplankton) in eutrophic conditions and a more scattered topology in oligotrophic conditions with dominance of links among microbes. Ecological-network models based on organism biomass and biological traits[86] and carbon flux estimates indicated that most species can play different roles in the food web in distinct trophic states (**D'Alelio *et al*., 2016b**) (Figure 1).

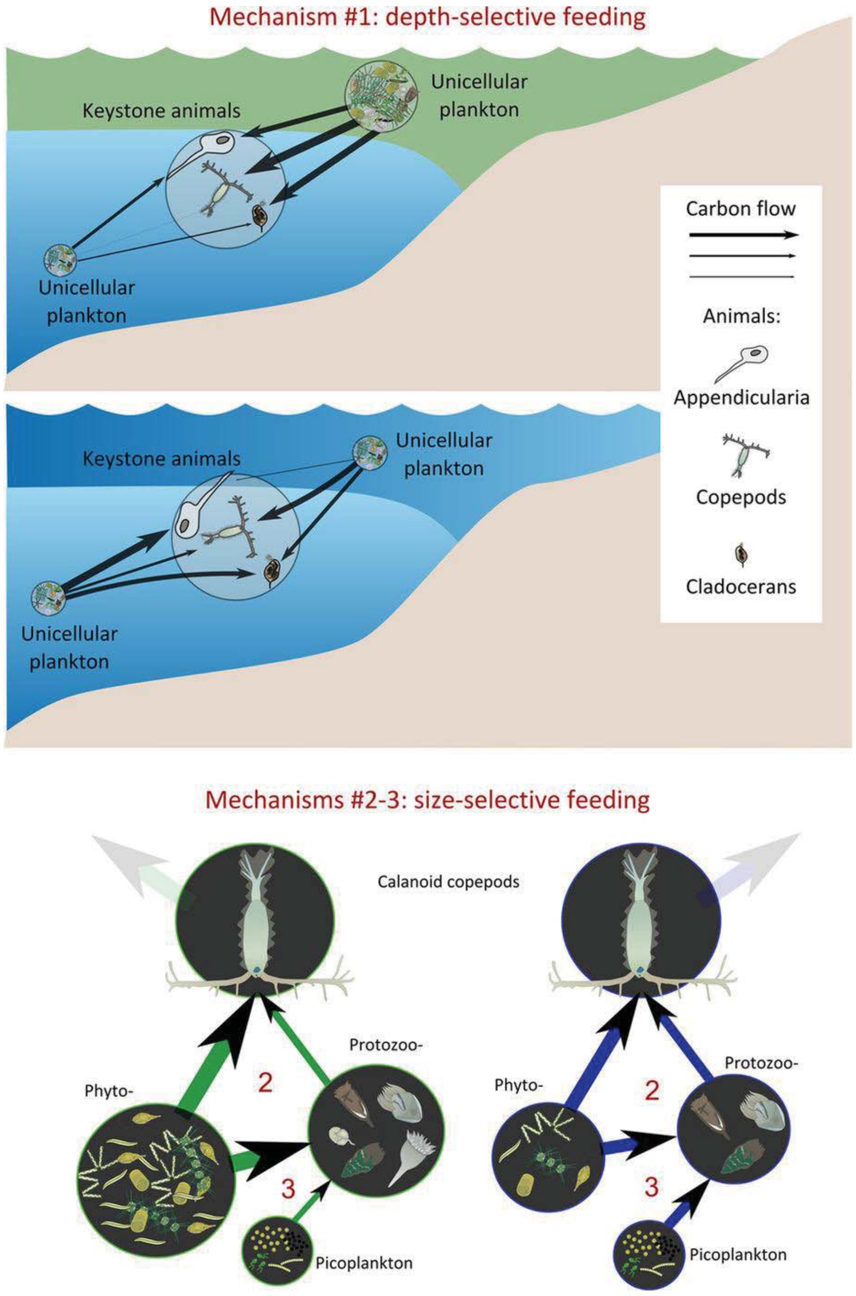

Figure 1. Schematic depiction of the main differences in trophic fluxes between the green (eutrophic) and blue (oligotrophic) states.

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[86] D'Alelio D, Montresor M, Mazzocchi MG, Margiotta F, Sarno D, Ribera d'Alcalà M. Plankton food webs: to what extent can they be simplified? ***Adv. Oceanogr. Limnol.*** (2016a) 7: 67-92.

Trophic niches largely overlap in eutrophic conditions, while specialization increases in oligotrophic conditions. In the transition from eutrophic to oligotrophic states, primary production decreased by seven-folds, while zooplankton production only by two-folds. Mechanisms of trophic plasticity at organismal level and flexibility of web topology at community level maintained high system functionality in terms of carbon fluxes despite trophic intermittency (D'Alelio *et al.*, 2016b).
This research has been funded by SZN FOE and RITMARE Flagship Project.

a2. Picoplankton (Casotti)

At station LTER-MC picoplankton are dominated numerically by heterotrophic bacteria, followed by cyanobacteria and picoeukaryotes. Picoeukaryotes follow total chlorophyll and larger phytoplankton trends, while cyanobacteria (*Synechococcus* and *Prochlorococcus)* appear to respond to other unknown factors and show peaks at surface (*Synechococcus*) and at depth (*Prochlorococcus*) in summer, probably due to the release from grazing pressure in this season (Figure 2). *Synechococcu*s, in addition, appears to profit of sudden freshwater inputs and is able to thrive also close to terrestrial-influenced areas[87]. Instead, total picoeukaryotes follow the general trend of larger phytoplankton even though they are also able to react rapidly to sudden local inputs. Sudden and short-lived peaks of *Synechococcus* and picoeukaryotes were recorded by a high-frequency (every 2 hours) scanning flow cytometer placed on a floating buoy in another coastal site of the Gulf of Naples[88], highlighting the fast dynamics of these microbes, which is missed by routine weekly sampling. Heterotrophic bacteria increase in number in low-salinity waters with high organic loads such as those influenced by rivers or discharges, but their high numbers do not always indicate disturbances or pollution, as shown during surveys along the eastern coasts of the Gulf[89].

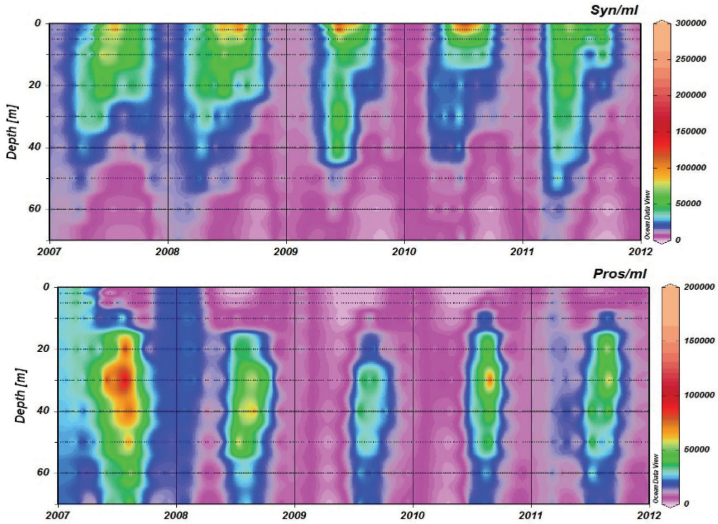

Figure 2. *Synechochoccus* (upper panel) and *Prochlorococcus* (lower panel) distribution at the LTER-MC station during 2007-2011. (Casotti *et al.,* unpublished).

The subpopulations identified based on their apparent Nucleic Acid content (HNA and LNA) segregate depending on areas and time of the year likely related to trophic characteristics of the water. Free planktonic viruses investigated during one year at the LTER-MC station have shown to be highly variable and significantly correlated to bacterial concentrations, suggesting that they are mainly composed of phages.
Both conventional and scanning high-frequency flow cytometry allowed to provide a better estimate of the number of subpopulations within the picoplankton in the Northern Adriatic Sea and improve our knowledge on their distribution and variability. Heterotrophic bacteria also appear to be very diverse and dominated by freshwater clades due to the influence of the Po River. These data are part of a larger database collected in the framework of the RITMARE Flagship Project and still

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[87] Trano AC, Balestra C, Casotti R. Analisi delle variazioni temporali delle comunità microbiche planctoniche in una zona costiera del Golfo di Napoli tramite citometria a flusso. ***Lettere GIC*** [g1] (2017) 26: 27-31.

[88] van Dijk MA, Passarelli A, Conversano F, Casotti R. Phytoplankton dynamics by autonomous high-frequency flow cytometry from a floating buoy in the Gulf of Naples. Proceedings of the IMEKO TC19 International Workshop "Metrology for the Sea", (2017) pp. 54-57.

[89] Casotti R, Balestra C, Van Dijk M, Passarelli A, Sigrist J, Hammes F . Real-Time microbial concentrations by automated on-line flow cytometry for marine coastal monitoring. Proceedings of the IMEKO TC19 International Workshop "Metrology for the Sea", (2017) pp. 50-53

under elaboration[90,91]. In general, these results highlight the need of revising actual beliefs on picoplankton as a background feature in marine systems and call for a deeper investigation of its functional and diversity features.

This research has been funded by SZN FOE (Premiale MARMIC and IRMA).

a3. Phytoplankton (Zingone)

The identification of phytoplankton successional patterns and of their driving factors is imposed by the pivotal role of species-specific properties in controlling ecosystem functions and at times posing human health risks. Based on light microscopy analysis of surface samples collected in 1984-2010, we determined species associations and their seasonal and interannual distribution at station LTER-MC with the aim of assessing the extent of their regularity and robustness and contribute to the still debated issue of the rule of assembly in phytoplankton communities. Seven main groups of species were identified using an r-mode clustering analysis, five of which were recurrent over the years in early spring, spring, early-summer, autumn and winter, respectively. With the exception of the winter group, which mainly included coccolithophores and silicoflagellates, diatoms were present in all associations. The occurrence of the different associations was mainly driven by the season (i.e., temperature). A seasonal trend in morpho-functional heterogeneity within the associations was evident, with the maximum diversity found within the early spring association and the minimum in the winter one. This pattern indicates a change in the rule of assembly of phytoplankton communities along the year and reflects a variations across the seasons of the environmental constraints, which are conceivably weaker in periods of more available resources (e.g., light, and nutrients), i.e. spring, allowing for different life-forms to coexist.

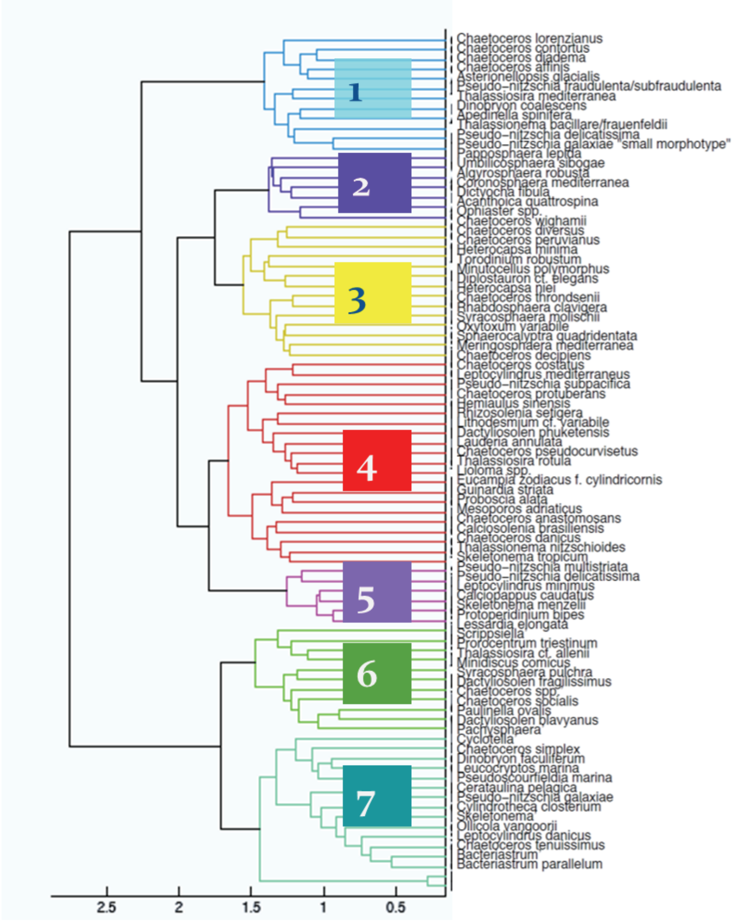

Figure 3. The 7 associations of phytoplankton species recurrently found in distinct seasons of the year over the years 1984-2010. Association n°1, repeatedly found in spring, had the highes morpho-functional diversity.

The role of diatom resting spore in the dynamics of blooms in the water column was investigated through germination experiments conducted on sediments samples collected in different periods of

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[90] Bastianini M, Riminucci F, Capotondi L, Barra E, Pasqual S, Casotti R, Trano AC, Van Dijk M, Celussi M, Fabbro C. (2017a) Rapporto sulle attività oceanografiche, biologiche e di manutenzione della stazione MEDA S1-GB svolte durante la campagna LTER-ANOC16 (26-30 aprile 2016) con N/O DallaPorta nel Mare Adriatico Settentrionale. Rapporto Tecnico CNR-ISMAR, N° 145, pp. 1-27

[91] Bastianini M, Riminucci F, Pansera M, Coluccelli A, Casotti R, Dal Passo E, Dametto L, Van Dijk M, Russo E, Titocci J, Pazzaglia J, Virgili S (2017b). Rapporto sulle attività biologiche, oceanografiche, geologiche e di manutenzione della stazione Boa E1 svolte durante la campagna INTERNOS17 (14-21 marzo 2017b) con N/O Minerva Uno nel Mare Adriatico centro-settentrionale. Rapporto Tecnico CNR-ISMAR N° 146, 2017, pp. 1-37.

the year at the station LTER-MC (**Montresor *et al.*, 2003**). This first time-series analysis of a coastal seed bank showed that the assemblage of viable diatoms in the sediment varies over the year, reflecting the diatom dynamics in the water column, but also species-specific characteristics in survival capability and likely the disturbance due to biotic and abiotic factors. The formation of resting stages was confirmed to be a widespread life strategy in coastal areas.

This research has been funded by SZN FOE and RITMARE Flagship Project.

A4. Zooplankton (Mazzocchi)

The temporal dynamics of mesozooplankton at station LTER-MC (1984-2006) was examined in the context of comparative analyses at basin and global scale. The absence of significant correlations between climate indices and local temperature or community abundance suggested that, in the coastal Mediterranean Sea, zooplankton are mainly driven by local conditions (**Berline *et al.*, 2012**). The variability in zooplankton phenology analysed at global scale indicated that the ‘‘earlier when warmer’’ response in phenology seen in most of the Atlantic and Pacific time series is less frequent in the Mediterranean, and is replaced in many species by a strong ‘‘later when warmer’’ pattern (**Mackas *et al.*, 2012**). In the Gulf of Naples, two abundant spring-early summer copepod species showed significant abbreviation of the population cycle in relation to spring or summer temperature anomalies. However, the time course of the whole copepod assemblages in the same two decades showed a significant resilience in their seasonal cycle despite the high variability in the environmental conditions (**Mazzocchi *et al.*, 2012**).

The seasonal and long-term abundance patterns of *Oithona similis*, one of the most abundant and ubiquitous marine copepods, were compared between the LTER-MC and L4 (Plymouth, NE Atlantic Ocean) long-term time series (Figure 4), showing that temperature is the main limiting factor for persistence and flourishment of this species in the spatial and temporal scales (**Castellani *et al.*, 2016**).

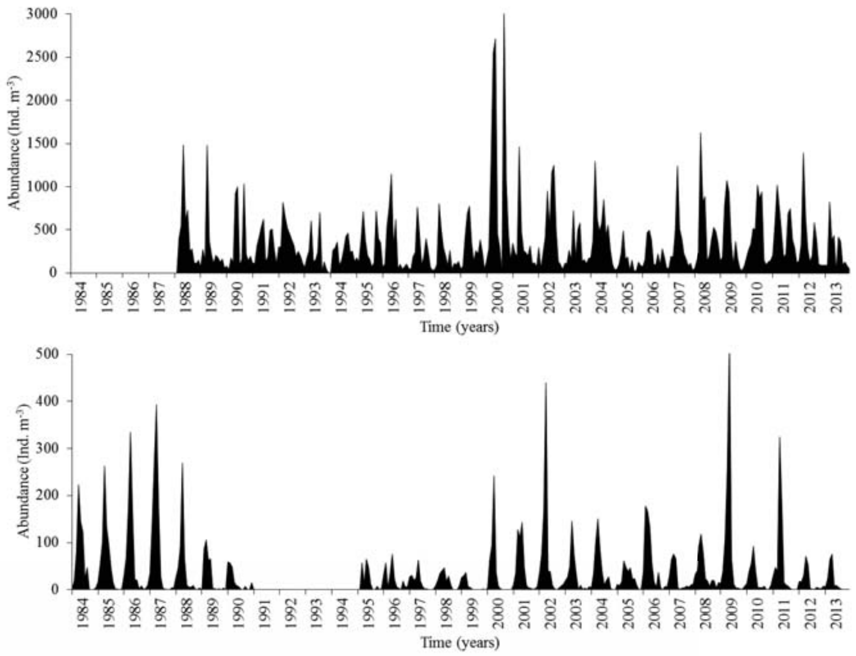

Figure 4. Long-term patterns of *Oithona similis* monthly mean abundance (Ind. m^{-3}) at L4 (Plymouth) (upper panel) and LTER-MC (Gulf of Naples) (lower panel).

The ecological niches of *Clausocalanus* congeneric species that are common and abundant in the Gulf of Naples were outlined from the species distribution along a latitudinal cline in the Atlantic Ocean (**Peralba *et al.*, 2016**) and were also used to explain their seasonal succession at station LTER-MC. To better identify the seasonal cycles of the numerous *Oncaea* and *Triconia* species at LTER-MC, the family Oncaeidae was investigated with morphological and molecular methods (**Di Capua *et al.*, 2017**).

To examine other possible factors responsible of the time-course of copepod populations, we focused on mortality, one of the most neglected aspects of zooplankton biology so far. Non-predatory mortality was estimated with the neutral red staining method to quantify copepod carcasses at group, species and gender levels throughout an annual cycle at station LTER-MC. Our results showed that carcasses accounted on average for about 10% of copepod abundance and non-predatory mortality rates differed among species and varied during their seasonal cycle (**Di Capua and Mazzocchi, 2017**).

This research has been funded by SZN FOE and RITMARE Flagship Project.

b. Plankton molecular ecology

b1. Picoplankton (Casotti)

A deeper focus on bacterial community composition in these years has highlighted a huge taxonomical diversity (Figure 5) and also an inverse relationship between abundance and potential activity, as also estimated from DNA and RNA sequencing data. Alphaproteobacteria dominate the community, with SAR11 (*Pelagibacter ubique*) thriving offshore and several members of Rhodobacterales at coastal stations. However, the most abundant clades are not necessarily the most active metabolically, and many so-called "rare" taxa appear to play an important role in the ecosystem, not only as "seed" bank from where species are relieved when condition become favorable, but also because they harbor a persistent functional pool of potential ecological diversity in terms of metabolic activity (**Richa *et al*., 2017**; **Thiele *et al*., 2017**).
This research has been supported by the SZN FOE (Premiale MARMIC) and RITMARE Flagship Project.

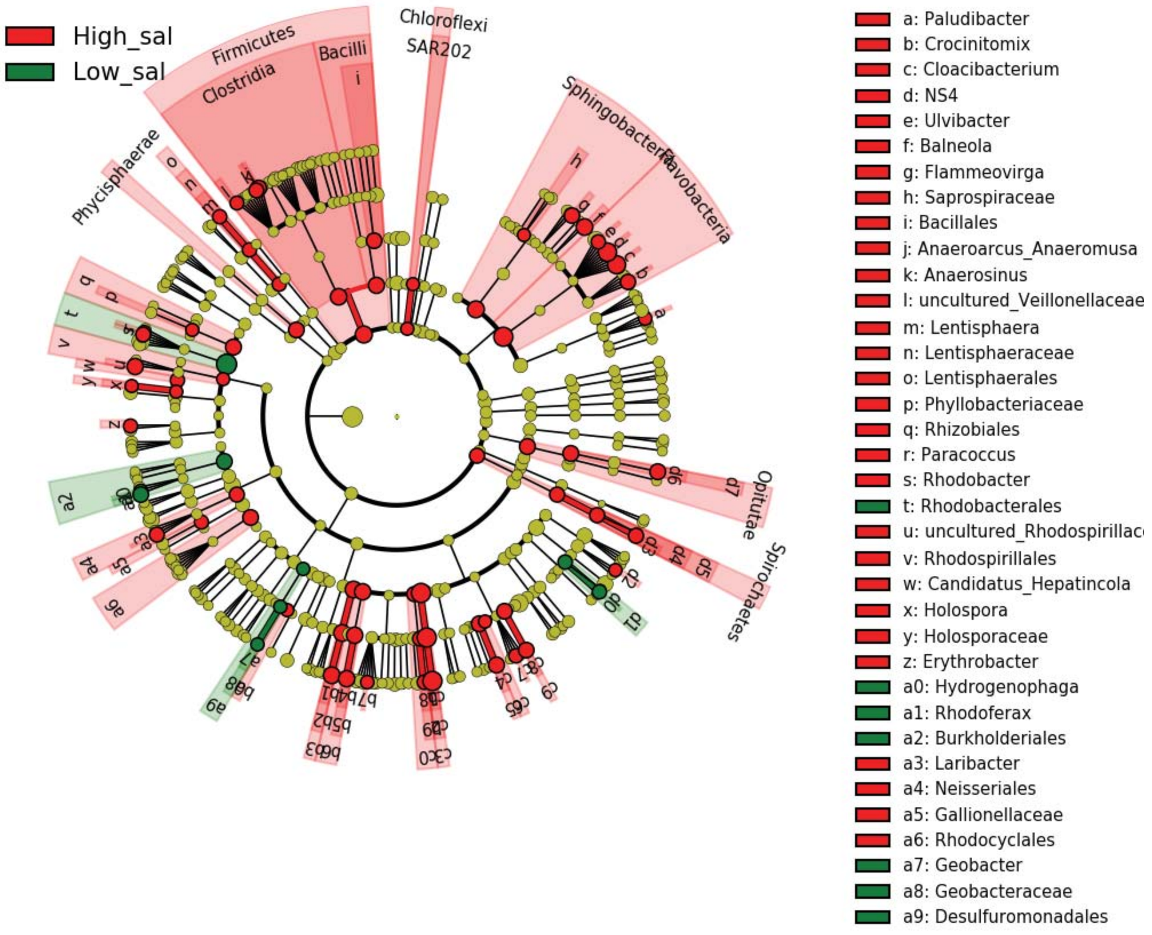

Figure 5. Cladogram of bacterial OTUs from the Gulf of Naples separating the ones observed in high-salinity vs low-salinity (Sarno river) waters (Richa *et al*., unpublished figure).

b2. Protists (Zingone)

Molecular analyses of environmental DNA have boosted the development of knowledge in marine protists, allowing for much deeper insights into spatial and temporal patterns of species and communities so far hardly covered with classic methods. DNA-metabarcoding coupled with clone libraries shed light on the species-specific seasonal cycles of toxic and non-toxic diatom of the genus *Pseudo-nitzschia,* which cannot be identified with optical methods due to high level of cripticity (**Ruggiero *et al*., 2015**). More recently, High Throughput Sequencing (HTS) has further enhanced this approach enabling the recovery of huge number of sequences from microbial samples. First HTS-metabarcoding results obtained at the LTER-MC site for 8 sampling dates over one year revealed a much higher diversity in protist communities than that detected so far with morphological methods, with more than 6,000 OTUs (**Piredda *et al*., 2016 a**). Patterns of the most abundant diatom taxa matched those obtained with classical methods and were related to environmental variability in terms of temperature, salinity and trophic status. A pilot study was conducted comparing diatom resting stages based on HTS data from sediments with results from Serial Dilution Cultures, which also showed a rather good match between morphological and molecular approaches (Figure 6a) (**Piredda *et al*., 2016 b**). Nonetheless, both cases highlighted the need for more complete reference databases to be able to exploit metabarcoding data. A much larger dataset encompassing 48 dates has also been analysed to investigate diversity and temporal patterns of selected group of species (Dinoflagellates), and key genera (the diatoms *Chaetoceros* and *Leptocylindrus*, see also the report of Theme # 9 'Biodiversity').

From the Gulf of Naples, HTS-metabarcoding studies have been extended across European seas to analyse the composition of coastal protist communities in the water column (**Massana *et al*., 2015)**, also addressing rare species (**Logares *et al*., 2014)**, and in the sediments, which showed a higher amount of unknown diversity compared to surface waters assemblages (**Forster *et al*., 2016)**. In the Ocean Sampling Day project, the photosynthetic component of the protist communities was investigated with two different barcodes (V4 and V9) at a subset of global coastal sites. Results showed that both markers capture similar diversity and spatial patterns for the different groups, although the V9 reference dataset is considerably more limited (**Tragin *et al*., 2017**).

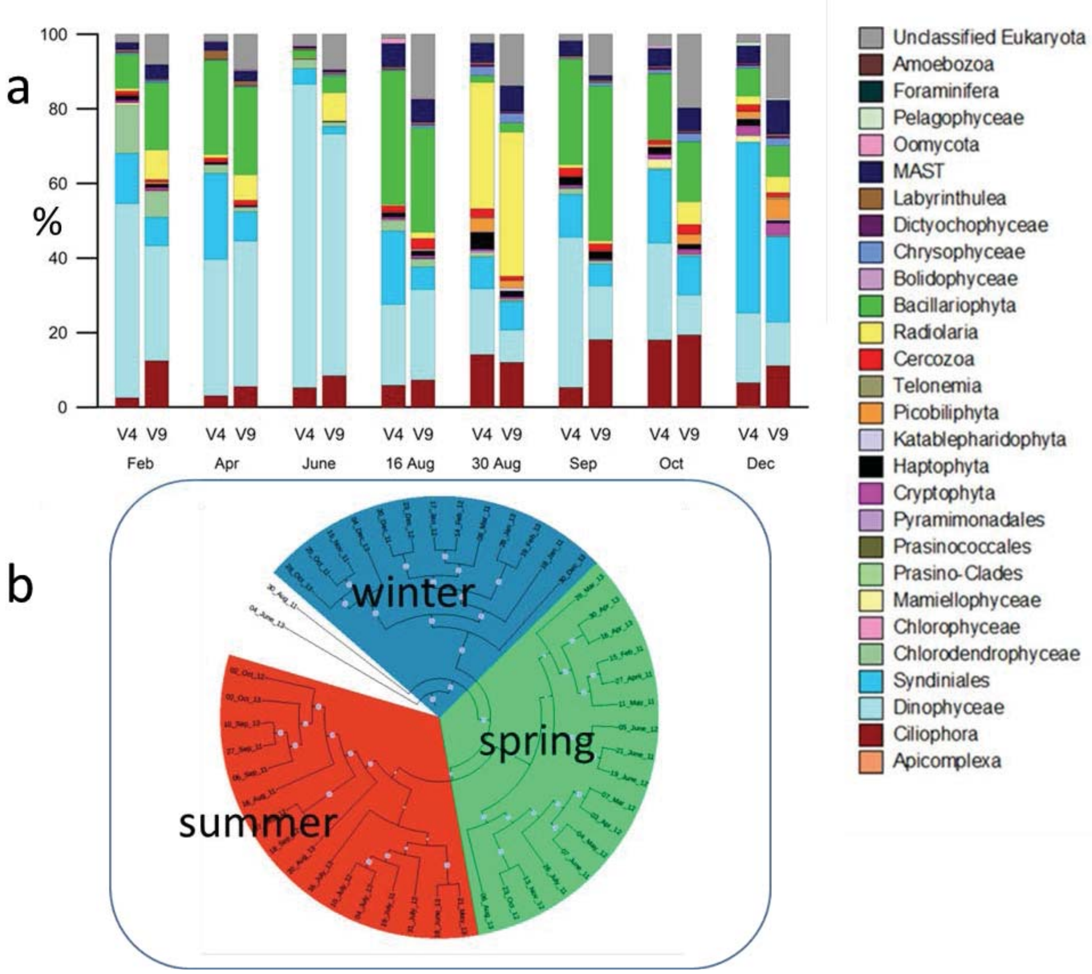

Figure 6. HTS-DNA metabarcoding of protist communities at LTER-MC. a) Relative contribution of different taxonomic groups on 8 sampling dates across one year; b) protist communities on 48 sampling dates over 3 years, showing a clear seasonal signal (Piredda *et al*., unpublished).

This research has been supported by the EU Projects MIDTAL and BioMarks (the latter providing HTS protist sequence across European seas), the MIUR-FIRB ‘Multitaxa approach to the study of Italian Diversity’, and the RITMARE Flagship Project.

c. Plankton responses to specific environmental and anthropic factors

c1. Iron Fertilization Experiment (Montresor)

Ocean iron fertilization experiments are based on the hypothesis that the addition of trace amounts of iron to iron-limited phytoplankton leads to blooms with subsequent mass sinking of organic matter and ultimately sequestration of significant amounts of atmospheric carbon dioxide (CO_2) in the deep sea and sediments. During a five-week long experiment carried out in a mesoscale eddy of the Antarctic Circumpolar Current, a large diatom bloom peaked in the fourth week after fertilization and was followed by mass mortality of several species that formed rapidly sinking aggregates. At least half the bloom biomass sank far below a depth of 1,000 m and a substantial portion was likely to have reached the seafloor (Figure 7) (**Smetacek *et al.*, 2012**). The bloom was comprised of various thin- and thick-shelled diatom species. The ecology of these silica-sinking species decouples silicon and carbon cycles in the iron-limited Southern Ocean, whereas carbon-sinking species, when stimulated by iron fertilization, export more carbon per silicon (**Assmy *et al.*, 2013, 2014**). During another iron fertilization experiment carried out in a cyclonic mesoscale eddy located along the Antarctic Polar Front in the Atlantic sector of the Southern Ocean, it was provided the first quantitative evidence of the major ecological relevance of copepods and their fecal pellets in the cycling of iron in silicate depleted areas (**Laglera *et al.*, 2017**).

This research was supported by the SZN FOE.

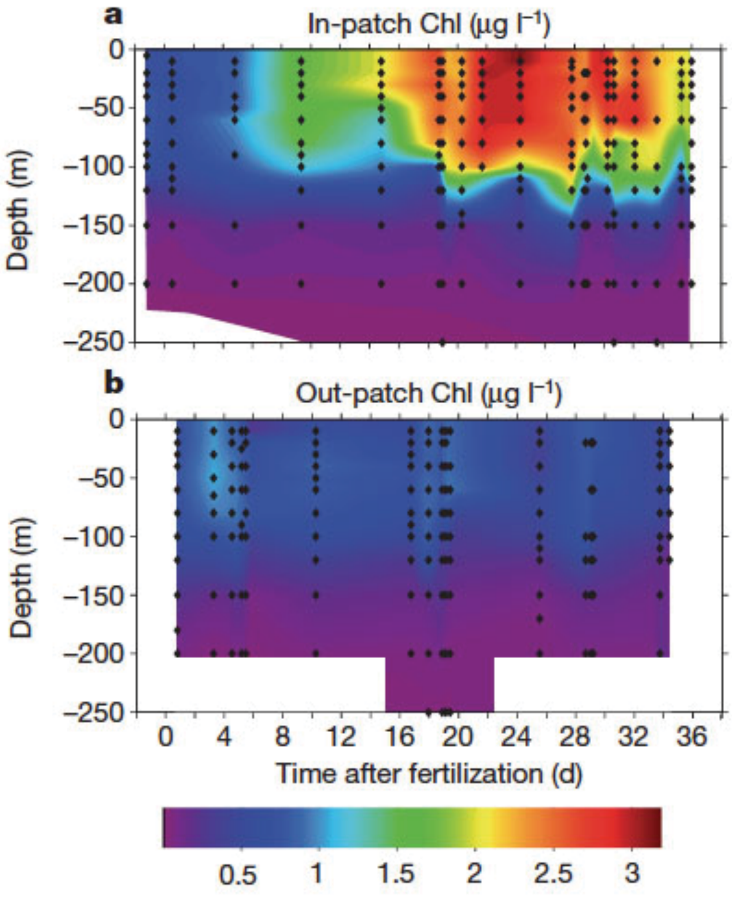

Figure 7. Chlorophyll *a* concentrations within the iron-fertilized patch (a) and outside it (b). Chlorophyll concentration reflect the growth, peak and demise phases of the diatom-dominated EIFEX bloom in the Atlantic sector of the Southern Ocean.

c2. Phytoplankton in the Ross Sea (Saggiomo, Margiotta)

Phytoplankton communities and primary production processes were studied in relation to physical and limiting factors in different environmental contexts, i.e., sea-ice, marginal ice zones and ice-free waters. In land fast ice of Terra Nova Bay during spring, sympagic diatom blooms occurred in the bottom and platelet ice, where biomass reached values up to three orders of magnitude higher than in sea-ice free waters. These two typologies of sea ice are characterized by different microalgal assemblages. High biomass of benthic diatoms accumulating in the bottom horizon of the land fast ice constitutes an important organic matter source for both pelagic and benthic food webs. By contrast, the planktonic species of the platelet-ice constitute an incubator for the late spring bloom in open waters of Terra Nova Bay (Figure 8) (**Saggiomo *et al.*, 2017**).

