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Project Report

The Interreg Project AdSWiM: Managed Use of Treated Wastewater for the Quality of the Adriatic Sea

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Abstract: The Italy-Croatia Cross Border Cooperation (CBC) Programme is the financial instrument supporting the cooperation between the two European Member States overlooking the Adriatic Sea. The first call for proposals was launched in 2017, identifying four priority axes of intervention. Subsequently, in 2019, the kick-off of the AdSWiM project "Managed use of treated urban wastewater for the quality of the Adriatic Sea" took place in Udine (IT). Adriatic marine waters are generally classified as good to excellent based on the Bathing Water Directive (2006/7/EC). Nevertheless, issues of low productivity or the lack of nutrients have been often suggested, especially on the Italian side. The project addresses the question of whether wastewater treatment plants (WWTPs) discharging to the sea, after applying appropriate pollution control and management technologies, can modulate the nutrient content of their effluents to support localized depleted areas. This idea is borrowed from one of the motivations that support the reuse of treated wastewater for irrigation, thus leading to the return of nutrients (nitrogen, phosphorus, potassium, etc.) to natural biogeochemical cycles. However, the hypothesis of modulating the nutrient composition of wastewater opens up to several critical aspects, including legislative and technological ones. Being aware of the delicate environmental implications, we have undertaken the project involving WWTPs, research centers, municipalities, and legal experts with the aim of investigating in detail the problems related to wastewater reuse, especially with regard to the content of nutrients. Our experimental approach aimed to evaluate appropriate and possibly new treatment technologies to reduce the microbial load and to implement chemical and microbiological tests on the treated wastewater. Results have shown that it can be tricky to draw decisive conclusions because (i) the wastewater management systems differ between the two sides of the Adriatic sea due to the different levels of technological development of WWTPs; (ii) the Italian and Croatian coasts deeply differ in geographic characteristics (i.e., topography, orography, current circuits, presence of rivers) and anthropogenic pressure (i.e., exploitation levels, population density); (iii) the new treatment technologies to lower bacterial contamination need further efforts to raise their technological level of readiness (TRL) and make them implementable in the existing WWTPs. However, in terms of chemical control methodologies, the proposed sensors and biosensors

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gave positive results, managing to decrease the detection limits for the measured parameters, and the tested technologies for microbiological monitoring were also effective. In particular, the latter was carried out by using recent molecular biology techniques, capable of resolving the microbiota in treated wastewater, which emerged to be strictly related to the features of the WWTPs.

Keywords: wastewater; granular biomass; monitoring; phosphate sensor; nutrients; metals; emerging pathogens; biosensors; remediation; purification plants

1. Context

Laws for the protection of the environment are becoming increasingly specific and well defined, especially those concerning the protection of water (Figure 1). Water is a fundamental component of daily life, as it is necessary for every type of human activity, from recreational to domestic ones, as well as for industrial and agricultural needs. Sustainable water management is the object of interest in a multiplicity of scientific, social, and political programs around the world. Yet, hydric resources are increasingly threatened in their health and hygienic quality, and, ultimately, their availability is at stake.

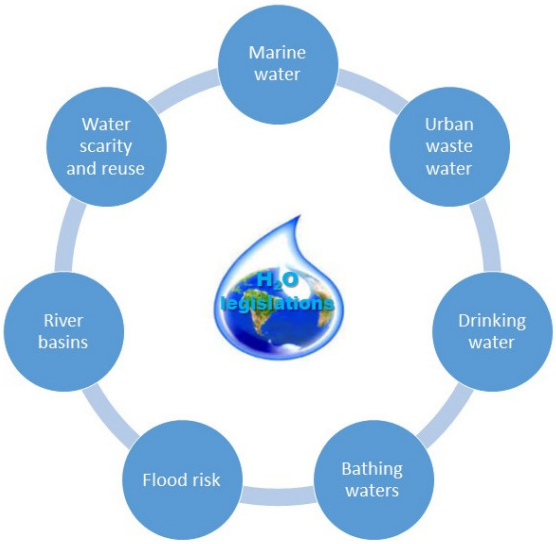

Figure 1. Legislations having "water" as target.

This problem concerns all water bodies, not just hydric resources intended for human consumption. Rivers, lakes, and marine water are increasingly reported to experience alterations in their chemical and physical characteristics, as well as in their hygienic quality. This is due to several reasons, including growing anthropic pressure, the ongoing climatic changes, and an increase in maritime traffic. Consequently, comparing the knowledge and sharing the implementation of actions may be an effective way to bolster interventions aimed at protecting waters, especially marine ones.

The water quality classification of the Adriatic Sea ranges from good to excellent, although hot spots for anthropic pollution and macronutrient imbalances have been identified in some areas [1]. The EU-Interreg IT-HR is a program devoted to the territorial cooperation. In this context, AdSWiM focused on WWTPs and their impact on the marine environment. From EU Directive 91/271/EEC to date, the 10th report published in September 2020 highlights the significant progress in the area of wastewater treatments with ever-higher levels of compliance.

Components of domestic/urban wastewater include several critical elements: pathogens, nutrients, suspended solids, salts, emerging contaminants (e.g., antibiotics, drugs, etc.), and the so-called "oxygen-consuming materials" (e.g., microbes and decomposing organic

waste). In addition, with the introduction of the concepts of sustainability and reuse, WWTP managers have been considering how to reduce their environmental impacts using new technologies. Wastewater reuse is a discussed topic in the context of the "unconventional" water sources debate. Reusing wastewater in irrigation is classically proposed as an option, due to its content in nutrients (nitrogen, phosphorus, potassium, etc.), which thus would return into the natural biogeochemical cycles. Therefore, it was reasoned that the off-shore discharging of treated wastewater (WWT) with modulated nutrient concentrations (i.e., based on the values of the receiving marine waters) can represent a way to support local marine life, while simultaneously reducing pollutants (thanks both to the implementation of the existing technologies or by adopting new ones). In this framework, the activities foreseen were organized (i) to measure by classical instrumental approaches chemical derivatives (i.e., nutrients and metal ions), and (ii) to optimize devices based on sensors and biosensors to a quick analysis of WWT toxicity and concentration of phosphate, for comparison/validation purposes. Moreover, new technologies for microbial load reduction and for the assessment of fecal bacteria and emerging pathogens (by recent molecular biology techniques) were tested. These analyses were performed both on WWT samples collected at the WWTPs and, almost simultaneously, on seawater samples collected close to the discharging line offshore (deliberately, no specific directives were followed in the organization of the sampling). We also examined and compared the cross-border legislation related to the management of WWTPs, as the EU directives need be integrated into the national/local organization.

The goal and challenge of the AdSWiM project was also to bring together cross-border research centers, universities, and municipalities with WWTP managers.

With our activities, we have investigated whether there were gaps between the microbiological/analytical data resulting from the application of individual directives (e.g., the Urban Waste Water Treatment Directive, the Bathing Water Directive), and the ecological quality of marine waters. We have also implemented dissemination and training actions, with a broad area of contents (see Supplementary Materials), involving students and teachers to highlight the importance of safeguarding the sea, its inhabitants, and its connected ecosystems (i.e., beaches and dunes). This manuscript represents a "project report" with the aim of presenting carried out activities, as well as suggesting further developments. Given the differences in each activity, the manuscript is organized according to the structure of the project rather than as a classical research paper.

