Memorandum of Understanding

Between

Italy, Greece, Spain, Tunisia

This Memorandum of Understanding (MOU) sets for the terms and understanding between the Partners: Italy, Greece, Spain, Tunisia to collaborate for research aimed also to be ready to application to coming Horizon projects on foodborne pathogens.

Background

The Mediterranean Sea

The Mediterranean Basin has a unique feature. It includes parts of Europe and Africa and extends into Asia, and, with the exception of the Suez Canal and the Strait of Gibraltar, it is entirely enclosed. The Mediterranean is surrounded by 22 countries, including Malta and Cyprus islands and also dotted by a myriad of islands. Its hydrographic basin is diverse, encompassing European and African countries. Its catchment area is 4,135 103 km^2, with the Nile and over 200 rivers **(Poulos et al., 2011)**. The Mediterranean Basin has a human population of 480 million, mainly concentrated near coasts. Livestock includes 26.3 million cattle, 126.2 million sheep, 33.9 million goats, camels, equines, and pigs, and 1.3 million dogs. Agriculture (highly developed) uses 80% of available water for irrigation, putting environmental pressure. The Mediterranean region, a popular tourist destination, primarily attracts 30% of international tourists during summer holidays (**Berrilli et al., 2023**).

The Mediterranean Sea: a hotspot for pathogens contamination

The ecological pressure caused by intense anthropic activities means that all marine environments can be considered reliable indicators (a sort of "litmus test") of several environmental threats including fecal contamination (**UNEP/MAP, 2010**). Wastewater outfalls and runoff from rural, suburban and urban landscapes can carry a variety of resistant forms of pathogens into waters, some used for agriculture (often untreated) - thus contaminating soil and produce - some for recreational areas, and others even for drinking water (**Noda et al., 2009**). Pathogens can reach estuaries and contaminate the seawater where they are filtered and concentrated by shellfish (most of them edible) or are swallowed by a range of marine animal hosts (**Fayer et al., 2004; Giangaspero et al., 2009; Aksoy et al., 2014; Ligda et al., 2019**).
Several marine animal inhabitants - and in particular shellfish thanks to their filtering ability - are considered important bio-sentinels for seawater contamination by pathogens of fecal origin **(Giangaspero et al., 2019; Bigot-Clivot, 2022)**. They can accumulate several pathogens of anthroponotic and/or zoonotic origin, and those which cause most concern are viruses (e.g., *Norovirus*, Hepatitis A virus), bacteria (e.g., pathogenic *Escherichia coli, Campylobacter jejuni, Salmonella* spp., *Vibrio vulnificus, Vibrio cholerae, Vibrio parahaemolyticus*), and protozoan parasites (including *Cryptosporidium, Cyclospora, Giardia* and *Toxoplasma*).

6. Effects of climate change

The Mediterranean is a particularly sensitive area, identified as a climate change hotspot. Given its status as a climate change hotspot, the Mediterranean is a region that needs special attention. In its Fifth Assessment Report, the Intergovernmental Panel on Climate Change identified the Mediterranean ecosystems among the most impacted by the consequences of the ever-increasing concentration of greenhouse gases in the atmosphere (https://eo4society.esa.int/regional-initiatives/mediterranean-regional-initiative/mediterranean-regional-initiative-overview/). In the Mediterranean Sea, *in situ* oceanographic and meteorological records, together with the most recent satellite observations, show an estimated warming between 0.6°C and 1°C over the last three decades. Recent satellite observations and *in situ* oceanographic and meteorological records indicate that the Mediterranean Sea has warmed by an estimated 0.6 to 1 degree Celsius over the past three decades. (**Lopez-Garcia, 2021**). It is known that 2 °C global warming would reduce rainfall by around 10%-15% and an increase of between 2°C and 4°C would reduce rainfall by up to 30% in Southern Europe. Water temperature is expected to rise between 1.8°C and 3.5°C by 2100 with hotspots in Spain and in the Eastern Mediterranean (https://www.unep.org/unepmap/resources/factsheets/climate-change).

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Climate change has a direct impact on parasite life cycles, increasing or decreasing development and survival of parasite stages in the environment and influencing the biology of their hosts (**Mignatti et al., 2016; Knapp-Lawitzke et al., 2016**). The intensity of rainfall favours the spread of resistant forms through contaminated waters (**Jiménez et al., 2010**). Drought, on the other hand, reduces the survival of parasitic forms but the consequent evaporation increases their concentration in water. The growing circulation of low-quality waters raises the risk of outbreaks.