During the austral summer of 2014, the considerable biomass and large-size phytoplankton indicated relevant alterations in the seasonal productivity. Moreover, the distribution of the main functional groups showed significant anomalies, represented by the unusual high abundance and distribution of *Phaeocystis antarctica* colonies in a wide upper mixed layer, contradicting the classic paradigm of Antarctic diatom accumulation in highly stratified waters (**Mangoni *et al.*, 2017**). The imbalance observed between phytoplankton standing stocks and primary consumers, independent of the phase of the bloom, might dramatically alter the fate of the summer primary production and the carbon export in the Ross Sea. The distribution of the carbonate system in surface waters showed complex spatial heterogeneity and was controlled primarily by phytoplankton activity rather than physical forcing (**Rivaro *et al.*, 2017**). The substantial changes recently observed in the Ross Sea will probably affect the entire Southern Ocean with consequences a global scale.

This research has been supported by National Plan for Antarctic Research (PNRA).

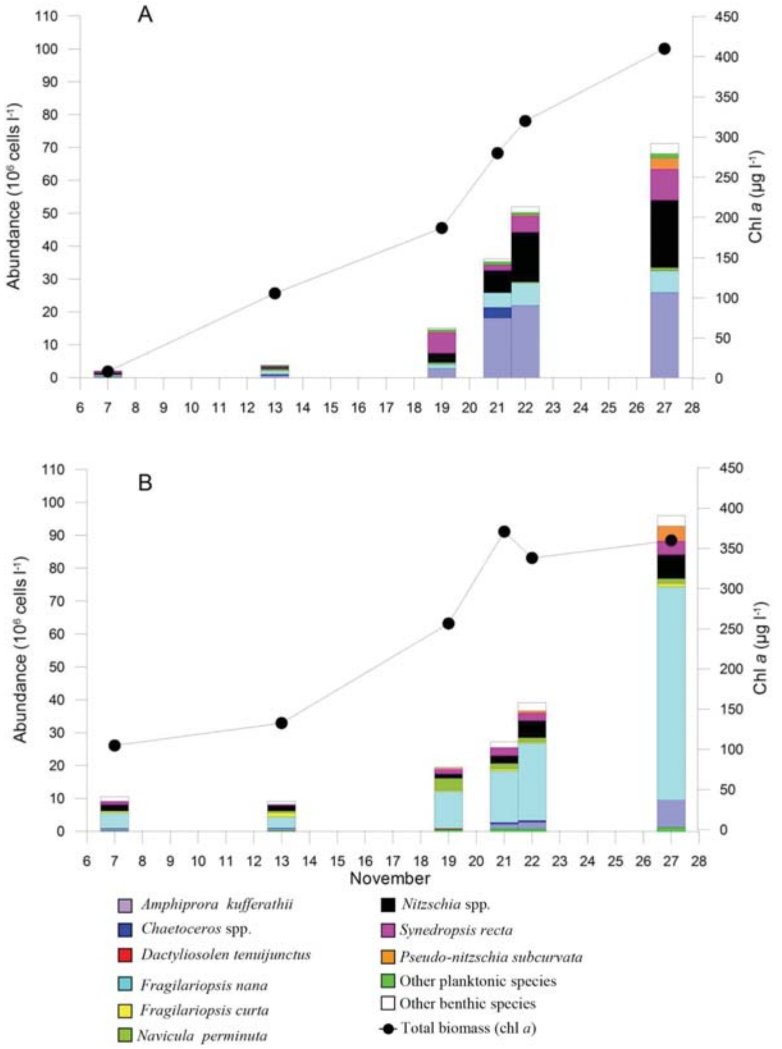

Figure 8. Temporal variation of diatom cell abundance and chlorophyll (chl) a concentration in (A) the bottom ice and (B) the platelet-ice layer in Terra Nova Bay, Antarctica in November 1999.

c3. Microplastics

Mesocosms (Brunet). A two-week experiment (INPUT) was carried out in June 2016 using six large mesocosms deployed in the coastal area of the Gulf of Naples to investigate the ecosystem responses to nutrient fertilization and microplastic addition. Microplastics (five types, 20-1000 µm size range) were poured into three mesocosms and the physical, chemical, biological and ecological variables were monitored daily. The preliminary results indicate that the distribution of microplastics along the water column was mainly related to hydrodynamic conditions and partly to their density. Microplastics seemed to determine changes in the abundance and activity of bacterial community, which affected the nutrient concentration, with a cascading effect on phytoplankton in terms of growth, diversity, size dominance and interactions with heterotrophic microbes and on microzooplankton. Viral abundance and production seemed also to be affected by changes in the microbial community. Microplastic fragments were found in the gut of mesozooplanktonic species, as well as in their faecal pellets but grazing experiments with a target copepod species indicated low rates of ingestion.

This research has been supported by SZN FOE.

Tara-Mediterranean (Mazzocchi). The preliminary data collected during the Tara-Mediterranean expedition and analysed within the Tara-Med Consortium show that abundant microplastic

fragments of different typologies were spread in all surveyed areas. Microplastics were much more abundant in the western than in the eastern basin and the coastal zones of Naples, Corsica and Marseille were identified as areas of particularly high plastic concentration. The high ratio of plastic to zooplankton abundance suggests a potential impact of microdebris on the numerous neustonic zooplankton taxa and their incorporation into the neustonic food web, with consequences on the pelagic biota in the most polluted areas.
This research has been supported by the SZN FOE.

d. Plankton dynamics at global scale

d1. Tara-Oceans (Iudicone, Ribera d'Alcalà, Zingone)

Tara-Oceans allowed an international multidisciplinary team to collect and analyse a huge dataset of eco-morpho-genetic data of total plankton ecosystems, from viruses to animals, during two circum-navigations of the world oceans. Over the last 10 years, Tara-Oceans have thus compiled the most comprehensive collection of homogenous samples and data for any planetary biome, and pioneered a systemic modeling of the world Ocean system based on actual biological data. The Tara Oceans project has generated the largest standardized eco-morpho-metaomics dataset available (>40 Terabases), including over 1,000 virus-, prokaryote-, and eukaryote-enriched metagenomes and metatranscriptomes, as well as 4 billion eukaryotic and prokaryotic metabarcodes from 3,000 size-fractionated plankton communities worldwide (to be added to over 13,000 environmental measurements). The results have had great scientific and societal impact, with notably the creation of the first global eco-systemics, publicly available database at EBI (European Bioinformatics Institute).
Marine plankton undergo a constant reset and re-organization of trans-kingdom communities at a much higher rate than in any other global biomes. Eukaryotic ribosomal diversity saturated at ~150,000 operational taxonomic units, about one-third of which could not be assigned to known eukaryotic groups (**De Vargas *et al.*, 2015**). The first global plankton interactome spanning all domains of life showed that biotic interactions predominate over environmental controls in shaping community structure and the prevalence of symbiosis *sensu lato* seems to challenge the classical view of food webs (**Clerissi *et al.*, 2015**; **Lima- Mendez *et al.*, 2015**). Applied systems-biology network approaches to identify euphotic plankton sub-communities and gene modules associated with carbon export from the upper photic zone to the ocean interior allowed to formulate insightful hypotheses about key ocean biogeochemical processes (**Guidi *et al.*, 2016**). The study of the impact of the Agulhas choke point on plankton ecosystems demonstrated the impact of large-scale circulation on transported communities across basins via the integration of *in situ* metagenomics data with the modelling of the physical transport (Figure 8) (**Villar *et al.*, 2015**). A biogeographic study showed that diatoms are an important and diversified component not only in coastal areas but also in open sea regions (**Malviya *et al.*, 2016**). The global analysis of the influence of ocean circulation on plankton biogeography showed the dominating role of currents in shaping plankton communities on a characteristic turnover time scale of one year (**Richter *et al.*, under revision**). The analysis of plankton network response, from genes to communities, to natural perturbations of the iron availability pointed to a large functional redundancy and consequent plasticity of ecological networks as result of the ecosystem substrate, a fluid in continuous movement (**Caputi *et al.*, under revision**). The Tara-Oceans dataset has also revealed a high diatom diversity in offshore areas based on HTS sequences and an interesting symbiotic association between diatom (*Fragilariopsis doliolus*) and tintinnid species (*Salpingella* spp.), whose distribution has been traced across the world seas (**Vincent *et al.*, 2017**). Collectively, all these initial studies represent unprecedented scale systems-biology analyses of a global ecosystem and are today an international reference.
This research has been supported by SZN FOE, EU-MicroB3, RITMARE Flagship Project.

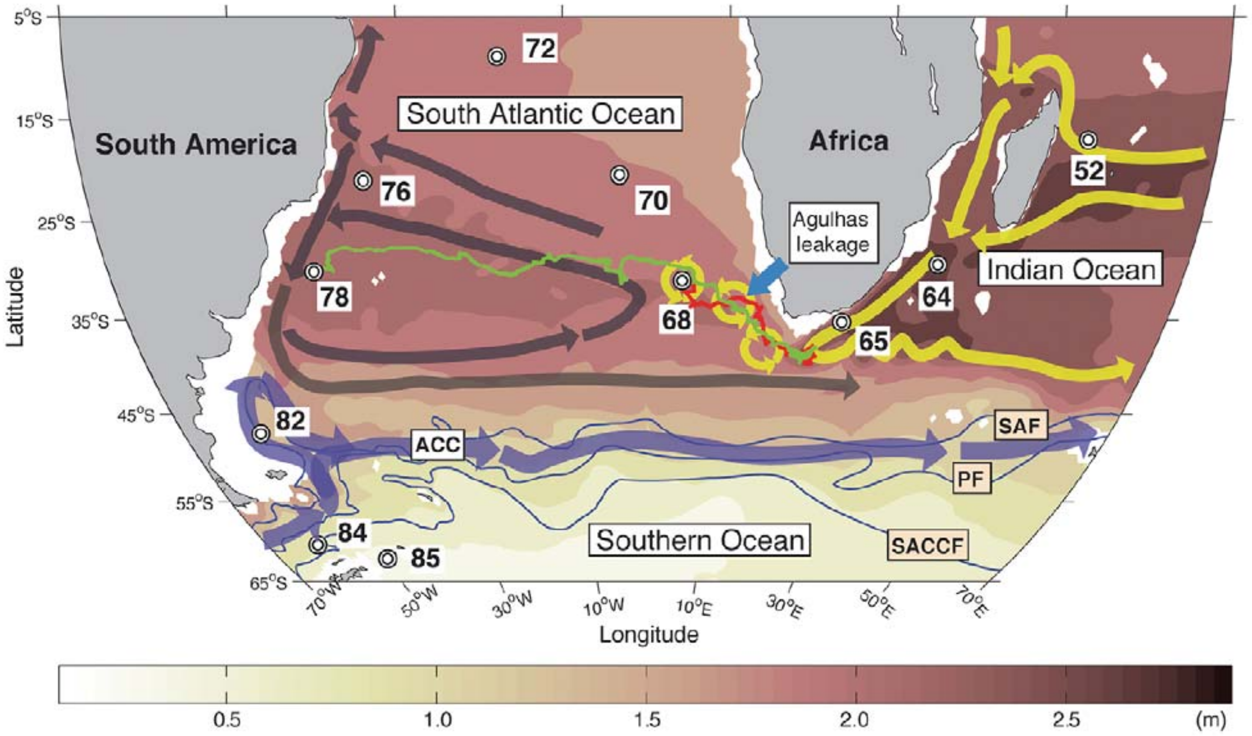

Figure 9. The oceanic circulation around the Agulhas choke point and location of Tara-Oceans stations that were used for the analysis of the interbasins global ocean plankton connectivity. The Agulhas Ring was sampled at St. 68.

d2. Impact of physical oceanography on biogeochemical cycles (Iudicone)

We tested the hypothesis that the physical environment sets both the biogeochemical and organismal responses by redistributing chemical tracers among water masses because of strong buoyancy forcing (from storms to large scale mixing). We have identified the mechanisms underlying the formation of the oceanic anthropogenic carbon inventory (e.g., **Iudicone *et al.*, 2016**) and completed a series of studies on i) the effect of storms and turbulence on global biogeochemical cycles and on the bloom dynamics (e.g., **Lacour *et al.*, 2017**), ii) the intensity of the vertical currents in the oceans (**Zhai *et al.*, 2017**), and iii) the settling velocity of phytoplanktonic organisms (**Olivieri *et al.*, 2014**). We completed a study on the interplay between the seasonal cycle of forcing and the plankton life strategy (reduced growth phases after the sexual reproduction).

This research has been supported by RITMARE Flagship Project, NSF, NOAA, EU-MicroB3.

4. Perspectives

a. Plankton ecology in the Gulf of Naples

Plankton populations are undergoing major changes at global level. For instance, increased temperature and progressive oligotrophication of surface waters are favouring smaller phytoplankton species, decreasing primary production. Moreover, crustacean populations are decreasing at global scale while outbreaks of ‘jellies’ occur more frequently along the coasts. Modifications of relative abundances along the food-web can be amplified by trophic relationships and induce important changes, especially at the top of the web. The latter changes have important implications on ecosystem services, e.g., provision of fish stocks.

We will analyse the three-decade (1984-2015) plankton dataset to extract information on different aspects of long-term plankton dynamics in the Gulf of Naples. With the general aim to identify trends, changes and shifts or resilience in the whole pelagic system or in any of its abiotic and biotic components, we will address: physical and biogeochemical variables (Margiotta, Ribera d'Alcalà *et al*.), pigments (Saggiomo *et al*.), picoplankton (Casotti, Balestra *et al.*), phytoplankton (Sarno, Zingone *et al.*), microzooplankton (Franzè, Percopo *et al*.), mesozooplankton (Mazzocchi, Di Capua *et al*.), copepod grazing (Mazzocchi *et al*.), copepod reproduction (Carotenuto *et al*.), plankton phenology (Mazzocchi, Zingone *et al*.), size distribution (Zingone *et al*.). The data analyses are in course and will be finalised for a series of manuscripts for a special issue of an ISI journal. Ecological network approaches will be applied to different time-phases (e.g., lower vs. higher temperature periods) to reveal possible long-term changes in the planktonic food web at LTER-MC (Ribera d'Alcalà, D'Alelio *et al*.).

Our long-term time series will be integrated with investigations on gelatinous zooplankton (D'Ambra). Hydromedusae will be identified at species level in the mesozooplankton samples and gelatinous macrozooplankton will be included in the regular weekly monitoring using a drone, which will allow to record and quantify the occurrence and distribution of jellies and large salps at station LTER-MC and in the whole Gulf of Naples.

Short-term responses of picophytoplankton will be addressed using an *in situ* scanning flow cytometer placed on the elastic beacon in front of the city of Naples. Challenges are represented by automated acquisition and data analysis through a software under construction. In addition, the short spatial scale of heterotrophic bacteria abundances will be investigated using high-frequency flow cytometry with the aim of identifying crucial sites along coastal areas that deserve closer investigation for their potential threat to human health in terms of pathogens. This involves technical improvements and the coupling of classical cultivation methods for the screening of potential pathogenic microbes (Casotti).

The research at station LTER-MC and other complementary studies conducted at a larger spatial scale in the Gulf of Naples will contribute to the establishment and reinforcement of a Marine Observatory in the Campania Region (www.osservatoriodelmare.it).

The establishment of a fully multidisciplinary, end-to-end, genomic-enabled observatory in the Gulf of Naples will be realized through a pilot project implementing the Tara-Oceans genomic protocols and pipelines within the routine sampling at station LTER-MC. As part of the observatory, a link with functional laboratory studies will be established via the set up at the SZN of genomic/ecological database that will include the Tara-Oceans database (NEMO project). The activity will be conducted within the newly created G7 working group on Augmented Observatories, directed by the SZN and involving several other institutions at international level.

b. Plankton molecular ecology

b1. Bacteria (Casotti)

The hidden functional diversity of marine heterotrophic bacteria will be explored by means of a combination of flow cytometry and molecular methods (CARD-FISH and High Throughput Sequencing. Metagenomics and metatranscriptomics datasets from the Gulf of Naples and the Northern Adriatic Sea are being analzyed and will provide useful information about community composition and potential metabolic activities of local communities and how they structure according to the environmental conditions.

b2. Protists (Zingone, Kooistra, Montresor, Sarno)

HTS-metabarcoding studies will continue with the analysis of a 3-years' dataset collected at station LTER-MC, to highlight temporal and spatial scale of the whole protist communities. Besides ribosomal V4 and V9 markers, this dataset also includes chloroplastic 16S data, which will be analysed in comparison with detailed pigment data (HPLC) to shed light especially on autotrophic flagellates, which are an important and poorly known component of the plankton community. Within a PhD project, HTS data will be fully integrated with the dataset of biological and environmental variables to address the relationships of taxonomic and functional protist diversity with seasonally and annually varying environmental conditions.

In addition, the HTS data will be used to assess possible changes in the population structure of selected *Chaetoceros* species over the seasons. The data will be compared with similar HTS datasets generated elsewhere to assess possible phylogeographic structuring within these species.

b3. Zooplankton (Stefanni)

This research activity new to SZN will be conducted by analysing data acquired in previous projects. Mesozooplankton will be characterized throughout the Adriatic Sea (46 stations, 42º-46º lat), using a highly variable fragment of the mtDNA COI and the V9 region of 18S rRNA genes. Sampling was carried out in the summer of 2014 and supported by the MEDIAS project and the RITMARE Flagship Project. A combined effort in identification of prey in gut contents by classical microscopy methods, coupled with a metabarcoding approach, will provide insights of alien species that contributes to the diet of small pelagic fish. With this aim, particular attention will be paid to detection and quantification of the copepod *Pseudodiaptomus marinus* in gut contents of anchovies and sardines in the northern Adriatic Sea. This project is supported by Lifewatch-Italy Call for interdisciplinary research projects at the distributed Laboratory of "Molecular Biodiversity". Metabarcoding analysis will also be applied to mesozooplankton throughout the Western Mediterranean Sea (26 stations) using a highly variable fragment of the mtDNA COI gene. Samples were collected during the Research Cruise VENUS3-OC2015 in August 2015 in the project Ocean Certain (EU-FP7 2013-2017 “Ocean Food web Patrol – Climate Effects: Reducing Targeted Uncertainties with an Interactive Network”).

c. Plankton responses to specific environmental and anthropic factors

c1. Phytoplankton in the Ross Sea (Saggiomo, Margiotta)

In the Ross Sea, additional studies will be conducted to assess the role of sea ice dynamics in shaping the sympagic communities at temporal and spatial scales. The ongoing P-ROSE Project aims to investigate pelagic communities (in terms of standing stocks and carbon transfer through the pelagic food web) as related to sea ice dynamics and the chemical and physical properties of water masses. Specific objectives of the project are to: i) identify signs and patterns of biological responses to current climate change; ii) provide an Ecosystem Based Approach for the assessment of the climate change consequences on biological stocks and productivity patterns; iii) explore future scenarios of change in the Ross Sea. An oceanographic cruise has been conducted in late-spring and early summer 2016, aimed at comparing the data acquired during several oceanographic cruises carried out by Italian Research Team from 1994 to 2014. Moreover, an oceanographic cruise will be carried out in December 2017-February 2018 on board the R/V Natalie Palmer, in the framework of CICLOPS Project, funded by the NSF. The main objective of this research is to determine whether iron and vitamin B12 dynamics are primarily responsible for causing shifts in phytoplankton community composition in Terra Nova Bay.

c2. Microplastics

Regarding the INPUT experiment, we plan to (i) complete the analyses of the results and finalize the entire database to test the hypotheses addressed until now, (ii) propose a schema of functioning and stress of the ecosystem under microplastic pollution. Successively, we plan to quantify the biogeochemical cycle of microplastics in a coastal area and to model numerically the ecosystem functioning under the microplastics pressure. From this study, we expect to address new questions regarding the microbial life in the pelagic “plastisphere” (**Brunet**).

The preliminary results of the numerous variables collected during the TARA-Mediterranean survey will be merged in targeted and global analyses to depict the presence and dynamics of microplastics and their effects on the pelagic system at basin scale. In particular, for neustonic zooplankton, the database will be completed with the analysis of samples collected during the night and of possible microplastic ingestion by target taxa (**Mazzocchi**).

The microbial communities associated with microplastics will be characterized based on the observation that, as soon as plastic enters the ocean, it is colonized by microbes, and bacteria at first. Therefore, the structure of the community should be a function of residency in the water and plastics may also represent carriers for potential pathogens. This research will be developed in the framework of the MicroMare project (**Casotti and Palumbo**).

Marine microbial communities can potentially harbour the metabolic machinery to degrade plastic polymers. Within the project "Ricerca il futuro – Davines", the potential of bacterial-mediated microplastic degradation will be explored by using indigenous marine and lagoon microbial communities from impacted coastal areas (**Quero**).

d. Plankton dynamics at global scale

d1. Tara-Oceans (Iudicone, Ribera d'Alcalà, Zingone)

Despite the discovery of thousands of new species and millions of new genes, we are just starting the integration of the complex heterogeneous Tara-Oceans data (DNA, images, environmental parameters) into a new, holistic understanding of the marine ecosystems. We need a significant amount of experimental and theoretical work to fully use the power of this massive amount of information, understand the interactions across scales, processes, and systems, to extract ultimate information on the eco-evolutionary dynamics of this complex adaptive ecosystem to predict the future of our ocean in our fast-changing world. The encouraging results on the role of the oceanic connectivity in setting up the plankton community composition and dynamics will thus be continued by exploiting a newly created database of gene mutations (Single-Nucleotide Polymorphisms) at global scale, with a specific focus on the rapidly changing Arctic communities. The analysis will inform on the global spatial patterns of transport via the accumulation/dispersal of mutations of plankton functional genes at global scale and the evaluation of the actual gene flow (and presence of metabolic barriers such as fast changes in temperature along the pathways). By developing new theoretical tools from the application of the complex system theories, this activity based on dedicated modelling will improve our understanding of interactions, adaptation and evolution of ocean's life in the context of ongoing climate changes. Plankton functional realised niches will be identified via the use of statistical tools from terrestrial macroecology and will be analysed in relation to the ecological network structures and their responses to the climate change.

d2. Impact of physical oceanography on biogeochemical cycles (Iudicone)

The role of micro- and macro-scale physics will be investigated using numerical, theoretical and experimental tools. A particular focus will be given to the study of the role of vertical velocities in the oceans and on their impact on the carbon export and sequestration and on the dispersal of plastics.

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6. List of grants

- **Biodiversitalia: Multitaxa** approach to the study of the biodiversity response to climate change in Italy (2012-2016)
- **CEFA**: Funzionamento degli ecosistemi costieri in un Oceano Antartico in cambiamento (2012-2017)
- **EU-BIODIVERSA-BioMarKs -** Biodiversity of Marine euKaryotes (2008-2011)
- **EU- Enveurope:** Environmental quality and pressure assessment across Europe: the LTER network as an integrated and shared system for ecosystem monitoring (LIFE08 ENV/IT/000399) (2012-2014)
- **NEMO** Premiale MIUR (2017-2019)
- **P-ROSE:** Plankton Biodiversity and Functioning of the Ross Sea Ecosystems in a changing Southern Ocean (2016-2019)
- **RITMARE**: The Italian research for the sea. Italian Flagship Project (2013-2018)
- **ROME**: ROss sea Mesoscale Experiment (2014-2017)

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8. Adaptive responses of benthos to environmental changes and multiple stressors

1. People involved

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2. Background, aims and objectives

Marine coastal habitats are amongst the most productive systems of the planet, yet they are undergoing rapid transformations in response to intensifying human activities and global change [92,93]The coastal environment benefits society by underpinning a wide range of activities, including fisheries, shipping, aquaculture, tourism, windfarms and recreation[94]. These activities provide important economic assets, but they also cause social and ecological impacts through increased habitat degradation, pollution and eutrophication[95]. Regional stressors cumulate with global threats such as ocean warming and acidification, increasing frequency and intensity of extreme climate events, sea-level rise and biological invasions[96,97].

Single and multiple stressors can affect single organisms, leading to range shifts, behavioral changes, altered phenology and local extinctions. Organismal responses to global environmental change are conventionally categorized as either ecological or evolutionary, the former including phenotypic plasticity and dispersal, while the latter entailing genetic change. Effects ultimately reflect on the biodiversity and structure and functioning of benthic communities at various spatial scales.

Recent biodiversity conservation actions and mitigation measures are aimed at reducing the impact of anthropogenic activities on the natural environment, for example the Aichi targets of the Convention for Biological Diversity (CBD), the European Marine Strategy Framework Directive (MSFD) and Water Framework Directive (WFD) and the Ecosystem Approach of the Mediterranean Action Programme (UNEP-MAP). A major concern is the high rate of species-level extinctions, often equated with loss of biodiversity, as well as extinctions of local populations.

The aim of the present research topic is to address ecosystem and organismal response to single and multiple stressors related to human pressure and global changes. Related research has been developed at different hierarchical scales, and benthic ecosystem structuring species such as seagrasses, have been studied.

Among the climate-related factors, temperature and ocean acidification (OA) are the ones mostly affecting the marine biota, although climate changes also have indirect effect on light regime and intensity, sediments and nutrients inputs from nearby terrestrial systems. Emissions of CO_2 volcanic origin represent suitable natural laboratories to study the medium and long term effects of Ocean Acidification on the benthic biota. Some of the research objective have been carried out at the natural CO_2 vents occurring at the Island of Ischia, while organismal response to changes in light and temperature has been studied across natural geographic and bathymetric clines and in mesocosm conditions.

The results obtained during the period 2012-2017 are in the following main research lines:

a. Seagrass adaptive response to environmental changes
b. Multilevel responses of marine macrophytes to a changing world
c. Adaptations of benthic communities and selected invertebrates to climate change with focus on ocean acidification
d. Analysis of responses of algal and invertebrate benthic assemblages to compounded global and local stressors
e. Effect of volatile compounds produced by micro- and macro-algae on the behavior of various invertebrates in relation to ocean acidification.

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3. Results

a. Seagrass response to environmental changes (Procaccini)

Seagrasses have been recognized in the European Union (EU) Water Framework Directive (WFD, Directive 2000/60/EC) as key coastal ecosystems. They are considered "ecosystem engineers" that form essential habitats for economically important fish and crustaceans, and provide crucial ecological services, including nursery grounds, sediment trapping and stabilization, nutrient filtering from coastal inputs, and carbon sequestration. Seagrass meadows and marine coastal habitats in general are undergoing rapid transformations in response to intensifying human activities and global change. The compounded effects of regional and global stressors erode the resilience (the ability of a system to withstand to and to recover from perturbations) of marine coastal ecosystems, including seagrass meadows and may ultimately cause transitions towards undesired states. This research line aims to understand seagrass adaptive response to single and multiple stressors and to identify early warning signals that the system is in distress, long before conditions become irreversible and possibly un-restorable.

Provision of genomic resources: The first complete sequencing of a seagrass genome (*Zostera marina*) revealed hints for plants adaptation to the marine environment and unique insights into the genomic losses and gains involved in achieving the structural and physiological adaptations required for marine lifestyle (Figure 1; **Olsen *et al.*, 2016**).

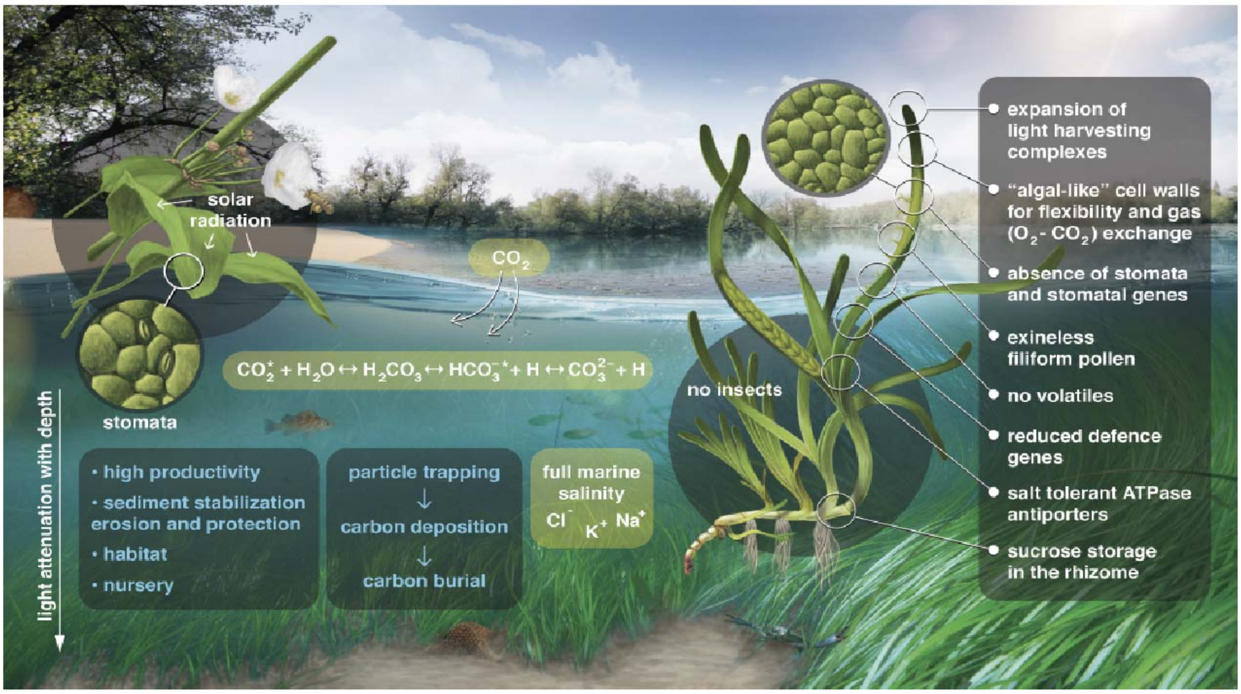

Figure 1. Conceptual summary of physiological and structural adaptations made by *Z. marina* in its return to the sea.

The first transcriptomes of the two most important Mediterranean seagrass species (*Posidonia oceanica* and *Cymodocea nodosa*) were obtained from plants in natural conditions and plants submitted to different abiotic stresses. Transcriptomic analyses from manipulative experiments characterized the molecular mechanisms that facilitate the persistence of Mediterranean seagrasses to transient warming (**Marin-Guirao *et al.*, 2017**) and ocean acidification (**Ruocco *et al.*, 2017**), while transcriptomics from natural plants identified the physiological adaptations which allowed *P. oceanica* plants to distribute along wide bathymetric gradients (**D'Esposito *et al.*, 2016**). Finally, a comparative transcriptomic analysis of *P. oceanica* leaves, male and female flower tissues addressed key biological and ecological issues related to flowering in marine plants (**Entrambasaguas *et al.*, 2017**).

Adaptive response: In the 2012 review (Seagrass ecophysiology meets ecological genomics: Are we ready?), Procaccini and collaborators summarize the state of art on the merging of ecological genomics with more classical approaches, looking at ecophysiology and morphology/phenology of marine plants. Since then, many progresses have been made. The response of *P. oceanica* and *C. nodosa* to changes in light, temperature and CO_2 has been analysed *in situ* and in mesocosms, by means of RNA-Seq and RT-qPCR, coupled with the study of changes in biochemistry, physiology and morphology. Overall results suggest that plants are adapted to the particular environmental conditions in which they grow and that different ecotypes differently respond to variable levels of the considered stressors. Deep and shallow *P. oceanica* plants show asynchrony in the daily regulation of gene expression following the daily light cycle and governing diel oscillations in key physiological processes (**Filisberto *et al*., 2015**; **Mazzuca *et al*., 2013**; **Procaccini *et al*., 2017**). They activate distinct response mechanisms to succeed in very different light climates (**Dattolo *et al*., 2013, 2014**), and save memory of their original response when transplanted in reciprocal conditions, supporting their adaptive divergence along the depth gradient (**Dattolo *et al*., 2017**). Similarly, the analysis of seagrass response to simulated heatwaves revealed contrasting strategies between species and between shallow and deep genotypes of the same species (*P. oceanica*). Their ability to sustain key physiological processes, such as photosynthesis and respiration (**Marin-Guirao *et al*., 2016**), or to activate antioxidant mechanisms (**Tutar *et al*., in press**) is on the basis of their different thermal tolerances. Moreover, the response of seagrasses to warming has been seen to be more plastic and complex than previously expected as derived from the first experimental evidence of flowering enhancement by temperature in *P. oceanica* (**Ruiz *et al*., in press**). Experiments conducted near natural CO_2 vents show, contrary to what was expected, that high CO_2 availability does not benefit plant productivity (**Olivè *et al.*, 2017**), but that increased nutrient supply enhanced *P. oceanica* production, re-establishing C/N balance (**Lauritano *et al*., 2015; Ravaglioli *et al*., 2017**). Mesocosm studies revealed in *C. nodosa* the overexpression of photosynthesis and respiration-related gene expression together with the activation of the biosynthesis of secondary metabolites (Ruocco *et al*., 2017). Multiple stressors, such as high CO_2 and high nutrients (Ravaglioli *et al.*, 2017) and high burial and high nutrients (**Ceccherelli *et al*., submitted**), show either antagonistic or synergistic effects. Potential early warning stress indicators have been identified in several studies combining plant responses at different levels of biological organization (e.g. Marin-Guirao et al., 2016; Tutar et al., in press). Some early molecular responses were identified to precede the deleterious effects observed much later on plant fitness and survival (Ceccherelli et al. submitted).