2. Challenges

In the context of the project, it was reasoned whether treated and safe wastewater could contribute to restoring localized marine areas affected by altered trophic chains due to low nutrient availability or imbalance. The aim of the project was to highlight the need to exploit the available knowledge and technologies to improve the control of the anthropic interactions with the marine environment. The consortium involved WWTPs, and the Italy-Croatia cross-border cooperation provided the opportunity of testing the project at pilot sites located on the two sides of the Adriatic Sea. Finding a solution for wastewater treatment and safe discharge is a difficult challenge because it entails integrated processes in which technical, economic, and financial considerations come into play. In particular, the AdSWiM project was organized around (i) new instruments to acquire a better knowledge and a better control upon the ecological state of marine ecosystems, (ii) innovative and environmentally friendly technologies of wastewater treatment, and (iii) changes in the regulations aimed at achieving higher standards of flexibility and care in every intervention.

On the practical point of view, the project was organized in six work packages (WPs), out of which three (WP 3, WP 4, and WP 5) were dedicated to experimentations, while the other three were for project preparation (WP 0), management (WP 1), and communication (WP 2). An initial literature survey helped gain more information about the health state of the Adriatic Sea. Data about the current distribution of nutrients, bacteria, and pollutants were collected, analyzed, and then used for activity planning, including experimental

design and sampling. The latter was organized also using mapping/modeling tools developed by project partners (WP 3). The project investigated new treatments to be applied in WWTPs with the aim of reducing the microbial loads; new analytical devices were developed to carry out measurements both in seawater and wastewater; and possibly, new chemical and microbiological parameters for maintaining and improving the environmental quality of the sea and the bathing water (WP 4) were also investigated. Reefballs®, which are artificial reefs used to restore marine sites as they offer support and protection for aquatic life, were submerged at sea along the discharging pipeline and are currently still under observation to confirm the development of life at site. The two legislative frameworks were compared, as even if the EU directives are the benchmark, the national organizations of the Italian and Croatian bodies are different, and they need to integrate the EU laws. Finally, in the interest of the project, we also mapped the decision-making bodies in the two States, to lay the foundations for a cross-border sharing in the management strategy of treatment plants in relation to the quality of Adriatic Sea waters (WP 5).

3. Activities Carried Out

3.1. Harmonization of the Knowledge, Project Areas Modeling and Mapping, and Activities Plan (WP 3)

For more than four decades, the EU has developed regulations to safeguard public health and ensure clean bathing waters. Although the classification and quality status of bathing waters is performed considering long-term monitoring, a "good quality" state of coastal waters allows a maximum number of 200 and 500 colony forming units (CFU)/100 mL for intestinal enterococci and *Escherichia coli*, respectively. The status of "excellent quality" is achieved by lowering these thresholds to 100 and 250 CFU/100 mL (Directive 2006/7/EC), respectively. The primary source of these indicators in bathing waters is represented by human activities and, to a lesser extent, warm-blooded animals. The spreading of these microorganisms in the marine environment may occur in several ways, such as through waste waters, run-off rain waters, bathers, swimmers, etc. Likewise, the EU legislation (Directive 2008/105/EC, Directive 2013/39/UE, D.Lgs 219/2010, D.Lgs 172/2015) provides for measures against chemical pollution of surface waters. There are two components: (i) the selection and regulation of substances of broad concern (the priority substances), and (ii) the selection by the Member States of substances of national or local concern (river-basin-specific pollutants). Member States were required to take actions to meet the quality standards in the EQSD (Environmental Quality Standard Directive) by 2015 as part of chemical status (Water Framework Directive Article 4 and Annex V point 1.4.3). For this purpose, a program of measures (according to Water Framework Directive Article 11) had to become operational by 2012. In this context, BW quality is of major concern in the EU policy. BW is not only essential for public health reasons. Clean unpolluted water is necessary to improve ecosystem resilience. Both can be achieved with more integrated and sustainable water resource management.

Within this context, the WP 3 "Harmonization of the knowledges, project areas modeling and mapping, activities planning" was built as a set of activities that were necessary to develop the project implementation.

First, a collection of existing microbiological data was implemented, pertaining both Croatian and Italian waters. Data generated in previous research and monitoring programs have been used to create a common and shared baseline knowledge in order to define the current cross-border status of bathing water quality and ecosystem features in general, in relation to fecal indicator bacteria (*Escherichia coli* and *Enterococci*). The criteria for the choices of datasets followed the project's main objectives and were identified as those areas in the proximity of WWTP outfall in seawater. The three areas were (i) the North Adriatic area, which includes the WWTPs of Lignano Sabbiadoro and San Giorgio di Nogaro, (ii) the Zadar area with the city's WWTP, and (iii) the Split area, with the point sources of Katalinića brig and Stobreč (Figure 2).

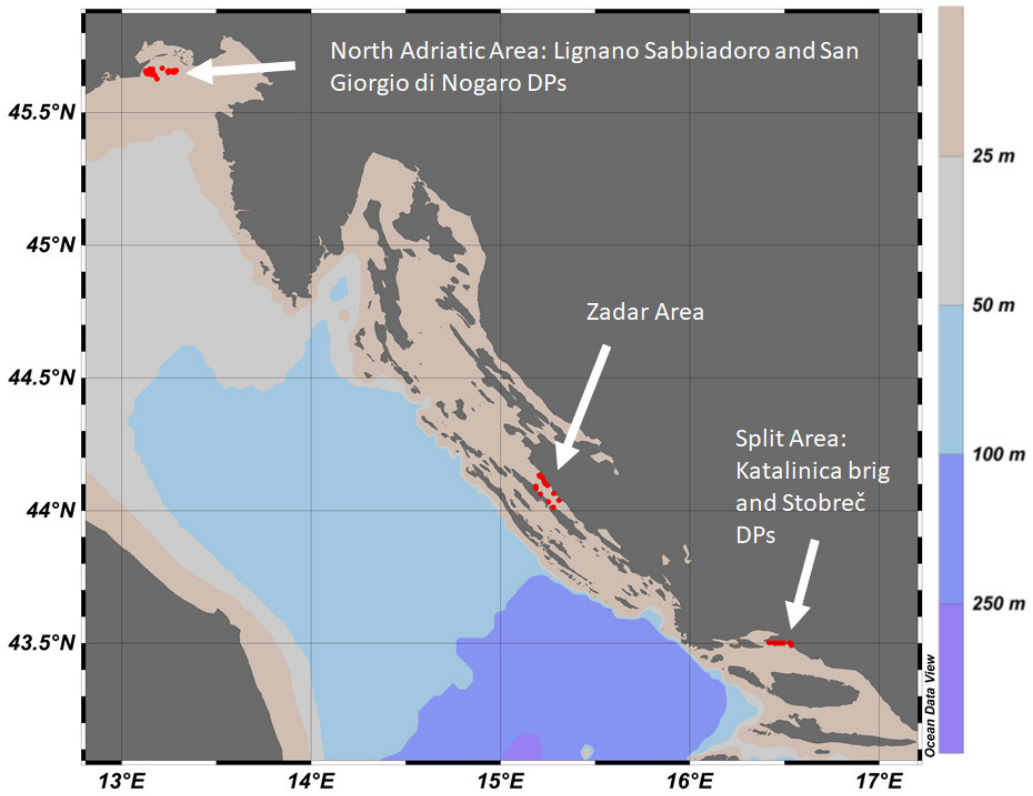

Figure 2. Location of the areas where existing data have been collected for the analyses. Red dots indicate the sampling stations where microbiological samples have been collected.