The protozoan pathogen *Giardia* (like other intestinal protist parasites, i.e., *Cryptosporidium*) is particularly affected by these changes (**Lal et al. 2013**). Climate change may affect the occurrence, distribution and transmission of *G. duodenalis* and other intestinal protozoa in the Mediterranean countries in different ways. The spread of oo/cysts in the environment is influenced by seasonal rainfall, which carries the cysts from land to water, including drinking water, with risk of waterborne giardiasis, and affects the flow of rivers leading to the sea contamination with this flagellated protozoan. Climatic conditions can also affect the geographic distribution and population density of humans and animal species that serve as suitable hosts and play an important role in the occurrence, distribution and transmission of intestinal protozoans (**Young et al., 2015**; **Brunn et al., 2019**).

The contamination and the risk of transmission for this and other pathogens is worsened by various socio-anthropological aspects - linked to climatic changes -, such as *i)* the greater number of people living in urban areas, particularly in the Mediterranean countries; *ii)* new food habits, locally produced or imported; *iii)* increased colonization by wild animals of the urban and peri-urban environment **(Pozio, 2020).**

Intestinal protozoan parasites as model for monitoring the marine contamination

Giardia duodenalis, and *Cryptosporidium* spp. are well-known causative agents of gastrointestinal disease in humans (particularly children) and animals worldwide. Infection

occurs via the fecal-oral route by ingestion of *G. duodenalis* cysts and *Cryptosporidium* oocysts. Eight major genetic groups of *G. duodenalis* (assemblages A through H) have been identified to date, of which assemblage A and to a lesser extent assemblage B are considered to be of zoonotic interest (**Ryan et al., 2019**). As for *Cryptosporidium* spp., currently, 42 species are recognized as valid, more than 20 species and genotypes have been identified in humans, but the majority of human cryptosporidiosis is caused by either the zoonotic *Cryptosporidium parvum* or the anthroponotic *Cryptosporidium hominis* (**Ryan et al., 2021**).
Cyclospora cayetanensis has emerged as an important cause of epidemic and endemic diarrheal disease in humans mostly in USA **(CDC, 2021).** Humans are probably the only host, and environmental dispersion of oocysts can contaminate water, food and soil.
Toxoplasma gondii is a cosmopolitan intracellular coccidian protozoan that infects all warm-blooded vertebrate species, including humans; wild and domestic felines are the definitive hosts and excrete *T. gondii* oocysts.
The environmentally resistant forms of all these protozoans are currently considered a major global foodborne problem and have been detected in several edible marine animal species worldwide. Moreover, a relationship between shellfish consumption and infection by *Cryposporidium* had been documented (**Sutthikornchai et al., 2016**).
All these pathogens may survive for extended periods in water, including sea water (**Gerard et al., 2019**) and the infective range dose is very low (10-100 oo/cysts).
Worldwide, those intestinal protozoans have been detected in farmed or wild shellfish and other marine animals, including in some areas of the Mediterranean Sea (**Giangaspero et al., 2005; 2014; Gómez-Couso et al., 2004; 2005; Putignani et al., 2011; Aksoy et al., 2014; Ghozzi et al., 2017; Hermosilla et al., 2018; Marino et al., 2019; Ligda et al., 2020; Santoro et al., 2020).**
PCR-based methods are now commonly used. Recently, **Ligda et al. (2019)** have standardized a technique to detect *Giardia* and *Cryptosporidium* in mussels.

From this perspective, all these protozoan parasites represent a valid model for understanding the "vulnerable nature" of the Mediterranean Sea and the effects of such contamination, which are capable of determining a vicious circle between animals, humans and the environment.

Changes, chance and reasons for Cooperation

1. Countries bordering the Mediterranean Sea share common identities but also display an extreme disparity in wealth, resources and health. In recent decades, the Mediterranean region has undergone an extensive and profound urban transformation linked to population growth, increasing rate of urbanization, agriculture, tourism, and profound social and economic changes. As a result, a huge amount of natural wetland habitats has been lost (**Fluet-Chouinard et al., 2023; (https://www.unep.org/unepmap/ne ws/news/working-healthy-coastal-wetlands-mediterranean**). As a further consequence of this high degree of anthropization, the Mediterranean Sea receives over 10 billion tonnes of industrial, animal and urban waste per year with little or no purification **(De Stefano, 2004; WTO, 2020).**
The Mediterranean population is predicted to increase to 572 million by 2030 and that of the coastal regions is expected to increase by over 180% by 2100 (**Reimann et al., 2018**). This entails an ongoing intensification of agricultural practices (crops and livestock), which will, on the one hand, result in greater use of water for irrigation and, on the other hand, have negative