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b. Adaptations of benthos to climate change with focus on ocean acidification

b1. Multi-level responses of macrophytes (Buia)

Ocean acidification is an emerging problem and marine macrophytes are expected to benefit from the increased levels of CO_2 in the water. However, studies conducted *in situ* along a pH gradient showed that only a suite is resilient to long-term naturally high concentrations of pCO_2[98,99]. Among them, the seagrass *Posidonia oceanica*, the invasive alien green alga *Caulerpa cylindracea* and the geniculate calcareous red alga *Jania rubens* grow, with different cover, both at current and low pH, while the brown alga *Sargassum vulgare* occurs only at very low pH. This research line aimed to evaluate different levels of resilience under future climatic scenarios in species with different evolutionary traits by using a multidisciplinary and integrated approach. Reciprocal transplants experiment among populations growing at lowered and current pH were conducted in order to highlight differences between long and short-term adaptive responses.

The study conducted on the dynamics of early algal settlements revealed a decrease in species diversity and coverage along the pH gradient and a shift in the succession of morpho-functional forms, with crustose calcareous species disappearing and turf algae dominating (**Porzio *et al.*, 2013**).

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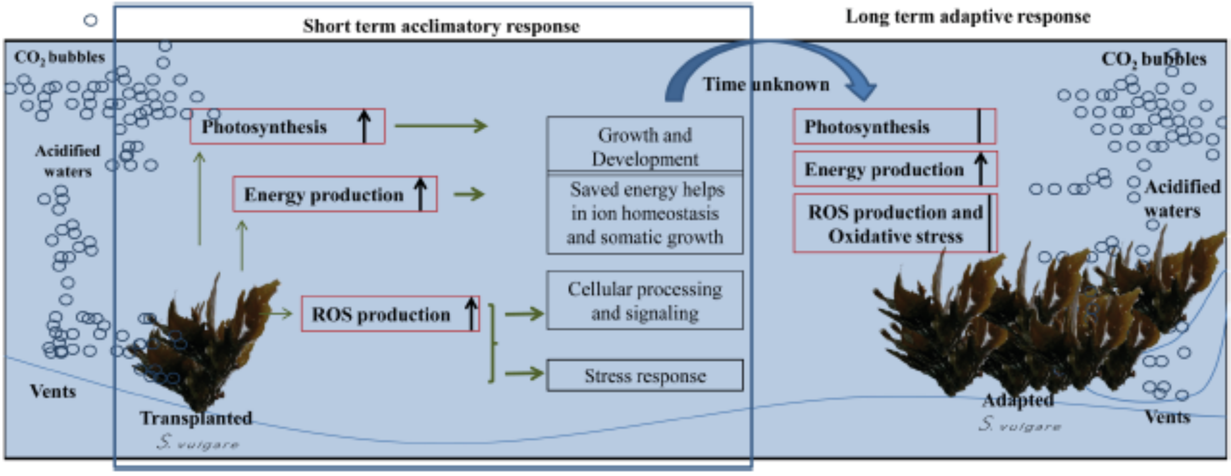

Figure 2. Summary of possible series of events happened to *S. vulgare* at the acidified site. (Red boxes: examined processes; black boxes: hypothesized processes.

In collaboration with A. Palumbo, I. Castellano and F.P. Patti, in the frame of an inter-laboratory PhD project, we identified, through a *de novo* transcriptomic analysis of “acid” and control *Sargassum* populations, the differentially expressed transcripts and the pathways affected under lowered pH/high-CO_2 conditions. A number of genes encoding proteins involved in cellular signaling, information storage and processing, and transposition were differentially expressed between the two conditions. Increased expression of transcripts involved in energy metabolism, photosynthetic processes, ion homeostasis, allocation of carbon to cell wall and carbon storage, was observed. Overall, these results indicate an adaptation of growth and development of *S. vulgare* at the CO_2 vents by increasing energy production to maintain ion-homeostasis and other cellular processes (**Kumar *et al*., 2017a**).

Moreover, a series of physiological and biochemical parameters, including photosynthetic rates, oxidative stress levels, antioxidant contents, activities of antioxidant enzymes and oxidative metabolism were measured in *S. vulgare* natural populations as well as in *in situ* reciprocal transplants from control to acidified site and *vice versa* (**Kumar *et al*., 2015**; **Kumar *et al*., 2017b**, Figure 2). The differences in the response of *S. vulgare* to acidification at different time scales indicate that regulating the inner oxidative processes this alga is capable to mitigate stress effects and that the natural population at the acidified site is adapted to live at the lowered pH (Kumar *et al.*, 2017b; **Porzio *et al*., 2017**).

The carbon and nitrogen metabolism was studied in above- and below-ground organs of *P. oceanica*. Under low pH, the plant is able to modulate the C and N metabolism. C:N ratio decreases in the leaves seemingly promoting grazing by herbivores. Dynamic C reserves (sucrose) are mobilized in view of a higher demand for shoot recruitment and for accumulation of N compounds whereas stable reserves (starch) are not affected; such a balance points to a buffering capacity against environmental perturbation. Acidification alters the isotopic composition of C and N in tissues and metabolites, possibly due to a quali-quantitative change in the supply of these elements (**Scartazza *et al*., 2017**).

Whilst *P. oceanica* did not show change in the photosynthetic activity between wild populations growing at normal and low pH conditions (Lauritano *et al*., 2015; Ravaglioli *et al*,, 2017), the performance of transplanted thalli of the erect calcareous alga *Jania rubens* was impaired under low pH whereas the carbonate skeleton content was not affected (**Porzio *et al*., submitted**).

This project was supported by SZN FOE and MIUR Italian RITMARE Flagship Project and by Regione Campania.

b2. Responses of benthic communities and selected invertebrates (Gambi, Teixido)

Climate change related stressors, such as increased temperature and ocean acidification, have increasingly proved to alter the biodiversity and structure and functioning of benthic communities at various spatial scales. Among the climate-related factors, ocean acidification (OA), defined as the other CO_2 problem" is an emerging stressor whose effects are still largely unknown for most marine organisms and the hierarchical levels of the biological organization. Emissions of CO_2 volcanic origin represent suitable natural laboratories to study the medium and long term effects of Ocean Acidification on the benthic biota. Around the coast of the island of Ischia, several marine vent' systems occur and, in addition to the well known vent's system at the Castello Aragonese, these new systems include various coastal habitat typical of the Mediterranean, such as a semi-dark cave, *Posidonia* beds, rocky reefs and coralligenous formations. The aim of this project is to investigate the biological and eco-physiological responses of selected benthic organisms robust or sensitive to OA, and the changes in the structure and functioning of benthic communities, by studying species/populations and habitat of the different vent's system around Ischia, as a proxy for near-future predicted scenarios of OA conditions at sea.

During the past 5 years, eco-physiological adaptation to low pH/high$p$$CO_2$ conditions (OA) of some species of polychaetes, molluscs and isopods was studied by means of transplants experiments conducted at the CO_2 vent's system off the Castello (Ischia). According to the species studied different responses were observed; sensitive species of isopods of the genus *Dynamene* and *Cymodoce* are able to maintain high energy metabolism when exposed to low pH but at the expenses of carbonic anhydrase (**Turner *et al.*, 2016**), as the sabellid polychaete *Sabella spallanzanii*, which however seem to be impaired by long-term exposure to OA in its oxidative stress and arsenic metabolism (Figure 3; **Ricevuto *et al.*, 2016**). Surprisingly also some species of calcifiers were able to survive on OA conditions.

Coralline algae settled in volcanic artificial tiles placed along the pH gradient of the Castello CO_2 vent's system, and followed up to 14 months, revealed that while the overall number of individuals is strongly reduced at low pH, the size and the mineralogy is not changed (Kamenos et al. 2016), suggesting the ability of these calcifying algae to buffer the negative effects of acidification.

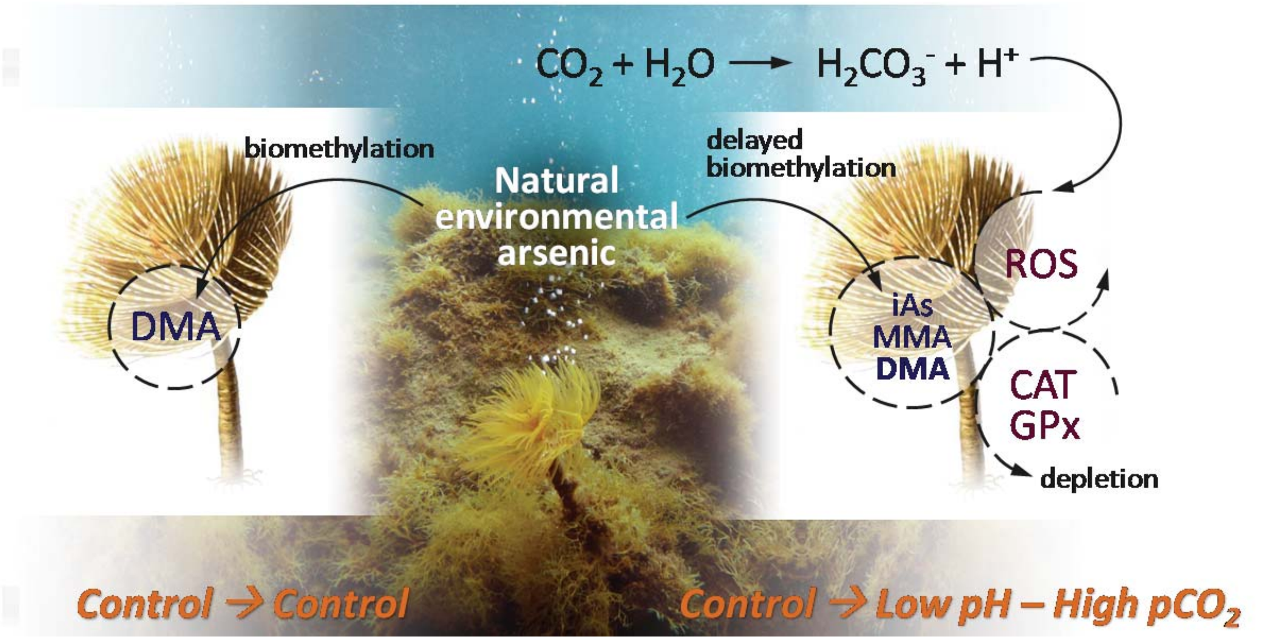

Figure 3. Ecophysiological and arsenic dynamics of the polychaete *Sabella spallanzanii* to transplant experiment in Ocean Acidification condition at the Castello CO_2 vent's system.

The bivalve *Pinna nobilis* juveniles transplanted in naturally acidified waters of the Castello vents, reveal a tolerance in parameters such as growth, mortality, oxygen consumption and mineralization at pH 7.7 compared to those ones at pH 7.2 (**Basso *et al.*, 2015**). Finally, two populations of the small calcifying spirorbid polychaete, *Simplaria* sp. living inside and outside the vents, when placed on low pH conditions in mesocosms, showed higher mortality, lower reproductive output and levels of maturation, and high tube growth when compared to individuals subjected to ambient pH conditions, suggesting that local adaptation to acidification has not occurred (**Lucey *et al.*, 2016**).

At population and evolutionary level we observed that two sibling species of the nereidid polychaete genus *Platynereis* showed a different habitat selection related to their different reproductive features, and exposure to OA conditions: the free spawner, *P. dumerilii* occurred (with at least two sibling species) under normal pH conditions, while the egg brooder and direct developer *P. massiliensis* was mainly recorded (with two sibling species) in acidified conditions within vent's systems (Ischia and Vulcano) (**Waege *et al.*, 2017**).
At community level, a study on the structural and trophic response of benthic macroinvertebrate community to low pH was finalized this year. The studied vent's system is located at the Vulcano island (Aeolian Archipelago) and demonstrated that ocean acidification is a driver factor of community simplification via the collapse of higher-order consumers and rise of lower-order consumers (**Vizzini *et al.*, 2017**). We have investigated 4 new habitat / sites around the coast of Ischia characterized by CO_2 venting and natural water acidification[100]. Field surveys in the new vent's systems allowed to assess pH variability and water chemistry, and identify (by visual and photographic censuses) benthic species and communities. These vents systems have been compared with various control areas (2 control zones not affected by venting for each acidified system) located at similar depth, and with similar habitat and overall environmental conditions. In particular, in the Mago's Cave vent system we set a transplant experiment with the dominant madreporarian species colonizing this area, *Astroides calycularis*, to compare its growth and biology. Mineralogy and trascriptomic responses will be compared with populations transplanted in control, normal pH zones.
Finally, a review summarizing the ecological research done at the Castello vents in the past 10 years has been recently finalized (**Foo *et al.*, in press**).
Project Funded by SZN FOE, RITMARE Flagship Co-Found (VulnerClima); "National Geographic Society" (Window to the future), "TOTAL Fundation" (High CO_2 Seas), Marie Curie fellowship Future4Oceans; Premiale NEMO (OGS & SZN).

b3. Effects on invertebrate behavior mediated by volatile compounds produced by micro- and macro-algae (Zupo)

This study is aimed at defining the effects of secondary metabolites on invertebrates, especially those performing as infochemicals, in order to define their ecological roles and to set possible biotechnologies based on chemical ecology techniques. The main topic is represented by the effect of volatile compounds produced by micro- and macro-algae on the behavior of various invertebrates in normal and acidified conditions.
Several VOCs are produced by diatoms and macroalgae when wounded. They may represent important infochemicals for invertebrates and fish. However, ocean acidification may change the physiology of the plants or the structure of the infochemicals, modifying the reactions of invertebrates (**Zupo *et al.*, 2015, 2017**). Thus their reactions, naturally evolved, may bring to a loss in fitness and a decrease of the natural populations. In this context we test the production of volatile compounds (in cooperation with the University of Cologne) by micro- and macro-algae in special bioreactors (devised ad hoc during the works for this project) allowing the control of pH and then we bioassay their activity on selected invertebrates, by measuring their chemotactic reactions. This research was supported by Acid.it project (OGS Premiale).

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[100] Gambi MC. Emissioni sommerse di CO_2 lungo le coste dell'isola d'Ischia. Rilievi su altre aree come possibili laboratori naturali per lo studio dell'acidificazione e cambiamento climatico a mare. ***Notiziario S.I.B.M***. (2014) 66: 67-79.

c. Responses of algal and invertebrate benthic assemblages to compounded global and local stressors (Bertocci)

Climate-related physical disturbance, such as that associated with severe storms, and nutrient inputs due to natural and/or anthropogenic activities occur concomitantly, but experimental tests of the simultaneous effects of changes in the regime of more than one perturbation are generally lacking. Filling this gap is the key to understand ecological responses of natural assemblages to climate-related change in the intensity and temporal patterning of physical disturbance combined with other global stressors. In the present study, such issues were addressed by means of a field experiment examining the responses to factorial manipulations of nutrient enrichment and of the mean intensity and temporal variability of storm-like mechanical disturbance, using benthic assemblages of tide-pools as model system. The examined response variables included mean abundance values and temporal variances of taxa with different life-traits (Figure 4, **Bertocci *et al.*, 2017 a**)

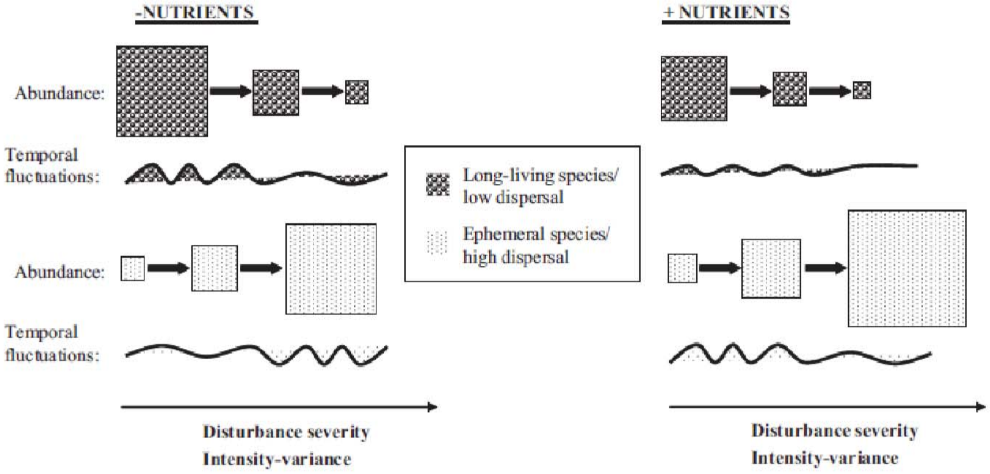

Figure 4. Schematic representation of how intensity and variance of mechanical disturbance and nutrient enrichment can affect patterns of abundance and temporal fluctuations of algal species with different life-traits.

Consistent negative effects of disturbance intensity were observed for the mean cover of long-living taxa (algal canopies and the polychaete *Sabellaria alveolata*), whose temporal fluctuations were also reduced by more severe mechanical stress. More resilient taxa (ephemeral algae, mostly green of the genus *Ulva*) increased under enriched conditions, particularly when low-intensity events were irregularly applied over time. Opposite effects of disturbance intensity depending on nutrient availability occurred on filamentous algae (e.g. red of the genus *Ceramium*). This suggests that, although nutrient enrichment stimulated the abundance of both algal groups, when this condition was combined with relatively mild physical disturbance the competitively superior ephemeral green algae tended to become dominant over filamentous red algae. Grazers were positively affected by nutrients, likely responding indirectly to more food available.

Project funded by RAP – Responses to Anthropogenic Perturbations: climatic and nutrient effects on rock pool assemblages - Reference: FCT 2009 Call for Proposals in All Scientific Domains - PTDC/MAR/111223/2009.

4. Perspectives

a. Seagrass response to environmental changes (Procaccini)

The future perspectives of the present research line go in different directions.
First, we are improving the available –omic resources in seagrasses. The sequencing of the first draft genome of four new species of seagrasses (*Posidonia oceanica, Cymodocea nodosa, Thalassia testudinum* and *Potamogeton crispus*) is ongoing under the support of JGI.
Second, we are further analysing the adaptive response of *P. oceanica* and *C. nodosa* to simulated heat waves (Reccam Project_MINECO, Spain). Cold-adapted and warm-adapted populations of the two species have been sampled along a latitudinal gradient and their differential response is analysed after exposure to heat stress in controlled conditions (mesocosms), using physiological, transcriptomic and metabolomics approaches. First results show differences in mechanisms for achieving C-balance and different capability of recovering after heat stress. After submitting plants to different levels of heat stress, we are also aiming at identifying genes that can represent early warning indicators of stress. The expression of genes involved in the flowering response to heat by Northern, cold-adapted *P. oceanica* populations is also in analysis, with the goal of detecting genes responsive of flowering induction.
Third, we are focussing on the plant response to multiple stressors, including regional human-driven stressors such as eutrophication and sediment accumulation. The combined effects of nutrient excess, combined with epiphyte loading (Tetris, PRIN 2013) and light stress are under analysis, both in natural and in mesocosm conditions. Moreover, the effects of industrial pollution on adult and juveniles of *P. oceanica* will be assessed in the framework of the project ABBaCo, together with a pilot transplantation study to evaluate the possibility of meadow restoration in polluted sites. A first experiment has been carried out in the new mesocosm system of SZN, especially designed for seagrasses, where *P. oceanica* plants have been kept at two different temperatures. We also aimed to assess if different parts of the same shoot respond in a different way and to define the most indicative leaf portion to analyse in order to standardize the response obtained in different experiments.
Fourth, we are looking at the effect of CO2 enrichment of the C-balance in *C. nodosa* plants kept in controlled conditions (GRASSMET FCT – Portugal). We will analyse genes theoretically involved in processes related to C/N budget, C and N assimilation (e.g. Rubisco and PEP carboxylase, nitrate reductase, nitrite reductase, glutamine synthetase and glutamate synthetase) as well as response to oxidative stress.
Finally, we are looking at the inherited adaptive response, evaluating the differences among juveniles kept in common gardens and collected in several populations along the distribution areal of *C. nodosa* (RESIGRASS, Ministerio de economia y competitividad, Spain). We also aim to start assessing the role of epigenetic mutation is affecting the response behavior of adult plants. This is especially important for clonal plants, such seagrasses, where epigenetic mutations can be at the basis of plasticity and persistence of long living genotypes.
We are also working at the integration of -omic approaches (metabolomics, proteomics and transcriptomics) in describing seagrass response to stress and exploring the application of a trait-based approach in seagrasses (Euromarine Foresight Workshop funded for 2018).

b. Adaptations of benthos to climate change with focus on ocean acidification

b1. Multi-level responses of macrophytes (Buia)

In collaboration with A. Palumbo, I. Castellano and F.P. Patti, *de-novo* sequencing analyses are in progress to examine the differential protein expression of *Sargassum vulgare* population at the acidified site compared to control populations. A label free shotgun proteomic analysis has been performed by Gabriella Tedeschi at the University of Milan and the proteomic platform at Filarete Foundation (Milan). The analysis at protein level will allow to have a better overview of the molecular mechanisms responsible for the life of *S. vulgare* at low pH.

Analyses of metabolomic data, performed by the group of Gerrit Beemster (University of Antwerp), are in progress to examine the differential content of the two populations regarding some metabolites, including sugars, fatty acids, amino acids, phenolics and mineral content.

Researches on short- and long-term effects of ocean acidification on the invasive green alga *Caulerpa cylindracea* are in progress. Physiological (chlorophyll - carotenoids contents, and photosynthetic performance) and ultrastructural analyses have been performed and will be compared with new data obtained from the congeneric species *Caulerpa prolifera*.

Transplants of *Posidonia oceanica* and *Cymodocea nodosa* from current to low pH have been performed: the analyses of ultrastructural changes in young meristematic leaves are in progress.

b2. Responses of benthic communities and selected invertebrates (Gambi, Teixido, Munari)

The aim of the future activities is to finalize the analysis on the benthic communities' composition, abundance pattern, distribution and functional traits in the Castello vents system, as well as in the 4 new vents systems and various control areas all located along the coast of Ischia.

In addition, we will finalize another project aimed to study phenology, epiphytes, microbiome and fungi associated to *Posidonia oceanica* living shoot and leaf detritus in some meadows subjected to ocean acidification in various CO_2 vent's systems of the island of Ischia (Vullatura, Castello) and Panarea (hot/cold points).

The analysis of the autoecological, eco-physiological and genetic (transcriptomic) responses of the Mediterranean madreporarian *Astroides calycularis* to ocean acidification and thermal stress will also be completed.

b3. Effects on invertebrate behavior mediated by volatile compounds produced by micro- and macro-algae (Zupo)

The analyses on the biochemical profiles of algae cultured in various conditions are still in progress. The data on the physiology of animals influenced by infochemicals will be used for the construction of models explaining future scenario in an acidified sea. The results on the physiology of *Cocconeis scutellum* (whose apoptogenic activity is null in acidified conditions) will be exploited to compare metabolomics profiles and shed light on the mechanisms investigated in the research line "Effects of organisms interactions on ecosystem functioning".

b4. Adaptive response of the sea urchin *Paracentrotus lividus* (Palumbo, Gambi)

Previous studies have reported that the sea urchin *Paracentrotus lividus* is present in the low pH zone at the Ischia CO_2 vents (mean pH ≈7.8-7.9), while it is absent in the extreme low pH zone (mean pH < 7.4)[101] [102]. Our aim is to characterize the population living at the acidified site compared to control populations with the objective to assess if it is adapted to life at low pH and what are the molecular mechanisms responsible.

In preliminary monitoring experiments by using a non-invasive tagging technique we revealed that the sea urchins are long-term resident at the low pH site. We have mainly focused the attention to immune cells, which are considered the sentinels of environmental stress. These cells have been characterized in terms of number and type, expression of stress markers, oxidative and nitrosative status, and antioxidant capacity. *De-novo* sequencing analyses are in progress to examine the differential protein expression of immunocytes of animals from low pH/acidified and control sites. These data, together with measurements of some physiological parameters (size, respiration and nitrogen excretion), will provide a better understanding of the molecular mechanisms used by the sea urchins in coping lowered pH environment in future predicted marine scenarios.

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[101] Kroeker KJ, Micheli F, Gambi MC. Ocean acidification causes ecosystem shifts via altered competitive interactions. ***Nature Clim. Change*** (2013a) 3: 156-159.

[102] Kroeker KJ, Gambi MC, Micheli F. Altered recovery dynamics result in homogenous assemblages in an acidified ocean. ***Proc. Nat. Acad Scie.*** (2013b) 110 (31): 12721-12726.

b5. Evolutionary relevance of ocean acidification and other stressors (Munari)

The fate and effects of environmental contaminants under global climate changes (GCCs) scenarios are still poorly studied. Till now, most research has focused on the evaluation of single stressors on a single life-stage. This approach does not take into account that multiple stressors, such as changes in environmental parameters and pollutants, act simultaneously in the environment, showing additional, synergistic or antagonistic effects on organisms, or inducing further unexpected effects. However, negative effects possibly imposed by environmental changes to marine organisms during different stages of their life history, can be mitigated somewhat by anticipatory parental effects. This kind of heritable plasticity may have important ecological and evolutionary consequences. The present study is then focused to investigate the selective pressure of ocean acidification and other human-induced environmental stressors on marine invertebrates using both CO_2 vents of volcanic origins around the cost of the island of Ischia, as natural laboratories, and more classic laboratory approaches.

The project will provide the opportunity to develop a new integrated approach to assess vulnerability of marine invertebrates to climate change and pollution, highlighting interactions between stressors throughout relevant phases of the animals' life history. The experimental setup will give new insight into potential transgenerational effects of the selected stressors and will represent a first attempt to fill the gap in the knowledge of risks for the maintenance of the wild populations in the future. In this regard, it has to be noticed that improving predictive ERA approaches is also pivotal to fulfil requirements in the EU Marine Strategy Framework aimed at defining environmental quality standards for marine coastal waters. The project will allow also to consider the role of regional constraints that may differently influence sensitivity of animals from the Gulf of Naples compared with those from other geographical areas.

b6. Phenotypic traits in the adaptability of marine Sponges (Nunez-Pons)

The state of the art of what is known about the mechanisms of adaptation in Porifera to acidified marine ecosystems is largely undiscovered, despite the fact that sponges are often forecasted as chief “winner” taxa in future marine benthic ecosystems. In general, there is a lack of knowledge about the biological processes underlying organisms' adaptability, but on top of that, research interconnecting the phenotypic processes that may lead to such acclimatization is totally lacking. Here we propose a multidisciplinary project integrating ecological, microbial, metabolic, chemical and isotopic studies to address strategies of acclimatization to Ocean Acidification in Porifera.

We have selected five target species displaying different levels of adaptation/tolerance to OA conditions and different ecological traits for our study.

Project co-funded by FOE-SZN and Polytechnic University Marche (Ancona)

c. Responses of algal and invertebrate benthic assemblages to compounded global and local stressors (Bertocci)

The study will focus on the responses of benthic organisms to current and predicted disturbances in habitats subjected to long-term anthropogenic pressure. The effects on coastal areas of human-induced perturbations, which occur typically on local scale, add to and potentially interact with events occurring at larger scales, including climate-change related disturbances. The post-industrial area of Bagnoli is an ideal case-study within such a general context, since the sessile assemblages therein are necessarily adapted to the stressful conditions due to the past polluting activities, but that could affect their resistance and resilience to additional pressure related with climatic events and the physical disturbance associated with the harvesting of organisms. However, the direction of such potential alterations is difficult to anticipate, as the adaptation to persistent stressful conditions could have made local organisms more resistant to further disturbance and capable of recovering faster from changes determined by new disturbance events, or, on the contrary, it could have made them more sensitive to disturbance and reduced their recovery ability.

Such alternative scenarios will be examined in the present project by means of two combined field experiments.

1. The first experiment will assess the responses of low-shore sessile assemblages from rocky substrates to events of mechanical disturbance simulating the physical impact of waves during extreme storms. Climate models and empirical observations indicate that such events will, in the near future, increase in intensity and tend to become more clustered in short periods, separated by prolonged ‘calm’ periods. In this context, patches of rocky substrate in both the area of Bagnoli and in reference areas far from it will be exposed to experimental combinations of mechanical disturbance varying in intensity (force applied) and temporal variance (regularly distributed vs. aggregated events over the course of the experiment), manipulated in a way suitable to separate the effects of the total intensity of disturbance applied during the course of the experiment and the effects of the temporal variability of the same total number of events. The experiment will last about 18 months, within which the mean frequency (number of events over time) of the experimental disturbance events will be established according to the number of natural events of extreme storms occurred in the study area in the last decade.

The examined responses will include changes in patterns of distribution, abundance and diversity of sessile assemblages, examined as mean values and temporal fluctuations over the entire experimental period. The comparison of such changes between the area of Bagnoli and reference areas will test the hypothesis that local organisms are characterized by different resistance ability to physical disturbance.

2. The second experiment will examine the recovery ability of assemblages dominated by different morpho-functional groups of organisms (e.g., mussel beds, turf-forming algae, encrusting algae) from the area of Bagnoli and reference areas, after increasing intensities of physical disturbance (e.g., harvesting of organisms, human trampling) ultimately capable of producing free patches of rocky substrate of increasing size. At the beginning of the experiment, patches of substrate of increasing size dominated by each morpho-functional group will be experimentally cleared of all organisms, then left untouched for 12 months. An analogous number of patches of the same sizes will be marked and left unmanipulated as control. During the course of the experiment, the patterns of re-colonization and recovery of organisms in cleared patches will be examined by quantifying the abundance of each taxon at consecutive times. The comparison of the recovery patterns among treatments and with the unmanipulated control in both the area of Bagnoli and in the reference areas will test for possible differences in the recovery ability of assemblages subject to different conditions.

Study funded within the ABBaCo project.

5. Publications (2012-2017; *corresponding author)

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6. List of grants

- **"Acid.it."** Progetto Premiale (OGS and SZN) (2016-2017).
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- **Marine biodiversity conservation** -POR- FESR Regione Campania (2015-2016).
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- **RECCAM**: Resiliencia de las praderas de angiospermas marinas al calentamiento global: un análisis basado en respuestas ecofisológicas, poblacionales y ecosistémicas - Spanish Govemment: "Proyectos de Investigación Fundamental No Orientada" (2014-2016).
- **RITMARE**: The Italian research for the sea. Italian Flagship Project of the Italian Ministry of University and Research (2013-2018).
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- **"Window to the Future"** National Geographic Society (2016).

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9. Biodiversity and functional mechanisms of marine organisms, from genes to populations

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2. Background, aims and objectives

The term ‘biodiversity’ is generally used to define the sum of organisms living in an ecosystem, but it encompasses multiple levels of organization: species, which are constituted by distinct populations of organisms, which have genes that determine their functional traits. The diversity of organisms, in turn, determines the structure and functioning of ecosystems, their resilience and capability to respond to disturbances. Biodiversity is considered as the main descriptor for marine water quality (Marine Strategy Framework Directive) and the ‘blue economy’ is based on a healthy ocean in which biodiversity is preserved and exploited in a sustainable way. The research addresses the following topics:

Protist diversity - High throughput sequencing (HTS) of rDNA marker regions, so called metabarcoding, has shown that the diversity of unicellular eukaryotes is severely underestimated. We investigate diversity of key unicellular organisms using an integrated approach that combines the study of their morphology, molecular fingerprint, physiology and life cycle traits. One important product are taxonomically curated reference sequences required for the interpretation of HTS data. We also explored protist diversity at the intra-specific level, studying the genetic structure of the model diatom *Pseudo-nitzschia multistriata.*

Functional diversity: focus on diatoms - To understand ‘biodiversity’ we have to understand the biology of the organisms and this is a stimulating challenge for protists. We have focused on diatoms, where we have set up molecular, genetic and genomic resources for different species, including the *de novo* sequence of the genome of the planktonic *P. multistriata.* Our research questions focus on i) the mechanisms that regulate specific life cycle phases, ii) the capability to perceive ‘signals’ deriving from the environment (turbulence), other organisms (sex), with the aim to define the major signaling pathways and physiological changes involved, iii) the role of specific secondary metabolites such as oxylipins and prostaglandins in diatom physiology.

Diversity and demographic structure of key marine species - The aim of this research is the study of microevolutionary processes that occur within single species at the population level and that affect species distribution in space and time. The higher or lower level of genetic diversity and connectivity between populations will also affect their response capability and resilience to external impact. We considered species with high ecological or commercial value, with different reproductive behaviors and ecological niches. We consider both animal and plant species. First, the seagrasses, whose valuable meadows provide important ecosystem services for humans; hence, the marine turtle *Caretta caretta*, an iconic endangered species; the tunicate *Ciona robusta*, an invasive species of polluted areas, model for developmental biology; two species of commercial interest, the cephalopod *Octopus vulgaris* and the teleost *Engraulis encrasicolus*, and benthic Polychaetes and molluscs.