For the analyses, only samples collected at a distance <5 km from the WWTPs outfalls have been considered and divided into three categories based on increasing distances from the source: (1) <500 m, (2) >500 m and <2000 m, and (3) >2000 m and <5000 m. The datasets, divided by geographical area, highlighted that most samples were characterized by fecal indicators <40 UFC/100 mL. Figure 3 shows the frequency distribution of all the data considered in the analysis. An outstanding assessment is the lack of data with microbe abundance higher than the threshold indicated by Directive 2006/7/EC as "good" quality water. It is also worth mentioning that most of the collected data (99.4% for *E. coli* and 99.2% for *Enterococci*) highlight an excellent quality of seawater in areas at a distance within 5 km from the WWTP outfalls. The subdivision of data according to the distance from the discharge points failed to identify the influence of WWTPs on the distribution of fecal indicators in all the selected areas. In fact, statistically significant (Mann-Whitney test) variations between categories 2 and 3 were found only in the Split area, at both sites. However, we recorder contrasting trends in Katalinića brig and Stobreč, with a higher abundance of *E. coli* in category 2 for Katalinića brig and higher abundance of *E. coli* and *Enterococci* in category 3 in for Stobreč. These results clearly indicate the need for dedicated sampling activities, which should not be based on coastal samples. In the Croatian areas, in fact, data were retrieved from bathing water monitoring programs that collect seawater very near the coast. This might introduce biases because the coast itself (run-off rain waters) and the high touristic fluxes at seaside holiday locations can both represent important sources of fecal bacteria, even at levels higher than WWTP discharging points. The overall outcomes of the analysis highlight the need for specific environmental monitoring programs in areas affected by wastewater discharge.

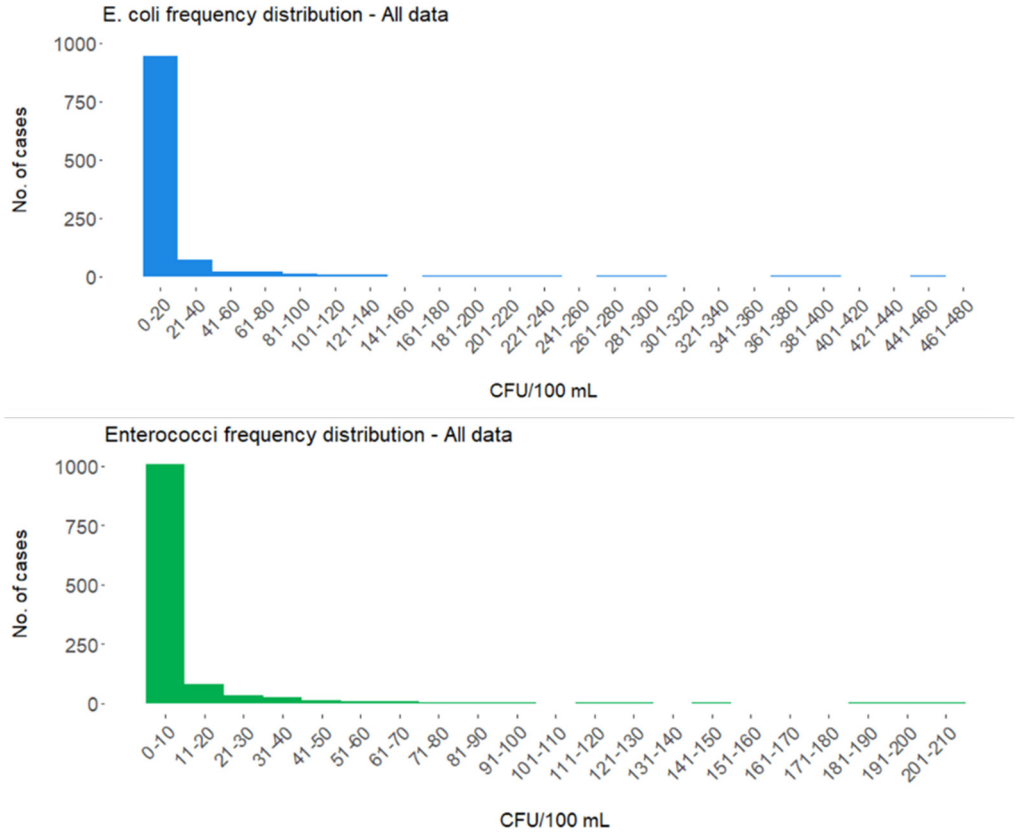

Figure 3. Frequency distribution of the abundances of *Escherichia coli* (blue) and *Enterococci* (green), considering all data collected at all sites.

Within the context of the WP 3 "Harmonization of the knowledge, project areas modeling and mapping", as well as for the microbiological analysis, an investigation on literature data on nutrients and trace elements was carried out to collect relevant information on the sites' object of investigation in the AdSWiM project.

Macronutrients (N, P, Si) play a crucial role in ocean surface waters stimulating the planktonic primary production [2,3]. Indeed, their concentrations are related to the evaluation of the trophic status of the water body and eutrophication phenomena. At sea, nutrients mainly come in from rivers and WWTPs outfalls [4]. Despite this, only a few studies investigate the impact of wastewater on the nutrient seawater levels, especially in the Adriatic Sea [5–11].

Bibliographic research on Elsevier's Scopus (www.scopus.com) (accessed on 22 March 2022) was conducted in all years until the cut-off date of 1 September 2021; the results are shown in Table 1.

EMODnet (European Marine Observation and Data Network) is a new key instrument to reach the GES (Good Environmental Status) in the European seawaters described in the MSFD (Marine Strategy Framework Directive), according to the objectives of the Blue Growth and the Inspire Directive (EU 2007/2/EC). The EMODnet chemistry portal is an open-access website that collects a vast dataset containing a large variety of chemical parameters, including nutrients, produced from many scientific publications and validated reports by public research institutes, private companies, or research groups. A large amount of georeferenced data about nutrient concentrations (grouped for parameter, position, and depth) is available thanks to EMODnet, making the comparison with the literature quick and easy. As a case study, the Gulf of Trieste (N-Adriatic area) was chosen, and data were extracted for the periods of summer 2001–2006, summer 2006–2011, summer 2011–2016, and summer 2016–2020, with depth set to "−10 m". Punctual values, expressed as μmol L^{-1}

obtained in the sites close to Marano Lagoon, were then converted into μg L^{-1} and are reported as min-max in Table 2.