effects on water resources, biodiversity, and the health of the landscape. The reduction and loss of wetlands will further exacerbate the impact caused by the coastal development of human activities, because coastal ecosystems can remove pathogens from contaminated water runoff and mitigate their effects before discharge to the sea (**Shapiro et al., 2010; Klohmann and Padilla-Gamino, 2022)**. Moreover, it is known that treatment plants can be inefficient, due to old technology used (mostly still chlorine-based), or are sometimes inactive due to operating costs, as has been seen, particularly, in some areas of the Mediterranean Basin. The implementation of sewage treatment plants must take into account technological advancement, and only the application of multi-barrier systems can ensure a more effective water purification.
All these factors, including the impacts of climate change, have the potential, individually and in synergy, to increase land-sea water flow and thereby increase the transport of land-borne pathogens and pollutants into the Mediterranean Sea.

2. Contributions on the presence of the above-mentioned parasites in sea water and marine animals inhabiting the Mediterranean Sea is largely lacking or patchy, and as said above, available only in a few Mediterranean coastal areas, as well as in several cases quite dated **(Putignani et al., 2011; Ghoneim et al., 2012; Hilles et al., 2014; Giangaspero et al., 2005; 2014; Ghozzi et al., 2017; Hermosilla et al., 2018; Tedde et al., 2019; Ligda et al., 2020).**
More importantly, for all the above-mentioned matrices, when data are available, samples were examined by different techniques.
Thus, detection of these pathogens in marine animals (shellfish, fish, marine mammals) indicates fecal contamination of seawater, and provides a good indication of the biological pollution of marine ecosystems.

PARTNERS

Partners of this MoU for research projects are among the protagonists of the debate in the most recent season of studies on these topics.

ITALY: The Italian Partners, i.e. University of Foggia (UNIFG) and University of Naples Federico II (UNINA), have a strong record of publishing on topics related with foodborne parasites and public health. Specifically, the research groups are experienced on detection of these protozoans in several food matrices, water and soil. Over the past years, the IT Partners have been involved as PIs/Co-PIs of National and International Projects) to conduct research on food-borne protozoans. Expertise of IT Partners in One Health and parasitology is also testified by the number of research articles and by the fact that UNINA leads the World Health Organization (WHO) Collaborating Centre for diagnosis of intestinal helminths and protozoa (WHOCC ITA-116).

GREECE: The Greek Partner is the Veterinary Research Institute, of the Hellenic Agricultural Organization (ELGO)-DIMITRA, located in Thessaloniki, Greece. The group has a long experience on topics related with foodborne and waterborne parasites and public health. Precisely they have a significant research record and publications on detecting *Cryptosporidum*, *Giardia* and *Toxoplasma* parasites in water, mussels and other matrices as well as the standardization of molecular techniques. Moreover, the group has experience in developing prediction models for the risk of exposure of such pathogens using modern tools such as machine learning and AI.

SPAIN: Spanish partners are represented by the Spanish National Research Council (CSIC), as the largest public research institution in Spain and one of the most renowned institutions in the European Research Area (ERA). CSIC is affiliated to the Ministry of Science and Innovation through the Secretary General for Research. Specifically, the group has experience in the control of some foodborne and waterborne parasites but also in the diagnosis of protozoan parasites infecting both livestock and companion animals, using different methods and including the latest molecular biology techniques.

TUNISIA:
Tunisian partners represented by the National Institute of Marine Sciences and Technologies (INSTM) and the Faculty of Pharmacy of Monastir (FPM), have significant publications on subjects pertaining to public health and foodborne parasites. In particular, the research teams have expertise finding these protozoans in bivalves and fish among other dietary matrices.

Purpose
This MOU will (purpose/goals of partnership) establish a relationship to co-operate on Mediterranean Sea "health status" through detection of emerging food and waterborne parasites in marine inhabitants and to work together for their mutual benefit, in this specific geographic context.
Tentative title: *Protozoan parasites in sea water and marine animals: the Mediterranean Sea in a climate change scenario*

OBJECTIVES
General objective
To promote collaboration research between Mediterranean countries and coordinated methods performance to understand in each participating countries the degree and the extent of the Mediterranean Sea pollution by faecal contamination from urban and/or livestock waste, and the degree and origin of on land environmental food contamination, by investigating the presence of *Giardia/Cryptosporidium/Toxoplasma/Cyclospora* from various food matrices to improve surveillance efforts, detection, genomic typing and environmental investigation.
Specific objectives
1. to investigate the hygienic-sanitary quality of the most farmed or wild species of shellfish present in each country;
2. to monitor the presence of the intestinal pathogens in fecal samples of other marine animals;
3. to investigate the prevalence of these pathogen resistance forms in soil, vegetables and fruits, including berries);
4. to monitor the possible presence of illegal urban and/or livestock discharges and the technology present in treatment plants and indirectly the removal efficiency of highly resistant protozoans from wastewater treatment plants.