3. Results

a. Protist diversity (Kooistra, Montresor, Sarno, Zingone)

A group on which considerable research has been carried out, also thanks to various international collaborations are the Chaetocerotaceae, a cosmopolitan family of marine planktonic diatoms, which includes two genera: *Chaetoceros* and *Bacteriastrum*. The family is very diverse and extremely abundant in all oceans including the LTER-MC site in the Gulf of Naples. Isolation of strains, ultrastructural study of their vegetative and resting stages and the sequence of LSU and SSU markers allowed to i) provide molecular information for species for which only morphological information was available, ii) detect, as expected, cryptic diversity in a number of taxa, iii) detect a number of species new to science (**Bosak and Sarno 2017; Gaonkar *et al*., 2017**, **Figure 1; Li *et al*., 2017; Balzano *et al*., 2016; Bosak *et al*., 2016a, b**) (Figure 1). This comprehensive study allowed to gain insights and new questions on species circumscription in protists, their biogeographic distribution, the information provided by different markers and ultimately the mechanisms through which diversification and speciation occur. A phylogenetic analysis based on LSU and SSU sequences will provide more than 200 taxonomically curated reference sequences for species of the genus *Chaetoceros*; the study is complemented by the analysis of the SSU-V4 barcode region that allows the discrimination of the vast majority of species.

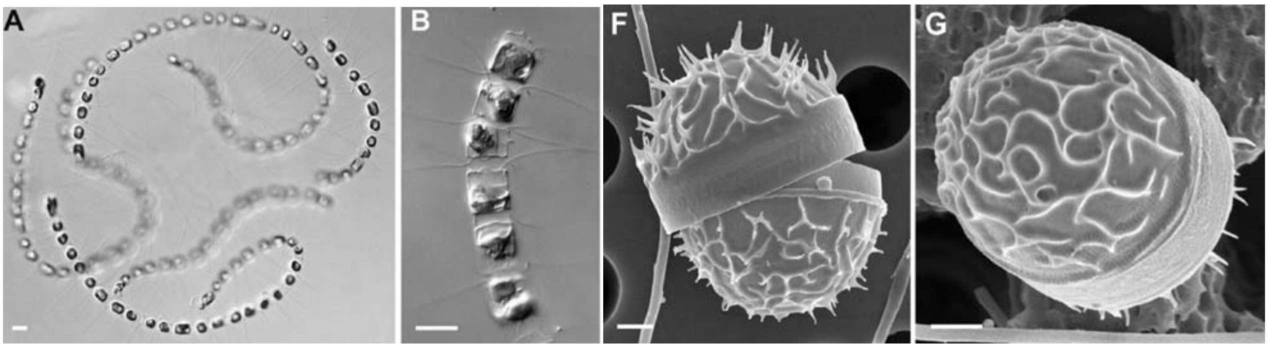

Figure 1. *Chaetoceros dichatoensis*, a cryptic species in the Chaetoceros socialis species complex: colonies of vegetative cells (A, B, light microscopy) and resting spores (F, G, SEM).

The Leptocylindraceae are the most ancient diatom family in which the comparison of numerous cultivated strains, shed light on the actual taxonomic composition of the family with the description of four new species (**Nanjappa *et al.,* 2013**). A first HTS biogeographic study mainly at the European scale just widened the diversity of the family by one unit, and showed a quite wide distribution of all taxa, which however at times differed in their ranges (Nanjappa *et al*., 2013).

Biochemical characters should be also taken into account to achieve a better species delineation and our studies show that this is a promising avenue to pursue, also in the perspective of biotechnological applications. As an example, oxylipin composition in each *Leptocylindrus* species reflected their phylogenetic relationships and also revealed the presence of a new metabolic

pathways for PuFA synthesis (**Nanjappa *et al.*, 2014**). A similar match has been reported for species of the genus *Pseudo-nitzschia*, which exhibit a rich and varied lipoxygenase metabolism of eicosapentaenoic acid (EPA), with a high level of specificity for oxylipin markers that generally corroborated the genotypic delineation, even among genetically closely related cryptic species (**Lamari et al., 2013**). Functional diversity provided by distinct metabolomic fingerprints was also detected between two cryptic *Chaetoceros* species (**Degerlund *et al.*, 2012; Huseby *et al.,* 2012**).
Toxic or harmful species represent a threat for public health and economy (**Anderson *et al.*, 2012**; **Berdalet *et al.*, 2013a, b)** and it is important to know their identity as well as to implement effective methods for their quantification in natural samples. The EC project MIDTAL was aimed at implementing microarrays for the identification of toxic microalgae and their toxins (**Medlin *et al.*, 2013; McNamee *et al.*, 2016).** Various *Pseudo-nitzschia* species were tested within the project, highlighting '*pros*' but also several '*cons*' for the microarray approach applied to this diatom genus (**Barra *et al.*, 2013a, b**). Interestingly, domoic acid was also detected in a species of the genus *Nitzschia* recorded in a Tunisian lagoon (**Bouchouicha *et al.*, 2012**).

This genus that includes several species capable of producing domoic acid Dinoflagellates represent another important group of marine protists that include various species capable of producing toxins (e.g., species of the genus *Azadinium*, **Percopo et al., 2013; Rossi *et al.*, 2017**, Figure 2) or harmful (e.g. **Escalera *et al.*, in press**).

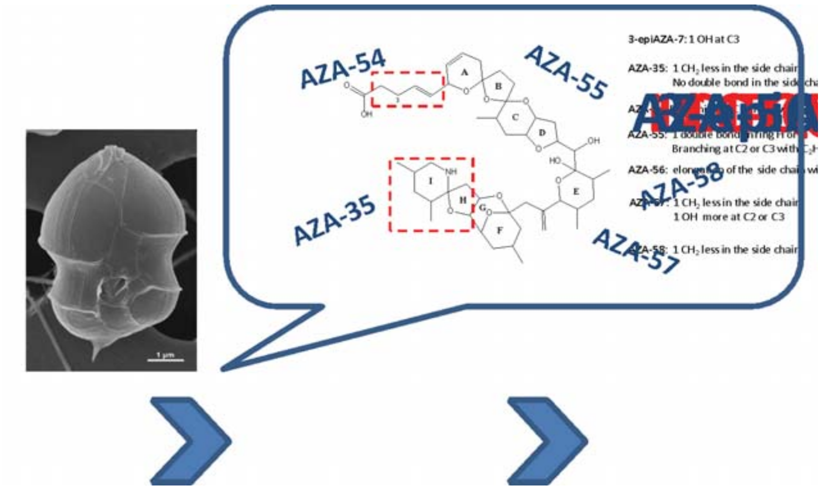

Figure 2. *Azadinium dexteroporum,* a dinoflagellate that produces azaspiracid toxins.

Phylogenetic analyses with various molecular markers, presence/absence of genes involved in the synthesis of saxitoxin, and the detailed study of the ultrastructure of the vegetative cells, allowed to revise the species included in the *Alexandrium tamarense* species complex (**John *et al.*, 2014a, b)**. A reference database (DinoREF) of the 18S rDNA of dinoflagellates is in the final steps of implementation. It includes more than 1500 taxonomically curated sequences (18S) belonging to 148 genera and will be integrated in the PR^2 database (**Guillou *et al.*, 2013)**. This latter one represented the first Protist Ribosomal Reference database for eukaryotes, which is now an open-source infrastructure. Ribosomal genes are not the only barcode regions explored for protists and a database has been implemented also for the 16S region of photosynthetic eukaryotes (**Decelle *et al.*, 2015**).
High genotypic diversity has been always recorded in population genetic studies of microalgae, but almost no information is available on the temporal scale. We investigated population genetic structure of the model diatom *P. multistriata* at LTER-MC over four consecutive years to explore possible changes over seasons and from year to year. Two main sub-populations were detected, alternating over the years, but with a similar genetic fingerprint within the multiple sampling points within each year (**Tesson *et al.*, 2013, 2014)**. The study was extended for two more years using a high number of microsatellite markers and we could document a brief but massive demographic and clonal expansion driven by strains of the same mating type (**Ruggiero *et al.*, in press)** (Figure 3). The analysis of the extended data set (6 years) indicated that the genetic fingerprint of *P. multistriata* changed over time with a nonlinear pattern, with intermittent periods of weak and strong diversification related to the temporary predominance of clonal expansions over sexual recombination, as inferred by the analysis of linkage disequilibrium.

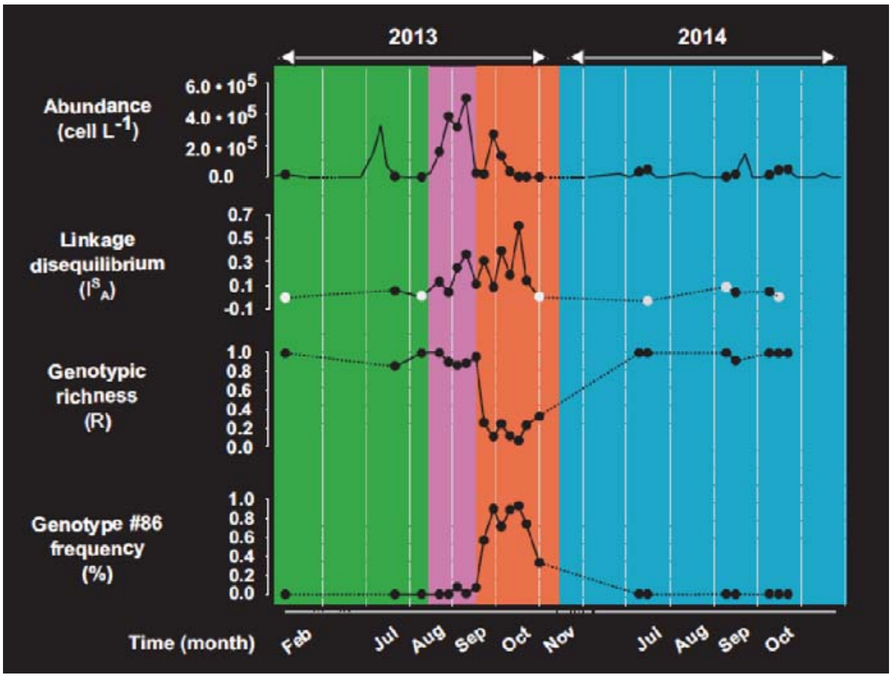

Figure 3. Population genetic structure of the pennate diatom *Pseudo-nitzschia multistriata* at station LTER-MC in the Gulf of Naples. A clonal expansion event was detected during the blooming season in 2013.

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b. Functional diversity: focus on diatoms

b.1 Towards new experimental models and tools for functional studies (Ferrante, Ribera d'Alcalà, Iudicone)

In order to broaden the range of species that can be an object of molecular studies, we sequenced the genome of the planktonic diatom *Pseudo-nitzschia multistriata* (GyPSy project) in collaboration with the Earlham Institute (UK), and produced an annotated genome available via a dedicated genome browser(http://apollo.tgac.ac.uk/Pseudo-nitzschia_multistriata_V1_4_browser/sequences, **Basu *et al*., 2017**). Several features of the genome have been explored, providing important data on the evolutionary history of *Pseudo-nitzschia* and diatoms in general. We also optimized techniques to obtain genetically engineered strains (Figure 4) (**Sabatino *et al*., 2015**) (EMBRIC project), and established the CRISPR/Cas9 technology in the model diatom *Phaeodactylum tricornutum* (DiaEdit project), a powerful tool to investigate gene function via loss of function approaches. These efforts allowed to expand the molecular, genetic and genomic resources available for diatoms. The availability of perturbation methods revealed for the first time the effects of the downregulation of the lipoxygenase gene, responsible for the production of oxylipins, important secondary metabolites.

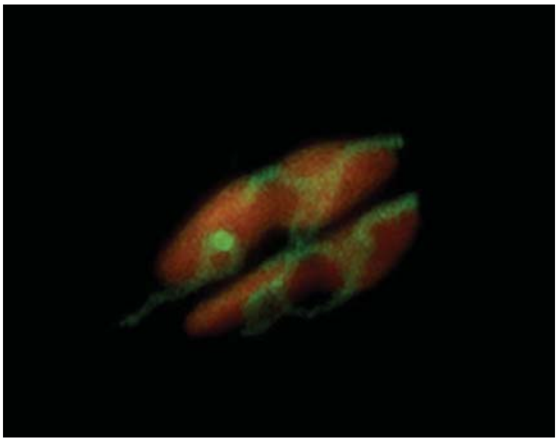

Figure 4. Fluorescent image of a pair of transgenic *Phaeodactylum tricornutum* cells transformed with a GFP fusion (green) protein. Chlorophyll autofluorescence from the plastid is shown in red.

Within the RITMARE Fragship Project, a dedicated, custom-built instrument called TURBOGEN was used to assess the effects of changes in marine turbulence on diatom biology (**Amato *et al*.,**

2016). It is known that diatoms thrive in turbulent environments, however, despite several experimental and numerical studies, if and how diatoms may profit from turbulence is still an open question. Several diatom species were tested using TURBOGEN: they actively responded to turbulence in non-limiting nutrient conditions by tuning their chain length but, more importantly, they displayed a control of chain formation that is different in different species (**Gherardi *et al*., 2016**). Differential expression analyses were conducted to study the transcriptional response of *Chaetoceros decipiens* to turbulence, and changes were observed in activating energy storage pathways like fatty acid biosynthesis (**Amato *et al*., 2017**). In addition, in experiments lasting 12 days, we observed that in turbulence *C. decipiens* continued to take up phosphorus and carbon even when silicon was depleted (**Dell'Aquila *et al*., 2017**). These findings indicate that turbulence affects diatoms in a more sophisticated fashion than what was accepted so far.

b2. Unraveling the bases of diatom life cycles (Ferrante, Montresor)

The particular structure of diatom cells and the modality in which cell divide cause a progressive average cell size size reduction in the population: this miniaturization process can be arrested by the formation of large-sized cells that occurs following the sexual phase (**Fuchs *et al*., 2013**; **Godhe *et al*., 2014**; **Montresor *et al*., 2016**)**.** The main reason for selecting *P. multistriata* as a model for genomic studies was the fact that the species is heterothallic, i.e. sex occurs only when two opposite mating types are in contact, and that its life cycle is controllable in the lab. Information derived from laboratory investigations showed that successful sexual reproduction can only be achieved when crossing parental strains in the exponential growth phase. Evidence was also provided for the fact that sexual reproduction is a density-dependent event and requires a threshold cell concentration to start, thus suggesting that a chemical cue may be involved in the communication between mating types (**Scalco *et al*., 2013, 2014)**. This prompted us to investigate the molecular bases of the chemical signaling during the early phases of sexual reproduction using a transcriptomic approach. Sexual reproduction impacted on cell cycle progression and induced an asymmetric response of the opposite mating types. G protein-coupled receptors and cyclic guanosine monophosphate (cGMP) are implicated in the response to sexual cues, which overall entails a modulation of cell cycle, meiosis-related and nutrient transporter genes, suggesting a fine control of nutrient uptake even under nutrient-replete conditions (**Basu *et al*., 2017**) (Figure 5).

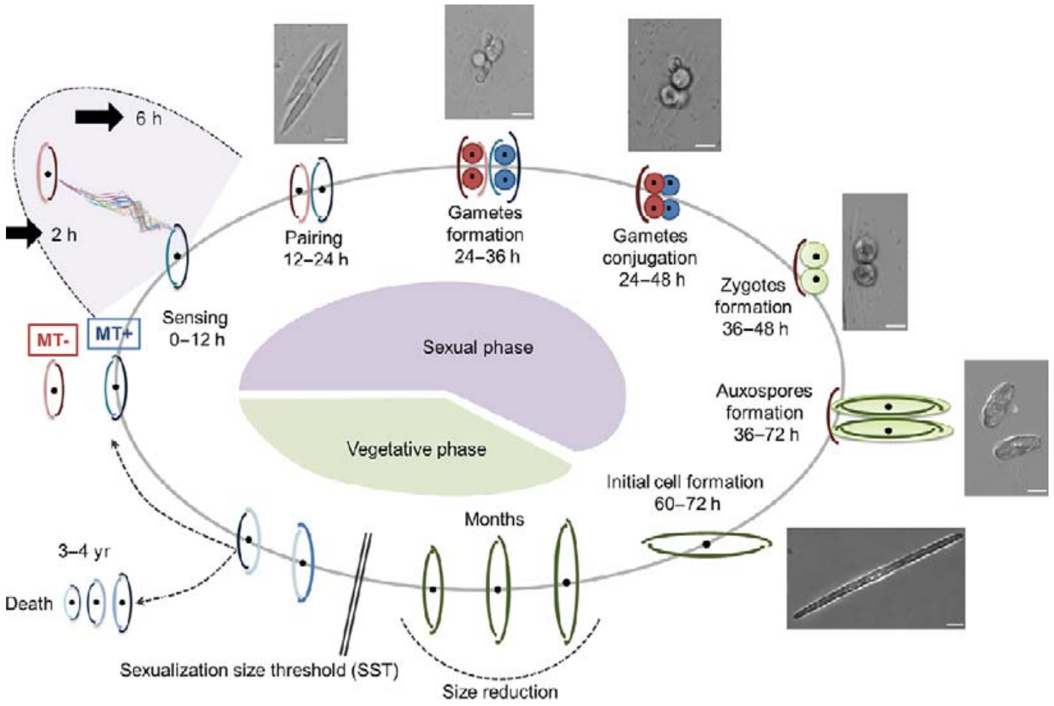

Figure 5. Schematic drawing of the life cycle of the model pennate planktonic diatom *Pseudo-nitzschia multistriata*; the signaling mechanisms during the early phases of the sexual phase are illustrated in Basu *et al*. (2017).

Various genetic sex-determining regions, either sex chromosomes or mating-types loci, have been detected in animal, plants and fungi, but almost nothing is known for unicellular microalgae. We aimed at investigating the mechanism that determine the mating type in our model diatom through a comparative transcriptomic approach of three MT+ and three MT- strains. Besides the important implications for ecological, population dynamics, and evolutionary studies, the results of this research will be of interest for biotechnological applications.

b3. Assigning a function to diatom genes (Ferrante, Romano, Zingone)

Transcriptomics has been widely used to explore the novelties deriving from the broad diversity of diatoms (**Keeling *et al*., 2014)**. A comparison of different *Pseudo-nitzschia* species allowed to identify specific and shared traits and to discover the presence of important genes such as the nitric oxide synthase (**Di Dato *et al*., 2015**). Exploring diatom genomes and transcriptomes, we also identified the diatom toolkit for meiosis (**Patil *et al*., 2015**). Through a comparative study of the transcriptomes of *L. danicus* and *L. aporus*, of which only the former is known to undergo sexual reproduction, several genes linked to the flagellum development were discovered, which paves the way to the exploration of these genes as hallmarks of sexual reproduction in metatranscriptomics datasets (**Nanjappa *et al*., 2017**).

An exploration of diatom genomes also allowed to identify genes involved in nitrogen uptake, revealing the presence of gene families with different members whose exact function remains to be defined (**Rogato *et al*., 2015**), The expression of different target genes related to programmed cell death (PCD) was evaluated through RT-qPCR in the cosmopolitan coastal centric diatom species *Skeletonema marinoi* under different experimental conditions. Results indicated that of the four gene studied, death specific protein (DSP) is a possible PCD marker induced by aging in this diatom species. In contrast, levels of DSP transcripts induced by silica starvation were relatively low compared to those induced by cell aging suggesting differential activation and/or regulation of the PCD machinery in response to different stressful conditions (**Orefice *et al*., 2015**). Bioinformatic analysis of the transcriptomes of two strains of the diatom *Skeletonema marinoi*, enabled the identification of sequences of three main enzymes involved in Prostaglandin (PG) biosynthesis, well known hormone-like mediators involved in many physiological and pathological processes in mammals. Results of gene expression evaluation showed that the PG pathway is differentially expressed in different strains, confirming clone variability of fatty acid- derivatives metabolism (**Di Dato *et al*., 2017**).

This research was supported by SZN FOE, GyPSy, DiaEdit, MetaTRAc, TuPRE and EMBRIC

c. Diversity and demographic structure of key marine species

c1. The marine turtle *Caretta caretta* (Hochscheid)

Our mission is to provide the scientific foundation for conservation and management actions to ensure the survival of these charismatic flagship species. Through advances in bio-logging technologies and their application to free-ranging marine turtles it was possible to deepen our understanding of behavioral plasticity in these marine reptiles by revealing that they individually adapt the best foraging strategy to make efficient use of marine resources (**Cheng *et al*., 2013; Hochscheid 2013; Hochscheid *et al*., 2013; Luschi *et al*., in press**). Using a multi-methodological approach that includes the monitoring of environmental and phenological data of the only stable nesting site in the Western Mediterranean, oceanographic modelling, molecular tools and photo identification, the existence of a Mediterranean hotspot for poleward expansion of loggerhead turtles due to global warming was identified. However, high mortality rates of post-hatchlings due to the persistence of low winter temperatures may hamper this possible new colonization event (Figure 6) (**Maffucci *et al*., 2016**).

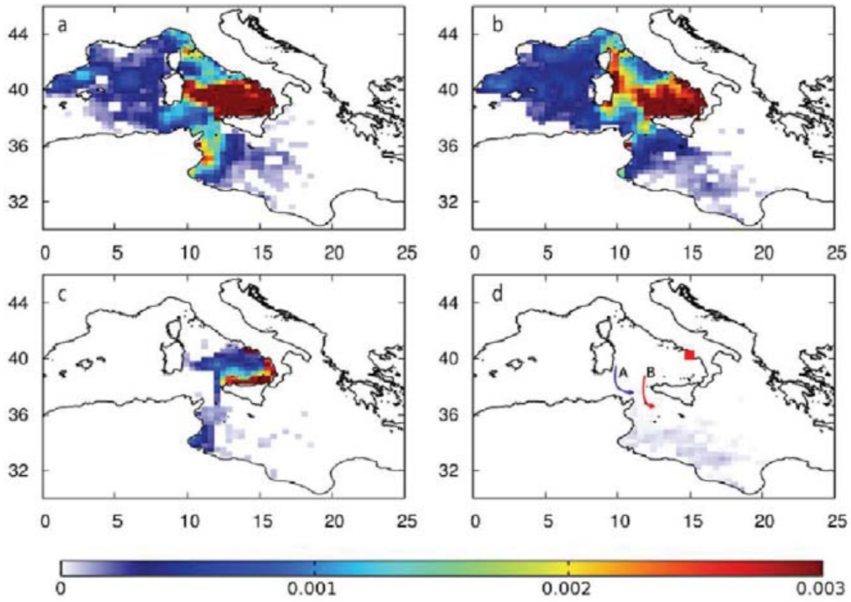

Figure 6. Modelled dispersion of loggerhead hatchlings of *Caretta caretta* departing from the SW Italian coast. Relative density (%) after one year of dispersal in 2007 (a, c) and in 2013 (b, d), obtained either without (a, b) and with (c, d) the mortality function based on SST (i.e. hatchlings experiencing mean SST < 15°C for more than 10 days had a 50% chance of survival). The winter (blue) and summer (red) southward conveyors connecting the south Tyrrhenian Sea with the Eastern Mediterranean are shown in Panel d.

The application of genetic tools made important contributions to understanding marine turtle population and evolutionary biology, by establishing connectivity between rookeries and foraging habitats, determining phylogeography and broad scale stock structure for loggerhead turtles in the Mediterranean and providing insight into differential human impacts among populations (**Kaara *et al*., 2016; Clusa *et al*., 2014; Saied *et al*., 2012**).

Providing of the biggest marine turtle research and rehabilitation center and an efficient regional stranding network knowledge was also advanced in the anatomy, physiology and health sector, including life-history related morphological changes, the effectiveness of new antibiotics, presences of new parasites, immunocytochemistry of blood cells, and prevalence of ingested marine litter (**Matiddi *et al*., 2017; Santoro *et al*., 2017; Rees *et al*., 2016; Nardini et *al*., 2015; Maffucci *et al*., 2013; Di Santi *et al*., 2012**).

This research was supported by SZN FOE, MolEcOC, TurtleDives, and Regione Campania.

c2. The ascidian tunicate *Ciona robusta* (Sordino)

Ciona robusta is a unique model chordate to address biological questions at the interface between population, evolutionary and developmental biology. This animal displays several traits of evolutionary interest, *e.g.*, conservative anatomy, high genetic polymorphism, cryptic speciation, metapopulation structure and invasive behavior. Some of these aspects depend on the ecology of this organism, which display a great ecophysiological tolerance and unpredictable colonization capabilities. We found that the genetic patterns of *C. robusta* in the Mediterranean and Atlantic seas do not reflect the geographic distribution of sampled population, with a substantial gene flow in a predominant east-ward migration pattern likely driven by human activities (Figure 7) (**Affinito *et al*., 2015**).

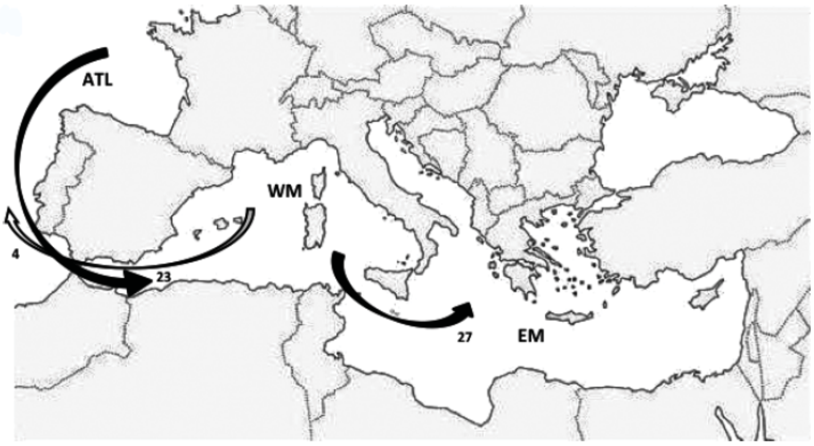

Figure 7. Dominant eastward gene flow among Atlantic and Mediterranean populations of *Ciona robusta*.

We also defined the seasonal ranges of sea surface temperature in which sampling size, animal size and reproductive status reach their highest values (**Arienzo *et al*., 2014; Caputi *et al*., 2015**). To advance research on this tunicate model species, we have developed a protocol for liquid nitrogen cryopreservation for the storage and distribution of genetic resources (**Sorrenti *et al*., 2014**), and described notions and methodologies that might be useful for the implementation of easy and tight procedures for mutations studies in *Ciona* (**Crocetta *et al*., 2015**). In conclusion, we integrate molecular, genetic, ecological, phylogenetic and developmental techniques to address conceptual and methodological problems about a talented marine organism that provides insights in specific aspects of evolutionary biology.

This research was supported by SZN FOE, EU-FP7 Assemble.

c3. The seagrasses (Procaccini)

Seagrass meadows are distributed all along the Mediterranean coastline and provide important ecosystem services. Nevertheless, seagrass meadows are in regression due to the double effect of global climate changes and regional human impact. Here we analyze seagrass population genetic and structure in the framework of planning management and restoration strategies and assessing the role of genetic diversity in population response to pressures. Moreover, we approached the study of the ecological factors underlying flowering, a strategy that potentially increases population genetic diversity.

Assessment of genetic diversity and connectivity among donor and transplantation sites in *Zostera noltii* highlighted the need for genetic assessments in putative donor areas and in remnant meadows near the transplantation area before transplantations were carried out, in order to assess the genetic distinctiveness between donor and acceptor sites and to select genetically polymorphic material. The lack of success of transplanted shoots could be due to an adaptation mismatch of the marine donor material to lagoon conditions or to low plasticity of the transplanted shoots (**Jahnke *et al.*, 2015b**). Studies on the potential (oceanographic) and realized (genetic) connectivity were carried out in the framework of the EU project Coconet. In the Adriatic Sea *Posidonia oceanica* population, realized dispersal does not necessarily match with the potential for dispersal. Still, both genetic and physical connectivity analyses show good agreement in identifying hotspots of connectivity (**Jahnke *et al.*, 2017**). Black Sea populations of *Zostera noltii* appear genetically isolated. The comparison between physical connectivity over the time-frame of nearly a decade and the assessment of different temporal scales of genetic connectivity also shows that infrequent long-distance dispersal affects the dynamics of populations (**Jahnke *et al.*, 2016**). Both studies call for ad hoc strategies for the design of networks of marine protected areas.

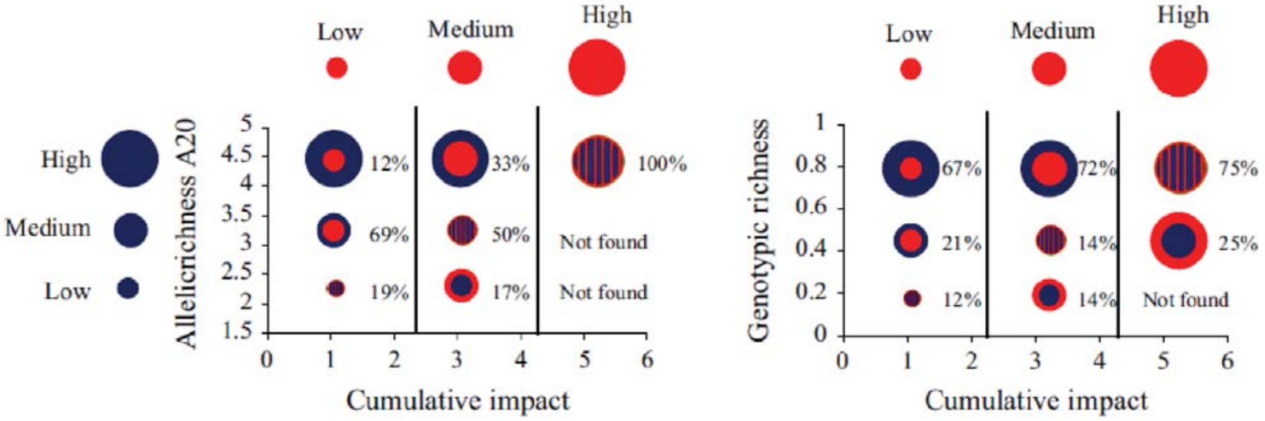

Figure 8. Low, medium and high cumulative impact (red) vs. low, medium and high allelic richness (A20; MLG = 20) and genotypic richness (R; N = 14; blue) in 74 *Posidonia oceanica* populations. Striped dots indicate that the genetic diversity measurement and impact were of the same category level.

A meta-analysis reveals a temporal mismatch between genetic diversity metrics and environmental status in *Posidonia oceanica.* A combination of mainly genetic but also ecological factors causes heterogeneous flowering patterns in *Posidonia oceanica* seascapes. A strong positive relationship

was found between the number of flowers and heterozygosity, adding evidence to the controversial association between heterozygosity and fitness (**Jahnke *et al*., 2015a**) (**Figure 8**).
This research was supported by SZN FOE, RITMARE Flagship Project and COCONET.

c4. Species of commercial interest: *Octopus vulgaris* and *Engraulis encrasicolus* (Procaccini)

Atlantic and Mediterranean stocks of the common anchovy *Engraulis encrasicolus* were found to be genetically distinct, as assessed by a small panel of informative SNPs markers specifically selected for the target species (**Catanese et al., 2016**). Both in the Atlantic and the Mediterranean Sea (Adriatic and Tyrrhenian Sea), anchovy distribution seems to be related to the presence of river outflows (**Ruggieri *et al*., 2016; Bonanno *et al*., 2016; Catanese *et al*., 2017**).
Mediterranean populations of *Octopus vulgaris* presented a weak genetic structure and high levels of microsatellite allelic richness and moderate heterozygosity (**De Luca *et al*., 2014, 2015, 2016**).
This research was supported by RITMARE Flagship Project.

c5. Polychaetes (Gambi)

A recent study on GC genomic pattern in polychaetes, highlighted that polychaetes with a motile life habit showed higher metabolic rate (MR) and higher genomic GC content, respect to sedentary/sessile forms **(Tarallo *et al.*, 2016)** (Figure 9**).** The fluctuation of both variables was not affected by the phylogenetic relationship of the species. Thus, present results further support that a very active lifestyle affects at the same time MR and GC, showing an unexpected similarity between invertebrates and vertebrates (e.i., teleosts).

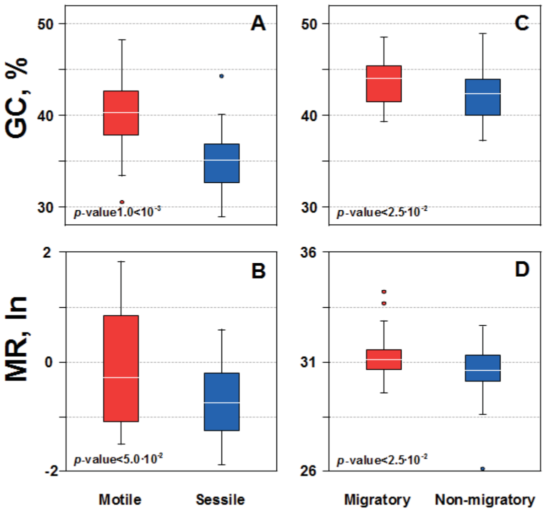

Figure 9. Boxplot of the average genomic GC-content and natural-log transformed specific metabolic rate for polychaetes (panels A and B) and teleosts (panels C and D). Red boxes: motile polychaetes and migratory fishes. Blue boxes: low-motile polychaetes and non-migratory fishes.