Table 1. Nutrients (ug/L) in Adriatic Sea from literature data.

Sampling Site (Year)	P-DIP	N-NO_2	N-NO_3	N-NH_3	Si-SiO_2	Reference
Northern Adriatic (1991)	3.1 ± 5.9	2.7 ± 2.9	21.9 ± 26.8	29 ± 18.9		[5]
Northern Adriatic (2003–2004)	5.85–25.09					[12]
Northern Adriatic (1994)	7.4 ± 0.9				70.8	[13]
Northern Adriatic (1993–1994)	3.7–6.5		14.43–46.6	6.4–14.3	20.2–118.8	[14]
Northern Adriatic surface	1.5–3.8		8.1–44.5		14.3–158.1	[15]
Northern Adriatic bottom	0.6–2.2		5.6–17.5		37.6–70.8	[16]
Gulf of Trieste (1992–1993)	<3–~30		<70–~350	~14–~70	<140~700	[17]
Gulf of Trieste (2006–2007)		~0–13	~0–122.6	<7.4–46.8	~0–191	[18]
Medium Adriatic (MSW, June–September)	2.51 ± 1.58	0.99 ± 0.90	7.9 ± 9.5	16.67 ± 8.231		[19]

Table 2. Nutrient levels in North Adriatic Sea found in EMODnet chemistry portal (μg L^{-1}).

Sampling Year	DIN	DIP	Si-SiO_2	Reference
2001–2006	10.4–19.2	0.7–1.8	39–56	EMODnet chemistry portal
2006–2011	~14	0.7–1.3	34–48	
2011–2016	~21.4	0.7–0.9	17–34	
2015–2020	15.5–27	0.6–0.8	18.5–21.5	

The data collected describe the state of art of the knowledge on inorganic nutrients in the Adriatic Sea, underlining the lack of information on the influence of sewage discharge on their dynamics. Therefore, the activities foreseen in the AdSWiM project are of paramount importance in filling this gap.

Concerning trace elements (i.e., Hg, Cd, Pb, As, Ni), it is essential to underline that the Mediterranean Sea is a semi-closed basin and that it is surrounded by some of the most heavily populated and industrialized countries in the world [20]. For this reason, it is particularly subjected to pollution events. Persistent contaminants, such as toxic metals and other elements, are here widespread by natural and anthropic pathways and are particularly concerning because of their bioaccumulation in sediments and biota, harboring a potential for biomagnification along the trophic web.

The Northern Adriatic is a vulnerable environment to pollution phenomena because of its closed morphology and shallow depth. A high concentration of Hg has been found both in sediments and waters due to long-term cinnabar (HgS) extraction from the Idrija mine, located in the Slovenian side of the Isonzo River drainage basin [21,22]. During mining operations, mineralized tailings generated by HgS roasting were discharged along the banks of the Idrija River, swept away by floodwaters towards the Isonzo River, and finally transported into the Gulf of Trieste [21]. In addition, the mining district of Raibl (Cave del Predil village, Northern Italy) and a wastewater discharge from a cement factory (near Kanal, Slovenia) can contribute to the transport of other metals (Cd, Pb, Zn, etc) from the Isonzo River to the Adriatic Sea [23]. Bibliographic research on Elsevier's Scopus was conducted in all years until the cut-off date of 24 September 2021. Only 30 papers have been published, with the effect of WWTPs never being evaluated in the area.

The most relevant results from the analysis of the literature about the areas of interest in the AdSWiM project are listed in Table 3.

Table 3. Dissolved metals levels in seawater (ng L^{-1}) from literature. Data are expressed as mean ± sd (min-max) (T: total concentration; nd: not determined).

Location, Sampling Year	Hg	Cd	Pb	As	Cr	Cu	Zn	Ni	Mn	Fe	Al	Reference
Marano Lagoon (Italy), 2004	4.1–52.4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	[22]
Gulf of Trieste (Italy), 2011–2012	0.2–15	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	[24]
Mljet National Park (Croatia), 2005–2008	0.5–24.2 (T)	6.4–18.7	14.6–55.2	nd	nd	169–391	83.6–1098	nd	nd	nd	nd	[25]
Southeast Adriatic coast (Montenegro) 2005–2007	200–2000	0–7400	1300–10,800	500–3100	nd	1400–19,700	400–36,100	2100–21,500	2400–20,000	3000–61,400	nd	[26]
Durres Bay (Albania), 1999–2002	76.6–101.1 (T)	121–187 (T)	165–539 (T)	nd	2992–3674 (T)	170–990 (T)	1111–5962 (T)	440–1078 (T)	66–308 (T)	605–1210 (T)	nd	[27]
Ancona (Italy), 2005	nd	14 ± 1	30 ± 6	nd	nd	400 ± 38	nd	nd	nd	nd	nd	[28]
Central Adriatic Sea, 2004	nd	15 ± 3	27 ± 12	nd	nd	597 ± 330	nd	nd	nd	nd	nd	[29]
Po Plume (Italy), 2002	nd	14 ± 5	68 ± 43	nd	nd	337 ± 159	nd	nd	nd	nd	nd	[30]
Mediterranean Sea, 1995	nd	7	nd	nd	nd	108	176	141	nd	nd	nd	[21]
Isonzo River (Italy)	<LOD-8.60	nd	<LOD-300	190–2310	<LOD-310	150–1770	<LOD-57,900	<LOD-810	1070–16,900	<LOD-6640	nd	[21]
Gulf of Trieste (Italy), 2012	1	nd	nd	1500	620	2610	4640	880	1540	12,500	nd	[31]
Venice Lagoon, 1992	nd	nd	4–30	nd	nd	240–560	nd	380–1130	270–1530	80–630	nd	[32]
Adriatic Sea, 1992–1995	nd	nd	nd	nd	nd	nd	nd	290–1170	1400–5660	280–1840	nd	[33]
Krka River (Croatia), 1997–2000	0.50–1.10	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	[34]
Zrmanja River	nd	nd	54,300–127,000	250–1740	220–110	<50–1040	nd	40–6360	130–3220	6140–10,100	nd	[35]
Gulf of Trieste, 1990–1999	<0.20–4.9	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	[36]

Therefore, investigating the extent of heavy metals pollution could represent an additional and very important indicator for the BW quality and our seas' general environmental status, as requested by the Marine Strategy Directive. However, information about metals pollution in the Adriatic Sea both in the literature and in EU/local/regional reports is scant, even if the Water Framework Directive 2000/60/EC has established provision for a list of Priority Substances (Annex X of the Directive). Decision 2455/2001/EC established the First list, and Directive 2008/105/EC (the Environmental Quality Standards Directive–EQSD) set the quality standards as required by Article 16(8) of the Water Framework Directive. Annex II to the EQSD replaced Annex X of the Water Framework Directive. Among these, Cd, Pb, and Hg are listed as Priority Substances to monitor in the water column. A 2019 report from ISPRA (https://annuario.isprambiente.it/ada/downreport/pdf/6436) (accessed on 28 April 2022) on Marine Strategy shows a metal survey in the Adriatic Sea, available just for 0.04% of the VA (valuation area).