This collaborative work will allow:
- to have a snapshot of the epidemiological situation in a single large area (the Mediterranean Sea), data and, implicitly, the responsibilities of different countries in fostering contamination.

- working together with the strengths and univocal nature of the project to ensure that use of resources is maximized, and results are shared by the laboratories.
- to provide more complete data to the European scientific community and Health Agencies, which are aware of the need to fill knowledge gaps.
- a more integrated marine environmental strategies to carry out regular protection programs supported by national and international authorities responsible for the conservation of this threatened and vulnerable sea.
- provide provides a unique opportunity for assessing the "health" condition of the Mediterranean Sea and the "*glocal*" impact of *foodborne protozoans.*

Details on the activities will be better defined in the EU proposal (see Reporting section) with wider objectives goals and wider country participation.

The above goals will be accomplished by undertaking the following activities:
(List and describe the activities that are planned for the partnership and who will do what)
IT, *GR, ES, TUNISIA partners:*
Each Partner in its own country will:
- provide the status of art in his/her country on the knowledge of the spread of the listed intestinal protozoan pathogens in marine environment (shellfish, other marine inhabitants) and genotypes circulating;
- provide the analytical methods used for the detection of protozoan parasites from the previously investigated matrices in terms of sampling and tools (microscopy, IF, kits, DNA extraction, purification, and molecular detection);
- collect samples of shellfish, fishes and other marine animals from their own marine environment;
- monitoring during sampling all climatic parameters;
- test samples according to **Ligda et al. (2019)** standardized method for mussels;
- accept young scientists for practicing in more advanced laboratories and share samples to be confirmed;
- record all water treatment plants at drainage basins level and classify them according to the technology used (chlorine, UV, ozone, membranes, multibarrier technologies, etc.) and report any presence and location of illegal urban and/or livestock discharges.

Reporting
(Record who will evaluate effectiveness and adherence to the agreement and when evaluation will happen)
The project will be candidate to an Horizon Europe Framework Programme (HORIZON) for the future Call), similar to the following:
CALL: https://ec.europa.eu/info/funding-tenders/opportunities/portal/screen/opportunities/topic-details/horizon-cl6-2024-farm2fork-01-4?tenders=false&callIdentifier=HORIZON-CL6-2024-FARM2FORK-01&pageNumber=2
THEME: Climate change and food safety: effects of climate change on food safety across food systems. HORIZON-CL6-2024-FARM2FORK-01-4
TENTATIVE TITLE: Mediterranean Safe Bivalve Food in a climate change scenario (Me.Sa.Bi.F).

The effectiveness, the efficiency, the relevance, the coherence and adherence to the agreement will be evaluated by the evaluators of the European General Union Directorates, once the Project will be submitted.

Funding
This MOU is not a commitment of funds but a baseline project for a Pillar for coming Horizon's projects on the farm to fork strategy for a fair, healthy and environment-friendly food system in line with the European Green Deal priorities.

Duration
This MOU is at-will and may be modified by mutual consent of authorized officials from:

Annunziata Giangaspero, University of Foggia, Italy
Laura Rinaldi, University of Naples Federico II, Italy
Alessandra Barlaam, University of Foggia, Italy
Smaragda Sotiraki, Veterinary Research Institute ELGO-DIMITRA, Thessaloniki, Greece
María Martínez Valladares, Spanish National Research Council (CSIC), Spain
Kemissa Ghozzi, National Institute of Marine Sciences and Technologies, Tunisia
Ibtissem Lahmar, Faculty of pharmacy of Monastir, Tunisia

This MOU shall become effective upon signature by the authorized officials from

Annunziata Giangaspero, University of Foggia, Italy
Laura Rinaldi, University of Naples, Italy
Alessandra Barlaam, University of Foggia, Italy
Smaragda Sotiraki, Veterinary Research Institute ELGO-DIMITRA, Thessaloniki, Greece
María Martínez Valladares, Spanish National Research Council (CSIC), Spain
Kemissa Ghozzi, National Institute of Marine Sciences and Technologies Tunisia
Ibtissem Lahmar, Faculty of pharmacy of Monastir, University of Monastir Tunisia

and will remain in effect until modified or terminated by any one of the partners by mutual consent.

In the absence of mutual agreement by the authorized officials from:

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this MOU shall end in December 2025.

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