Studies on alien species reported the invasive behavior of two congeneric species of the genus *Branchiomma* (Sabellidae), in some coastal biotopes, with *B. luctuosum* introduced from the Red Sea and *B. bairdi* from the Caribbean Sea (**Arias *et al*., 2013; Mastrototaro *et al*., 2015).** The two species show features of the life cycle/reproductive habit which can explain their higher success respect to native ecologically equivalent worm's species. Spatial variability of selected polychates (Eunicidae), borers of the seagrass *Posidonia oceanica* sheaths, highlight a high small scale patchiness of these invertebrates, which seems not related to the local plant shoot density or the pristine conditions of the meadow **(Vasapollo *et al*., 2015).**

c6. Molluscs (Crocetta, Patti)

The taxonomy of two different genera (*Ocinebrina* and *Ocenebra*) belonging to the family Muricidae was investigated, resulting in the description of new species in the *Ocinebrina aciculata* complex (**Crocetta *et al*., 2012**; **Barco *et al*., in press**) and the discovery of cryptic diversity in the *Ocenebra edwardsii* complex (**Barco *et al*., 2015**). The taxonomy of the Mediterranean species of Retusidae was explored, resulting in: i) three species excluded from the Mediterranean fauna; ii) a re-evaluation of the taxonomic affinity of a species; iii) a nomenclatural change in a species (**Crocetta *et al*., 2015a**; **Crocetta and Tringali, 2015**). Studies on alien species compared the degree of implementation of marine biodiversity indicators by European countries in relation to the Marine Strategy Framework Directive (MSFD) (**Hummel *et al*., 2015**), and a step-by-step taxonomic review of the alien species occurring in several Mediterranean countries culminated in updated faunal lists of alien molluscs occurring in Italy (**Crocetta, 2012**; **Crocetta *et al*., 2013a**) and Greece (**Crocetta *et al*., in press**). Molecular work has been also carried out on different alien Mediterranean molluscs: i) barcoding of the family Haminoeidae revealed an overlooked invasion by the japanese bubble shell *Haminoea japonica* (**Crocetta *et al*., 2013a**; **Hanson *et al*., 2013**); ii) barcoding of alien oysters revealed years-long misidentifications within the genera *Alectryonella* and *Dendostrea* (**Crocetta *et al*., 2015b**); iii) the origin and dispersal pathway of the sea slug *Aplysia dactylomela* was traced (**Valdés *et al*., 2013**). A review of molluscan fauna from Lebanon was conducted, resulting in three articles published: i) "opisthobranchs" (now heterobranchs) yielded 13 new records (**Crocetta *et al*., 2013b**; **Tsiamis *et al*., 2015**; **Crocetta and Bitar 2017**); ii) bivalves yielded 24 new records. In addition, comparison between historical and present data revealed rarefaction or extinction of native habitat formers, replaced by alien ones (**Crocetta *et al*., 2013c**); iii) Polyplacophora, Scaphopoda, and Cephalopoda yielded 6 new records (**Crocetta *et al*., 2014**). Ecological studies on the Mediterranean molluscan biota have been carried out in the Sabaudia Lake (Italy), confirming with zoological data the local shift from a freshwater lake to a marine-influenced lagoon (**Macali *et al*., 2013**), and in Slovenia, where the Species-Area Relationship (SAR) model was tested with Mollusca living in association with the habitat former *Cladocora caespitosa*[103].

Figure 10. The alien nudibranch *Godiva quadricolor* and its egg masses from the Fusaro Lake (Naples, Tyrrhenian Sea).

4. Perspectives

a. Protist diversity (Kooistra, Montresor, Sarno, Zingone)

Inference of multigene phylogenies of Chaetocerotaceae. Results of the sequencing of nuclear encoded LSU and SSU rRNA genes of ca. 300 strains of Chaetocerotaceae from all over the world enabled inference of well resolved phylogenies of this family of planktonic diatoms. Yet, some of the basal clades were not well resolved. Currently trees are inferred not only from nuclear (LSU and SSU), but also from multiple plastid (rbcL, psbA) and mitochondrial (COI) gene regions to better resolve the phylogeny of the family, which then can be used for reconstructing the morphological evolution and to assess the order in which novel character states were acquired.

Chaetoceros used as a model to explore mechanisms of diversification/speciation in protists. As next step we explore phylogeographic and seasonal patterning within what we believe to be selected, widely distributed species that occur year-round. To this aim we use sufficiently fast evolving markers to establish haplotype networks. As input we use both Sanger sequences from multiple geographic strains of the selected species as well as meta-barcode haplotypes belonging to these species, gathered from NGS sequence data from these sites (Ocean Sampling Day) and different seasons from the station LTER-MC (BioDiversItalia).

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[103] Pitacco V*, Crocetta F, Orlando-Bonaca M, Mavrič B, Lipej L. The Mediterranean stony coral *Cladocora caespitosa* (Linnaeus, 1767) as habitat provider for molluscs: colony size effect. ***J. Sea Res.*** (2017) 129: 1-11.

In dinoflagellates, species of the genus *Tripos* have been proposed as indicators of ocean warming. The species diversity and phylogenetic relationships in this genus will be explored using a single-cell approach because these species cannot be maintained easily in culture. Taxonomically validated 18S sequences from these cells will be added to the reference database DinoREF in order to improve the detection of individual species of this important genus in metabarcode data of the LTER-MC.

The biogeographic study on species of the genus *Leptocylindrus* will be followed by a closer analysis on several coastal and offshore metabarcodes datasets collected in different projects (Tara Oceans, Ocean Sampling Day and BioMarks); preliminary results lead to the discovery of a higher diversity in most species in the genus, with new species and/or genetically distinct populations.

b. Functional diversity: focus on diatoms (Ferrante, Montresor, Romano)

The study of gene function will benefit from the established protocols for genome editing, we plan to address the role of nitrogen transporters and of other important genes in diatoms (OvoA) using loss of function approaches, mostly with the CRISPR/Cas9 technology. New funding (ASSEMBLE+) will allow to transfer this technology from the model diatom *P. tricornutum* to other ecologically relevant species such as *Pseudo-nitzschia*.

In order to confirm preliminary data on a putative sex locus in diatoms, more genomes will be sequenced and functional validations will be employed.

Signal transduction mechanisms are still poorly defined in marine phytoplankton, including diatoms. Yet, understanding how cells sense and integrate environmental signals is fundamental to explain population dynamics, community interactions and ecosystem functioning. Studies will continue to explore these alleys, focusing on signaling during sexual reproduction, on the interaction with grazers and with other biotic components of the ecosystem.

The existence of a canonical animal pathway synthesizing most of the known prostaglandins and their metabolites in marine unicellular eukaryotes opens new intriguing perspectives on the evolution and role of these molecules in the marine environment as possible intracellular or cell-to-cell signaling mediators, eventually influencing population dynamics in the plankton.

Another important trait of diatom life cycles is the capability of some species to produce resting stages, which are usually considered as survival strategies to overcome unfavorable environmental conditions. We are investigating various cues that may induce the formation of resting stages in *Chaetoceros socialis* an important species at LTER-MC and in the world oceans. Nitrogen limitation is an effective trigger and has been selected as the experimental set up for an RNA-seq approach that will provide information on the molecular responses at two time points: when cells perceive the stimulus and when they are transformed to spores.

In the warm water species *Leptocylindrus aporus*, transcriptomic analysis of strains grown at different temperature will be conducted to shed light on the role of transposable elements in the adaptations to cold temperatures, whereas intra and interspecific functional diversity will be investigated in a phylogenomics and comparative transcriptomic study. The information on functional traits gathered for individual species will be used in the analysis of metatranscriptomic datasets collected at LTER MC and within Tara Oceans.

c. Diversity and demographic structure of key marine species

c1. The marine turtle *Caretta caretta* (Hochscheid)

A current ongoing study uses loggerhead turtles as oceanographic platforms to obtain detailed depth profiles of temperature, salinity and chlorophyll concentrations of the Mediterranean Sea. Two principal objectives are pursued, first, these data will be compared to the ARGO data from the Global Ocean Observing System to evaluate the usefulness of marine turtles as oceanographic sampling platforms; second, they will be used to identify those features of oceanic habitats to which loggerhead turtles are attracted and which can be used to explain and predict distribution patterns for various climate change scenarios. The latter will be addressed also in coastal areas, where new data transmission pathways through modern communication tools are being explored to monitor fine-scale movement patterns of marine turtles and environmental parameters along their trajectories.

To further investigate the impact of climate change on the processes and dynamics of marine turtle nesting range expansion to northern latitudes, we will monitor intensively the nesting sites in the Central Tyrrhenian Sea to establish the factors that influence nest site selection, the nesting phenology, the reproductive success, and the origin of turtles.

c2. The ascidian tunicate *Ciona robusta* (Sordino)

To study the ability of *C. robusta* to invade coastal ecosystems we will address the question if the genetic structure is shaped by temporal environmental changes by correlating physiological, toxicological, genetic and seawater variables over time. Then, we aim to understand the colonization history of this species in the Mediterranean Sea using nuclear and mitochondrial markers under different evolutionary forces.

c3. The seagrasses (Procaccini)

We will explore the assessment of the inter-annual variability of anchovies stocks and define a genetic tagging for regional catches.

We will also carry out an assessment of population genetic structure and diversity of *Posidonia oceanica* (seagrass) along the coasts of Turkey, in relation with the existence of environmental clines and potential (oceanographic) connectivity.

Outlier analysis and a genome-wide transcriptome analysis to mine for genes underlying adaptation to environmental factors in *P. oceanica* are ongoing. We study populations along a latitudinal and a bathymetric gradient in which key environmental factors known to play a central role in *P. oceanica* fitness and survival (Jahnke M, D’Esposito D, Orrù L, Lamontara A, Mazzuca S, Procaccini G, Orsini L. Combining a genome scan and a transcriptome approach to assess adaptive responses to global warming in the seagrass *Posidonia oceanica*. BMC Genomics, submitted 6/6/2017)

c5. Polychaetes (Gambi)

Studies on alien polychaete species of the genus *Branchiomma* (Sabellidae) will continue by using, in addition to morphological analyses, also genetic tools (barcoding) to disentangle the taxonomy of another alien species, closely related to *Branchiomma bairdi,* and recently reported in the Mediterranean Sea. At taxocoene level, taxonomic analyses of polychaetes associated to *Posidonia oceanica* meadows are in progress to study the spatial variability of these organisms in meadows submitted to different degree of disturbance and influence of stressors, such as human impact and ocean acidification.

c6. Molluscs (Crocetta, Patti)

We are completing work on the genus *Conopleura*, the genera *Cerithiopsis* and *Retilaskeya*, and the taxon *Bursa scrobilator*, and the same holds for an additional new species to be described within the genus *Ocenebra*. Further taxonomic work based on shell morphology and/or anatomical data coupled with molecular data is being carried out in collaboration with other Italian and European researchers on the Mediterranean taxa of the genera *Conus*, *Haliotis*, and *Muricopsis*, as well as on the worldwide taxonomy of the species of the genus *Bursatella* and their allied taxa. At the same time, the taxonomic composition of molluscs community associated with the algae will be analysed using innovative barcoding techniques. Finally in order to solve the taxonomic ambiguity of some species, morphologic and genetic characterization of marine molluscs will be carried out through sequence analysis of target genes using the double digestion RADSeq technique in collaboration with the Phycology Research Group of the Ghent University.

With the main aim to genetically characterize the local mussel species and to study human impacts on the fishing economy in Campania, *Mytilus galloprovincialis* from different mussel farms, natural environment and local fish markets are being analysed to detect the presence of cryptic and/or invasive species and to identify any food adulteration, such as the replacement of species.

General studies on alien species yielded results on the European horizon scanning and on the consistency of the different impact assessments of alien invasive species. Work is being carried out in collaboration with other Italian and European researchers on alien species, including *Anadara kagoshimensis*, *Aplysia parvula*, *Bursatella leachii*, *Mya arenaria*, and several *Okenia* taxa. With the aim to raise up knowledge on the Mediterranean molluscan biota in countries with low taxonomic expertise, the fourth and last part of molluscan fauna from Lebanon, covering Gastropoda, is being finalized. In addition, two wide projects covering invertebrates, including molluscs, are being carried out in Cyprus.

Two papers mostly based on taxonomy and nomenclature are also being carried out, one covering molluscan taxa introduced by an almost unknown Italian malacologist, Raffaello Bellini, that widely worked in the Gulf of Naples, and one covering molluscan taxa described from the Gulf of Naples though the centuries.

Finally, in collaboration with other research groups from Naples and Ischia, three main projects are being carried out: i) ecological studies on the mollusca associated with *Cystoseira* algae; ii) faunal biota and economical value of the taxa living in the Regno di Nettuno MPA; iii) GC genomic pattern in Mollusca.

5. Publications (2012-2017; *corresponding author)

2017

Amato A, Dell'Aquila G, Musacchia F, Annunziata R, Ugarte A, Maillet N, Carbone A, Ribera d'Alcalà M, Sanges R, Iudicone D, Ferrante* MI. Marine diatoms change their gene expression profile when exposed to microscale turbulence under nutrient replete conditions. ***Sci. Rep.*** (2017). doi:10.1038/s41598-017-03741-6.

Barco A, Aissaoui C, Houart R, Bonomolo G, Crocetta F, Oliverio M*. A new species of the small mussel drills genus Ocinebrina Jousseaume, 1880 (Mollusca: Gastropoda: Muricidae), with comments on the identity of the type species. ***J. Molluscan Stud.*** (2017) in press.

Basu S et al. (including Russo MT, Vitale L, Casotti R, Montresor M, Sanges* R, Ferrante* MI). Finding a partner in the ocean: molecular and evolutionary bases of the response to sexual cues in a planktonic diatom. ***New Phytol*** (2017) doi: 10.1111/nph.14557.

Berdalet* E, Montresor M, Reguera B, Roy S, Yamazaki H, Cembella A, Raine R. Harmful algal blooms in fjords, coastal embayments, and stratified systems: Recent progress and future research. ***Oceanography*** (2017) 30: 46-57.

Berdalet* E et al. (including Montresor M). A new program to promote international research, observations, and modeling of harmful algal blooms in aquatic systems. ***Oceanography*** (2017) 30: 70-81.

Bosak* S, Sarno D. The planktonic diatom genus *Chaetoceros* Ehrenberg (Bacillariophyta) from the Adriatic Sea. ***Phytotaxa*** (2017) 314: 001–044.

Catanese G et al. (including Watteaux R, Botte V, Mazzocchi MG, Di Capua I, Iudicone D*, Procaccini G*). Insights on the drivers of genetic divergence in the European anchovy. ***Sci. Rep.*** (2017) 7: 4180.

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Dell'Aquila G, Ferrante MI, Gherardi M, Cosentino Lagomarsino M, Ribera d'Alcalà M, Iudicone* D, Amato* A. Nutrient consumption and chain tuning in diatoms exposed to storm-like turbulence. ***Sci. Rep.*** (2017) 7, 1828.

Di Dato* V, Orefice I, Fontanarosa C, Amoresano A, Ianora A, Romano G. 2017. Animal-like prostaglandins in marine microalgae ***ISME J.*** (2017) doi:10.1038/ismej.2017.27.

Escalera L*, Italiano A, Pistocchi R, Montresor M, Zingone A. *Gonyaulax hyalina* and *G. fragilis* (Dinoflagellata), two taxa associated with 'mare sporco', are the same species. ***Phycologia*** (2017) in press.

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6. List of grants

- **BIODIVERSITALIA '**Approccio multitaxa allo studio delle risposte della biodiversità italiana al cambiamento climatico' (2013-2016) MIUR-FIRB (see Research Topic 'Plankton variability in relation to environmental, climate, and anthropogenic forcing: patterns of diversity, structure and functioning')
- **COCONET '**Towards COast to COast NETworks of marine protected areas (from the shore to the high and deep sea), coupled with sea-based wind energy potential' (2012-2015) EU-FP7, P.I. Gabriele Procaccini
- **DiaEdit** 'Development of genetic tools for the establishment of routine genome editing in the marine diatom *Phaeodactylum tricornutum*' (2015-2018) The Gordon and Betty Moore Foundation's Marine Microbiology Initiative, P.I. Maria Immacolata Ferrante
- **EMBRIC '**European Marine Biological Research Infrastructure Cluster' (2015-2019) EU H2020-EU.1.4.1.1 Grant n 654008
- **GyPSy** 'A forward genetic screen in the marine planktonic diatom *Pseudo-nitzschia multistriata'* Marie Curie Career Integration Grant (2011-2015) EU-FP7-PEOPLE-2011-CIG, P.I. Maria Immacolata Ferrante
- **Joint Genome Institute** 'A deep transcriptomic and genomic investigation of diatom life cycle regulation (Contract No. DE-AC02-05CH11231) to Wim Vyverman, transcriptomes of *Pseudo-nitzschia multistriata*
- **Lifewatch-Italy**: 'An investigation of the genetic basis of toxin production in the diatom species *Pseudo-nitzschia multistriata'* (2014) Lifewatch-Italy. Genome sequencing and transcriptomes of *P. multistriata.* PI Maria Immacolata Ferrante
- **MetaTRAc** 'METATRAnsCriptomics of a waxing and waning plankton bloom in the Gulf of Naples' (2013-2014) MIUR Premiale, P.I. Adriana Zingone
- **MIDTAL** 'MIcroarrays for the Detection of Toxic Algae' (2009-2012) EU-FP7 P.I. Wiebe Kooistra
- **MMETSP** 'The Marine Microbial Eukaryote Transcriptome Sequencing Project' Gordon and Betty Moore Foundation (Grant #2637 to the National Center for Genome Resources) transcriptomes of *Pseudo-nitzschia arenysensis, P. delicatissima, Leptocylindrus danicus, L. aporus, Skeletonema marinoi*
- **MolEcOC** 'Approaching inter- and intra-individual variability by molecular ecology for the technology transfer of basic research on marine model organisms (*Octopus* and *Caretta*)' (2012-2013) MIUR Premiale P.I. Sandra Hochscheid and Graziano Fiorito
- **RITMARE** 'La Ricerca ITaliana per il MARE' Italian Flagship Project (2013-2018), P.I. Maurizio Ribera d'Alcalà
- **TuPre** 'Gene expression changes in response to abiotic and biotic stimuli in diatoms: Turbolence and PREdation. CoFound RITMARE-Marie Curie (2014-2016), P.I. Alberto Amato, Mariella Ferrante
- **TurtleDives** Comparison of dive behavior and performance of two marine turtle species inhabiting different environments (2010-2012) Bilateral Cooperation Program (Taiwan/Italy), P.I. I-Jiunn Cheng, Flegra Bentivegna

- **VulnerClima** and **Window to the Future -** New volcanic CO_2 vents provide a glimpse of the potential impacts of ocean acidification. CoFound RITMARE-Marie Curie (2014-2016),P.I. Teixido, Gambi.

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10. Effects of organism interactions on ecosystem functioning

1. People involved

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Francesco Esposito	Staff scientist	SZN	since 2000
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2. Background, aims and objectives

In any ecosystem, organisms live together in an ecosystem and depend on one another. They have many different types of interactions with each other, and many of these interactions are critical for their survival. This line of research aims to elucidate the nature (direct, indirect), sign (negative, neutral, positive), and type (additive, synergistic, antagonistic) of ecological interactions among marine organisms. Single research lines focus on either plankton or benthos organisms, but with many overlaps, for instance when model organisms are used to test hypothesis and to investigate mechanisms of action or when similar classes of secondary metabolites are investigated in both the plankton and the benthos.

In the plankton, one category of interactions describes the different ways organisms obtain their food and energy. Zooplankton interact with prey, predators and mates and in turn are affected by the characteristics of the environment. These interactions are critical for the planktonic food web and for large-scale ocean dynamics (Mazzocchi, Uttieri). Copepod reproduction can be affected upon feeding on diatoms, due to a particular class of secondary metabolites, the oxylipins, which trigger specific responses investigated at the gene expression level (Carotenuto, Ianora). Oxylipins, in turn, modulate population dynamics and community composition of closeby microbes, including diatoms themselves, by triggering the production of reactive oxygen and nitrogen species which lead to death (Casotti). These and other (some still unknown) compounds mediate plant-animal interactions also in the benthos (Zupo) and several of them are interesting candidates for biotechnological purposes.

In the benthos, bottom-up effects related to environmental changes and top-down effects of predators can be important in determining structure and function of vegetated habitats formed by engineering macrophytes. Their persistence, decline or loss also have relevant consequence on the organization of their associated communities, because they benefit from the physical structure provided by the structuring plant and from its influence on major biological processes (predation, food-supply, recruitment). Macrofaunal invertebrates associated with *Posidonia oceanica* are investigated in CO_2 vents, representing a proxy for scenarios of future changes, so as the effect of sea urchin predation on macroalgae (Buia).

Within the long-term interactions, symbiosis is also investigated in the benthos with a focus on acclimation, and adaptation exchange of nutritional and bioactive/allelopathic metabolites between host and associated microbiota and also on the role of bacteria in triggering diseases of marine organisms (Núñez Pons). Among microbe-host interactions, many are beneficial and play an essential role in the health and physiology of diverse marine organisms. These interactions are studied for their capacity to drive flows of energy and matter within their ecosystems, and for their important contribution to nutrients cycling and ecosystem functioning (Cardini).

Interactions link organisms into ecological networks, and ecological theory predicts that the number, type and redundancy of such linkages determine ecosystem stability and resilience. To uncover the complexity of ecological networks field observations are compared with known natural history of model organisms, focusing in particular on grazing and predation (Musco/Vega Fernández).

3. Results

a. Microscale interactions in zooplankton (Mazzocchi, Uttieri)

The swimming behavior of target copepod species abundant in Mediterranean coastal waters was analysed using a specific 3D optical equipment and a high-speed camera set up at the SZN. The behavior of *Clausocalanus furcatus* was analysed in the three dimensional space with the Power Spectral Density (**Bianco *et al.*, 2013**). Our results revealed that the copepod's swimming is based on a limited repertoire of basic kinematic modules but exhibits greater plasticity than previously thought, which may explain its success both in oligotrophic and eutrophic environments. The analysis of self-overlap in swimming trajectories of the co-occurring calanoids *C. furcatus* and *Temora stylifera* showed that the geometry of trajectories differs according to species, gender, and environmental conditions (**Bianco *et al.*, 2014**) (Figure 1). Low self-overlap increases the foraging efficiency of the animals at short length scales while it can yield enhanced predation risk, and therefore lower zooplankton fitness on larger length scales.

The motion behavior can also increase the invasive potential of a given species, as it was demonstrated for the calanoid *Pseudodiaptomus marinus*. Although being reported as epibenthic during the day, this species has evolved a fully planktonic strategy in the central part of Lake Faro (Sicily), due to the presence of a permanent anoxic hypolimnion (**Sabia *et al.*, 2015**). The behavioral and ecological traits of *P. marinus* from Lake Faro have been integrated with molecular descriptions using ITS2 marker, which suggested the presence of a new morphotype (**Sabia *et al.*, 2017**).

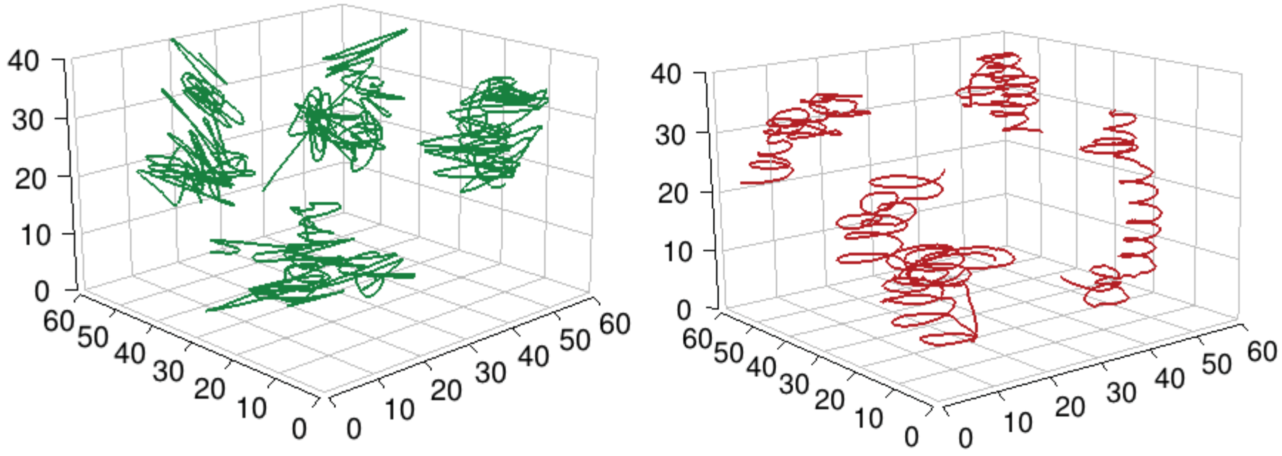

Figure 1. Swimming trajectories of *Clausocalanus furcatus* (left) and *Temora stylifera* (right), recorded in a three-dimensional space (mm).

The small-scale behavior of *Temora stylifera* was investigated by high-speed video recording in relation to the presence of diatoms having different size and morphology. The results indicate that *T. stylifera* shows a clear behavioral plasticity with species-specific responses, which allow this copepod to adjust rapidly to changes in the food environment and to exploit short-lived phytoplankton blooms (**Makadik *et al.*, 2017**).

The motion of adult males and females of *Paracalanus parvus* were characterised using several metrics (swimming speed, symbolic analysis) to depict sex-specific behavioral features. These results have then been used to implement an Individual Based Model (IBM) through which analysing the motion-dependent predation rate by a virtual chaetognath predator (Uttieri *et al.*, in prep.). This work has highlighted the importance of nesting real observations and numerical descriptions in order to understand predator-prey interactions and their cascading effects on the trophic relationships in pelagic environments.

This research was supported by SZN FOE and by the flagship program RITMARE Flagship Project.

b. Chemically-mediated diatom-copepod interactions (Carotenuto, Ianora)

We have shown that the oxylipin-producing diatom *Skeletonema marinoi* inhibited the expression of a series of genes involved in stress response, aldehyde detoxification, and control of apoptosis in the copepod *Calanus helgolandicus* (**Lauritano *et al.*, 2012**), thus, providing a mechanistic explanation to the reduced egg hatching success and induction of apoptotic due to this diatom. We have also revealed a biogeography difference in the response of copepods to toxic algae, both at physiological and gene expression levels (**Lauritano *et al.*, 2013**). A similar reduced hatching rate and altered gene expression, was also induced by the same *S. marinoi* strain in the copepod *Calanus sinicus*, a cold-temperate species that supports secondary production of important fisheries in the Northwest Pacific Ocean (**Lauritano *et al*., 2015**). We have also generated and made publicly available 947 Expressed Sequence Tags (ESTs) from *C. helgolandicus* females fed on *S. marinoi* using the Suppression Subtractive Hybridization technique (**Carotenuto *et al*., 2014**). This study showed that *S. marinoi* activated a generalized Cellular Stress Response (CSR) mechanism in *C. helgolandicus*, by inducing over-expression of molecular chaperones and signal transduction genes that protected the copepod from the immediate effects of the diatom diet (Figure 2).

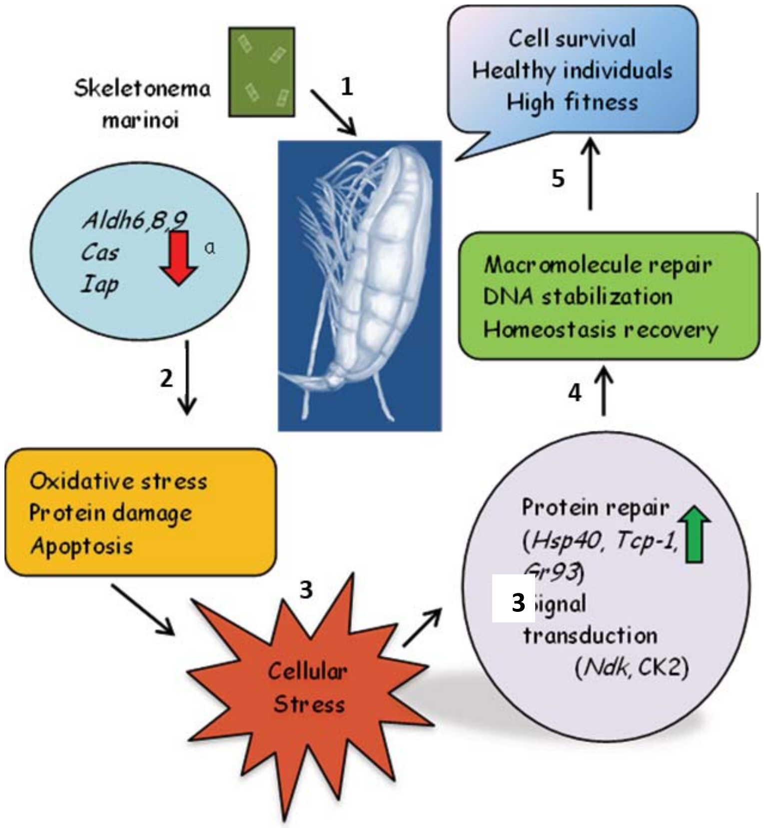

Figure 2. Hypothetical cascade effects of *Skeletonema marinoi* feeding on *Calanus helgolandicus*. Ingestion of *S. marinoi* (1) induced down-regulation of aldehyde dehydrogenases genes (ALDH 6, 8 and 9), cellular apoptosis susceptibility (CAS) and inhibitor of apoptosis (IAP) genes, leading to oxidative stress, protein damage and apoptosis (2). This elicited a Cellular Stress Reponse (CSR) (3) characterized by over-expression of molecular chaperones (HSPs, TCP- 1, gr93) and signal transduction pathways genes (NDK, CK2), which re-established cellular homeostasis and macromolecular structure and function (4). The net effect is high *C. helgolandicus* cell viability and fitness (5).

Functional genomics studies on calanoid copepods are still in its infancy[104]. Therefore, we have recently optimized a high-quality RNA extraction protocol for Next Generation Sequencing of copepod transcriptome (**Asai *et al.,* 2015**), and have a generated a *de-novo* assembled *C. helgolandicus* transcriptome by RNA-Seq (in prep.).

During the last years, we have also reported the harmful effect of non-volatile diatom oxylipins on copepod reproduction during natural phytoplankton blooms in the Norther Adriatic Sea. We have shown that inter-annual variations in diatom species composition and oxylipin production correlates well with year-to-year differences in copepod hatching success *in situ* (Ianora et al. 2015), with concomitant change in expression levels of key stress-related genes in copepods (**Ianora *et al*., 2016)**.

This research was supported by SZN FOE.

c. The ecological role of Polyunsaturated Aldehydes in the plankton (Casotti)

We have demonstrated that PolyUnsaturated Aldehydes (PUA) are released in nature during demise of diatom blooms and that diatom cell lysis similarly occurs in response to stress during late stages of blooms (**Ribalet *et al*., 2014**). This confirms that the process of PUA release is ecologically significant, and confirms results on PUAs shaping local microbial communities[105]. Organisms thriving in close vicinity of a lysing diatom are invested with relevant concentrations of

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[104] Amato A*, and Carotenuto Y. "Planktonic calanoids embark into the 'omics' era." In Trends in Copepod Studies - Distribution, Biology and Ecology, edited by M. Uttieri. New York: Nova Science Publishers, Inc. (2017) in press, ISBN: 978-1 53612-593-1.

[105] Casotti R. L'ecologia microbica: dai genomi ai biomi e le interazioni tra gli organismi mediate dai metaboliti secondari. ***Biol. Mar. Medit.*** (2012) 19(1): 2-5.

these metabolites and must have adopted strategies to cope with them[106]. PUA-resistant bacteria have been isolated and shown to react to PUAs by increasing the degree of saturation of their membranes fatty acids (**Pepi *et al*., 2017**). In addition, other non-volatile oxylipins are suspected to affect copepod reproduction (**Ianora *et al*., 2015**), suggesting that PUAs are only the tip of the iceberg for what diatom secondary metabolites are concerned.

We have also found that the effects of PUAs on diatoms themselves are species and PUA-specific (**Gallina *et al*,. 2014**), involving O_2* generation and SOD activity in H_2O_2 formation (**Gallina *et al*., 2016**), triggering also death-specific gene expression[107]. This is particularly relevant for population dynamics at sea, during blooms, when cell lysis increases and PUA are released. As a consequence, higher local PUA concentrations accumulate, which in turn induce intracellular ROS generation that ultimately leads to cell death and bloom decay (Figure 3).

This research was supported by SZN FOE and by the flagship program RITMARE Flagship Project.