Besides the importance of generating a shared knowledge on the concentration of fecal indicator bacteria, nutrients, and metals, the analysis of existing datasets was performed with the aim to understand the pathways of pollutants spreading at sea from WWTPs. For this purpose, hydrodynamic models have been created both for the northern Adriatic and the Dalmatian coasts (Croatia). The generation of these models should have served in the construction of a monitoring plan at sea aiming at implementing "classical" laboratory procedures with innovative analytical methods and devices to be developed in Work Package 4. However, due to the extreme variability of marine currents in the study areas, the choice of analyzing seawater samples collected only in the very proximity of the outfall of WWTPs pipelines has been taken. Finally, all the partners agreed on the sampling strategy and on activities to be carried out on seawater and treated wastewater samples. These included the analysis of macronutrients [37], metals [38], fecal indicator bacteria (classical approach), emerging pathogens (both enumeration of bacteria of the genus *Pseudomonas* and DNA-based analyses of the whole bacterial community to highlight unconventional, potentially pathogenic microbes), and antibiotic-resistant bacteria [36]. Water samples were also collected to develop novel analytical procedures in the field of chemical measurements, disinfection, and ecotoxicology.

3.2. Innovative Analytical Solutions for Microbiological Control and Treatment of Urban Wastewaters (WP4)

The objective of Work Package 4 was to implement technological solutions to control the impact of wastewater discharge into seawater. According to the Water Framework directive, the quality definition of a water body is based on a wide array of chemical, physical, and biological parameters. The hygienic quality of the marine environment and the balance of a given aquatic ecosystem (i.e., the maintenance of their biodiversity and productivity) are related to the availability of nutrients (phosphorus, nitrogen, organic matter) and their ratio to each other. The project addresses the question of whether controlled use and management of urban wastewater is profitable to maintain and/or improve BW quality. The planned activities are carried out according to the sampling/monitoring plan defined in WP3. Through the development and optimization of novel analytical devices and methods, this WP tested the profitability of innovative solutions in the microbiological control of UWWT. The results have been made available to stakeholders, including the general public as well as governmental and research institutions.

3.2.1. WP 4.1: New Treatments to Reduce the Microbial Loads of Treated Wastewater

In relation to the first WP topic (WP 4.1 Innovative actions in WW treatments test at WWTP level), the application of two technologies for reducing microbial load in WWTPs was evaluated: photodisinfection and granular biomass. Photodisinfection requires a light source, a photosensitizing molecule (PS), and molecular oxygen. Upon irradiation, PS converts oxygen into active oxygen species, which possess a strong bactericidal effect. Immobilization of the photosensitizer on a solid support resulted in photoactive materials suitable for disinfection. The aim was to prepare and optimize a photoactive material, using PVC as a binder, spiked with a newly synthesized photoactive molecule to be used as a chemical-free water disinfection technique in WWTPs.

Granular biomasses have been used in the aerobic step of wastewater treatment in both Italian and Croatian WWTPs. The aerobic granular biomass is an aggregate of microbial origin that does not coagulate under reduced hydrodynamic shear. The objective of this task was to select a suitable treatment process that improves the nitrification process and the energy efficiency of the treatment. Both treatment processes were found to be efficient, but their readiness level (TRL) was not high enough to proceed with the pilot phase (see Section 3.2.6).

3.2.2. WP 4.2: Characterization of Treated WW and of the Seawater in Proximity of the Offshore Discharging Line

In the framework of this task (Innovative Analytical Methods/Devices IAMD), a characterization of treated wastewater and seawater near the offshore discharge with respect to nutrients (nitrate, nitrite, ammonium, phosphate, and silicate) and metal ions (e.g., Pb, Cd, As, Hg) in conjunction with hydrological variables (temperature (°C), salinity (g L^{-1}), electrical conductivity (µS cm^{-1}), pH, dissolved oxygen (ppm), Eh (mV), chlorophyll a, turbidity (NTU)) was performed. To achieve this goal according to our EP, we collected samples of treated wastewater in the Italian plants of Lignano Sabbiadoro, San Giorgio di Nogaro, and Francavilla al Mare, as well as in Croatian ones in Zadar, Katalinića brig, and Stobreč. Seawater samples were gathered in proximity of the emitting points. Both wastewater and seawater samples were collected at monthly intervals during the bathing season (between April and September) in 2019 and 2020.

The measurements were performed using classical analytical methods. For nutrient analyses, samples were filtered in situ with 0.45 µm mixed esters of cellulose filters. N-NO_3, N-NO_2, N-NH_3, P-PO_4, and Si-SiO_2 concentrations were determined colorimetrically using the Systea EasyChemPlus discrete analyzer. In detail, the following methods were used for each of the dissolved nutrients analyzed: N-NO_3 ref. National Environmental Methods Index 9171 Nitrate via V(III) reduction; N-NO_2 ref. United States Environmental Protection Agency (EPA) Method # 354.1; N-NH_3 ref. APHA Standard Methods for the Examination of Water and Wastewater 4500-NH_3 G-Automated Phenate Method; P-PO_4 ref. International Standard Organization 15923-1; and Si-SiO_2 ref. APHA Standard Methods for the Examination of Water and Wastewater 4500-SiO_2) [39].

As for metals, samples were filtered through decontaminated cellulose mixed esters (0.45 µm pore size), diluted with ultrapure grade HCl 2% (*v*/*v*), and analyzed with an AFS Titan 8220 spectrofluorometer (Fulltech Instruments, Rome, Italy) [40].

Both nutrients and potentially toxic elements (PTE) concentrations were below the limits, confirming the good environmental status of the northern Adriatic Sea waters. PTE concentration did not significantly differ between wastewater and seawater samples, although other parameters (e.g., nutrient concentration and microbial indicators) need to be investigated to identify possible synergistic effects.

Some differences were observed in the composition of the WWTP effluents between Italian and Croatian plants because of the different source of waters converging to the depuration plants [41–43].

However, on both sides of the basin, nutrient concentration dynamics in WWTP-affected areas highlighted a limited impact of the treated effluent on inorganic nutrients stocks in the surroundings of WWTP outflows. This pattern was likely due to the rapid dilution of the treated sewage plume in the adjacent marine waters.

While these analyses were carried out using standard operation procedures, this kind of monitoring is well suited for the employment of bio- and electrochemical sensors [44,45]. Indeed, these sensors are less affected by interference and can be miniaturized, and are therefore suitable for on-site detection. In light of these properties, the classical analytical methods used in this WP will be compared with new analytical devices developed in WP 4.3 and 4.4 for the measurement of nutrients (phosphate) and toxicity (algal biosensors) (see section below).

3.2.3. WP 4.3: An Electrochemical Sensor (E-Sensor) for Phosphate Detection with Improved Detection Limit

The standardized method for the detection and quantification of dissolved inorganic phosphorus (DIP) is based on the color change of a phosphomolybdate complex, commonly known as the molybdenum blue method [46,47]. This method is not practical for in situ detection because the reaction between the molybdate anions (from a molybdate ion stock solution) and the DIP occurs in solution and requires an incubation step of at least 30 min. In particular, the molybdenum blue method is not sensitive enough for

surface seawater samples [48]. In this study, a plastic electrode based on graphite, a PVC binder [49,50], and an in-house synthesized organic derivative of molybdate anion (MOP) [51] was prepared as the first approach to develop an electrochemical DIP sensor (Figure 4A). Prior to electrode preparation, surface waters of the northern Adriatic Sea were characterized, and nutrient concentrations were measured during a whole season using classical analytical techniques [41,42]. After determination of the baseline nutrient content, the electrode was prepared and optimized for its use as a phosphate sensor.

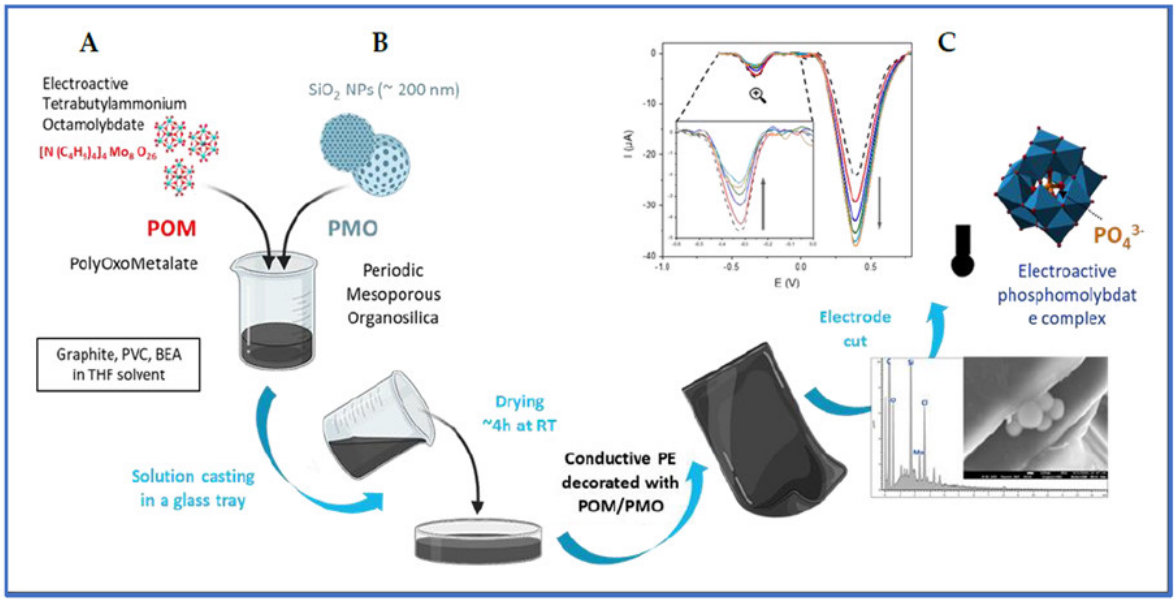

Figure 4. Scheme for the E-sensor preparation for phosphate detection in the two formulations adopted (**A**,**B**) and signal of enhanced sensitivity measured in presence of DIP (**C**).

The detection limit (LOD) obtained was 5 nM, about 10–15 times lower than the limit achievable with the routinary approach. The low manufacturing cost of the electrodes, as well as the portability of the technology, opens the possibility of using this method to monitor phosphate content in oligotrophic seawater samples, thus achieving one of the goals of the AdSWiM project. The E-sensor was further optimized by adding periodic meso-porous organosilica (PMO) nanoparticles (Figure 4B) to the plastic electrode, which locally enrich the electrode surface with MOP, increasing sensitivity as the DL is now 1 nM DIP (Figure 4C). The E-sensor is used on both wastewater samples and seawater from the above sampling sites (Figure 2, Table 2) with a 98% recovery rate.

3.2.4. WP 4.4: Algae Based-Biosensors to Assess the Global Biotoxicity of Wastewater Samples

An electrochemical biosensor was developed and successfully applied to bacteria monitoring for the assessment of global biotoxicity of wastewater samples. Specifically, whole cells of the green photosynthetic alga *Chlamydomonas reinhardtii* were immobilized on nanomodified screen-printed carbon black electrodes to detect *Escherichia coli* as a case study bacterium. The biosensing principle is based on the bacteria's ability to increase the algae's oxygen evolution, which leads to an increase in current signals due to the algae's response to the illuminated target when integrated into an amperometric transduction system. Indeed, bacteria can decrease the photosynthetic oxygen tension in the algal microenvironment, causing them to overproduce oxygen. This biosensor was tested in standard solutions and wastewater samples to optimize the analytical parameters such as detection limit, linear concentration range, interference studies, and matrix effect, with satisfactory results for the assessment of global biotoxicity in the water matrix of wastewater treatment plants [52].

An optosensor based on quantum dot nanoparticles functionalized with artificial peptides mimicking the D1 protein from the photosystem II of the green alga *C. reinhardtii* was developed for monitoring herbicides from agricultural sources that may be present in wastewater. Biomimetic 50- and 70-mer peptides were projected by computational modeling, synthesized by automated synthesis, and characterized by fluorescence spectroscopy and circular dichroism. The molecule with the best properties in terms of structural stability and sensitivity to atrazine, a case study herbicide, was immobilized on quantum dots, and the analytical parameters—including detection limit, linear concentration range, interference studies, and matrix effect—were optimized.

3.2.5. WP 4.5: Emerging Pathogens and Risk Assessment for Marine Environment

The emerging pathogen survey was designed with the premises that (i) only a limited number of pathogens are considered in surveillance measures (*E. coli* and *Enterococci*) and (ii) antibiotic-resistant bacteria (ARB) pose an increasing threat to human health and aquaculture. Nevertheless, prior to the implementation of AdSWiM, no data on ARB presence and distribution were available for the Adriatic Sea. To fill these gaps, two molecular biology approaches were performed. We collected samples of treated wastewater in the Italian plants of Lignano Sabbiadoro, San Giorgio di Nogaro, and Francavilla al Mare, as well as in Croatian ones in Zadar, Katalinića brig, and Stobreč. Seawater samples were gathered in proximity of the emitting points. Both wastewater and seawater samples were collected at monthly intervals during the bathing season (between April and September) in 2019 and 2020. DNA collected from these samples was subjected to various analytical procedures.

To study the whole bacterial community in each sample, the 16S rRNA gene was amplified using the latest technology, and the amplicons were sequenced (Illumina sequencing) to identify the most abundant bacterial genera. The taxonomic lists obtained were then analyzed to determine the relative abundance of the putatively pathogenic microbes (Figure 5).