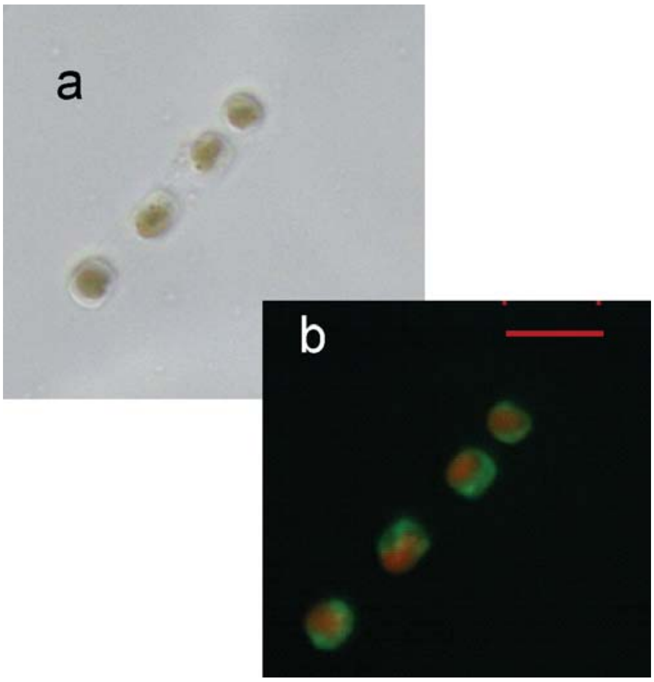

Figure 3. Micrographs of Skeletonema marinoi exposed to the PUA octadienal (a) under transmitted light (b) epifluorescence. The green fluorescence indicates the production of Reactive Oxygen Species (ROS) upon exposure to the PUA. Scale bar is 10 µm.

d. Effect of secondary metabolites on the physiology of benthic marine organisms (Zupo)

The results obtained during the period 2012-2017 are focused on the study of the effects of secondary metabolites produced by the benthic diatom *Cocconeis scutellum* (Figure 4) and by Cyanobacteria of the genus *Halomicronema* living associated to *Posidonia oceanica* leaves, on the physiology and survival of benthic invertebrates and other model planktonic crustaceans. Concerning the first activity, the separation of the *C. scutellum* extracts has reached a final stage, obtaining a fraction containing a single compound whose activity has been confirmed on the shrimp *Hippolyte inermis*. This highly lipophilic compound contains 21 carbon atoms but its detailed chemical structure has not been elucidated yet (**Maiban *et al*., 2014**; **Zupo *et al*., 2014**)

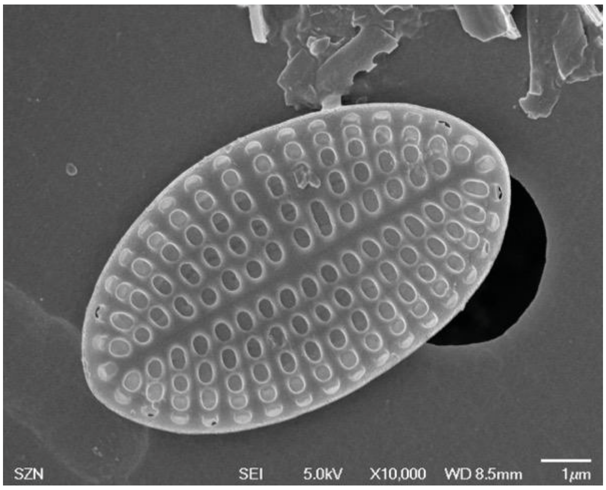

Figure 4. *Cocconeis scutellum var. posidoniae*, one of the active diatoms in the sex reversal of *Hippolyte inermis.*

As for the effects of Cyanobacteria of the genus *Halomicronema*, we have shown that this genus constitutively produces (by continuous leaching)

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[106] Balestra C, Galasso C, Casotti R*. Effect of algal polyunsaturated aldehydes on a natural marine bacterial community from the Gulf of Naples. ***Cytometry A*** (2013) 83A: doi:10.1002/cyto.a.22412.

[107] Gallina A*, Chung C-C, Casotti R. Expression of death-related genes and ROS production in *Skeletonema tropicum* upon exposure to the polyunsaturated aldehyde octadienal. ***Advances in Oceanography and Limnology*** (2015) 6(1/2): 13-20.

toxic compounds that are active against protozoans and metazoans. We tested their effect on the parasite copepod *Lepeophtheirus salmonis* (salmon louse), on worm parasites of zebrafish (*Dactylogyrus* sp.), and on the toxic dinoflagellate *Ostreopsis ovata*, and observed a reduced survival and/or growth in presence of the cyanobacteria exudates. Our preliminary results also indicated that this Cyanobacteria produce a clear toxic effect both in *Brachionus* adults and in *Paracentrotus lividus* embryos at similar concentrations. The same concentrations were also active against the salmon louse *L. salmonis*, inducing high mortality in dose-dependent time lags. We hypothesize that this Cyanobacteria genus may have a role in controlling the life-cycle of these benthic organisms.

This research was supported by SZN FOE.

e. Plant-animal interactions in benthic vegetated systems (Buia, Patti)

We have investigated the bottom-up effects induced by ocean acidification on the assemblage of macrofaunal invertebrates associated to *Posidonia oceanica* meadow at Castello Aragonese site (Ischia Island), characterized by natural volcanic CO_2 vents. Our results demonstrated that the overall abundance of motile invertebrates associated to *P. oceanica* was almost double in acidified stations in comparison to control ones (Figure 5) One unexpected feature was the lack of decline of most heavy calcifiers (molluscs above all) in response to acidification. Also, no overall difference in species richness between pH zones was detected.

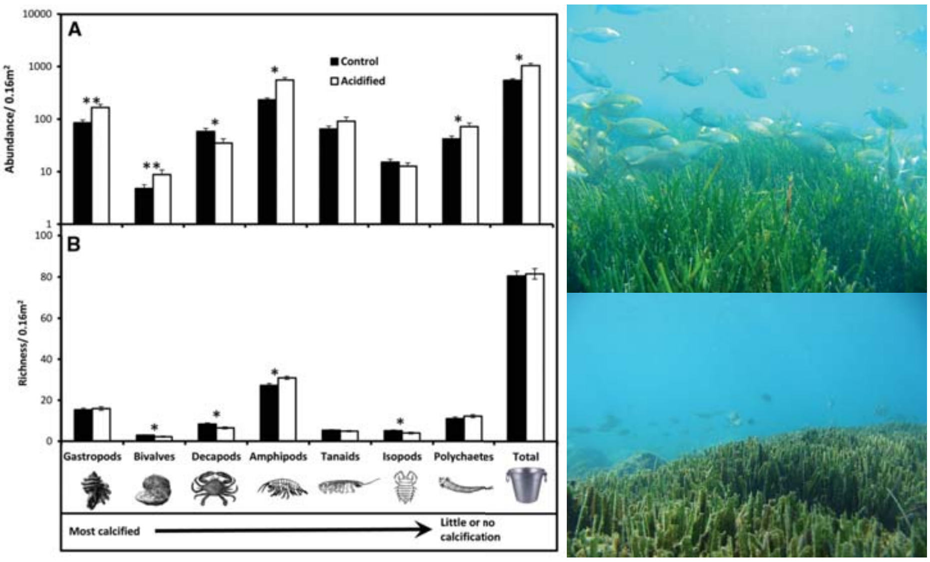

Figure 5. Density and richness of taxonomic groups along a gradient of acidification (left) and structural changes of the habitat complexity (right).

These results suggest that no negative effect is exerted by ocean acidification on the invertebrate assemblage as a whole. Furthermore, a number of system attributes, e.g. habitat complexity as partially represented by seagrass shoot density and/or the quality of seagrass epiphytes, seem to be enhanced under ocean acidification. These effects on the plant component point to a major, although indirect, role of acidification in shaping the associated animal communities (**Garrard *et al.*, 2014**).

We also explored the top-down mechanism of seaweeds control by predators, and its effects in determining the structure and function of vegetated habitat. In particular, we devised a food choice experiment to investigate the feeding preferences of the sea urchin *Paracentrotus lividus* among four different brown macroalgae of the genus *Cystoseria*, *C. amentacea, C. brachycarpa, C. compressa*, widely distributed in the Gulf of Naples, in relation to their chemical content. The seagrass *Posidonia oceanica* has been added in the experiment as a control and because it is a common food item for the sea urchin.

The phenolic composition and biological activities of the macroalgae of the genus *Cystoseira* and of the seagrass *Cymodocea nodosa* as been related to the associated molluscs community in the Gulf of Naples (**Kolsi *et al.*, 2017**; **Gara *et al.*, 2016**). The species were also studied for the human protection functions by seaweed sulphated polysaccharide (**Kolsi *et al.*, 2016**; **Kolsi *et al.*, 2015**).

This research was supported by SZN FOE and by the flagship program RITMARE Flagship Project.

f. Unexpected trophic interactions among benthic organisms (Musco, Vega Fernández)

In order to elucidate pathways of ecological networks, we assessed if the native, generalist sea urchin *Paracentrotus lividus* can provide resistance to invasion by alien seaweeds of the genus *Caulerpa*, as hypothesized on theoretical grounds. Our research unfolded that, even though the sea urchin actively consumes and even displays positive selection for the alien seaweeds (Noè et al. in press), the combined consumption of two different species (*Caulerpa cylindracea* and *C. taxifolia* var. *distichophylla*) provokes a synergistic decrease in sea urchin grazing performance after some days. This reveals both the limitation of short term experimentation, and how multiple interactions can substantially differ from simple ones through non-linear effects. Further evidence was gathered from a manipulative predation experiment revealing that a suite of different native predators was highly efficient in reducing the survivorship of individuals of an alien invasive crab within marine protected areas, confirming the so-called enemy-release hypothesis (**Noè *et al.*, submitted**). These experiments highlight the opportunity that biological invasions offer to test ecological theories in the field. In addition, contrasting experimental outcomes reveal case sensitiveness and points to the relevance of facilitated processes (i.e. grazing deterrence, predation).

Furthermore, we propose the new term kleptopredation to describe the predation upon the cnidarian *Eudendrium racemosum* by se sea slug *Cratena pellegrina* (Willis et al. in press). This is based on the collected evidence that cnidarian polyps (hydranths) are used by the sea slug as a vehicle to access zooplankton grazers from the water column. As such, kleptopredation consists in diet subsidizing by parasitizing the host ability to capture its prey (Figure 6).

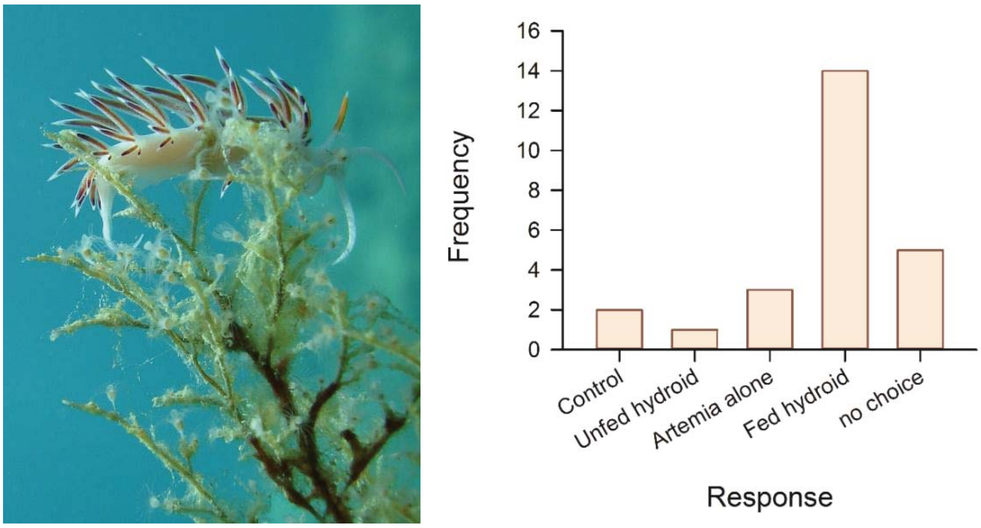

Figure 6. Left: *Cratena pellegrina* feeding on *Eudendrium racemosum* hydrants. Right: Results of single-choice behavioral trials (n = 25) with sea slugs moving towards food items contained into mesh-bags: unfed hydroid branches, *Artemia salina* naupli, hydroid branches fed with *A. salina naupli*, and lack of any choice. Control: empty mesh-bags.

In addition, we described the previously unknown predation of the relative large mauve stinger, the jellyfish *Pelagia noctiluca*, by the orange coral *Astroides calicullaris* through the collective involvement of numerous small polyps belonging to different colonies (**Musco *et al.*, under revision**). This highlights the existence of new pathways of energy transfer from the pelagic compartment to the benthic one, also increasing the estimated rate of benthic carbon storage (Figure 7).

Figure 7. A mauve stinger (*Pelagia noctiluca*) seized by several polyps belonging to different colonies of the orange coral (*Astroides calycularis*).

g. Marine symbiosis for health, stress and disease (Núñez Pons)

Marine reefs are ecologically complex biogenic formations, and the fitness of the building blocks, the scleractinian corals, is key for the health of the whole ecosystem. Disease in animals involves an interaction between agent, host and environment. In reef corals, one of the major threats globally is climate warming, causing bleaching through the loss of microalgal *Symbiodinium* dinoflagellate zooxanthellae (**Núñez-Pons *et al*., 2017**). This has concomitant effects in the coral holobiont and its microbiome communities, yet the patterns of this unbalance are still not well defined. In order to investigate symbiont dynamics during thermal acclimation, we exposed *Exaiptasia* anemones to Gradual Thermal Stress (GTS) and Heat Shock (HS) exposures and monitored chlorophyll and symbiont dynamics to test the phenotypic plasticity of these photosynthetic holobionts. GTS enhanced chlorophyll concentrations and decreased *Symbiodinium* proliferation. A recovery period after GTS returned chlorophyll to lower concentrations and symbiont divisions to higher rates. HS triggered a stress response characterized by intense symbiont declines through degradation and expulsion, algal compensatory proliferation, and chlorophyll accumulation. Anemones pre-exposed to GTS displayed more acute signs of symbiont paucity after HS, demonstrating that recurrent stress does not always induce bleaching-resistance. We firstly document *Symbiodinium* C and D, along with the Clade B1 in *Exaiptasia* anemones. C subclades found in outdoor specimens faded under laboratory exposures. Clade D emerged after HS treatments, especially after GTS pre-exposure. This highlights the thermo-tolerance of D subclades found in *E. pallida* and shows that bleaching-recovery can involve shifts of background symbiont phylotypes. This study enlightens the capability of *Exaiptasia* anemones to acclimate to gradually increased temperatures, and explores into how thermal history influences in subsequent stress tolerance in symbiotic cnidarians.

Over the past decade, by global warming, severe syndromes of unusual lethality have annihilated sea stars raising awareness globally. Echinoderms are key components in benthic biocenoses, thus significant die-offs can alter whole ecosystems. We described the first case study of a disease-like syndrome affecting Antarctic *Asteroidea* (**Núñez-Pons *et al*., submitted**). Histological analyses evidenced ulceration, inflammation and necrosis in diseased animals. Bacterial and fungal diversity were consistently lower in the lesion fronts, and revealed different composition at several taxonomic levels in healthy versus diseased tissues. This suggested a microbiome dysbiosis that could contribute in the development of the symptoms. Microbial analysis and microscopy failed to reveal putative pathogens, thus we inclined on viruses as potential infectious agents.

There are also pathogen driven disorders, especially those that induce tissue loss (white syndrome, WS). During the recovery from an intense bleaching episode that took place after the remarkably warm summer of 2014, which affected ~80% of the reefs in Kane'ohe Bay, a devastating outbreak of WS also hit populations of *Montipora capitata* (**Núñez-Pons *et al*., under revision**). Colonies were tagged in the field and subsampled at different time-points from the climax of the bleaching through the recovery for MISeq sequencing of the 16S V4, ITS-1 and ITS-2 regions. This study exposes our data tracing the populations of *Symbiodinium*, fungi, and bacteria in bleached and resistant corals following this incisive event, and contrasts these microbial community patterns with those associated to colonies with WS.

h. The biogeochemistry of beneficial host-microbe interactions (Cardini)

Beneficial host-microbe interactions are ubiquitous in nature and play an essential role in the health and physiology of diverse marine organisms. Thanks to their expanded metabolic repertoire, host-microbe interactions can drive flows of energy and matter within their ecosystems, importantly contributing to nutrients cycling. However, environmental stressors such as climate change increasingly threaten the physiological function of the holobiont (the assemblage of all participants in the symbiosis, forming an ecological unit). Our work focuses on the biogeochemistry of host-microbe interactions, as well as the physiological and ecological consequences of these dynamics. We investigate in particular the carbon and nitrogen cycles and the role of microbes in symbiosis with benthic hosts. A key goal is to provide a mechanistic understanding of how the mutualism leads to improved physiological performance of the host, and how it contributes to ecosystem functioning.

To this end, we use a combination of underwater fieldwork for sample collection and incubation experiments with living organisms. Nutrient and oxygen measurements, acetylene reduction assays and stable isotope probing are used to quantify fluxes.

Our research demonstrated the importance of N_2-fixing bacteria (diazotrophs) in sustaining primary productivity of the invasive marine plant *Halophila stipulacea* (**Cardini *et al*., 2017**). *Halophila stipulacea* spread throughout the oceans, aided by human activity and climate change, and colonized areas in the Mediterranean and in the Caribbean. Our study suggests that the association of this plant with diazotrophs could further contribute to its invasiveness in areas with variable N concentrations.

In a similar study, we investigated the activity of diazotrophs associated with two common macroalgae from the Gulf of Aqaba (Northern Red sea), and found that whereas increased sea surface temperatures correlated with increased N_2 fixation rates, so did declining ambient dissolved inorganic nitrogen (DIN) availability (**Tilstra *et al.*, 2017**). These results suggest that increasing temperatures caused by ocean warming may boost N_2 fixation by macroalgae-associated diazotrophs which in turn would provide an additional N source for algal growth. At the same time, input of N from allochthonous sources may cause macroalgae-associated diazotrophs to be outcompeted by other bacteria.

4. Perspectives

a. Microscale interactions in zooplankton (Mazzocchi, Uttieri)

As future perspectives, real 3D tracks recorded for *Clausocalanus furcatus* will be used to test the applicability of macroscopic metrics routinely used in statistical mechanics (mean square displacement, temporal correlation functions), for distinguishing short-time ballistic motion and Fickian diffusion. These descriptors prove invaluable in providing concise information even in presence of strongly heterogeneous individual behavior.

Statistical mechanics tools will also be used to evidence any potential effect played by different crowding conditions on the behavior of *Paracalanus parvus*. In particular, the average squared velocities extracted from 3D observations will be used to recognize potential differences in the pattern of movement as induced by the massive presence of congeneric individuals in a limited volume.

Individual-scale investigations, moreover, promote the understanding of the mechanisms by which zooplankters interact with other individuals and, as an effect, how this structures the pelagic communities in the ecosystem. Attention will be focused on the behavior of key species as a trade-off between acquiring food and avoiding predators, for a better understanding of the mechanistic interactions between trophic levels.

b. Chemically-mediated diatom-copepod interactions (Carotenuto, Ianora)

Future research activity will be focused on the functional annotation of the *de-novo* assembled *Calanus helgolandicus* transcriptome to decipher the molecular mechanisms underlying the reproductive failure and teratogenesis in copepods due to diatoms and oxylipins. We will also investigate the *in situ* reproductive and molecular responses of copepod species collected at the Long-Term Ecological Research station LTER-MC in the Gulf of Naples, together with the analysis of the metabolic profiles of co-occurring phytoplankton community in the sampling station, during one-year seasonal cycle. Our aim is to elucidate the ecological role of diatom infochemicals on plankton dynamics in the Gulf of Naples (The Open University-SZN PhD course, XVIII Cycle, 2016-2019, Ennio Russo). Several bloom-forming diatom species occurring in the Gulf of Naples in fact, produce active oxylipins; yet, little or no information exists on the reproductive and molecular responses of co-occurring copepods. Investigating the chemically-mediated diatom-copepod interactions in the Gulf of Naples, may help to understand the seasonal dynamic of the zooplankton in relation to phytoplankton blooms in this area.

c. The ecological role of Polyunsaturated Aldehydes in the plankton (Casotti)

Based on the observation that bacteria live in close vicinity, and often attached to diatoms, future research will investigate the taxonomic identity of bacteria found attached to diatoms in culture and *in situ*, in order to selectively test their metabolic properties and identify possible degradation pathways. For natural samples, SEM will be used to visualize the bacteria attached to diatoms and High Throughput Sequencing for their taxonomical characterization through sequencing of hypervariable regions of the 16S ribosomial gene. The relationship between diatoms and bacteria will also be investigated using co-culturing of the PUA-producing diatom *Skeletonema marinoi* and 6 strains isolated during a *Skeletonema* bloom in the Northern Adriatic Sea (Pepi *et al.* 2017).

d. Effect of secondary metabolites on the physiology of benthic marine organisms (Zupo)

In the future, we aim at identifying the chemical structure of the active compound isolated in *Cocconeis scutellum*, thanks to a new PhD Project started in cooperation with the University of Cologne (Germany). We also aim at elucidating the mechanism of action of this compound, in order to design new specific apoptogenic compounds, to be used for biotechnological applications (pharmaceutical, aquaculture, etc.). In the future, we plan to perform a first characterization of the active compound(s) produced by the Cyanobacteria *Halomicronema*, by separating several fractions of the culture medium by HPLC; preliminary results indicate that the active compound is quite hydrophilic and is probably a polypeptide. In the future, it is possible that natural compounds produced by this Cyanobacteria genus could represent an interesting solution to naturally controlling various aquaculture pests.

e. Plant-animal interactions in benthic vegetated systems (Buia)

In the future, we will perform the analysis of a three-year data set of the grazing pressure of a key herbivore in relation to *Posidonia oceanica* features at natural CO_2 vents.
We will also analyse the data related to the feeding behavior of the sea urchin *Paracentrotus lividus* in term of consumption of single or combined items, total phenolic content and C/N ratio.
We will compare the levels of phenolic compounds in brown macroalgae with a multi-analysis approach from the interaction studies of several marine invertebrates associated with habitat-forming seaweeds.

f. Unexpected trophic interactions among benthic organisms (Musco, Vega Fernández)

Future research is directed towards:

- identification of cues driving animal behavior
- trade-offs involved in optimal foraging strategies
- facilitation in the context of biological invasions
- anti-predatory mechanism as grazing deterrence
- indirect effects like the redistribution of grazing intensity
- evolution of gregarism,
- complexity of the ecological networks
- relative importance of energy transfert from the pelagic realm to the benthic one in the net energy budget of the system.

g. Marine symbiosis for health, stress and disease (Núñez Pons)

As future perspectives, we plan to:
- Improving the methods for genetic characterization of associated marine fungi.
Fungal microbial symbionts are supposed to be highly important partners for nutrition and for their impressive capacity to produce bioactive metabolites that the host can then use as alellochemicals. We will develop an optimized novel technique called ‘PCR clamping’. This technique will allow a sequencing depth necessary to characterize fungal communities, which up to now have been greatly overlooked in marine organisms. Our target organisms are sponges and corals.

Sequencing characterization of associated fungi will be accompanied by microscopical evidence of fungi morphologies within the target holobionts.
- Studying the adaptability of sponge and microbiomes to ocean acidification

Here we propose a multidisciplinary project integrating ecological, microbial, metabolic, chemical and isotopic studies to address the following objectives: a) to identify acclimatization strategies adopted by Porifera under the influence of future scenarios of OA; b) to disentangle the coupling of microbiome and metabolism in sponge holobionts, with emphasis on bioactive products; c) to estimate the potential repercussions of "acidified sponge assemblages" in the ecosystem structure and nutrient recycling.

h. The biogeochemistry of beneficial host-microbe interactions (Cardini)

Novel approaches are needed to investigate the role of complex and diverse host-microbe interactions within their habitats. In the future, we plan to combine *in situ* investigations with manipulation and mesocosms experiments to disentangle the role of single environmental conditions. By integrating across biological scales and maintaining an environmental context, this work will help determine the capacity of symbiotic marine organisms to shape their ecosystems and to withstand and adapt to a changing marine environment.

5. Publications (2012-2017; *corresponding author)

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6. List of grants

- **Crowdfunding Platform** "Staying Alive: How to microbes help corals survive warming oceans?" (April-December 2015).
- **GGI - Smithsonian Institute Grant Program** "'Symbioses on the Rocks': Exploring Microbial and Nutritional Couplings of Coral Reef Architects" (2016-2017).
- **RITMARE**: The Italian research for the sea. Italian Flagship Project of the Italian Ministry of University and Research (2013-2018).

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11. Environmental assessment, management and restoration

1. People involved

Name	*Position*	*Funding*	*Period*
Iacopo Bertocci	Staff scientist-Term contract	SZN	since 2016
Massimiliano Bottaro	Staff scientist	SZN	since 2017
Maria Cristina Buia	Staff scientist	SZN	since 1989
Raffaella Casotti	Staff scientist	SZN	since 2000
Paola Cirino	Staff scientist	SZN	since 1989
Maria Costantini	Staff scientist	SZN	since 2010
Fabio Crocetta	Staff scientist	SZN	since 2017
Claudia Di Somma	Staff scientist	SZN	since 2008
Maria Cristina Gambi	Staff scientist	SZN	since 1983
Sandra Hochscheid	Staff scientist	SZN	since 2001
Daniele Iudicone	Staff scientist	SZN	since 2009
Fulvio Maffucci	Staff scientist-Term contract	SZN	since 2017
Francesca Margiotta	Staff scientist	SZN	since 2009
Luigi Musco	Staff scientist-Term contract	SZN	since 2016
Francesco Paolo Patti	Staff scientist	SZN	since 2010
Milva Pepi	Staff scientist-Term contract	SZN	since 2017
Maurizio Ribera d’Alcalà	Staff scientist	SZN	since 1981
Maria Saggiomo	Staff scientist	SZN	since 2012
Vincenzo Saggiomo	Staff scientist	SZN	1969-2012
Diana Sarno	Staff scientist	SZN	since 1994
Francesco Terlizzi	Staff scientist	SZN	since 2016
Alfonso Toscano	Staff scientist	SZN	since 1994
Adriana Zingone	Staff scientist	SZN	since 1989
Valerio Zupo	Staff scientist	SZN	since 1994
Cecilia Balestra	Postdoctoral fellow	MarMIC/RITM./SZN	since 2007
Vincenzo Botte	Postdoctoral fellow	RITMARE	since 2016
Lucia Porzio	Postdoctoralfellow	Campania Region	2015-2016
Luciana Sabia	Postdoctoral fellow	SZN/Terna	since 2016
Mark Van Dijk	Postdoctoral fellow	S&TMed/RIT./IN-OGS	since 2014
Alexandre Epinoux	PhD student	SZN/ISMAR-CNR	since 2017
Daniele Grech	PhD student	SZN	2014-2016
Mirko Mutalipassi	PhD student	SZN	since 2015
Fabio Badalamenti	Affiliated scientist	CNR-IAMC	since 2017
Tomas Vega Fernandéz	Affiliated scientist	CNR-IAMC	since 2015

2. Background, aims and objectives

The marine environment is characterised by a high complexity, stemming from the presence of diverse habitats and high biodiversity, which is at the base of goods and services provided by the system. However, marine areas are facing significant increasing threats, which include biological, physical, chemical pollution and habitat destruction, all together causing loss of biodiversity. Anthropogenic pressure is the main cause of ecological changes. These are driven by the increasing request for new economic and social development, which implies an acceleration in exploitation of marine resources worldwide. In order to mitigate environmental changes, policy-makers seek to develop strategies to protect, conserve and manage the marine realm.

The SZN is increasingly required to provide solutions to environmental problems. We are contributing to the assessment and management of the marine environment in the Mediterranean Sea following the indications of the Marine Strategy Framework Directive 2008/56/EC (MSFD), aiming at achieving the Good Environmental Status for all European marine waters by 2020. Such an approach is framed within the National Research Plan (Blue growth) and Horizon 2020 "Protection of the marine environment as a source of food, energy and biotechnology and the choice of the tools necessary for decision-makers and politicians".

Main objectives of this research topic are:

a. implementing the assessment status of the marine coastal environment;
b. analyzing the response of marine assemblages to anthropogenic pressure;
c. studying the effects of single and multiple stressors on marine organisms;
d. providing management tools for the marine system;
e. suggesting and planning restoration plans for degraded marine habitats.

3. Results

a. Environmental assessment: gaps and perspectives

The European Union adopted in 2008 the Marine Strategy Framework Directive (MSFD), which is to guide the future maritime policy and aims to achieving or maintaining a Good Environmental Status (GES) in each of the European regional seas. Moreover, the MSFD requires an assessment of how humans use the marine environment and the development of action plans and measures to achieve the GES for all European waters by 2020. According to the MSFD, part of the research conducted in the last years at the SZN was aimed at identifying knowledge gaps and at developing new approaches and methodologies for the correct evaluation of the GES. In particular, information on historical conditions and long-term series need to be used as reference to understand the effects of anthropogenic pressures and their consequences on the amount of resources a healthy ecosystem can provide. SZN researchers have been involved in international working groups aiming at implementing the application of the MSFD at a regional scale (Mediterranean Sea) and at finding new fast and coast-effective approaches for assessing the environmental status.

a1. Synthetic indicators for eutrophication (Margiotta, Saggiomo)

The need for assessing the trophic status of aquatic ecosystems is a priority due to the serious impacts caused by worldwide eutrophication phenomena[108]. The distribution of phytoplankton communities, in terms of size fractions and relative contribution of main phytoplankton functional groups (e.g., diatom to flagellate ratio), was investigated across the Po river plume in the Northern Adriatic Sea, where a highly dynamic frontal zone separates an in shore from an offshore system (**Mangoni *et al.*, 2013**) and in three other different sectors of the Adriatic Sea, characterized by different trophic conditions (**Mangoni *et al.*, 2017**).

Both studies revealed the utility of the *Fp-ratio*, index of trophic status by pigment signatures. The results show that the *Fp* well defines different trophic conditions, from eutrophic to oligotrophic, as

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[108] Cloern JE. Our evolving conceptual model of the coastal eutrophication problem. ***Mar. Ecol. Prog. Ser.*** (2001) 210: 223–253.

it significantly associate with salinity, Dissolved Inorganic Nitrogen concentrations, total chlorophyll biomass and percentage of microphytoplankton size fraction (Figure 1).
This research was supported by SZN FOE.

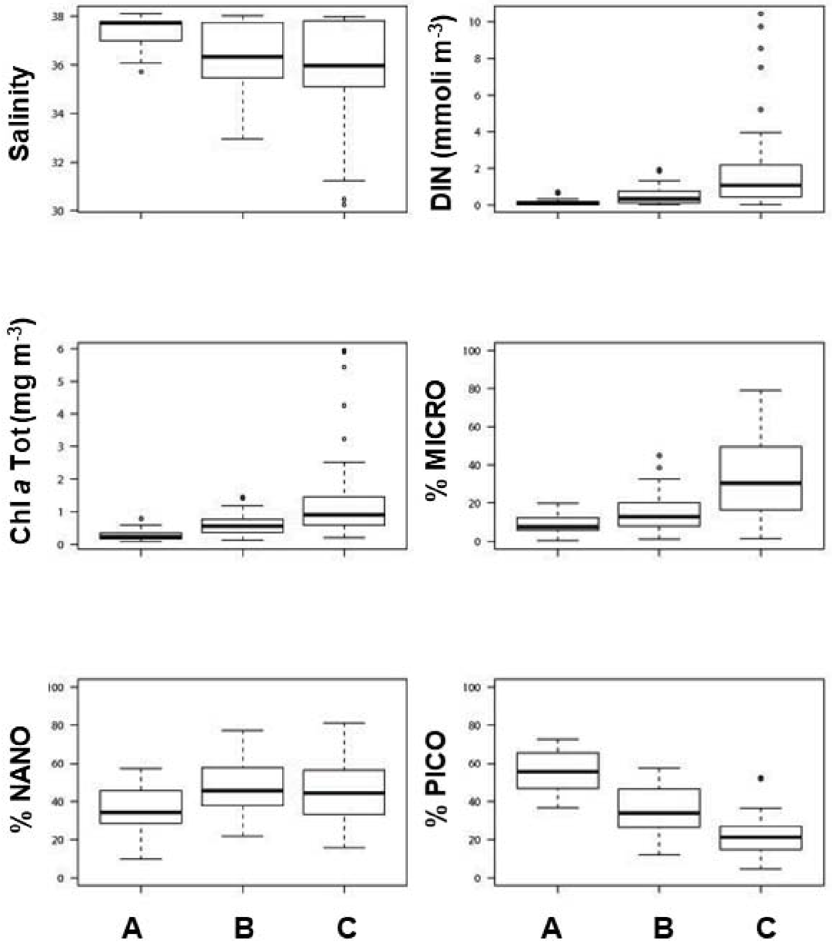

Figure 1. Distribution of salinity, Dissolved Inorganic Nitrogen (DIN), total biomass (Tot Chla), percentage of micro-, nano- and pico-fractions in the three different identified classes by means of Fp-index in the Northern Adriatic Sea (boxplot by classes).

a2. Vegetated ecosystems: Assessment & Management (Buia)

The aim of this research line is to identify knowledge gaps on vegetated coastal systems at different geographical scales.
In the framework of the PERSEUS project, a meta-analysis of existing literature on pressure/impact/knowledge gaps was performed for the Southern European Seas (**Crise *et al.*, 2015**). Data on Biodiversity, Non Indigenous Species, Fisheries, Marine Food Webs, Eutrophication, Sea Floor Integrity, Hydrological Conditions, Contaminants, Contaminants in Fish and Seafood, Marine Litter, and Underwater Noise were included in the analysis. In general, the analysis revealed (a) low data availability, (b) fragmented knowledge on biodiversity and ecosystem functioning, (c) limited and heterogeneous knowledge on the processes relating pressures and impacts, (d) the need for long-term time series datasets and (e) the need for establishing suitable monitoring networks. Adaptive scenario analyses incorporating also the socio-economic component and decision support systems have been recognized as relevant tools that should be used for the assessment of the response of marine ecosystems, whose uncertainty is and will be an intrinsic feature of such 'highly non-linear' complex systems.
Studies conducted on vegetated coastal systems revealed that most of the alien species present along the Italian coast are closely associated to the hot spot areas of introduction, and only a small fraction of them spread across natural marine habitats (**Corriero *et al.*, 2015)**.