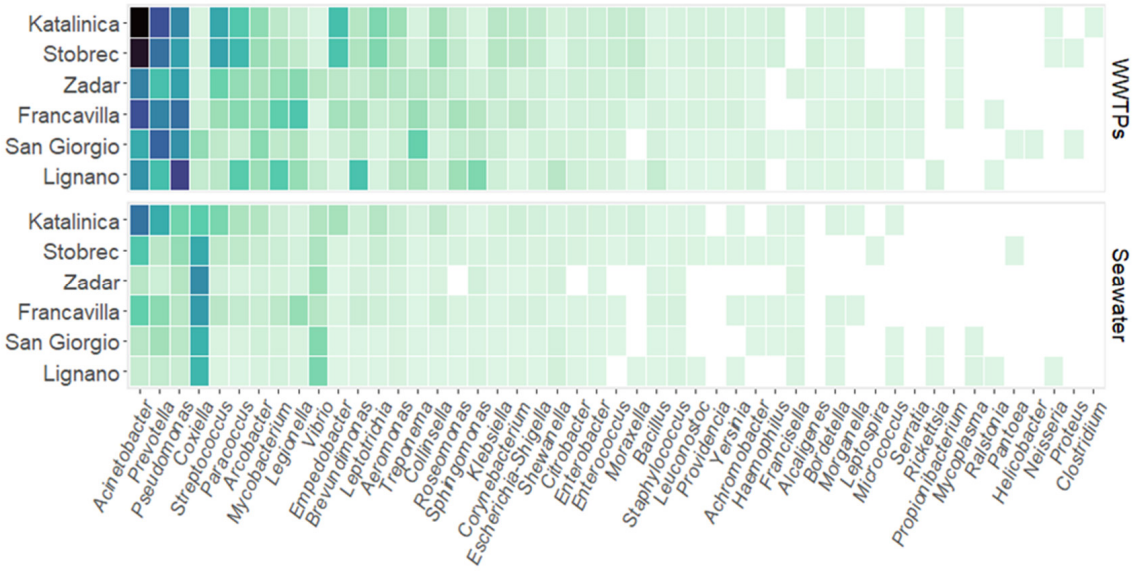

Figure 5. Potential pathogenic bacteria in treated sewage (WWTPs) and seawater samples. Out of the 60 genera investigated, *Bacteriodes*, *Borrelia*, *Brucella*, *Burkolderia*, *Campylobacter*, *Chlamydia*, *Laclercia*, *Listeria*, *Orientia*, and *Salmonella* were not found in the dataset. ASV raw counts were log(1 + x) transformed to improve visualization. Tiles represent the average count over time per site. Higher values correspond to darker shades. White tiles indicate 0 reads.

The second approach was based on the quantification of a specific group of genes (q-PCR) that confer antibiotic resistance to bacteria. The genes tested were *sul*2 (resistance to sulfonamides), *tet*A (resistance to tetracyclines), *erm*B (resistance to macrolides), *qnr*S

(resistance to quinolones), $bla_{\text{OXA-48}}$, $bla_{\text{CTX-M}}$, bla_{TEM} (resistance to β-lactams), *mcr*-1 (multidrug resistance), and *intl*1 (representative of anthropogenic pollution). Some of the results obtained are reported in Fonti et al. [43].

3.2.6. WP 4.6 Assessment of Feasibility and General Considerations from the Results of Each Task

Finally, in WP 4.6, all the results gathered in WP 4 were harmonically analyzed. A feasibility assessment was conducted to identify strengths and weaknesses, difficulties faced, innovative and positive elements, and possible alternative scenarios. In addition, this phase evaluated the different products of the project activity to determine the value of their possible technical implementation. The products generated by AdSWiM consisted of concrete actions (analysis and equipment development), research results (mapping of emerging pathogens and micronutrients), and proposals for new reference models for ecological indices.

The optimized devices (i.e., the E-sensor and the algae-based biosensor) had different TRLs, but overall, the main objectives of optimization and testing in real samples of treated wastewater and seawater were achieved. The electrochemical sensor for detection of orthophosphate is easy to fabricate and inexpensive (i.e., few cents/electrode). The detection limit of 1 nM is several orders of magnitude lower than that of currently available classical approaches (20 nM). For these reasons, it would be interesting to further optimize this sensor to learn more about phosphate dynamics and its impact on the marine communities, and in turn on bathing and ecological quality of water bodies. However, a collaborative effort of research as well as technical support is needed to bring this electronic sensor to a higher level. In contrast, the biosensor for biotoxicity assessment is a device that is now completed and can prove itself in an operational environment. A further effort is required to identify potential markets for this product and to define its placement for its future commercialization.

Two treatments were evaluated to reduce the microbial load of the wastewater, namely photodisinfection and the use of granulated biomass. Photodisinfection was found to be effective in the laboratory, but further work is needed to transfer this approach to a pilot plant and subsequently to in situ deployment. The cost of preparing the photoactive material proved to be the main limiting factors. Granular biomasses are very effective and have been used in wastewater treatment plants in Italy and Croatia. It turned out that granular biomasses, to work properly, need individual optimization in relation to the characteristics of the wastewaters to be treated. All the aspects propaedeutic to an operational use in field conditions were studied and evaluated during the project implementation, and protocols for proper and effective application were established.

The study on emerging pathogens in the WWTPs and seawater shows that wastewater treatment technologies need to be improved to avoid the transfer of genetic material from the WWTPs to the seawater, and that special monitoring programs are needed at the WWTP discharge points to the sea. The same findings emerged from metals and chemical contaminants monitoring. These measurements (of both microorganisms and chemicals) were collected in a data set that is freely available at the web address referenced below and is useful for the implementation of the already available databases (e.g., EMODnet).

3.3. Technologies and Strategies for Managing WWTPs Guidelines Definition and Cross-Borders Strategies (WP5)

Finally, WP5 is structured in three different main activities aiming to promote transboundary cooperation and dialog between project partners around depuration technologies, management, and legislation.

The pilot plants involved in this project have very different characteristics, in terms of both waters treated and technological facilities. However, despite being in different countries, all the facilities discharge into the Adriatic Sea, and the different features (e.g., trophic regimes, water mass circulation patterns, anthropogenic pressure, land use in the catchment basins, etc.) of the two sides of the Adriatic Sea must be considered when modeling the

technological solutions. All these aspects were evaluated taking into account the legal frameworks of the two countries and were analyzed and summarized by the legal experts engaged on purpose.

The Republic of Croatia has been a full member of the European Union since 1 July 2013. It should be emphasized that in the process of joining the European Union, the Republic of Croatia had to harmonize its national legislation dealing with environmental protection and water management with the European Union body of law. This resulted in the obligation to meet all the requirements arising from the alignment with the *acquis communautaire*.

As a member state of the European Union, the Republic of Croatia has the right to access funds from the Structural Funds and the Cohesion Fund of the European Union. The main purpose of these funds is to provide financial assistance in meeting the requirements arising from the European Union legislation that Croatia has transposed into its national legislation, i.e., the Accession Treaty signed upon accession to the European Union.

As set out in the Marine Strategy Framework Directive, three important implementation steps were taken in 2012:

(1) Member States submitted reports on the initial assessment of the current environmental status of marine waters environmental status (Article 8 of the Marine Strategy Framework Directive);

(2) Member States defined what good environmental status (GES) means for the waters of the respective marine regions and subregions (Article 9 of the Marine Strategy Framework Directive);

(3) Member States set environmental targets and corresponding indicators to guide their progress towards achieving good environmental status by 2020 (Article 10 of the Marine Strategy Framework Directive).

In this context, Croatia is currently intensively engaged in the upgrade of its depuration plants. The main strengths of the Croatian WWTP network are due to i) the good environmental characteristics of the recipient water bodies, ii) to the placement of outfall pipes at considerable depth, and iii) to the limited inflow of waters used for industrial and agricultural processes. The weaknesses are related to the lack of biological treatment and no nutrient or sludge removal from sewages. In addition, the WWTP system collects a combination of rainfall runoff and sewage wastewater.

On the Italian side, all treatment steps possible for this type of plants (i.e., mechanical, chemical, and biological) are applied in the considered WWTPs. Current efforts are aimed at improving the knowledge of the environmental status of the recipient water body, to implement remediation measures (the installation of submerged Reefballs is one of the results achieved by the AdSWiM project), and to evaluate the feasibility of resources recovery from the treated wastewaters. In this framework, the evaluation of new treatments to reduce the sewage microbial load and the investigation about phosphate discharge and its eventual recovery must be considered. The survey on the legislative framework and the administrative organization of integrated water management, with particular focus on water purification and sewer management, suggested some considerations to improve the coordination between Italy and Croatia in this sector.

Due to the common derivation of the national standards from the EU regulations, there is a substantial uniformity in the relevant legislative guidelines between Italy and Croatia. For this reason, finding any significant differences in the compliance to keep the concentration of chemicals below legislative thresholds in treated waters to be discharged at sea is improbable. However, there are significant differences in the way the two countries manage the entire water cycle, the integrated water system, and the agencies in charge of water quality control. Indeed, while the Italian administrative model is characterized by substantial uniformity, on the Croatian side, the same uniformity is not found in the organization of the operational, management, and control schemes. These discrepancies show that the possibility of proposing a common legal framework enhancing the protection of the Adriatic Sea in consideration of the local needs is obviously complicated and, in the end, it still falls under EU authority. However, the stipulation of a series of agreements

between the Italian agencies responsible for integrated water management and Croatian administrative bodies responsible for water purification appears to be feasible.

These agreements should be oriented towards the exchange of information about the treatment steps and technologies used and about the quality of the recipient water bodies, aiming to define and share best practices between the two countries.

This constant exchange of information can lead to the drafting of targeted strategies, highlighting common elements and differences in approach. In this regard, the purpose of the AdSWiM project was to connect WWTPs managers and technologists on both sides of the border to favor the bottom-up development of an integrated water management strategy, a dialogue that can be shared with, and expanded to, regional, national, and European authorities.

4. Conclusions and Considerations

In this project, we addressed many different issues which concerned both technology and research, as well as aspects of dissemination and involvement, especially of the younger generations [53].

It was possible to create a link between different working areas, integrating diverse knowledge and skills, highlighting the importance of stimulating dialogues among different contexts. Monitoring, maintaining, and improving the environmental quality are tasks that can be successfully carried out only through a multidisciplinary approach and networking with decision makers.

Our activities showed that the alternative technologies that were evaluated to reduce microbial contamination from WWTPs are not mature enough to be applied with general protocols into relevant environments (i.e., WWTPs); major and specific development projects must be undertaken to further develop these approaches. We also demonstrated that the use of molecular tools for monitoring WWTPs microbial communities is a highly effective approach and useful to profile the microbial population which is selected by the inlet waste. By this approach, some health-related issues, such as the occurrence of antibiotic resistance in sewage, can be monitored. Finally, the new low-cost, fast-response devices for the monitoring of phosphate concentration and toxicity have given results comparable to those obtained by conventional analytical methods. Furthermore, these devices have improved detection limits and lower sample pre-treatment procedures, preluding to their large-scale utilization for monitoring purposes. However, the cross-border verification and comparison of the current treatment technologies and facilities is still very uneven. Croatia is now involved in a significant upgrade of its WWTPs, which at present operate, in general, without secondary or further steps of treatment. This will greatly reduce the impact from WWTPs on the marine environment, with effects that will need to be evaluated over time.

The survey on the legislative aspects regulating the management of the treatment processes with respect to the quality of the Adriatic Sea confirmed that essentially both countries are respecting EU requirements. However, the question of whether WWTPs effluents could be used to modulate nutrient (i.e., phosphate) concentration into an oligotrophic sea is still open. Indeed, from the physical, biological, and chemical point of view, the parameters controlling the whole aquatic systems are difficult to predict, particularly in consideration of the consequences of the climate change we are experiencing.

From a legislative perspective, it emerged that in Croatia, the OTAMB (Optimal Territorial Ambit Management Body) is the entity eventually involved in starting a process of tailoring of the legislation in relation to the features of the Adriatic Sea. Its Italian counterpart is "Ausir" because it is representative of the entire area involved and connects the regional authority and the integrated water managers. On the other hand, the OTAMB must also have capabilities on a district basin conference level, because this government body coexists with the state administration; in other words, OTAMB is the subject able to interact with counterparts on the Croatian side and, at the same time, on the level of the community that must be interacted with in order to definitively determine, if necessary, exemption arrangements and the contents thereof.

Therefore, during the institutional conference, the best solutions can be drafted to be brought to the attention of the European Commission, following discussion with the Croatian (state) partner.

The operational structure can be summarized as follows:

- The OTAMBs dialogue with the Croatian bodies that carry out the same functions (perhaps a "permanent round table" or something similar can be proposed);
- Each OTAMB interprets requests from the integrated water system managers and returns the results drafted by the "permanent round table" to them;
- The OTAMBs are invited to participate in the permanent institutional conference, partly for the purpose of contributing what has come out during the "permanent round table" to the discussion;
- The permanent institutional conference invites the relevant Minister (State representative, in Italy the Minister of Environment) to be the spokesperson (with their Croatian counterpart) at the EU Commission.

It is a difficult course, but if followed steadfastly, it may produce meaningful results over time. Moreover, it emerged that this solution is fitting with the two countries here involved and with their territorial peculiarities.

Supplementary Materials: Video: https://www.youtube.com/watch?v=uXTTaosEl04 (accessed on 14 June 2022); https://www.youtube.com/watch?v=JzvtSwiuYEU (accessed on 14 June 2022); https://www.youtube.com/watch?v=97cW3d4VmYg (accessed on 14 June 2022); https://www.youtube.com/channel/UClkAN1VGaIuaVJ7gArxlL1A (accessed on 14 June 2022).

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Abbreviations

BW: bathing water, EMODnet: European Marine Observation and Data Network, EQSD: Environmental Quality Standards Directive, GES: Good Environmental Status, MSFD: Marine Strategy Framework Directive, TRL: Technological Level of Readiness, WWT: treated wastewater, WWTP: wastewater treatment plant.

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