The revision of the occurrence of Fucales algae in the Gulf of Naples [109] has been updated and the current shallow belts and deep forests of these brown algae have been surveyed.

Their decline in the area has been confirmed and it seems to be mainly due to the huge coastal transformation[110,111] for shallow species, and to illegal fishnets for the deepest ones (Figure 2).

This research was supported by SZN FOE, by RITMARE Flagship Project, by the Campania Region (montitoring of *P. oceanica*), by the EC Project PERSEUS and by LTER-I.

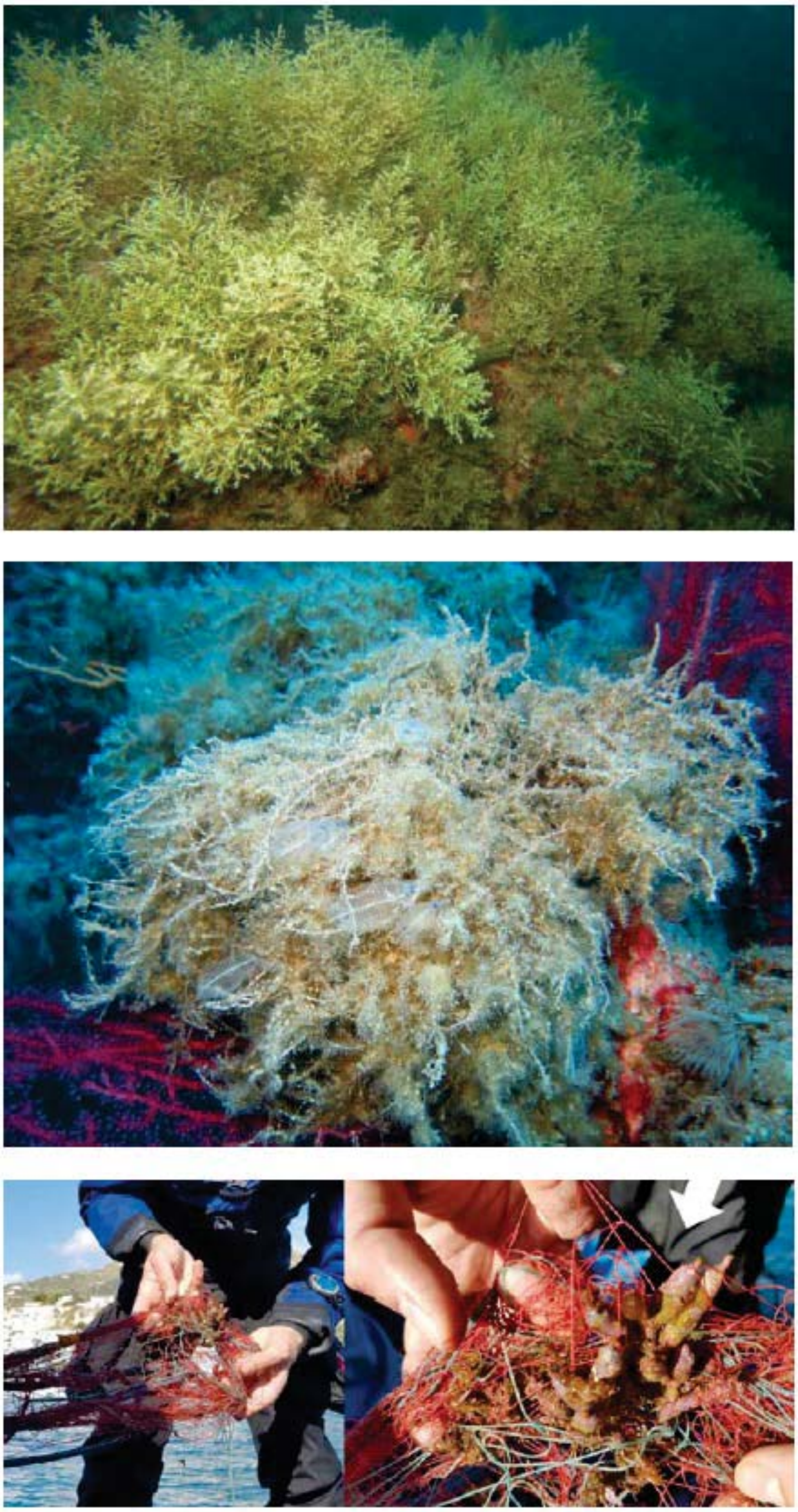

Figure 2. *C. brachycarpa* forests from 1 to 15m depth, an isolated thallus of *C. zosteroides* between 28 and 35 m depth and a thallus of the same species entagled in the nets.

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[109] Buia MC*, Chiarore A, Mulas M, Porzio L. Historical changes in the algal diversity in the Gulf of Naples. In: Özhan E. (ed), Proceedings of the Global Congress on ICM: Lessons Learned to Address New Challanges***, EMECS 10 – MEDCOAST 2013 Joint Conference*** (2013), 30 Oct-03 Nov, Marmaris, Turkey, MEDCOAST, Mediterranean Coastal Foundation, Dalyan, Mugla, Turkey, vol 2: 837-846 (ISBN 978-605-88990-9-4).

[110] Grech D, Chiarore A, Fioretti S, Kumar A, Mulas M, Buia MC, Patti FP. The importance of an integrated approach in the study, conservation and restoration of Fucales, ***Biol. Mar. Medit***.(2015) 22: 32-33.

[111] Grech D, Patti FP, Chiarore A, Mulas M, Buia MC*. Coastal transformation and marine habitat loss. In: E Ozhan (Ed), Proceedings of the Twelfth International ***Conference on the Mediterranean Coastal Environment, MEDCOAST*** '15, 06-10 October 2015, Varna, Bulgaria: 495-505. ISBN 978-605-85652-3-4, 978-605-85652-4-1.

a3. New insights for neglected habitats: rocky shores and bioconstructions (Bertocci, Musco, Vega Fernandez)

It this therefore evident that baseline biodiversity studies are thus of paramount importance in the MSFD view, in order to provide the knowledge needed to assess both the GES and its changes. Biogenic reefs are valuable marine habitats which are a focus of protection according to European legislation. A study was carried out along the South Sicilian coast on biogenic reefs produced by tube-dwelling polychaetes of the genus *Sabellaria* (Figure 3) (**Bertocci *et al*., 2017a**). This study addresses some of the current knowledge gaps by quantifying and comparing multi-scale patterns of abundance and distribution of two habitat-forming species (*Sabellaria alveolata* and *S. spinulosa*) and their associated fauna along 190 km of coast on the Italian side of the Sicily Channel. Findings contribute to meet a crucial requirement of any future effective protection strategy, i.e., identifying relevant scales of variation to be included in protection schemes aiming at preserving representative samples not only of target habitats and organisms, but also of the processes driving such variability.

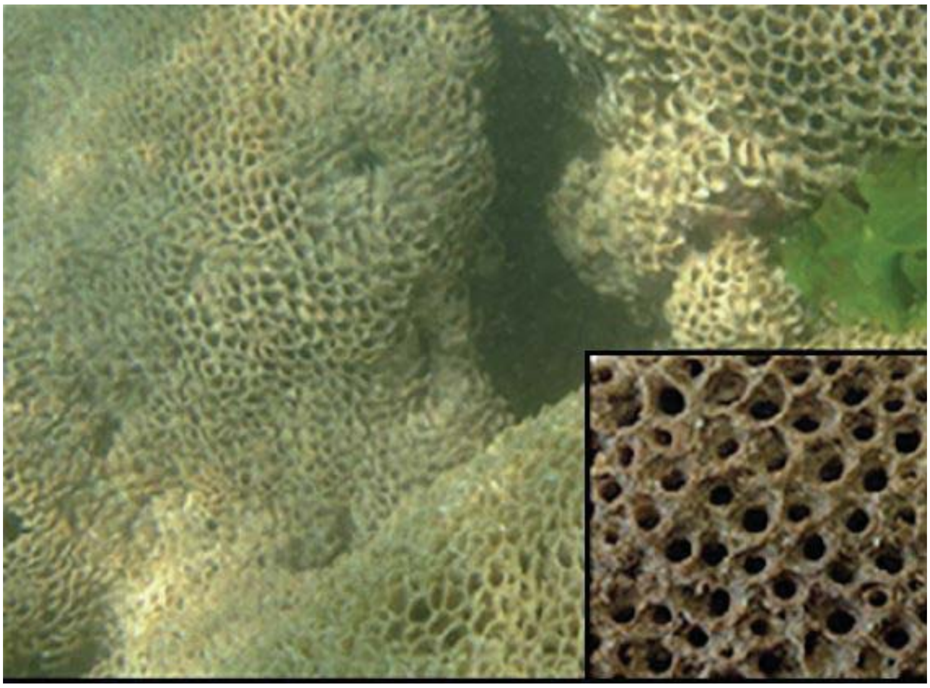

Figure 3. *Sabellaria alveolata* reef in the Sicily Channel.

Following the above-mentioned need of baseline biodiversity studies, a recent paper (**Hamdy *et al.,* 2017**) analyzed spatial temporal variation of crustacean assemblages associated to shallow rocky reefs along the Mediterranean coast of Egypt, unexpectedly revealing poor diversity probably due to the combination of both local unfavourable natural environmental features and human pressure. This is a baseline assessment has noticeable importance for understanding the predicted future changes of biodiversity for the area due to the enlargement of the Suez Canal and the consequent increase in alien invasions.

Intertidal assemblages were analyzed in order to verify the hypotheses that (i) urbanization was associated to different patterns of variation between urban and extra-urban environments; (ii) such patterns were consistent across mainland and insular systems, spatial scales from 10s cm to 100s km, and a three-months period. Several trends emerged: (i) a more homogeneous distribution of most algal groups in the urban compared to the extra-urban condition and the opposite pattern of most invertebrates; (ii) smaller/larger variances of most organisms where these were, respectively, less/more abundant; (iii) largest variability of most response variables at small scale; (iv) no facilitation of invasive species by urbanization and larger cover of canopy-forming algae in the insular extra-urban condition. Present findings confirm the acknowledged notion that future management strategies will require to include representative assemblages and their relevant scales of variation associated to urbanization gradients on both the mainland and the islands (**Bertocci *et al*., 2017b**).

a4. Biological invasions in the MSFD perspective (Musco, Vega Fernandez)

The difficulty in teasing apart the effects of biological invasions from those of other anthropogenic perturbations has however hampered our understanding of the mechanisms underpinning the global biodiversity crisis. A manipulative study carried out at several Mediterranean locations (**Bulleri *et al.,* 2016**), tested the hypothesis that the effects of invasive seaweeds on native biota tend to be more negative in relatively pristine than in human-impacted environments. More specifically, based on the scores provided by recently published maps of global human impact the study assessed which out of land-based and sea-based cumulative human impact scores was a better predictor of the direction and magnitude of the effects of this seaweed on extant and recovering native assemblages. Results suggested that competition from the invasive non-indigenous *Caulerpa cylindracea* is an important determinant of benthic assemblage diversity in pristine environments, but less so in species-poor assemblages found at sites exposed to intense disturbance from land based human activities. This suggest that measures to reduce the establishment and spread of *C. cylindrace*a should be implemented in areas little impacted by land-based human activities, that should be considered a priority for preserving the biodiversity of Mediterranean shallow rocky reefs.

b. New methodologies for integrated environmental management

b1. Real-time assessment of phytoplankton and bacteria (Casotti)

High--frequency, on line flow cytometry using Cytobuoy bv technology for picophytoplakton and a prototype for heterotrophic prokaryotes has been used connected to the water supply of the RV Vettoria while sailing (Figure 4), providing full spectrum of pico and nano-phytoplankton in the Gulf of Naples every 2 to 10 min (100 m to 1 km, depending on the boat speed). The same system has been used during cruises in the Northern Adriatic Sea[112,113] and in the Venice Lagoon, revealing details patterns of phytoplankton distribution that would have missed using the classical discrete sampling approach. In the Gulf of Naples, this integrated system has been used to map microbial concentrations (including heterotrophic bacteria) along the coast from Naples harbor to Sorrento paeninsula, sampling every 10 min, corresponding to one sample every km, approximately. A new program routine can achieve one sample every 4 min, which should increase spatial resolution considerably.

Figure 4. High-frequency flow cytometric setup for the detection of picophytoplankton (CytoSense, right) and heterotrophic planktonic bacteria (onCyt, left) on board the RV Vettoria.

The results[114] show two main critical areas along the coast, where total bacterial concentrations

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[112] Bastianini M, Riminucci F, Capotondi L, Barra E, Pasqual S, Casotti R, Trano AC, Van Dijk M, Celussi M, Fabbro C. Rapporto sulle attività oceanografiche, biologiche e di manutenzione della stazione MEDA S1-GB svolte durante la campagna LTER-ANOC16 (26-30 aprile 2016) con N/O DallaPorta nel Mare Adriatico Settentrionale. ***Rapporto Tecnico CNR-ISMAR*** (2017) N° 145, pp. 1-27.

[113] Bastianini M, Riminucci F, Pansera M, Coluccelli A, Casotti R, Dal Passo E, Dametto L, Van Dijk M, Russo E, Titocci J, Pazzaglia J, Virgili S. Rapporto sulle attività biologiche, oceanografiche, geologiche e di manutenzione della stazione Boa E1 svolte durante la campagna INTERNOS17 (14-21 marzo 2017) con N/O Minerva Uno nel Mare Adriatico centro-settentrionale. ***Rapporto Tecnico CNR-ISMAR*** (2017) N° 146, 2017, pp. 1-37.

[114] Casotti R, Balestra C, Van Dijk M, Passarelli A, Sigrist J, Hammes F. Real-Time microbial concentrations by automated on-line flow cytometry for marine coastal monitoring. **Proceedings of the IMEKO TC19 International Workshop "Metrology for the Sea"** (2017) pp. 50-53

accumulate, one at S. Giovanni, where a urban waste collector has broken and discharges urban freshwater at one mile from the coast, the other in correspondence of the Sarno river, one of the most polluted rivers in Europe. At these sites also samples for classical microbiological analyses have indicated a recent faecal contamination. These results call for the validity of the flow cytometric method that is able to map a considerable area in a short time, detecting anomalous situations that can be subject to further more complex analyses to be proven dangerous for human or animal health.

This research was supported by MIUR Premiale (IRMA), RITMARE Flagship Project- PON EMSO MedIT (support for buoy purchase).

b2. Environmental modelling (Iudicone)

Environmental modelling has been performed mainly through the use of an ocean model, that is a numerical code that allows to predict temperature, salinity and currents in a region of arbitrary shape and the capability of handling open boundaries. The tool used for this modelling is the Regional Ocean Modelling System (ROMS [115]), a free-surface, terrain-following, primitive equations ocean model widely used by the scientific community for a diverse range of applications. A specific data handler has been written to prepare the data for the code starting from a variety of sources (Mediterranean Forecasting System, ECMWF, river data, etc.) and to post-process the output. The data handler allows for a simple handling of multiple nested grids, giving the capability of obtaining high resolution in regions of specific interest. The ROMS code has been coupled with a sediment transport model that allows the user to define an arbitrary number of sediment classes, each one with its own property (for example grain size and settling velocity). The sediment model has been modified to allow the simulation of open sea discharge of sediments and has been used in two practical applications as required by external conventions.

In a first project, the model has been used to simulate the impact of the open-sea deposal of sediments dredged from the Salerno Harbour (Italy), allowing to analyze the dispersion of finer grain sediments due to currents. The performed simulations have been used to select the point of minimal impact for future deposal of dredged sediments in the Gulf of Salerno.

In the second project the model has been coupled with a simple nutritional approach of acquaculture waste to analyze the impact of a fish farm in the Cilento area (Italy) in terms of Nitrogen and Phosphorous dispersion in the environment. In this case three nested grids were used to reach a 100 m resolution, needed to represent the coastal area in the vicinity of the fish farm. The simulations performed have shown that, due to the strong currents in the area, the impact of the fish farm on the coast appears to be minimal.

A second line of research in environmental modelling is based on the use of an Individual Based Model (IBM), that is with a model where individuals are treated autonomously, allowing to dissect for each of them the cumulative effect of a few key biological traits characterizing the species, the responses elicited by the time varying flow field and the interactions with the other organisms. A new IBM model has been built using the principles of object-oriented programming, where the information is stored and processed in each object and the different objects may interact with each other in different forms[116], and used to predict the impact of turbulence on the feeding rates of copepods[117].

This research was supported by RITMARE Flagship Project, Autorità Portuale di Salerno and LPA.

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[115] Haidvogel DB *et al.*, Ocean forecasting in terrain-following coordinates: Formulation and skill assessment of the Regional Ocean Modeling System**, Journal of Computational Physics** (2008) 227: 3595-3624.

[116] MarianiP, Botte V, Ribera d'Alcalà M. An object oriented model for the prediction of turbulence effects on plankton, ***Deep Sea Research II*** (2005) 52: 1287-1307.

[117] Mariani P, Botte V, Ribera d'Alcalà M. A numerical investigation of the impact of turbulence on the feeding rates of Oithona davisae, ***Journal of Marine Systems*** (2008), 70: 273-286.

c. Management and restoration

c1. Environmental restoration (Musco, Vega Fernandez)

Within the framework of ecosystem-based management, restoration appears as a sensible option to counteract the global decline of coral reefs. Contrary to what is done in the tropics since decades, attempts at restoring coral reefs have never been done in the Mediterranean area. A study carried out at SZN revealed that culturing of coral fragments may provide a number of novel colonies that can be replanted. In fact, an environmental friendly method for the restoration of reefs of the Mediterranean endemic scleractinian *Astroides calycularis* has been tested (Figure 5). This coral suffers from to anthropogenic impact, particularly from damage due to accidental contacts by SCUBA divers, and it is expected to suffer from sea storms of increasing power under the projected climate change scenarios. Corals of opportunity (i.e. dislodged colonies found alive on the seabed) appeared as a useful resource for the restoration of *A. calycularis* reefs (Figure 6), given that the fragment based transplant technique is effective for this species as it is for other massive corals. The applied technique provides the opportunity to restore rocky reefs, even the very shallow ones, through direct transplant of coral fragments, thus making reef restoration a feasible option in ecosystem-based management plans for this species (**Musco *et al.*, 2017**).

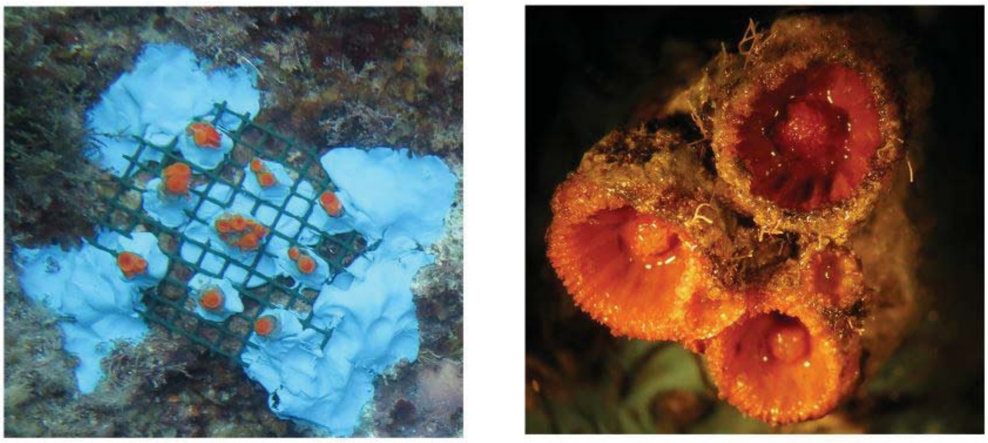

Figure 5. *Astroides calycularis* transplanted fragments.

Figure 6. *Astroides calycularis* reef.

c2. The SZN: a reference for environmental issues

The scientific knowledge accumulated over the years, along with the numerous datasets collected and the network of international collaborations, have made the Stazione Zoologica a reference for different stakeholders, including local Institutions and agencies and private enterprises that are involved and interested in environmental and health issues concerning the marine realm. Accordingly, the request for collaboration and consultancy has increased over the years, resulting in a series of projects concerning a wide range of environmental problems, listed in the following, and aimed at:

- protecting human health from risks posed by blooms of the toxic benthic dinoflagellate *Ostreopsis* cf. *ovata* (Zingone);
- contributing to definition of GES in the framework of the MSFD (Zingone);
- monitoring the water column along the Campania Region to fill the gaps for of the Western Mediterranean Sub-region in the framework of the MSFD (Casotti);
- monitoring the effects of reopening of the conenctions to the sea of the Fusaro and Miseno Lagoons on the hydrological dynamics and assessing possible changes in the trophic status (M. Saggiomo);
- monitoring the spontaneous colonization of *P. oceanica*, the benthonic communities and the physico-chemical properties of sediments along the underwater gas pipeline (Margiotta, Buia);
- monitoring the marine environment possibly affected by the power cable laying for the connection of Capri Island to the National Power Network (Casotti);
- characterizing the area identified for disposal of dredged sediments from Salerno harbour (Iudicone, Margiotta);
- modelling the dispersion of Nitrogen and Phosphorus produced by two fish farming plants (Iudicone);
- assessing the risk of colonisation of harmful benthic microalgae following the building of groynes (Zingone);
- developing new approaches for the removal and remediation of contaminated sediments and restoration of marine habitats (V. Saggiomo, Musco).

4. Perspectives

a. Environmental assessment: gaps and perspectives (Buia)

The assessment and management of vegetated ecosystems will be addressed as following:

- a meta-analysis of existing literature on pressure/impact/knowledge gaps for the Gulf of Naples has been already done and the pertinent manuscript is in preparation;
- a georeferenced map of *Cystoseira* and *Sargassum* spp distribution along the neapolitan upper littoral fringe will be elaborated;
- transplants of *Cystoseira* spp will be performed in order to try to restore native lost habitats;
- data on the structure of the *P. oceanica* meadow off Lacco Ameno (Ischia)(LTER-LA site) along a depth gradient will be analyzed in order to detect changes in the spatial anisotropy of shoot density at pluridecadal scale;
- surveys on the main seagrass meadows around the Ischia island has been performed, Synthetic ecological indices have been applied in order to characterize the health status of these systems and the manuscript is in preparation;
- the value of the natural capital of the MPA “Regno di Nettuno” will be assessed applying a biophysical and trophodynamic environmental accounting model based on emergetic criteria.

b. New methodologies for integrated environmental management

b1. Real-time assessment of phytoplankton and bacteria (Casotti)

As future perspectives, we plan:

- To further develop a software routine for the automated analysis of the large amount of data generated by the scanning high-frequency cytometry, both on board and on buoys. This step is much needed, considering the big data produced and the subjectivity of manual analysis of numerous cellular clusters detected.
- To use high-frequency flow cytometry to different sites in order to better elucidate the responses of picoplankton to environmental changes at the short space and time scale. This will be coupled with estimates of growth rates of phytoplankton from scattering and autofluorescence, in order to estimate their contribution to biogeochemical fluxes.
- To further explore potentiality of high-frequency bacterial detection, coupled with classical microbiology, to increase resolution and to find better indicators of pollution events.

b2. Environmental modelling (Iudicone)

We are preparing a hindcast high resolution simulation over 15 years to study the processes that regulate the variability of the marine populations in the Gulf of Naples. In collaboration with ISMAR CNR we are preparing a new dataset of the local forcings that wi be used to force the model and to reanalyse the time series of measurements at MC LTER.
We will then implement the Grand Unified Darwin model (collaboration with MIT) to directly simulate the plankton diversity.

b3. Intelligent operating devices for integrated environmental services (Zupo)

The study is aimed at devising and realizing new integrated intelligent systems based on the Internet of Things. Intelligent devices will be produced or both indoor and outdoor use in cooperation with a private firm for engineering of structures and addition of specific software. The new inventions, including an intelligent photobioreactor, an intelligent aquaculture tank, various intelligent probes for coastal monitoring, an intelligent submersed cage for aquaculture and special probes to be allocated on board sea turtles, will be produced applying new paradigms of information technology and networking (IoT). At the moment, the Ministry of Economical Development (MISE) has approved the project and a first trials have started to devise new “intelligent cages” to be deployed in Procida to continue another funded project (ModRes). In particular, we draw the details of a cage for hosting young sea urchin, able to follow the development of individuals, control the internal environmental conditions, activate pumps for the circulation of the water when needed and inform operators of critical conditions needing diver’s assistance.

c. Management and restoration of coastal marine habitats (most of the SZN staff involved)

During the next 3 years also thanks to the project ABBaCo the SZN will carry out a multidisciplinary study at Bagnoli-Coroglio, a post-industrial marine area which represent a model of management and restoration for similar degraded Mediterranean areas. Particularly, the complete description of the area according to the MSFD will provide an exhaustive view of the present status of the marine environment from the bacterial to the magafauna diversity, including species having economic and commercial value. Special emphasis on alien species and their effects on the resident communities will be given. Moreover, to fulfil the MSDF requirements the physical-chemical status of the water column will be particularly checked as well as the integrity of the sea bed. The actual presence oif pollutants, their spread within the food web and the risk associated to human consumption of contaminated sea food will be evaluated and particular attention will be given to sea waste, especially microplastic and their relevance in the coastal area of the gulf of Naples. An extensive acoustic survey will provide the first screening of the amplitude of acoustic pollution in the area. As restoration requires the inversion of the degradation trend back toward pristine conditions, thanks to the SZN biological collections, and especially to the SZN historical library, we will define the benchmark of the coastal areas within the Gulf of Naples, and provide historical maps of distribution of valuable species and habitats, and diversity. Special regard to habitat formers such as sea grasses and biogenic habitats will be given.

Such an information is of paramount importance for the planning of restoration actions including the restoration of *Posidonia oceanica* meadows, coral reefs, and the introduction of key species which will be attemped by pilot studies in the Bagnoli bay.

Future research includes recovery of habitat-forming organisms, marine protected areas and fishery reserves, and maritime territory management. The prospected activities are embedded in the wider context of marine spatial planning and found application in the sustainable and resilient exploitation of natural resources and of other uses of the sea. These are research lines created on demand by public society, which ask for improved environmental quality, management efficiency, and fair partition of their costs and benefits deriving from management actions.

d. Fish biology, ecology and conservation (Bottaro)

Cartilaginous fishes and Antarctic teleosts will be investigated focusing on their sensory ecology, geographical distribution and conservation. In addition, studies will be conducted on elasmobranchs by analysing their historical ecology and spatial ecology and their conservation through an ecosystem approach to fishery.

5. Publications (2012-2017; *corresponding author)

2017

Bertocci I*, Arenas F, Cacabelos E, Martins GM, Seabra MI, Álvaro NV, Fernandes JN, Gaião R, Mamede N, Mulas M, Neto AI. Nowhere safe? Exploring the influence of urbanization across mainland and insular seashores in continental Portugal and the Azorean Archipelago. ***Mar. Pollut. Bull.*** (2017) 114: 644-655.

Bertocci I*, Badalamenti F, Lo Brutto S, Mikac B, Pipitone C, Schimmenti E, Vega Fernández T, Musco L. Reducing the data-deficiency of threatened European habitats: spatial variation of sabellariid worm reefs and associated fauna in the Sicily Channel, Mediterranean Sea. ***Mar. Environ. Res.*** (2017) 130: 325-337.

Mangoni O, Lombardo R*, Camminatiello I, Margiotta F, Passarelli A, Saggiomo M Phytoplankton community to assess the environmental status of the Adriatic Sea via non-linear partial least squares regression. ***Qual. Quant.*** (2017) 51: 799-818.

Musco L,* Prada F, D'Anna G, Galasso NM, Pipitone C, Fernández TV, Badalamenti F. (2017) Turning casualty into opportunity: fragmenting dislodged colonies is effective for restoring reefs of a Mediterranean endemic coral. ***Ecol. Eng.*** 98: 206-212.

Hamdy R, Langeneck J*, Atta MM, Dorgham MM, El-Rashidy HH, Musco L Diversity and ecology of crustaceans from shallow rocky habitats along the Mediterranean coast of Egypt. ***Mar. Biodiv.*** (2017) 1-13.

2016

Bulleri F*, Badalamenti F, Iveša L, Mikac B, Musco L, Jaklin A, Rattray A, Vega Fernández T, Benedetti-Cecchi L The effects of an invasive seaweed on native communities vary along a gradient of land-based human impacts. ***PeerJ*** (2016), 4: e1795.

2015

Corriero, G* et al. (including Buia MC, Gambi MC, Giangrande A, Guglielmo R, Lorenti M, Mazzocchi MG, Patti FP, Sarno D, Scipione MB, Zingone A, Zupo V) Ecosystem vulnerability to alien and invasive species: a case study on marine habitats along the Italian coast. (2015). ***Aquatic Conserv. Mar. Freshw. Ecosyst***. 26: 392-406.

Crise A* *et al.* (including Buia MC, Mazzocchi MG, Ribera d'Alcalà M) A MSFD complementary approach for the assessment of pressures knowledge and data gaps in Southern European Seas: The PERSEUS experience. ***Mar. Poll. Bull.*** (2015) 95: 28-39.

2013

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6. List of grants

- **EU PERSEUS**, 2012-2015.
- **MIUR** Premiale Integrated (IRMA), 2015-2017.
- **ModRes**. Progetto Bandiera SZN, 2017-2018.
- **Monitoring of *Posidonia oceanica*** funded by Campania Region, 2015-2016.
- **INTENSE**. Fondi MISE, 2018-2020.
- **MIUR-FISR-ABBaCo** 2017-2020.
- **PON** EMSO MedIT, 2015-2016.
- **Research contract funded by Comune di Pozzuoli** (environmental rehabilitation and improvement of the Campi Flegrei lakes), 2015-2017.
- **Research contract funded by Terna Rete Italia** (monitoring the potential effects of power cable laying on marine environment), 2014-2017.
- **Research contract funded by ISPRA**-Marine Strategy, 2013-2014.
- **Research contract funded by LPA** (modelling the dispersion of Nitrogen and Phosphorus produced by two fish farming plants), 2017.
- **Research contract funded by Autorità Portuale Salerno** (characterization of disposal area), 2016-2017.
- **Research contract funded by Ischia Gas** (monitoring the effects of gas pipeline construction), 2016-2019.
- **RITMARE**: The Italian research for the sea (2013-2018).

7. List of external collaborators

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-Sabrina Lo Brutto, Università di Palermo

-Olga Mangoni, Università di Napoli ‘Federico II’

-Gustavo Martins, Universidade dos Açores, Portugal

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12. Blue biotech: application of science and technology for the production of knowledge, goods and services from marine biological resources

1. People involved

Name	*Position*	*Funding*	*Period*
Christophe Brunet	Staff scientist	SZN	since 2000
Ylenia Carotenuto	Staff scientist	SZN	since 2010
Immacolata Castellano	Staff scientist-Term contract	SZN	since 2016
Paola Cirino	Staff scientist	SZN	since 1989
Maria Constantini	Staff scientist	SZN	since 2010
Isabella D’Ambra	Staff scientist	SZN	since 2017
Enrico D’Aniello	Staff scientist-Term contract	SZN	since 2017
Salvatore D’Aniello	Staff scientist	SZN	since 2010
Francesco Esposito	Staff scientist	SZN	since 1987
Mariella Ferrante	Staff scientist	SZN	since 2010
Adrianna Ianora	Staff scientist	SZN	since 1983
Weibe Kooistra	Staff scientist	SZN	since 2008
Chiara Lauritano	Staff scientist-Term contract	SZN	since 2017
Luigi Musco	Staff scientist-Term contract	SZN	since 2016
Anna Palumbo	Staff scientist	SZN	since 1980
Giovanna Romano	Staff scientist	SZN	since 1989
Clementina Sansone	Staff scientist-Term contract	SZN	since 2017
Paolo Sordino	Staff scientist	SZN	since 2000
Alfonso Toscano	Staff scientist	SZN	since 1994
Elisabetta Tosti	Staff scientist	SZN	since 1989
Valerio Zupo	Staff scientist	SZN	since 1994
Valera Di Dato	Postdoctoral fellow	SZN	since 2015
Giulia Fasano	Postdoctoral fellow	Università del Sannio	since 2013
Christian Galasso	Postdoctoral fellow	SZN/MARCAN	since 2017
Alessandra Gallo	Postdoctoral fellow	SZN/ABBaCo	since 2015
Francesco Manfelotto	Postdoctoral fellow	SZN/MARCAN	since 2017
Valwria Nittoli	Postdoctoral fellow	PONa3/SZN	since 2013
Ida Orefice	Postdoctoral fellow	SZN/MARCAN	since 2017
Monia T. Russo	Postdoctoral fellow	EMBRIC/DiaEdit	since 2016
Valeria Sabatino	Postdoctoral fellow	EMBRIC	since 2014
Mariarita Brancaccio	PhD Student	SZN	since 2017
Filippo Dell’Anno	PhD student	SZN	since 2016
Marie-Lyne Macel	PhD student	SZN	since 2017
Kevin Martinez	PhD student	MarPipe	since 2017
Alfonsina Milito	PhD student	SZN	since 2015
Mirco Mutalipassi	PhD student	SZN	since 2015

Nadia Ruocco	PhD student	SZN	since 2015
Arianna Smerilli	PhDStudent	SZN	2014-2017
Maria L. Chiusano	Affiliated scientist	University of Naples	since 2015
Daniela Giordano	Affiliated scientist	CNR	since 2015
Alberto Pallavicini	Affiliated scientist	University of Trieste	since 2017
GianLuigi Russo	Affiliated scientist	CNR	since 2016
Antonio Terlizzi	Affiliated scientist	University of Trieste	since 2016
Cinzia Verde	Affiliated scientist	CNR	since 2015

2. Background, aims and objectives

The Blue biotechnology research activity is aligned with the SZN PTA and Vision 2015-2025 to study marine organisms for new molecules for biomedical and biotechnological applications and with the objectives of Horizon 2020 Blue Growth regarding “Health and Wellbeing”.

The socio-economic and scientific potential of marine resources still remain insufficiently exploited by modern biotechnology. Scientific studies have most often focused on the determination of the biogeography and richness of marine organisms (biodiversity) or ecosystem functioning, while largely ignoring the aspects of ecosystem services and sustainable exploitation. The enormous biodiversity of these ecosystems offers multiple opportunities for bio-prospecting and exploitation of marine organisms, as demonstrated by the growing number of therapeutants of marine origin currently in clinical use for cancer and pain management. Currently there is also great interest in marine-derived products as nutraceuticals and cosmeceuticals because of their beneficial effects on human health. These often have drug-like properties (hence the term -ceutical) and contain active ingredients such as vitamins, phytochemicals, enzymes, antioxidants and essential oils which are finding uses as natural additives in foods, as nutritional supplements including colour additives and antioxidants, and as vitamins, oils and cofactors which enhance general well-being.

Research activities conducted under the theme “Blue biotechnology” are multidisciplinary and aim to (i) survey marine micro/macroalgae and invertebrates (crustaceans, sponges, molluscs and cnidarians) to achieve optimized and sustainable production of relevant biomass and high added-value compounds for pharmaceutical, cosmetic and nutraceutic applications, (ii) investigate and apply solutions for the decontamination of polluted marine habitats through bioremediation, a strategy based on the capability of microorganisms, fungi and green plants to enhance natural processes involved in the removal of contaminants thereby reducing their ecological risk.

Regarding the first research activity, the main aim is the targeting of groups of bioactive secondary metabolites (e.g. polyketides, terpenes and small peptides), pigments (e.g. carotenoids and phycobiliproteins) and other primary products (e.g. oils, polysaccharides, aminoacids, peptides) that may find important pharmaceutical, cosmeceutical and nutraceutical applications, including identification of target species, characterization of chemical structures, bioactivity screening, determination of mode of activity and enhanced production of novel or already identified metabolites belonging to the target groups of compounds, but only where there exists a high probability of successful commercial potential. These objectives are being achieved using an interdisciplinary approach that includes ecology, marine biology, biochemistry, cell and molecular biology, and bioinformatics. In addition, studies are also aimed at defining the optimal culturing conditions for various marine organisms to be used for biotechnological investigations. Some species already established as models for scientific research (e.g. *Paracentrotus lividus* and *Ciona intestinalis*) will be completely cultured, from gametes to the adults, in order to provide organisms for various biotech research activities at the Stazione Zoologica.

The results obtained during the period of the 2012/2017 had been subdivided into five main research lines:

a. Marine pharmacology (including cosmeceuticals and nutraceuticals),
b. Microalgal growth optimization and bioactive compound modulation,
c. Functional genomics applied to biotechnology.
d. Environmental biotechnology (bioremediation),

e. Optimization of maintenance and production of marine organisms for biotechnological applications.

3. Results

a. Marine pharmacology (including cosmeticals and nutraceuticals)

a1. Bioactive compounds from microalgae (Ianora, Romano)

Researchers at the SZN were involved in 3 National Projects (PONREC 2007-2013) having the aim of discovering new bioactive compounds from marine microalgae, with antitumor (PON01_02782), anti-infective, anti-inflammatory (PON01_02093) and adjuvant (PON01_00117) activities. Ten microalgal species were identified with antitumor activity, 7 species with adjuvant properties and 1 species with anti-inflammatory activity. The species that showed the most promising activity were grown in 70/100 Litre photobioreactors in order to have sufficient amount of biomass for isolation (through bioassay-guided fractionation) and characterization of bioactive molecules. In collaboration with ICB-CNR, a molecule with promising antitumor activity has been isolated and characterized that is able to induce a specific cell death mechanism through the activation of an autophagic pathway (patent application under consideration). A new adjuvant for vaccines has also been isolated from the marine diatom *Thalassiosira weissflogii;* (**Manzo *et al.,* 2017**) as well as a possible anticancer extract from the dinoflagellate *Alexandrium andersoni* which induces cell death in lung and colorectal tumor cell lines (**Sansone *et al*., 2017**). Moreover, a marine microalga (*Tetraselmis suecica*) with strong antioxidant, repairing and anti-inflammatory activities on human cells has been identified (**Sansone *et al*., 2017**) and patented (Table 1) and a new fast method has been implemented for the early diagnosis of cell fate by transcript analysis (Patent, Table 1). Another outcome of the activities in the frame of these PON projects was the creation of a new company (BioSEArch s.r.l.) as a Spin-off of the SZN and CNR (https://www.biosearchsrl.com/copia-di-home).

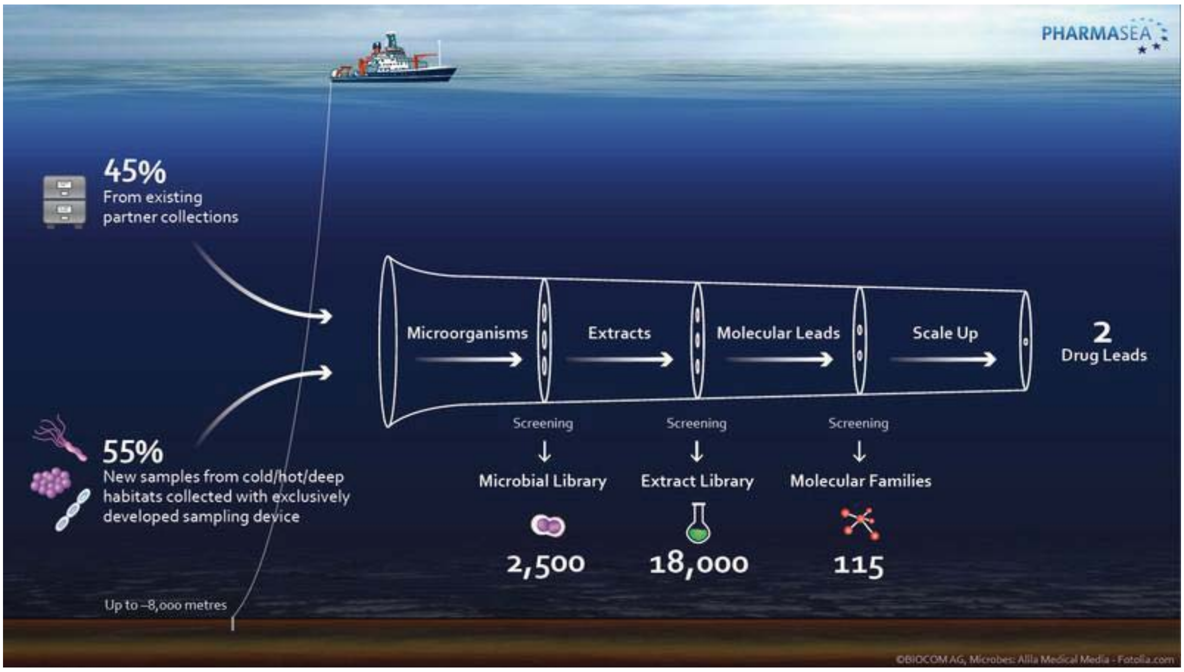

Figure 1. The pipeline of the PharmaSea Project.

During the EU FP7 PharmaSea project (2012-2017, Figure 1), 70 microalgal species were isolated, genotyped using 18S and 28S ribosomal subunit sequencing, and deeply investigated for possible antimicrobial, anti-convulsant and anti-inflammatory activities. Results showed that 16 species had antimicrobial activity, 15 species had neuroactive activity and 6 species showed anti-inflammatory activity. Part of the results have been already published by **Lauritano *et al.* (2016)** and 2 other papers have been submitted, one on a compound with anticonvulsant activity isolated from the diatom *Skeletonema marinoi* (**Brillatz *et al.,* submitted** to Marine Drugs) and another on two diatoms with antituberculosis activity (**Lauritano *et al.,* submitted** to Scientific Reports). In addition, a dinoflagellate with strong antifungal activity and a diatom with strong anti-inflammatory activity have been identified. Fractionation and dereplication have been performed and chemical partners (i.e. University of Aberdeen, Scotland and University of TromsØ,Norway) are identifying the compounds responsible for these activities (Papers in preparation).

a2. Bioactive compounds from sponges (Costantini)

Sponges have been considered a gold mine for the discovery of marine natural products during the past 50 years, with about 4851 compounds identified to date, contributing to nearly 30% of all marine natural products discovered so far (**Ruocco *et al.,* 2016a,b; Romano *et al.,* 2017a; Ruocco *et al*., 2017b)**. Extracts from the marine sponge, *Geodia cydonium*, were tested on normal human breast epithelial cells (MCF-10A) and human breast cancer cells (MCF-7). Results showed that this extract has no cytotoxic effects on both cell lines whereas it induces a decrease in levels of VEGF and five pro-inflammatory cytokines (CCL2, CXCL8, CXCL10, IFN-γ, and TNF-α) only in MCF-7 cells (**Costantini *et al.*, 2017**). This anti-inflammatory effect highlights the potential of *G. cydonium* for future drug discovery against major diseases such as breast cancer (**Zuppa *et al.*, 2014; Costantini *et al.,* 2015**). Studies are being conducted to identify the compound(s) responsible for this activity with chemistry partners from the University of Naples.

a3. Bioactive compounds from sea urchins (Palumbo, Cirino, Brunet)

Other studies conducted at the SZN have allowed the isolation and characterization of bioactive molecules from sea urchins such as ovothiols, sulfur-containing amino acids, and thiohistidine derivatives. Ovothiol A is produced in millimolar amounts by sea urchin eggs and protects embryos from the high oxidative burst at fertilization and from environmental cues, when released in sea water (**Castellano *et al.*, 2016**). Ovothiol A has also been shown to reduce proliferation of human liver carcinoma cells through the activation of an autophagic pathway (**Russo *et al.*, 2014**). Recently, ovothiol A has also been shown to act as an anti-oxidative/ inflammatory compound in pathologies related to cardiovascular diseases and endothelial dysfunction (Figure 2**; Patent,** Table 1**)**.

Moreover, the non-harvested sea urchin *Arbacia lixula* is being investigated as a new marine source for the production of astaxanthin, a natural pigment known for its protective effect against neurodegenerative diseases. The study clearly demonstrates that specific cultivation of *A. lixula* leads to astaxanthin-rich eggs (**Patent,** Table 1, paper in preparation).

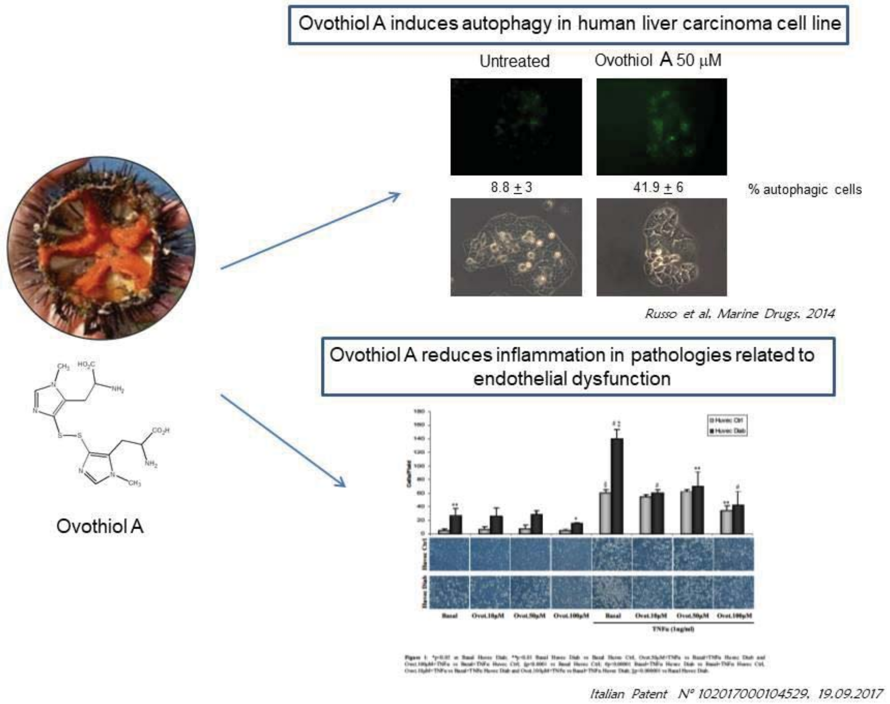

Figure 2. Biological activity of ovothiol

Studies are in progress to further investigate the role of ovothiols in sea urchin development. Preliminary results indicated that the enzyme responsible for the synthesis of ovothiols, OvoA, shows a specific temporal and spatial expression during development. A strong immunopositivity appears in specific cells and tissues. To assess the functional significance of ovothiol formation, perturbation experiments will be performed by morpholino microinjection (SZN funded PhD-OU project 2015-2018).

Moreover, the non-harvested sea urchin *Arbacia lixula* is being investigated as a new marine source for the production of astaxanthin, a natural pigment known for its protective effect against neurodegenerative diseases. The study clearly demonstrates that specific cultivation of *A. lixula* leads to astaxanthin-rich eggs (Patent, Table 1).

b. Microalgal growth optimization and bioactive compounds modulation (Brunet)

Microalgal production under different physiological conditions has been investigated (**Barra *et al.*, 2014**) and a light system modulation for the maximisation of growth and modulation of carbon metabolism in microalgae has been patented (Table 1). The system is able to modulate light intensity, photoperiod, light spectrum and light distribution, i.e. the frequency of variability of the intensity (and spectrum) over the daylight.

Carbon metabolism (carbohydrates, lipids and proteins) is strongly affected by light sensed by cells, showing the possibility to significantly modulate the nutritional state of the cells with light. The production of carotenoids, mainly xanthophylls, and vitamins or antioxidant molecules and capacity of the cells are tuned both by light intensity and spectrum (**Orefice *et al.*, 2016; Smerilli *et al.*, 2017**). Recent results show that diatoms possess an efficient photoprotective and antioxidant network to cope with light changes. While the activity of antioxidant enzymes such as catalase, ascorbate peroxidase and superoxide dismutase was enhanced under red+blue light, antioxidant molecules like total phenolic content and ascorbic acid and the protective xanthophyll cycle pigments were produced under high monochromatic blue light (Smerilli et al., 2017). Preliminary results indicate the high antioxidant capacity of the photoprotective xanthophyll diatoxanthin. These results provide valuable insights for manipulation of the light environment in order to maximise production of bioactive molecules in diatom cultures.

c. Functional genomics applied to biotechnology (Ferrante, Lauritano)

1. Gene-mining and –omics approaches are being used in order to identify enzymes with biotechnological applications from microalgae (Figure 3**; Lauritano *et al.*, 2017**). Type I β-ketosynthase, involved in the synthesis of secondary metabolites called polyketides, have been identified in the dinoflagellate *Amphidinium carterae*. In addition, L-asparaginase (used for the treatment of leukemia and for acrylamide reduction in food industries) and cellulase (useful for biofuel production and other industrial applications) have been identified for the first time in this species, giving new insights into possible biotechnological applications of microalgae.

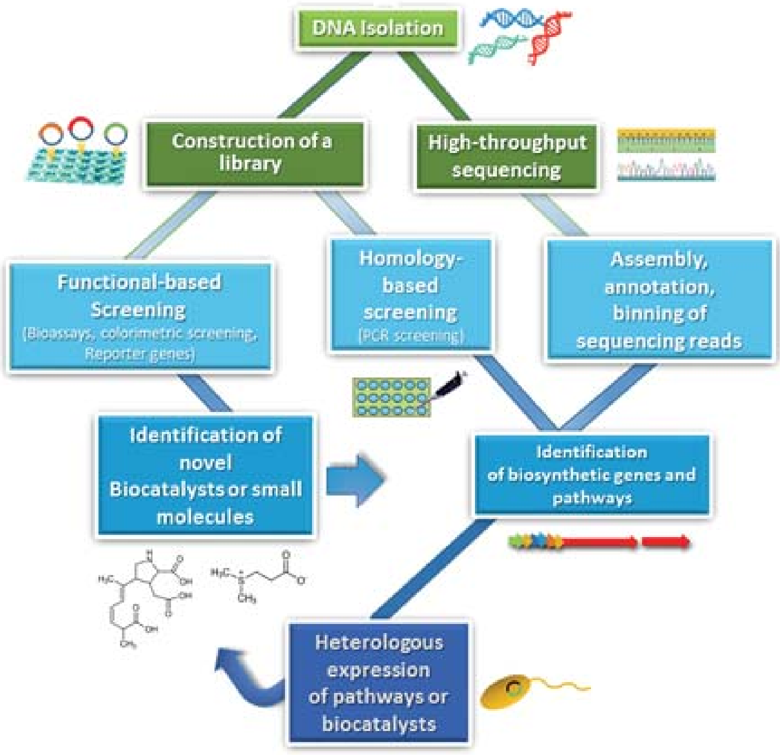

Figure 3. Example of molecular biology approach for natural product discovery.

2. Through the transcriptome analysis of the diatom *Skeletonema marinoi* we have recently demonstrated the presence of Prostaglandins (PGs) in this microalgae (see Di Dato et al 2017 in section on biodiversity and functional mechanisms). These have several therapeutic and research applications but their organic chemical synthesis has elevated costs. We thus are studying the feasibility of PG production from *S. marinoi* that can easily and massively be grown in bioreactors making possible the accumulation of large amounts of cells from which prostaglandins can eventually be extracted.
3. Different methods (RNAi, gene overexpression, fluorescent tagging) are being applied to produce diatom strains with increased, reduced or abolished expression of target genes involved in the production of specific compounds (EMBRIC project, DiaEdit project). Importantly, the DiaEdit project has allowed to set up the CRISPR/Cas9 technology for gene knockouts, a diatom mutant strain defective in the synthesis of a lipase is currently being characterized.

d. Optimization of maintenance and production of marine organisms for biotechnological applications (Zupo)

To achieve optimized and sustainable production of relevant biomass and high added-value compounds for pharmaceutical, cosmetic and nutraceutic applications, we developed and patented a new Automated device for the production of marine organisms (**Patent,** Table 1). This device, scalable and totally automatic, allows for the production of animal and plant biomasses for scientific and biotechnological purposes.

In synthesis:

- In the last three years, SZN researchers have deposed 7 patents, regarding technical developments, new bioactive molecules or new biological sources for bioactive molecules harvesting or production (Table 1).

Table 1: list of patents developed by SZN researchers involved in Blue Biotechnology research activities

Brunet C, Corato F	Pholia: a light system for aquatic organisms"	EUROPEAN PATENT AGENCY, 2015. REFERENCE: EP2883950; APPLICATION NUMBER Ep 20130196793 20131212.
Palumbo A, Pandolfi A, Castellano I, Di Tomo P.	Oviothols for the treatment of chronic low-grade systemic inflimmation and the related patologies	Italian Patent Application, N° 102017000104529, 19.09.2017.
Zupo V., Mutalipassi M	New Automatic Devise To Culture Model Organisms	Pct/Ib2016/052128
Sansone C, Ianora A, Romano G, Galasso C.	The *Tetraselmis Suecica* Crude Extract Reduces Oxidative Stress And Induces Repairing Mechanisms In Human Cells	Italian Patent N° 102016000124434 (07.12.2016)
Fontana A, Ianora A, Sansone C, Romano G, Cutignano A, Nuzzo G	Glycox A New Marine Derived Molecule Inducing Autophagic Cell Death In Human Cancer Cells	Patent In Preparation
Cirino P, Toscano A, Sansone C, Brunet C	Method For Improving Astaxanthin Production By The Sea Urchins In Culture Through The Food And Preparation Method Thereof	Italian Patent Application N° 102017000053831
Sansone C, Ianora A, Chiusano Ml, Romano G, Galasso C	A New Fast Method For The Early Diagnosis Of Cell Fate By Transcripts Analysis	Patent Pending

- A new spin-off was created in 2016. BioSearch srl (https://www.biosearchsrl.com/) is the first innovative start-up in Italy for research and development of active compounds from marine microalgae for pharmaceutical, cosmeceutical and nutraceutical applications. BioSearch is a spin-off company of two academic institutions, the Stazione Zoologica Anton Dohrn and the Italian National Research Council. The current portfolio of products includes a new adjuvant for the preparation of vaccines, a new active compound with anticancer properties and a natural preparation for topical use for skin repairing and as an antiageing agent.

4. Perspectives

With the birth of the new department dedicated to the Blue biotech research activities by the end of this year, we expect that these activities will rapidly grow, thanks to: common laboratories and infrastructures, recruitment of researchers, technicians and technologists on well defined themes regarding: (i) bioactive compounds for pharmaceutical, cosmeceutical or nutraceutical applications, (ii) environmental biotechnology, (iii) biomaterials.

One of the objectives for the immediate future is to increase the number of patents and their use by biotechnological companies.

Specific objectives are reported subdivided in five main research lines:

a. Marine pharmacology (including cosmeceuticals and nutraceuticals),
b. Microalgal growth optimization and bioactive compound modulation,
c. Functional genomics applied to biotechnology,
d. Environmental biotechnology (bioremediation),
e. Optimization of maintenance and production of marine organisms for biotechnological applications,
f. New biomaterials from marine organisms.

a. Marine pharmacology (including cosmeticals and nutraceuticals) (Ianora, Costantini, Palumbo, Romano)

Our aim is:

1. To structurally identify the compounds isolated from microalgae within the EU funded PharmaSea project having antifungal, anti-tuberculosis, anti-inflammatory activities.
2. To characterize the interaction of ovothiols with a recently identified molecular target, highly expressed in some lines of human cancer cells, and considered an attractive target for cancer chemotherapy (SZN co-funded PhD-OU program 2017-2020).
3. To further fractionate the organic extract from the sponge *G. cydonium* to identify and chemically characterize the compound(s) responsible for cytotoxic effects on breast cancer. Once isolated, the compound will also be tested on other cancer cell lines.
4. To further investigate the anticancer activity discovered after initial screenings of different macroorganisms. For example, the immunomodulatory effect of *Corallium rubrum* mucus is being studied on human dendritic cells in a co-culture with naïve cells. Dendritic cells treated with mucus are able to induce Th17 differentiation in naïve cells indicating the production of potential anti-inflammatory compounds in mucus. Extracts of the polychaetes *Syllis prolifera* and *Diopatra neapolitana* show antiproliferative activity on cancer cell lines (A549 and BeWo), without affecting normal cell lines (Wi38 and Beas 2B). These extracts are being further fractioned in order to isolate the bioactive compounds. The gastropod mollusc *Hexaplex trunculus* is being investigated for the production of antiproliferative compounds against human lung adenocarcinoma cells that do not affect the viability of normal human lung fibroblasts.
5. To investigate the dermoprotective role of ovothiols in skin tissues considering that similar compounds (2-thiohistidines) are already known for their photoprotective properties.
6. To identify the biosynthetic pathway responsible for the production of astaxanthin in the sea urchin *A. lixula*.
7. To isolate the active compound(s) produced by a species of Cyanobacteria isolated in the gulf of Naples, in cooperation with the University of Koeln. The activity of the secondary metabolites (mainly polypeptides) will be characterized and the use for various biotechnological applications will be evaluated.
8. To use the zebrafish *Danio rerio* and the tunicate *Ciona robusta* in drug discovery screenings aiming to identify anti-inflammatory and immunomodulatory compounds of marine origin. The

teleost *Danio rerio*, commonly known as zebrafish, is now a well-established model organism in biomedical research. This organism presents highly conserved molecular pathways and experimental evidence showed that in some cases it recapitulates a symptomology closer to humans, with respect to other vertebrate models. Based on this organism, a drug discovery platform for anti-inflammatory high-throughput screening has been recently established. Thanks to deep genetic and cellular characterization and a rich computational and genomic toolbox, *Ciona* represents a new frontier of investigation through the development of large-scale ad hoc assays to identify new bioactive natural products with therapeutic potential.

b. Microalgal growth optimization and bioactive compounds modulation (Brunet, Palumbo)

1. To build and set-up a new generation of photobioreactors for microalgal mass cultivation to maximize the production of bioactive compounds.
2. To optimize the nutrient concentrations of the microalgal culture medium for maximizing the growth and duration of the growth phase.
3. To enhance the synthesis of vitamins and antioxidants molecules by microalgae to be used in cosmeceuticals or nutraceuticals, for example, by overexpressing enzymes involved in the carotenoid biosynthetic pathway in order to enhance carotenoid production in the diatom *Phaeodactylum tricornutum*.
4. To identify and characterize the structure of ovothiols produced by diatoms and optimize their production under different light conditions (e.g. for the diatom *Skeletonema marinoi*) (SZN funded PhD-OU project 2015-2018)
5. To identify and characterize the structure of ovothiol in an advanced engineered system overexpressing OvoA in *P. tricornutum*.

c. Functional genomics applied to biotechnology (Ferrante, Palumbo)

1. Genomic and transcriptomic approaches will be used in order to identify enzymes/gene clusters involved in the synthesis of bioactive metabolites with biotechnological applications (e.g. cosmetics, pharmaceutics and bioremediation) from marine planktonic organisms. For example, a recent study identified in the dinoflagellate *Amphidinium carterae* Type I β-ketosynthase, L-asparaginase and cellulose by using a transcriptomic approach (Lauritano et al., 2017). A similar approach will be used for already sequenced phytoplankton genomes and transcriptomes.
2. We are also continuing the transcriptome and genome mining of the diatom *Thalassiosira rotula* and we already found the biosynthetic pathway for the monoterpene secologanin, which occupies a central role in plants for the synthesis of alkaloids. In addition, secologanin has been demonstrated to have a number of pharmaceutical properties (paper in preparation).
3. On-going in-silico search of OvoA-like proteins from available transcriptomes and genomes of marine organisms have revealed that the biosynthetic pathway leading to ovothiol production is widespread in marine environments, especially in sulphur oxidizing bacteria, microalgae, and several edible species of marine invertebrates (Castellano et al., 2016). Therefore, further comparative transcriptomic and genomic analysis will be carried out to find new sources of the molecule and to optimize engineered biosynthetic pathways of ovothiols (SZN co-funded PhD- OU project 2017-2020).
4. With the information generated by the different approaches mentioned above, it will be possible to identify specific pathways to target with the available technologies, including the revolutionizing CRISPR/Cas9 technology, in order to enhance strain properties.
5. Green fluorescent proteins from marine organisms will be investigated. Most planktonic marine organisms produce biofluorescence for intra- and inter-species communication, reproductive purposes, predator-prey interactions and extended vertical migration with an impact on the trophic and biogeochemical flows at sea. GFP are currently used as markers for molecular biology and cellular studies and for screening in human medicine; nevertheless, there is still great interest in finding new fluorescent probes, as for example the urgent need for biosensors (engineered GFP) with a high sensitivity to different pollutants,

toxins, and stress factors. SZN aims to a) conduct a computational search for GFP-like proteins from available transcriptomes and genomes of marine organisms; b) biochemically and spectrally characterize new and engineered fluorescent proteins for biomedical applications.

d. Environmental biotechnology (bioremediation) (Ianora, Musco, Tosti)

This novel experimental field will focus on new biotechnological solutions, based on biostimulation and bioaugmentation strategies for the treatment of contaminated marine sediments coming from the Sito di Bonifica di Interesse Nazionale Bagnoli-Coroglio and from the harbor of Naples. This biotechnological approach, using ability of microbial communities to degrade contaminants or reduce their toxicity, would provide solutions for the environmental friendly treatment of contaminated sediments by removing or biodegrading contaminants thus reduce their toxicity. Such an approach will create waste with lower environmental impact allowing for the restoration/rehabilitation of impacted coastal areas, thus creating the basis for economic and social requalification. Within the framework of ABBaCo, the SZN has begun sampling this year and the experimental phase and preliminary results are expected to be achieved early in next year.
Specific objectives are:
1) To develop new eco-friendly approaches for the treatment of contaminated marine sediments;
2) New environmental tools for environmental remediation, available for companies in this sector.

e. Optimization of maintenance and production of marine organisms for biotechnological applications (Zupo)

1. Based on results obtained at the laboratory scale, a plant for the production of marine organisms will be set in Procida with the cooperation of a private firm (Echinoidea srl). This large-size plant, providing both ground laboratories and field controlled areas for the marine productions, will provide resources for the production of large biomasses of marine organism, starting with the mass cultivation of sea urchins, especially of the species *Paracentrotus lividus,* and *Ciona intestinalis* as sources of secondary metabolites (e.g. Ovothiol) and model organisms for scientific research.
2. A new project (INTENSE) is starting, aimed at the study of new “intelligent tanks” and “intelligent photobioreactors” to be used for the mass culturing of marine organisms, useful for various biotechnological purposes, for the extraction of chemicals, production of biomaterials and for specific investigations in different fields of research.
3. We will set standard methods for the continuous cultivation of tunicate *Botryllus shlosseri* and set standard methods to use this organism as a quick bioindicator of environmental quality, for the rapid assessment of the presence of pollutants, apoptogenic compounds, allochemicals, etc.

f. New biomaterials from marine organisms (D’Ambra)

Objective of this study will be the development of new biomaterials from marine organisms such as collagen from jellyfish will for applications in tissue regeneration and drug delivery systems.
In contrast with the negative interactions of jellyfish with human activities that induce people to look at them as “pests” or at least a nuisance, these cnidarians have been recently re-qualified by a restricted number of scientists as a potential yet underexploited source of bioactive compounds. Preliminary analytical tests indicated that the collagen extracted from the scyphomedusa *Rhizostoma pulmo* is more similar to human collagen than bovine and porcine collagen. This suggests that jelly-collagen may have extensive biomedical applications because it may reduce the potential of rejection by the human body.
During summer and fall 2017, conspicuous swarms of *R. pulmo* have been reported in the Gulfs of Pozzuoli and Napoli and samples have been collected to perform preliminary analyses, to extract the collagen and define its amino acid composition.
We will continue the extraction of collagen from scyphomedusae, especially from *R. pulmo*, providing a readily exploitable product. Indoor and collaborative research with external partners will be conducted to develop biotechnological applications of this product. It is worth to note that the

biomass availability would not represent a limiting factor since the occurrence of scyphomedusae outbreaks appears to be increasing. In addition, outbreaks of scyphomedusae and other jellyfish will be monitored at the SZN using a drone, which will make it easier to locate the target organisms in real time.

5. Publications (2012-2017; *corresponding author)

2017

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6. List of grants

- MIUR **ABBACCO; "**Restauro Ambientale e Balneabilità del SIN Bagnoli-Coroglio" (2017-2020). PI: Musco, Saggiomo
- EU INFRAIA **ASSEMBLE Plus**; "Association of European Marine Biological Laboratories Expanded" (01/10/2017 - 30/09/2021). PI: Kooistra, Borra.
- EU H2020 **EMBRIC** "European Marine Biological Research Infrastructure Cluster to promote the Blue Bio-Economy" (01/06/2015 – 31/05/2019). PI: Kooistra, Ferrante.
- H2020-INFRADEV **pp2EMBRC** "European Marine Biology Resource Centre preparatory phase 2" (01/10/2015 to 30/09/2016). PI: Kooistra, Borra.
- MIUR **ExPO** "Exploring the biotechnological Potential of marine Organisms" (2017-2019) PI: Romano, De Luca, Nunez Pons.
- MIUR **Food&Health** (01/01/2015- 31/12/2016). PI: Brunet, Costantini, Ianora
- Progetto Bandiera SZN **MARCAN** "Marine biotechnology for new cosmeceuticals and nutraceuticals" (01/10/17 – 30/09/18). PI: Ianora
- EU Marie Curie ITN **MARPIPE "**Improving the flow in the pipeline of the next generation of marine biodiscovery scientists" (01/11/16 – 31/10/20). PI: Ianora
- Progetto Bandiera SZN **ModRes** New model organisms for the scientific research: culture, ecology, physiology and genomic characterization. (2017-2018). PI: Zupo
- EU FP7 **OCEAN MEDICINES** (01/12/15 – 30/11/19). PI: Ianora
- EU EUROFLEETS2 **Pharmadeep** "New pharmaceuticals from the deep Antarctic" (20th-26th of December 2016). PI: Ianora.
- EU FP7 **PHARMASEA "**Increasing Value and Flow in the Marine Biodiscovery Pipeline". (October 2012 – March 2017). PI: Ianora
- MIUR-PONREC **PON01_02093 – SANOFI** "Study of new technologies and technological platforms for the improvement of production processes of active pharmaceutical ingredients of industrial interest and search for new bioactive molecules from natural sources"(2011-2014). PI: Ianora
- MIUR-PONREC **PON_01_00117-NOVARTIS** "Antigens and Adjuvants for Vaccines and Immunotherapy "(2011-2015). PI. Ianora
- MIUR-PONREC **PON_01_02782 –BIOGEM** "New nanotechnological strategies for the development of drugs and diagnostic tools directed to circulating cancer cells (CTC)" (2011-2015). PI. Ianora

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